ISOZYME-SELECTIVE METABOLISM OF ETHYL CARBAMATE BY CYTOCHROME P450 (CYP2E1) AND CARBOXYLESTERASE (HYDROLASE A) ENZYMES IN MURINE LIVER MICROSOMES

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ABSTRACT:

Cytochrome P450 and carboxylesterase enzymes have been implicated in the metabolism of the carcinogen ethyl carbamate (EC). In this study, we have used a murine liver microsomal system to investigate the relative contributions of P450 and carboxylesterase isozymes to hepatic metabolism of EC. N-Nitrosodimethylamine (NDMA) demethylation and p-nitrophenyl acetate (PNA) hydrolysis were used as catalytic markers of CYP2E1 and carboxylesterase enzymes, respectively. Incubation of liver microsomes with EC (1 mM) produced slight but significant decreases in NDMA demethylation and PNA hydrolysis activities. Incubation of microsomes with paraoxon (PAX), a general carboxylesterase inhibitor, or phenylmethylsulfonyl fluoride (PMSF), a specific inhibitor of hydrolase A, produced decreases of 85 and 45%, respectively, in carboxylesterase activities; neither of the inhibitors elicited alterations in levels of NDMA demethylation. Reaction of microsomes with either PAX or PMSF and then with EC exacerbated the reduction (285%) of NDMA demethylation, and this loss corresponded to decreases in immunodetectable CYP2E1 content. The reduction in PNA hydrolysis activity induced by PAX, PMSF, or EC correlated with decreased immunodetectable hydrolase A in liver microsomes; however, reaction with PAX and not PMSF or EC resulted in loss of immunoreactivity for hydrolase B. These data correlated with levels of covalent binding of [ethyl-14C]EC to liver microsomes, which were significantly elevated in incubations conducted with PAX or PMSF. Antibody inhibition of the CYP2E1 enzyme significantly reduced levels of binding to microsomal proteins, compared with control levels. These results are consistent with the premise that EC is metabolized by CYP2E1 and hydrolase A in liver microsomes of mice.

EC, commonly known as urethane, is a water- and lipid-soluble aliphatic ester that is well established as a carcinogen in experimental animals, inducing tumors in a variety of tissues, including liver, lung, mammary gland, and lymphoid tissue (Nettleship et al., 1943; Tannenbaum and Silverstone, 1958; Mirvish, 1968). The carcinogenic effect is not associated with the parent compound but is ascribed to metabolism of EC to reactive intermediates, of which VC epoxide has been proposed to be the carcinogenic metabolite (Dahl et al., 1978). Although the epoxide has not yet been directly detected, the pathways of EC metabolism have been identified. Cytochrome P450-dependent metabolism of EC is implicated in the bioactivation pathway; initial oxidation to VC is followed by an additional oxidative step to produce VC epoxide (Guengerich and Kim, 1991). Findings from experiments with human liver microsomes indicated that the oxidative metabolism of both EC and VC is catalyzed by the P450 isozyme CYP2E1 (Guengerich and Kim, 1991; Guengerich et al., 1991). 1,N6-Ethenoadenosine adducts were formed in incubations of human liver microsomes with EC and VC when incubations were conducted in the presence of adenosine and an NADPH-generating system (Guengerich et al., 1991). Adduct formation was inhibited in incubations performed with microsomes that had been previously incubated with the CYP2E1-selective inhibitor diethyldithiocarbamate. This inhibitory effect was also observed in incubations carried out with microsomes that had been preincubated with a CYP2E1-inhibitory antibody (Guengerich et al., 1991). A similar P450-mediated mechanism is also supported by findings from animal studies. Clearance of EC from blood, as reflected by levels of binding of radiolabeled metabolites to liver proteins, was increased in mice that had been previously treated with acetone and in mice that had been treated chronically with ethanol (Kurata et al., 1991a,b). Both of these solvents are potent inducers of CYP2E1, but they also induce enzymes of the CYP2B subfamily (Johansson et al., 1988). In other studies, elevation of covalent binding of EC metabolites to hepatic macromolecules was not detected in rodents treated with the CYP2E1 inducer pyridine, in conjunction with [ethyl-14C]EC (Page and Carlson, 1994; Carlson, 1994). The lack of exacerbation of EC activation under conditions of CYP2E1 induction raises a question regarding the contribution of this P450 enzyme to EC metabolism and the mechanisms involved.

