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Lindane-Induced Generation of Reactive Oxygen Species and Depletion of Glutathione do not Result in Necrosis in Renal Distal Tubule Cells

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Lindane is a chlorinated hydrocarbon pesticide, currently used in prescription shampoos and lotions to treat scabies and lice infestations. Lindane is known to be nephrotoxic; however, the mechanism of action is not well understood. In other organ systems, lindane produces cellular damage by generation of free radicals and oxidative stress. Morphological changes were observed in lindane-treated Madin–Darby canine kidney (MDCK) cells indicative of apoptosis. Lindane treatment induced time-dependent reactive oxygen species (ROS) generation. Onset of ROS generation correlated with an initial increase in total glutathione (GSH) levels above control values, with a subsequent decline in a time-dependent manner. This decline may be attributed to quenching of free radicals by GSH, thereby decreasing the cellular stores of this antioxidant. Necrotic injury was assessed by measuring lactate dehydrogenase (LDH) leakage from the cell after lindane exposure. No significant LDH leakage was noted for all concentrations tested over time. Generation of ROS and alterations in cellular protective mechanisms did not result in necrotic injury in MDCK cells, which corresponds with our morphological findings of lindane-induced apoptotic changes as opposed to necrosis in MDCK cells. Thus, lindane exposure results in oxidative damage and alterations in antioxidant response in renal distal tubule cells, followed by cell death not attributed to necrotic injury.

Lindane, the γ-isomer of hexachlorocyclohexane, is a highly lipophilic organochlorine pesticide used for domestic and agricultural purposes, most notably as a crop seed treatment. Human contact with lindane occurs primarily through the oral route, including consumption of contaminated food and water, as well as through general hand-to-mouth activity. Additional routes of exposure include inhalation and dermal contact, with the latter largely associated with pharmaceutical applications of lindane (Agency for Toxic Substances and Disease Registry, 2005). The U.S. Environmental Protection Agency recently discontinued registration of lindane for use on crops. However, given the recalcitrant nature of lindane, residues of this pesticide may remain in regions where it is applied, contaminating exposed soil and watersheds for extended periods of time. Currently, use of lindane is predominantly as a component of prescription lotions and shampoos for treatment of scabies and lice infestations.

Studies showed that lindane is toxic to the central nervous system (CNS), reproductive system, and cardiovascular system, as well as the liver and kidneys (Ananya et al., 2005; Agency for Toxic Substances and Disease Registry, 2005; Eldefrawi & Eldefrawi, 1987; Saradha et al., 2008; Videla et al., 1990, 1997, 2000). The adverse CNS effects of lindane are attributed to antagonism of the central γ-aminobutyric acid/benzodiazepine ($GABA_A$/BZD) receptor, which inhibits Cl^- channel flux (Eldefrawi et al., 1985; Eldefrawi & Eldefrawi, 1987; Griffith & Wooley, 1989). In other organs, exposure to lindane induces reactive oxygen species (ROS) generation and oxidative damage (Ananya et al., 2005; Garcia-Fernandez et al., 2002; Sahoo et al., 2000; Videla et al., 1990, 1997, 2000). Free radical generation is a key component of lindane's mechanism of toxicity, resulting in altered cellular function, injury, and death.

Studies reported in vitro and in vivo lindane-induced lipid peroxidation in human serum samples, rat liver, rat cerebral hemisphere, and Chinese hamster ovary (CHO)-K1 cells (Banerjee et al., 1999; Sahoo et al., 2000; Garcia-Fernandez et al., 2002; Srivastava & Shivanandappa, 2005). Lipid peroxidation indicates free radical-induced tissue damage, which results in cell

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membrane damage and release of cellular contents (Kappus, 1985; Aust, 1994; Thamilselvan et al., 2000). Lindane treatment of in vitro rat liver cells results in generation of ROS (Barros et al., 1998; Junqueira et al., 1988; Videla et al., 2000). Induction of oxidative stress in these test models is correlated with dysregulation of endogenous cellular antioxidants that serve to protect from adverse effects associated with ROS generation. Lindane-induced depletion of glutathione γ-glutamylcysteinylglycine (GSH) in rat brain regions (cortex, midbrain, cerebellum, brainstem) is consistent with findings in the rat cerebral cortex and CHO-K1 cells (Sahoo et al., 2000; Garcia-Fernandez et al., 2002; Srivastava & Shivanandappa, 2005). Depletion of GSH may be a direct function of exposure to toxicants, such as lindane, or it may be a consequence of toxicant-induced ROS production resulting in exhaustion of available GSH stores.

