Diglycolic Acid Is the Nephrotoxic Metabolite in Diethylene Glycol Poisoning Inducing Necrosis in Human Proximal Tubule Cells In Vitro

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Diethylene glycol (DEG), a solvent and chemical intermediate, can produce an acute toxic syndrome, the hallmark of which is acute renal failure due to cortical tubular degeneration and proximal tubular necrosis. DEG is metabolized to two primary metabolites, 2-hydroxyethoxyacetic acid (2-HEAA) and diglycolic acid (DGA), which are believed to be the proximate toxicants. The precise mechanism of toxicity has yet to be elucidated, so these studies were designed to determine which metabolite was responsible for the proximal tubule cell death. Human proximal tubule (HPT) cells in culture, obtained from normal cortical tissue and passaged 3–6 times, were incubated with increasing concentrations of DEG, 2-HEAA, or DGA separately and in combination for 48 h at pH 6 or 7.4, and various parameters of necrotic and apoptotic cell death were measured. DEG and 2-HEAA did not produce any cell death. DGA produced dose-dependent necrosis at concentrations above 25 mmol/l. DGA did not affect caspase-3 activity and increased annexin V staining only in propidium iodide-stained cells. Hence, DGA induced necrosis, not apoptosis, as corroborated by severe depletion of cellular adenosine triphosphate levels. DGA is structurally similar to citric acid cycle intermediates that are taken up by specific transporters in kidney cells. HPT cells, incubated with N-(p-aminocinnamyl)lanthanilic acid, a sodium dicarboxylate-1 transporter inhibitor showed significantly decreased cell death compared with DGA alone. These studies demonstrate that DGA is the toxic metabolite responsible for DEG-induced proximal tubular necrosis and suggest a possible transporter-mediated uptake of DGA leading to toxic accumulation and cellular dysfunction.

Key Words: diethylene glycol; nephrotoxicity; proximal tubular necrosis; hydroxyethoxyacetate; diglycolic acid; oxybisacetate.

Diethylene glycol (DEG; 2,2′-oxybisethanol; CAS RN 111-46-6) is an industrial solvent that may present a significant health challenge because of its presence in commercial products, which can be mistakenly or intentionally ingested (Marraffa et al., 2008). Most modern poisoning cases have involved illegal substitution as a solvent in place of propylene glycol or glycerin. The most known such poisoning epidemic occurred in the United States back in 1937, where 105 people died from acute renal failure after ingesting a sulfanilamide preparation that had been dissolved using 72% DEG (Wax, 1995). Unfortunately in countries that lack strict enforcement of drug safety regulations, epidemic poisonings with DEG are still occurring (Schep et al., 2009), such as that which occurred in Nigeria in late 2008 resulting in 84 deaths of children under the age 3 after ingesting a teething syrup contaminated with DEG (Alkahtani et al., 2010).

DEG poisoning affects the renal, hepatic, and nervous systems and leads to renal failure, liver toxicity, and peripheral neuropathies, with kidney damage and failure being the predominant feature (Alfred et al., 2005; Cantarell et al., 1987; Hasbani et al., 2005; Rollins et al., 2002). The mechanisms that are responsible for DEG toxicity have not been fully elucidated, although metabolism of DEG appears to be important. Studies administering nontoxic doses (0.5–5 g/kg) of carbon-14 labeled DEG to rats and dogs concluded that ~70–80% of the radiolabeled DEG was excreted in the urine unchanged, and the remaining 10–30% was excreted as urinary 2-hydroxyethoxyacetic acid (2-HEAA) (Lenk et al., 1989; Mathews et al., 1991). However, in rats treated with toxic doses of DEG, our recent studies have revealed the minor presence of an additional metabolite diglycolic acid (DGA) (Besenhofer et al., 2010) at about 50-fold lower levels than that of 2-HEAA. No additional anionic metabolites were observed, suggesting that DGA may be a terminal metabolite of DEG. It has been suggested that DEG, or perhaps the 2-HEAA metabolite, may be the proximate toxicant(s) causing target organ toxicity because they attain relatively high urinary concentrations in DEG-treated animals (Mathews et al., 1991; Wiener and Richardson, 1989). However, it has recently been confirmed that administration of fomepizole (4-methylpyrazole), an alcohol dehydrogenase inhibitor, prevented both DEG metabolism and the subsequent target organ toxicity in rats (Besenhofer et al., 2010). This study provided definitive evidence that a metabolite is responsible for the toxicity observed in DEG poisonings. Subsequent studies have shown that, although DGA is a minor metabolite in the urine and blood, it is markedly accumulated in the kidney to levels as high as those of 2-HEAA (Besenhofer et al., 2011) Nevertheless, the specific metabolite responsible for the target organ toxicity...
could not be identified in those studies, and identification of the toxic metabolite is a necessary component for determining the mechanism of toxicity.

