Oxidation of Vinyl Carbamate and Formation of 1,N6-Ethenodeoxyadenosine in Murine Lung

Poh-Gek Forkert, Martin Kaufmann, Gordon Black, Raymond Bowers, Heidi Chen, Kathy Collins, Ashish Sharma, and Glenville Jones

Departments of Anatomy and Cell Biology (P.-G.F., G.B., K.C., A.S.), Biochemistry (M.K., G.J.), and Chemistry (R.B.), Queen’s University, Kingston, Ontario, Canada; and Food Research Division, Bureau of Chemical Safety, Health Canada, Ottawa, Ontario, Canada (H.C.)

Received November 9, 2006; accepted February 9, 2007

ABSTRACT:

Vinyl carbamate (VC) is derived from ethyl carbamate, a carcinogen formed in fermentation of food and alcoholic products. We have undertaken studies to test the hypothesis that an epoxide generated from VC oxidation leads to formation of 1,N6-ethenodeoxyadenosine (εdAS). We have developed approaches using liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry for identification and quantitation of εdAS. Scanning and fragment ion analyses confirmed the identity of εdAS based on the molecular ion [M + H]+ m/z 276 and the specific fragment ion m/z 160. Chemical oxidation of VC in reactions containing 2′-deoxyadenosine produced εdAS with 1H NMR, chromatographic, and mass spectral characteristics identical to those of the authentic εdAS, suggesting DNA alkylation by the VC epoxide. Subsequent studies evaluated formation of εdAS in incubations of murine lung microsomes or recombinant CYP2E1 with VC. The formation of εdAS in incubations of lung microsomes or recombinant CYP2E1 with VC was dependent on protein concentrations, CYP2E1 enzyme levels, and incubation time. The rates of εdAS formation were highly correlated with VC concentrations. Peak rates were produced by lung microsomes and recombinant CYP2E1 at 3.0 and 2.5 mM VC, respectively. In inhibitory studies, incubations of VC were performed using lung microsomes from mice treated with the CYP2E1 inhibitor diallyl sulfone (100 mg/kg, p.o.). Results from these studies showed significantly decreased εdAS formation in microsomes incubated with VC, with an inhibition of 70% at 3.0 mM. These findings suggested that CYP2E1 is a major enzyme mediating VC oxidation, leading to the formation of a metabolite that alkylates DNA to form the εdAS adduct.

Vinyl carbamate (VC) is a metabolite and structural analog of ethyl carbamate (EC), a byproduct of fermentation found in alcoholic beverages and a variety of food products including yogurt, bread, soya sauce, cheese, and apple cider vinegar (Ough, 1976; Battaglia et al., 1990; Zimmerli and Schlatter, 1991). The highest concentrations of EC (100–20,000 ng/g) were detected in alcoholic beverages including stone-fruit brandies (plums, apricots, or cherries), sherry, and bourbon. It is also found as a natural constituent in tobacco at concentrations ranging from 310 to 375 ng/g (Schmeltz et al., 1978).

It has been estimated that the mean daily intake of EC from various food items in adults is approximately 10 to 20 ng/kg body weight (Zimmerli and Schlatter, 1991). The carcinogenicity of EC and the formation of lung adenomas in animals were first reported in 1943 (Nettleship et al., 1943). Subsequent studies in mice demonstrated that EC induces tumors in a variety of tissues including the skin, liver, lung, mammary gland and lymphoid tissue (Mirvish, 1968). A long latent period of about 1 year was generally required for tumor development. An exception was in the lung, where tumors were formed more rapidly and were manifested in about 2 to 6 months (Mirvish, 1968; Shinkin and Stoner, 1975). A similar spectrum of tumors is induced by both EC and VC. However, comparative studies revealed that VC is more carcinogenic than EC; lung tumors induced by VC are 20- to 50-fold greater in number than are induced by EC, depending on the route of exposure (Dahl et al., 1978, 1980). Moreover, VC but not EC is mutagenic to Salmonella typhimurium strains TA 1535 and TA100 (Dahl et al., 1978, 1980; Leithauser et al., 1990). However, more recent studies have identified the mutagenicity of EC, using the transgenic Muta Mouse and lacZ as the reporter gene (Williams et al., 1998). Nevertheless, these findings indicated that VC has greater carcinogenic...
potential than EC, and further that the lung is an important and possibly a primary target tissue.

