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Contribution of reactive oxygen species to *para*-aminophenol toxicity in LLC-PK$_1$ cells

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ABSTRACT

para-aminophenol (PAP) causes nephrotoxicity by biochemical mechanisms that have not been fully elucidated. PAP can undergo enzymatic or non-enzymatic oxidation to form reactive intermediates. Using modulators of reactive oxygen species (ROS), the role of ROS in PAP toxicity in LLC-PK$_1$ cells was investigated. ROS formation was determined using a fluorescein derivative and viability using alamarBlue. Following treatment of cells with PAP, ROS formation occurred prior to loss of cell viability. Several modulators of ROS were used to identify the pathways involved in PAP toxicity. Viability was improved with catalase treatment, while viability was decreased when cells were treated with superoxide dismutase (SOD). Both catalase and SOD exert their effects outside of cells in the incubation medium, since there was no evidence of uptake of these enzymes in LLC-PK$_1$ cells. In cell-free incubations, hydrogen peroxide (H_2O_2) was produced when 0.5 mM PAP was included in the incubation medium. Further, SOD greatly increased and catalase greatly decreased H_2O_2 production in these cell-free incubations. These data suggest that H_2O_2 formed in the incubation medium contributes to loss of viability following PAP treatment. When cells were coincubated with 0.5 mM PAP and tiron, pyruvate, bathocuproine, 1, 10-phenanthroline, or dimethylthiourea (DMTU), ROS formation was decreased. However, there was minimal improvement in cell viability. Paradoxically, DMTU exacerbated PAP-induced loss of viability. These data suggest that ROS are generated in cells exposed to PAP but these species are not the predominant cause of cellular injury.

Introduction

para-aminophenol (PAP) is a nephrotoxic compound that may be encountered as a component of hair dyes (Burnett and Goldenthal, 1988) or a metabolite of acetaminophen (Yan et al., 2000). The mechanisms involved in PAP toxicity are not entirely clear. PAP undergoes non-enzymatic oxidation in aqueous solutions to form numerous by-products including benzoquinoneimine and *para*-aminophenoxy radical (Josephy et al., 1983). Inhibitors of CYP450, flavin monooxygenase, 5-lipoxygenase, and prostaglandin H synthase-associated cyclooxygenase-1 did not prevent toxicity in LLC-PK$_1$ cells incubated with PAP (Gonzalez and Tarloff, 2004). Coincubation or preincubation of LLC-PK$_1$ cells with reduced glutathione (GSH) or ascorbate significantly attenuated PAP-induced toxicity (Hallman et al., 2000). These data suggest that PAP undergoes non-enzymatic oxidation in LLC-PK$_1$ cells. However, the nature of the reactive species formed in cells during PAP exposure has not been thoroughly investigated.

Harmon and co-workers (2005, 2006) observed formation of 4-hydroxynonenal-adducted proteins when rat renal cortical slices were incubated with PAP, suggesting that lipid peroxidation accompanied toxicity. GSH depletion occurred following PAP exposure in both in vitro (Harmon et al., 2005) and in vivo studies (Shao and Tarloff, 1996), suggesting that PAP induced oxidative stress. Reactive oxygen intermediates (ROS), such as superoxide anion, may be generated during oxidative stress (Harmon et al., 2005). While covalent binding of radiolabel from PAP has been reported (Crowe et al., 1979), the ability of PAP to generate ROS has not been investigated previously.

The present study was designed to determine if ROS are formed when LLC-PK$_1$ cells are exposed to PAP, what types of ROS (superoxide anion, hydrogen peroxide (H_2O_2), or hydroxyl radicals) are formed, and the role of those intermediates in PAP toxicity. LLC-PK$_1$ cells are spontaneously immortal proximal tubule cells that have been used in numerous toxicity studies (Holohan et al., 1988; Baliga et al., 1998; Park et al., 2002). In our previous studies, LLC-PK$_1$ cells were used to determine toxicity of PAP and the role of antioxidants in preventing toxicity (Hallman et al., 2000).

Methods

Cell culture and assays. LLC-PK$_1$ cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Cells were cultured in Dulbecco's Modification of Eagles Medium/Ham's F-12 50/50 (DMEM/F12) mix supplemented with L-glutamine, 15 mM HEPES (Fisher Scientific, Pittsburgh, PA), 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 100 U/ml penicillin–100 μg/ml streptomycin. Cells were incubated in a humidified atmosphere at 37 °C in 5% CO_2/95% air.

