Inhibition of Metabolism of Diethylene Glycol Prevents Target Organ Toxicity in Rats

Lauren M. Besenhofer,* Patrick A. Adegboyega,+ Michael Bartels,‡ Mark J. Filary,§ Adam W. Peral,§ Marie C. McLaren,* and Kenneth E. McMartin*†‡

*Department of Pharmacology, Toxicology and Neuroscience, 1To whom correspondence should be addressed at Department of Pharmacology, Toxicology and Neuroscience, Louisiana State University Health Sciences Center—Shreveport, Louisiana 71130; and †Department of Pathology, Louisiana State University Health Sciences Center—Shreveport, Louisiana 71130; and ‡Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, Michigan 48674

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Diethylene glycol (DEG) is an industrial chemical, the misuse of which has led to numerous epidemic poisonings worldwide. The mechanism of its toxicity has not been defined as to the precise relationship between the metabolism of DEG and target organ toxicity. The purpose of this study was to investigate the mechanism for the acute toxicity of DEG, and the effect of the alcohol dehydrogenase inhibitor 4-methylpyrazole (fomepizole), by determining the relationship between accumulation of DEG or its metabolites and the resulting kidney and liver toxicity. Rats were treated by oral gavage with water, 2 g/kg DEG (low dose), 10 g/kg DEG (high dose), or 10 g/kg DEG + fomepizole, and blood and urine were collected over 48 h. Rats treated with high-dose DEG had metabolic acidosis, increased BUN and creatinine, and marked kidney necrosis, noted by histopathology. A minor degree of liver damage was noted at the high dose. After low and high doses of DEG, 2-hydroxyethoxyacetic acid (HEAA) was the primary metabolite in the urine, with only minor amounts of urinary diglycolic acid (DGA). Small amounts of ethylene glycol (EG), but not oxalate or glycolate, were observed in the urine. Treatment with fomepizole blocked the formation of HEAA and DGA and the development of metabolic acidosis and the kidney and liver toxicity. These results indicate that the mechanism for the target organ toxicity results from metabolites of DEG, and not DEG itself nor formation of EG from DEG, and that fomepizole may be a useful antidote for treating DEG poisoning.

Key Words: diethylene glycol; fomepizole; nephrotoxicity; hepatotoxicity; hydroxyethoxyacetate; diglycolic acid; oxysibacetate.

In 1937, the Sulfanilamide Elixir Disaster in the United States resulted in 105 deaths when DEG was used as a solvent to dissolve the antimicrobial drug sulfanilamide (Wax, 1995). This event precipitated the promulgation of the Food, Drug, and Cosmetic Act of 1938 in the United States, but in countries lacking strict drug regulation enforcements, epidemic poisonings involving misuse of DEG still occur. The most recent epidemic of DEG poisoning occurred from August 2008 until January 2009 in Nigeria, where 54 children younger than 3 years died after exposure to a liquid teething medication with DEG as a solvent (Abubukar et al., 2009). This episode and others in the past 20 years (Barr et al., 2007) emphasize the need for research on DEG toxicity, including identifying the toxic metabolite and understanding its mechanism of action and development of an antidote.

The target organs for acute DEG toxicity are the kidney, liver, and nervous system. Clinical manifestations of DEG poisoning include metabolic acidosis, marked increases in BUN and serum creatinine, anuria, increased liver enzymes, hepatomegaly, and facial nerve paralysis (Alfred et al., 2005; O’Brien et al., 1998). If left untreated, patients may succumb to the syndrome resulting in coma or death within 2–7 days.

The metabolism of DEG has been well studied, but only at doses that are nontoxic to animals, using oral doses (0.5–5 g/kg) of [14C]-DEG in rats and dogs. These studies found that about 70–80% of the radiolabeled DEG was excreted in the urine unchanged and another 10–30% was determined to be urinary 2-hydroxyethoxyacetic acid (HEAA) (Heilmair et al., 1993; Lenk et al., 1989; Mathews et al., 1991; Wiener and Richardson, 1989). Ethylene glycol (EG) and its metabolites, glycolic acid, glyoxylic acid and oxalic acid, were not detected in any of these studies. Further evidence that DEG does not undergo ether cleavage to become two EG molecules has been reported in DEG-intoxicated patients because oxalate crystals, a hallmark of EG poisoning, are not found in the urine or kidney tissues (Alfred et al., 2005; Brophy et al., 2000; Okuonghae et al., 2008).
et al., 1992). Rather, DEG appears to be oxidized to the intermediate metabolite 2-hydroxyethoxyacetaldehyde and then further to HEAA, which can form a six-member ring, 1,4-dioxan-2-one, under acidic conditions (Lenk et al., 1989). As seen in Figure 1, HEAA might be further oxidized to diglycolic acid (DGA) (also known as oxybisacetic acid), but no studies to date have detected this potential final metabolite in DEG-treated animals. Inhibition of the enzyme alcohol dehydrogenase (ADH) in rats treated with DEG by either ethanol (Durand et al., 1976) or pyrazole (Wiener and Richardson, 1989) decreases the metabolic acidosis and the excretion of HEAA compared with animals receiving only DEG. Despite these studies, the metabolism of DEG has never been adequately elucidated in a way that explains the mechanism of acute toxicity to organs like the kidney.

