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# Contribution of Reactive Oxygen Species to *para*-Aminophenol Toxicity in LLC-PK$_1$ Cells

**Brooke D. Foreman** and **Joan B. Tarloff**
*Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy*

## 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 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 byproducts 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

Author Contact Information: Joan B. Tarloff, Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, 600 South 43rd Street, Philadelphia, PA 19104, Voice 215-596-8983, Fax 215-895-1161, E-mail j.tarlof@usp.edu.

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 seeded in 24-well plates at a density of $1.0 \times 10^5$ 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, menadione 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-$H_2$DCFDA) (Molecular Probes, Eugene, OR). CM-$H_2$DCFDA 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 $1.0 \times 10^5$ 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 0.5ml 10μM CM-$H_2$DCFDA 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 units/ml), catalase (1,000 units/ml) or vehicle for 1 h. Cells were scraped from dishes, lysed with 100μl 0.1% triton-X 100 containing protease inhibitor cocktail (Sigma Chemical Company, St, Louis, MO), sonicated, and assayed for protein (Bradford, 1976). Protein samples (50μg/well) were mixed with LDS sample buffer (Invitrogen, Carlsbad, CA) and reducing agent and boiled for 10 minutes. Samples were loaded onto a NuPAGE 4–12% Bis-Tris precast gel (Invitrogen, Carlsbad, CA) and run at 150V for 1.25 h. Proteins were transferred to a PVDF membrane at 30V 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 (1:2,000 dilution) and bovine liver catalase (1:2,000 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 (1:2,000 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 units/ml), tiron (0–10mM), catalase (0–1,00 units/ml), pyruvate (0–10mM), mannitol (0–75mM), DMTU (0–40mM), bathocuproine (0–10mM) or 1,10-phenanthroline (0–0.25mM) for 1 h at 37° C. Cells were then co-incubated with PAP (0.5mM) 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 co-incubated with PAP (0.5mM) 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 units/ml) or catalase (1000 units/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 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 $H_2$DCFDA 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 $H_2$DCFDA 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 units 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 (Figure 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 units SOD/ml, viability was reduced to even lower values than with PAP alone (Figure 1).

Tiron is a cell-permeable superoxide anion scavenger (Vetter et al., 2003). In the presence of 4 through 10 mM tiron, fluorescence in PAP-treated cells was significantly less than in cells incubated with PAP in the absence of tiron (Figure 2). Although formation of ROS was significantly reduced by tiron, viability in cells coincubated with tiron and PAP was not significantly improved (Figure 2).

### Effect of catalase and pyruvate

Catalase converts $H_2O_2$ to water. In the presence of 100 through 1000 units catalase/ml, $H_2$DCFDA fluorescence was significantly reduced compared with cells incubated with PAP in the absence of catalase (Figure 3). Along with reduced ROS formation, viability in cells coincubated with 0.5 mM PAP and either 500 or 1000 units catalase/ml was significantly improved (Figure 3).

Pyruvate has been used as a cell-permeable $H_2O_2$ scavenger (Salahudeen et al. 1991; Nath et al., 1995). In the presence of 4 through 10 mM pyruvate, $H_2$DCFDA fluorescence was significantly reduced compared with cells incubated with PAP in the absence of pyruvate (Figure 4). Although ROS formation was significantly decreased, there was no difference in viability of cells coincubated with 0.5 mM PAP and any concentration of pyruvate (Figure 4).

### Cellular catalase and SOD determination

SOD exacerbated PAP-induced loss of viability (Figure 1) whereas tiron, another superoxide anion scavenger, was ineffective (Figure 2). Catalase attenuated PAP-induced loss of viability (Figure 3) whereas pyruvate, another $H_2O_2$ scavenger, was ineffective (Figure 4). These disparities and the large sizes of both SOD (32.5 kDa) and catalase (250 kDa) called into question whether or not these enzymes were internalized in LLC-$PK_1$ cells. Cells were incubated in the presence or absence of catalase or SOD and enzyme content was analyzed by Western blotting. As shown in Figure 5, cells incubated in the absence of catalase or SOD contained measurable amounts of both enzymes. Signal intensities were unchanged when cells were incubated with 300 units SOD/ml or 1000 units catalase/ml.

### PAP-induced $H_2O_2$ formation in cell-free incubations

Incubation of 0.5 mM PAP in cell culture medium in the absence of cells caused formation of $H_2O_2$ in excess of that formed in the absence of PAP (Figure 6). SOD (300 units/ml) significantly increased the amount of $H_2O_2$ formed in incubations containing PAP without influencing $H_2O_2$ concentration in incubations without PAP. In contrast, catalase (1000 units/ml) reduced the amount of $H_2O_2$ detected in the presence or absence of PAP to below control values (Figure 6). The effect of catalase to reduce $H_2O_2$ content was due to its enzymatic activity as heat-inactivated catalase (1000 units/ml) was ineffective (data not shown).

### Effects of metal chelators

Phenanthroline is often used as a chelator of iron, similar to deferoxamine (Millar et al., 2000). In the presence of 0.1 through 0.25 mM phenanthroline, fluorescence was significantly reduced compared with cells incubated with PAP in the absence of phenanthroline (Figure 7). Despite reduced ROS formation, viability in cells coincubated with 0.5 mM PAP and any concentration of phenanthroline was not significantly different from viability of cells incubated with PAP alone (Figure 7).

Bathocuproine is used as a metal chelator and displays a relative preference for copper (Coloso et al., 1990). In the presence of 6 through 10 mM bathocuproine, fluorescence was significantly reduced compared with cells incubated with PAP in the absence of bathocuproine (Figure 8). Although ROS formation was significantly decreased, there was no difference in viability of cells coincubated for 4 h with 0.5 mM PAP and any concentration of bathocuproine (Figure 8).

