Propylene Glycol-Mediated Cell Injury in a Primary Culture of Human Proximal Tubule Cells

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Propylene glycol (propane-1,2-diol; PD) is a widely used solvent for intravenous drugs. Clinical studies have reported serious side effects, including the development of renal insufficiency in patients receiving PD as drug vehicle. Despite such clinical reports, the data on the toxicity of PD in isolated renal cells are limited. Using primary cultured human proximal tubule (HPT) cells as an in vitro model, we have previously shown the acute toxic effects of PD in HPT cells (Morshed et al., Fundam. Appl. Toxicol. 23, 38-43, 1994). Since most cases of clinical toxicity are noted after prolonged administration of PD, the current studies were designed to investigate the toxicity of repeated exposure of PD in HPT cells. The onset of toxicity was determined using 10-50 mM racemic, sinister, and rectus PD (rac-, S-, and R-PD, respectively) for periods up to 6 days. Cytotoxicity was noted by decreases in thymidine incorporation, in mitochondrial metabolic activity, and in lysosomal accumulation of neutral red. Exposure of HPT cells to 50 mM PD produced toxic responses, while at 10 mM, responses were not significantly greater than those of osmotic controls. The toxicity was caused by a PD-specific mechanism and by a secondary mechanism without any enantiomeric specificity. The HPT cell toxicity was associated with a 35% increase in cellular thiobarbituric acid-reactive substances and a 20% decrease in glutathione. These findings suggest the development of a mild, subacute toxicity in normally proliferating HPT cells at concentrations that could be achieved in human plasma when PD is used as a drug vehicle. © 1998 Society of Toxicology.

Propylene glycol (synonym: propane-1,2-diol, PD) is a 50:50 racemic (rac-) mixture of sinister (S-) and rectus (R-) PD and is present in many drugs and intravenous (iv) formulations, including the anesthetics (Van de Weile et al., 1995). As a solvent and vehicle, PD is incorporated into oral, topical, and injectable medications at levels up to 96% (Demey et al., 1984). In addition, PD is used in cosmetics and processed foods (Ruddick, 1972), as a substitute for carbohydrates in the animal diet (Levine et al., 1985), and in the cryopreservation of embryos and tissues (Damien et al., 1989; Lasalle et al., 1985; Ng et al., 1988). The World Health Organization recommends a total load of 1875 mg PD per day for a 75-kg individual. However, despite its apparently innocuous nature, clinical abnormalities such as lactic acidosis (Kellner and Bailey, 1985; Bedichek and Kirschbaum, 1991), increased hyperosmolality (Bekaris et al., 1979), hemolysis (Van de Wiele et al., 1995), and renal insufficiency (Kulick et al., 1980; Glasgow et al., 1983; Levy et al., 1995) have been reported in patients intoxicated with PD. At the time of estimation, the plasma PD concentrations in these patients ranged from 60 to 120 mM (Van de Wiele et al., 1995; Bedichek and Kirschbaum, 1991; Kellner and Bailey, 1985; Demey et al., 1984; Glasgow et al., 1983). Of these well-documented side effects, data on the renal toxicity of PD are limited and less conclusive. Although few studies (Kulick et al., 1980; Glasgow et al., 1983) have previously speculated on a role of PD in producing renal abnormalities in vivo, only one study has shown an effect of PD in producing renal insufficiency in hospitalized patients (Levy et al., 1995). Whether such clinical manifestations are due to PD's direct effect on renal cells is not known.

Assessment of renal PD toxicity in vivo could be complicated by PD's simultaneous metabolism or elimination via hepatic, renal, and gastrointestinal routes. Such difficulty in elucidating PD's renal effect could be overcome using an in vitro renal cell culture system. Studies using primary cultured human proximal tubule (HPT) cells as an alternative model to test PD's acute toxicity have shown significant cellular injury and membrane damage within 15 min of PD exposure (Morshed et al., 1994). These studies were conducted using PD concentrations ranging from 61 to 263 mM (i.e., 5 to 20 g/L, the highest dose being equivalent to 9% of the in vivo LD50 dose). The studies by Levy et al. (1995) suggest that PD probably causes renal insufficiency at much lower doses when infused continuously. These patients, who received a total load of 52-90 g PD in a 24-h delivery period, had body weights ranging from 46 to 77 kg. The direct experimental evidence of prolonged (subacute) PD toxicity in renal cells is lacking. Moreover, whether such subacute PD toxicity would differ from that of the acute...
toxicity is not known, as differential toxicities for the rac-, S-, or R-PD (synonym: DL-, L-, and D-PD, respectively) were observed during acute PD exposure (Morshed et al., 1994).