In a parallel pathway believed to be implicated in detoxication, carboxylesterase enzymes have been shown to mediate the hydrolysis
of EC to ethanol, ammonia, and CO₂ (Skipper et al., 1951; Kaye, 1960; Mirvish and Kaye, 1964; Nomier et al., 1989). Treatment of mice with pyridine caused a 3-fold increase in CYP2E1-dependent NDMA demethylase activity, and this correlated with a 2-fold increase in metabolism of EC to CO₂, in liver microsomes (Page and Carlson, 1994). This increased metabolism of EC to CO₂ has been attributed to increased metabolism by CYP2E1. However, CO₂ is also produced when EC is metabolized by the carboxylesterases, suggesting that these enzymes may also be involved in the enhanced metabolic conversion. The dual roles of CYP2E1 and the carboxylesterases in in vivo metabolism are also supported by findings that show that levels of CO₂ are significantly inhibited in microsomal preparations incubated with either the CYP2E1 inhibitor diethyldithiocarbamate or the esterase inhibitor PAX (Page and Carlson, 1994). Hence, the relative contributions of the two enzyme systems to EC metabolism have not been clearly delineated.

In the present study, we have used a microsomal system to investigate the metabolism of EC by P450 and carboxylesterase enzymes in murine liver. We have used carboxylesterase inhibitors, protein immunoblotting, and measurements of enzyme catalytic activities to assess the relative contributions of CYP2E1 and carboxylesterase isozymes to EC metabolism. We have further determined levels of covalent binding of EC to liver microsomal proteins, to estimate the formation of metabolites that correspond to alterations in enzyme activities. Our results support the metabolism of EC by CYP2E1 and the carboxylesterase isozyme hydrolase A in liver microsomes from mice. Furthermore, metabolism via the CYP2E1 pathway is increased by inhibition of hydrolase A, suggesting an important contribution of this carboxylesterase isozyme to EC metabolism.

Materials and Methods

Treatment of Animals. Female CD-1 mice (body weight, 20–25 g) were obtained from Charles River Canada (St. Constant, Quebec, Canada). The animals were maintained on a 12-hr light/dark cycle and were given free access to food (Purina Rodent Chow) and drinking water. The animals were acclimated for a minimum of 7 days before being randomly assigned to an experimental group. Mice were killed by cervical dislocation, for preparation of microsomes.

Materials. Chemicals and reagents were purchased from suppliers as follows: Aldrich Chemical Co. (Montréal, Québec, Canada), EC, dimesone, and sodium acetate; New England Nuclear Co. (Boston, MA); [14C]formaldehyde (>95% radiochemical purity; specific activity, 10 μCi/mmol); Sigma Chemical Co. (St. Louis, MO), PAX, p-nitrophenol, PNA, 4-nitrocatechol, and PMSF; ICN Chemical Co. (Costa Mesa, CA). Eco-Lite scintillation fluid; Bio-Rad Laboratories (Hercules, CA), [14C]NDMA. Incubations were performed for 10 min at 37°C in a shaking water bath. The reaction was initiated by addition of 1 mM PAX in DMSO; this amount was previously shown to inhibit carboxylesterase-mediated hydrolysis of PNA (Morgan et al., 1994a; McCracken et al., 1993). Incubation was carried out for an additional 20 min at 25°C. The reactions were terminated by cooling of the samples on ice, after which the microsomes were washed and recovered. The microsomal pellet was resuspended in 1 ml of buffer, and incubations with EC and/or enzyme assays were performed. For preincubations with PMSF, a final concentration of 50 μM in DMSO was used; this amount was shown in previous studies to inhibit hydrolase A selectively (Morgan et al., 1994b).