The purpose of this study was to determine which of the metabolites, 2-HEAA, DGA, or both in combination, was responsible for the kidney toxicity using cultured human proximal tubule (HPT) cells to model the major kidney region targeted in DEG toxicity (Guo et al., 2007; Schep et al., 2009). Because, DGA, not 2-HEAA, appeared to cause the most necrotic damage to HPT cells, further studies used inhibitors of several organic anion transporters (OATs) to determine if HPT cell death was dependent upon inhibition of DGA transport into the proximal tubule cell.

MATERIALS AND METHODS

Materials. DEG (97.5%) and DGA (98%) were purchased from Sigma-Aldrich (St Louis, MO). 2-HEAA (97%; lot no. CX1866) was custom synthesized by Isotech. Growth media (Dulbecco’s Modified Eagle’s Medium, DMEM, and Ham’s F-12 Medium, F-12), fetal bovine serum, and other cell culture grade chemicals were purchased from Gibco Life Technologies (Grand Island, NY). Purified bovine collagen type I was purchased from Vitrogen (Palo Alto, CA). Media supplements (insulin, transferrin, selenium, hydrocortisone, and epidermal growth factor) were obtained from BD Collaborative Biomedical Products (Bedford, MA), whereas triiodothyronine was obtained from Sigma Chemical (St Louis, MO). Ethidium homodimer and the EnzChek Caspase-3 Assay Kit #1 were purchased from Invitrogen (Carlsbad, CA). The lactate dehydrogenase (LDH)-cytotoxicity assay kit II and Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit were purchased from BioVision (Mountain View, CA). The cell death detection ELISA and adenosine triphosphate (ATP) Bioluminescence Assay Kit HS II were purchased from Roche Applied Science (Indianapolis, IN). N-(p-aminocinnamoyl)anthranilic acid (ACA), 2,3-dimethylsucinate (2,3-DMS), probenecid (PBD), and succinic acid as well as all other chemicals were obtained from Sigma-Aldrich. The cellular protein assay (bicinchoninic acid method) was provided by Pierce Chemical (Rockford, IL).

Cell culture. HPT cells were isolated from kidneys removed by nephrectomy because of tumor or trauma. Healthy tissue from the outer cortical region was removed, whereby it was remote from any pathologic alterations, as judged by the institutional pathologist. The Institutional Review Board for Human Research (Louisiana State University Health Sciences Center, Shreveport, LA) approved these studies on these tissues. HPT cells were isolated using a collagenase-DNase digestion, filtration, and centrifugation technique that produced a suspension of primarily PT cells (Todd et al., 1996). The suspensions were cultured in neutral bovine collagen-coated flasks in a serum-free mixture of Dulbecco’s modified Eagle medium and Ham’s F-12 nutrient mixture (50:50) with growth factors (insulin-transferrin-selenium mixture, hydrocortisone, triiodothyronine, epidermal growth factor, and glutamine) and grown for 5–7 days until confluent. For experiments, cells were subcultured into 24-well collagen-coated plates using trypsin-EDTA (0.25%) solution. HPT cells retain the properties of the proximal tubule in vivo as indicated by their polarized boundaries when grown on membrane inserts (Morshead et al., 1997) and retention of transport properties associated with the proximal tubule (sodium-directed glucose transport across the apical membrane). HPT cells also sustain such enzyme activities as succinate dehydrogenase, nicotinamide adenine dinucleotide/ nicotinamide adenine dinucleotide phosphate dehydrogenase, LDH, glucose-6-phosphatase, alkaline phosphatase, γ-glutamyltranspeptidase that are important for metabolism in the proximal tubule (Detrisac et al., 1984; Kempson et al., 1989; Triulli et al., 1985). HPT cells sustain oxidative respiration activity in culture over at least four passages, although somewhat reduced from the activity in freshly seeded cells (Luttropp et al., 2011). HPT cells in culture to confluency maintain the expression and activity of a number of transport proteins, including OATs, organic cation transporters, and multidrug resistance proteins suggesting transport competence of the cells (Brown et al., 2008; Lash et al., 2006). The expression and activity of drug metabolizing enzymes are significantly preserved in cultured HPT cells (Lash et al., 2008). These studies have shown that expression of such enzymes families as cytochrome P450s, glutathione S-transferases, uridine diphosphate glucuronosyltransferases, and sulfotransferases is kept at significant levels atconfluence, albeit somewhat reduced from fresh tissue, suggesting that these cells remain metabolically active in culture.