### Effects of mannitol and dimethylthiourea (DMTU)

Mannitol is a poorly permeable hydroxyl radical scavenger (Seo et al., 2006). In the presence of 15 through 75 mM mannitol, fluorescence was not significantly different from that in cells

incubated with PAP in the absence of mannitol (data not shown). Viability in cells coincubated with 0.5 mM PAP and any concentration of mannitol was not significantly different from viability of cells incubated with PAP alone (data not shown).

DMTU is a cell-permeable hydroxyl radical scavenger (Fox, 1984). In the presence of 20 through 40 mM DMTU, fluorescence was significantly reduced compared with cells incubated with PAP in the absence of DMTU (Figure 9). Despite significantly reduced ROS formation, viability of cells coincubated with 0.5 mM PAP and 30 through 50 mM DMTU was significantly reduced compared with cells incubated with PAP in the absence of DMTU (Figure 9).

## Discussion

PAP cytotoxicity was attenuated when antioxidants such as ascorbate and glutathione were included in the incubation medium, suggesting that oxidation is an important contributor to PAP toxicity (Hallman et al., 2000). PAP undergoes non-enzymatic oxidation in aqueous solutions to form numerous byproducts including benzoquinoneimine and *para*-aminophenoxy radical (Josephy et al. 1983). These reactive intermediates may be responsible for mitochondrial damage and covalent binding observed with PAP (Crowe et al., 1977, 1979; Kruidering et al., 1994). However, PAP has also been shown to cause lipid peroxidation, measured as 4-hydroxynonenal-adducted proteins (Harmon et al., 2005, 2006). The ability of PAP to generate reactive oxygen intermediates has not been investigated previously. In the present study, PAP-induced loss of viability was correlated with formation of ROS that oxidize $H_2$DCFDA to a fluorescent product. Based on these results, experiments were conducted using a concentration of PAP (0.5 mM) that reduced viability by about 60% within 4 h and generated ROS within 2 h to a level about 200-fold greater than control (untreated) cells. Two time points were used because there was no evidence for significant loss of viability after a 2 h exposure to PAP (measured using lactate dehydrogenase release or alamarBlue fluorescence, data not shown) while cell loss at 4 h due to PAP toxicity could bias flow cytometry analysis. Thus, changes in ROS formation were determined prior to changes in viability, allowing investigation of potential correlations between ROS formation and viability using scavengers of various reactive intermediates.

### Extracellular autoxidation and PAP toxicity

SOD and catalase are important enzymes involved in protecting cells from oxygen toxicity. SOD converts superoxide into $H_2O_2$ while catalase converts $H_2O_2$ to water and molecular oxygen. SOD dramatically increased ROS formation and decreased viability when cells were incubated with PAP (Figure 1) whereas catalase significantly reduced ROS formation and improved viability when cells were incubated with PAP (Figure 3).

The extent to which SOD or catalase are internalized has been questioned (Yonehana and Gemba, 1995; Doroshow 1986). In rat hepatocytes incubated with SOD, intracellular SOD activity was increased about 4-fold (Kyle et al., 1988). Further, SOD-mediated protection from hypoxia and reoxygenation injury in hepatocytes was attenuated when inhibitors of endocytosis were included in the incubation medium (Kyle et al., 1988). LLC-$PK_1$ cells incubated with cyclosporine A in the presence of catalase formed significantly less $H_2O_2$ and released significantly less LDH than cells incubated with cyclosporine A alone (Nishida et al., 2003), suggesting that catalase was internalized to some extent. LLC-$PK_1$ cells do not accumulate either enzyme in excess of constitutive expression levels under the experimental conditions (Figure 5). Cell-free incubations with 0.5 mM PAP confirmed that $H_2O_2$ is produced in aqueous solution (Figure 6). The ability of catalase to decrease $H_2O_2$ in cell-free incubations is consistent with the improved viability observed when LLC-$PK_1$ cells were incubated with PAP and catalase (Figure 3). The greatly enhanced $H_2O_2$ production in cell-free incubations in the

presence of SOD (Figure 6) suggests that one of the products of PAP autoxidation is superoxide anion.

From these data, we can construct the following scenario for PAP toxicity. PAP undergoes nonenzymatic oxidation in the incubation medium to form unstable *p*-aminophenoxy radical (Josephy et al., 1983). Products formed likely include superoxide anion, which, in the presence of SOD, undergoes conversion to $H_2O_2$, and $H_2O_2$ itself, which in the presence of catalase undergoes conversion to water and carbon dioxide. $H_2O_2$ is more permeable than superoxide anion (Watanabe and Forman, 2003) and diffuses into LLC-$PK_1$ cells, accounting for the increases in fluorescence and decreases in viability seen when cells were incubated with SOD (Figure 1). In contrast, catalase in the incubation medium was able to prevent $H_2O_2$ from accumulating and tended to protect cells from PAP-induced loss of viability (Figure 3). Extracellular formation of $H_2O_2$ is exaggerated with SOD or attenuated with catalase (Figure 6). In the case of SOD, excessive $H_2O_2$ formation and diffusion may overwhelm cellular protective mechanisms such as intracellular SOD and glutathione peroxidase while in the case of catalase, extracellular and intracellular concentrations of $H_2O_2$ are likely reduced. A similar scenario has been proposed for anticancer quinones such as doxorubicin and mitomycin C (Doroshow 1986).

## Intracellular events in PAP toxicity

PAP itself is accumulated by rat renal epithelial cells (Li et al., 2005) and presumably by LLC-$PK_1$ cells, so a portion of toxicity is due to intracellular events. Formation of superoxide anion may occur in the cytoplasm of cells incubated with PAP since tiron, a cell-permeable superoxide scavenger (Vetter et al., 2003) significantly reduced $H_2$DCFDA fluorescence (Figure 2A). However, the correlation between superoxide anion formation and toxicity was not apparent as viability was unaltered by tiron (Figure 2B). In contrast to extracellular formation of $H_2O_2$, this ROS does not seem to be an important intracellular trigger for toxicity with either toxicant since coincubation with pyruvate, a putative $H_2O_2$ scavenger (Salahudeen et al., 1991; Nath et al., 1995) had no effect on viability (Figure 4B). Formation of $H_2O_2$ did contribute to ROS-associated fluorescence with PAP since pyruvate at concentrations of 4 mM or greater significantly reduced fluorescence.

