TOXICITY OF IFOSFAMIDE AND ITS METABOLITE CHLOROACETALDEHYDE IN CULTURED RENAL TUBULE CELLS

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SUMMARY

Renal injury is a common side effect of the chemotherapeutic agent ifosfamide. Current evidence suggests that the ifosfamide metabolite chloroacetaldehyde may contribute to this nephrotoxicity. The present study examined the effects of ifosfamide and chloroacetaldehyde on rabbit proximal renal tubule cells in primary culture. The ability of the uroprotectant medication sodium 2-mercaptoethanesulfonate (mesna) to prevent chloroacetaldehyde-induced renal cell injury was also assessed. Chloroacetaldehyde (12.5–150 μM) produced dose-dependent declines in neutral red dye uptake, ATP levels, glutathione content, and cell growth. Coinadministration of mesna prevented chloroacetaldehyde toxicity while pretreatment of cells with the glutathione-depleting agent buthionine sulfoximine enhanced the toxicity of chloroacetaldehyde. Ifosfamide (1000–10 000 μM) toxicity was detected only at concentrations of 4000 μM or greater. Analysis of media collected from ifosfamide-treated cell cultures revealed the presence of several ifosfamide metabolites, demonstrating that renal proximal tubule cells are capable of biotransforming this chemotherapeutic agent. This primary renal cell culture system should prove useful in studying the cause and prevention of ifosfamide nephrotoxicity.

Key words: chloroacetaldehyde; ifosfamide; sodium 2-mercaptoethanesulfonate; nephrotoxicity; glutathione.

INTRODUCTION

Ifosfamide is a chemotherapeutic agent that is being used with increasing frequency to treat pediatric malignancies. Initially, ifosfamide therapy was limited by the side effect of severe hemorrhagic cystitis. This complication has been circumvented by the concurrent use of sodium 2-mercaptoethanesulfonate (mesna), a synthetic thiol that combines with reactive ifosfamide metabolites to form stable nontoxic thioether compounds (13,16). However, despite mesna, approximately 40% of ifosfamide-treated children develop a permanent subclinical renal tubulopathy and 5% have a persistent De Toni-Debre-Fanconi syndrome (6,16). This syndrome is caused by a generalized dysfunction of renal proximal tubule cells and is defined clinically by excessive urinary excretion of glucose, amino acids, phosphate, bicarbonate, and other solutes handled by this nephron segment. Growth failure, rickets, and progressive renal failure are sequelae of this disorder.

Ifosfamide is a prodrug that must first be biotransformed by the cytochrome P450 system before it can exert its therapeutic or toxic effects (16,23,24). Ring hydroxylation produces 4-hydroxyifosfamide (HOIF) which is then converted into the active alkylating agent ifosfamide mustard (IPM) and acrolein, the putative cause of toxic effects (16,23,24). Ring hydroxylation produces 4-hydroxyifosfamide (HOIF) which is then converted into the active alkylating agent isophosphoramide mustard (IPM) and acrolein, the putative cause of hemorrhagic cystitis. Ifosfamide also undergoes considerable chloroethyl side chain oxidation yielding N2-dechloroethylifosfamide (N2D) and N4-dechloroethylifosfamide (N3D) together with concomitant release of the coproduct chloroacetaldehyde (CAA). We have previously shown that CAA causes renal injury in both the isolated and in vivo perfused rat kidney (20,25).

The mechanism by which CAA causes kidney damage is not known. In rat liver and kidney, CAA toxicity is associated with intracellular glutathione and ATP depletion (18,20). Ifosfamide metabolites, including CAA, deplete tissue glutathione stores in humans suggesting a similar method of toxicity (7). In this study, we examined the effect of ifosfamide, CAA, and mesna on kidney cell function, ATP levels, and glutathione content using primary cultures of rabbit renal proximal tubule cells. In addition, the ability of these cells to metabolize ifosfamide was evaluated.

MATERIALS AND METHODS

Isolation and culture procedures. Proximal tubule cells were isolated and cultured as previously described (22). Male 2.0–2.5-kg New Zealand white rabbits (Becken’s Farms, Sanborn, NY) were sacrificed by CO2 narcosis. The kidneys with the renal artery intact were immediately removed and washed with sterile culture medium (a 50:50 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 further supplemented with 15 mM HEPES buffer, 20 mM NaHCO3, 5 μg bovine insulin per ml, 5 μg human transferrin per ml, and 5 × 10−6 M hydrocortisone). The renal artery was cannulated with a sterile, blunt 20-gauge needle and kidneys were perfused with phosphate-buffered saline (PBS) until clear of blood. The kidneys were then perfused with a 0.5% solution of iron oxide until black. The iron oxide is trapped in glomeruli facilitating the subsequent isolation of proximal tubules. After perfusion, kidneys were decapsulated. The cortex was removed, minced into 1–2 mm pieces, and homogenized in a sterile Dounce homogenizer. The resulting homogenate was poured onto a 253-μm mesh screen that was placed in series over a 85-μm screen and washed with culture medium. Tubules and glomeruli retained on top of the 85 μm screen were removed and resuspended in a 50-
ml tube containing culture medium and a magnetic stir bar. Glomeruli associated with iron oxide adhered to the stir bar which was then removed. To disrupt basement membrane, the tubules were then incubated with 0.05 mg collagenase per ml and 0.05 mg trypsin inhibitor per ml for 2 min at 23°C. After digestion with collagenase, the tubules were washed twice by centrifugation, resuspended in culture medium, and inoculated into 35-mm plastic tissue culture dishes. Culture dishes were maintained at 37°C in an incubation chamber with a humidified 5% CO2/95% air environment. Medium was changed after 24 h and every 48 h thereafter. All experiments were conducted 6–8 d after plating when confluent monolayers had been achieved.