Unfortunately for patients with acute DEG poisoning, there are no good therapies for treating the toxicity because of the lack of knowledge about the mechanism. The accumulation of DEG or its metabolites has never been specifically correlated with the observed kidney and liver toxicities nor is the metabolism of toxic doses of DEG well understood. Although inhibition of ADH appears to decrease the formation of HEAA, no studies have conclusively shown a concomitant decrease in target organ toxicity. The lack of conclusive evidence identifying toxic metabolites has made it impossible to begin understanding the mechanism of toxicity of DEG, thus making the treatment of poisoned individuals extremely difficult. The continued possibility of illicit use of DEG in pharmaceuticals worldwide, as well as the availability of DEG in consumer products in the United States, renders more urgent the efforts to understand DEG toxicity and identify efficacious antidotes.

The purpose of the following experiments was to link the time course of toxicity with the accumulation of toxic metabolites and to investigate whether fomepizole, an ADH inhibitor that is effective in the treatment of methanol or EG poisoning (Brent, 2009), diminishes the formation of metabolites and reduces DEG-related toxicity in vivo.

MATERIALS AND METHODS

**Materials.** DEG, provided by Shell Chemicals, was analyzed for purity by gas chromatography (GC) with flame ionization detection prior to dosing. The DEG contained DEG (99.78%), EG (0.05%), and triethylene glycol (0.08%). Synthetic standards of DEG and oxalate, with chemical purities of 100% and 99.6%, respectively, were obtained from Fluka Chemical Corporation (Milwaukee, WI). Standards of EG, DGA, and glycolate with chemical purities of 99.8, 99.4, and 100%, respectively, were obtained from Sigma-Aldrich Corporation (St Louis, MO). A standard of HEAA with a chemical purity of 99% was obtained from Isotec Incorporated (Miamisburg, OH). The internal standards D₆-DEG, D₆-EG, D₆-DGA, D₆-HEAA, ¹³C₂-glycolate, and ¹³C₂-oxalate were all obtained from Isotec Incorporated.

Fomepizole is the generic name for the pharmaceutical formulation of 4-methylpyrazole (4-MP). For practical purposes, the two terms represent the same chemical because the chemical form of fomepizole in the United States is the free base 4-MP. Although free base 4-MP (99%; Sigma-Aldrich) was used in these studies, the term fomepizole is used in the article for its clinical relevancy.

Ethidium homodimer (Invitrogen, Carlsbad, CA) was diluted to 5 µmol/l in pH 7.4 buffer containing 115mM NaCl, 5.5mM glucose, 16mM NaHCO₃, 1mM MgCl₂, 0.2mM NaHPO₄, 0.8mM NaH₂PO₄, 1mM CaCl₂, and 5mM KCl (Edwards et al., 2007). All other chemicals were bought from Sigma-Aldrich.

**Animal protocol.** Adult male Wistar rats (425–475g; Harlan, Indianapolis, IN) were implanted with chronic indwelling jugular catheters 10 days prior to the start of the experiment to minimize stress to the animal during blood collections (Goeders and Guerin, 1996). Catheters were flushed every other day with streptokinase and sterile heparinized saline until the start of the experiment at which time they were flushed after every collection. Animals were randomly placed into one of four treatment groups with six rats per group, including 10 g/kg DEG, 2 g/kg DEG, water, or 10 g/kg DEG plus fomepizole. DEG was administered by oral gavage at time 0, and fomepizole (15 mg/kg) was administered ip as a 10 mg/ml solution in saline at 15 min and then at 12, 24, and 36 h (10 mg/kg) for a total of four ip doses. Animals were then housed in metabolic cages for 48 h for urine collection. Standard conditions of humidity, temperature (25°C ± 2°C), and light (12:12-h light-dark) were maintained in the animal room, and all rats were allowed free access to food (normal rat chow) and water. The animal protocols were approved by the Institutional Animal Care Committee (Louisiana State University Health Sciences Center—Shreveport [LSUHSC-S]) and were in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.”

**Urine collection and analysis.** Urine was collected at 4, 8, 12, 24, 36, and 48 h over ice to minimize degradation of urinary metabolites. Metabolic cages were rinsed with deionized water between collections, and the rinses were

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**FIG. 1.** Metabolic pathway for DEG based on previous animal studies and on the results presented in this report. Metabolites in lined boxes have been observed following administration of DEG to animals; those in dashed boxes are theoretical intermediates. Because fomepizole reduced the amount of EG in vivo, its use is shown as coming from the aldehyde or acid intermediate, rather than from DEG itself. ALDH = aldehyde dehydrogenase. DGA is also known as oxybisacetic acid.
saved and analyzed to ensure that there was no loss of urinary metabolites. Immediately after collection, the urines were vortexed and the volume and pH was measured and recorded. Two 1 ml aliquots of vortexed urine were transferred to cryogenic vials and stored at −80°C until metabolite analysis. The remaining urine was allowed to settle for 30 min on ice, and then, two 1 ml aliquots of clean urine were transferred to microtubes and stored at −80°C until needed. The remaining urine was stored at 4°C for later use if necessary.

