Effect of phenobarbital on the level of translatable rat liver epoxide hydrolase mRNA

drug-metabolizing enzymes/phenobarbital induction/cell-free protein synthesis/immunoprecipitation

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ABSTRACT Liver poly(A)+RNA isolated from untreated and phenobarbital-treated rats has been translated in the rabbit reticulocyte cell-free system in order to determine the level of translationally active epoxide hydrolase (EC 3.3.2.3) mRNA. The in vitro translation systems were immunoprecipitated with rabbit IgG prepared against purified epoxide hydrolase, and the amount of epoxide hydrolase synthesized by the lysate programmed with control and phenobarbital poly(A)+RNA was quantitated. The level of translatable epoxide hydrolase mRNA is increased 3-fold after chronic phenobarbital administration. This level of induction correlates well with the 5-fold induction in catalytic activity of epoxide hydrolase (using styrene 7,8-oxide as substrate) in microsomes isolated from phenobarbital-treated rats. Therefore, we suggest that chronic phenobarbital administration increases the amount of functional epoxide hydrolase in rat liver microsomes by way of an increase in the translatable mRNA level encoding for the enzyme. We do not know whether the increase in mRNA is the result of increased transcription or messenger stability.

Cytochrome P-450 and epoxide hydrolase (EC 3.3.2.3) are integral membrane proteins of the endoplasmic reticulum and function in concert in the metabolism of various drugs, mutagens, and carcinogens (1–5). Although barbiturates such as phenobarbital have been shown to increase the level or activity of cytochrome P-450 and epoxide hydrolase in rat liver (6–8), it was unknown whether this increase was related to an increase in the level of translatable mRNA for these proteins. With the recent advances in the purification of multiple species of cytochrome P-450, the development of specific antibodies to those different forms (9–11), and the refinements made in cell-free translation systems, it became possible to examine the molecular basis of induction of cytochrome P-450 by various xenobiotics. As a result, studies from a number of laboratories (12–16) have demonstrated that the level of functional mRNA for the phenobarbital-inducible species of cytochrome P-450 in rats is increased by 12–16 hr after a single injection of phenobarbital.

Despite the recent advances in the purification and characterization of rat liver epoxide hydrolase, only recently have any studies focused on the capacity of mRNA isolated from phenobarbital-treated rats to direct the synthesis of epoxide hydrolase (17, 18). Both of these brief communications reported that the primary translation product of epoxide hydrolase appears to be synthesized in cell-free systems as the mature enzyme. These findings suggest that, unlike many secreted proteins and some membrane proteins, epoxide hydrolase appears not to require a cleavable signal sequence for insertion into the endoplasmic reticulum membrane.

In the present investigation we have utilized cell-free protein synthesis and specific immunoprecipitation to quantitate the level of translationally active rat liver epoxide hydrolase mRNA after chronic phenobarbital administration and have correlated the mRNA level with the catalytic activity of the enzyme in microsomes isolated from phenobarbital-treated animals. Our data indicate that the functional mRNA level and the catalytic activity of epoxide hydrolase are induced by phenobarbital to similar extents.

METHODS

Preparation and Characterization of Antibodies Against Purified Epoxide Hydrolase. Epoxide hydrolase was isolated from microsomes of phenobarbital-treated rats by the method of Knowles and Burchell (19) as modified by Guengerich et al. (20). Antibody against rat liver epoxide hydrolase was raised in rabbits by intradermal injections (eight sites), 200 μg per animal, of purified enzyme mixed with Freund's complete adjuvant. Booster injections consisting of 70 μg of epoxide hydrolase mixed with Freund's incomplete adjuvant were given at approximately 7-day intervals. Rabbit IgG was partially purified from the antiserum by affinity chromatography on DEAE-Affi-Gel blue.

Antibody specificity was determined by Ouchterlony double immunodiffusion (21) and Laurell rocket immunoelectrophoresis (22).

