INCREASED BIOACCUMULATION OF URETHANE IN CYP2E1−/− VERSUS CYP2E1+/+ MICE

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ABSTRACT:

Urethane is a fermentation by-product and a potent animal carcinogen. Human exposure to urethane occurs through consumption of alcoholic beverages and fermented foods. Recently, CYP2E1 was identified as the primary enzyme responsible for the metabolism of [14C]-carbonyl-labeled urethane. Subsequently, attenuation of urethane-induced cell proliferation and genotoxicity in CYP2E1−/− mice was reported. The present work compares the metabolism of single versus multiple exposures of CYP2E1−/− and CYP2E1+/+ mice to 14C-ethyl-labeled urethane. Urethane was administered as a single 10 or 100 mg/kg gavage dose or at 100 mg/kg/day for 5 consecutive days. CYP2E1+/+ mice administered single or multiple doses exhaled 78 to 88% of dose as 14CO2/day. CYP2E1−/− mice eliminated 30 to 38% of a single dose as 14CO2 in 24 h and plateaued after day 3 at ~52% of dose/day. The concentrations of urethane-derived radioactivity in plasma and tissues were dose-dependent, increased as a function of the number of doses administered, and were significantly higher in CYP2E1−/− versus CYP2E1+/+ mice. Whereas urethane was the main chemical found in the plasma and tissues of CYP2E1−/− mice, it was not detectable in CYP2E1+/+ mice. In conclusion, multiple dosing led to considerable bioaccumulation of urethane in mice of both genotypes; however, greater retention occurred in CYP2E1−/− versus CYP2E1+/+ mice. Furthermore, greater bio-accumulation of 14C-ethyl-labeled than [14C]-carbonyl-labeled urethane was observed in mice. Comparison of the metabolism of ethyl- versus carbonyl-labeled urethane was necessary for tracing the source of CO2 and led us to propose for the first time that C-hydroxylation is a likely pathway of urethane metabolism.

Urethane is formed as a by-product of fermentation and is therefore found at appreciable levels in alcoholic beverages and foods, such as bread and cheese (Benson and Beland, 1997). Earlier reports have suggested that the mean daily intake of urethane in adults ranges between 10 and 20 ng/kg b.wt., with bread as the primary source. Furthermore, the FDA has reported that the mean daily intake of urethane detected from 45 ng/slice to 117 ng/slice (Benson and Beland, 1997). Depending on the extent and type of alcoholic beverage consumed, the mean daily intake of urethane can increase 3- to 60-fold (Zimmerli and Schlatter, 1991). Urethane is also a component of tobacco leaves and mainstream smoke. Smoking 20 cigarettes/day can increase the daily intake of urethane by 2-fold (Zimmerli and Schlatter, 1991). In the past, human exposure to urethane was also experienced through the use of pesticides, fungicides, cosmetics, and textiles (IARC, 1974). In the 1940s, this chemical was used as a hypnotic and as an antineoplastic agent to treat chronic leukemia and multiple myeloma (Zimmerli and Schlatter, 1991).

Urethane is a potent animal carcinogen that was classified as “reasonably anticipated to be a human carcinogen” (NTP, 2000). Previous studies have shown that independent of animal species or strain and regardless of the route of exposure, urethane undergoes rapid systemic distribution, and greater than 85% of an administered dose was eliminated in the expired air as CO2, within 8 h in genetically intact mice (Skipper et al., 1951; IARC, 1974; Yamamoto et al., 1988, 1990; Nomeir et al., 1989; Hoffler and Ghanayem, 2003; Hoffler et al., 2003). Until recently, the accepted hypothesis regarding urethane metabolism presumed that enzymatic hydrolysis via esterase was the primary metabolic route, whereas N-hydroxylation and pathways mediated by cytochromes P450 (P450s) played minor roles (Fig. 1). Recent studies conducted in this laboratory using CYP2E1−/− mice, however, demonstrated for the first time that cytochrome P4502E1 (CYP2E1), and not esterase, was the principal enzyme responsible for greater than 96% of [14C]-carbonyl-labeled urethane metabolism to 14CO2 (Hoffler et al., 2003). It was also shown that P450s other than CYP2E1 and esterase contributed approximately 3 and less than 1%, respectively (Hoffler et al., 2003). In the absence of CYP2E1-mediated metabolism, the half-life of urethane was considerably increased (0.8 h in CYP2E1+/+ versus 22 h in CYP2E1−/− mice), and significantly higher blood and tissue concentrations of urethane-derived radioactivity were detected in CYP2E1−/− versus CYP2E1+/+ mice (Hoffler et al., 2003).

