Pulmonary Bioactivation of 1,1-Dichloroethylene Is Associated with CYP2E1 Levels in A/J, CD-1, and C57BL/6 Mice

POH-GEK FORKERT, STEPHANIE M. BOYD, and JUDITH B. ULREICH

Department of Anatomy and Cell Biology, Queen’s University, Kingston, Ontario, Canada (P.G.F., S.M.B.); and Department of Surgery, University of Arizona, Tucson, Arizona (J.B.U.)

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ABSTRACT

1,1-Dichloroethylene (DCE) elicits lung cytotoxicity and selectively targets Clara cells of bronchioles. The toxic effects are ascribed to CYP2E1-mediated formation of reactive intermediates including the DCE epoxide. Here we tested the hypothesis that differential CYP2E1 levels in the lungs of A/J, CD-1, and C57BL/6 mice lead to differences in the extents of DCE bioactivation and lung damage. Our results showed that lung CYP2E1 levels differed significantly in the three murine strains, and followed the rank order A/J > CD-1 > C57BL/6. Covalent binding of [14C]DCE to lung proteins in A/J mice was significantly higher than in either CD-1 or C57BL/6 mice. HPLC analysis of lung cytosol from DCE-treated mice showed that 2-S-glutathionyl acetate, a glutathione (GSH) conjugate derived from the epoxide (conjugate [C]), was the major metabolite formed. Levels of [C] detected in cytosol from A/J and CD-1 mice were significantly higher than in C57BL/6 mice. Immunohistochemical staining for [C] was pronounced in the lungs of A/J mice, was lower in CD-1 mice, and was lowest in C57BL/6 mice. Levels of GSH were similar in the lungs of all untreated mice. However, significant reduction in GSH was found in DCE-treated mice, with decreases comparable in all three strains. Bronchiolar Clara cell damage was more severe in A/J and CD-1 mice than in C57BL/6 mice. These results showed differences in CYP2E1 levels in the lungs of A/J, CD-1, and C57BL/6 mice that correlated with the extent to which the DCE epoxide is formed as well as with the severity of lung cytotoxicity.

1,1-Dichloroethylene (DCE), a monomeric intermediate used in the manufacture of plastic products, elicits a pneumotoxic lesion that selectively targets Clara cells of the bronchiolar epithelium (Forkert and Reynolds, 1982). Previous studies have confirmed that the mechanism responsible for the lung damage is mediated by cytochrome P450-dependent metabolism of DCE to reactive intermediates (Okine and Gram, 1986; Forkert et al., 1987). The primary metabolites formed from DCE in rat liver microsomal incubations have been identified as 2,2-dichloroacetaldehyde, DCE epoxide, and 2-chloroacetyl chloride (Liebler and Guengerich, 1983; Costa and Ivanetics, 1984; Liebler et al., 1985, 1988). In more recent studies, we demonstrated that the DCE epoxide-derived conjugate [C] was the major metabolite generated in vivo, whereas conjugate [B] was formed at minimal amounts (Forkert, 1999). These results indicated that the epoxide is the most plausible candidate for mediating the toxic effects of DCE.

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ABBREVIATIONS: DCE, 1,1-dichloroethylene; [A], S-(2,2-dichloro-1-hydroxyethyl)glutathione; [B], 2-(S-glutathionyl)acetetyl glutathione; [C], 2-S-glutathionyl acetate; [D], S-(2-chloroacetyl)glutathione; BSA, bovine serum albumin; DASO2, diallyl sulfone; GSH, glutathione; PCA, perchloric acid; PBS, phosphate-buffered saline; PNP, p-nitrophenol; P450, cytochrome P450; HPLC, high performance liquid chromatography.
significantly inhibited the CYP2E1 enzyme, decreased the generation of 2,2-dichloroacetaldehyde and the epoxide, and protected from DCE-induced bronchiolar Clara cell cytotoxicity (Forkert et al., 1996b). Moreover, other studies have identified differing quantities of CYP2E1 in the lungs of female and male mice (Forkert et al., 1996a). Levels of CYP2E1-dependent p-nitrophenol (PNP) hydroxylase activity, were more than 50% higher in female than in male mice, and these amounts correlated with formation of DCE epoxide that was about 2-fold higher in the former than in the latter. These findings are consistent with involvement of CYP2E1 in the bioactivation of DCE.