Studies showed that lindane is nephrotoxic, but the mechanism of action remains unclear. Andrews and Gray (1990) demonstrated that lindane exposure in Long-Evans hooded rats resulted in increased kidney weight and urinary lactate dehydrogenase (LDH), and decreased urinary Ca^{2+}. Lindane was also found to produce increased excretion of glucose in the urine and hypertrophy of the renal tubule epithelia in male Wistar rats (Srinivasan et al., 1984). Lindane was shown to alter $GABA_A$ receptor activity in human embryonic kidney cells and to produce calcium flux in Madin-Darby canine kidney (MDCK) cells (Nagata et al., 1996; Lu et al., 2000). These findings are evidence of variable signal pathways for lindane-induced nephrotoxicity. The purpose of the present study was to evaluate lindane-induced oxidative damage via generation of ROS and dysregulation of the intracellular antioxidant GSH in MDCK cells as an in vitro model of renal distal tubule cell toxicity.

MATERIALS AND METHODS

Cell Culture

MDCK cells were used as a model of renal distal tubule cell response for the in vitro mechanistic evaluation of lindane toxicity. Cells obtained from the American Type Culture Collection (Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Gaithersburg, MD) supplemented with 10% newborn calf serum (HyClone, Logan, UT), 5×10^{-8} *M* hydrocortisone (Sigma Chemical Company, St. Louis, MO), and 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenious acid (BD Biosciences, Bedford, MA).

Chemicals

Dimethyl sulfoxide (DMSO), oxidized GSH, GSH reductase, hydrogen peroxide, lindane, trichloroacetic acid (TCA), and *N*-acetylcysteine (NAC) were obtained from Sigma Chemical Company.

Cellular Morphology Analysis

MDCK cells were grown on 35-mm^2 tissue culture dishes and treated once the cells reached 75–80% confluency. Cells were treated with DMSO (vehicle control), 0.5 µM hydrogen peroxide (H_2O_2) (positive control), or lindane (0.1, 1, 10, or 100 µ*M*). Morphological changes were observed at 15 and 30 min and 1, 2, 4, 6, 12, and 16 h after treatment using a Nikon Phase Contrast 2 microscope (Nikon, Melville, NY).

Assessment of Oxidative Stress

Concentrations for H_2O_2 and lindane were determined through literature searches on lindane-induced in vitro toxicity. The data obtained from the morphological analysis was used to model the concentration-response of MDCK cells for both H_2O_2 and lindane. MDCK cells were grown on 96-well plates (30,000 cells/well) until 80% confluent, washed with Hanks balanced salt solution (BSS), and pretreated with 50 µl of 100 µ*M* 2',7'-dichlorofluorescein diacetate (DCF-DA) in serum-free medium for 30 min. After aspirating the DCF-DA, the cells were washed with Hanks BSS. The cells were treated with DMSO, 100–500 µ*M* H_20_2, or 10 or 100 µ*M* lindane alone or after pretreatment with NAC in triplicate for 15 min, 30 min, 1 h, or 2 h (n = 4). Fluorescence was measured in live cells at Ex/Em of 490/526 nm at 37°C using a Tecan SpectraFluor microplate reader (Tecan, Durham, NC) (Vincent et al., 2004; Wang & Joseph, 1999). Data are presented as the proportion of the untreated control for each time point.

Microassay for Measurement of Total GSH

To measure total GSH levels in MDCK cells, cells were grown in triplicate on 96-well plates until they were 80–85% confluent (n = 4). Cell medium was aspirated and replaced with serum-free DMEM. Cells were then treated with DMSO, 500 µ*M* H_2O_2, and 10 or 100 µ*M* lindane and incubated for 15 or 30 min and 1, 2, 6, 12, or 24 h. GSH levels in the cells were measured as described by Allen et al. (2001). The cell medium was discarded after treatment, and 200 µl of 0.01 *M* phosphate-buffered saline (PBS) (pH 7.6) was added to each well and centrifuged at 180 × g at 4°C for 3 min, repeating the wash cycle once. Ice-cold Triton X-100 was added to each well, and plates were placed on a plate shaker at room temperature. An aliquot of cell solution was transferred to a new 96-well plate for protein analysis. Trichloroacetic acid (5%) was added to the cells on the original plate and was allowed to shake at room temperature for 2 min. An aliquot of the cell solution was added to a new 96-well plate for the total GSH analysis. Glutathione standards were made (2–10 µ*M* GSH), and added to new 96-well plates in triplicate to be used as control for GSH measurements. A GSH reaction buffer containing 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide phosphate (NADPH), and GSH reductase was added to each well of the new plate, and absorbance was read at 405 nm

for 5 min at 30-s intervals using a Tecan SpectraFluor microplate reader. Total GSH concentration in MDCK cells was calculated using the rate of change in absorbance over the linear portion of the reaction for the standards and unknowns (Allen et al., 2001).