Previous studies suggested that the carcinogenicity of EC is associated with its metabolism to VC and subsequently to VC epoxide (VCO), a metabolite that has been proposed to be the ultimate carcinogenic species (Dahl et al., 1978, 1980; Ribovich et al., 1982). Other studies using human liver microsomes identified oxidation of both EC and VC and implicated CYP2E1 in their metabolism (Guengerich and Kim, 1991; Guengerich et al., 1991). The results of more recent studies in murine and human lung also supported oxidation of EC and VC by the CYP2E1 enzyme (Lee and Forkert, 1997, 1999; Forkert et al., 2001). Metabolism of both EC and VC generated 1,N\(^6\)-ethenodeoxyguanosine and 3,N\(^6\)-ethenocytidine adducts in hepatic RNA and 7-(2-oxoethyl)guanine adducts in hepatic DNA of rats and mice (Ribovich et al., 1982; Miller and Miller, 1983; Scherer et al., 1986). In subsequent studies, VCO was synthesized and found to react with calf thymus DNA to form two major guanine adducts, N\(^6\)-deoxyadenosine, 1,N\(^6\)-ethenodeoxyguanosine, and one minor adenine adduct, 1,N\(^6\)-ethenodeoxyadenosine (edAS) (Park et al., 1993). More recent studies have identified the formation of edAS and 3,N\(^6\)-ethenodeoxycytidine in liver and lung DNA of several strains of mice treated with EC or its metabolites (Fernando et al., 1996). These data supported the proposal that VCO is a reactive species responsible for mediating DNA alkylation, leading to the formation of DNA adducts.

In this investigation, we have extended the findings of previous studies and adopted an in vitro approach for experiments designed to further elucidate the metabolism of VC and the mechanisms that mediated DNA alkylation. We have undertaken studies to test the hypothesis that oxidation of VC by CYP2E1 leads to the formation of an epoxide that alkylates DNA, resulting in the formation of edAS in murine lung. Using an approach based on high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), we have performed studies to determine whether chemical oxidation of VC in reactions supplemented with 2'-deoxyadenosine (dAS) leads to edAS formation. The proposed scheme for this metabolic pathway is illustrated in Fig. 1. We have subsequently carried out studies to investigate dAS formation in incubations of murine lung microsomes with VC. The involvement of CYP2E1 in VC metabolism was also investigated using incubations of recombinant rat CYP2E1 (rCYP2E1) and lung microsomes from mice treated with the CYP2E1 inhibitor, diallyl sulfone (DASO\(_2\)). Our results demonstrated that VC oxidation has a critical role in dAS formation. The edAS adduct was also generated from VC in incubations of lung microsomes and rCYP2E1. These results demonstrated that VC oxidation, mediated mainly by CYP2E1, and subsequent DNA alkylation leads to production of edAS in murine lung.

Materials and Methods

Chemicals and Reagents. Chemicals were purchased from suppliers as follows: 2'-deoxyadenosine, 1,N\(^6\)-ethenodeoxyadenosine, NADPH, K\(_2\)HPO\(_4\), and BSA, Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada); KCl, EDTA, and acetonitrile, Fisher Scientific (Ottawa, ON, Canada). Synthesis of DASO\(_2\) was carried out by Colour Your Enzyme (Bath, ON, Canada). Recombinant rat CYP2E1 expressed in human \(\beta\)-lymphoblastoid microsomes was obtained from BD Biosciences Discovery Labware (Bedford, MA). All solvents used for sample preparation and HPLC and LC-MS analyses were of HPLC grade and were purchased from Caledon Laboratories (Georgetown, ON, Canada). Details and results of the synthesis of VC and 2'-deoxy-2-deuterodeoxoadenosine (DDAS) are described in the Supplemental Data.

Chemical Oxidation of VC and Synthesis of edAS. Oxidation of VC was performed according to the following method. VC (0.5 mM) was added to \(m\)-chloroperoxybenzoic acid (mCPBA) (0.51 mM) dissolved in CH\(_2\)Cl\(_2\) (3.0 ml). The reaction mixture was stirred for approximately 25 min. During this time a saturated solution of dAS (0.2 mM) was prepared in water (3.0 ml), added to the reaction mixture, and stirred vigorously for 12 h at room temperature. The aqueous layer was then separated and the organic layer was extracted with water (three times, 3.0 ml). The combined aqueous layers were washed with ether (four times, 10 ml) to remove unreacted mCPBA and the side product \(m\)-chlorobenzoic acid. The aqueous layer was lyophilized in vacuo and resuspended in water containing 5% acetonitrile. Controls, comprising samples in which mCPBA or VC and mCPBA were omitted, were processed in parallel with the experimental samples and were subjected to identical protocols. All the samples were then subjected to HPLC analysis for purification of the edAS adduct and subsequent analyses by LC-MS, LC-MS/MS, and \(^1\)H NMR.