**Blood collection and analysis.** Approximately 1 ml of blood was collected via the indwelling jugular catheter at 4, 8, 12, 24, 36, and 48 h into heparinized syringes and immediately placed on ice. Catheters were flushed as described above after every collection. Heparinized whole blood was analyzed for pH, pCO₂, and pO₂ by a blood gas analyzer; blood bicarbonate concentrations were calculated by the analyzer. The remaining blood was transferred to separator tubes (ref 365956; BD, Franklin Lakes, NJ), inverted six times, then centrifuged at 12,000 × g for 5 min to isolate the plasma, which was then transferred to a microtube and stored at 4°C until analysis. Plasmas were analyzed for a basic metabolic panel, including markers of renal function (urea nitrogen [BUN] and creatinine) and of liver function (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) by the LSUHSC-C Clinical Laboratory.

**Etidium homodimer perfusion.** At 48 h, the animals were anesthetized with sodium pentobarbital (50 mg/kg, ip), and the left kidney was perfused with 5μl etidium homodimer for 20 min following the method of Edwards et al. (2007). Etidium homodimer is a fluorescent dye that has low membrane permeability in live cells, but once inside cells can bind to DNA with a high affinity. Therefore, the fluorescence of internalized etidium homodimer can be used as a specific marker for necrotic cell death in situ. Perfused kidneys were removed, cut into four sections, frozen in Optimal Cutting Temperature medium (Tissue Tek), and stored at −80°C until use. Three 5-μm sections were cut and affixed to a microscope slide, and then, the tissues were permeabilized with ice-cold methanol and counterstained with 1μM 4′,6-diamidino-2-phenylindole (DAPI) for 30 min. The stained tissues were visualized with a tetramethyl rhodamine isothiocyanate filter to view the etidium homodimer-stained (necrotic) cells and with a DAPI filter to view all cells. The degree of fluorescent staining was assessed by scoring each tissue section with a number from 0 to 15, with 0 indicating no necrosis and 15 indicating extensive renal tubule staining. Scores for three tissue slices were averaged to get a final score for each animal. The person scoring (M.C.M.) the slices was blinded as to the animal treatment.

**Histopathology of unperfused tissues.** At the end of the perfusion, the right (unperfused) kidney and the whole liver were collected for further histopathological studies. For each tissue, a 1-mm slice was fixed in 10% neutral buffered formalin. The tissues were washed and dehydrated in isopropanol and xylene and then embedded in molten paraffin wax. Four-micrometer sections were cut and stained with hematoxylin and eosin. Tissues were examined with light and polarizing microscopy to visualize tissue necrosis and to look for the presence of calcium oxalate crystals. The observer (P.A.A.) was blinded as to the animal treatment.

**Determination of metabolite concentrations in urine.** DEG and EG were determined by an existing method that employs chemical derivatization with pentafluorobenzoyl chloride (PFBCl) followed by separation with gas chromatography and detection by negative chemical ionization mass spectrometry (GC-NCI-MS) (Pottenger et al., 2001), with a few modifications, primarily in sample preparation. Sample preparation for DEG analysis involved initial 50-fold dilution of a 25-μl urine aliquot with Milli-Q water. A 40 μl aliquot of the diluted urine was added to 2 ml of Milli-Q water (saturated with NaCl), and a 20 μl aliquot of D₄-DG internal standard solution (1000 μg/ml) added. The sample was then derivatized with 100 μl of 5 N NaOH, 25 μl PFBCl, and 1 ml toluene (vortex-heated at 45°C × 30 min). A 200 μl aliquot of the toluene layer was removed after centrifugation and analyzed by GC-NCI-MS. Sample preparation for EG analysis involved addition of a 100 μl aliquot of urine and a 25 μl aliquot of D₆-EG internal standard solution (1000 μg/ml) to 2 ml of Milli-Q water (saturated with NaCl). The sample was then derivatized with 100 μl of 5 N NaOH, 50 μl PFBCl, and 1 ml toluene (vortex-heated at 45°C × 30 min). A 200 μl aliquot of the toluene layer was removed after centrifugation and analyzed by GC-NCI-MS, which was conducted on an Agilent 5973 GC/MSD (San Jose, CA) as follows: injection volume 1 μl, split flow 35 ml/min, split ratio 25:1; temperature gradient elution on an Agilent HP-5MS capillary column (30m × 0.25mm id × 0.25μm film) initial hold at 100°C for 0.5 min, ramp at 20°C/min to 280°C, hold for 0.5 min (helium carrier gas at 15 psi); transfer line temperature 300°C, MS quadrupole temperature 150°C, MS source temperature 230°C; ionization gas = methane; quantitation ions for analyte/internal standard pairs: DEG 494/502 amu, EG 450/454 amu at 100 msec/ion/scan. The achieved limits of quantitation (LOQ) were 10 μg/ml for DEG and 50 μg/ml for EG.

