Oxidative Metabolism of Cyclophosphamide: Identification of the Hepatic Monoxygenase Catalysts of Drug Activation

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ABSTRACT

Cytochrome P-450-catalyzed activation of cyclophosphamide to alkylating metabolites was studied in isolated rat liver microsomes and purified, reconstituted P-450 enzyme systems in order to identify the major enzymatic catalysts of drug activation in both uninduced and drug-induced liver tissue. P-450 form PB-4 (P-450 gene IIB1) activated cyclophosphamide with high efficiency ($V_{\text{max}} \text{(app)} = 18.2 \text{ nmol metabolite/min/mmol}$ P-450; $K_{\text{m}} \text{(app)} = 0.16 \text{ mmol}$ via the formation of 4-hydroxycyclophosphamide, which was quantitatively trapped as a bisulfite adduct then characterized following its conversion to cyano derivatives. Antibodies to P-450 PB-4 inhibited cyclophosphamide activation catalyzed by phenobarbital-induced adult male rat liver microsomes (specific activity, 5.4 nmol metabolite/min/mg liver microsomes) in a selective and near quantitative (>80%) fashion; little or no inhibition was obtained using antibodies inhibitory towards six other rat hepatic P-450 forms. Cyclophosphamide activation catalyzed by uninduced adult male rat liver microsomes (specific activity, 0.68 nmol/min/mg), although not inhibited by anti-P-450 PB-4 antibodies, was partially inhibited (~60%) by antibodies to P-450 PB-1 (gene IIC6) and more completely inhibited (>95%) by antibodies reactive with both P-450 PB-1 and P-450 2c (gene IIC11). Consistent with these observations, P-450 PB-1 and P-450 2c both activated cyclophosphamide at moderate rates in reconstituted systems (turnover, 1.6-2.7 nmol metabolite/min/mmol P-450), while seven other pure hepatic P-450 forms exhibited significantly lower activities (turnover <0.5 nmol metabolite/min/mmol P-450). Further studies revealed that the changes in liver microsomal cyclophosphamide activation rates with age and sex and in response to in vivo administration of cisplatin primarily reflect changes in the levels of P-450 forms PB-1 and 2c. These studies establish that P-450 forms PB-1, 2c, and PB-4 are the major catalysts of cyclophosphamide activation in rat hepatic tissue and that the modulation of microsomal cyclophosphamide activation with development and in response to drug exposure largely reflects alterations in the levels of these three hepatic P-450 enzymes.

INTRODUCTION

The phase-nonspecific alkylating agent CPA has a high therapeutic index and a broad spectrum of activity against a variety of human tumors. This drug also has significant immunosuppressive activity and is used clinically in the treatment of autoimmune diseases and for renal and bone marrow transplantations (1). Cyclophosphamide itself is devoid of alkylating activity and must first undergo bioactivation by hepatic cytochrome P-450. Initially, CPA is hydroxylated by P-450 at C-4. The 4-hydroxy-CPA thus formed exists in equilibrium with the ring-opened aldosphamide, which spontaneously eliminates acrolein to form phosphoramid mustard (Fig. 1). Phosphoramid mustard possesses significant DNA-alkylating activity, and appears to be the therapeutically significant metabolite (2). Acrolein, on the other hand, covalently binds to and thereby inactivates several hepatic enzymes, including P-450 (3).

Although the metabolism of CPA has been studied extensively in vivo and in vitro, the specific P-450 enzymes contributing to its bioactivation have not been identified. Cyclophosphamide activation is inducible by phenobarbital, both in rats and in humans (4, 5), suggesting that the major CPA 4-hydroxylase is a phenobarbital-inducible P-450 enzyme. Consistent with this finding, Mariniello et al. (6) observed that the major phenobarbital-inducible P-450 enzyme of rat liver metabolizes CPA efficiently in reconstituted systems in a manner consistent with the 4-hydroxylation pathway. However, product analyses carried out by these same investigators indicated that the major reaction catalyzed by that P-450 results in hydrolysis of the chlorine substituents of CPA (Fig. 1), with only minimal hydroxylation at the C-4 position of CPA observed (7). The present studies were therefore carried out to clarify the role of the major phenobarbital-inducible rat hepatic P-450 (designated P-450 PB-4) in CPA activation. The results obtained establish that P-450 form PB-4 (P-450 gene IIB1) is the major catalyst of CPA activation in phenobarbital-induced rat liver microsomes and that it does catalyze CPA 4-hydroxylation with high efficiency. In uninduced liver microsomes, however, the P-450 enzymes designated PB-1 (gene IIC6) and 2c (gene IIC11) are shown to be the predominant catalysts of CPA activation.