An earlier study conducted by Lawson and Pound (1973) demonstrated that covalent binding of urethane to DNA in vivo requires the oxidation of the ethyl carbons. In turn, it was suggested that CYP2E1-mediated oxidation of the ethyl moiety of urethane results in the formation of vinyl carbamate and subsequently vinyl carbamate epoxide (Fig. 1), leading to the formation of DNA, RNA, and protein adducts. It was hypothesized that the manifestation of these events led

ABBREVIATIONS: P450, cytochrome P450; HPLC, high performance liquid chromatography; VC, vinyl carbamate; VCE, vinyl carbamate epoxide.
to urethane-caused mutagenesis and carcinogenesis. Therefore, the overall objective of our work is to assess the relationship between CYP2E1-mediated metabolism and urethane-induced mutagenicity and carcinogenicity. In particular, the present studies were undertaken to compare the metabolism and disposition of single versus multiple doses of \(^{14}\text{C}\)-ethyl-labeled urethane in CYP2E1\(^{+/−}\) and CYP2E1\(^{+/+}\) mice, compare the results of these studies with our earlier data on the metabolism and disposition of \(^{14}\text{C}\)carbonyl-labeled urethane, and assess the potential bioaccumulation of \(^{14}\text{C}\)-ethyl-labeled urethane-derived radioactivity as a result of multiple exposures and in comparison to the bioaccumulation of \(^{14}\text{C}\)carbonyl-labeled urethane (Hoffler et al., 2003). The use of \(^{14}\text{C}\)-ethyl versus -carbonyl-labeled urethane was necessary to identify the source of the urethane-derived CO\(_2\) and characterize the retained metabolites.

Materials and Methods

Chemicals. [Ethyl-\(^{14}\text{C}\)]Urethane, specific activity 56 mCi/mol, was obtained from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled urethane with purity greater than 98% was purchased from TCI America (Portland, OR). Using high performance liquid chromatography (HPLC), the radiochemical purity of ethyl-labeled urethane was determined to be greater than 99%.

Animals and Treatment. Male CYP2E1\(^{+/+}\) (wild-type) and CYP2E1\(^{−/−}\) (CYP2E1-null) mice were 8 to 9 months old and ranged in weight from 28 to 42 g. Mice were obtained from a colony developed at the National Cancer Institute (Bethesda, MD) and were re-derived and bred at Charles River Laboratories, Inc. (Wilmington, MA) (Lee et al., 1996; Hoffler et al., 2003). Animals were individually housed in an animal facility with a 12-h light/dark cycle and fed NIH #31 diet and water ad libitum throughout the study. All animal care and experimentation were conducted according to National Institutes of Health guidelines (U.S. Department of Health and Human Services, 1986). Dosing solutions were made in tap water using a mixture of radiolabeled and unlabeled urethane. All urethane-dosing solutions were made fresh and administered by gavage at either 10 or 100 mg/kg, delivering 100 \(\mu\)Ci/kg in a dose volume of 10 ml/kg.

Experimental Design. Groups (4–8 animals each) of CYP2E1\(^{−/−}\) and CYP2E1\(^{+/+}\) mice were administered urethane by gavage as follows. 1) CYP2E1\(^{+/−}\) and CYP2E1\(^{+/+}\) mice received a single 10- or 100- mg dose of \(^{14}\text{C}\)-ethyl-labeled urethane/kg and were euthanized 24 h after urethane administration. 2) CYP2E1\(^{−/−}\) mice received 100 mg of \(^{14}\text{C}\)-ethyl-labeled urethane/kg/day for 5 consecutive days and were euthanized 24 h after the last dose.

