INACTIVATION OF CYTOCHROME P-450 (CYP2E1) AND CARBOXYLESTERASE (HYDROLASE A) ENZYMES BY VINYL CARBAMATE IN MURINE PULMONARY MICROSONES

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ABSTRACT:
We tested the hypothesis that vinyl carbamate (VC) is metabolized in vitro by cytochrome P-450 and carboxylesterase enzymes in murine lung. Incubations with VC and an NADPH-generating system produced a 50% decrease in N-nitrosodimethylamine (NDMA) demethylation and a corresponding loss in the amounts of immuno-detectable CYP2E1. Preincubation of microsomes with a CYP2E1 inhibitory antibody or the CYP2E1-selective inhibitor diallyl sulfone (DASO₂) inhibited demethylation activity; no alterations were detected upon subsequent exposure to VC. Carboxylesterase-mediated hydrolysis of p-nitrophenol acetate was reduced by 22% in microsomes incubated with VC. Decreased carboxylesterase activity also was detected in microsomes incubated with phenylmethylsulfonyl fluoride (PMSF), an inhibitor of hydrolase A, a carboxylesterase isozyme. No change in enzyme activity was detected when microsomes were subsequently incubated with VC.

The loss in carboxylesterase activity correlated with decreased immunodetectable hydrolase A in microsomes incubated with VC, PMSF, or PMSF and VC. The reduction in VC-induced NDMA demethylation activity was increased to 85% of the control in microsomes previously incubated with PMSF, and this corresponded with a marked decrease in CYP2E1 immunoreactivity in the immunoblots. Covalent binding of VC to proteins was detected in microsomes incubated with VC and an NADPH-generating system. Binding was inhibited in microsomes preincubated with either an inhibitory CYP2E1 antibody or DASO₂. In contrast, binding levels were augmented in microsomes preincubated with PMSF. These data supported VC metabolism by CYP2E1 and hydrolase A in murine lung microsomes and is consistent with involvement of CYP2E1 and hydrolase A in the activation and detoxication of VC, respectively.

Vinyl carbamate (VC)² is a primary metabolite derived from metabolism of ethyl carbamate (EC, urethane), a naturally occurring dietary constituent that is present in fermented products and alcoholic beverages (Ough, 1976; Battaglia et al., 1990). Serious concern has been raised regarding the potential carcinogenic risk posed by exposure to EC and VC, particularly in view of the large amounts of EC used as a cosolvent in analgesic drugs in Japan between 1950 and 1975 (Nomura, 1975; Miller, 1991). VC but not EC was found to be mutagenic to Salmonella typhimurium (Dahl et al., 1978, 1980; Leithauser et al., 1990). VC also exhibited greater carcinogenic potency than EC at similar doses and produced numbers of lung tumors that were 20- to 50-fold greater than those elicited by EC, depending on the route of exposure (Dahl et al., 1978, 1980). Moreover, VC-induced formation of etheno-DNA adducts in livers and lungs of mice were at levels that were 3-fold higher than those produced by EC (Fernando et al., 1996). These findings indicated that the toxic and carcinogenic potential of EC is linked not only with the parent compound but also with its metabolic derivative VC.

The toxic and carcinogenic effects of EC and VC are believed to be associated with their metabolism to a reactive intermediate. It was postulated that oxidation of EC leads to the formation of VC, which is oxidized further to produce VC epoxide, a metabolite that has been proposed to be the ultimate carcinogenic species (Fig. 1) (Dahl et al.,