The potential acid metabolites (oxalic acid, glycolic acid, DGA, and HEA) were determined using ion chromatography with detection by negative electrospray ionization mass spectrometry (IC-ESI-MS). Aliquots of urine samples were diluted 50-fold with deionized water. A 0.02 ml aliquot was further diluted to 1.0 ml with deionized water containing internal standards (final concentrations: −1−2 μg/ml each of 13C₁-GA, 13C₂-OX, D₂-DGA, and D₃-HEA). Diluted urine samples were analyzed via ion chromatography on a Dionex ICS-3000 Series RFIC system (Sunnyvale, CA) with suppressed conductivity and mass spectral detection. Ion chromatography conditions were RFIC EluGen cartridge EGC II KOH; Dionex IonPac AG11-HC guard column (4 × 50 mm), and Dionex IonPac AS11-HC analytical column (4 × 250 mm); column temperature 30°C; eluent flow 1.0 ml/min (split 50% to MS); injection volume 25 μl; gradient: 10 min linear ramp from 1 to 5 mM KOH, 11 min linear ramp from 5 to 60 mM KOH, 4.2 min hold at 60 mM KOH; suppressor ASRS-ULTRA II 4-mm at 138 mA. Mass spectrometry conditions were: Agilent LC/MSD SL operated in the selected-ion-monitoring mode; negative-ion electro-spray ionization; nitrogen drying gas at 350°C and 10 l/min; nebulizer pressure 40 psi; HV capillary 4000 V; fragmentor 70 V; EMV gain 2.0; quantitation ions for analyte/internal standard pairs: GA 75/76 amu, OX 89/91 amu, HEA 119/125 amu, DGA 133/137 amu at 146 msec/ion/scan. The achieved LOQ were 50 μg/ml for glycolate, 25 μg/ml for HEA, 26 μg/ml for oxalate, and 26 μg/ml for diglycolate in urine.

**Statistics.** Differences between treatment groups and time points were assessed with two-way ANOVA with Bonferroni post-hoc test. To compare differences between treatment groups only, one-way ANOVA with Tukey post-hoc test was used. All analyses were performed using GraphPad Prism 5 for Windows. Tests were considered significant if \( p < 0.05 \).

**RESULTS**

**Animal Observations: Clinical Signs and Mortality**

Animals were monitored throughout the time course of the experiment for behavioral signs that would indicate morbidity, including lack of eating or drinking or decreased responsiveness to stimuli. Within 4 h after DEG administration, all rats having received DEG appeared intoxicated with decreased balance and loss of motor control, but all animals returned to normal behavior by 12 h. Two rats treated with 10 g/kg DEG were lethargic starting at 36 h but still responsive when handled during blood collection. One rat treated with 10 g/kg DEG + fomepizole appeared to have small amounts of blood in his urine starting at 4 h and continuing until 48 h. Upon examination during the perfusion, the two animals treated with high dose noted above had grossly enlarged kidneys, whereas the one rat given DEG + fomepizole had several pustules on the surface of the kidneys. No other animals exhibited any overt signs of morbidity, and there was no mortality up to 48 h.
Metabolic Acidosis

Metabolic acidosis was assessed using the parameters of blood pH and blood bicarbonate (Fig. 2). By 4 h after a high dose of DEG and continuing until the endpoint at 48 h, both blood pH and blood bicarbonate were significantly decreased from controls ($p < 0.05$). Rats treated with a low dose of DEG had slightly decreased blood pH at 4–12 h with no change in bicarbonate; urine pH in these rats was significantly decreased ($p < 0.05$) at 8, 12, and 24 h, indicating a small degree of acidosis (data not shown). Concomitant administration of fomepizole (DEG + fomepizole) completely blocked the metabolic acidosis so that blood pH and bicarbonate remained similar to that of control animals throughout the experiment (Fig. 2). Plasma was also analyzed for concentrations of electrolytes, such as sodium, potassium, chloride, calcium as well as glucose levels. Treatment with DEG did not affect the plasma electrolytes or glucose at any of the time points (data not shown).

Renal Injury

BUN and plasma creatinine were the primary parameters used to assess renal injury (Fig. 3). At 36 and 48 h after DEG dosing, both BUN and creatinine were significantly increased in rats treated with the high dose of DEG compared with control animals ($p < 0.05$). Rats treated with low doses of DEG or with high-dose DEG plus fomepizole did not experience any increases in BUN or creatinine. Urine volume was also measured at each time point (Fig. 4). Rats treated with both high-dose DEG and high-dose DEG + fomepizole experienced significantly increased diuresis until 12 h ($p < 0.05$), whereas rats treated with low-dose DEG experienced only a slight diuresis until 4 h ($p < 0.05$). During killing, the unperfused kidney was removed and weighed and the kidney/body weight (bw) ratio was calculated as another marker of kidney injury. Rats treated with 10 g/kg DEG had significantly higher kidney/bw ratios (0.010 ± 0.001) than rats treated with 2 g/kg DEG, control rats, or rats treated with DEG + fomepizole (0.007 ± 0.001, 0.007 ± 0.001, 0.008 ± 0.0003, respectively; $p < 0.05$).

Renal Histopathology

The major pathological abnormalities, noted in the kidneys of rats treated with DEG alone, consisted of multifocal acute tubular necrosis with cell death and sloughing of the epithelial lining of the cortical tubular portion of the nephrons. The renal damage appeared to be dose dependent, being slight and patchy in distribution at the low dose, with worsening severity and extent at the high dose (Fig. 5). At a dose of 2 g/kg DEG, the cortical damage involved about 15% of the cortex in only one of the six rats compared with tubular necrosis involving the entire cortex in most animals that received 10 g/kg DEG (Fig. 6). Five of the six rats treated with high-dose DEG showed kidney pathology, whereas only one of the six rats treated with DEG + fomepizole showed any necrotic pathology (Table 1). When samples were examined under polarizing light microscopy, calcium oxalate crystals were not evident in kidney tissues of any rats treated with DEG (data not shown).