Immediately after urethane administration, mice were housed in individual glass metabolism cages (Wyse Glass Specialties, Inc., Freeland, MI) for 24 or 120 h, which allowed for the separate collection of urine, feces, and exhaled radioactivity. A vacuum system was attached to the glass cages that permitted the passage of air through the cage at a flow rate of 0.6 to 0.8 l/min. Air passing through the cage was initially passed through solid calcium sulfate and soda lime to reduce moisture and CO\(_2\) content, thus extending the efficiency of the trapping solutions over time. Air exiting the cage was passed through a series of traps. The first trap was an activated charcoal trap (SKC Inc., Eighty Four, PA) used to adsorb organic volatiles exhaled by urethane-treated mice. A second trap containing 400 ml of ethanol was used to capture exhaled organic volatiles that escape adsorption by the charcoal. Air was then passed through a trap containing approximately 400 ml of a 7:3 (v/v) mixture of ethylene glycol monomethyl ether and ethanolamine for collection of expired CO\(_2\). CO\(_2\) traps were changed at 1, 4, 8, 16, and 24 h after urethane administration in the single-dose study. For the 5-day study, \(^{14}\text{CO}_2\) traps were changed at 2, 4, 8, 16, and 24 h after each daily dose of urethane. Ethanol traps were changed every 24 h. Urine, feces, and charcoal traps were collected every 24 h after dosing. Urine and charcoal traps were stored at \(-80^\circ\text{C}\) to be analyzed at a later time.

At the end of the holding periods, mice were euthanized by CO\(_2\) asphyxiation, blood was collected from animals by cardiac puncture, and major tissues were dissected and stored at \(-60^\circ\text{C}\) to \(-80^\circ\text{C}\) for analysis at a later time. Tissue and whole blood samples weighing between 25 and 50 mg were sampled in triplicates and \(^{14}\text{C}\) content was quantitated via oxidation to \(^{14}\text{CO}_2\) using a PerkinElmer Tri-Carb sample oxidizer (PerkinElmer Life and Analytical Sciences, Boston, MA). Collected blood from 24 and 120 h was centrifuged to separate red blood cells and plasma, and urethane-derived radioactivity was also quantitated using the tissue oxidizer. Feces were air-dried, ground to a fine powder, weighed, and similarly analyzed in triplicate. Charcoal traps were opened; then, charcoal contents were weighed and analyzed in triplicates using the sample oxidizer. Recovery of radioactivity from the sample oxidizer was greater than 95%. Oxidized samples as well as triplicate aliquots of the \(^{14}\text{CO}_2\) and charcoal trapping solutions (1 ml) were changed at 2, 4, 8, 16, and 24 h after urethane administration and stored at \(-80^\circ\text{C}\) to be analyzed at a later time.

HPLC Analysis of Plasma, Liver Homogenates, and Urine. The metabolite profiles of plasma, liver homogenates, and urine samples from CYP2E1\(^{+/+}\) and CYP2E1\(^{−/−}\) mice treated with 100 mg of \(^{14}\text{C}\)-ethyl-labeled urethane/kg for 24 h or daily for 5 consecutive days were analyzed by HPLC. Individual plasma, liver homogenates, and urine samples were centrifuged at 14,000 g for 20 min at 4°C, and 100 \(\mu\)l of supernatant were directly injected into the HPLC apparatus. Small aliquots (10 \(\mu\)l) of plasma and urine supernatants were also analyzed for total radioactivity using a Beckman Coulter Model LS 9800 scintillation counter (Beckman Coulter, Fullerton, CA).

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ml/min. Parent urethane was identified in the urine, plasma, and liver homogenates by comparing the retention times of these biological samples to the retention time of 14C-ethyl-labeled urethane standard. Furthermore, biological samples were spiked with 14C-ethyl-labeled urethane and analyzed using HPLC to confirm the presence of parent compound.

**Statistical Analysis.** Group mean comparisons were performed using Student’s t test, two-tailed, assuming equal variances. Values were considered statistically significant at P ≤ 0.05.