Fig. 1. Proposed pathway of EC and VC metabolism.
1978, 1980; Park et al., 1990, 1993). This proposed metabolic pathway involving two oxidative steps has been supported by data from studies of EC and VC in human liver microsomes that implicated the cytochrome P-450 isozyme CYP2E1 in their metabolism (Guengerich and Kim, 1991; Guengerich et al., 1991). Although the liver is a major target of EC- and VC-induced carcinogenicity, the lung appears to be a highly susceptible tissue. A latent period of about 1 year was required for manifestation of hepatic tumors, whereas lung tumors developed more rapidly and were found 2 to 6 months after EC treatment (Tannenbaum, 1964; Mirvish, 1968; Shimkin and Stoner, 1975). It thus is of importance to determine the underlying basis for this particular susceptibility of the lung to EC-induced tumor formation and to identify the mechanisms involved in EC and VC bioactivation. As an initial step to this end, our previous studies have investigated the lung metabolism of EC (Forkert and Lee, 1997). Data from these studies supported a central role for CYP2E1 in catalyzing the metabolism of EC in murine lung microsomes. An objective of the present study is to determine whether lung CYP2E1 is also involved in VC metabolism and to confirm whether both steps of the oxidative pathway of EC metabolism are mediated by CYP2E1.

Although metabolic activation is of importance in manifestation of toxicity and carcinogenicity, the detoxication pathway is also of relevance. In our recent studies, we reported that the catalytic activity of carboxylesterases, a class of enzymes that metabolize ester substrates, was significantly decreased in lung microsomes reacted with EC, suggesting that the carboxylesterases are involved in EC metabolism (Forkert and Lee, 1997). The end products of EC metabolism by the carboxylesterases are ethanol, ammonia, and CO₂, indicating that this metabolic pathway is associated with detoxication (Boyland and Rhoden, 1949; Kaye, 1960; Mirvish and Kaye, 1964; Nomier et al., 1989). This assertion is supported by studies that showed that, when carboxylesterase enzymes were inhibited with either paraoxon or phenylmethylsulfonyl fluoride (PMSF), CYP2E1-dependent oxidation of EC was enhanced and produced significantly higher levels of covalent binding of [¹⁴C-ethyl]-EC to microsomal proteins (Forkert and Lee, 1997). The structural and chemical characteristics common to EC and VC implicated the carboxylesterases in VC metabolism and, hence, detoxication. However, the extent and involvement of this enzyme system in VC metabolism in the lung has not been identified and characterized.

In the present study, we have used a microsomal incubation system to investigate the metabolism of VC in the lungs of mice. We have measured enzyme catalytic activities for CYP2E1 and carboxylesterase enzymes and used enzyme inhibition and immunoinhibition experiments to investigate the involvement of these enzyme systems in VC metabolism. Protein immunoblotting was used to further confirm the potential isozyme-selective metabolism of VC by cytochrome P-450 and carboxylesterase enzymes. In addition, covalent binding of [¹⁴C-carbonyl]-VC was measured to assess magnitudes of formation of reactive intermediates from VC.

Materials and Methods

Treatment of Animals. Female CD-1 mice of 20 to 25 g body weight were purchased from Charles River Canada (Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS). A goat polyclonal antibody raised against CYP2E1 was obtained from Oxford Biomedical Research Inc. (Oxford, MS). All other chemicals were of reagent grade and were purchased from standard commercial suppliers.

Preparation of Microsomes. Lungs from 10 mice were pooled for each sample and were prepared using differential centrifugation as described previously (Lee and Forkert, 1995). Microsomes were resuspended in 100 mM K₂HPO₄ buffer containing 1.5 mM EDTA, pH 7.0. Aliquots (250 µl) were placed in Eppendorf tubes, layered over with argon, frozen in liquid nitrogen, and stored at −70°C. Protein concentrations were determined using the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Microsomal Incubations with VC. In preliminary experiments, microsomal incubations performed at 37°C yielded results that were highly variable. This may have been due to the instability of VC at this temperature. Incubations performed at 25°C produced data that were highly reproducible. Therefore, all subsequent incubations with VC were performed at 25°C. Reaction mixtures consisted of 3 mg of lung microsomal protein suspended in 100 mM K₂HPO₄ buffer containing 1.5 mM EDTA, pH 7.0, and an NADPH-generating system (7.5 mM glucose 6-phosphate, 2 U of glucose 6-phosphate dehydrogenase, 5.0 mM MgCl₂, and 0.4 mM NADP⁺). The final incubation volume was 1 ml. The mixtures were preincubated for 3 min at 25°C with agitation, and the reaction was initiated by addition of VC dissolved in water. Incubation vessels were promptly sealed with Teflon stoppers and incubations were continued for an additional 30 min. Reactions were terminated by cooling the samples on ice. Microsomes were washed in 100 mM K₂HPO₄ buffer, pH 7.0, and centrifuged at 105,000g for 30 min at 0°C. The resulting microsomal pellet was resuspended in 1 ml of 100 mM K₂HPO₄ buffer, pH 7.0, and the microsomal suspension was used for the enzyme assays. To assess for linearity of reactions, a series of incubations with lung microsomes and VC was performed, with substrate concentrations ranging from 100 µM to 10 mM and incubation times ranging from 10 to 180 min. Based on the data obtained, an incubation time of 30 min and a VC concentration of 0.5 mM were used for subsequent incubations.