Enzyme Assays. Microsomal carboxylesterase activity was determined by measuring the hydrolysis of PNA to p-nitrophenol, as described in previous studies (Morgan et al., 1994a,b). A volume of 1 ml of 100 mM K₂HPO₄ buffer, pH 7.0, containing 1.5 mM EDTA and 150 μg of microsomal protein was placed in a 1.5-ml sample cuvette, and the reaction was initiated by addition of 1 mM PNA in DMSO. The hydrolysis of PNA to p-nitrophenol was determined by comparison with a standard calibration curve relating absorbance levels to known amounts of p-nitrophenol.

NDMA demethylation was used for estimation of the catalytic activity of the CYP2E1 enzyme and was determined using a radiometric assay (Hong et al., 1989). The [14C]NDMA was purified by using a Dowex-1-bisulfite column and the procedures described in our previous studies (Forkert et al., 1996). The enzyme assay was carried out in a total volume of 1 ml, and reaction mixtures contained 100 mM K₂HPO₄ buffer, pH 7.0, 1.5 mM EDTA, 3 μg of microsomal protein, and components of an NADPH-generating system, as detailed previously. The reaction mixtures were preincubated for 3 min at 37°C with gentle agitation, after which the reaction was initiated by addition of 40 μM [14C]NDMA. Incubations were performed for 10 min at 37°C in a shaking water bath, and the reactions were terminated by cooling on ice and addition of a solution containing 1 M sodium acetate, pH 4.5, 100 mM HCHO, and 400 mM dithiothreitol. The incubation vessels were capped, and the samples were vortex-mixed and boiled for 5 min. The [14C]formaldehyde formed was then extracted into hexane (4 ml). After an additional extraction with H₂O, the hexane layer (2 ml) was added to scintillation fluid, and levels of radioactivity were determined. Formation of [14C]HCHO was measured by relating sample counts to those of known amounts of [14C]HCHO.

Protein Immunoblotting. Microsomes were subjected to SDS-polyacrylamide gel electrophoresis as described previously (Forkert et al., 1994).
Samples were electrophoretically separated on an 8.5% gel and then transferred to a 0.45-μm nitrocellulose membrane filter. The membrane was immersed for 2 hr at room temperature with 5% nonfat dry milk in Tris with 500 mM NaCl. The membrane was then incubated overnight with a monoclonal antibody for CYP2E1 (Mab 1-98-1) (Park et al., 1986) or polyclonal antibodies for hydrolase A (1:500) or hydrolase B (1:1500) (Morgan et al., 1994a,b), or hydrolase B (1:1500) (Morgan et al., 1994a,b). The antibodies were diluted in Tween 20-Tris-buffered saline containing 1% gelatin. After thorough rinsing in buffer to remove unbound antibodies, the nitrocellulose membrane was incubated for 2 hr with IgG conjugated to alkaline phosphatase (1:1000). The membrane was then immersed in a solution containing p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt. The apparent molecular weights of the protein bands that reacted with the antibodies were estimated by reference to the position of relative molecular weight standards.

Immuno inhibition of CYP2E1. An inhibitory anti-CYP2E1 antibody (Mab 1-91-3) (Ko et al., 1987) was used for the immuno inhibition experiments. A Mab specific for egg white lysozyme (HyHel 9) (Smith-Gill et al., 1994a,b) was used as a control antibody to assess nonspecific reactions. The experiments were performed by using the protocol described in our previous studies (Lee and Forkert, 1994). Reaction mixtures contained 3 mg of microsomal protein and a Mab protein/microsomal protein ratio of 0.5, in a total volume of 1 ml. The incubations with the Mab were performed at room temperature for 30 min, with gentle agitation. After reaction with the Mab, components of an NADPH-generating system were added and the reaction mixtures were preincubated for 3 min at 25°C. The reaction was then initiated by addition of EC (1 mM) in H2O, and the incubations were carried out for an additional 60 min at 25°C in a shaking water bath. The reactions were terminated by cooling of the samples on ice.