MATERIALS AND METHODS

Materials. [4-14C]Cyclophosphamide was purchased from New England Nuclear, and [chloroethyl-1H] CPA and [4-14C]endrost-4-ene-3,17-dione were from Amersham. Unlabeled CPA, calf thymus DNA, BSA, dexamethasone, isoniazid, and NADPH were from Sigma. 4-HydroperoxycPA was kindly provided by Dr. M. Colvin, Johns Hopkins Oncology Center.

Liver Microsomes. Adult male and female Fischer 344 rats (Taconic Farms, Germantown, NY) were untreated or were treated with phenobarbital, β-naphthoflavone, isoflurane, or clofibrate as described previously (8). Dexamethasone induction was achieved by 4 daily injections of the drug in corn oil at 100 mg/kg i.p. with the animals sacrificed on day 5. Isoniazid was administered at 0.1% (w/v) via drinking water for 10 days prior to sacrifice. Cisplatin (6 mg/kg) was administered by injection (i.v.) with animals sacrificed on day 0 (control group), day 7, or day 14 in experiments carried out by Dr. G. A. LeBlanc of this laboratory. In the experiment shown in Fig. 6, uninduced male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) ranging from 2 to 14 weeks of age were used. Liver microsomes were prepared as described elsewhere (9). Protein concentrations were determined with the Bio-Rad protein assay reagent using BSA as standard.

P-450 Enzymes. Hepatic P-450 enzymes were purified to apparent homogeneity from Sprague-Dawley rats, as summarized in earlier publications (Ref. 10, and references therein). P-450 form g (11) was kindly provided by Dr. J. Goldstein, National Institute of Environmental Health Sciences, and P-450 form RLM2 (12) by Dr. F. Gonzalez, National Cancer Institute. Designations given to the principal rat hepatic P-450 enzymes described in this study can be related to the formal names of the respective P450 enzymes as follows: P-450 PB-1 (IIC6, P450g); P-450 2c (IIC11, P450c); P-450 PB-4 (IIB1, P450a).

Received 6/8/88; revised 10/27/88, 1/27/89; accepted 2/2/89.

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1 Supported in part by Grant BC-462 from the American Cancer Society.

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3 The abbreviations used are: CPA, cyclophosphamide; P-450, cytochrome P-450, BSA, bovine serum albumin; PB, phenobarbital; MAb, monoclonal antibody. The terms P-450 form g and P-450 enzyme are used interchangeably.

4 A summary of alternative designations given by other investigators to the individual rat hepatic P-450 enzymes described in this study is provided under "Materials and Methods."
standardized gene designations (13) and the P-450 protein nomenclatures used by other investigators, as follows: P-450 PB-4 (gene IIB1) = b or PB-B; P-450 PB-5 (gene IIB2) = e or PB-D; P-450 PB-1 (gene IIC6) = k or PB-C; P-450 2c (gene IIC11) = h, UT-A, RLM5, male, or 16a. For a more detailed listing of these and other rat hepatic P-450 designations, see our Table 3, and Table 1 of Ref. 14.

**BSA-binding Assay for Cyclophosphamide Activation.** Activation of $[^{14}C]CPA$ was measured by quantitation of the binding of $[^{14}C]$acrolein to BSA using a modification of a previously described method for assaying the binding of $[^{14}C]$acrolein to microsomal protein (6). Standard microsomal incubation samples contained 0.1 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 0.5 mM $[^{14}C]CPA$ (0.5 mCi/mmol), 60 µg hepatic microsomal protein, and 3 mg BSA in a volume of 0.4 ml. Assays were carried out with liver microsomes isolated from uninduced or phenobarbital-induced adult male rats unless indicated otherwise.

Samples were preincubated at 37°C for 4 min prior to initiation of the reaction by addition of the NADPH (1 mM). The reaction was terminated after 45–60 min by addition of ice-cold 5% trichloroacetic acid (3 ml). After 10 min on ice, tubes were centrifuged, the supernatant was discarded, and the protein pellets were washed twice with 5% perchloric acid (1 ml), followed by ethanol (1 ml) and then ether (1 ml). Excess ether was allowed to evaporate and the pellets were digested with 0.3 M NaOH (0.5 ml) for 10–20 min, with sonication. Water (0.5 ml) was added to the digested pellets prior to scintillation counting.

Purified P-450 enzymes (10 pmol) were reconstituted with purified (15) rat liver NADPH P-450 reductase (0.7 unit) [1 unit = 1 µmol cytochrome c reduced/min at pH 7.7, 30°C] and dilauroylphosphatidylcholine (2 µg) (total volume, 20–30 µl) for 15 min at 20–22°C before addition to the standard assay mixture (in place of liver microsomes). Assays were performed in 0.5 mM potassium phosphate (pH 7.4) 0.1 mM EDTA for 45 min at 37°C.