Cell exposures to assess necrotic and apoptotic cell death. Growth media was removed, and confluent HPT cell cultures were washed twice with warm PBS. Each chemical (DEG, 2-HEAA, or DGA (all at 0–100 mmol/l)) was first dissolved in growth media, which were then adjusted to pH 6 or 7.4 by the addition of HCl or NaOH (to mimic acidic or neutral conditions); these media were added to separate wells in triplicate, and the cells were incubated for up to 48 h under standard conditions (5% CO2, 90% humidity, 37°C). These concentrations were chosen because these metabolites can reach concentrations as high as 200 mmol/l in the urine of DEG-treated rats (Bensenhofer et al., 2010). A 48 h incubation time was chosen based on pilot time course experiments with all three compounds, in which increased DGA-induced cell death was not observed at earlier time points (6 and 24 h). After the incubations, the media were collected to measure LDH release, and cells were treated as described below to assess ethidium homodimer (EtHD) uptake as two measures of necrotic cell death. In separate experiments, cells were incubated with DEG, 2-HEAA, and DGA at the concentrations described above for 48 h at pH 6, and apoptosis was assessed using caspase-3 activity and a cytoplasmic mononuclearcytosomal detection ELISA as initial parameters for apoptosis. To assess effects on annexin V/propidium iodide (PI) staining and on cellular ATP levels, cells were incubated with a 50 mmol/l DGA solution in complete media at pH 6 for multiple time points.

Measurement of ethidium homodimer (EtHD) uptake. EtHD uptake and fluorescence is a quantitative measure of cell membrane damage and necrotic cell death. After treatment of cells and removal of treatment media, cells were incubated with a 2 μmol/l EtHD solution, and EtHD fluorescence was measured as described previously (Guo and McMartin, 2005).

Measurement of LDH release. As a second parameter of necrotic cell death, LDH released into the media was measured using the LDH-cytotoxicity assay kit II according to manufacturer’s instructions (BioVision). To normalize this parameter for potential cell confluency differences, the cells were solubilized with 0.1% Triton X-100 and cellular protein analyzed using the bicinchoninic acid method. For experiments in which ATP was measured, cells were solubilized using the lysis reagent provided in the kit.

Measurement of caspase-3 activity. Cells were incubated as described above, media were collected and cells were lysed to measure intracellular caspase-3 activity using fluorometric substrate aspartate-glutamate-valine-aspartate-7-amino-4-methylcoumarin with the EnzChek Caspase-3 Assay Kit #1 (Invitrogen, Carlsbad, CA). The assay was carried out according to manufacturer’s instructions.

Cell death detection ELISA. Cells were treated as described above, and detection of mononucleosomes and oligonucleosomes in the cytoplasmic fractions of treated cell lysates was done using the Cell Death Detection ELISA Kit according to manufacturer’s instructions (Roche Applied Science).

Measurement of annexin V and propidium iodide staining. Annexin V and PI staining was determined using flow cytometry as described previously with slight modifications (Goldberg et al., 1999; Schutte et al., 1998). Assay was performed using the Annexin V-FITC Apoptosis Detection Kit. Briefly, cells were exposed to a 50 mmol/l DGA solution in pH 6 growth media for 1, 2, 4, 6, 12, 24, and 36 h, and media were removed. Adherent cells were released with 0.25% trypsin-EDTA and collected by centrifugation at 1000 rpm for 10 min at 4°C. The cell pellet was resuspended in 1 ml of complete media and centrifugation. The resuspended pellet was resuspended in 500 μl binding buffer and incubated with annexin V (10 μl/ ml) and PI (10 μl/ml) for 15 min. Annexin V-FITC binding was analyzed by flow cytometry (λex = 488 nm, λem = 530 nm) using FL1 signal detector (FL1) and PI staining by the phycoerythrin emission signal detector (FL2). For each measurement, 20,000 events were counted.
Measurement of ATP levels.  Cellular ATP levels were measured using the ATP bioluminescence assay kit HS II in which the enzyme luciferase catalyzes the conversion of ATP and luciferin to adenosine monophosphate and bioluminescent light. Cells were exposed to a 50-mmol/l DGA solution in pH 6 growth media for 1, 2, 4, 6, 12, 24, and 36 h. After the incubations, the media were collected to measure LDH concentrations as above, then cells were lysed, and cellular ATP levels were measured as per manufacturer’s instructions (Roche Applied Science).