Covalent Binding of [ethyl-14C]EC to Liver Microsomes. Covalent binding was determined by equilibrium dialysis, using procedures described in our previous studies (Forkert et al., 1986). Microsomal incubations were performed as described above. The reaction mixtures, containing 3 mg of microsomal protein and an NADPH-generating system in a volume of 1 ml, were preincubated for 3 min at 25°C, after which the reaction was initiated by addition of 1 mM EC (0.15 μCi of [ethyl-14C]EC; specific activity, 50 mCi/mmol) in H2O. The incubation vessels were poured on ice and incubated for 60 min at 25°C in a shaking water bath; the reactions were terminated by cooling on ice and addition of 4% SDS (0.5 ml). The samples were then transferred to polypropylene tubes and immersed in a boiling water bath for 15 min. The boiled samples were permitted to cool at room temperature, transferred to dialysis tubing, sealed, and dialyzed overnight against 500 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 0.1% SDS. Aliquots (250 μl) of the dialyzed samples were then solubilized overnight in Soluene (2 ml). After the addition of glacial acetic acid (300 μl) and aqueous scintillation fluid (15 ml), levels of radioactivity of the dialysate were determined. The difference between the amounts of radioactivity in the dialysate and the buffer was regarded as the quantity of covalently bound EC in the sample.

Statistical Analysis. Data are expressed as mean ± SD and were analyzed by one-way analysis of variance, followed by pairwise multiple comparisons with the Student-Newman-Keuls test. Significant differences between experimental groups were set at a value of p < 0.05.

Results

Effects of EC and/or Carboxylesterase Inhibitors on CYP2E1 Catalytic Activity. Microsomal incubations conducted in the absence of an NADPH-generating system or with only the vehicle produced no alterations in the levels of NDMA demethylase activity (table 1). However, incubations with EC in the presence of an NADPH-generating system caused a slight but significant decrease in NDMA demethylation. There were also no changes in enzyme activity levels in microsomes incubated with only the carboxylesterase inhibitors PAX or PMSF (table 1). However, when the microsomes were preincubated with PAX or PMSF and then incubated with EC, the amounts of NDMA demethylase activity were significantly decreased. The magnitudes of reduction were similar with both inhibitors and were estimated for PAX and PMSF to be about 60 and 70% of control, respectively.

Effects of PAX, PMSF, and/or EC on Carboxylesterase Activity. Microsomal carboxylesterase activity was estimated by determining the hydrolysis of PNA to p-nitrophenol. Incubations of microsomes with EC elicited a slight but significant decrease in carboxylesterase activity, compared with levels in incubations without EC (table 2). Incubations of liver microsomes with PAX caused an 85% reduction in PNA hydrolysis, compared with incubations containing only the vehicle. When microsomes were preincubated with PAX and then with EC, the EC-induced alterations in enzyme activity were abolished. An inhibitory effect was also observed for microsomes incubated with PMSF, although the 45% decrease in hydrolysis was considerably less than the amounts detected in microsomes incubated with EC (table 2). Similar to the results obtained with PAX, preincubation with PMSF elicited no additional changes in the level of hydrolysis when the microsomes were subsequently incubated with EC.

Protein Immunoblotting. Protein immunoblotting of liver microsomes for CYP2E1 revealed a single band of about 51 kDa (fig. 1). This is similar to the apparent molecular mass of this P450 isozyme identified in murine liver microsomes in our previous studies (Lee and Forkert, 1994). Alterations in the quantities of immunodetectable CYP2E1 were not detected in blots prepared with microsomes incubated in the presence of PAX (fig. 1A, lanes 3 and 7) or microsomes incubated with EC in the presence of an NADPH-generating system (fig. 1A, lanes 4 and 8). The content of CYP2E1 protein was similar to that determined for microsomes in the control incubation, which consisted of microsomes incubated with only an NADPH-generating system (fig. 1A, lanes 2 and 6). In contrast, decreased levels of immunodetectable CYP2E1 were found in microsomes incubated with PAX and then with EC (fig. 1A, lanes 5 and 9). Similar to the results obtained from blots of microsomes incubated with PAX, the amounts of CYP2E1 protein were not different from those in micro-

<table>
<thead>
<tr>
<th>Experiment</th>
<th>NDMA Demethylase Activity (nmol [14C]HCHO/min)</th>
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<tbody>
<tr>
<td>−NADPH + EC</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>−PAX + EC</td>
<td>0.97 ± 0.03</td>
</tr>
<tr>
<td>+PAX + EC</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>−PAX + EC</td>
<td>0.77 ± 0.01</td>
</tr>
<tr>
<td>+PAX + EC</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>−NADPH + EC</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>−PMSF + EC</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>+PMSF + EC</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>−PMSF + EC</td>
<td>0.78 ± 0.01</td>
</tr>
<tr>
<td>+PMSF + EC</td>
<td>0.32 ± 0.04</td>
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</table>

a Incubations were conducted in the presence of an NADPH-generating system unless otherwise indicated.