RNA Isolations. Male Sprague–Dawley rats (120–170 g) were used in all experiments. Control animals were given water ad lib, phenobarbital-treated rats were given sodium phenobarbital in their drinking water (1 mg/ml) for 8 days before sacrifice. Total liver RNA was isolated by the guanidine thiocyanate/cesium chloride procedure (23). Livers were rapidly homogenized in 4 M guanidine thiocyanate (Tridom, Hauppauge, NY)/0.5% sodium N-laurylsarcosine/25 mM sodium chloride, pH 7.0/0.1 M 2-mercaptoethanol. The homogenate was layered over 1.2 ml of 5.7 M cesium chloride buffered with 0.1 M sodium EDTA (pH 7.0) and centrifuged for 20 hr at 40,000 rpm and 20°C in a Beckman SW 60 rotor. The RNA pellets were resuspended in 7.5 M guanidine hydrochloride (Bethesda Research Laboratories, Rockville, MD)/5 mM dithiothreitol and buffered in 0.025 vol of 1 M sodium citrate at pH 7.0. The RNA pellets were heated at 68°C until dissolved and precipitated by addition of 0.025 vol of 1 M acetic acid and 0.5 vol of absolute ethanol. The precipitated RNA was centrifuged at 10,000 rpm for 10 min at 10°C in a SS-34 rotor. The final RNA pellet was washed in absolute ethanol and centrifuged at 10,000 rpm for 10 min.

The pellet was dried with nitrogen and resuspended in sterile water at a concentration of 20–30 A260 units/ml. The RNA suspension was heated for 2 min at 68°C, rapidly cooled to room temperature, and adjusted to (final concentration) 0.5 M in lithium chloride, 0.5% in NaDodSO₄, 1 mM in EDTA, and 10 mM in Tris–HCl (pH 7.4). The RNA was passed over an oligo(dT)-
cellulose column (type T-3, Collaborative Research, Waltham, MA) that had been equilibrated with the same buffer. Poly(A)+ RNA was eluted from the column with 10 mM Tris-HCl, pH 7.4. The solution was adjusted to 0.2 M in potassium acetate (pH 5.0) and precipitated overnight with 2 vol of absolute ethanol. The precipitated poly(A)+ RNA was centrifuged at 10,000 rpm for 30 min and +5°C in a SS-34 rotor. The pellet was lyophilized and resuspended in sterile water.

Cell-Free Protein Synthesis. Total liver poly(A)+ RNA was translated for 60 min in a micrococcal nuclease-treated rabbit reticulocyte lysate system (24). The reaction mixture (90 μl) contained 30 μl of lysate, 25 mM Hepes (pH 7.4), 1 mM creatine phosphate, 48 mM KCl, 87 mM K acetate, 1.2 mM MgCl₂, amino acids (without methionine) at 50 μM each, 20 μCi of [35S]methionine (1100 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels), and 1.00 μg of poly(A)+ RNA. To assess the incorporation of [35S]methionine into protein, 2 μl of each translation mixture was spotted onto Whatman 3 MM filter paper and the paper was placed into ice-cold 10% trichloroacetic acid. The filter papers were boiled for 15 min in 5% trichloroacetic acid and then rinsed thoroughly with cold 5% trichloroacetic acid, 100% ethanol, ethanol/ether, 1:1 (vol/vol), and, finally, ether. The filter papers were dried and placed in scintillation vials; 500 μl of Protosol (New England Nuclear) was added to each vial and the vials were incubated for 30 min at 55°C. The vials were then cooled to room temperature and 10 ml of Econofluor was added in preparation for liquid scintillation counting. Aliquots (5–10 μl) of the translation mixtures were also subjected to NaDodSO₄/polyacrylamide gel electrophoresis on 7.5% gels (25).