Measurement of succinate dehydrogenase activity. Succinate dehydrogenase activity was measured spectrophotometrically as described with slight modifications (Cimen et al., 2010). Briefly, mitochondria (1 mg/ml) were preincubated in a buffer containing 300-mmol/l mannitol, 6-mmol/l KH₂PO₄, 14-mmol/l K₂HPO₄, 10-mmol/l KCl, 5-mmol/l MgCl₂, pH 7.2. Sodium azide (40 mmol/l) was added prior to the addition of 500 μmol/l 2,6-dichlorophenolindophenol (DCPIP). Sodium succinate and DGA (both at 50 mmol/l) were added immediately before measuring enzymatic activity by monitoring the reduction of DCPIP at 600 nm.

Cell exposures with potential DGA uptake inhibitors. Cells were exposed to DGA (0, 30, or 50 mmol/l) at pH 6 as described above. In experiments where cells were cocultivated with PBD (100 mmol/l), 2,3-DMS (1 mmol/l) or succinate (10 mmol/l) and DGA, the coinucubation was 48 h. However, in ACA treatment groups, ACA (1 mmol/l) was added 15 min prior to DGA addition, then the subsequent 48 h incubation. EtHD uptake was assessed as described above.

Statistical analysis. Data were compared by one-way ANOVA, with either Tukey’s or Dunn’s post hoc tests to compare differences between individual groups or differences between individual groups and the control, respectively. A significance level of p < 0.05 was used. All analyses were performed using GraphPad Prism 5 for Windows. Single experiments were conducted using triplicate wells of a single cell isolate per concentration of compound. Experiments were then replicated with multiple cell isolates (tissue source). Results are expressed as a mean of multiple experiments ± SEM, where n represents the number of tissue sources.

RESULTS

Diglycolic Acid (DGA) Is the Only Metabolite That Produces a Dose-Dependent Necrosis in Cultured Human Proximal Tubule Cells

To confirm the in vivo results that DEG itself is not toxic and produces renal toxicity via a metabolite, DEG in concentrations up to 100 mmol/l did not produce necrotic cell death in HPT cells—no increase in LDH release or EtHD uptake (data not shown). 2-HEAA, also did not produce necrotic cell death at either pH 6 or 7.4, as indicated by no significant EtHD uptake (Figs. 1A and B) nor any LDH release (data not shown). In contrast, HPT cells incubated with DGA at 50 and 100 mmol/l showed significant (p < 0.05) increases in EtHD uptake (Fig. 1C) as well as in LDH release in a concentration-dependent manner (Fig. 1E). Linear regression analyses confirmed a positive relationship between increasing DGA concentrations and increases in EtHD uptake (r = 0.990 and 0.962 at pH 6 and 7.4, respectively) as well as increases in LDH release (r = 0.999 for both pH 6 and 7.4). Increases in necrotic cell death parameters following DGA incubation did not attain statistical significance until 50 mmol/l concentrations. However, visual inspection of the data suggests the possibility that cell death may be occurring at concentrations lower than 50 mmol/l. Interestingly, cells appeared to be more sensitive to DGA toxicity at pH 6 than at pH 7.4, as indicated by significant increases in EtHD uptake at 50 mmol/l. Although appearing higher at pH 6, LDH release was not significantly different between pH groups at any DGA concentration (compare Figs. 1C and D; Figs. 1E and F).

Because proximal tubule cells are exposed to both the metabolites and the parent compound at the same time during DGA poisoning, combinations of DGA and HEAA or HGA produced a different degree of cell death (Figs. 2A and B), especially when compared with DGA alone. Furthermore, incubations with both metabolites did not increase the degree of cell death compared with DGA alone (Fig. 2C).