Microsomal Incubations with the Carboxylesterase Inhibitor PMSF. Incubation mixtures consisted of 3 mg of lung microsomal protein suspended in 1 ml of 100 mM K₂HPO₄ buffer containing 1.5 mM EDTA, pH 7.0, in a final incubation volume of 1 ml. The mixtures were preincubated for 3 min at 25°C with gentle agitation. The reaction was initiated by addition of 25 µM PMSF in dimethyl sulfoxide, and the incubation continued for an additional 20 min at 25°C. The reactions were terminated by cooling the samples on ice. Microsomes were then washed and centrifuged at 105,000g for 30 min at 0°C. The resulting microsomal pellet was resuspended in buffer and incubated with VC and/or carboxylesterase activity was determined.

Microsomal Incubations with Dialyl Sulfone (DASO₂). Reaction mixtures consisted of 3.0 mg of lung microsomal protein suspended in 1 ml of 100 mM K₂HPO₄ buffer containing 1.5 mM EDTA, pH 7.0, in a final volume of 1 ml. Components of the NADPH-generating system were added and the mixtures were preincubated for 3 min at 37°C with gentle agitation. The reaction was initiated by the addition of 1 mM DASO₂ in water, and the mixtures were
incubated for an additional 30 min at 37°C with agitation. The reaction was terminated by placing the vessels on ice. The microsomes subsequently were washed, recovered, and resuspended in 100 mM K$_2$HPO$_4$ buffer. The microsomal suspension was then used for incubation with VC and/or measurement of NDMA demethylase activity.

**Enzyme Assays.** Catalytic activity of CYP2E1 was determined by measuring demethylation of [14C]-N-nitrosodimethylamine (NDMA), as described previously (Hong et al., 1989). Reaction mixtures consisted of 3.0 mg of microsomal protein suspended in 1 ml of 100 mM K$_2$HPO$_4$ buffer containing 1.5 mM EDTA, pH 7.0, and components of an NADPH-generating system, as detailed previously. Samples were preincubated for 3 min at 37°C, and the reaction was initiated by addition of 40 μM [14C]NDMA (specific activity 40 mCi/mmoll) in water. Reactions were carried out for 10 min at 37°C with agitation and were terminated by cooling the samples on ice. Radiolaabeled formaldehyde ([14C]HCHO) was extracted into hexane; 2 ml of this hexane solution was added to 18 ml of Eco-lite scintillation fluid, and levels of radioactivity were determined by liquid scintillation spectroscopy. The formation of [14C]HCHO was quantified by relating sample cpm to those determined for known amounts of [14C]HCHO.

Carboxylesterase activity was determined by measuring the conversion of p-nitrophenyl acetate (PNA) to p-nitrophenol (PNA), as described previously (Morgan et al., 1994). Briefly, lung microsomes (300 μg) suspended in 1 ml of 100 mM K$_2$HPO$_4$ buffer, pH 7.0, were placed in a spectrophotometric cuvette, and the reaction was initiated by addition of 10 μl of 100 mM PNA (final concentration of 1 mM) at room temperature. The hydrolysis of the substrate to PNA was determined by an increase in absorbance at 410 nm. The amounts of PNA formed were determined by relating absorbance levels at 410 nm to those of known amounts of PNP.