The inability to protect LLC-$PK_1$ cells from toxicity by including pyruvate is at odds with previous reports in which pyruvate provided partial protection from PAP toxicity in rat renal cortical slices (Harmon et al., 2006). It is possible that differences in route of entry might contribute to the disparity. Renal cortical slices are oriented with basolateral membranes toward the incubation medium and luminal membranes collapsed (Kirkpatrick and Gandolfi, 2005) so that PAP and pyruvate would be entering cells across the basolateral membrane. In contrast, cultured cells are oriented with the luminal membrane exposed to the incubation medium and the basolateral membrane largely inaccessible (Ford, 2005). It is possible that differences in route of entry for PAP might lead to differences in susceptibility to or mechanism of toxicity. For example, $H_2O_2$ caused LDH release in opossum kidney cells at much lower concentrations (0.5 mM) than required in rabbit renal slices (5 mM) while isolated rabbit proximal tubules exhibited no damage with $H_2O_2$ at concentrations up to 3 mM (Kim et al., 1998). In studies comparing $H_2O_2$ and *tert*-butylhydroperoxide toxicity, Park and co-workers (2003) found marked differences in concentrations required to elicit LDH release and the ability of antioxidants to protect damage when comparing rabbit renal cortical slices and primary cultured rabbit proximal tubule cells. They suggested that the mechanism of $H_2O_2$ toxicity may differ depending on the test system employed (Park et al., 2003).

Hydroxyl radicals are highly damaging due to their reactivity. Metal chelators phenanthroline (for iron; Millar et al., 2000) and bathocuproine (for copper; Coloso et al., 1990) were used to determine if Fenton chemistry accounted for production of hydroxyl radicals when cells were

incubated with PAP. ROS-associated fluorescence was significantly decreased with either chelator (Figures 7 and 8). However, the reductions in ROS were not associated with improved viability in cells incubated with PAP. Deferoxamine, another iron chelating agent, was ineffective in protecting rabbit proximal tubules from PAP toxicity (Lock et al., 1993). The results with phenanthroline echo those obtained with tiron and suggest that intracellular events associate with PAP toxicity are independent of iron- or copper-catalyzed reactions.

Scavengers of hydroxyl radicals include poorly permeable compounds such as mannitol (Seo et al., 2006) and permeable compounds such as DMTU (Fox, 1984). Mannitol was ineffective in decreasing ROS-associated fluorescence or improving viability in cells treated with PAP. Thus, formation of hydroxyl radicals apparently does not occur in the incubation medium or hydroxyl radicals formed in the incubation medium are not able to diffuse into cytosol to influence toxicity. For cells incubated with PAP, hydroxyl radicals contribute to ROS-associated fluorescence since incubation with DMTU reduced cell-associated fluorescence (Figure 9). Paradoxically, viability was significantly reduced by coincubation with DMTU and PAP. The decrease in cell viability with DMTU was not due to toxicity of the scavenger itself since the toxicity was not seen in cells incubated with DMTU alone. DMTU has been used with opossum kidney cells (Park et al., 2003) and LLC-$PK_1$ cells (Yonehana and Gemba, 1995) with no evidence of toxicity. The interaction between PAP and DMTU is perplexing and will require further study.

In summary, PAP undergoes oxidation in incubation medium to generate ROS such as $H_2O_2$ and superoxide. Increased extracellular $H_2O_2$ formation potentiates PAP-induced loss of viability and decreased extracellular $H_2O_2$ formation partially protects cells from loss of viability. Once inside cells, PAP generates ROS including superoxide and possibly hydroxyl radicals. However, these ROS do not contribute to toxicity since chemicals that prevent ROS generation have minimal impact on viability.

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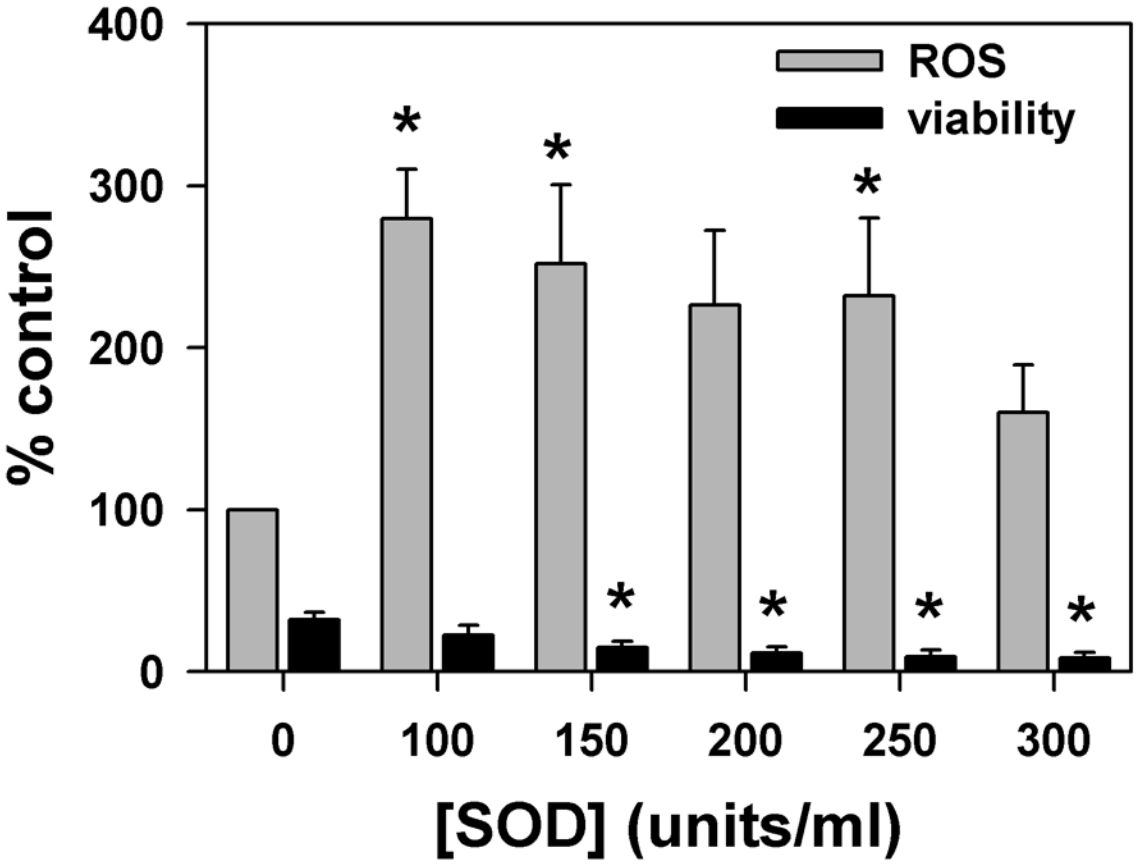