No DGA Metabolite Appears to Induce an Apoptotic Event

Although DGA or 2-HEAA did not produce any necrosis, studies using all three compounds were done to address the possibility that one might produce apoptotic cell death. DEG did not produce apoptosis as determined by a lack of caspase-3 activation, as well as no production of oligonucleosomes in samples treated with DGA alone (data not shown). Furthermore, 2-HEAA did not induce caspase-3 activation nor did it increase the amount of nucleosomal fragments even at very high concentrations (Figs. 3A and B). DGA significantly (p < 0.05) increased the amount of nucleosomal fragments at 50 mmol/l but not at 100 mmol/l; however, DGA did not increase caspase-3 activation at any concentration (Figs. 3C and D).

Flow cytometric analysis of apoptosis showed that cells exposed to 50 mmol/l of DGA at multiple time points up to 36 h had significant increases in PI staining, indicating a cellular necrotic event (data not shown). Annexin V staining was only present in cells that also showed increased PI staining, indicating that these cells had experienced a late-apoptotic, early-necrotic event (data not shown), but not apoptosis per se.

DGA Induces a Time-Dependent Decrease in Cellular ATP Concentrations That Precedes the Increased LDH Release

Severely, decreased ATP production, most likely due to mitochondrial dysfunction, can cause necrotic cell death. In HPT cells exposed to 50 mmol/l of DGA, a time dependent decrease in ATP concentrations was observed (Fig. 4). This decrease preceded a time dependent increase in LDH release. Interestingly, ATP concentrations fell rapidly such that significant ATP loss was observed beginning at 2 h, with further depletion up to 36 h. The increase in LDH release did not attain statistical significance until 36 h.

Small Molecule Inhibitors of Sodium Dicarboxylate Transporter-1, but Not Sodium Dicarboxylate Transporter-3, Reduce DGA-Induced Cell Death in Human Proximal Tubule Cells

DGA accumulated markedly in the kidney tissues of rats treated with high doses of DGA, suggesting a role for concentrative
uptake of DGA (Besenhofer et al., 2011). Both the low affinity sodium dicarboxylate-1 transporter (NaDC-1) and the high affinity sodium dicarboxylate-3 transporter (NaDC-3) are primarily localized to the proximal tubule cell (apical and basolateral membrane, respectively) and can be involved in concentrative uptake of various dicarboxylates. Coincubation of HPT cells with 1 μmol/l ACA, a potent NaDC-1 inhibitor (IC$_{50} <$ 15 μmol/l) (Pajor and Randolph, 2007), decreased the degree of cell death by about 50% when compared with 50-mmol/l DGA alone (Fig. 5A). The coincubated values were reduced to the value of the control group ($p > 0.05$). Coincubation of HPT cells with 1-mmol/l 2,3-DMS, a competitive NaDC-3 inhibitor (Burckhardt et al., 2005) did not decrease DGA-induced cytotoxicity (Fig. 5B). In addition, HPT cells were coincubated with DGA and 100 μmol/l PBD, a nonspecific OAT inhibitor. In these studies, the amount of cellular necrosis appears to be somewhat lower in the PBD-treated cells as compared with that induced by 50-mmol/l DGA alone (Fig. 6), but the difference was not statistically significant.

**FIG. 1.** DGA, but not 2-HEAA, induces necrotic cell death in HPT cells. Cells were incubated with indicated concentrations of 2-HEAA (A, B) or of DGA (C, D) at pH 6 (A, C, E) or pH 7.4 (B, D, F) for 48 h. Cell death determined by EtHD uptake (A–D) or LDH release (E, F). Data are represented as mean ± SEM ($n = 3$ for A and B; $n = 5$ for C and D; $n = 4$ for E and F). Asterisk (*) indicates significant difference from media only controls as determined by a one-way ANOVA followed by Dunnett’s post hoc test, $p < 0.05$. Pound sign (#) indicates significant difference at pH 6 (C) when compared at pH 7.4 (D) (Student’s $t$-test; $p < 0.05$).
**Coincubation with Succinate Decreases DGA-Induced Cell Death**

DGA has high structural similarity to metabolic intermediates such as succinate, which has high affinity for dicarboxylate transporters (Pajor, 1999, 2000; Wright, 1985). Results indicate that coincubation of 10-mmol/l succinate and 30-mmol/l DGA significantly reduced the cell death produced by 30-mmol/l DGA treatment alone (Fig. 7). Further, the combination was not different from the control. At high DGA concentrations (50 mmol/l), succinate had no effect on DGA-induced cell death.