Cell viability was measured using alamarBlue (Trek Diagnostics, Cleveland, OH). In previous studies, loss of viability determined using alamarBlue paralleled loss of intracellular lactate dehydrogenase activity (Kendig and Tarloff, 2007), suggesting that loss of viability measured with alamarBlue represents cytotoxicity. LLC-PK$_1$ cells were

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seeded in 24-well plates at a density of 1.0×10^5 cells/ml and allowed to attach overnight. Cells were incubated for 4 h at 37 °C in serum- and phenol red-free DMEM/F-12 containing PAP as previously described (Hallman et al., 2000). Following treatments, cells were rinsed with Hank's balanced salt solution (HBSS) and incubated with 1 ml/well HBSS containing 5% alamarBlue for 2 h. Fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using a Perkin-Elmer HTSoft 7000 series plate reader (Wellesley, MA). Viability was calculated as a ratio of fluorescence measured in treated as compared to untreated cells and expressed as a percentage. Untreated cells were those incubated in the absence of any treatments (PAP or interventions) and viability of these cells was set at 100%.

Cellular oxidative stress was measured using 5-(and 6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes, Eugene, OR). CM-H_2DCFDA is a cell-permeable, non-fluorescent dye that, once inside the cell, is cleaved by intracellular esterases to an impermeable product (DCFH-DA) that is oxidized by ROS to a fluorescent product (DCF). LLC-PK_1 cells were seeded in 60 mm dishes at a density of 1.0×10^5 cells/dish and allowed to attach overnight. Cells were incubated for 2 h at 37 °C in serum- and phenol red-free DMEM/F12 containing PAP. Following treatment, cells were rinsed with HBSS and incubated with 0.5 ml 10 μM CM-H_2DCFDA for 1 h at 37 °C in the dark (Nishida et al., 2003). Cells were rinsed twice with HBSS and treated with 0.25% trypsin/0.1% EDTA until cells detached. Cells were resuspended in 0.6 ml BD FACSFlow™ sheath fluid and fluorescence was determined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Measurements were taken at an emission wavelength of 515 nm–545 nm after excitation of cells at 488 nm with an argon ion laser. Data were acquired and analyzed using CellQuest Pro software. For each analysis 10,000 events were recorded. Data were calculated as a percent of mean fluorescence in pretreated cells as compared to cells treated with only PAP. Pretreated cells were those incubated with scavengers followed by treatment with PAP. Mean fluorescence of PAP-treated cells was set at 100%.

PAGE and Western blot analysis. Western blotting was used to determine the ability of LLC-PK_1 cells to internalize SOD and catalase present in the incubation medium. LLC-PK_1 cells were plated in 100 mm dishes and allowed to attach overnight. Cells were incubated with serum-free medium containing SOD (300 U/ml), catalase (1000 U/ml) or vehicle for 1 h. Cells were scraped from dishes, lysed with 100 μl 0.1% Triton-X 100 containing protease inhibitor cocktail (Sigma Chemical Company, St, Louis, MO), sonicated, and assayed for protein (Bradford, 1976). Protein samples (50 μg/well) were mixed with LDS sample buffer (Invitrogen, Carlsbad, CA) and reducing agent and boiled for 10 min. Samples were loaded onto a NuPAGE 4–12% Bis–Tris precast gel (Invitrogen, Carlsbad, CA) and run at 150 V for 1.25 h. Proteins were transferred to a PVDF membrane at 30 V for 1 h. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) overnight at 4 °C. Membranes were incubated with primary antibody for bovine erythrocyte SOD (1:2000 dilution) and bovine liver catalase (1:2000 dilution) (Chemicon International, Temecula, CA) for 1 h. After multiple washes with 5% nonfat dry milk in TBST to remove excess primary antibody, membranes were incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit IgG antibody (1:2000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Membranes were washed multiple times with TBST to remove excess secondary antibody. Immunoblots were visualized using enhanced chemiluminescence (GE Healthcare Life Sciences, Buckinghamshire, England) and blots were imaged using a Storm Gel and Blot Imaging System (GE Healthcare Life Sciences, Buckinghamshire, England).

Oxidative stress and viability with ROS interventions. To determine if PAP-induced loss of viability was dependent on ROS, cells were coincubated with PAP in the presence of SOD, tiron, catalase, pyruvate, bathocuproine, phenanthroline, mannitol or dimethylthiourea (DMTU). Cells were rinsed with 1.0 ml HBSS and preincubated with SOD (0–300 U/ml), tiron (0–10 mM), catalase (0–1000 U/ml), pyruvate (0–10 mM), mannitol (0–75 mM), DMTU (0–40 mM), bathocuproine (0–10 mM) or 1,10-phenanthroline (0–0.25 mM) for 1 h at 37 °C. Cells were then coincubated with PAP (0.5 mM) or vehicle for 2 h. Cellular fluorescence was measured using flow cytometry to determine the effect of scavengers on ROS formation. None of the pretreatments altered ROS formation in the absence of PAP.

To determine if PAP-induced loss of viability was affected by ROS formation, the same scavengers were used. Cells were rinsed with 0.5 ml HBSS and incubated in dye-free, serum-free DMEM/F12 media at 37 °C with concentrations of SOD, tiron, catalase, pyruvate, mannitol, DMTU, bathocuproine or 1,10-phenanthroline, as described above, for 1 h. Cells were then coincubated with PAP (0.5 mM) or vehicle for 4 h at 37 °C. Cells were rinsed with HBSS and were immediately assessed for cellular viability using 5% alamarBlue. None of the pretreatments altered cell viability in the absence of PAP.