Animal Treatment. Female CD-1 mice, weighing 25 to 28 g, were purchased from Charles River Canada (St. Constant, QC, Canada). The mice were maintained on a 12-h light/dark cycle and were allowed free access to water and food (Mouse Diet 5015; PMI Nutrition International Inc., Brentwood, MO). The mice were acclimatized to laboratory conditions for 1 week before being used for experiments. For inhibition studies, mice (\(n=40\)) were treated with 100 mg/kg DASO\(_2\) (p.o.) in water, whereas control mice (\(n=40\)) were treated with only water. After 2 h, the mice were sacrificed, lungs tissues excised, frozen in liquid nitrogen, and stored at \(-80^\circ\)C.

Preparation of Microsomes. Lungs from 40 mice were pooled for each microsomal sample. This pooling is essential for obtaining sufficient tissue for preparation of the lung microsomes. Microsomes were prepared by differential
centrifugation as described previously (Forkert et al., 2006). Lung tissue was minced and homogenized in 4 volumes of ice-cold phosphate-buffered KCl (139 mM KCl, 100 mM K$_2$HPO$_4$, 1.5 mM EDTA; pH 7.4). The homogenate was subjected to centrifugation at 12,000g for 20 min at 2°C. The supernatant was then centrifuged at 105,000g for 60 min. The final pellet was homogenized using 400 µl of buffer, and aliquots were frozen in liquid nitrogen and stored at −80°C. Microsomal protein concentrations were determined by using the Bradford assay, using bovine serum albumin as the standard (Bradford, 1976).

**Formation of edAS in Lung Microsomal Incubations.** In the lung microsomal incubations, reaction mixtures in a total volume of 250 µl contained microsomal protein in 0.1 M phosphate buffer, pH 7.4, 1.0 mM NADPH, and 12.5 mM dAS. The incubations were carried out at 37°C. After the incubation, the samples were placed on ice and 250 µl of ice-cold acetonitrile was added. The proteins were precipitated and removed by centrifugation at 15,000g for 3 min. Experiments for determination of the protein concentration-curve were carried out with VC (1.0 mM), an incubation time of 20 min, and 0 to 5 mg of microsomal protein per ml. The time course experiments were performed with VC (1.0 mM) and 2.0 mg/ml microsomal protein, using incubation times of 0 to 125 min. The concentration studies were carried out with 0 to 3.0 mM VC, 2 mg/ml microsomal protein, and an incubation time of 40 min.

**Formation of edAS by rCYP2E1.** Rates of formation of edAS by CYP2E1 were determined in incubations containing rCYP2E1 coexpressed with NADPH-cytochrome P450 reductase. Reaction mixtures in a final volume of 250 µl of 0.1 M phosphate-buffered saline, pH 7.4, contained 10 pmol of rCYP2E1, 1.0 mM VC in water, and were preincubated for 3 min at 37°C. The reaction was initiated by addition of 1.0 mM NADPH and the incubations were carried out for an additional 20 min. Reactions were terminated by immersion into liquid nitrogen. To determine edAS formation as a function of CYP2E1 enzyme levels, incubations of VC (1.0 mM) were performed with amounts of enzyme ranging from 0.25 to 60 pmol and an incubation time of 20 min. Time course studies were carried out in the incubations of VC (1.0 mM) with rCYP2E1 (10 pmol), using incubation times of 0 to 80 min. Concentration-response studies were performed using 10 pmol of rCYP2E1, an incubation time of 20 min, and VC concentrations ranging from 0 to 4.0 mM.

**Formation of edAS by Lung Microsomes from DASO$_2$-Treated Mice.** Incubations with VC were performed with lung microsomes from mice that were treated 2 h previously with DASO$_2$ (100 mg/kg p.o.) in water. Incubations with lung microsomes from mice treated with equivalent volumes of the vehicle served as controls. Reaction mixtures in a final volume of 250 µl contained microsomal protein (2.0 mg/ml) in 0.1 M phosphate buffer, pH 7.4, 1.0 mM NADPH, 12.5 mM dAS, and a range of VC concentrations (0–3.0 mM). The incubations were carried out at 37°C for 40 min.