**Figure 1.**

Effect of SOD on $H_2$DCFDA 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 $H_2$DCFDA (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.

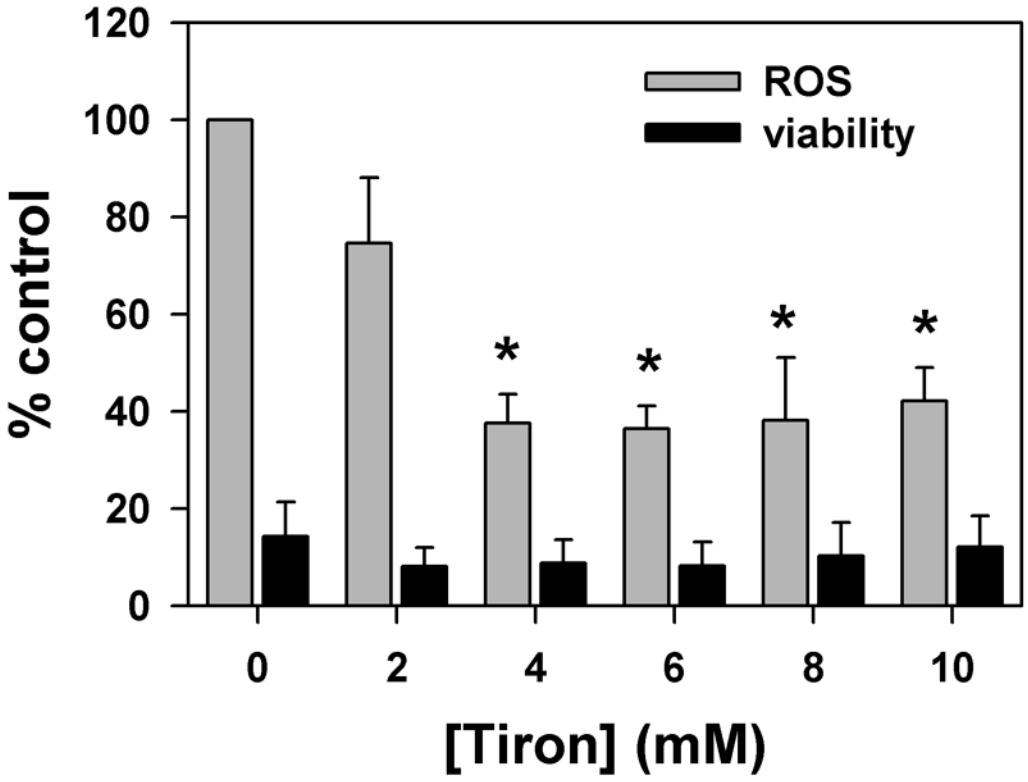


**Figure 2.**

Effect of tiron on $H_2$DCFDA fluorescence and viability in LLC-$PK_1$ cells treated with PAP. Cells were preincubated with the indicated concentrations of tiron for 1 h Cells wereincubated with the indicated concentrations of tiron and 0.5 mM PAP for 2 h, and then stained for 1 h with $H_2$DCFDA (gray bars). Other cells were incubated with PAP in the presence or absence of tiron 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 232.5 ±70.3 AFU. Asterisks indicate means that are significantly different from values in cells treated with PAP in the absence of tiron.