In this study, we have addressed these questions by examining the toxicity of repeated exposures of rac-, S-, and R-PD in HPT cells. The HPT cellular model is valuable for in vitro toxicity testing because the kidney is a frequent target of xenobiotic toxicity (Hazan-Martin et al., 1989; Sens et al., 1989). The toxicity parameters were carefully selected to obtain a reliable assessment of subacute toxicity. These include studies of cell proliferation (thymidine incorporation into DNA), mitochondrial metabolic activity [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT], and cellular viability (lysosomal accumulation of neutral red, NR, by viable uninjured cells). Furthermore, PD’s effects on cellular thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) were studied as indicators of plasma membrane integrity and vital cellular detoxication mechanisms.

**METHODS**

**Materials.** [methyl-³H]Thymidine (20 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA). N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine sodium salt (TOOS), S-PD, and R-PD were obtained from Fluka (Switzerland). Glucose 6-phosphate dehydrogenase and phosphoglucoisomerase were obtained from Boehringer Mannheim (New York, NY). All other reagents were from Sigma (St. Louis, MO) unless otherwise mentioned.

**Cell culture.** HPT cells were isolated from human kidneys that were removed because of renal adenocarcinoma or trauma or from kidneys unable to be used in transplantation. Immediately after surgery, healthy tissue from the outer cortical region, remote from any pathological alterations as judged by the surgical pathologist, was removed. No record was kept of the patient, and the studies were exempted by the Institutional Review Board for Human Research (Louisiana State University Medical Center). HPT cells were isolated by an enzyme dissociation method using a collagenase–DNase mixture as described by Blackburn et al. (1988) and cultured as described by Morshed et al. (1994). For toxicity testing, HPT cells at passages 4–8 were subcultured in plastic culture wells (Coming Instruments) coated with bovine dermal collagen (Celltrix Labs, Palo Alto, CA). HPT cells have been shown to retain morphologic, electrophysiologic, and transport properties associated with the proximal tubule for at least 10 passages (Detrisac et al., 1984; Blackburn et al., 1988).

**Toxicity studies.** The cytotoxicity of PD during the proliferative stages of cell growth (1–5 days) was assessed by exposing the cells to 0 (regular growth medium containing no PD, mannitol, or L-glucose) or 10–50 mM PD (treated) or mannitol (equimolar control cultures) or 10–50 mM rac-, S-, and R-PD (treated cultures). After a single oral administration of 29 mmol/kg, peak blood PD concentrations (at 1–2 h) were 17 mM in rats (Morshed et al., 1988) and 41 mM in rabbits (Morshed et al., 1991). Plasma PD concentrations in intoxicated human patients were 120 mM (Demey et al., 1984) and 58 mM (Bedichek and Kirschbaum, 1991), both measured on the second day after initiation of PD-containing medications. Thus, the concentrations used in the present study were below the reported plasma PD concentrations in PD intoxications. An isotonic solution of 263 mM PD was first prepared in 5 mM N,N,N,N,N-trimethyl-1-proline-N²-ethanesulfonic acid (Hepes)–2.5 mM Tris, pH 7.2, containing 1.9 mM NaCl. This stock solution was sterilized using syringe-fitted filters (0.2 μm porosity) and was diluted with the standard growth medium to achieve the indicated concentrations. In addition to the regular cultures used as controls, equimolar control cultures were run simultaneously using 10–50 mM concentrations of mannitol or L-glucose. Stock solutions of isotonic mannitol or L-glucose were prepared and diluted with the growth medium as described for PD. Thus, the control and treated media remained osmotically similar. HPT cells were exposed to standard growth medium containing no PD, mannitol, or L-glucose during the first 24 h after cell seeding (day 0) to allow for cell attachment. Thereafter, the medium was replaced daily with media containing 0–50 mM concentrations of PD (treated) or mannitol or L-glucose (control). The cytotoxicity was evaluated by studying the incorporation of thymidine into DNA, the mitochondrial metabolism of MTT, the accumulation of NR into the lysosomes, and the changes in cellular levels of TBARS and GSH.