**HPLC Purification of edAS.** Samples generated from the chemical synthesis of VCO and the rCYP2E1 incubations were redissolved in 95% water/acetonitrile and subjected to HPLC purification against the standards, dAS, and edAS. HPLC purification was conducted on an Alliance 2695 separations module (Waters, Milford, MA) using a Waters 250 × 4.6 mm C$_18$ column (Beckman Coulter, Fullerton, CA). The gradient was started at 95:5% water/acetonitrile and brought to 86:14% over 16 min at a flow rate of 0.4 ml/min. The mobile phase was returned to its initial conditions of 95:5% water/acetonitrile over 3 min at a flow rate of 0.4 ml/min and subsequently over 6 min at a flow rate of 1 ml/min. The duration of each run was 25 min. A 5.0-min time delay between samples was included to ensure proper reequilibration of the HPLC column. Under these conditions, the authentic edAS and the edAS adduct eluted at 18.93 min, whereas dAS eluted at 16.87 min. Fractions that possessed spectral and/or chromatographic properties identical to those of the authentic edAS were collected, and samples of each type were pooled separately and evaporated to dryness.

**Identification of DNA Adducts.** The pooled fractions from the chemical synthesis, lung microsomal incubations, and rCYP2E1 incubations were redissolved in 95:5% water/methanol and subjected to LC-MS/MS analysis using an HPLC system similar to that described above, connected in-line with a Quattro Ultima mass spectrometer (Waters) in positive ion mode. Separation was facilitated by a gradient solvent system starting at 95:4:1% water/methanol/1% glacial acetic acid and terminating at 59:40:1 over 25 min on a Zorbax SB-C$_18$ column (3.5 µm, 2.1 × 150 mm; Agilent Technologies, Palo Alto, CA), using a flow rate of 0.12 ml/min. Initially, the instrument was set to establish the molecular weight of the fractions in scanning or MS1 mode (set to scan from 50 to 600 m/z), against the authentic dAS, edAS, and dAS (synthesis described in Supplemental Data). MS2 analysis was subsequently used to establish the fragmentation pattern of the selected MS1 parent, using collision-induced dissociation with argon gas at a pressure of 30 mbar and a collision energy of 20 eV, in which specific molecular fragments were detected over a range of 50 to 450 m/z. The identification of the DNA adduct in our experimental preparations was based upon a comparison of UV spectral properties, chromatographic retention time, and characteristic ions from MS1 and MS2, with the authentic edAS.

**Quantitative Analysis of DNA Adducts.** Samples generated from incubations of lung microsomes or rCYP2E1 were divided into two aliquots. DDAS (100 ng) was dispensed into one aliquot of each sample, as an internal standard. The aliquots were analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode, in which the abundance of the characteristic MS2 ions (D–F) from selected parent MS1 ions (A–C) were measured for dAS (A), the authentic edAS used as a standard (B), and DDAS used as the internal standard (C).

**Results**

**Identification of edAS by LC-MS/MS.** In this study, we have developed a LC-MS/MS protocol to establish the identity of the DNA adduct formed in incubations of VC with lung microsomes and rCYP2E1 as well as under conditions of VC oxidation using mCPBA. The MS1 mass spectral analysis of dAS revealed the molecular ion m/z 252 [M + H]$^+$ (Fig. 2A). Similar analysis for the authentic edAS, which was used as the standard, was identified by the molecular ion m/z 252 [M + H]$^+$. The pooled fractions from the chemical synthesis, lung microsomal incubations, and rCYP2E1 incubations were redissolved in 95:5% water/methanol and subjected to LC-MS/MS analysis using an HPLC system similar to that described above, connected in-line with a Quattro Ultima mass spectrometer (Waters) in positive ion mode. Separation was facilitated by a gradient solvent system starting at 95:4:1% water/methanol/1% glacial acetic acid and terminating at 59:40:1 over 25 min on a Zorbax SB-C$_18$ column (3.5 µm, 2.1 × 150 mm; Agilent Technologies, Palo Alto, CA), using a flow rate of 0.12 ml/min. Initially, the instrument was set to establish the molecular weight of the fractions in scanning or MS1 mode (set to scan from 50 to 600 m/z), against the authentic dAS, edAS, and dAS (synthesis described in Supplemental Data). MS2 analysis was subsequently used to establish the fragmentation pattern of the selected MS1 parent, using collision-induced dissociation with argon gas at a pressure of 30 mbar and a collision energy of 20 eV, in which specific molecular fragments were detected over a range of 50 to 450 m/z. The identification of the DNA adduct in our experimental preparations was based upon a comparison of UV spectral properties, chromatographic retention time, and characteristic ions from MS1 and MS2, with the authentic edAS.