**Immunoinhibition Studies.** mAb Ab 1-91-3, an inhibitory mAb specific for CYP2E1 (Park et al., 1986), was used to inhibit lung CYP2E1 (Lee and Forkert, 1995). mAb HyHel 9, a mAb specific for egg white lysozyme (Smith-Gill et al., 1982) was used as a control antibody to assess for nonspecific reactions. Incubations with antibodies were performed at a mAb to microsomal protein ratio of 0.5, as described in our previous studies (Lee and Forkert, 1995). Briefly, reaction mixtures containing 3.0 mg of microsomal protein in a volume of 1 ml 100 mM K$_2$HPO$_4$ buffer, pH 7.0, were incubated with the appropriate mAb at room temperature for 30 min with gentle agitation. Thereafter, components of the NADPH-generating system were added and the mixtures were preincubated for 3 min at 25°C. The reaction was initiated by addition of 0.5 mM VC in water, and the mixtures were incubated for an additional 30 min at 25°C. The reactions were terminated by cooling the samples on ice. The microsomes were then washed and recovered, and NDMA demethylase activity or levels of covalent binding were determined.

**Protein Immunoblotting.** After incubation with PMSF and/or VC, microsomal samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on an 8.5% gel as described previously (Forkert et al., 1994). The protein samples were then transferred to a 0.45-μm nitrocellulose membrane for 1 h at 12 V in 25 mM Tris-HCl, 192 mM glycine, pH 8.3, and 20% (v/v) methanol. The membrane subsequently was incubated for 2 h in a blocking solution consisting of 5% nonfat dried milk in 20 mM Tris/500 mM NaCl, pH 7.5. After thorough rinsing in buffer, the membrane was incubated overnight with a goat anti-rabbit liver CYP2E1 polyclonal antibody (1:200) or a rabbit anti-rat liver hydroxylase A or B (1:1500) (Morgan et al., 1994). The membrane then was rinsed in buffer, reacted for 2 h with an IgG conjugated to alkaline phosphatase, and immersed in a solution containing p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt. The apparent molecular weight of immunodetectable protein bands was estimated by reference to the position of prestained molecular weight standards.

**Covalent Binding of [14C-carboxyl]-VC to Lung Microsomes.** Covalent binding was determined by using the equilibrium dialysis method as described in our previous studies (Forkert et al., 1986). Reaction mixtures consisted of 3.0 mg of microsomal protein suspended in 1 ml of 100 mM K$_2$HPO$_4$ buffer containing 1.5 mM EDTA, pH 7.0, and components of an NADPH-generating system; the mixtures were preincubated for 3 min at 25°C. The reaction was initiated by addition of 0.5 mM VC (0.30 μCi [14C-carboxyl]-VC, specific activity 1.5 mCi/mmol) in water. Incubation vessels were capped with Teflon stoppers and incubated for an additional 30 min at 25°C with gentle agitation.

![Fig. 2. Effect of VC concentrations on CYP2E1-dependent [14C]NDMA demethylase activity in lung microsomes.](image)

Reaction mixtures contained 3.0 mg of microsomal protein and an NADPH-generating system in a final incubation volume of 1 ml of 100 mM K$_2$HPO$_4$ buffer, pH 7.0. The mixtures were preincubated for 3 min at 25°C, and the reaction was initiated by addition of VC in water. The incubations were carried out for 30 min, after which the microsomes were washed and reharvested, and [14C]NDMA demethylase activity was determined. Data are expressed as mean ± S.D. of quadruplicate determinations performed on three to four different microsomal samples. Data were analyzed using one-way ANOVA followed by the Student–Newman–Keuls test; p < .05.