The selective Clara cell damage evoked by DCE and the high level of covalent binding of DCE metabolites to the Clara cells suggested that bioactivation occurs in situ within this cell type (Forkert et al., 1990). This assertion is consistent with findings from previous studies showing that the CYP2E1 protein and mRNA are localized preferentially within the Clara cells (Forkert, 1995). Significantly, results from immunohistochemical studies indicated that the epoxide is formed and localized within the Clara cells (Forkert, 1999). Furthermore, pretreatment of mice with DASO2 inhibited formation of the epoxide, and markedly decreased the level of immunoreactivity in the bronchiolar epithelium and Clara cells. These findings supported the premise that the cell-selective cytotoxicity ensuing after DCE exposure is mediated in situ metabolism of DCE to reactive intermediates including the epoxide within the Clara cells. These data further supported the contention that the DCE epoxide is the reactive species responsible for the Clara cell damage.

In preliminary studies, we have serendipitously identified strain-related differences in CYP2E1 levels in A/J and C57BL/6 mice. Here we have tested the hypothesis that DCE metabolism differs in these strains of mice, leading to differences in the severity of DCE-induced lung cytotoxicity. These studies were also performed in CD-1 mice, an outbred strain that is more economical and that is commonly used in experimental studies. To identify and to evaluate these differences in A/J, CD-1, and C57BL/6 mice, we have determined lung CYP2E1-dependent PNP hydroxylase activity, measured covalent binding of [14C]DCE to lung proteins, and estimated formation in vivo of conjugate [C]. We have also determined levels of GSH in the lungs of the three strains of mice under control conditions and after treatment with DCE. In addition, we have determined the lung distribution of formation of the DCE epoxide and evaluated the histopathological alterations ensuing from DCE exposure.

Materials and Methods

Chemicals and Reagents. Chemicals and reagents were obtained from suppliers as follows: 1,1-dichloroethylene (DCE) (>99% purity), phosphoric acid (85%, v/v), and GSH (Aldrich Chemical Co., Montreal, Quebec, Canada); Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA); bovine serum albumin (BSA), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, p-nitrophenol, 4-nitrocatechol, glutaraldehyde (50% aqueous), paraformaldehyde, hydrogen peroxide (30%, v/v), 3,3'-diaminobenzidine tetrahydrochloride, γ-glutamyl glutamate, NADP⁺, and NADPH (Sigma Chemical Co., St. Louis, MO); [14C]DCE (99% pure by gas liquid chromatography, specific activity 11.3 nCi/nmol, (Amersham Pharmacia Biotech, Arlington Heights, IL); Universal scintillation fluid (ICN Chemical Co., Costa Mesa, CA); Spectra-por-3 dialysis tubing, 3500 molecular weight cut-off (Fisher Scientific, Nepean, Ontario, Canada); sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Hamilton, Ontario, Canada); biotinylated goat anti-rabbit IgG, avidin-biotin blocking reagent (Vector Laboratories, Inc., Burlingame, CA); streptavidin-conjugated to horseradish peroxidase (Zymed Laboratories, San Francisco, CA). The DCE epoxide-derived GSH conjugate [C] used as a standard for metabolite identification was synthesized as described (Dowsley et al., 1985). The conjugate glycine-glutaraldehyde-BSA that is used as a blocking...
Strain-Related Bioactivation of 1,1-Dichloroethylene in Mice