**Quantitative Analysis of DNA Adducts.** Samples generated from incubations of lung microsomes or rCYP2E1 were divided into two aliquots. DDAS (100 ng) was dispensed into one aliquot of each sample, as an internal standard. The aliquots were analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode, in which the abundance of the characteristic MS2 ions from selected parent MS1 ions (A–C) were measured for dAS (A), the authentic edAS used as a standard (B), and DDAS used as the internal standard (C).
m/z 276 (Fig. 2B). The synthesized DDAS, applied as the internal standard, was characterized by the production of the molecular ion m/z 277, which accounts for the introduction of a single deuterium atom in the authentic 277, which was characterized by the production of the molecular ion m/z 276 (Fig. 2B). The synthesized DDAS, applied as the internal standard (m/z 277→160), was produced in the presence of lung microsomes (A and D), in incubations of VC with rCYP2E1 (B and E), or synthesized by VC oxidation, using mCPBA, in the presence of dAS (C and F).

Fig. 3. Mass spectral analyses of chemical and biological samples for formation of ezAS. The ezAS adduct was identified on the basis of its characteristic MS1 spectra (A–C) and MS2 fragmentation patterns (D–F) in positive ion mode. The ezAS adduct was generated in incubations of VC with lung microsomes (A and D), in incubations of VC with rCYP2E1 (B and E), or synthesized by VC oxidation, using mCPBA, in the presence of dAS (C and F).

The slight discrepancy between the retention time of the authentic ezAS and the adduct detected by the same MRM transition in the microsomal and rCYP2E1 incubations (0.32–0.33 min) is due to the presence of excess dAS in the sample, even after an initial HPLC purification step before LC-MS/MS. Some contaminating dAS (m/z 252) in the prepurified adduct sample derived from incubations with rCYP2E1 was also evident (Fig. 4B). Taken together, the chromatographic retention times, and characteristic MS1 and MS2 ions confirmed that ezAS is produced in incubations of VC and dAS with both lung microsomes and rCYP2E1.

Chemical Oxidation of VC and Synthesis of ezAS. The DNA adduct produced from dAS in the presence of VC oxidized with mCPBA produced identical MS1 and MS2 spectra (Fig. 3, C and F), similar to those for the authentic ezAS (Fig. 2, A and B). The spectra were also similar to those obtained in the incubations of VC with lung microsomes (Fig. 3, A and D) and with rCYP2E1 (Fig. 3, B and E). The chromatographic retention time for ezAS formed under the oxidative conditions of VC was 18.60 min, and was similar to those identified for the adduct produced in the lung microsomal and rCYP2E1 incubations, which were 18.61 min and 18.76 min, respectively (Fig. 4B).

The identity of the ezAS adduct formed via chemical oxidation of VC in the presence of dAS was confirmed by the spectroscopic data. ¹H NMR (600 MHz, D₂O): 9.08 (1H, s, H₂), 8.37 (1H, s, H₈), 7.94 (1H, s, H₁₀ or H₁₁), 7.53 (1H, s, H₁₀ or H₁₁), 6.54 (1H, t, J = 6.6 Hz, H₅), 4.10 (1H, m, H₄), 3.76 (1H, dd, J = 12.6, 3.4 Hz, H₃), 3.70 (1H, dd, J = 12.6, 4.7 Hz, H₃), 2.87 (1H, pentet, J = 13.8, 6.8 Hz, H₂), 2.55 (1H, m, H₂). These spectroscopic data are in agreement with those obtained for the authentic ezAS. ¹H NMR (400 MHz,
D₂O): 9.10 (1H, s, H₂), 8.42 (1H, s, H₈), 7.97 (1H, s, H₁₀ or H₁₁), 7.58 (1H, s, H₁₀ or H₁₁), 6.59 (1H, t, J \( /H₁₀05\) 6.6 Hz, H₁), 4.70 (1H, m, H₃), 4.19 (1H, m, H₄), 3.85 (1H, dd, J = 12.5, 3.5 Hz, H₅₀), 3.79 (1H, dd, J = 12.5, 4.9 Hz, H₅₁), 2.94 (1H, pentet, J = 14, 6.8 Hz, H₂ₐ), 2.63 (1H, dd, J = 14, 6.3, 3.9 Hz, H₂₅₂).

Formation of edAS in Lung Microsomal Incubations. Incubations of lung microsomes with VC and NADPH showed that edAS formation was dependent on protein concentrations ranging from 0 to 5 mg of microsomal protein, 1.0 mM VC, and an incubation time of 20 min (A). Time-dependent studies were performed using 2.0 mg of microsomal protein, 1.0 mM VC, and incubation times of 0 to 125 min (B). Concentration-dependent studies were carried out using 2.0 mg/ml microsomal protein, an incubation time of 40 min, and VC concentrations ranging from 0 to 3.0 mM (C). All incubations contained 1.0 mM NADPH and were carried out at 37°C.