Evidence of Necrosis by Ethidium Homodimer Uptake

Staining of necrotic renal tubule cells via in situ perfusion was achieved using ethidium homodimer uptake. This technique is useful for visualizing necrotic cells in specific areas of the nephron and is sensitive enough to provide evidence of necrosis before gross markers of renal injury like BUN and creatinine are increased (Edwards et al., 2007). Animals treated with 10 g/kg DEG showed a greater degree of fluorescence than any of the other treatment groups, primarily focused in the cortical proximal tubule cells (Fig. 7). Ethidium homodimer staining was scored for each rat to assess the magnitude of the renal necrosis among the treatment groups (Fig. 7). The average score for rats treated with the high dose of
DEG was 12.1 ± 1.2, which was significantly greater than that for control rats (3.1 ± 1.7), low-dose DEG-treated rats (4.2 ± 1.2) and DEG + fomepizole-treated rats (3.1 ± 1.2; p < 0.05).

Liver Injury

To assess liver injury, AST and ALT levels were measured in the plasma (Fig. 8). By 36 h, AST was significantly increased only in high-dose DEG-treated rats compared with control rats (p < 0.05). ALT was not different between groups. Albumin, alkaline phosphatase, as well as both total and conjugated bilirubin were also measured in the plasma as markers of liver injury, but no differences were found among the treatment groups (data not shown).

Liver Histopathology

Injury in the liver was characterized by variable diffuse microvesicular fatty change (steatosis), with a minimally dose-dependent pattern (Fig. 9). Five of the six rats treated with high-dose DEG showed some degree of liver pathology characterized by steatosis with one rat having severe steatosis that was most marked in the perivenular region (Fig. 9, Table 1). Liver steatosis in all the other treatment groups was considered mild or moderate. The steatosis seen in the control rats indicates that a mild degree of steatosis is normal in these rats.

Urinary Metabolites

An initial screening of the high-dose sample urine was done using IC-MS with suppressed conductivity detection to look for the presence of anionic metabolites. HEAA was the major peak observed in the suppressed conductivity chromatogram; no other peaks, besides those observed as endogenous compounds in control urine, were observed in the high-dose urines (data not shown). Subsequently, more sensitive GC-MS and IC-MS techniques were used to quantify the concentrations of DEG and the metabolites EG, HEAA and DGA (Fig. 10) in the urine as described in the “Materials and Methods” section. Concentrations of DEG were highest at 4 h (about 450mM), then decreased throughout the time course and were completely eliminated from the urine by 48 h for the high-dose group and by 36 h for the low-dose group (Fig. 10A). DEG excretion in the urine followed first-order kinetics with calculated half-life of 5.8 h for 2 g/kg DEG and 8.5 h for 10 g/kg DEG. Rats treated with DEG + fomepizole had significantly higher concentrations of urinary DEG compared with those treated with DEG alone (p < 0.05, starting at 8 h).

For rats treated with high-dose DEG, the metabolite HEAA peaked at about 200mM in the urine between 12 and 24 h (Fig. 10B). Interestingly, the urinary HEAA concentrations in rats treated with low-dose DEG peaked at 8 h and were roughly equivalent to those in the high dose rats up to 8 h. HEAA was eliminated from the urine by 36 h in low dose–treated rats but was still present in urines at 48 h in rats treated with high-dose DEG. Concomitant treatment with fomepizole (DEG + fomepizole) completely blocked the metabolism of DEG to HEAA, thus HEAA was not increased in the urine from this treatment group. DGA was detected in rats treated with both low and high doses of DEG (Fig. 10C), but at concentrations

FIG. 3. Treatment with high-dose DEG produces kidney injury by 36 h as assessed by BUN (A) and plasma creatinine (B). Data are represented as means ± SEM (n = 6 for all treatment groups). Asterisk indicates significant difference from control rats (p < 0.05).

FIG. 4. Urine volume is increased in rats treated with high-dose DEG, regardless of fomepizole treatment. Data are represented as means ± SEM (n = 6 for all treatment groups). Asterisk indicates significant difference from control rats (p < 0.05).
that were 50-fold lower than those of HEAA. Urinary DGA peaked at 12 h at 4mM for low-dose DEG-treated rats and at 24 h at 4mM for high-dose DEG-treated rats and was still present at the endpoint of the experiment at 48 h. Treatment with DEG þ fomepizole completely blocked the formation of DGA. Because DEG has been theorized to be metabolized to EG, urinary EG concentrations were also measured (Fig. 10D). EG concentrations steadily increased until they peaked at about 7mM at 12 (low dose) or 24 h (high dose). Interestingly, concomitant treatment with fomepizole decreased the concentration of EG in the urine (compared with rats treated with DEG alone), starting at 12 h. Neither glycolate nor oxalate was excreted in the urines of the DEG-treated rats at concentrations higher than those measured in the control rats (data not shown).

Analyses of these metabolite time-concentration profiles for area under the curve (AUC) showed that the duration of exposure to DEG was doubled by concomitant treatment with fomepizole (Table 2). These data also showed that, although peak HEAA concentrations were similar after the low and high doses of DEG, the duration of exposure to elevated concentrations of HEAA was almost three times greater after the high-dose exposure. Concomitant treatment with fomepizole diminished the exposure of the high-dose DEG-treated rats to HEAA, DGA, and EG (Table 2).