2 Activity of NDMA demethylase was determined by measuring the formation of [14C]HCHO, as described in Materials and Methods. Data are expressed as mean ± SD of quadruplicate determinations from three or four different microsomal preparations. Pairwise multiple comparisons of experimental groups were performed by the Student-Newman-Keuls test (p < 0.05).
TABLE 2
Effects of PAX, PMSF, and/or EC on carboxylesterase activity in marine liver microsomes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Carboxylesterase Activity* (mmol p-nitrophenol protein/min)</th>
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<tbody>
<tr>
<td>−PAX − EC</td>
<td>1.90 ± 0.06</td>
</tr>
<tr>
<td>−PAX + EC</td>
<td>1.59 ± 0.04</td>
</tr>
<tr>
<td>+PAX − EC</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>+PAX + EC</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>−PMSF − EC</td>
<td>1.79 ± 0.05</td>
</tr>
<tr>
<td>−PMSF + EC</td>
<td>1.42 ± 0.06</td>
</tr>
<tr>
<td>+PMSF − EC</td>
<td>0.98 ± 0.05</td>
</tr>
<tr>
<td>+PMSF + EC</td>
<td>0.90 ± 0.04</td>
</tr>
</tbody>
</table>

* Carboxylesterase activity was determined spectrophotometrically by measuring the conversion of PNA to p-nitrophenol at 410 nm. Data are expressed as mean ± SD of quadruplicate determinations from three or four different microsomal preparations. Pairwise multiple comparisons of experimental groups were performed by the Student-Newman-Keuls test (p < 0.05).

Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with an anti-CYP2E1 Mab. Microsomes were incubated with EC or PAX; B, microsomes were incubated with EC or PMSF. A, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 6, control; lanes 3 and 7, PAX; lanes 4 and 8, EC; lanes 5 and 9, PAX and EC. B, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 6, control; lanes 3 and 7, PMSF; lanes 4 and 8, EC; lanes 5 and 9, PMSF and EC. A and B, Lanes 2–5 and 6–9, 1 and 3 μg of microsomal proteins, respectively.

Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with an anti-hydrolase A polyclonal antibody. A, Microsomes were incubated with EC or PAX; B, microsomes were incubated with EC or PMSF. A, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 5, control; lanes 3 and 6, PAX; lanes 4 and 7, EC. B, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 3, control; lanes 3 and 6, PMSF; lanes 4 and 7, EC. A and B, lanes 2–4 and 5–7, 0.1 and 0.25 μg of microsomal proteins, respectively.

Microsomal proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and reacted with an anti-hydrolase B polyclonal antibody. The microsomes were incubated with EC, PAX, or PMSF. A, Lane 1, molecular weight standards; lanes 2 and 5, control; lanes 3 and 7, PAX; lanes 4 and 7, EC. B, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 6, control; lanes 3 and 7, EC. A and B, lanes 2–4 and 5–7, 0.1 and 10 μg of liver microsomal proteins, respectively.

FIG. 2. Protein immunoblotting for hydrolase A in marine liver microsomes.

Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis and reacted with an anti-hydrolase A polyclonal antibody. Microsomes were incubated with EC or PAX; B, microsomes were incubated with EC or PMSF. A, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 5, control; lanes 3 and 7, PAX; lanes 4 and 7, EC. B, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 3, control; lanes 3 and 6, PMSF; lanes 4 and 7, EC. A and B, lanes 2–4 and 5–7, 0.1 and 0.25 μg of microsomal proteins, respectively.