**Thymidine incorporation.** The incorporation of [³H]thymidine into DNA was studied during the proliferative phase of HPT cell growth (days 1–5). HPT cells were seeded in 12-well culture plates for 24 h and then fed daily as described above. At 6 h after each day’s feeding, 1 μCi of [³H]thymidine was added per well. After another 18 h, the medium was removed and each well was washed three times with ice-cold pH 7.4 buffer (Morshed et al., 1994). The cells were solubilized by adding 1 ml of 0.1 N NaOH for 1.5 h and an aliquot was counted in 5 ml scintillation cocktail to determine the amount of labeled tritium. Because initial studies showed inhibition of cell growth by rac-PD by 3 days (see Results), the stereoselective effects of rac-, S-, and R-PD on cell growth were determined by treating the cells for the first 3 days and then adding thymidine as above. In this study, equimolar mannitol-containing media (10–50 mM) were added in the control cultures.

**MTT assay.** In HPT cells that were treated with PD for 3 days, the reduction of the soluble MTT tetrazolium salt to an insoluble MTT formazan product by mitochondrial succinate dehydrogenase activity (Mossman, 1983) was measured. The assay serves as a measure of overall energy metabolism and detects only living cells, thereby quantifying relative cell number in cytotoxicity studies. HPT cells were seeded in 96-well plates for 1 day and then fed the standard growth medium containing 0 (regular culture) or 10–50 mM rac-, S-, and R-PD (treated) for 3 days. In this study, L-glucose-containing media (10–50 mM) were added in the control cultures. On day 4, when the wells were nearly confluent, the MTT assay was performed according to the method of Mossman (1983) as modified by Denizot and Lang (1986).

**NR dye uptake.** NR uptake was performed to assess the cytotoxic effects of PD, based on the observation that NR, a supravital dye, is taken up by viable, uninjured cells and accumulates in the lysosomes (Borenfreund and Puerner, 1985). Individual wells of a 96-well plate were seeded with 1000–5000 cells and maintained on standard growth medium for 3 days, by which time the wells were 80–90% confluent. Then, the cells were fed with media containing 0–50 mM PD or with equimolar concentrations of L-glucose. After another 48 h, the control and the PD-containing media were replaced with similar fresh media (0.2 mL/well) which also contained 50 μg of NR/mL. Cells were incubated in these media for 3 h, by which time lysosomal accumulation of NR was maximum. Then, media were removed, the wells were washed three times with ice-cold phosphate-buffered saline (PBS), and the dye was then extracted from the cells with 0.2 ml of a solution of 1% acetic acid–50% ethanol for 10 min at room temperature. After brief agitation, the absorbance was measured in a microplate reader at 555 nm. This absorbance has shown a linear relationship with the number of surviving cells (Borenfreund and Puerner, 1985).