**DGA Does Not Appear to Directly Inhibit Succinate Dehydrogenase Enzyme Activity**

Addition of 50-mmol/l DGA in isolated mitochondria in the presence of 50-mmol/l sodium, succinate does not decrease DCPIP reduction, indicating that in the presence of high DGA concentrations, succinate dehydrogenase is still capable of oxidizing succinate to fumarate and no inhibition is observed (data not shown).

**DISCUSSION**

Although DEG poisoning can involve several major organ systems, DEG causes the most extensive damage to the kidneys, targeting mainly the proximal tubule and cortical region (O’Brien et al., 1998; Schep et al., 2009). Recent studies have confirmed that the renal toxicity of DEG results because of its metabolism to toxic metabolites (Besenhofer et al., 2010). The majority of literature examining DEG poisonings has suggested that 2-HEAA is the likely toxic metabolite responsible for DEG toxicity with no mention of any role of DGA (Alfred et al., 2005; Ferrari and Giannuzzi, 2005; Kraut and Kurtz, 2008; Mathews et al., 1991; O’Brien et al., 1998). However, the present study has shown that DGA is much more toxic than 2-HEAA to human kidney cells in vitro, and thus represents the first study to demonstrate the likelihood that DGA is the metabolite responsible for the kidney toxicity observed in DEG poisoning. Data in an in vivo study (Besenhofer et al., 2011) support this observation. In rats treated with toxic doses of DEG, the mean 2-HEAA and DGA concentrations in the kidney tissue were similar, both equivalent to 4 mmol/l. However, mean concentrations of DGA in the blood were only 0.04 mmol/l, thus kidney levels were 100 times higher indicating a selective concentrative uptake of DGA into the kidney. In contrast, concentrative uptake of 2-HEAA into the kidney was not observed.

In the present study, DGA produced toxicity in HPT cells at concentrations above 25 mmol/l, suggesting a fairly low potency and questioning the relevance of these concentrations. The toxic concentrations appear to be much higher than the mean kidney concentrations found in rats treated with single...
doses of DEG (4 mmol/l) (Besenhofer et al., 2011). However, there were individual rats in that study which showed DGA concentrations in the kidney about 13 mmol/l. Although DGA was toxic to HPT cells at 50 mmol/l concentrations and not at 12.5 mmol/l (see Figs. 1C and D), these in vitro concentrations are only fourfold more than what has been observed in vivo. One explanation for the low apparent potency in vitro may be an interspecies difference in sensitivity in that the human cells may be less sensitive than rat kidney cells (thus HPT cells require higher DGA concentrations to elicit damage). Also, the rats were administered single doses of DEG, whereas most DEG intoxications occur following multiple ingestions over 48–72 h, where it is likely that repeated doses enhance the accumulation of DGA to higher concentrations than after single doses. Lastly, although DGA may appear to have low potency in these studies, it is distinctly more potent than the alternative (2-HEAA).

HPT cells were exposed to increasing concentrations of all three compounds at both pH 6 and 7.4 to mimic the acid-base situations that occur in DEG poisoning pathologies. Animals and humans exposed to DEG have acidic blood and urine pH (Besenhofer et al., 2010), indicating a metabolic acidosis. Thus, these pHs in vitro were chosen to mimic these in vivo counterparts. Also, because DGA and HEAA are weak acids, the reduced pH would likely enhance the intracellular diffusion of these weak acids, possibly leading to an increased toxicity.

**FIG. 3.** 2-HEAA does not induce apoptosis, while DGA increases nucleosomal fragmentation but with no increase in caspase-3 activity. HPT cells were incubated at pH 6 for 48 h with the indicated concentrations of 2-HEAA (A, B) or of DGA (C, D). Cells were assessed for caspase-3 activity (A, C) or nucleosomal fragmentation (B, D). Data are represented as mean ± SEM (n = 3). Asterisk (*) indicates significant differences from media only controls as determined by a one-way ANOVA followed by Dunnett’s post hoc test, p < 0.05.