**TABLE 1**

<table>
<thead>
<tr>
<th>Degree of Tissue Injury in DEG-Treated Rats</th>
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<tr>
<td>Treatment group</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>2 g/kg DEG</td>
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<tr>
<td>10 g/kg DEG</td>
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<tr>
<td>10 g/kg DEG + fomepizole</td>
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Note: Data are represented as numbers of rats assigned to each of the following categories: (-) no injury, (+) mild injury, (++) moderate injury and (+++) severe injury with n = 6 for each treatment group. Asterisk indicates damage that was classified as acute pyelonephritis.

**FIG. 5.** Hematoxylin and eosin images of cortical renal tissue for each of the treatment groups shows little to no tissue damage for rats treated with vehicle (control) (A), 2 g/kg DEG (B), and 10 g/kg DEG + fomepizole (D), but severe tissue injury for rats treated with 10 g/kg DEG (C). Magnification ×400 for all images.

**FIG. 6.** Representative hematoxylin and eosin images of cortical renal tissue for each of the treatment groups shows little to no tissue damage for rats treated with vehicle (control) (A), 2 g/kg DEG (B), and 10 g/kg DEG + fomepizole (D), but severe tissue injury for rats treated with 10 g/kg DEG (C). Magnification ×400 for all images.

**DISCUSSION**

The treatment for DEG toxicity remains mostly palliative because the mechanisms of DEG toxicity are not well understood. Previous studies in animals have explained the metabolism (Lenk et al., 1989; Mathews et al., 1991; Wiener and Richardson, 1989) or toxicokinetics (Heilmair et al., 1993) of nontoxic doses of DEG (< 5 g/kg bw) but would not have revealed toxic metabolites that may accumulate at high doses. Furthermore, no studies have convincingly demonstrated that
inhibitors of DEG metabolism can block the target organ toxicity of DEG (Borron et al., 1997; Brophy et al., 2000; Wiener and Richardson, 1989). The present experimental design has overcome these deficiencies to demonstrate that the mechanism for the kidney and liver injury in rats involves the formation of toxic metabolites from DEG. We have used fomepizole to unequivocally demonstrate that the metabolites of DEG, but not DEG itself, are responsible for the observed toxicity in rats. Furthermore, our results suggest that HEAA is most likely the key toxic metabolite because it is found in the urine at concentrations 50× greater than those of other metabolites such as DGA.

FIG. 7. Ethidium homodimer (EthD) staining, indicative of necrosis, is increased in rats treated with high-dose DEG. At 48 h, the left kidney was perfused with 5µM EthD that binds to DNA in cells with compromised cell membranes and fluoresces. Perfused kidneys were frozen in Optimal Cutting Temperature medium and then 5-µm slices were cut and counterstained with DAPI to stain all cells. Each pair of images are the same field of view for DAPI (left) and EthD (right) staining. (A and B) Control rats. (C and D) 2 g/kg DEG rats have some EthD uptake, but only in single cells. (E and F) 10 g/kg DEG rats have extensive EthD uptake, especially in cortical tubules. (G and H) 10 g/kg DEG + fomepizole have little to no EthD uptake, limited only to single cells. (I) EthD staining was scored from 0 to 15, with 0 indicating no necrosis and 15 indicating extensive tubule necrosis. Data are represented as means ± SEM (n = 6 for each treatment group). Asterisk indicates significant difference from control, low dose, and DEG + fomepizole-treated rats (p < 0.05).

FIG. 8. Markers of liver injury AST (A) and ALT (B) in rats treated with high-dose DEG. Data are represented as means ± SEM (n = 6 for each treatment group). Asterisk indicates significant difference from control rats (p < 0.05).
Fomepizole is a useful tool to delineate the role of the metabolism of DEG in its toxicity. Although fomepizole can also inhibit cytochrome P450 isozymes (Chow et al., 1992), this occurs only at doses (100 mg/kg) well above those at which it inhibits ADH (10 mg/kg) (Jacobsen et al., 1996). Thus, at the doses in this study (which are the same as those used to treat methanol and EG poisoning; Brent, 2009; Brent et al., 1999), fomepizole acts primarily as an ADH inhibitor. Because DEG is thought to be initially metabolized by ADH, fomepizole was used to block this metabolic step to assess whether DEG itself or its metabolites were responsible for DEG toxicity. Rats that were treated with high-dose DEG + fomepizole had no increases in BUN or creatinine, and only one of the six rats showed mild kidney necrosis by pathological analysis. In contrast, rats treated with high-dose DEG alone had significantly elevated markers for kidney injury, as well as marked renal necrosis in five of the six high-dose DEG-treated rats. DEG + fomepizole-treated rats showed no increases in liver enzymes, and only limited steatosis was noted by histopathology, whereas increased AST and steatosis were observed in high-dose DEG-treated rats. Analysis of the urinary metabolites from high-dose DEG and DEG + fomepizole-treated rats indicated that fomepizole completely blocked the metabolism of DEG to HEAA and DGA. Also, because fomepizole blocked DEG metabolite formation, the concentrations of urinary DEG in DEG + fomepizole-treated rats were markedly greater for a longer time than in rats treated with high-dose DEG alone, as confirmed by the AUC analysis. Although it has been suggested that DEG itself may be

FIG. 9. Liver injury is evident in rats treated with high-dose DEG. Representative hematoxylin and eosin images of liver tissue show no injury in rats treated with vehicle (control) (A), low-dose DEG (B), and high-dose DEG + fomepizole (D). Injury in rats treated with high-dose DEG (C) is characterized by steatosis that is most marked in the perivenular regions. Magnification ×400 for all images.