Formation of edAS in Lung Microsomal Incubations. Incubations of lung microsomes with VC and NADPH showed that edAS formation was dependent on protein concentrations ranging from 0 to 5.0 mg/ml (Fig. 5A). Peak adduct formation was found in incubations containing 3.0 mg of microsomal protein. In time-dependent studies, edAS production from VC was incremental from 0 to 60 min. Prolongation of the incubation time to 125 min resulted in no further increase in edAS production but rather resulted in a decline (Fig. 5B). Based on the data obtained from the protein concentration and time course studies, microsomal incubations with a range of VC concentrations were carried out under conditions of linearity, and were performed with 2.0 mg/ml microsomal protein and an incubation time of 40 min. The rates of edAS formation were highly correlated \( (R^2 = 0.9940) \) with VC concentrations (0.25–3.0 mM) used in the microsomal incubations (Fig. 5C). The peak rate (210.8 ± 4.8 pmol/min/nmol P₄₅₀) of edAS production in the incubations was achieved at a VC concentration of 3.0 mM.

Formation of edAS by rCYP2E1. Formation of edAS was detected in incubations of rCYP2E1 with VC. Production of edAS was dependent on rCYP2E1 concentrations of 2.5 to 40 pmol used in the incubations (Fig. 6A). Although maximal edAS formation was achieved in incubations containing 40 pmol of rCYP2E1, saturation was found at about 20 pmol of enzyme. In time course experiments, edAS formation was dependent on the duration of the incubations, and was incremental from 0 to 30 min (Fig. 6B). Adduct formation...
reached saturation at incubation times of 30 to 60 min and declined at 80 min. On the basis of these data, incubations of rCYP2E1 with a range of concentrations of VC (0.25–4.0 mM) were carried out under conditions of linearity, and were performed for 20 min, using 10 pmol of rCYP2E1 enzyme. The rates of edAS production increased between VC concentrations of 0.25 to 2.5 mM, achieved saturation at a concentration of 2.5 mM (207.7 ± 21.7 pmol/min/10 pmol rCYP2E1), and remained at a plateau from 2.5 to 4.0 mM. The results demonstrated a high correlation ($R^2 = 0.9108$) between the rates of edAS formation and the concentrations of VC used in the incubations (Fig. 6C).

**Formation of edAS by Lung Microsomes from DASO2-Treated Mice.** Incubations with VC (0–3.0 mM) were also carried out with lung microsomes from control mice and mice treated with DASO2 (100 mg/kg p.o.). In incubations of control lung microsomes with VC, the levels of edAS formation were concentration-dependent and were maximal in reactions containing the highest VC concentration of 3.0 mM (Fig. 7). Regression analysis revealed a high correlation ($R^2 = 0.9510$) between the VC concentrations used in the incubations and magnitudes of edAS formation. In incubations of lung microsomes from DASO2-treated mice, the levels of edAS formation were also concentration-dependent but were lower than those in incubations using microsomes from the untreated controls. Regression analysis also showed a high correlation ($R^2 = 0.9883$) between VC concentrations and production of edAS. Comparative analysis of edAS levels in the lung microsomal incubations revealed significant decreases in all the incubations containing microsomes from DASO2-treated mice, relative to the controls. The extent of inhibition by DASO2 was similar regardless of the VC concentrations used in the microsomal incubations; a decrease of approximately 70% of the control levels was detected for all VC concentrations.

**Discussion**

This study has produced definitive physicochemical data that have validated our working hypothesis that oxidation of VC leads to the formation of VCO that can alkylate DNA to form edAS (Fig. 1). We have adopted an approach using LC-MS and LC-MS/MS to investigate formation of edAS from VC. The identification of edAS was confirmed based on the MS1 (m/z 276) and MS2 (m/z 160) ions (Figs. 2, B and E; and Fig. 3), as well as the chromatographic retention times of the samples (Fig. 4). A critical component of this study was the synthesis of the internal standard DDAS ([Fig. 2, C and F], which was integral to quantitation of the formation of edAS under various experimental conditions. In conjunction with NMR analysis, the results have provided validation for the application of the LC-MS and LC-MS/MS approach for qualitative and quantitative characterization of the edAS adduct.