**FIG. 4.** DGA produces time-dependent decreases in ATP levels in HPT cells, occurring prior to the increase in LDH release. Cells were incubated at pH 6 with 50-mmol/l DGA for the indicated times. Cellular ATP concentrations (solid line) and LDH release (dashed line) were determined. Data are represented as mean percent of control ± SEM (n = 3). The mean cellular ATP concentrations and LDH release in the controls were 2262 ± 228 mmol/mg protein and 25 ± 14 units/mg protein, respectively. Asterisk (*) indicates significant differences from controls as determined by a one-way ANOVA followed by Dunnett’s post hoc test, p < 0.05.
Furthermore, dicarboxylate transporter upregulation has been reported at acidic pH (Pajor, 2000), suggesting possibly enhanced DGA uptake via these transporters into the cell. In fact, HPT cells were significantly more sensitive to DGA-induced toxicity at low pH, as evident by a higher cell death in pH 6 samples, especially at the 50-mmol/l concentrations.

The renal damage produced by DEG in vivo is primarily severe cortical necrosis (Ferrari and Giannuzzi, 2005; Hebert et al., 1978), localized mainly in the proximal tubular cells (Besenhofer et al., 2010). Our finding that the apparent toxic metabolite of DEG, DGA, produced a necrotic cell death rather than apoptosis is therefore consistent with previously reported in vivo findings. DGA increased markers of cell permeability loss (LDH release and EtHD uptake). Although DGA did increase DNA fragmentation after 48 h exposure as measured by the ELISA method, it did not activate caspase-3 activity. The DNA fragmentation could represent a mixture of apoptosis and necrosis. However, DGA also did not increase another classical marker of apoptosis, annexin V, except in the same population of cells that also took up PI. These latter results are usually interpreted as “late apoptosis-early necrosis,” but the weight of evidence from all these parameters indicates that DGA induced necrotic cell death. This conclusion is further substantiated by the severe depletion of cellular ATP, which would abrogate the likelihood of apoptosis (Breggia and Himmelfarb, 2008).

DGA is available commercially as a white odorless solid and is useful in many industrial synthetic reactions (Bruner and Sherwood, 1949). Because of an appearance similar to granulated sugar, a fatal accidental ingestion of DGA has occurred (Roscher et al., 1975) and this case appears to be the only reported human exposure in the literature. Within 24 h of ingestion, the patient reported to the hospital in acute renal failure. FIG. 5. Small molecule NaDC-1, but not NaDC-3, transporter inhibitors reduce DGA-induced cell death. HPT cells were incubated with DGA (30 or 50 mmol/l), alone or in combination with the NaDC-1 inhibitor, ACA (1 μmol/l) (A), or in combination with the NaDC-3 inhibitor, 2,3-DMS (1 mmol/l) (B). Data are represented as mean percentages ± SEM (n = 6 for A; n = 3 for B) Asterisk (*) indicates significant difference from media only controls. Pound sign (#) indicates significant difference from 50-mmol/l DGA alone group (one-way ANOVA followed by Tukey’s post hoc test, p < 0.05).

FIG. 6. OAT inhibitors do not significantly reduce DGA-induced cell death. HPT cells were incubated with DGA (30 or 50 mmol/l), alone or in combination with the general OAT inhibitor, PBD (100 μmol/l). Data are represented as mean percentages ± SEM (n = 6). Asterisk (*) indicates significant difference from media only controls. (one-way ANOVA followed by Tukey’s post hoc test, p < 0.05).

FIG. 7. Succinate reduces DGA-induced cell death. HPT cells were incubated with DGA (30 or 50 mmol/l), alone or in combination with succinate (10 mmol/l). Data are represented as mean ± SEM (n = 3 per concentration). Asterisk (*) indicates significant differences from media only controls as determined by a one-way ANOVA followed by Tukey’s post hoc test, p < 0.05.
failure, with elevated levels of hepatic enzyme markers. Despite repeated hemodialysis, the patient eventually developed cardiopulmonary failure and succumbed. Autopsy confirmed massive renal cortical necrosis and centrolobular hepatic necrosis. Thus, in a single-isolated case, DGA ingestion is known to lead to an acute renal failure and heptotoxicity similar to that reported in DEG ingestions.