**Results**

**Metabolism and Disposition of a Single Dose of 14C-Ethyl-Labeled Urethane.** Within 24 h after a single administration of either 10 or 100 mg/kg 14C-ethyl-labeled urethane, approximately 80% of the total administered dose was eliminated in the expired air of CYP2E1+/+ mice as 14CO2 (Table 1; Fig. 2). In contrast, CYP2E1−/− mice receiving the same treatment eliminated less than 40% of the total dose as 14CO2 in 24 h (Table 1; Fig. 2). Whereas 14C-ethyl-labeled urethane-derived radioactivity, eliminated as organic volatiles in CYP2E1+/+ mice, comprised less than 1% of either the high or low doses, elimination in CYP2E1−/− mice via this route accounted for 3 and 4.2%, respectively (Table 1). In both CYP2E1+/+ and CYP2E1−/− mice, fecal excretion of 14C-ethyl-labeled urethane-derived radioactivity was negligible and accounted for less than 1% of the total administered urethane doses (Table 1). No significant differences were evident in the percentage of dose excreted as 14C-ethyl-labeled urethane-derived radioactivity in the urine regardless of the genotype or dose administered, 3.1 to 4.7% of dose (Table 1). However, HPLC analysis of urine identified greater than 62% of the 14C-ethyl-labeled radioactivity as unmetabolized urethane in CYP2E1-null mice, whereas in CYP2E1+/+ mice, the majority of the urinary radioactivity (>70%) was metabolites, with parent urethane accounting for the remainder.

Notable differences and similarities were evident when comparing the current data on the metabolism and disposition of a single dose of 14C-ethyl- versus our earlier work using 14C-carbonyl-labeled urethane in CYP2E1−/− and CYP2E1+/+ mice (Hoffler et al., 2003). Regardless of the location of the radiolabel and independent of dose, exhalation of urethane-derived CO2 in CYP2E1+/+ mice increased in a dose-dependent manner and plateaued within 8 h of exposure (Fig. 2; Hoffler et al., 2003). An approximately 50% decrease in urethane-derived 14CO2 exhalation was determined at both the 10 and 100 mg/kg doses of either 14C-ethyl- or carbonyl-treated CYP2E1−/− mice (Fig. 2; Hoffler et al., 2003). Although the average rate of 14CO2 exhalation (percentage of dose per hour) remained relatively constant in CYP2E1−/− mice during the entire 24 h after exposure to either the high or low 14C-ethyl- or carbonyl-labeled urethane doses, CYP2E1+/+ mice administered 14C-ethyl-labeled urethane experienced a minor delay in the production of exhaled 14CO2 in the first 4 h after treatment (data not shown) in comparison to [14C]carbonyl-labeled urethane (Hoffler et al., 2003). At the end of 24 h, it was evident that the cumulative percentage of doses eliminated as exhaled 14CO2 slightly decreased in mice treated with 14C-ethyl-labeled urethane in contrast to mice similarly treated with the 14C-carbonyl-labeled chemical (Table 1; Hoffler et al., 2003). Regardless of the location of the radiolabel, elimination of urethane-derived radioactivity as exhaled organic volatiles, as urinary or fecal metabolites, was considered to be via minor pathways. However, significant differences in the disposition of 14C-ethyl-labeled urethane-derived radioactivity were observed in both CYP2E1+/+ and CYP2E1−/− mice administered 10 mg/kg in comparison to mice exposed to [14C]carbonyl-labeled urethane at the same dose. CYP2E1+/+ mice treated with 10 mg/kg 14C-ethyl-labeled urethane exhibited a 3-fold increase in the cumulative percentage of dose eliminated as organic volatiles, in comparison to CYP2E1+/+ mice administered 10 mg/kg 14C-carbonyl-labeled urethane (Table 1; Hoffler et al., 2003). Furthermore, 10 mg/kg 14C-ethyl-treated CYP2E1+/+ mice eliminated a 2-fold higher percentage of dose as urinary metabolites than did its [14C]carbonyl-treated counterparts. On the other hand, CYP2E1−/− mice administered the same 14C-ethyl-labeled urethane treatment exhibited a 2-fold decrease in the percentage of dose eliminated via renal excretion in comparison to 10

**TABLE 1**

**Summary of ethyl-labeled urethane (U) disposition 24 h after 10 or 100 mg/kg administration by gavage**

Values are presented as cumulative percentage of dose and are the mean ± S.E. of three to eight mice.