An important objective of this study was to confirm that oxidation of VC leads to the production of a metabolite, presumably the reactive VCO, which reacts with DNA to form edAS (Fig. 1). We have carried out chemical experiments in which VC was oxidized with mCPBA and then reacted with dAS. The DNA adduct produced from this reaction demonstrated mass spectral (Fig. 3, C and F) and chromatographic properties (Fig. 4B) similar to those of the authentic edAS (Fig. 2, B and E), thus confirming that edAS was formed. In the biological experiments, chromatographic and mass spectral analyses of samples from the lung microsomal incubations supplemented with DASO2 confirmed that the edAS adduct was generated from VC in a manner that was dependent on protein concentrations (Fig. 5A) and incubation time (Fig. 5B). The production of edAS was highly correlated ($R^2 = 0.9940$) with VC concentrations (Fig. 5C). These results have established the ability of lung microsomes to catalyze P450-dependent formation of edAS in our experiments. Furthermore, our results have shown that edAS production in incubations of VC with rCYP2E1 was dependent on enzyme concentrations (Fig. 6A) and incubation time (Fig. 6B), and was highly correlated ($R^2 = 0.9108$) with VC concentrations (Fig. 6C). Taken together, these findings have provided evidence to support a role for CYP2E1 in VC oxidation and the subsequent production of edAS (Fig. 1).

Previous studies have identified the garlic constituent DASO2 as an inhibitor of the CYP2E1 enzyme in the lungs of mice (Forkert et al., 1996). Treatment of mice with DASO2 (100 mg/kg p.o.) elicited a 75% inhibition of lung p-nitrophenol (PNP) hydroxylation, a catalytic activity associated with CYP2E1. The loss of PNP hydroxylase activity coincided with a decrease in immunodetectable lung microsomal CYP2E1 protein. Hydroxylation of chlorzoxazone, a prototypic substrate for the CYP2E1 enzyme, was decreased by 60% in lung microsomes from mice treated with DASO2 (100 mg/kg p.o.) (Simmonds et al., 2004a). The mechanism of CYP2E1 inactivation has been ascribed to formation of an epoxide (1,2-epoxypropyl-3,3′-sulfonylethene) from oxidation of DASO2, leading, in part, to production of the heme adduct N-alkylprotoporphyrin IX in incubations of lung microsomes or recombinant rat CYP2E1 (Forkert et al., 2000; Black et al., 2006). These findings implicated CYP2E1 in the metabolism of DASO2, resulting in CYP2E1 inactivation and inhibition of the metabolism of CYP2E1 substrates. Here, we have carried out inhibitory studies using DASO2 to verify that CYP2E1 catalyzes the formation of edAS under biological conditions. The results showed significant inhibition of edAS formation in incubations of VC (0.25–3.0 mM) with lung microsomes from DASO2-treated (100 mg/kg p.o.) mice (Fig. 7). Irrespective of the amounts of VC used in the incubations, the extent of inhibition was approximately 70% of the corresponding control levels, suggesting that this was the maximum extent inhibitable by DASO2. Taken together, these data suggested that CYP2E1 is responsible, in large measure, for the production of edAS in the lung microsomal incubations.

Although our CYP2E1 inhibition studies with DASO2 supported the role of this P450 in catalyzing the formation of VCO and subsequently edAS, the specificity of the inhibitory effect of DASO2 on P450 enzymes must also be addressed. We have previously determined the effects of DASO2 on the rates of PNP hydroxylation by rCYP2E1 and recombinant goat CYP2F3 (rCYP2F3). The results...
showed that incubation of rCYP2E1 and rCYP2F3 with DASO decreased hydroxylase activities by 90 and 70%, respectively (Simmonds et al., 2004a). Moreover, incubation of DASO with rCYP2E1 and rCYP2F3 decreased hydroxylation of chlorozoxazone by 90 and 77%, respectively. These findings demonstrated that DASO inhibited both CYP2E1 and CYP2F3, and additionally suggested the possibility that the CYP2F enzyme, in addition to CYP2E1, may be involved in VC metabolism. In light of these data and our current observation that 30% of the activity remained after maximal inhibition with DASO (Fig. 7), we carried out preliminary experiments to determine whether recombinant mouse CYP2F2 or recombinant rat CYP2F4 could also be involved in VC metabolism, leading to edAS formation. The CYP2F enzyme is expressed selectively in the lung, with relatively little expression in the liver (Hakkola et al., 1994; Shultz et al., 1999). We have incubated VC (1.0 mM) and dAS (12.5 mM) with amounts of recombinant mouse CYP2F2 and recombinant rat CYP2F4 ranging from 0.25 to 40 pmol, using procedures described previously (Simmonds et al., 2004b). Our chromatographic and mass spectral analysis failed to detect formation of the edAS adduct by either the rCYP2F2 or the rCYP2F4 enzyme. Taken together, these findings suggested that CYP2E1 plays a major role in the production of edAS in the lung microsomal incubations. These results do not, however, preclude the participation of other P450 isofoms, as yet unidentified, in VC metabolism.