The samples were cooled on ice, after which 0.5 ml of 4% SDS was added. The samples then were transferred to polypropylene tubes and placed into a boiling water bath for 15 min. The boiled microsomal proteins were cooled slowly at room temperature, transferred to dialysis tubing, and dialyzed overnight in 500 ml of 0.1% SDS/100 mM K$_2$HPO$_4$ buffer, pH 7.0. Aliquots (250 μl) of the dialyzed samples were solubilized overnight in 2 ml of Soluene. After the addition of glacial acetic acid (300 μl) and Eco-Lite scintillation fluid (15 ml), levels of radioactivity of dialyzed samples were determined by liquid scintillation spectroscopy. The difference in the levels of radioactivity of the dialysate and the buffer represented the amounts of covalently bound VC in the sample.

**Statistical Analysis.** All data are expressed as mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test to identify significant differences between experimental groups. The level of significance was set at p < .05.

**Results.**

**Effects of PMSF and/or VC on NDMA Demethylase and Carboxylesterase Activities.** Levels of NDMA demethylase activity were decreased significantly in lung microsomes incubated with VC in the presence of NADPH compared with those obtained in reactions in which NADPH was omitted (Fig. 2). The alterations were concentration-dependent. Demethylase activities decreased progressively in microsomes incubated with VC in amounts ranging from 100 μM to 1 mM compared with those in control incubations performed with VC alone (0.34 ± 0.02 nmol [14C]HCHO/mg protein/min) or with only NADPH (0.33 ± 0.09 nmol [14C]HCHO/mg protein/min). Saturation was achieved at a concentration of 1.0 mM VC, and further decreases detected at concentrations ranging from 1.0 mM to 10 mM were slight (Fig. 2). Time-dependent alterations in NDMA demethylase activity also were determined and were detected at incubation periods of up to
Core activity by about 70%, compared with levels in microsomes that were not reacted with the mAb. Lung microsomes incubated with VC and NADPH caused a significant decrease (50%) in demethylation, compared with the levels detected in microsomal incubations conducted in the absence of NADPH or VC; these data are consistent with those of the preceding experiments (Table 1). When microsomes were incubated with the mAb and VC, the level of demethylase activity was similar to that detected in microsomes that were incubated with the mAb alone. Incubations with the nonspecific mAb HyHel 9 (Smith-Gill et al., 1982) and VC produced levels of demethylase activity that were similar to those in microsomes incubated with only VC.

Our recent studies have demonstrated that the garlic derivative, DASO₂, is an efficacious inhibitor of lung CYP2E1 and, hence, the metabolism of CYP2E1-selective substrates (Forkert et al., 1996). In view of these findings, we were interested in investigating the possibility that DASO₂ is also an effective inhibitor of VC metabolism and in further confirming that CYP2E1 has an important role in its biotransformation. The results of these studies are summarized in Table 2. NDMA demethylase activity was inhibited by 50% in microsomes incubated with 1 mM DASO₂ and NADPH compared with the level in microsomes incubated in the absence of DASO₂. A similar 50% decrease in demethylase activity was also detected in microsomes incubated with VC and NADPH in comparison with the amounts in microsomal incubations containing VC and not DASO₂ or NADPH. The amount was similar to that obtained in microsomes incubated with DASO₂ and VC.

Covalent Binding of [1⁴C-carbonyl]-VC to Lung Microsomal Proteins. Results of the covalent binding studies are summarized in Table 3. Covalent binding of [1⁴C-carbonyl]-VC to lung microsomal proteins was significantly greater in preparations incubated in the presence of VC and NADPH compared with the level detected in incubations conducted with VC in the absence of NADPH. The magnitude of binding in microsomes that were preincubated with the CYP2E1 mAb was not significantly different from that obtained in incubations carried out with VC in the absence of both the mAb and NADPH. The amount of binding is significantly lower than the amount detected in microsomes incubated with VC and NADPH. In control incubations performed with the nonspecific mAb HyHel 9, the binding level was similar to that obtained in microsomes reacted with VC and NADPH.

Binding levels were decreased by about 50% when microsomes were preincubated with DASO₂, an inhibitor of CYP2E1 (Brady et al., 1991a,b; Forkert et al., 1996), and subsequently incubated with VC. In an additional experiment, we measured binding in microsomes preincubated with PMSF, a carboxylesterase inhibitor (Morgan et al., 1994), before incubation with VC. The level of binding was increased by about 35% in these microsomes.