FIG. 3. Protein immunoblotting for hydrolase B in marine liver microsomes.

Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis and reacted with an anti-hydrolase B polyclonal antibody. The microsomes were incubated with EC, PAX, or PMSF. A, Lane 1, molecular weight standards; lanes 2 and 5, control; lanes 3 and 7, PAX; lanes 4 and 7, EC. B, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 3, control; lanes 3 and 7, EC. A and B, lanes 2–4 and 5–7, 0.1 and 10 μg of liver microsomal proteins, respectively.

FIG. 1. Protein immunoblotting for CYP2E1 in marine liver microsomes.

Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with an anti-CYP2E1 Mab. A, Microsomes were incubated with EC and/or PAX; B, microsomes were incubated with EC and/or PMSF. A, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 6, control; lanes 3 and 7, PAX; lanes 4 and 8, EC; lanes 5 and 9, PAX and EC. B, Microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 6, control; lanes 3 and 7, PMSF; lanes 4 and 8, EC; lanes 5 and 9, PMSF and EC. A and B, Lanes 2–5 and 6–9, 1 and 3 μg of microsomal proteins, respectively.

In samples of liver microsomes incubated with only the vehicle (fig. 2A, lanes 2 and 5). However, the amounts of hydrolase A protein were decreased in microsomes incubated with PAX (fig. 2A, lanes 3 and 6) or with EC (fig. 2A, lanes 4 and 7), compared with those determined for the controls. The amounts of protein detected for hydrolase A were also diminished in microsomes incubated with PMSF (fig. 2B, lanes 3 and 6) or EC (fig. 2B, lanes 4 and 7); the reductions appeared to be similar in extent for both of these chemical compounds (fig. 2B, lanes 2 and 5). The decrease in hydrolase A immunoreactivity was also detected in blots prepared with microsomes incubated with either PAX or PMSF plus EC (data not shown). The protein blots for hydrolase B showed that a band was visible only when the amounts of protein loaded were considerably larger than those for hydrolase A (figs. 2 and 3). Compared with the controls (fig. 3, lanes 2 and 6), the protein content was decreased only in microsomes incubated with

Protein immunoblotting of liver microsomes for hydrolase A and hydrolase B revealed bands of approximately 57 kDa and 59 kDa, respectively (figs. 2 and 3); these were similar in apparent molecular mass to those detected in rat liver microsomes, as reported in previous studies (Morgan et al., 1994a, b). Hydrolase A was immunodetectable
PAX (fig. 3, lanes 3 and 7) and not in microsomes incubated with either PMSF (fig. 3, lanes 4 and 8) or EC (fig. 3, lanes 5 and 9).

Covalent Binding of [ethyl-14C]EC to Liver Microsomes. Data from the covalent binding experiments, which are summarized in table 3, demonstrated that binding of [ethyl-14C]EC to liver microsomes was significantly greater in incubations containing an NADPH-generating system, compared with incubations conducted in the absence of this system. Quantities of binding were similar for microsomes incubated with only PAX or PMSF. However, when microsomes were preincubated with either PAX or PMSF and then with EC, levels of binding were significantly increased. Table 4 summarizes the data obtained from the antibody inhibition studies that were performed to assess the contribution of CYP2E1 to EC binding; this was accomplished by reacting microsomes with an anti-CYP2E1 Mab (Mab 1-91-3) before incubation with EC and an NADPH-generating system. The results revealed significant reduction in levels of binding, compared with those found in control incubations in which microsomes were incubated with EC alone or with an NADPH-generating system. Binding levels in incubations performed with [ethyl-14C]EC and an NADPH-generating system were not significantly different from those for microsomes reacted with a nonspecific antibody (Mab HyHel 9) and EC.

Discussion

Studies of the metabolic disposition of EC have implicated at least two parallel pathways of metabolism, one of which participates in bioactivation and the other in detoxication. Data for the activation pathway have primarily been derived from studies in human liver microsomes, and these indicate that CYP2E1 is, in part, responsible for EC oxidation to VC epoxide (Guengerich and Kim, 1991; Guengerich et al., 1991). On the other hand, data for the detoxication pathway have resulted from studies in experimental animals and indicate hydrolysis of EC to the end products ethanol and CO₂ (Skipper et al., 1951; Kaye, 1960; Mirvish and Kaye, 1964). In the present studies, we have used a murine microsomal experimental system to investigate P450- and carboxylesterase-mediated oxidation and hydrolysis, respectively, in the same animal model and to evaluate the extent to which these two pathways are involved in EC metabolism.