<table>
<thead>
<tr>
<th>[Ethyl-14C]U</th>
<th>100 mg/kg</th>
<th>10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1+/+</td>
<td>CYP2E1−/−</td>
<td>CYP2E1+/+</td>
</tr>
<tr>
<td>CO2</td>
<td>87.5 ± 2.9</td>
<td>87.5 ± 3.6</td>
</tr>
<tr>
<td>Organic volatiles</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>Urine</td>
<td>3.1 ± 0.5</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Feces</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Total percentage of dose</td>
<td>91.3</td>
<td>82.8</td>
</tr>
</tbody>
</table>

Neg, negligible; value was less than 1%.

* CYP2E1−/− values that are statistically different from corresponding CYP2E1+/+ values (P ≤ 0.05).

**FIG. 2.** Effects of dose and time on cumulative 14CO2 exhalation in CYP2E1−/− and CYP2E1+/+ mice after gavage administration of 14C-ethyl-labeled urethane. Values are presented as cumulative percentage of dose and are the mean ± S.E. of four to eight mice. a and b denote significant differences in 14CO2 elimination by the two genotypes of mice at 100 and 10 mg/kg; c denotes a significant difference of 14CO2 elimination at the 2nd hour between the 10 and 100 mg/kg treated CYP2E1+/+ mice.
mg/kg [14C]carbonyl-treated CYP2E1-null mice (Table 1; Hoffler et al., 2003). With regard to the cumulative percentage of dose excreted as fecal metabolites, discernible differences were not apparent in mice treated with either 14C-ethyl- or [14C]carbonyl-labeled urethane (Table 1; Hoffler et al., 2003).

In general, the concentration of 14C-ethyl-labeled urethane-derived radioactivity detected in the blood and tissues increased in a dose-dependent manner in both mouse genotypes, but were considerably higher in CYP2E1−/− mice, independent of the location of the radiolabel (Table 2). Significant distinctions, however, were observed in the blood and tissues of CYP2E1+/+ mice. Twenty-four hours after exposure to 14C-ethyl-labeled urethane, a 3- to 16-fold increase in the concentration of 14C-ethyl-labeled urethane-derived radioactivity was detected in the blood and tissues of these mice versus CYP2E1+/+ mice treated with [14C]carbonyl-labeled urethane (Table 2; Hoffler et al., 2003).

Metabolism and Disposition of 14C-Ethyl-Labeled Urethane Administered Daily for 5 Consecutive Days. Inhibition of urethane metabolism to 14CO2 continued after multiple doses of urethane (Fig. 3). Exhalation of 14CO2 in CYP2E1+/+ mice after each daily dose of 100 mg of 14C-ethyl-labeled urethane/kg exhibited a pattern similar to that seen after a single dose (Fig. 3). Maximum 14CO2 exhalation was achieved in CYP2E1+/+ mice after the second day of dosing, and steady-state levels were maintained with approximately 85% of the daily dose eliminated as expired 14CO2. In CYP2E1−/− mice, maximum exhalation was delayed and observed after 3 days (approximately 52% of the daily doses) (Fig. 3). An average of 28 and 13% of the five cumulative 14C-ethyl-labeled urethane doses was eliminated as urethane-derived radioactivity in the urine and organic volatiles of CYP2E1−/− mice, respectively. CYP2E1+/+ mice, however, eliminated merely 10 and 3.5% of the five doses, respectively, via these routes.