Protein Immunoblotting. The CYP2E1 polyclonal antibody recognized a single protein band of Mᵋ 51,000 in murine lung microsomes (Fig. 4A), and this protein species is consistent with that detected in our previous studies (Lee and Forkert, 1995). The content of immunodetectable CYP2E1 was diminished significantly in microsomal samples previously incubated with VC and NADPH compared with protein samples incubated with only the vehicle or only PMSF. When microsomes were incubated with PMSF and subsequently with VC, immunoreactivity for CYP2E1 was virtually abolished.

Protein immunoblotting for the hydroxylase A protein revealed a single band of Mᵋ 57,000 in microsomal samples from murine lung (Fig. 4B) and is similar to the apparent molecular weight of the protein species identified in our previous studies (Forkert and Lee, 1997). The content of immunodetectable hydroxylase A was decreased significantly in samples previously incubated with either PMSF, VC, or PMSF and VC, compared with the amount detected in microsomal samples not reacted with the mAb.
TABLE 1

<table>
<thead>
<tr>
<th>Exp.</th>
<th>NDMA Demethylation*</th>
<th>Exp.</th>
<th>PNA Hydrolysis*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nmol [14 C]HCHO/mg protein/min</td>
<td></td>
<td>μmol PNP/mg protein/min</td>
</tr>
<tr>
<td>− PMSF − VC − NADPH</td>
<td>0.32 ± 0.01</td>
<td>− PMSF − VC − NADPH</td>
<td>0.52 ± 0.02</td>
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<tr>
<td>− PMSF − VC + NADPH</td>
<td>0.34 ± 0.03</td>
<td>− PMSF − VC − NADPH</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>+ PMSF − VC + NADPH</td>
<td>0.32 ± 0.05</td>
<td>+ PMSF − VC − NADPH</td>
<td>0.24 ± 0.01^d</td>
</tr>
<tr>
<td>+ PMSF + VC + NADPH</td>
<td>0.16 ± 0.01^a</td>
<td>− PMSF + VC − NADPH</td>
<td>0.41 ± 0.02^c</td>
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<tr>
<td>+ PMSF + VC − NADPH</td>
<td>0.05 ± 0.05^b,c</td>
<td>+ PMSF + VC − NADPH</td>
<td>0.21 ± 0.01^b,c</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± S.D. of quadruplicate determinations performed on three to four different microsomal preparations and were analyzed by one-way ANOVA and the Student–Newman–Keuls test; p < .05.
^Significantly different from (− PMSF − VC − NADPH), (− PMSF − VC + NADPH), and (+ PMSF − VC − NADPH).
^Significantly different from (− PMSF − VC + NADPH).
^Significantly different from (− PMSF − VC − NADPH) and (− PMSF − VC + NADPH).
^Significantly different from (− PMSF + VC − NADPH).