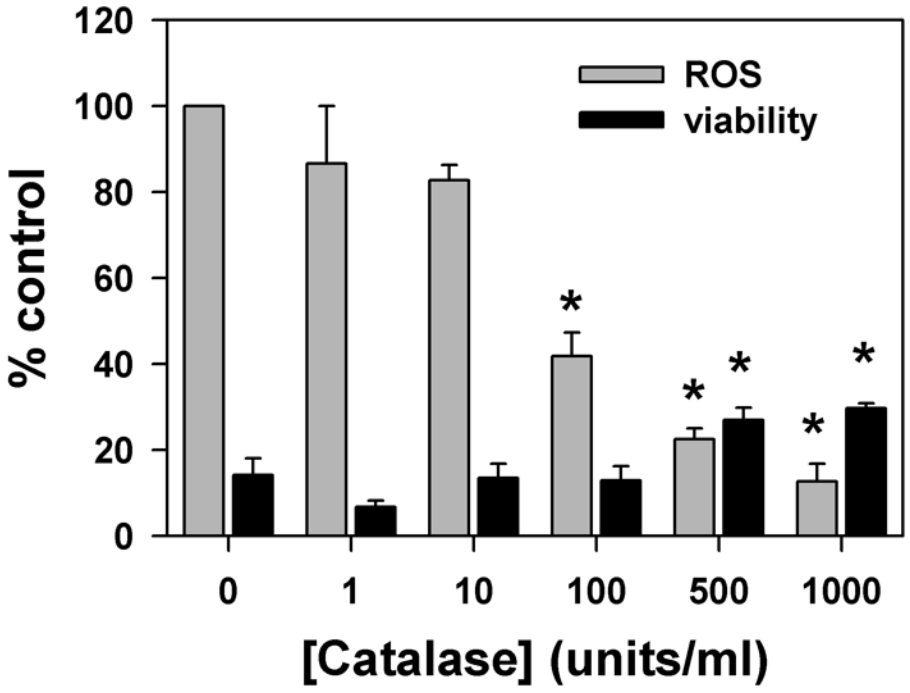


**Figure 3.**

Effect of catalase on $H_2$DCFDA fluorescence and viability in LLC-$PK_1$ cells treated with PAP. Cells were preincubated for 1 h with the indicated concentrations of catalase. Cells were incubated with 0.5 mM PAP and the indicated concentrations of catalase for 2 h, and thenstained for 1 h with $H_2$DCFDA (gray bars). Other cells were incubated with PAP in the presence or absence of catalase 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 2643.8 ± 504.9 AFU. Asterisks indicate means that are significantly different from values in cells treated with PAP in the absence of catalase.

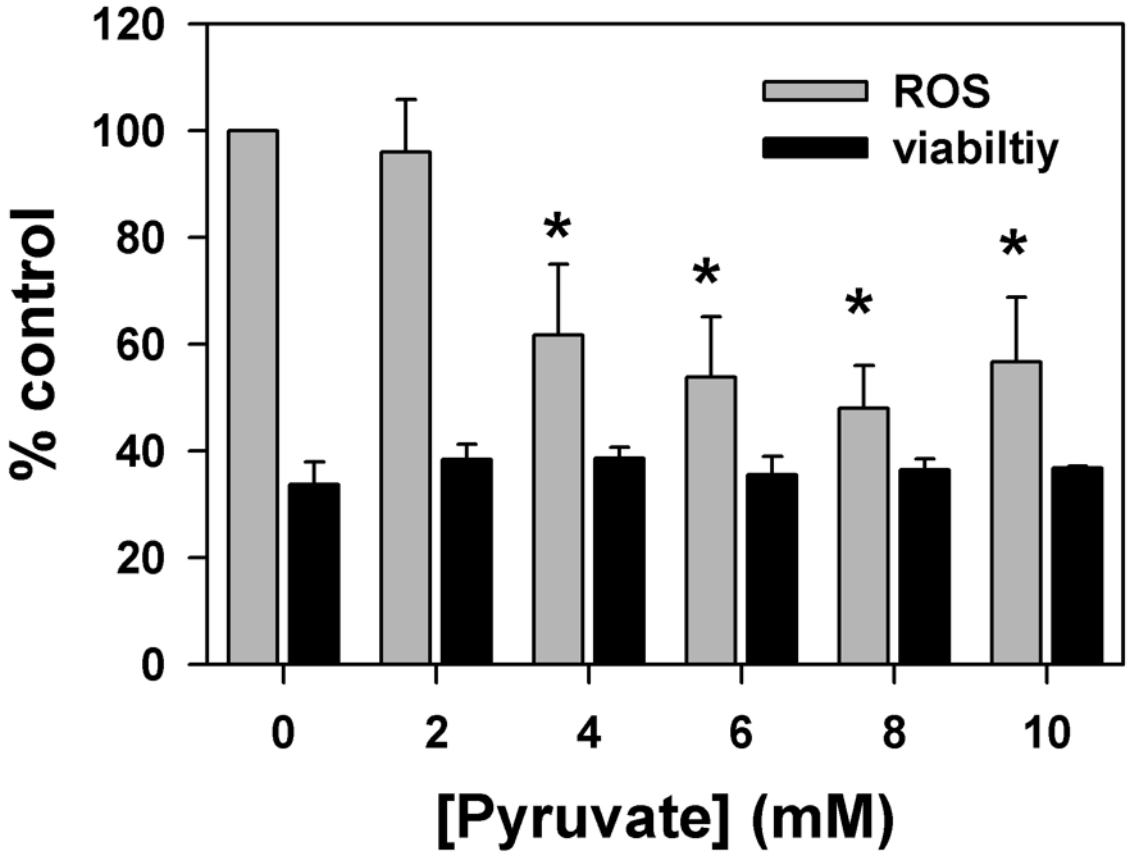


**Figure 4.**

Effect of pyruvate on $H_2$DCFDA fluorescence and viability in LLC-$PK_1$ cells treated with PAP. Cells were preincubated for 1 h with the indicated concentrations of pyruvate. Cells were incubated with 0.5 mM PAP and the indicated concentrations of pyruvate for 2 h, and then stained for 1 h with $H_2$DCFDA (gray bars). Other cells were incubated with PAP in the presence or absence of pyruvate for 4 h, and then assessed for viability (black bars). Values represent means ± SE of 3 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 1490.5 ± 605.5 AFU. Asterisks indicate means that are significantly different from values in cells treated with PAP in the absence of pyruvate.