Although elimination of urethane-derived radioactivity via renal excretion was considered a minor metabolic route, there were significant differences in the HPLC profiles of urinary metabolites from CYP2E1−/− and CYP2E1+/+ mice (Fig. 4). Twenty-four hours after a single dose of 14C-ethyl-labeled urethane was administered to CYP2E1+/+ mice, approximately 28% of the total radioactivity excreted in the urine was identified as unmetabolized urethane. In CYP2E1−/− mice, urethane comprised greater than 70% of the total radioactivity excreted in the urine (Fig. 4). Furthermore, after day 1, urethane constituted less than 10% of the total urinary radioactivity in CYP2E1+/+ mice, whereas in CYP2E1−/− mice, it comprised 60 to 70% through day 5 (Fig. 4).
in both CYP2E1\textsuperscript{+/+} and CYP2E1\textsuperscript{+/-} mice were markedly higher in mice administered five \textsuperscript{14}C-ethyl-labeled urethane exposures than in those receiving a single treatment (Table 2). HPLC analysis showed that most of the urethane-derived radioactivity detected in the plasma and liver homogenates of CYP2E1\textsuperscript{+/-} mice that received a single \textsuperscript{14}C-ethyl-labeled urethane dose was parent compound (Fig. 5). In CYP2E1\textsuperscript{+/+} mice administered five daily doses, approximately 83 to 88\% of the urethane-derived radioactivity identified in the plasma and liver homogenates was unmetabolized urethane (Fig. 5). Parent urethane was not detected in plasma and tissues of CYP2E1\textsuperscript{+/-} mice administered either a single or multiple urethane dose. In comparing the current data on the bioaccumulation of \textsuperscript{14}C-ethyl-labeled versus \textsuperscript{[14C]}carbonyl-labeled urethane (Hoffler et al., 2003), it was clear that \textsuperscript{14}C-ethyl-labeled urethane has a greater tendency to be retained in mice of both genotypes.

Discussion

Numerous studies investigating urethane's metabolism and disposition have suggested the involvement of two pathways. The first entails hydrolysis via esterase and was thought to be responsible for greater than 90\% of urethane's metabolism to \textsuperscript{14}CO\textsubscript{2} (Fig. 1; Skipper et al., 1951; Kaye, 1960; Nomeir et al., 1989; Salmon and Zeise, 1991). The second pathway, which was considered minor, proposed dehydrogenation of urethane to form vinyl carbamate (VC) followed by P450-mediated oxidation of this intermediate to form vinyl carbamate epoxide (VCE) (Fig. 1; Dahl et al., 1978, 1980; Guengerich and Kim, 1991). Contrary to earlier assertions, a recent report from this laboratory using \textsuperscript{[14C]}carbonyl-labeled urethane showed for the first time that CYP2E1, and not esterase, was the principal enzyme responsible for greater than 96\% of urethane's metabolism (Hoffler et al., 2003). The contributions of other P450s and esterase were estimated at approximately 3 and 0.5\% of the dose, respectively (Hoffler et al., 2003). Lawson and Pound (1973) suggested that covalent binding to mouse liver DNA in vivo requires the oxidation of the ethyl moiety of urethane. This finding, in addition to our recent report demonstrating an essential role for CYP2E1 in urethane metabolism and an increase in the half-life of urethane in CYP2E1\textsuperscript{+/-} versus CYP2E1\textsuperscript{+/+} (Hoffler et al., 2003) mice, prompted this investigation. The objectives of the current studies included 1) investigating the metabolism and disposition of \textsuperscript{14}C-ethyl-labeled urethane in CYP2E1\textsuperscript{+/+} and CYP2E1\textsuperscript{+/-} mice and comparing these data to our earlier results using \textsuperscript{[14C]}carbonyl-labeled urethane (Hoffler et al., 2003); 2) assessing the consequences of lack of CYP2E1 on the metabolism and disposition of single versus multiple doses of \textsuperscript{14}C-ethyl-labeled urethane in mice of both genotypes; and 3) characterizing the bioaccumulation of \textsuperscript{14}C-ethyl-labeled urethane in mice of both genotypes after multiple doses.