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FIG. 10. Urinary DEG (A) and its metabolites, HEAA (B), DGA (C), and EG (D) from rats treated with DEG. Data are represented as means ± SEM (n = 6 for each treatment group). Asterisk indicates significant difference between high-dose DEG-treated group and DEG + fomepizole-treated group (two-way ANOVA followed by Bonferroni post-hoc test, p < 0.05). Statistical analysis also showed that, for HEAA (10B), for DGA (10C), and for EG (10D), there were no significant differences between the DEG + fomepizole group and the control groups.
responsible for the organ toxicity (Scalzo, 1996), our results clearly demonstrate that parent DEG itself is not nephrotoxic or hepatotoxic, but rather that metabolites are responsible for the organ toxicity.

Clinically, fomepizole is administered by slow iv infusion (Brent et al., 1999). Previous studies have shown that identical doses of fomepizole by iv infusions and by oral dosing produce equivalent pharmacokinetics in human subjects (Marraffa et al., 2007). Also oral dosing in humans (Jacobson et al., 1996) and ip administration in animals (McMartin et al., 1975) are capable of producing effective inhibition, suggesting that ip dosing is an adequate route of administration. Thus, in these studies, we administered fomepizole by ip dosing to avoid the 30 min iv infusion that would otherwise be necessary. Administration of fomepizole was begun 15 min after DEG ingestion to insure the complete blockage of DEG metabolism throughout the experiment. However, patients intoxicated with DEG are rarely able to be treated this quickly because DEG intoxication is not easily recognized, and thus, the use of fomepizole as an antidote for DEG poisoning must be more fully studied using clinical scenarios. Despite this, fomepizole may be a useful antidote for treating acute DEG poisoning and has been used with some success clinically (Boron et al., 1997; Brophy et al., 2000).

In the past, some studies have suggested that EG might be a primary metabolite of DEG (Durand et al., 1976; Hebert et al., 1978; Winik et al., 1978). In this low concentrations (25–50× smaller than those of HEAA). The levels of EG found in the DEG-treated groups were statistically higher than the trace levels found in the control group (Fig. 10D); also the total AUC for these groups was markedly elevated over that in the control group (Table 2). EG was not likely a contaminant of the initial DEG (measured at only 0.05%) that was given to the rats, as has been suggested for previous studies (Wiener and Richardson, 1989), because its concentrations were low at 4 h and increased to a peak at 12–24 h in rats treated with DEG (Fig. 10D). If EG was an initial contaminant, urinary EG concentrations would have been highest at 4 h (like DEG) and then decreased throughout the time course. The timing of urinary EG excretion suggests that EG was probably not produced by either cleavage of DEG, but rather by ether cleavage of either HEAA or 2-hydroxyethoxyacetaldehyde, via CYP-450-based O-dealkylation (as noted by the dashed arrows in Fig. 1). Additionally, EG concentrations were significantly reduced in rats treated with DEG + fomepizole, suggesting that inhibition of ADH decreased formation of EG from the DEG metabolites that were initially produced by ADH. Despite the presence of EG in urines of DEG-treated rats, it is unlikely that EG metabolism contributed to the observed renal toxicity because no oxalate crystals, the metabolite responsible for kidney damage in EG poisoning (Guo et al., 2007), were observed in the kidney tissue. Also, the primary metabolites of EG, glycolate and oxalate, were not increased in the urine from DEG-treated rats. The Wistar rat was specifically selected for these studies because it is the rat strain most sensitive to oxalate accumulation from EG exposure (Cruzan et al., 2004). As such if DEG were to induce oxalate crystal accumulation, it would have occurred most likely in the sensitive Wistar rat. The lack of oxalate accumulation in the DEG-treated Wistar rat thus corroborates the most recent clinical studies, which have reported no oxalate crystals and no increases in urinary oxalate in DEG-intoxicated humans (Alfred et al., 2005; Brophy et al., 2000; Ferrari and Giannuzzi, 2005).

The metabolism of low-dose DEG (Mathews et al., 1991; Wiener and Richardson, 1989) and high-dose DEG (our results) in rats appears to be similar, in that the parent compound was the most abundant compound excreted in the urine (450 mmol/l) followed by HEAA (200 mmol/l). Previous studies have not reported any urinary DGA excretion or overt toxicity at doses below 5 g/kg, whereas small amounts of DGA were excreted after the high toxic doses in our studies. Because HEAA was the most abundant metabolite (at 50× greater concentrations than DGA) in the rats that had marked renal and hepatotoxicity, it is the most likely candidate responsible for the observed toxicity. We also noticed a variation in target organ toxicity and metabolite concentrations among the rats treated with high dose that further suggested that HEAA was the toxic metabolite. One of the six animals treated with high-dose DEG had normal BUN and creatinine levels and unremarkable renal pathology; this rat also had markedly lower urinary HEAA concentrations compared with the five other rats in the treatment group.