**DNA-binding Assay.** Microsomal activation of $[^{3}H]CPA$ was monitored by a modification of a previously described assay for $[^{3}H]$phosphoramide mustard binding to DNA (6). Standard incubation samples were similar to those used for the BSA-binding assay except that $[^{3}H]CPA$ (0.5 mM, 1.2 mCi/mmml) was used, samples contained 100 µg hepatic microsomal protein and calf thymus DNA (1 mg) was included in place of BSA. Incubations were carried out as described for the BSA-binding assay except that the reaction was terminated by extraction with chloroform:isoamyl alcohol (95:5) (0.4 ml). The organic phase was back-extracted with 0.1 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA (2 × 0.2 ml) and the DNA was then precipitated from the combined aqueous phases by addition of ethanol (1.7 ml) and 5 mM NaCl (15 µl), followed by storage at −20°C for at least 1 h. The DNA precipitate was washed with 70% aqueous ethanol containing 30 mM NaCl and then dissolved in 10 mM Tris-Cl, pH 8.0, containing 1 mM EDTA (1 ml) prior to scintillation counting.

**Binding of $[^{3}H]$ to BSA and binding of $[^{3}C]$ to DNA under conditions of chloroethyl-$[^{3}C]CPA$ or 4-[14C]CPA metabolism was demonstrated to be negligible (data not shown), indicating that the assays described above are specific for acrolein (BSA-binding assay) and phosphoramide mustard (DNA-binding assay), respectively, and that significant covalent binding of the primary metabolites 4-hydroxy-CPA and aldo phosphamide does not occur in either case.

Isolation of Aldophosphamide. Incubation samples were prepared as for the BSA-binding assay except that BSA was omitted and samples contained 5 mM NaHSO₃ to trap aldo phosphamide as a bisulfite addition compound (Fig. 3, below). Samples were incubated for 45 min at 37°C and then extracted with chloroform:isoamyl alcohol (95:5) (3 × 1 ml) followed by ethyl acetate (2 × 1 ml). Potassium cyanide was added to the aqueous residue (0.2 M final concentration), which was extracted for 15 min at 22°C and then extracted with ethyl acetate (3 × 1 ml). Extracts were evaporated under N₂ and then spotted on aluminum-backed silica gel thin layer chromatography plates which were eluted by two developments in acetonitrile:formamide (3:1). Resolved metabolites were visualized by autoradiography or using a 4-(p-nitrobenzyl)pyridine spray reagent (16) and identified by comparison to a mixture of unlabeled aldophosphamide cyanohydrin and cis- and trans-4-cyano-CPA, prepared by reaction of 4-hydroxy-CPA with 10 mM KCN as described by Fenselau et al. (17).

**Antibody Inhibition Experiments.** Inhibition of CPA activation by monoclonal and polyclonal antibodies was determined by the BSA-binding assay. Complete reaction mixtures (minus NADPH) were preincubated with antibody for 30 min at 22°C prior to the initiation of CPA metabolism by addition of NADPH. Mouse monoclonal antibodies raised against individual rat hepatic P-450 enzymes were prepared and characterized with respect to their P-450 enzyme specificities as described previously (18) and were kindly provided by Drs. S. Park and H. Gelboin, National Cancer Institute. MAb B4 (clone 4-29-5) is reactive with the closely related P-450 forms PB-4 and PB-5, and MAB D1 (clone 1-68-11) is reactive with P-450 forms PB-1 and 2c. Specificities of the other antibodies used as controls (e.g., Fig. 5, below) are described in Ref. 18. Antibodies were purified from mouse ascites fluid by the caprylic acid/ammonium sulfate precipitation method of Mc Kinney and Parkinson (19).

**Androstenedione Hydroxylation.** Microsomal androstenedione hydroxylation was determined using 50 µM $[^{4}H]_{2}$, androstenedione, 60 µg microsomal protein, and 1 mM NADPH in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4)-0.1 mM EDTA (0.4 ml). Hydroxylated metabolites were extracted, separated by thin layer chromatography, and quantitated by scintillation counting as described previously (20).

**Immuunoquantitation of Microsomal P-450 PB-1 and P-450 2c.** Hepatic microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blotting analysis using immunoadsorbed (21) rabbit anti-P-450 antibodies. Individual P-450 forms were visualized by incubation with rabbit anti-P-450 primary antibody and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Protein bands were quantitated by scanning photographic negatives of the blots with a laser densitometer as detailed elsewhere (22).