After gavage administration, urethane was rapidly absorbed and distributed to all major tissues. Current work showed that metabolism and elimination of \textsuperscript{14}C-ethyl-labeled urethane via CO\textsubscript{2} was the predominant pathway in CYP2E1\textsuperscript{+/+} mice, although to a lesser
measure than in CYP2E1+/+ mice treated with a similar dose of [14C]carbonyl-labeled urethane (Hoffler et al., 2003). In CYP2E1+/+ mice administered [14C]-ethyl-labeled urethane, 73 to 77% of the dose was exhaled as 14CO2 within 8 h (Fig. 2), whereas CYP2E1+/− mice treated with [14C]carbonyl-labeled urethane eliminated 90 to 94% of the dose via the same route in 8 h (Hoffler et al., 2003). In CYP2E1−/− mice and regardless of the location of the label, animals exhaled 15 to 20% of the administered dose of urethane as 14CO2 within 8 h after dosing. On average, 38% of a single [14C]-ethyl- or [14C]carbonyl-labeled urethane dose was exhaled as 14CO2 in 24 h after exposure of CYP2E1−/− mice. Variations in the percentage of dose eliminated as organic volatiles, or urinary and fecal metabolites were minor when comparing the disposition of [14C]-ethyl- versus [14C]carbonyl-labeled urethane. These data confirmed our earlier findings that CYP2E1 plays an essential role in the metabolism of urethane. Furthermore, these data showed that a greater percentage of [14C]carbonyl-labeled urethane doses was metabolized to CO2 versus [14C]-ethyl-labeled urethane. The decrease in CO2 elimination was associated with greater retention of 14C-ethyl-labeled urethane in blood and tissues of CYP2E1+/+ mice. The majority of tissues known to be targets of urethane had exceedingly higher concentrations of radioactivity than did CYP2E1+/+ mice treated with [14C]-carbonyl-labeled urethane. These tissues included the brain (16-fold), spleen (14-fold), liver and thymus (13-fold), and lung (12-fold). These data are in agreement with those from the earlier work of Lawson and Pound (1973), which concluded that metabolites of the ethyl- and not the carbonyl carbons of urethane bind to mouse DNA in vivo.

Bioaccumulation of 14C-ethyl-labeled urethane-derived radioactivity increased as a function of the number of doses administered to CYP2E1+/+ and CYP2E1−/− mice. However, in the absence of CYP2E1-mediated metabolism, a significantly greater retention of 14C-ethyl-labeled urethane-derived radioactivity was detected in CYP2E1−/− versus CYP2E1+/+ mice. HPLC analysis showed that whereas urethane was the primary chemical in the plasma and liver homogenates of CYP2E1−/− mice, it was not identifiable in CYP2E1+/+ mice. In a subsequent study using doses as high as 1000 mg/kg, significant prolongation of urethane-induced anesthesia was observed only in CYP2E1−/− mice administered urethane (unpublished data), confirming that the anesthesia was caused by unmetabolized urethane. Recent studies in this laboratory also demonstrated significant inhibition of urethane-induced genotoxicity and cell proliferation in CYP2E1−/− versus CYP2E1+/+ mice (Hoffler et al., 2005). This finding supports the hypothesis that CYP2E1-mediated metabolism, presumably via epoxidation across the ethyl carbons, is a prerequisite for the induction of genotoxicity and carcinogenicity by urethane.

Metabolism of both 14C-ethyl- and [14C]carbonyl-labeled urethane in CYP2E1+/+ mice produced considerable amounts of CO2. However, until recently, it was thought that enzymatic hydrolysis of urethane via esterase to ethanol and subsequent metabolism via alcohol and aldehyde dehydrogenases was the primary route for the formation of CO2 (Fig. 1). Because our recent findings indicated that the contribution of esterase to urethane metabolism was less than 1% of the dose (Hoffler et al., 2003), it was concluded that ester cleavage was insufficient to justify the high percentage of 14C-ethyl-labeled urethane that converted to 14CO2, as well as to explain the potent carcinogenicity of this chemical. As a result, we proposed for the first time that C-hydroxylation, catalyzed primarily by CYP2E1, is an important contributor to urethane metabolism (Fig. 1). This pathway explains 14CO2 production in mice treated with [14C]-ethyl-labeled urethane and presents a second and/or alternate metabolic route for the generation of 14CO2 from [14C]carbonyl-labeled urethane (Hoffler et al., 2003). It was reported that P450s could oxidatively cleave esters, leading to the formation of aldehydes or ketones from the alcohol moiety of the C-hydroxylated metabolite (Guengerich, 1987, 2001; Guengerich et al., 1988; Peng et al., 1995). Cleavage of many alkyl esters such as ethyl acetate, ethyl formate, and ethyl propionate via CYP2E1 has also been reported (Peng et al., 1995). Additionally, C-hydroxylation may provide another route for the formation of vinyl carbamate via dehydration of α-hydroxyurethane (Fig. 1).