Experimental design. Confluent monolayers of proximal tubule cells were exposed to ifosfamide (1000–10,000 μM) alone or to chloroacetaldehyde (12.5–150 μM) and mesna (100–1000 μM) alone or in combination for 16–72 h. Subsequently, neutral red dye uptake, cell glutathione content, cell ATP levels, and cell growth were measured as described below. To determine the influence of cell glutathione content on CAA toxicity, monolayers were pretreated for 24 h with 50 μM of the glutathione synthesis inhibitor buthionine sulfoximine (BSO), exposed to 50 μM CAA for 16 h and assayed for neutral red dye uptake. Finally, monolayers were exposed to 1000 μM ifosfamide for 6 h and media collected for metabolite analysis as described below. The concentration of ifosfamide used was approximately 10–100-fold greater than that found in patients’ serum during ifosfamide treatment (23,25).

The concentrations of CAA used (12.5–150 μM) compare with serum CAA concentrations ranging from 10–109 μM and urine concentrations of up to 220 μM measured in patients receiving ifosfamide (23,25).

Neutral red assay. Cell viability was determined by neutral red dye uptake (4). This dye is taken up by viable cells and stored in lysosomes. Dye is then extracted and uptake quantitated by spectroscopy. A 0.33% (w/v) stock solution of neutral red was prepared in PBS, filtered by gravity, and added to culture media to achieve a final concentration of 50 μg/ml. This medium was then applied to culture plates and incubated for 3 h. Medium was then removed by aspiration and monolayers were gently washed with PBS. Neutral red dye taken up by cells was then extracted by the addition of 1% acetic acid/50% ethanol to each culture plate. After 60 min, the extracted neutral red dye was removed and absorbance at 540 nm was measured. The percentage of neutral red uptake by treated cells was calculated by dividing the A540 in treated cells by the A540 in control cells and multiplying by 100%.

Cell growth determination. To determine the effect of CAA on cell growth, cultures were seeded in 12-well plates and exposed to CAA. Medium was changed after 24 h and every 48 h thereafter. All experiments were performed on at least three separate culture dishes and averaged for each experiment. Results were expressed as mean ± SEM of at least four experiments. Means were compared by analysis of variance with the Scheffe post hoc test and significant difference from the control at P < 0.05. **P indicates a significant difference from results at preceding lower chloroacetaldehyde concentration at P < 0.05.

Cell ATP levels. ATP levels were measured with the luciferin-luciferase assay as previously described (2). Cells were solubilized with 0.5 ml 0.5% Triton X-100, acidified with 0.1 ml of 0.6 M perchloric acid, and placed on ice. The cell lysate was diluted with 10 mM potassium phosphate buffer containing 4 mM MgSO4; 0.5 ml of this buffer was added to 1 ml of 50 mM sodium arsenate buffer containing 20 mM MgSO4 to which 25 μl of 40 mg luciferin-luciferase per ml was added. Light emission was recorded after 20 s in a beta scintillation counter accepting signals out of coincidence. Cell protein content was determined by the method of Bradford (5) with bovine serum albumin as standard on a portion of the cell sample and ATP levels normalized for protein content of the monolayer.

Glucose uptake. Glucose transport studies were performed as previously described (15). Transport medium contained (in mM): 137 NaCl, 4.7 KCl, 0.44 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, 4 glutamine, 10 HEPES buffer, and 0.1 mg bovine serum albumin per ml. To achieve sodium-free medium, sodium was replaced isosmotically by choline. Cell monolayers were washed three times with substrate-free transport medium before incubation in uptake medium containing alpha-[3H]methyl-β-glucose (0.5 μCi/ml), a nonmetabolizable analogue of glucose that shares the apical sodium-dependent glucose pathway in the mammalian proximal tubule. Reactions were terminated at 60 min by our removing solutions and rapidly washing three times with ice-cold isonicotinic mammmal. Cells were then solubilized in 0.1 N NaOH for 90 min and aliquots sampled for protein and liquid scintillation counting. We calculated sodium-dependent uptake values measured in the presence of choline from those obtained in the presence of sodium. Glucose transport was normalized to cell protein content and expressed as pmol/mg cell protein per unit time.

Glutathione content. Total glutathione content was measured in deproteinized cell lysates with a commercial kit (GSH-400, R & D Systems, Minneapolis, MN) and normalized with respect to the protein content of the monolayer.

Cell growth determination. Primary rabbit kidney proximal tubule cell cultures treated with either ifosfamide (1000 μM) or CAA (25–150 μM) in either the presence or absence of mesna (1000 μM) were subcultured by trypsinization as previously described (22). Cells were dislodged by incubation with a 0.05% trypsin–0.5 mM EDTA solution in PBS. Trypsin activity was terminated by the addition of a 0.1% soybean trypsin inhibitor solution in PBS. Harvested cells were washed by centrifugation, resuspended in culture medium, and inoculated onto 35-mm plastic tissue culture dishes at 10,000 cells/plate. One wk later, cells were harvested from each plate and counted. Results are means ± SE of three experiments. *P indicates a significant difference from the control at P < 0.05. **P indicates a significant difference from results at preceding lower chloroacetaldehyde concentration at P < 0.05.

Results

CAA effects. Treatment with CAA impaired neutral red dye uptake by and growth of proximal tubule cells in a dose-dependent fashion (Fig. 1). Exposure to increasing concentrations of CAA produced a progressive impairment in glucose uptake (Table 1) and declines in cell ATP levels (Table 1) and glutathione content (Fig. 2). Pretreatment of monolayers with BSO significantly reduced cell glutathione...