**RESULTS**

**CPA Activation Catalyzed by Purified P-450 Form PB-4.** The metabolism of $[^{14}C]CPA$ catalyzed by purified P-450 form PB-4 was investigated in a reconstituted enzyme system using a BSA-binding assay which quantitates $[^{14}C]$acrolein, formed by decomposition of the primary metabolite, 4-hydroxy-CPA (Fig. 1). Initial experiments with purified, reconstituted P-450 PB-4 revealed an unusual time dependence for enzyme activity, with...
maximal rates of CPA metabolism not observed unless samples were incubated for at least 30 min (Fig. 2). This apparent acceleration of the reaction with time may reflect an accumulation of the initial hydroxylation product (4-hydroxy-CPA or the ring-opened aldophosphamide) due to its slow or incomplete decomposition to acrolein. This acceleration was most striking in samples incubated for shorter reaction times and at low phosphate buffer concentrations, conditions that would favor a slower decomposition of the initial hydroxylated metabolites (cf. Ref. 23). In practice, therefore, enzymatic incubations were carried out for 45 min and in buffer containing at least 0.1 M potassium phosphate to minimize the influence of 4-hydroxy-CPA decomposition rates on measured enzyme activities.

Apparent kinetic parameters for CPA metabolism catalyzed by purified, reconstituted P-450 PB-4 were found to be: V\text{max} = 18.2 ± 2.9 nmol metabolite bound to BSA/min/nmol P-450 and K\text{m} = 162 ± 31 μM. This apparent V\text{max} is 3.8-fold higher and the apparent K\text{m} is 2.7-fold lower than the values determined previously for a similar P-450 enzyme preparation using an assay that measures the conversion of CPA to polar metabolites (6). The formation of 4-hydroxy-CPA as an intermediate in the P-450-catalyzed activation of CPA to acrolein was confirmed by trapping the metabolite aldophosphamide with sodium bisulfite, followed by conversion of the bisulfite adduct to the ring-opened aldophosphamide cyanohydrin and the diastereomeric cis- and trans-4-cyano-CPA (see Fig. 3), which were extracted and chromatographed on TLC (see Table 1 and “Materials and Methods”).

Role of P-450 PB-4 in Microsomal CPA Activation. Experiments were carried out to ascertain whether P-450 PB-4 makes a significant contribution to microsomal CPA activation. CPA activation was quantitated in liver microsomes containing various levels of P-450 PB-4, i.e., microsomes prepared from untreated adult male and adult female rats and from rats pretreated with various P-450 inducers. CPA activation was assayed in these microsomes by the BSA-binding assay and also by a complementary assay which measures activation of [chloroethyl-3H]CPA to [3H]phosphoramid mustard by alkylation of calf thymus DNA (Table 2). Using both of these methods, CPA activation was determined to be markedly elevated (up to 10-fold increase) in phenobarbital-induced microsomes, which contain elevated levels of P-450 PB-4 (e.g., Ref. 21). Isoaasfole induced CPA activation to a lesser extent (~2-fold elevation of enzyme activity) and, correspondingly, P-450 PB-4 levels are significantly lower following induction with this chemical compared to phenobarbital (21). CPA activation was also induced in response to pretreatment with clofibrate and to a lesser extent with dexamethasone (4- and 1.6-fold elevation, respectively) (Fig. 4A); these drugs also induced P-450 PB-4 severalfold (data not shown). In contrast, liver microsomes isolated from

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Fig. 2. Turnover number for P-450 PB-4-catalyzed CPA activation. Time dependence and effect of phosphate concentration. Turnover numbers for CPA activation catalyzed by purified, reconstituted P-450 PB-4 were determined as a function of time at each of 3 phosphate buffer concentrations using the BSA-binding assay. Data points represent the mean of multiple determinations (n = 5 for 0.5 M KPi potassium phosphate (squares), n = 6 for 0.1 M potassium phosphate (circles), and n = 2 for 0.03 M potassium phosphate (triangles)).

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Fig. 3. Formation of aldophosphamide cyanohydrin and cis- and trans-4-cyano-CPA following bisulfite trapping of aldophosphamide. Aldophosphamide was trapped in high yield as a water-soluble bisulfite addition compound and then converted to the indicated mixture of organic soluble cyanide derivatives as detailed in Table 1.
rats induced with β-naphthoflavone (inducer of P-450 forms c and d) and isoniazid (inducer of P-450) activated CPA at rates similar to those catalyzed by uninduced liver microsomes. The observed profile for microsomal CPA activation was highly similar to the profile for P-450 PB-4-dependent (18, 21) microsomal androstenedione 16β-hydroxylation (Fig. 4B), strongly suggesting that the induction of CPA metabolism following administration of phenobarbital, isosafrole, and clofibrate may be a direct consequence of the elevation of microsomal P-450 PB-4 levels by these agents.