Immuno precipitation and Quantitation of Epoxide Hydrolase. Epoxide hydrolase was immunoprecipitated from the translation mixtures as described (18) with formalin-fixed, heat-inactivated Staphylococcus aureus cells. Immunoprecipitates were subjected to NaDodSO₄/polyacrylamide gel electrophoresis on 7.5% gels. [3H]Methionine-labeled epoxide hydrolase was detected by fluorography (26). In order to quantify the level of translatable epoxide hydrolase mRNA, the region of the gel corresponding to the position of purified epoxide hydrolase was excised and dissolved in 50% perchloric acid/30% hydrogen peroxide at 60°C. Aquasol-2 was then added to the solubilized gel and total radioactivity was determined by liquid scintillation counting. Background radioactivity was determined by excising gel regions above and below the radiolabeled epoxide hydrolase band and dissolving the slices as just described. Values obtained were then subtracted from the radioactivity obtained in the slices containing epoxide hydrolase.

Determination of Epoxide Hydrolase Activity. Epoxide hydrolase activity was measured in liver microsomes isolated from untreated and phenobarbital-treated (8 days) rats by the method of Oesch et al. (27) with [3H]styrene oxide as substrate. Protein was determined by the method of Lowry et al. (28) with bovine serum albumin as standard.

RESULTS

The purified epoxide hydrolase preparation utilized in this study for antibody production consisted of a single major band on NaDodSO₄/polyacrylamide gel electrophoresis with an estimated molecular weight of 50,000 (Fig. 1), which is similar to values reported previously (19, 20, 29–31). The purified enzyme had a specific activity of 577 nmol/min per mg of protein with styrene 7,8-oxide as substrate. When rabbit anti-epoxide hydrolase antiserum was allowed to react against either the purified enzyme or detergent-solubilized microsomes isolated from untreated or phenobarbital-treated rats, a single immunoprecipitin band formed after either Ouchterlony double immunodiffusion or Laurell rocket immunoelectrophoresis (Fig. 2). Ouchterlony double-immunodiffusion gels also revealed lines of identity between the purified enzyme isolated from phenobarbital-treated rats and detergent-solubilized microsomes isolated from untreated or phenobarbital-treated rats. These latter data suggest immunoochemical identity between the purified enzyme and the forms present in control and induced microsomes.

Quantitation of the Translatable mRNA Level for Rat Liver Epoxide Hydrolase. As reported (18) poly(A)+ RNA isolated from untreated and phenobarbital-treated rats have similar translational activities in the rabbit reticulocyte lysate system. Incorporation of [35S]methionine into total protein increased as a function of the amount of RNA added to the lysate. However, we generally observed a decrease in incorporation at RNA concentrations >50 μg/ml. When the translation products directed by poly(A)+ RNA isolated from phenobarbital-treated rats were analyzed by NaDodSO₄/polyacrylamide gel electrophoresis and autoradiography, we noted an increase in [35S]methionine incorporation into a polypeptide(s) with a molecular weight of 50,000–52,000 (Fig. 3). This molecular weight range corresponds to the size of both epoxide hydrolase and the phenobarbital-inducible species of cytochrome P-450: 50,000 and 52,000, respectively (32–35). We have reported previously (18) that phenobarbital enhances the synthesis not only of a polypeptide(s) of molecular weight 50,000–52,000 but also of two additional polypeptides.

**FIG. 1.** NaDodSO₄/polyacrylamide gel electrophoresis of purified epoxide hydrolase (lane B, 5 μg) used in this study for antibody preparation and characterization. Lane A, molecular weight markers: bovine serum albumin (68,000), ovalbumin (45,000), and carbonic anhydrase (30,000). The estimated molecular weight of purified epoxide hydrolase is 50,000.
FIG. 2. Ouchterlony double immunodiffusion performed in an agar gel from Hyland Laboratories, Costa Mesa, CA. Wells 3 and 5, purified epoxide hydrolase; wells 1 and 2, microsomes isolated from untreated rats and phenobarbital-treated rats, respectively; center well, antiserum against epoxide hydrolase (60 mg/ml); well 4, phosphate-buffered saline. Incubation was at room temperature for 24 hr. Rat liver microsomes were solubilized in 0.25 M sucrose/0.01 M Tris-HCl, pH 7.4/0.2% Emulgen/0.5% sodium cholate. The protein concentrations for the purified enzyme and microsomes isolated from untreated and phenobarbital-treated rats were 0.77, 9.4, and 4.7 mg/ml, respectively.