We have evaluated the contribution of CYP2E1 to EC metabolism by estimation of P450 isozyme-selective catalytic activities, protein immunoblotting, and determination of covalent binding of EC to hepatic macromolecules. Our results showed a significant decrease in CYP2E1-dependent NDMA demethylase activity in liver microsomes incubated with EC in the presence of an NADPH-generating system (table 1). This decrease (20% of control) was not reflected in any visible diminution in CYP2E1 protein content (fig. 1). Neither were there alterations in NDMA demethylase in microsomes incubated with either PAX or PMSF (fig. 1), indicating that the carboxylesterase inhibitors had no effect on the CYP2E1-selective enzyme activity. However, when the microsomes were incubated with PAX or PMSF and then reacted with EC, the EC-induced decreases in NDMA demethylation activity were exacerbated, yielding about 60 and 70%, respectively, of the control levels (table 1). This loss of enzymic catalytic activity correlated with marked decreases in immunodetectable CYP2E1 protein content (fig. 1), indicating that inhibition of the detoxication pathway by PAX and PMSF exacerbated the bioactivation pathway with CYP2E1. These results suggested that the VC epoxide formed from EC is of sufficient reactivity to alkylate the apoprotein of CYP2E1 at the site of formation. This concept is supported by results from our immunoblots, which revealed no loss of apparent protein when the blots were prepared with an anti-CYP2E1 polyclonal antibody (data not shown). In contrast, a decrease in protein content was visible in the blots prepared with an anti-CYP2E1 Mab (fig. 1), suggesting that a limited number of epitopes are affected. Furthermore, some of the electrophilic metabolite produced must escape the site of formation, leading to alkylation of hepatic RNA and DNA and generation of adducts in rodents (Ribovich et al., 1982; Miller and Miller, 1983; Scherer et al., 1986; Leithauser et al., 1990).

The role of the CYP2E1 enzyme in EC metabolism is further supported by our covalent binding data, which showed that incubation with an anti-CYP2E1 Mab before reaction with EC produced significant inhibition of EC binding to microsomal proteins (table 4). Other studies have also produced data supporting a role for CYP2E1 in EC metabolism; the CYP2E1 inhibitor diethyldithiocarbamate inhibited the formation of ethenoadenosine in hepatic RNA of mice (Leithauser et al., 1990). These data suggested that, although the contribution of CYP2E1 to EC metabolism may not be substantial, as assessed by loss of catalytic activity, the reactive intermediate formed is highly reactive and the biological effects are significant.

Metabolism of EC by the carboxylesterases results in the production of ethanol, ammonia, and CO₂ (Nomier et al., 1989). It is of interest that, although purified porcine liver carboxylesterase converted EC efficiently to CO₂, EC had no effect on the rate of PNA hydrolysis (table 4) or the detoxication pathway. This result might indicate that the hydrolase A isoenzyme of carboxylesterase is not involved in the metabolism of EC.

Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Covalent Bindinga (pmol [ethyl-14C]EC/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+EC − NADPH</td>
<td>4.20 ± 0.29</td>
</tr>
<tr>
<td>+EC + NADPH</td>
<td>14.53 ± 0.74</td>
</tr>
<tr>
<td>+PAX + EC + NADPH</td>
<td>22.56 ± 1.41</td>
</tr>
<tr>
<td>+PMSF + EC + NADPH</td>
<td>23.20 ± 3.51</td>
</tr>
</tbody>
</table>

a Data are expressed as mean ± SD of quadruplicate determinations performed with three or four different microsomal preparations and were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test (p < 0.05).