Antibody raised to purified P-450 PB-4 and inhibitory to its catalytic activities (MAb B4; see “Materials and Methods”) was then used to probe directly for the participation of P-450 PB-4 in CPA metabolism catalyzed by these rat liver microsomes. About 80% of the CPA activation activity of these microsomes was specifically inhibited by this antibody (PB + MAb B4; Fig. 5A), with little or no inhibition obtained using a nonspecific antibody (MAb Ly) or using antibodies reactive with several other P-450 forms (P-450 forms PB-1, 2c, PB-2a, c, d, and j; data not shown).4 Under these same incubation conditions, however, less than 20% of CPA activation catalyzed by liver microsomes prepared from uninduced (--) adult male rats were incubated with varying amounts of purified monoclonal antibodies reactive with P-450 PB-4 (MAb B4), lysozyme (MAb Ly; used as a control for the effects of nonspecific antibody) (4) or P-450s PB-1 and 2c (MAb D1), P-450s c and d (MAb A1), P-450 J, or P-450 PB-2a (C2) (8) for 30 min at 22°C as described under “Materials and Methods.” Residual CPA activity was then measured in comparison to samples incubated without antibody using the BSA-binding assay. Uninhibited activities corresponded to 0.49 and 3.77 nmol metabolite/min/mg for the experiments shown with uninduced (UT) and phenobarbital-induced liver microsomes, respectively.

Table 2. Microsomal activation of CPA to acrolein and to phosphoramide mustard: effects of sex and monooxygenase induction

<table>
<thead>
<tr>
<th>Liver microsomes</th>
<th>14C bound to BSA</th>
<th>Induction</th>
<th>3H bound to DNA</th>
<th>Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT male</td>
<td>0.68 ± 0.12</td>
<td>=1.0</td>
<td>0.043 ± 0.012</td>
<td>=1.0</td>
</tr>
<tr>
<td>PB male</td>
<td>5.37 ± 0.07</td>
<td>7.9</td>
<td>0.337 ± 0.015</td>
<td>7.8</td>
</tr>
<tr>
<td>ISF male</td>
<td>1.50 ± 0.23</td>
<td>2.2</td>
<td>0.083 ± 0.005</td>
<td>1.9</td>
</tr>
<tr>
<td>UT female</td>
<td>0.33 ± 0.08</td>
<td>=1.0</td>
<td>0.039 ± 0.006</td>
<td>=1.0</td>
</tr>
<tr>
<td>PB female</td>
<td>3.58 ± 0.57</td>
<td>10.8</td>
<td>0.196 ± 0.037</td>
<td>5.0</td>
</tr>
<tr>
<td>ISF female</td>
<td>0.73 ± 0.22</td>
<td>2.2</td>
<td>0.069 ± 0.015</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Activities were determined under standard conditions with the BSA-binding assay (using [14C]CPA as substrate) or with the DNA-binding assay (using [3H] CPA as substrate) (see “Materials and Methods”). Higher activities obtained with the BSA-binding assay reflect low efficiency of phosphoramide binding to DNA under the conditions used. Activities are given as mean ± SE for n = 3 or 4 individual rats in each treatment group.

* Microsomes were isolated from adult male or adult female rats which were untreated (UT) or induced with phenobarbital (PB) or isosafrole (ISF).

* Fold increase by PB or ISF compared to the corresponding untreated group.

Fig. 4. Influence of monooxygenase inducers on microsomal CPA activation. Liver microsomes isolated from adult male rats that were either untreated (UT) or induced with PB, β-naphthoflavone (BNF), isoniazid (ISN), dexamethasone (DEX), clofibrate (CLF), or isosafrole (ISF) were assayed for their ability to activate [4-14C]CPA to acrolein (A), using the BSA-binding assay as described under “Materials and Methods.” Parallel assays for microsomal androstenedione 16β-hydroxylation activity (B) were also performed. Data are presented as mean ± SE (bars) for n = 3-4 rats/group.