FIG. 3. NaDodSO4/polyacrylamide gel electrophoresis of [35S]methionine-labeled translation products directed by total liver poly(A)+RNA isolated from untreated (lane B) and phenobarbital-treated (lane C) rats. The protein synthesized in the absence of exogenously supplied poly(A)+RNA. Equivalent amounts of radioactivity from the translation mixtures programmed with poly(A)+RNA were layered on the gel. The arrow denotes the 50,000–52,000 molecular weight range, where a major increase in [35S]methionine incorporation is seen. The molecular weight markers were bovine serum albumin (68,000), ovalbumin (45,000), and carbonic anhydrase (30,000).

with molecular weights of approximately 29,000 and 27,000. Our total liver poly(A)+RNA preparations do direct the synthesis of polypeptides with molecular weights ranging up to 200,000. However, because of the length of exposure of the x-ray film to the radioactive gel, these products cannot be seen in Fig. 3.

In order to quantitate the amount of epoxide hydrolase synthesized by the rabbit reticulocyte lysate programmed with total liver poly(A)+RNA, translation mixtures were subjected to immunoprecipitation with rabbit anti-epoxide hydrolase IgG. Antigen–antibody complexes were recovered with formalin-fixed, heat-inactivated S. aureus cells. We found that pretreatment of the translation mixtures with an aliquot of the S. aureus cells before immunoprecipitation was absolutely necessary to lower nonspecific binding to the cells.

The immunoprecipitates obtained from control and phenobarbital poly(A)+RNA translations were analyzed by NaDodSO4/polyacrylamide gel electrophoresis. Analysis of the fluorograms of the dried gels revealed a major band of radioactivity associated with a polypeptide that had an approximate molecular weight of 50,000 (Fig. 4). This polypeptide comigrated exactly with purified epoxide hydrolase which is in agreement with previous studies from our laboratory (18) as well as Kasper's laboratory (17). When an excess of unlabeled epoxide hydrolase was added to the translation mixtures before immunoprecipitation, no radioactive polypeptide was observed. Furthermore, as found previously (18), when antibody to epoxide hydrolase was replaced with nonimmune IgG, no radioactive polypeptide corresponding to epoxide hydrolase was immunoprecipitated from the translation mixtures. The immunoprecipitated epoxide hydrolase could be visualized by fluorography more readily in translation mixtures directed by liver poly(A)+RNA isolated from phenobarbital-treated rats compared to that from untreated rats (compare lanes A and B in Fig. 4). However, upon longer exposure of the x-ray film to the gel, radioabeled epoxide hydrolase directed by control poly(A)+RNA became readily detectable (Fig. 4, lane C).

The level of mRNA specific for epoxide hydrolase was quantitated by comparing the amount of radioactivity that comigrated with authentic epoxide hydrolase with the total radioactivity incorporated into protein. It is clear from this type of analysis that approximately 3 times more epoxide hydrolase was synthesized by translation systems programmed with poly(A)+RNA isolated from phenobarbital-treated rats than by translation systems programmed with poly(A)+RNA isolated from untreated rats (Table 1). These data indicate that the level of functional mRNA for epoxide hydrolase is increased after chronic phenobarbital administration to rats. In addition, the induction of epoxide hydrolase mRNA after chronic phenobarbital administration found in this study corresponds exactly with the increase observed after acute administration of the drug (17).