TABLE 2

<table>
<thead>
<tr>
<th>Exp.</th>
<th>NDMA Demethylation*</th>
<th>Exp.</th>
<th>NDMA Demethylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol [14 C]HCHO/mg protein/min</td>
<td></td>
<td>nmol [14 C]HCHO/mg protein/min</td>
</tr>
<tr>
<td>− mAb − VC − NADPH</td>
<td>0.31 ± 0.01</td>
<td>− DASO₂ − VC + NADPH</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>+ mAb + VC + NADPH</td>
<td>0.10 ± 0.01^c,e</td>
<td>+ DASO₂ − VC + NADPH</td>
<td>0.15 ± 0.03^b</td>
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<tr>
<td>− mAb + VC − NADPH</td>
<td>0.33 ± 0.04</td>
<td>− DASO₂ − VC − NADPH</td>
<td>0.31 ± 0.04</td>
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<td>− mAb + VC + NADPH</td>
<td>0.17 ± 0.05^b</td>
<td>− DASO₂ + VC + NADPH</td>
<td>0.16 ± 0.02^d</td>
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<tr>
<td>+ mAb + VC + NADPH</td>
<td>0.09 ± 0.01^c,e</td>
<td>+ DASO₂ + VC + NADPH</td>
<td>0.17 ± 0.01^d</td>
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<tr>
<td>+ HyHel 9 + VC + NADPH</td>
<td>0.18 ± 0.01^b</td>
<td>+ DASO₂ + VC + NADPH</td>
<td></td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± S.D. of quadruplicate determinations from three or four different microsomal preparations and were analyzed by one-way ANOVA and the Student–Newman–Keuls test; p < .05.
^Significantly different from (− mAb − VC + NADPH) and (+ mAb + VC + NADPH).
^Significantly different from (− mAb + VC + NADPH) and (+ mAb + VC − NADPH).
^Significantly different from (− mAb − VC − NADPH) and (− mAb + VC − NADPH).
^Significantly different from (− mAb + VC − NADPH) and (− mAb + VC + NADPH).

TABLE 3

<table>
<thead>
<tr>
<th>Exp.</th>
<th>pmol [14 C-carbonyl]-VC/mg protein*</th>
<th>Exp.</th>
<th>pmol [14 C-carbonyl]-VC/mg protein*</th>
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<tbody>
<tr>
<td>− mAb + VC − NADPH</td>
<td>1.90 ± 0.07</td>
<td>− DASO₂ − VC − NADPH</td>
<td>2.81 ± 0.23</td>
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<td>− mAb + VC + NADPH</td>
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<td>+ mAb + VC + NADPH</td>
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<td>3.47 ± 0.76</td>
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<td>+ HyHel 9 + VC + NADPH</td>
<td>5.74 ± 0.27^b</td>
<td>+ PMSF + VC + NADPH</td>
<td>9.74 ± 0.53^d</td>
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</tbody>
</table>

*Data are expressed as mean ± S.D. of quadruplicate determinations from three or four different microsomal preparations and were analyzed by one-way ANOVA and the Student–Newman–Keuls test; p < .05.
^Significantly different from (− mAb + VC − NADPH) and (+ mAb + VC + NADPH).
^Significantly different from (− mAb + VC − NADPH) and (+ mAb + VC + NADPH).
^Significantly different from (− DASO₂ − VC − NADPH) and (+ DASO₂ + VC + NADPH).
^Significantly different from (− DASO₂ + VC − NADPH).

Discussion

It was postulated first by Dahl et al. (1978) that EC is oxidized to VC, which undergoes an additional oxidation step to produce VC epoxide, a metabolite that has been proposed to be the ultimate carcinogenic species (Fig. 1). Because of the high susceptibility of the lung to EC- and VC-induced tumor formation, it was of interest to determine whether a similar oxidative pathway is also involved in the lung metabolism of both of these carbamates. As an initial step to this end, our previous studies have investigated the metabolism of EC in lung microsomal incubations (Forkert and Lee, 1997). Our findings showed that EC metabolism was mediated by cytochrome P-450, inasmuch as NADPH was required for production of a reactive metabolite(s) that binds to lung microsomal proteins. This binding was increased significantly in microsomes preincubated with paraoxon, an inhibitor of the carboxylesterases, and indicated that inhibition of the detoxication pathway led to enhancement of EC oxidation and activation (Forkert and Lee, 1997). In the present studies, we have investigated the lung metabolism of VC to establish whether the metabolic events involving VC oxidation and detoxication are similar to those identified for EC.