Table 4

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Covalent Bindinga (pmol [ethyl-14C]EC/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−Mab + EC − NADPH</td>
<td>3.09 ± 0.26</td>
</tr>
<tr>
<td>−Mab + EC + NADPH</td>
<td>12.34 ± 1.50</td>
</tr>
<tr>
<td>+Mab + EC + NADPH</td>
<td>4.42 ± 0.42</td>
</tr>
<tr>
<td>+HyHel 9 + EC + NADPH</td>
<td>13.61 ± 1.38</td>
</tr>
</tbody>
</table>

a Liver microsomes were incubated with an anti-CYP2E1 inhibitory antibody (Mab 1-91-3) or a nonspecific antibody (Mab HyHel 9) at an antibody protein/microsomal protein ratio of 0.5.

b Data are expressed as mean ± SD of quadruplicate determinations from three or four different microsomal preparations. Pairwise multiple comparisons of experimental groups were performed by the Student-Newman-Keuls test (p < 0.05).

c Significantly different from −Mab + EC − NADPH and +Mab + EC + NADPH.

Significantly different from +EC − NADPH.

Significantly different from +EC + NADPH.
hydrolyase B (table 2; figs. 2 and 3). This EC-induced decrease was abrogated in microsomes incubated with PAX, suggesting that the carboxylesterase enzymes inhibited by PAX were involved in EC metabolism (table 2). This assumption is supported by the results from protein immunoblotting, which showed that PAX elicited a decrease in the protein contents of both hydrolyase A and hydrolyase B carboxylesterases (figs. 2A and 3). Hydrolysis of PNA was also significantly reduced in microsomes incubated with PMSF (table 2) and was correlated with decreased amounts of immunodetectable hydrolyase A (fig. 2B) but not hydrolyase B (fig. 3). These findings are consistent with the metabolism of EC by the hydrolyase A carboxylesterase enzyme. However, the participation of other forms of carboxylesterase enzymes in EC metabolism cannot be excluded.

The concept that carboxylesterase enzymes have a role in the detoxication and degradation of EC is supported by findings showing that inhibition of carboxylesterase activities by PAX and carbaryl decreased the metabolism of EC and reduced covalent binding to hepatic proteins (Yamamoto et al., 1990). These data indicated that inhibition of the carboxylesterase pathway by PAX and carbaryl did not increase the bioactivation pathway, suggesting a lack of relationship between these two pathways. More recent studies reported that inhibition of the CYP2E1 enzyme by diethylthiocarbaminate diminished the production of CO₂, as did incubation with PAX (Page and Carlson, 1994). However, because CO₂ is a byproduct of EC metabolism by both P450 and the carboxylesterase enzymes, the link between these two systems is not clearly evident. The results of our experiments demonstrated that, whereas NDMA demethylation was not altered by incubation of microsomes with PAX or PMSF, incubation with PAX or PMSF in conjunction with EC decreased the levels of enzyme activity (table 1), indicating that the bioactivation pathway is significantly augmented by reaction with the carboxylesterase inhibitors. Because reduction in the amounts of NDMA demethylation activity (table 1) and inactivation of the CYP2E1 protein (fig. 1) were substantial, the contribution of the carboxylesterases to EC metabolism is likely significant. These data are consistent with the results from our covariant binding studies; binding levels were significantly higher in microsomes reacted with EC in conjunction with either PAX or PMSF, compared with levels in microsomes reacted with EC alone (table 3). These findings suggested that detoxication by the microsomal carboxylesterases contributed significantly to EC metabolism. Our results also showed that the magnitudes of binding were not different for PAX and PMSF. Whereas PAX inhibited carboxylesterase enzymes, including hydrolyases A and B, and PMSF selectively inhibited hydrolyase A, the similarity in the inhibition of binding levels produced by PAX and PMSF suggested an important role for hydrolyase A in EC metabolism. Our results also suggested that other carboxylesterase isoforms, such as hydrolyase B, do not contribute significantly to EC metabolism.

In summary, our results support the metabolism of EC by both CYP2E1 and hydrolyase A. Inhibition of the carboxylesterases, and in particular hydrolyase A, exacerbated EC bioactivation by CYP2E1, suggesting that this carboxylesterase isoform plays an important role in EC detoxication.

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References