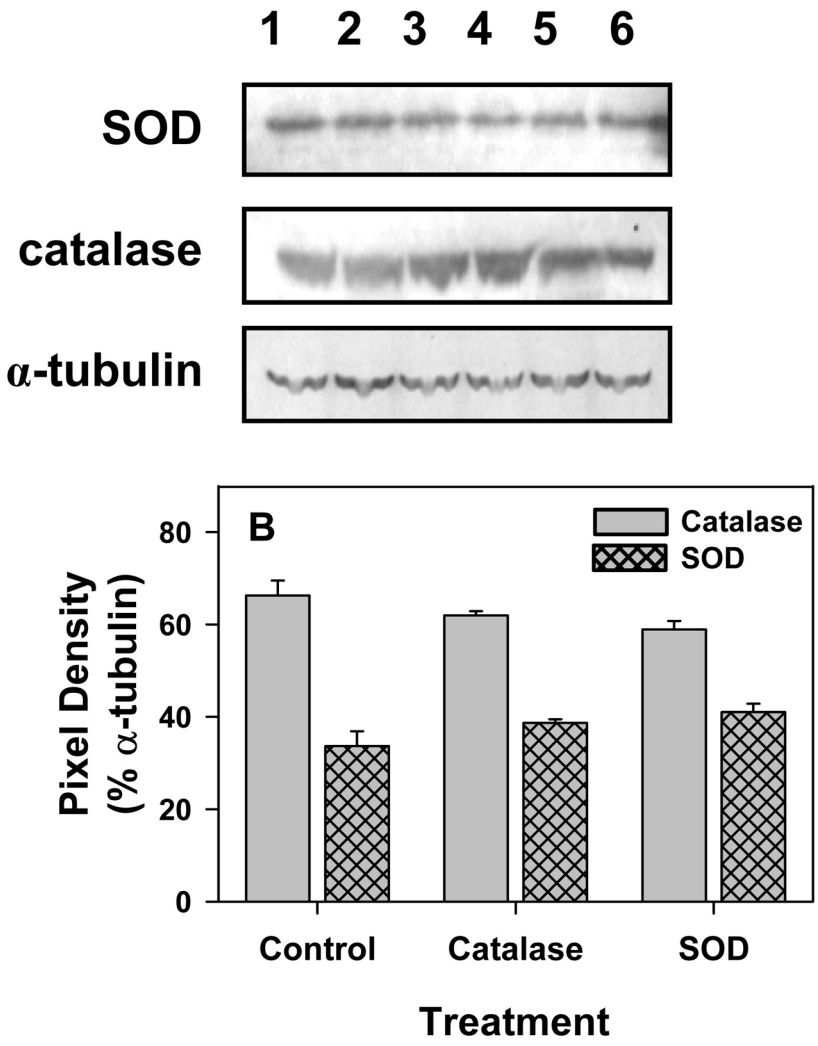


**Figure 5.**

SOD and catalase concentrations in LLC-PK$_1$ cells. **A:** Cells were incubated in the presence or absence of 300 units SOD/ml or 1000 units catalase/ml for 1 h, then lysed and processed for PAGE and Western blotting. Blots were probed with antibodies to SOD, catalase and α-tubulin for a loading control. Lanes 1 and 4 contain lysates from cells incubated with SOD, lanes 2 and 5 contain lysates from cells incubated with catalase, and lanes 3 and 6 contain lysates from cells incubated in the absence of either enzyme. **B:** Densitometric analysis of SOD and catalase content of LLC-PK$_1$ cells following incubation in the presence or absence of enzymes. Band intensities are expressed relative to α-tubulin. Bars represent means ± SE of 3 experiments.

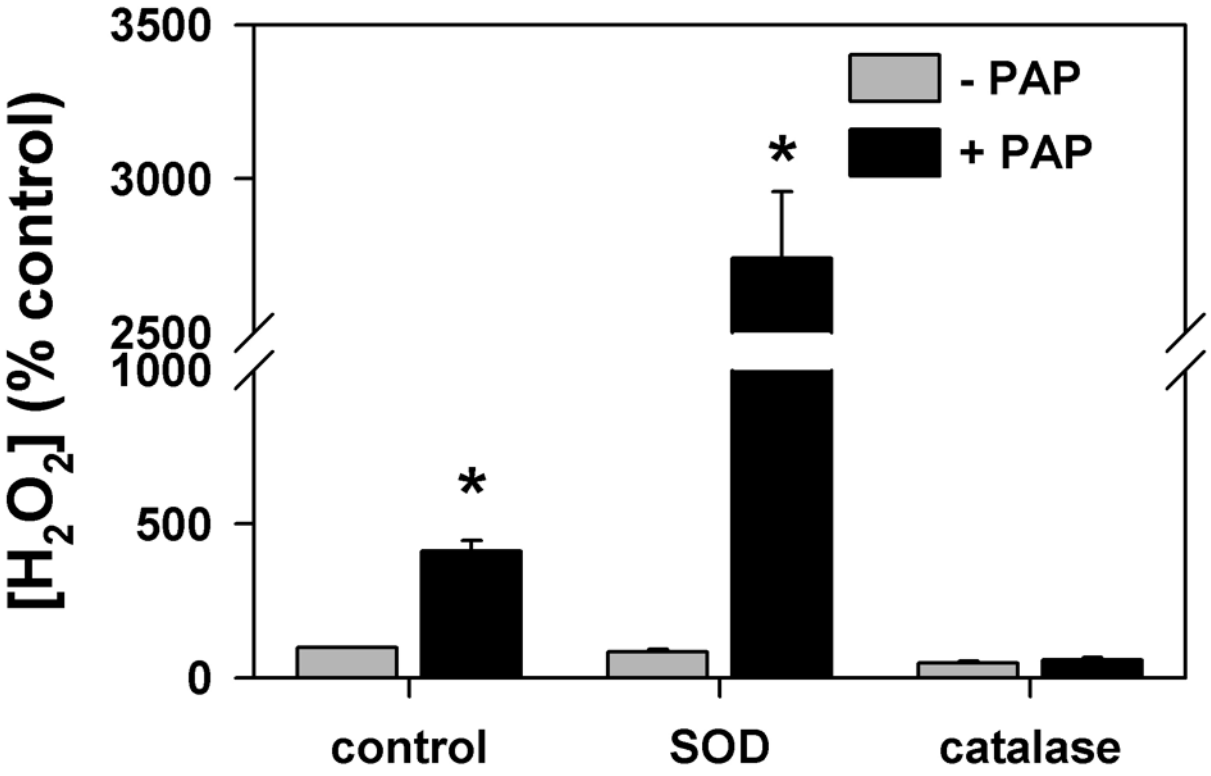


**Figure 6.**

PAP-induced $H_2O_2$ formation in cell-free incubations. PAP was incubated in the presence or absence of SOD (300 units/ml) or catalase (1000 units/ml) for 1 h in culture medium at 37 °C in an atmosphere of 5% $CO_2$/95% air and $H_2O_2$ concentrations were determined at the end of the incubation period. Values represent means ± SE of three independent experiments, expressed as a percentage of $H_2O_2$ fluorescence determined in culture medium in the absence of PAP. Asterisks indicate mean values that are significantly different from incubations in the absence of PAP.