In immunohistochemical studies, we have applied a polyclonal antibody raised against a hapten consisting of the chemically synthesized conjugate [C] cross-linked by glutaraldehyde to the carrier protein BSA (Forkert et al., 1997). Previous studies have established that this antibody recognizes [C] as well as sites of binding of the epoxide to cysteine residues of proteins (Forkert, 1999). For the sake of convenience and ease of reporting, we have designated herein the proteins detected by this antibody as conjugate [C], but with the assumption that proteins containing cysteine residues are also detected. Immunohistochemical studies for detection and localization of conjugate [C] were performed in lung tissues from all three strains of mice treated with DCE (125 mg/kg, i.p.) or the vehicle. The immunohistochemical experiments were carried out as described in our previous studies (Forkert, 1999). Briefly, tissues were fixed with 4% paraformaldehyde containing 2% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.3. Immunohistochemical localization of conjugate [C] was performed in paraffin-embedded tissue sections using the avidin-biotin complex technique as described (Forkert, 1999). The distribution of [C] was visualized by development in 3,3′-diaminobenzidine (0.05% in 0.1% hydrogen peroxide in PBS). Tissue sections were then dehydrated, cleared, and mounted.

Histopathology. Lung cytotoxicity in A/J, CD-1, and C57BL/6 mice was assessed at 24 h following DCE treatment (50, 75, 125, and 175 mg/kg, i.p.). Lung tissue was prepared for histopathologic evaluation as previously described (Forkert, 1995), with minor modifications. Lungs were fixed by intratracheal instillation and vascular perfusion through the left ventricle with 4% paraformaldehyde in 0.1 M Sorenson’s phosphate buffer (12.0 mM NaH2PO4, 69.0 mM Na2HPO4), pH 7.4. Tissues were processed and embedded in paraffin, using standard procedures. Lung sections (5 μm) were stained with hematoxylin and eosin.
Statistical Analysis. Data are expressed as mean ± S.D. Statistical analysis was performed by one-way or two-way analysis of variance followed by the Tukey test to identify significant differences between experimental groups (p < 0.05).

Results

Levels of Lung CYP2E1. Hydroxylation of PNP was used as an index of the catalytic activity of the CYP2E1 enzyme in the lungs of A/J, CD-1, and C57BL/6 mice. Our results showed that hydroxylase activity was highest in A/J mice, intermediate in CD-1 mice, and was lowest in C57BL/6 mice (Fig. 2). The levels in the lungs of A/J mice were about 20 and 40% higher than those in the lungs of CD-1 and C57BL/6 mice, respectively.

Covalent Binding of [14C]DCE to Lung Proteins. Covalent binding of [14C]DCE to proteins in lung homogenates was detected in A/J, CD-1, and C57BL/6 mice. Levels of DCE binding to lung proteins in A/J mice were significantly higher than those detected in CD-1 and C57BL/6 mice. Regression analysis of the relationship between PNP hydroxylase activity and covalent binding of [14C]DCE in the lungs of the three strains of mice showed a highly positive correlation between these two parameters (r^2 = 0.9555) such that increased hydroxylase activity coincided with enhanced binding levels.
the supernatant was subjected to reversed-phase HPLC analysis. Aliquots (250 μl) were isolated 1 h after treatment. Cytosolic proteins were precipitated, and 100 μl of the column effluent were collected, and levels of radioactivity were determined. Data are expressed as mean ± S.D. of triplicate determinations from three different cytosolic fractions.

### Table 1

Levels of conjugate [C] in cytosol from lungs of A/J, CD-1, and C57BL/6 mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conjugate [C] (nmol/mg protein)</th>
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<tr>
<td>A/J</td>
<td>0.36 ± 0.04*</td>
</tr>
<tr>
<td>CD-1</td>
<td>0.36 ± 0.03*</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0.12 ± 0.04</td>
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* Significantly different from levels of conjugate [C] in lung cytosol from C57BL/6 mice (p < 0.001).

(Fig. 3). Hence, binding levels were highest in A/J mice, occurred at intermediate levels in CD-1 mice, and were found at lowest levels in C57BL/6 mice.