Bioactivation of chemicals via dehydrogenation and subsequent P450-mediated oxidation is not unique to urethane. Aflatoxin B2 and some alkenylbenzene carcinogens such as safrole are metabolized via this pathway (Miller and Miller, 1983). The dehydrogenation product of urethane, VC, was shown to be a more potent carcinogen and mutagen than either ethyl N-hydroxycarbamate (N-hydroxyurethane) or urethane (Dahl et al., 1978, 1980). Initial studies by Dahl et al. (1978, 1980) demonstrated difficulties in identifying VC as a metabolite of urethane either in vivo or in vitro. However, subsequent studies using gas chromatography-mass spectrometry were successful in characterizing VC as a microsomal oxidation product of urethane (Guengerich and Kim, 1991). At present, isolation of VC in vivo has not been achieved. Oxidation of the ethyl moiety enables binding of the ethyl carbons to tissue macromolecules that may in turn foster adduct formation, and subsequent mutagenesis and tumorigenesis, presumably through VCE. Because of the highly reactive nature of epoxides, VCE has been termed as the ultimate carcinogenic metabolite of urethane. Numerous studies have identified etheno adducts of DNA and RNA in target tissues of urethane-induced carcinogenicity (Ribovich et al., 1982; Park et al., 1990; Fernando et al., 1996). In comparison to urethane, VC- and VCE-derived adducts were increased in target versus nontarget tissues (Ribovich et al., 1982; Miller and Miller, 1983; Park et al., 1990; Fernando et al., 1996). Collectively, these studies established that the formation of adducts resulted from the bioactivation of urethane at the ethyl carbons and not its hydrolysis product, ethanol.

In conclusion, current work supports our earlier findings demonstrating the predominance of CYP2E1 in urethane metabolism. In comparison to our earlier work using [14C]-carbonyl-labeled urethane (Hoffler et al., 2003), current data demonstrated that there is greater retention of [14C]-ethyl-labeled urethane-derived radioactivity in the blood and tissues of CYP2E1+/+ mice in association with a decrease in the exhalation of 14CO2. Furthermore, bioaccumulation of [14C]-ethyl-labeled urethane increased as a function of the number of administered urethane doses. Regardless of the position of the radio-label or the number of urethane doses, there was significantly greater bioaccumulation in CYP2E1−/− versus CYP2E1+/+ mice. Whereas the majority of [14C]-ethyl-labeled urethane-derived radioactivity found in the blood and tissues of CYP2E1−/− mice was unmetabolized urethane, parent compound was not identified in CYP2E1+/+ mice. Collectively, our current and earlier studies suggested that both carbonyl- and ethyl-carbons were extensively incorporated into urethane-derived CO2. We therefore proposed for the first time that urethane undergoes CYP2E1-mediated C-hydroxylation. This pathway explains CO2 production from the ethyl or carbonyl carbons of urethane and leads to the formation of VCE. Recent studies in this laboratory demonstrated significant attenuation of urethane-induced genotoxicity and cell proliferation in CYP2E1−/− versus CYP2E1+/+ mice (Hoffler et al., 2005). Additionally, LN6-ethenoadenosine adduct formation in the lungs of CYP2E1−/− mice exposed to urethane was reduced in comparison to urethane-treated CYP2E1+/+ mice (our unpublished data). These findings support the hypothesis that
CYP2E1-mediated metabolism, presumably via the epoxidation across the ethyl carbons, is a prerequisite for the induction of genotoxicity and carcinogenicity by urethane.

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