PAP-induced H_2O_2 formation and effects of SOD and catalase. To confirm that PAP in aqueous solution formed H_2O_2, 0.5 mM PAP was incubated in a 24-well culture plate in serum- and phenol red-free DMEM/F-12 at 37 °C in an atmosphere of 5% CO_2/95% air for 1 h. In addition, some wells contained SOD (300 U/ml) or catalase (1000 U/ml) in the presence or absence of 0.5 mM PAP. At the end of the incubation period, H_2O_2 concentration was determined using the Amplex Red hydrogen peroxide assay kit (Molecular Probes). In this assay, Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in the presence of horseradish peroxidase to form fluorescent resorufin. Fluorescence was read in a plate reader at excitation wavelength of 530 nm and an emission wavelength of 590 nm. For each experiment, a standard curve was generated using 0–5 μM H_2O_2. Data are expressed as a ratio of fluorescence measured in the presence of PAP as compared to fluorescence in the absence of PAP and expressed as a percentage. Fluorescence in the absence of PAP was set at 100%.

Statistical analysis. All data are expressed as a mean ± standard error (SE) where n denotes the number of independent experiments. Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's or Student–Newman–Keuls post hoc test. p values of less than 0.05 were considered significant.

Results

Viability and ROS formation

LLC-PK_1 cells were incubated for 4 h with various concentrations of PAP to determine viability using alamarBlue. At a concentration of 0.5 mM, PAP reduced viability to about 30 ± 4% of control. This concentration was used in subsequent experiments to test the effects of ROS scavengers on viability. Viability with 0.5 mM PAP ranged from 14.2 ± 7.1% to 32.2 ± 4.5% of control. Viability was always measured after a 4 h incubation period with toxicants and compared with untreated cells plated at the same density and time as the treated cells.

To determine if PAP caused formation of reactive oxygen species (ROS), a fluorescein derivative was used to stain cells. PAP produced concentration-dependent increases in H_2DCFDA fluorescence when measured after 2 h incubation, prior to the loss of viability (data not shown). At a concentration of 0.5 mM, PAP increased H_2DCFDA fluorescence to about 19,000 ± 2000 arbitrary fluorescence units (AFU). Fluorescence of untreated control cells averaged 7.6 ± 0.4 AFU. Fluorescence of treated cells was variable and ranged from 168.3 ± 11.1 to 2643.8 ± 504.9 AFU. Fluorescence was always measured after a 2 h incubation period and was compared with fluorescence of cells treated with PAP in the absence of scavengers.

Effects of superoxide dismutase and tiron

SOD converts superoxide anion to H_2O_2. In the presence of 100 through 250 U SOD/ml, DCFDA fluorescence in PAP-treated cells was significantly greater than in cells incubated with 0.5 mM PAP in the absence of SOD (Fig. 1). Along with increased formation of ROS, viability in cells coincubated with SOD and PAP was significantly reduced. In the presence of 150 through 300 U SOD/ml, viability was reduced to even lower values than with PAP alone (Fig. 1).

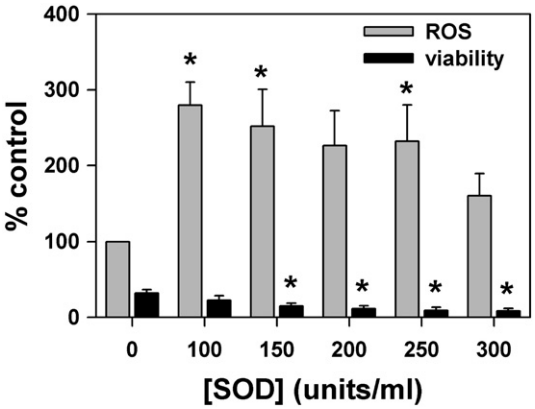

Fig. 1. Effect of SOD on H_2DCFDA fluorescence and viability in LLC-PK_1 cells treated with PAP. Cells were preincubated with the indicated concentrations of SOD for 1 h. Cells were incubated with 0.5 mM PAP and the indicated concentrations of SOD for 2 h, and then stained for 1 h with H_2DCFDA (gray bars). Other cells were incubated with PAP in the presence or absence of SOD for 4 h, and then assessed for viability (black bars). Values represent means ± SE of 4 experiments and are expressed as a percentage of PAP-treated cells for fluorescence or untreated control cells for viability. Mean fluorescence in cells treated with 0.5 mM PAP was 168.3 ± 11.1 arbitrary fluorescence units (AFU). Asterisks indicate means that are significantly different from cells treated with 0.5 mM PAP in the absence of SOD.