Fig. 5. Selective inhibition of microsomal CPA activation by P-450 forms-specific antibodies. Liver microsomes (40 μg) prepared from uninduced (--) or phenobarbital-induced (-----) adult male rats were incubated with varying amounts of purified monoclonal antibodies reactive with P-450 PB-4 (MAb B4), lysozyme (MAb Ly; used as a control for the effects of nonspecific antibody) (A) or P-450s PB-1 and 2c (MAb D1), P-450s c and d (MAb A1), P-450 J, or P-450 PB-2a (C2) (8) for 30 min at 22°C as described under “Materials and Methods.” Residual CPA activity was then measured in comparison to samples incubated without antibody using the BSA-binding assay. Uninhibited activities corresponded to 0.49 and 3.77 nmol metabolite/min/mg for the experiments shown with uninduced (UT) and phenobarbital-induced liver microsomes, respectively.
least 10-fold more active than the other P-450 enzymes studied, the P-450 forms designated 2c, PB-1, RLM2, and PB-5 all showed significant levels of CPA metabolism, while 6 other P-450s catalyzed very low levels of CPA activation or were inactive (=0.1-0.2 nmol/min/nmol P-450). P-450 PB-5 is unlikely to contribute significantly to CPA activation in uninduced liver microsomes since the anti-PB-4 antibody used (MAb B4) is highly cross-reactive with PB-5 (18) yet did not inhibit CPA metabolism in these microsomes (Fig. 5A). Similarly, polyclonal antibodies inhibitory toward P-450 RLM2 and P-450 3 (data not shown) as well as monoclonal antibodies reactive with several other P-450 enzymes (Fig. 5B) were noninhibitory toward CPA activation catalyzed by uninduced liver microsomes. In contrast, a monoclonal antibody reactive with PB-1 and 2c (MAb D1) (and perhaps other members of the P-450 family IIC) inhibited this microsomal activity by >95% (Fig. 5B), suggesting that one or more of these P-450s mediates this reaction in uninduced liver microsomes. Although the higher turnover exhibited by P-450 2c as compared to P-450 PB-1 (Table 3) might suggest that P-450 2c makes the more important catalytic contribution in the uninduced liver microsomes, the activity of purified P-450 PB-1 can be markedly stimulated by cytochrome b5 (24). Indeed, CPA activation catalyzed by P-450 PB-1 was enhanced 4- to 6-fold when cytochrome b5 was included in the reconstituted system (turnover, 1.57 nmol metabolite/min/nmol P-450 in the presence of cytochrome b5) while P-450 2c and P-450 PB-4 showed much smaller increases (20-50% elevation). Thus, P-450 forms PB-1 and 2c both catalyze CPA activation in purified, reconstituted systems. Both these P-450s are present at significant levels in adult male rat liver (21), and they therefore are catalytically competent to contribute to cyclophosphamide activation in uninduced liver microsomes.

Contributions of P-450 Forms PB-1 and 2c to CPA Activation in Uninduced Liver Microsomes. Antibody inhibition experiments carried out using polyclonal anti-P-450 PB-1 antibodies that exhibit low cross-inhibitory activity toward microsomal P-450 2c (10) indicated that ~60% of CPA activation catalyzed by uninduced adult male microsomes is mediated by P-450 PB-1 (data not shown). However, P-450 forms PB-1 and 2c exhibit a high degree of immunochemical cross-reactivity (10, 25) and it is therefore difficult to identify unambiguously the individual contributions of these two P-450s to microsomal CPA activation using this approach alone. Further evidence for the contribution of P-450 PB-1, as well as that of P-450 2c, to microsomal CPA activation was obtained in experiments based on (a) the distinctive sex-dependence and hormonal regulation of these two P-450s (10, 21) and (b) their differential response to in vivo administration of the anticancer drug cisplatin (26).

P-450 2c is male specific and developmentally induced at puberty (10) while P-450 PB-1 is expressed at similar levels in adult rats of both sexes (21). Examination of the age- and sex-dependent expression of microsomal CPA activation activity in liver microsomes isolated from male and female rats ages 2-14 weeks revealed similar microsomal CPA activation rates in both sexes through development, with a significant increase in activity seen between 2 and 4 weeks (Fig. 6). In addition, CPA activation catalyzed by each of these microsomal preparations was at least 85% inhibited by MAb D1, which is reactive with both P-450 PB-1 and P-450 2c. This age and sex dependence of microsomal CPA activation is characteristic of P-450 PB-1 (which is markedly elevated from 2 to 4 weeks of age), but not P-450 2c, since the latter enzyme is undetectable in females and is detectable in males only after its induction at puberty (5 to 6 weeks of age) (10).

Although these findings confirm that P-450 PB-1 is a major catalyst of liver microsomal CPA activation in immature and adult female rats as well as in immature male rats, they leave unanswered the question whether in adult males P-450 2c also contributes to this activity. This question was addressed by monitoring the effects of in vivo pretreatment of adult male rats with cisplatin, which results in a marked suppression of P-450 2c and a moderate elevation of P-450 PB-1 (while having no appreciable effect on P-450 PB-4) in isolated liver microsomes (26). These analyses revealed that microsomal CPA activation is suppressed by 54% 7 days after cisplatin administration, even though microsomal P-450 PB-1 levels are largely unaffected (Fig. 7). Microsomal levels of P-450 2c were, however, suppressed by ~90%. These observations suggest that P-450 PB-1 and P-450 2c make similar contributions to CPA activation in

| Table 3 Cyclophosphamide activation catalyzed by purified P-450 enzymes* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| P-450 enzyme | P-450 gene | nmol metabolite/min/nmol P-450 |
| PB-4 | IIb1 | 26.1 ± 3.8 |
| 2c | Iic11 | 2.72 ± 0.38 |
| PB-5 | Iib2 | 0.61 ± 0.03 |
| RLM2 | Iia2 | 0.50 ± 0.22 |
| PB-1 | Iic6 | 0.26 ± 0.09 |
| c | Ia1 | 0.16 ± 0.09 |
| 2d | Iic12 | 0.14 |
| 5 | Iia3 | 0.11 |
| g | Iic13 | 0.04 |
| PB-2a | Iia1 | 0.02 |
| d | Ia2 | 0.02 |

* Activities determined with purified P-450 enzymes at 500 μM [14C]CPA, 0.5 mM potassium phosphate buffer (pH 7.4), and 45 min incubation at 37°C. P-450 gene nomenclature is based on Ref. 13.