DGA is a four carbon dicarboxylic acid containing a central ether bond linking two monomethylcarboxyl groups (Fig. 8). This structure is strikingly similar to various endogenous compounds, including citric acid (tricarboxylic acid [TCA]) cycle intermediates, which are avidly collected and utilized by renal proximal tubule cells (Wright, 1985). Metabolic intermediates, as well as various pharmaceuticals and environmental xenobiotics, can be transported into and out of kidney cells by cell membrane bound transporters. Accumulation of dicarboxylic anion intermediates can be mediated by NaDC, particularly NaDC-1 and NaDC-3, and possibly, though less likely, by OAT transporters distributed apically and basolaterally on the proximal tubule cell membrane (Pajor, 1999; Sweet, 2005). The low-affinity Na-DC-1 has a broad specificity for dicarboxylic and tricarboxylic acids and its major function is to reabsorb TCA cycle intermediates from the tubular filtrate following glomerular filtration. This process occurs by coupling the movement of sodium down a concentration gradient to the sequestration of substrate. These transported intermediates are then transferred to the mitochondria for metabolism. In contrast to the basolateral, high affinity NaDC-3 has a much lower transport capacity than NaDC-1 and seems to prefer 4- and 5-carbon terminal dicarboxylates in the trans-configuration, including glutarate and α-ketoglutarate as its main transport substrates (Pajor, 1999). One of the major functions of NaDC-3 is indirect secretion of endogenous and exogenous organic anions through sodium-coupled uptake. In this process, NaDC-3 provides the majority of the intracellular α-ketoglutarate utilized to exchange organic anions transported by OAT-1/3 (Burckhardt et al., 2005). In addition, OATs play a role in the influx and efflux of organic acids, which can be sequestered inside the cell and induce toxicity via accumulation to high levels (Sweet, 2005). As such, DGA, which has been filtered from the plasma at the glomerulus, might be transported by the apical NaDC-1 or an OAT from the tubular filtrate across plasma membranes into proximal tubular cells; DGA may be transported across the basolateral membrane by the NaDC-3. Interestingly, when HPT cells were coincubated with ACA to inhibit NaDC-1, the toxicity of DGA was reduced, whereas the NaDC-3 inhibitor, 2,3-DMS, produced no decrease in DGA-induced cytotoxicity. This result suggests that NaDC-1 is a prime candidate for intracellular DGA transport and that the basolateral NaDC-3 transporter plays a very small, if any role at all. One potential explanation for the ability of ACA to reduce the cell death produced by DGA would be that it reduces the intracellular uptake of DGA by the putative transporters NaDC-1. One caveat regarding the ACA results is that ACA is also a potent phospholipase A2 inhibitor (Pajor and Randolph, 2007), albeit at higher ACA concentrations than what inhibits NaDC-1. It is possible that ACA could instead inhibit phospholipase A2 activity, thus altering calcium homeostasis, which might then lead to a reduction in cell death (Caro and Cederbaum, 2003; Pajor and Randolph, 2007). The small nonsignificant effect of PBD by a concentration that should inhibit OATs (0.1 mmol/l) suggests that OATS play only a minor role in DGA uptake.

Succinate is a key metabolic intermediate that is transported across the plasma membrane by NaDC-1, NaDC-3, and/or OAT (Pajor, 1999). Succinate at a concentration of 10-mmol/l prevented cell death caused by 30-mmol/l DGA, but not by 50-mmol/l DGA, suggesting a form of competitive inhibition between the two substrates. The amelioration of DGA-induced cell death by succinate could be due to a simple competitive inhibition of DGA transport via the NaDCs or OAT, thereby decreasing the level of DGA inside the cell and hence, its toxicity. Alternatively, the interpretation of how succinate may interfere with the elicitation of cell death by DGA in vitro is complicated by a possible interaction in the mode of action underlying cell necrosis. For example, DGA might be toxic by out-competing natural substrates, such as succinate, for intracellular access thereby starving the cell of vital nutrients. Hence, cotreatment with succinate might restore sufficient internalization of succinate to allow energy production, thus counteracting the antitransport effect of DGA. Also, if DGA is acting as a competitive inhibitor of the TCA cycle (see below), cotreatment with succinate might overcome this effect of DGA and thereby reduce DGA-induced cell death. The fact that DGA does not appear to directly inhibit succinate dehydrogenase suggests that the latter mechanism for the succinate-induced reduction of DGA toxicity is unlikely. Although the effect of succinate is consistent with inhibition of DGA uptake (similar to

FIG. 8. Structural comparisons between DGA and other metabolic dicarboxylates, succinic acid (TCA cycle intermediate), fumaric acid (TCA cycle intermediate), glutaric acid (intermediate in the breakdown of the amino acids: lysine, hydroxylysine, and tryptophan), malonic acid (competitive succinate dehydrogenase inhibitor).
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