**Measurement of cellular TBARS and GSH.** The cellular contents of TBARS and GSH were measured after growing the cells for 0–6 days in the presence of 0–50 mM rac-, S-, and R-PD (treated) or mannitol (equimolar control). For these studies, cells were seeded in 100-mm-diameter collagen-coated petri dishes (0.7–1.5 million cells per dish) in standard growth medium for 24 h, after which the medium was replaced with control or treated media for 1–5 days exposure. After each treatment period, the old medium was removed, and the cell monolayer was washed three times, each time using 10 ml alcohol to prevent initiation of membrane lipid peroxidation during sample processing. The cell monolayers were scraped using 1.5 mL cold PBS–BHT. Cells for 0 day treatment were seeded into dishes containing 0–50 mM PD or mannitol media, immediately placed on ice, and then collected into...
The potential for PD to cause HPT cellular injury was assessed by determining its effect on the incorporation of thymidine into DNA, initially using rac-PD only (Fig 1). Thymidine incorporation was inhibited by rac-PD at concentrations between 10 and 50 mM PD compared with the respective 0 mM PD controls (p < 0.05). Inhibition of cellular growth proliferation was observed from days 2 through 5 with 25 and 50 mM PD (p < 0.05), while with 10 -->
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FIG. 2. Cytotoxic effects of rac-, S-, and R-PD on HPT cells. Concentration-dependent effects of PD on cell proliferation (left panel), on mitochondrial metabolic activity (center panel), and on cell viability (right panel) by RS-, S-, and R-PD. Cell proliferation was measured by the incorporation of [3H]thymidine into DNA, cellular mitochondrial activity by the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and cell viability by the amount of neutral red (NR) accumulated in lysosomes by the viable uninjured cells. Additional controls were 10–50 mM concentrations of mannitol (left) and 10–50 mM concentrations of L-glucose (other panels). Each point represents the mean ± SEM of three separate isolates with quadruplicate incubations. Asterisks indicate a significant difference compared with no-PD controls; letters (same letters are not significantly different) indicate a significant difference among the equimolar mannitol (or L-glucose)- and PD-treated cells (p < 0.05).

mM PD, this inhibition was significant only after 4 days of treatment (p < 0.05). The effects of PD were not studied beyond day 5 because cell proliferation in control cultures was apparently arrested. By day 5, a confluent monolayer was revealed by microscopic examination and thymidine incorporation into DNA had also reached an apparent saturation in control cells (Fig 1).

Further investigations on the stereoselective effects of PD in causing HPT cell injury were carried out by treating the cells with rac-, S-, and R-PD for 3 days. Cellular injury was evaluated by studying the incorporation of thymidine into DNA (Fig. 2, left panel), the mitochondrial metabolism of MTT (center panel), and the lysosomal accumulation of NR (right panel). Inhibition of all three parameters of toxicity followed an apparent dose-dependent pattern without any dependency on the stereospecific actions of PD. At a concentration of 50 mM PD (rac-, S-, or R-), cellular thymidine incorporation was inhibited about 50%, MTT metabolism about 40%, and NR uptake about 25%. Mannitol at 50 mM also inhibited these parameters (p < 0.05) but to a significantly lesser extent. For example, the effect of 50 mM PD was significantly greater than the effect of mannitol (p < 0.05, denoted by a and b), suggesting that PD may affect HPT cell growth by a specific mechanism. This is especially evident at the 25 mM concentrations, where PD significantly inhibited all these parameters (p < 0.05), but mannitol had no effect.

The time course for TBARS formation (left panel) and GSH depletion (right panel) after treating the cells with 50 mM PD is shown in Fig 3. These cells, when grown in the presence of PD, showed an increased TBARS formation (p < 0.05) and GSH depletion (p < 0.05) that was significant after 2 days of treatment. These effects remained relatively similar for up to 6 days of growth. The concentration-dependent effects of PD (Fig. 4) on cellular contents of TBARS (Fig. 4A) and GSH (Fig. 4B) were studied after treating the cells with 10, 25, and 50 mM PD for 3 days, because preliminary studies indicated no significant effects prior to that time. Increased TBARS formation and GSH depletion were significant at 50 mM levels of PD (p < 0.05) when compared with the cultures treated with equimolar concentrations of mannitol. Mannitol by itself had no significant effect on TBARS and on GSH at these concentrations when compared with the cultures fed with regular growth medium.

The data presented in Fig. 5A show the levels of L-lactate and pyruvate and the L/P ratio during 1–5 days of cell growth. The calculated L/P ratio served as an indicator of cellular lactate–pyruvate homeostasis and thus of the redox metabolism. The L/P ratio varied within a narrow range in control cultures during 1–5 days of cellular growth (Fig. 5A). The PD-treated cultures showed a decreased L/P ratio during 3–5 days of cell growth (p < 0.05), but also had lower amounts of protein as a result of growth inhibition. The media lactate and pyruvate levels were, therefore, normalized with the respective protein data (Fig. 5B; 3-day data are shown only). The normalized data revealed similar changes, i.e., the L/P ratio in the treated cultures was decreased during 3–5 days of growth (p < 0.05). The data on the effects of S- and R-PD on L-lactate and pyruvate were similar to those for rac-PD and therefore are not shown. D-Lactate was not detected in media from cultures that were
treated with 10–50 mM rac-, S-, or R-PD. The level of D-glucose also remained similar in the rac-, S-, or R-PD-treated cultures compared with the control cells (data not shown).