The possible cross-reactivity of MAb D1 with P-450s f and g (and perhaps other unidentified members of the P-450 IIC subfamily that may be immunochemically related to P-450s PB-1 and 2c) is not known. P-450s 2c and PB-4 showed much smaller increases (20-50% elevation) than that exhibited by P-450 2c as compared to P-450 PB-1 (24). Indeed, CPA activation catalyzed by P-450 2c (10) indicated that ~60% of CPA activation catalyzed by uninduced adult male microsomes is mediated by P-450 PB-1 (data not shown). However, P-450 forms PB-1 and 2c exhibit a high degree of immunochemical cross-reactivity (10, 25) and it is therefore difficult to identify unambiguously the individual contributions of these two P-450s to microsomal CPA activation using this approach alone. Further evidence for the contribution of P-450 PB-1, as well as that of P-450 2c, to microsomal CPA activation was obtained in experiments based on (a) the distinctive sex-dependence and hormonal regulation of these two P-450s (10, 21) and (b) their differential response to in vivo administration of the anticancer drug cisplatin (26).

P-450 2c is male specific and developmentally induced at puberty (10) while P-450 PB-1 is expressed at similar levels in adult rats of both sexes (21). Examination of the age- and sex-dependent expression of microsomal CPA activation activity in liver microsomes isolated from male and female rats ages 2-14 weeks revealed similar microsomal CPA activation rates in both sexes through development, with a significant increase in activity seen between 2 and 4 weeks (Fig. 6). In addition, CPA activation catalyzed by each of these microsomal preparations was at least 85% inhibited by MAb D1, which is reactive with both P-450 PB-1 and P-450 2c. This age and sex dependence of microsomal CPA activation is characteristic of P-450 PB-1 (which is markedly elevated from 2 to 4 weeks of age), but not P-450 2c, since the latter enzyme is undetectable in females and is detectable in males only after its induction at puberty (5 to 6 weeks of age) (10).

Although these findings confirm that P-450 PB-1 is a major catalyst of liver microsomal CPA activation in immature and adult female rats as well as in immature male rats, they leave unanswered the question whether in adult males P-450 2c also contributes to this activity. This question was addressed by monitoring the effects of in vivo pretreatment of adult male rats with cisplatin, which results in a marked suppression of P-450 2c and a moderate elevation of P-450 PB-1 (while having no appreciable effect on P-450 PB-4) in isolated liver microsomes (26). These analyses revealed that microsomal CPA activation is suppressed by 54% 7 days after cisplatin administration, even though microsomal P-450 PB-1 levels are largely unaffected (Fig. 7). Microsomal levels of P-450 2c were, however, suppressed by ~90%. These observations suggest that P-450 PB-1 and P-450 2c make similar contributions to CPA activation in
BIOACTIVATION OF CYCLOPHOSPHAMIDE BY HEPATIC P-450

Fig. 7. Effects of in vivo cisplatin treatment on rat liver microsomal CPA activation and P-450 2c and P-450 PB-1 protein levels. Liver microsomes (80 µg) isolated from rats sacrificed 0, 7, or 14 days after a single i.v. injection of cisplatin (6 mg/kg) (26) were assayed for CPA activation using the BSA-binding assay. P-450 2c and P-450 PB-1 levels in these same microsomes were immunoquantitated by Western blotting. Data are presented as mean ± SD (bars) for n = 4 individual rats at each time point and are expressed relative to the untreated control group.

DISCUSSION

The studies described in this report establish that CPA activation is initiated by a cytochrome P-450-dependent 4-hydroxyl reaction and furthermore provide a detailed identification of the specific P-450 enzymes that contribute to CPA activation in liver microsomal systems. P-450 form PB-4 was shown to be the major catalyst of CPA activation in phenobarbital-induced rat liver. CPA activation catalyzed by this enzyme was shown to proceed via the 4-hydroxylation pathway, as evidenced by the trapping in high yield of 4-hydroxy-CPA as its cyanol cyanohydrin derivatives (Fig. 3), and confirmed by the high turnover number of P-450 PB-4 for formation of the 4-hydroxy-CPA decomposition product acrolein (Fig. 1). In contrast, previous studies using a similar P-450 enzyme preparation had identified the hydrolysis product didechlorodiiodo-CPA (Fig. 1) as the principal metabolite of CPA and detected only small amounts of 4-hydroxy-CPA (7). Duplication of the reaction conditions described in that study (7) consistently failed to yield didechlorodiiodo-CPA in our hands. The reasons for this discrepancy are unclear. It is possible that under the conditions of extensive metabolism used in the earlier study (65–80% substrate conversion) there was significant buildup of hydrogen peroxide and/or hydroxyl radicals, leading to formation of didechlorodiiodo-CPA by a chemical rather than an enzymatic pathway. Alternatively, a P-450 form distinct from P-450 PB-4 and present in the enzyme preparation used in the previous study (7) may have been responsible for the observed formation of didechlorodiiodo-CPA.