In order to correlate the level of translationally active mRNA with the induction of catalytic activity of epoxide hydrolase, we determined the activity of the enzyme in microsomes with styrene 7,8-oxide as substrate. The activity was linear over a wide range of protein concentrations (Fig. 5) and was approximately 5 times higher in microsomes isolated from phenobarbital-treated (8 days) rats compared to microsomes isolated from untreated rats (Table 1). Therefore, the increase in catalytic activity of epoxide hydrolase in isolated microsomes is in good agreement with the induction we have observed in the functional mRNA level for the enzyme in the liver.
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**DISCUSSION**

In this investigation we found that chronic phenobarbital administration results in an increase of translationally active rat liver mRNA for epoxide hydrolase. Furthermore, the relative increase in the level of mRNA, approximately 3-fold, correlates fairly well with the induction of catalytic activity, approximately 5-fold, in microsomes isolated from phenobarbital-treated rats. Although we have suggested that the increase in epoxide hydrolase mRNA leads to an increased level of the enzyme, we have not directly quantitated the amount of epoxide hydrolase in microsomes isolated from untreated (○) and phenobarbital-treated (●) rats. The catalytic activity was determined with styrene 7,8-oxide as substrate and is expressed as nmol of styrene glycol formed per minute. An average value for all the protein concentrations is shown in Table 1.

![Graph showing catalytic activity of epoxide hydrolase](image)

**Table 1.** Induction of rat liver epoxide hydrolase mRNA by phenobarbital

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cpm in total protein $^*$</th>
<th>cpm in immunoprecipitated epoxide hydrolase, × 10$^{-4}$</th>
<th>Catalytic activity $^+$</th>
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<tr>
<td>None</td>
<td>12.2</td>
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<tr>
<td>Phenobarbital</td>
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<tr>
<td>None</td>
<td>11.4</td>
<td>0.37</td>
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</tr>
<tr>
<td>Phenobarbital</td>
<td>11.4</td>
<td>1.22</td>
<td></td>
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</tbody>
</table>

$^*$ Values are corrected for endogenous protein synthesis in the rabbit reticulocyte lysate in the absence of exogenous mRNA.

$^+$ Catalytic activity is defined as nmol of styrene glycol formed per min per mg of protein.
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a translatable form, or just simply increased message stability. The third possibility has been proposed as a general mechanism accounting for the increases in mRNA levels induced by phenobarbital (41, 42). However, if this were the case, the mechanism by which the rate of mRNA degradation is decreased must be selective because only the level of a few mRNAs are affected. Furthermore, Gonzalez and Kasper (17) have found that cor- dycepin, an RNA synthesis chain terminator, can completely inhibit the increase in epoxide hydrolase mRNA after an acute administration of phenobarbital. Therefore, the inhibition of epoxide hydrolase mRNA by cor-dycepin and the rapid increase these authors found in functional epoxide hydrolase mRNA sug- gest that the increase might be due in part to increased transcrip- tion. However, we would like to stress that the mechanism (increased transcription, message stability, or precursor process- ing) responsible for the increase in epoxide hydrolase mRNA may be totally different when animals are treated chronically with phenobarbital as opposed to an acute administration.

The use of a translational assay for quantitating mRNA levels is also subject to certain reservations. For example, different mRNA species may be translated with different efficiencies in the in vitro system. As a result, the absolute percentage of epoxide hydrolase synthesized relative to the total protein might not accurately reflect the percentage of mRNA for that specific protein in the total RNA preparation. However, we do believe that the relative differences in mRNA levels found for epoxide hydrolase in untreated versus phenobarbital-treated rats is valid. The concentration of mRNA for epoxide hydrolase in the total RNA preparation could be determined more accurately if a specific probe for epoxide hydrolase sequences were available.

The development of a specific probe for epoxide hydrolase will allow us not only to examine the molecular basis of induc- tion of the enzyme by various xenobiotics but also to understand more fully the expression of the epoxide hydrolase gene during development. In addition, because epoxide hydrolase activity is markedly increased in hyperplastic nodules and in hepatomas by hepatocarcinogens (43, 44), a thorough understanding of the organization and expression of the epoxide hydrolase gene will also provide a greater insight into the molecular mechanisms underlying liver tumorogenesis.

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