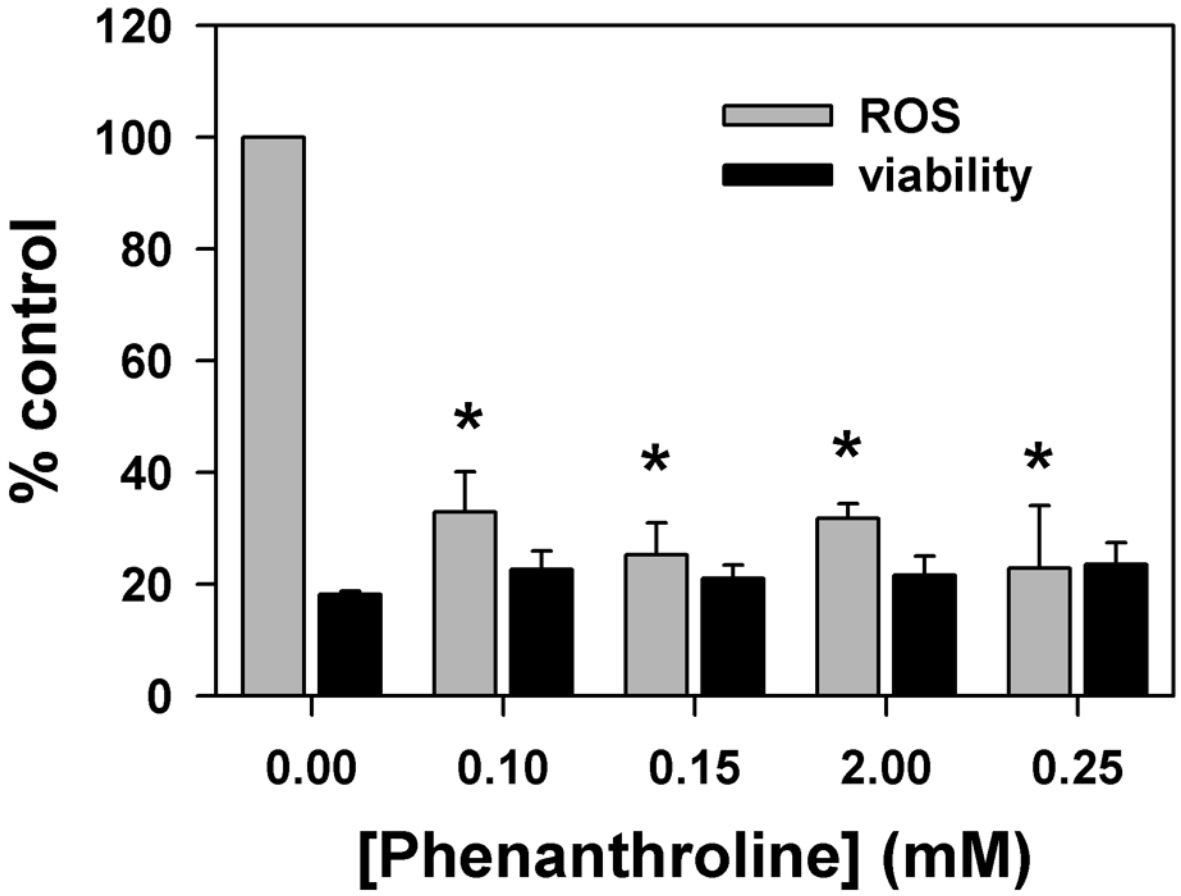


**Figure 7.**

Effect of phenanthroline on $H_2$DCFDA fluorescence and viability in LLC-$PK_1$ cells treated with PAP. Cells were preincubated for 1 h with the indicated concentrations of phenanthroline. Cells were incubated with 0.5 mM PAP and the indicated concentrations of phenanthroline for 2 h, then stained for 1 h with $H_2$DCFDA (gray bars). Other cells were incubated with PAP in the presence or absence of phenanthroline for 4 h, and then assessed for viability (black bars). Values represent means ± SE of 3 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 1304.3 ± 341.2 AFU. Asterisks indicate means that are significantly different from values in cells treated with PAP in the absence of phenanthroline.