**Formation of Conjugate [C]**. Representative radiochromatograms of conjugate [C] detected in lung cytosol isolated from the lungs of the three strains of mice are depicted in Fig. 4. The peak for [C] eluted at 7.5 min on the column and was similar to the retention time detected for the synthesized standard (not shown). A smaller peak eluted at 2 to 4 min, and this peak has been identified previously as containing glycolic acid and formaldehyde, both of which are believed to be degradation products of DCE epoxide (Dowsley et al., 1995). The amounts of conjugate [C] detected in cytosolic fractions isolated from the lungs of the three strains of mice are summarized in Table 1. Levels in cytosolic samples from CD-1 and A/J mice were comparable, and both were significantly higher than those from C57BL/6 mice. The level in C57BL/6 mice comprised only about 30% of the amounts detected in CD-1 and A/J mice.

**Glutathione Content**. The GSH contents in the lungs of A/J, CD-1, and C57BL/6 mice were determined to assess whether GSH levels contributed to potential differences in DCE metabolism in the three murine strains. Levels of GSH were similar in the lungs of all three strains of untreated mice (Fig. 5). Treatment of mice with DCE produced significant decreases in GSH levels, and these were comparable in all murine strains tested, with decreases ranging from 40 to 60% of control levels. Hence, no strain-related differences in GSH levels were observed in either untreated or DCE-treated mice.

**Detection and Localization of Conjugate [C] in Lung Tissue**. Studies using immunohistochemical procedures were performed to detect and to localize conjugate [C] in lung tissues from the three strains of mice. Immunohistochemical experiments revealed no specific staining in the lungs of all control untreated mice (Fig. 6a). In tissue sections from the lungs of DCE-treated mice, staining was found mainly in the bronchiolar epithelium and was localized in the Clara cells. The amounts of immunohistochemical staining for conjugate [C] varied in the lungs of C57BL/6, CD-1 and A/J mice. Staining was highest in the bronchiolar epithelium of lungs from A/J mice and was concentrated in the apical cytoplasms of the Clara cells (Fig. 6c). The levels of staining in the bronchiolar epithelium of CD-1 mice were slightly lower than seen in the lungs of A/J mice (Fig. 6b). However, staining in the lungs of C57BL/6 mice was low and confined to the distal apices of the Clara cells (Fig. 6d).

**Histopathology**. Histopathologic observations revealed normal morphology in the lungs of all untreated mice, with the usual distribution of ciliated and nonciliated Clara cells (Fig. 7a). In the experimental groups, mice were administered 50, 75, 125, or 175 mg/kg of DCE. Vacuolated Clara cells were apparent 24 h after treatment of A/J and CD-1 mice with 50 mg/kg DCE, whereas this vacuolization was manifested in only the occasional Clara cell in C57BL/6 mice. Vacuolation of Clara cells was more severe after treatment of A/J (Fig. 7c) and CD-1 (Fig. 7b) mice with 75 mg/kg DCE and was more severe than seen in the lungs of C57BL/6 mice (Fig. 7d). Treatment with 125 and 175 mg/kg produced denudation of airway epithelium in A/J and CD-1 mice, and in many cases, the bronchioles were devoid of an epithelial lining. Treatment of C57BL/6 mice with 125 mg/kg elicited foci of denuded airway epithelium, and an increase of the dose to 175 mg/kg produced marked exfoliation of epithelial cells. Hence, bronchiolar damage was similar in the lungs of A/J and CD-1 mice and was more severe than in the lungs of C57BL/6 mice. Differences in the histopathologic effects elicited by DCE in the three strains of mice were more evident at lower than higher chemical doses.

### Discussion

Our previous studies have produced data establishing a major role for CYP2E1 in DCE metabolism (Lee and Forkert, 1995; Dowsley et al., 1996; Forkert, 1999). Here, we have investigated strain-related differences in CYP2E1 levels in the lungs of A/J, CD-1, and C57BL/6 mice. Our results showed that lung CYP2E1 was present at significantly higher levels in A/J than in either CD-1 or C57BL/6 mice (Fig. 2). In view of the central role of CYP2E1 in DCE metabolism, we reasoned that DCE metabolism is likely to be augmented in A/J mice and diminished in C57BL/6 mice. Our findings showed that covalent binding of [14C]DCE, which represents in part an indirect index of metabolite formation, was significantly higher in A/J mice than in either CD-1 or C57BL/6 mice (Fig. 2). Regression analysis revealed a highly positive correlation between PNP hydroxylase activity and
binding levels in the three strains of mice (Fig. 3), suggesting that the extents of DCE metabolism are linked to levels of lung CYP2E1 available for bioactivation.