To determine whether the alterations of lactate and pyruvate reflect an effect of PD on HPT cellular metabolism or its own bioconversion in the cells, additional studies were conducted using 0–50 mM PD-containing isosmotic growth medium with the omission of glucose and pyruvate (see Methods). Under such conditions, the production of glucose and lactate in the control cultures was 13.5 ± 1.5 and 19.1 ± 2.2 μM, respectively. The production of glucose and lactate was similar in the treated cells (10–50 mM PD), thus suggesting the absence of the PD metabolism in HPT cells.

FIG. 3. Time course for the PD-induced production of thiobarbituric acid-reactive substances (TBARS, left) and depletion of glutathione (GSH, right) in HPT cells. HPT cell cultures were incubated with 50 mM mannitol (O) or rac-PD (●) for 0–6 days. The levels of TBARS and GSH were estimated from the sonicated cells as described under Methods and were expressed/mg of cellular protein. Each point represents the mean ± SEM of three separate isolates with duplicate incubations. Asterisks indicate a significant difference compared with equimolar mannitol controls (p < 0.05).

FIG. 4. Concentration dependence of the PD-induced production of TBARS (left) and depletion of GSH (right) in HPT cells. HPT cell cultures were incubated with 0–50 mM mannitol (O) or rac-PD (●) for 3 days. The levels of TBARS and GSH were estimated from the sonicated cells as described under Methods and were expressed/mg of cellular protein. Each point represents the mean ± SEM of three separate isolates with duplicate incubations. Asterisks indicate a significant difference compared with equimolar mannitol controls (p < 0.05).
The major objective of the present studies was to examine the toxicity of repeated exposures of PD in primary cultured HPT cells. These studies have provided data that help explain how prolonged use of PD can cause renal insufficiency, as observed in vivo when PD has been used as drug vehicle (Levy et al., 1995). Our recent studies in HPT cells have provided evidence of PD's acute toxicity in the kidney at relatively high dose levels (131-263 mM; Morshed et al., 1994). The present studies show that PD, when used repeatedly, exerted its toxic effects at lower doses (25-50 mM). Unlike the acute toxicity, the repeated toxicity in HPT cells did not occur by an enantiomer-specific mechanism. Mechanistically, therefore, subacute toxicity of PD differed from acute toxicity in HPT cells. One caveat of these studies is that the subacute effects of PD were observed on HPT cells primarily during the proliferative stage in culture (days 1-5). Hence, the effects in this study represent a toxic effect on normally growing cells, rather than a cytotoxic effect per se. In our previous study (Morshed et al., 1994), acute PD treatment had cytotoxic effects on cells at the confluent, nonproliferating stage. Although renal cells in vivo are not normally proliferating, the observations in the present study appear to be the first evidence of a subacute toxicity of PD in human renal cells and support the earlier findings that PD may have toxic potential in the kidney (Levy et al., 1995; Morshed et al., 1994; Watson et al., 1987; Glasgow et al., 1983; Kulick et al., 1980).