Data from the present studies showed that lung metabolism of VC is cytochrome P-450-dependent: substantial binding of VC to proteins was detected in lung microsomes incubated in the presence of NADPH compared with the level obtained in incubations conducted in the absence of NADPH (Table 3). Parallel studies performed to investigate the role of CYP2E1 in VC oxidation showed that levels of NDMA demethylase activity were decreased significantly in lung microsomes incubated with VC and NADPH, compared with those detected in incubations conducted in the absence of NADPH (Tables 1 and 2). This loss of enzyme activity coincided with a marked...
Microsomal proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with antibodies for CYP2E1 (A) or hydrolase A (B). A, lanes 6 to 9 and 2 to 5 contained 3.0 and 5.0 μg of protein, respectively. B, lanes 2 to 5 and 6 to 9 contained 0.5 and 1.0 μg of protein, respectively. A and B, microsomal proteins were loaded as follows: lane 1, molecular weight standards; lanes 2 and 6, control; lanes 3 and 7, PMSF (25 μM); lanes 4 and 8, VC (0.5 mM); lanes 5 and 9, PMSF (25 μM) and VC (0.5 mM). All incubations with VC were conducted in the presence of NADPH.

An objective of the present studies is to determine whether, as in the case of EC, microsomal carboxylesterases have a role in lung metabolism of VC. These studies also were undertaken to identify the potential isozyme-selective metabolism of VC. Our results showed a slight but significant decrease (22%) in carboxylesterase activity in microsomes incubated with VC compared with the constitutive level in lung microsomes (Table 1). When the microsomes were reacted with PMSF and then with VC, this decrease was exacerbated and comprised 50% of the level detected in microsomes incubated with VC alone. These data suggested that the amount of carboxylesterase activity inhibited by PMSF was substantial and prevented hydrolysis of VC by the carboxylesterases. These findings coupled with the loss of immunodetectable hydrolase A supported contributions of the carboxylesterases toward both EC metabolism and detoxication of EC. Our recent studies have investigated the involvement of the microsomal carboxylesterases in EC metabolism in lung microsomes (Forkert and Lee, 1997). These data together with the loss of immunodetectable hydrolase A but not hydrolase B (Fig. 3) supported EC metabolism by microsomal carboxylesterases and, in particular, by hydrolase A.

A and B, whereas PMSF selectively inhibits hydrolase A (Morgan et al., 1994). Both of these agents inhibited microsomal carboxylesterase activity to such an extent that EC could not be metabolized by this enzyme system (Forkert and Lee, 1997). These data together with the results of studies using paraoxon and carbaryl as metabolic inhibitors (Yamamoto et al., 1990). Carboxylesterase activity was inhibited by these compounds and coincided with decreased covalent binding of VC to liver proteins. Because the extent of binding generally is regarded as a measure of reactive metabolite formation, these data...
suggested that activation is depressed by reaction with the carboxylesterase inhibitors, leading to decreased EC binding. More recent studies have reported decreased production of CO₂ from EC in lung microsomes incubated with paraaxon (Page and Carlson, 1994). However, CO₂ is also a product generated from P-450-mediated metabolism of EC (Park et al., 1993; Kemper et al., 1995), and it is not clear through which pathway the CO₂ is generated and what the relative contributions are from the carboxylesterase and oxidative pathways.

In our recent studies with EC, it was shown that levels of covalent binding to lung proteins were increased by about 95 and 85% in microsomes preincubated with paraaxon and PMSF, respectively (Forkert and Lee, 1997). These increases produced by paraaxon and PMSF correlated with decreases of about 80 and 60% in levels of carboxylesterase activity, respectively. These data are consistent with a role of detoxication for the carboxylesterases, and inhibition of these enzymes led to enhanced activation, resulting in increased generation and binding of a VC metabolite(s). However, the carboxylesterases may not represent the only pathway of detoxication. Recent studies have reported that in vitro generation of 1,N⁶-ethenoadenosine from the VC epoxide is inhibited by glutathione; adduct formation is decreased further by the addition of glutathione S-transferases (Kemper et al., 1995). The relative extents to which the carboxylesterases and glutathione are involved in EC and VC detoxication are not known and remain to be investigated.

In summary, our results are consistent with important roles for CYP2E1 and hydroxyl A in bioactivation and detoxication of VC, respectively, and modification of either metabolic pathway leads to alterations in the extent to which reactive intermediates are formed, as assessed by covalent binding of VC to lung proteins.

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