The human relevance of the studies with VC and EC has a historical perspective. EC was used as a cosolvent for analgesic and sedative drugs in Japan between 1950 and 1975, and it was estimated that the total dose of EC administered to a 60-kg patient was approximately 0.6 to 3.0 g (Nomura, 1975). This period represented 25 years during which millions of humans were administered “the largest doses of a pure carcinogen that is on record” (Miller, 1991, p. 1323). Today, human exposures occur inadvertently via the consumption of fermented foods and alcoholic beverages (Ough 1976; Battaglia et al., 1990). In this study, the results from incubations of lung microsomes with VC revealed that the rate of edAS production was not fully saturated at a concentration of 3.0 mM (Fig. 5C). In the case of the incubations of rCYP2E1 with VC, saturation was achieved at a concentration of 2.5 mM. These findings suggested that formation of edAS occurs at a slow rate and at relatively high VC concentrations, suggesting that VC may not pose a carcinogenic risk in humans because exposures are predictably at extremely low levels, especially when VC exposure is via generation from EC. However, the potential adverse effects of VC exposure in human lung remain to be investigated and characterized. Nonetheless, in reference to EC, Miller and Miller (1983, p. 13) have reiterated that “it is possible that even low life-time intakes of so-called ‘weak’ carcinogens could induce cancers in some humans with especially predisposing genetic backgrounds and dietary habits”. Ethyl carbamate has exhibited moderate to weak life-time intakes of so-called ‘weak’ carcinogens could induce cancers in some humans with especially predisposing genetic backgrounds and dietary habits”. Ethyl carbamate has exhibited moderate to weak carcinogenic analog of ethyl carbamate. Ethylene glycol, ethyl-n-hydroxy carbamate and ethyl-n-hydroxycarbamate. Cancer Res 40:1194–1203. Dahlg, Miller, Miller, and EC (1978) Vinyl carbamate as a carcinogen and a more carcinogenic analog of ethyl carbamate. Cancer Res 38:3793–3804. Fernando RC, Nair J, Barbin A, Miller JA, and EC (1996) Detection of 1,3-ethenedoxygenosine and 1,3-ethenedoxygenosine by immunofluorescence/P3-positlabelling in lung and liver DNA of mice treated with ethyl carbamate (urethane) or its metabolites. Carcinogenesis 17:1711–1718. Fernott KG, Lee RP, Dowley TF, Hong Y-J, and Ulreich JB (1996) Protection from 1,1-dichloroethylenide-induced Clara cell injury by diallyl sulfone, a derivative of garlic. J Pharmaco Exp Ther 277:1655–1671. Fernott KG, Lee RP, and Reid K (2001) Involvement of CYP2E1 and carboxylesterase enzymes in vinyl carbamate metabolism in lung microsomes. Drug Metab Dispos 29:258–263. Fernott KG, Millen B, Lash LJ, Putt DA, and Ghanayem BI (2006) Pulmonary bronchiolar cytotoxicity and formation of dichloroacetyl lysine protein adducts in mice treated with trichloroethylene. J Pharmacol Exp Ther 316:520–529. Fernott KG, Premdas PD, and Bowers RJ (2000) Epoxide formation from diallyl sulfone, a derivative of garlic. J Pharmacol Exp Ther 298:225–233. Hernandez LG and Forkert PG (2007) In vivo mutagenicity of vinyl carbamate and ethyl carbamate in lung and small intestine of A/J (Big Blue) transgenic mice. Int J Cancer 120:1464–1471. Lee RP and Fernott KG (1997) Metabolism of ethyl carbamate by pulmonary cytochrome P450 and carboxylesterase isozymes: involvement of CYP2E1 and hydrolase A. Toxicol Appl Pharmacol 146:245–254. Lee RP and Fernott KG (1999) Inactivation of cytochrome P450 (CYP2E1) and carboxylesterase (Hydrolase A) enzymes by vinyl carbamate in murine pulmonary microsomes. Drug Metab Dispos 27:233–239. Leizhauser MT, Lien A, Stewart BC, Miller EC, and Miller JA (1990) 1,3-Ethenoxygenosine formation, mutagenicity and murine tumor induction as indicators of the generation of an
electrophilic epoxide metabolite of the closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. *Carcinogenesis* **11**:463–473.


Address correspondence 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