Peak HEAA concentrations in rats treated with high dose (~200 mmol/l) were not markedly greater than those in rats treated with low dose (~175 mmol/l), yet the differences in toxicity were great between the two doses. For example, 2 g/kg DEG produced minimal toxicity in rats, whereas 10 g/kg DEG produced significant kidney and liver toxicity. However, as shown by the AUC analysis, the duration of exposure to HEAA was markedly longer in rats treated with high dose, suggesting that the biological response to the toxicant may be duration dependent rather than concentration dependent. Although our data suggest that HEAA is the toxic metabolite, they are not

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**TABLE 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>2 g/kg DEG</th>
<th>10 g/kg DEG</th>
<th>10 g/kg DEG + fomepizole</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEG</td>
<td>28</td>
<td>2293</td>
<td>7577</td>
<td>15,888</td>
</tr>
<tr>
<td>HEAA</td>
<td>16</td>
<td>2593</td>
<td>6175</td>
<td>324</td>
</tr>
<tr>
<td>DGA</td>
<td>7</td>
<td>98</td>
<td>118</td>
<td>11</td>
</tr>
<tr>
<td>EG</td>
<td>26</td>
<td>136</td>
<td>213</td>
<td>83</td>
</tr>
</tbody>
</table>

*Note.* The time-concentration profiles (Fig. 10) were analyzed for the area under the respective curves using Graph-Pad Prism. The numbers represent the total AUC computed for each metabolite given the respective treatments.
definitive because the accumulation of metabolites in the target organ tissues is still unknown. Additionally, although DGA concentrations in the urine were relatively low, there are no data on the potency of either metabolite, so it is not known whether lower concentrations of DGA might cause injury. The present studies, by determining the urinary concentrations of the potential toxic metabolites from DEG-treated rats, provide key information on the relevant concentrations of these metabolites that should be examined in in vitro studies of toxicity.

An epidemic of DEG-induced acute renal failures in Haiti in 1996 resulted in all patients displaying significantly elevated liver enzymes and markers for kidney injury (O’Brien et al., 1998). The nephrotoxic effects of DEG in this study were similar to those that have been reported in previous animal studies (Durand et al., 1976; Hebert et al., 1978) and in clinical cases (Ferrari and Giannuzzi, 2005; O’Brien et al., 1998; Schep et al., 2009), basically increased markers of renal failure plus histopathological evidence of renal damage. In addition, the ethidium homodimer uptake studies have demonstrated that the renal necrosis resides primarily in the proximal tubule cell, suggesting that metabolites of DEG are capable of inducing proximal tubular cell death. The results from our study indicate that the kidney was the major target and that the liver injury appeared to be secondary. Pathology reports showed that liver steatosis only occurred in those animals with the most severe kidney injury. Additionally, only AST levels were elevated in high-dose DEG rats, whereas ALT remained unchanged. If the liver was a primary target of DEG toxicity, we would have expected to observe significant increases in both AST and ALT (like in humans—O’Brien et al., 1998) as well as more severe histopathology. It is not clear why AST levels were increased without concomitant changes in ALT levels. Possibly, there is lesser hepatotoxicity with a single dose of DEG as opposed to the repeated doses reported in most human cases (discussed below).

The toxic dose of DEG in humans has been estimated to be about 0.2 g/kg bw and a lethal dose about 1–1.5 g/kg bw (Brophy et al., 2000; O’Brien et al., 1998). The dose that was necessary to produce extensive renal injury in rats (10 g/kg bw) was significantly higher than that expected to cause lethality in humans, whereas the low dose (2 g/kg bw) produced only a minor transient acidosis and not any kidney injury in rats. Although the determination of toxic or lethal doses in humans is difficult and often inaccurate (because of the use mostly of second-hand information and faulty memories), there may still be a discrepancy in dose-response between rats and humans. One explanation for such a discrepancy might be a greater sensitivity in humans to DEG toxicity, so that smaller doses in humans cause more damage. Another explanation is related to the dosing regimen. In most epidemic DEG poisonings, patients are subjected to multiple small doses of DEG over several days before clinical symptoms develop (Ferrari and Giannuzzi, 2005; O’Brien et al., 1998). In our study, a single acute dose of DEG was administered, but large doses were necessary to observe metabolism and target organ toxicity of DEG. It is possible that repeated small doses of DEG (1 g/kg or less) given to rats would have effects similar to those seen in humans exposed to multiple small doses. Finally in our studies, rats were exposed to a very pure DEG preparation, whereas human exposures have involved DEG as an industrial-grade adulterant that might have contained other potentially nephrotoxic chemicals, in particular EG. Thus, the toxic doses of DEG per se may have been underestimated in humans.

In conclusion, this study has convincingly shown that the mechanism for the liver and kidney toxicity in rats administered high doses of DEG involves formation of unique toxic metabolites. Blocking the metabolism of DEG via concomitant administration of the ADH inhibitor fomepizole prevented target organ toxicity demonstrating that the metabolites of DEG, and most likely HEAA, were responsible for the toxicity. These studies confirmed that, in rats, DEG is not metabolized to EG in sufficient amounts to induce the DEG-associated renal toxicity rather that high toxic doses of DEG were exclusively metabolized by ADH to other nephrotoxic metabolites. As such, inhibition of ADH should be the clinical objective for treatment of DEG-intoxicated humans if treatment can be applied early after ingestion.

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REFERENCES