The concentrations of rac-, S-, and R-PD (10-50 mM) used in these studies were below those observed in clinical overdoses. In reported studies on human patients, repeated doses of PD, administered as a drug vehicle, have resulted in serum PD concentrations in the range of 60-120 mM at the time of estimation (Bedichek and Kirschbaum, 1991; Demey et al., 1984). Initial studies of the toxicity of PD involved measurement of HPT cell growth, as indicated by the incorporation of tritiated thymidine into DNA in the presence of rac-PD. While no inhibition of thymidine incorporation occurred on day 1, inhibition of thymidine incorporation occurred at 25 and 50 mM PD during the 2- to 5-day period. In order to study the nature of PD toxicity, subsequent studies included S and R enantiomers in addition to rac-PD. Toxicity parameters were selected to detect early signs of disturbances in HPT cells that can ultimately lead to cell death. These early signs include thymidine incorporation into DNA (cell proliferation), mitochondrial metabolic activity (reduction of MTT), and the lysosomal accumulation of NR (cell viability/integrity). The cytotoxicity of PD during the initial growth phase (thymidine or MTT methods) evidently occurred by a PD-specific mechanism as well as...
by a nonspecific mechanism. The fact that mannitol and 1-glucose (50 mM) significantly inhibited the parameters of cell proliferation suggests a nonspecific mechanism at high osmolar concentrations. However, the effects caused by PD were significantly greater than those observed with mannitol or 1-glucose, suggesting a PD-related effect. Moreover, 25 mM concentrations of mannitol or 1-glucose had no effect, while PD did have a significant toxic effect on these cells. Studies of the lysosomal accumulation of NR reflect the effects of PD when the cells were nearly confluent. Even with this cell culture model, PD produced a significant toxic effect compared with the controls. The effects by rac-, S-, or R-PD on all three parameters were similar, suggesting a lack of stereospecificity in the subacute effects of PD.

The effects of PD on the three toxicity parameters appeared to be concentrationally related. By day 3, the effects at 10 mM were not significantly different from those of osmolar controls, while those at 25 and 50 mM were significantly different (Fig. 2). The effects at 50 mM appeared to be greater than those at 25 mM, although this was not shown statistically by our analysis. Note that PD at 10 mM did decrease thymidine incorporation after longer treatment (Fig. 1).

To test whether cytotoxicity was associated with alterations in cellular membrane integrity and detoxification mechanisms, we estimated the levels of TBARS and GSH, respectively, during 0–6 days growth period. Our previous studies demonstrated significant membrane perturbations (increased leakage of lactate dehydrogenase and of preloaded chromium) when the HPT cells were exposed to 263 mM isotonic PD for 2 h (Morshed et al., 1994). Despite such membrane perturbations, acute exposure of HPT cells to 263 mM PD for 6 h did not result in increased TBARS production nor depletion in GSH (unpublished observation). In contrast, the present studies show that repeated exposures to PD increased the levels of TBARS and decreased the levels of GSH, suggesting that the subacute toxicity might differ from the acute toxicity in HPT cells. The effects of PD on these parameters were minor, with only about 20–40% change. Hence, the physiologic relevance of such small changes can be questioned.

To examine the contribution of PD metabolism in the development of HPT cellular subacute toxicity, we assessed the media levels of L-lactate, pyruvate, the L/P ratio, and D-glucose. PD-induced lactic acidosis appears to be one mechanism by which PD exerts its systemic toxicity (see Morshed et al., 1991, and the included citations). The exposure conditions in this study did not produce convincing evidence of PD metabolism by HPT cells. Initial studies using physiological growth medium indicated an interaction of PD with the L/P ratio, thus suggesting an effect of PD on the HPT cellular redox state. Subsequent studies using more controlled conditions (i.e., the growth medium was not supplemented with exogenous lactate, pyruvate, or glucose) revealed production of lactate and glucose, but this effect was not due to the metabolism of PD. These data, therefore, suggest PD's interaction with the HPT cellular energy metabolism rather than its own bioconversion in the cells.

In summary, this study showed a mild, subacute toxicity of PD on normal human renal cells at concentrations below the serum concentrations often observed during PD intoxication studies. In clinical situations, PD exposure as a pharmaceutical solvent often occurs in infants that are nutritionally or metabolically compromised or in adults that have renal or hepatic dysfunction. In those situations, even the mild toxicity noted in these normal cells might be significant. Mechanistically, the subacute toxicity differed from the acute toxicity in its effects on levels of lactate, TBARS, and GSH. The subacute PD toxicity also differed from the acute toxicity (Morshed et al., 1994) because the subacute toxicity was exerted by an apparently nonstereospecific mechanism. The interaction of PD with lactate and pyruvate metabolism paralleled the inhibition of cell growth; this effect was apparently mediated by PD itself. Together, the data suggest that tissues, in which PD metabolism is relatively low, but are exposed to PD for prolonged time, can be potential targets of PD toxicity. Although in vivo renal PD toxicity studies are limited, these in vitro toxicity data suggest a mild renal toxicity.

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