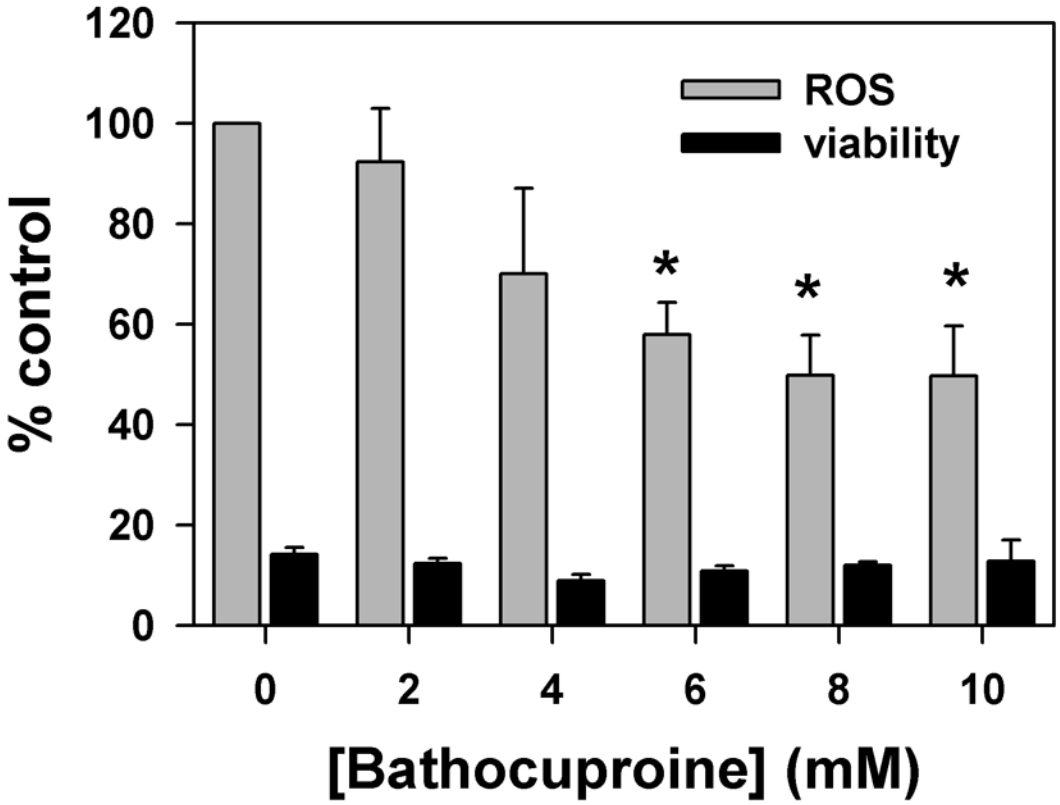


**Figure 8.**

Effect of bathocuproine on $H_2$DCFDA fluorescence and viability in LLC-$PK_1$ cells treated with PAP. Cells were preincubated for 1 h with the indicated concentrations of bathocuproine. Cells were incubated with 0.5 mM PAP and the indicated concentrations of bathocuproine for 2 h, then stained for 1 h with $H_2$DCFDA (gray bars). Other cells were incubated with PAP in the presence or absence of bathocuproine for 4 h, and then assessed for viability (black bars). Values represent means ± SE of 3 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 1603.4 ± 419.4 AFU. Asterisks indicate means that are significantly different from values in cells treated with PAP in the absence of phenanthroline

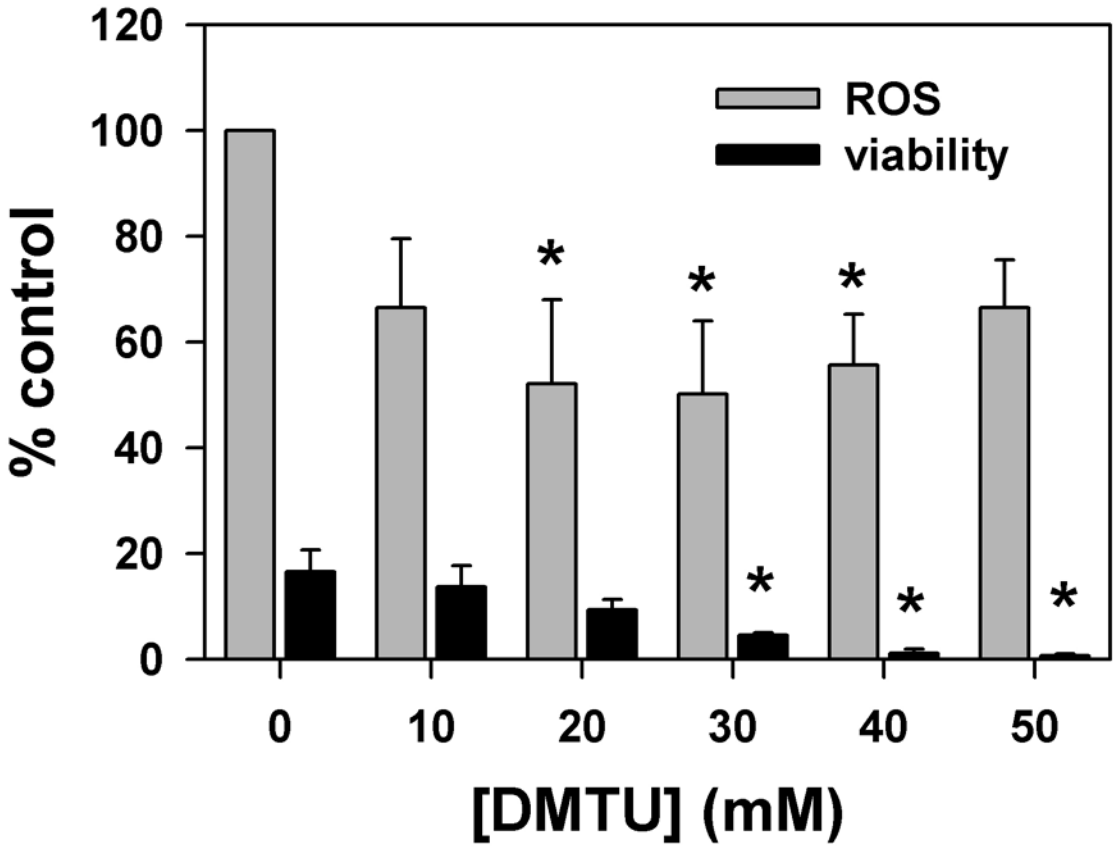


**Figure 9.**

Effect of DMTU on $H_2$DCFDA fluorescence and viability in LLC-$PK_1$ cells treated with PAP. Cells were preincubated for 1 h with the indicated concentrations of DMTU. Cells were incubated with 0.5 mM PAP and the indicated concentrations of DMTU for 2 h, then stained for 1 h with $H_2$DCFDA(gray bars). Other cells were incubated with PAP in the presence or absence of DMTU for 4 h, and then assessed for viability (black bars). Values represent means ± SE of 3 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 647.1 ± 365.6 AFU. Asterisks indicate means that are significantly different from values in cells treated with PAP in the absence of DMTU.