Previous studies have confirmed that the metabolites formed from DCE metabolism in vitro and in vivo are the secondary metabolites [B] and [C] (Dowsley et al., 1996; Forkert, 1999). As expected, conjugates [B] and [C] were formed at markedly lower levels under in vivo than in vitro conditions. In this in vivo study, we have found that conjugate [C] was the major metabolite detected in lung cytosol from mice treated with DCE, whereas [B] was formed at negligible levels. These results are in agreement with those reported in our previous in vivo studies (Forkert, 1999), which revealed that conjugate [C] was the major metabolite found in lung cytosol from DCE-treated mice (Fig. 4). Our studies also demonstrated that levels of [C] detected in lung cytosol were significantly higher in A/J and CD-1 mice than in C57BL/6 mice (Fig. 4 and Table 1). These findings indicated that the DCE epoxide is generated to a significantly greater extent in the lungs of A/J and CD-1 mice than in C57BL/6 mice. These discrepancies are likely not due to different magnitudes of conjugation of the epoxide to GSH.

Levels of lung GSH under control conditions were similar in all the three strains of mice (Fig. 5). Moreover, treatment with DCE produced significant decreases in GSH levels that were comparable in all three strains of mice. Hence, the quantities of conjugate [C] detected in lung cytosol from the DCE-treated mice most likely represented the amounts of DCE epoxide generated and were not due to differing rates of GSH conjugation. However, the possibility exists that the extent of generation of the GSH conjugates may be influenced also by potential differing rates of DCE distribution to the lungs of the three strains of mice.

Selective damage of Clara cells is induced by exposure to a broad spectrum of chemicals, including trichloroethylene (Forkert et al., 1985), naphthalene (Mahvi et al., 1977), carbon tetrachloride (Boyd et al., 1980), 4-ipomeanol (Boyd, 1977), bromobenzene (Forkert, 1985) and DCE (Forkert and Reynolds, 1982). It has been postulated that this cell-specific lesion is mediated by reactive intermediates produced in situ within this cell type. This concept emanated from studies showing that the cell-specific lesions are associated with preferential covalent binding of metabolites and localization of high concentrations of P450 enzymes within the Clara cells.
(Boyd, 1977; Serabjit-Singh, 1980; Forkert et al., 1990). Relevant in the context of DCE metabolism are results from previous lung cell isolation studies showing that binding of [14C]DCE is 4-fold higher in cell fractions enriched in Clara cells than in fractions enriched in alveolar type II cells or in mixed cells from whole lung (Forkert et al., 1990). Also pertinent is the finding that CYP2E1 resides preferentially within the Clara cells (Forkert, 1995). In more recent immunohistochemical studies, conjugate [C] was localized predominantly within the Clara cells and was markedly diminished in lung sections from mice pretreated with DASO₂ to inhibit CYP2E1 (Forkert, 1999). In this investigation, the immunohistochemical experiments produced results confirming preferential staining of the Clara cells in DCE-treated mice (Fig. 6). This staining was highest in lung sections from A/J mice, and was lowest in those from C57BL/6 mice. Intermediate amounts of staining were found in bronchioles from CD-1 mice and were consistent with levels detected in this murine strain in previous studies (Forkert, 1999). These results suggested that the DCE epoxide is formed and conjugated within the Clara cells. It should be reiterated that, since our antibody also detects the DCE epoxide bound to cysteine-containing proteins, the amounts of immunohistochemical staining seen in the Clara cells should represent signals for both these proteins and [C]. Importantly, the findings indicated that the epoxide is formed to the greatest extent in the Clara cells of A/J mice, is formed at intermediate levels in those of CD-1 mice, and is formed at relatively low levels in those of C57BL/6 mice.