CPA activation via 4-hydroxylation was determined in this study using a BSA-binding assay that was based on a previously described microsomal protein-binding assay (6). Use of BSA to bind [%]C acrolein generated by decomposition of 4-hydroxy-[%]C CPA allowed detection of CPA activation with high sensitivity. Typical yields of BSA-bound metabolites were increased ∼50-fold over values obtained by binding to microsomal protein (6). BSA has been shown to facilitate decomposition of 4-hydroxyl-CPA (27), and this may contribute to the higher yields that were obtained with this assay as compared to our DNA binding assay, which measures the parallel decomposition product, [%]H phosphoramidate mustard. In all cases assay samples had to be incubated for at least 45 min in order to obtain maximal rates of metabolism, suggesting a slow equilibration of 4-hydroxyl-CPA with aldophosphamide or perhaps slow decomposition of the latter compound to acrolein under our assay conditions (cf. Ref. 28).

Although P-450 PB-4 was found to be the major catalyst of CPA activation in phenobarbital-induced liver microsomes, our experiments showed that this enzyme is not a significant contributor to drug activation in uninduced liver samples. Rather, two proteins of P-450 gene subfamily IIC (P-450 PB-1 and P-450 2c) were found to make important contributions to CPA activation in the uninduced microsomes. This was demonstrated (a) by reconstitution experiments using the purified P-450 enzymes, (b) by antibody inhibition experiments, and (c) by examination of the age and sex dependence of microsomal CPA activation and the perturbation of microsomal CPA activation and P-450 expression in response to in vivo administration of cisplatin. This conclusion is also consistent with a previous demonstration that multiple P-450 forms present in phenobarbital-induced microsomes can metabolize CPA in reconstituted systems (29). In contrast, several P-450 enzymes which effectively hydroxylate other drugs and xenobiotics, including polycyclic hydrocarbon-inducible P-450s (gene subfamily IA) and P-450 forms induced by synthetic steroids and macrolide antibiotics (gene subfamily IIIA), did not catalyze CPA 4-hydroxylation at significant rates. Although the three CPA hydroxylases identified in the present study (P-450s PB-4, PB-1 and 2c) are most prominent in hepatic tissue, at least one of these enzymes (P-450 PB-4) is constitutively expressed in lung and other organs, where it is also inducible by phenobarbital (30). Interestingly, the expression and phenobarbital inducibility of P-450 PB-4 has also been observed in carcinogen-induced liver nodules as well as in neoplastic lesions in kidney (31, 32), indicating that CPA may indeed be subject to activation within target tissues. Although human P-450 enzymes that exhibit significant structural similarity to rat P-450 enzymes PB-1 and 2c have been identified (33, 34), it remains to be established whether they contribute significantly to CPA activation in human tissues.

The anticancer drug cisplatin has been shown to markedly affect the expression of a number of hormonally-regulated P-450s in rat liver (26) including P-450 2c, an adult male-specific steroid hormone 16α-hydroxylase that is also active in xenobiotic metabolism. The present demonstration that P-450 2c makes a significant contribution to CPA activation in adult male rats accounts for the observed decrease in CPA hydroxylation activity in response to cisplatin administration. Although activation of CPA does not appear to be rate-limiting in either rodents or humans, as judged by the invariant concentration × time curve for the activated metabolites over a range of CPA activation rates (35), decreased effectiveness can occur when activation is compromised (4). A similar decrease in
effectiveness might exist when activation is compromised by the administration of cisplatin. Whether or not cisplatin similarly compromises CPA activation in humans when these drugs are administered in combination chemotherapy is unknown.

Finally, although enhanced activation of CPA does not appear to increase its therapeutic efficacy (4, 5, 35), activation of the isomeric drug ifosfamide does appear to be limiting, at least under high dose therapy protocols (36). Ifosfamide activation is also inducible by phenobarbital (37), but recent studies indicate that P-450 form PB-4 is not the major microsomal enzyme catalyzing that activation reaction. Further studies on the metabolism of this clinically useful agent are in progress.

REFERENCES


Oxidative Metabolism of Cyclophosphamide: Identification of the Hepatic Monooxygenase Catalysts of Drug Activation

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