Evaluation of the relative severities of Clara cell toxicity in A/J, CD-1, and C57BL/6 mice was facilitated by our dose-response studies using doses of 50 to 175 mg/kg DCE. Histopathologic observations of lung sections from DCE-treated mice revealed that the severities of bronchiolar Clara cell damage was incremental with dose in all strains of mice. However, the onset of Clara cell cytotoxicity differed in the three strains of mice. Vacuolization of Clara cells was evident following treatment of A/J and CD-1 mice with 50 mg/kg DCE. Damage to Clara cells was augmented when the dose was increased to 75 mg/kg DCE, and at doses of 125 to 175 mg/kg, injury was severe, resulting in extensive areas of airway devoid of epithelial lining cells. In C57BL/6 mice, the
cytotoxic effects of DCE exposure were less severe, and vacuolated Clara cells were not observed at the low dose of 50 mg/kg DCE. Vacuolation of a few Clara cells was evident following treatment with a DCE dose of 75 mg/kg (Fig. 7d), and epithelial cell sloughing was observed at doses of 125 and 175 mg/kg. Treatment with the highest DCE dose of 175 mg/kg evoked airway lesions that were comparable in all three strains of mice. These results indicated that Clara cell cytotoxicity was relatively more severe in A/J and CD-1 mice than in C57BL/6 mice. These data coincided with the extents of covalent binding of DCE to lung proteins; the levels in A/J mice amounted to about 150% of those detected in C57BL/6 mice (Fig. 2). In this context, previous studies have demonstrated that magnitudes of DCE binding are proportional to the amounts of DCE administered and are associated with severities of Clara cell cytotoxicity (Moussa and Forkert, 1992). Taken together, our results are consistent with these previous reported findings and confirmed the higher levels of DCE metabolism and enhanced susceptibility of A/J mice, relative to those manifested in C57BL/6 mice. The results with CD-1 mice suggested that this outbred murine strain might be classified tentatively as a strain susceptible to cytotoxicity induced through activation of CYP2E1-selective substrates.

It is well established that the liver is highly responsive to the inducing effects of certain chemicals including ethanol, acetone, isopropanol, and pyridine (Coon and Koop, 1987; Kaul and Novak, 1987; Forkert et al., 1994). Severalfold increases in hepatic CYP2E1 are achieved, depending on the inducing chemical and route of exposure (Forkert et al., 1991, 1994). In contrast, the lung is refractory to induction by chemicals that are prototypic CYP2E1 inducers in the liver (Ronis et al., 1996). In our hands, significant increases in the CYP2E1 enzyme were not observed in the lungs of mice treated with acetone, pyridine, ethanol, isoniazid, and cigarette smoke (unpublished data). The results of this study have demonstrated that strain-related differences in CYP2E1 levels manifested in the lungs of A/J, CD-1, and C57BL/6 mice produced amounts of DCE metabolism that are significantly different from one another and was especially evident in A/J and C57BL/6 mice, strains in which CYP2E1 levels are the most discrepant (Fig. 2). These findings suggested that, although the quantity of CYP2E1 in the lungs of A/J mice was only about 40% higher than in C57BL/6 mice, this difference was of sufficient magnitude to produce a significant difference in DCE metabolism in these two strains of mice. This is presumably due to the observation that CYP2E1 is localized primarily in the Clara cells, and hence a 40% increase within a single cell type represented a substantial amount. This assumption is underscored by data showing that Clara cells are more susceptible to DCE-induced cytotoxicity than hepatocytes, even though CYP2E1 content in the lung comprises only a small fraction of the quantities in the liver. These findings indicated that the strains of mice used in this study are appropriate models for investigating metabolism of CYP2E1 substrates. An additional advantage to using these models is the lack of requirement for maneuvers that produce metabolic perturbations in related enzyme systems including GSH and/or other P450 enzymes.

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Send reprint requests to: Dr. Poh-Gek Forkert, Department of Anatomy and Cell Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6. E-mail: forkertp@post.queensu.ca