Determination of Lindane-Induced Cytotoxicity

MDCK cells were grown to confluence on 35-mm^2 tissue culture dishes for analysis of lactate dehydrogenase (LDH) activity. Cells were treated with DMSO, 50 μ*M* H_2O_2, and 10 and or 100 μ*M* lindane for 1, 2, 4, 8, 24, or 48 h. Culture medium was removed from each dish and reserved for analysis of lindane-induced LDH leakage from the cell into the culture medium. Phosphate buffer (0.01 *M*) was added to the dishes and cells were scraped from the matrix using a rubber policeman. The cell suspension was sonicated and centrifuged for 5 min, and the cell suspension and reserved culture medium were analyzed spectrophotometrically at 340 nm. The percent LDH leakage was calculated using the formula (LDH in medium)/(LDH in medium + LDH from cells) × 100.

Statistical Analysis

To assess lindane-induced time- and concentration-dependent generation of ROS, depletion of GSH and the LDH leakage assay data were analyzed using Student's *t*-test. The results of the experiments are expressed as the mean proportion of the untreated control. The results of the latter experiments are expressed as mean GSH concentrations. Experimental treatment results were considered to be statistically significant when the probability of chance occurrence was $p < .05$. Statistical significance was determined through comparison of treated cells with untreated. Microplate experiments were carried out in triplicate in four independent experiments. In addition, the cellular morphology and LDH leakage analyses were completed as sets of four experiments.

RESULTS

Morphological Analysis of MDCK Cells

Exposure to lindane resulted in time- and concentration-dependent morphological changes indicative of programmed cell death, but not necrosis in MDCK cells. Cell death was not observed in untreated control cells (Figure 1A) or vehicle control-treated cells. Cells treated with H_2O_2 showed early signs of damage at 2 h (Figure 1B). Initial morphological changes occurred within 30 min after treatment with lindane. Specifically, chromatin condensation was observed in cells treated at concentrations of 10 μ*M* and 100 μ*M*. At 2 h after treatment, lindane-treated cells at these higher concentrations exhibited formation of apoptotic bodies and lifting off of the plate (Figure 1, C and D), whereas cells treated at the two lower concentrations (0.1 μ*M* and 1 μ*M*) showed early hallmarks of apoptosis, including cell shrinkage and chromatin condensation (data not shown).

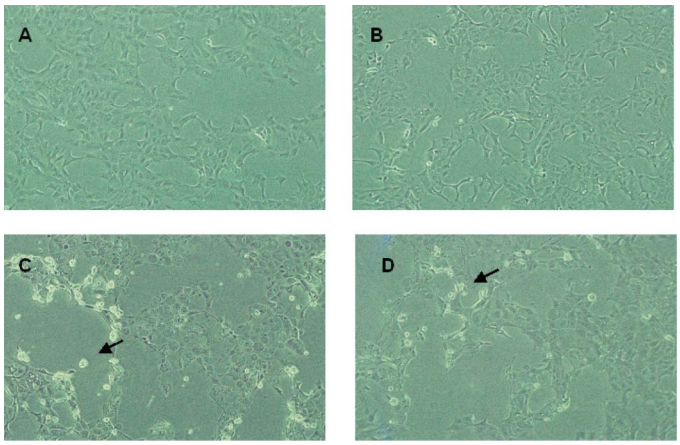

FIG. 1. Morphological changes in MDCK cells. Cellular morphology in MDCK cells after treatment with (A) untreated control, (B) 50 μ*M* H_2O_2, (C) 10 μ*M* lindane, or (D) 100 μ*M* lindane for 2 h using a Nikon Phase Contrast 2 microscope (10×).

Generation of ROS

Data showed that exposure of MDCK cells to lindane induced statistically significant, time-dependent generation of ROS (Figure 2). A statistically significant increase in ROS generation was observed with exposure of MDCK cells to the vehicle control (DMSO), although the increase in free radical production was not as high as treatment with lindane. At 15 min after lindane treatment a statistically significant peak in ROS generation was measured for all concentrations of lindane, with the highest spike in ROS generation occurring in MDCK cells treated with the positive control (H_2O_2) and the highest concentration of lindane (100 μ*M*) (Figure 2). At later time points, the ROS generation declined but continued to exceed levels of ROS measured in the untreated control cells. A significant difference in ROS generation between the lindane- and H_2O_2-treated cells was not found.

The effect of pretreatment of MDCK cells with the antioxidant NAC to protect the cells from free radical production was examined. Pretreatment with NAC attenuated ROS generation in cells treated with lindane at the highest concentration (100 μ*M*). By 2 h after treatment, ROS generation decreased to levels similar to those in the untreated control cells. Pretreatment with NAC had no effect in cells treated with H_2O_2 or lindane at the lower concentration of lindane (10 μ*M*) (Figure 3).

Total GSH Measurement

Treatment with both 10 and 100 μ*M* lindane produced statistically significant, concentration-dependent depletion of total GSH after a 15-min incubation period, with a decline in

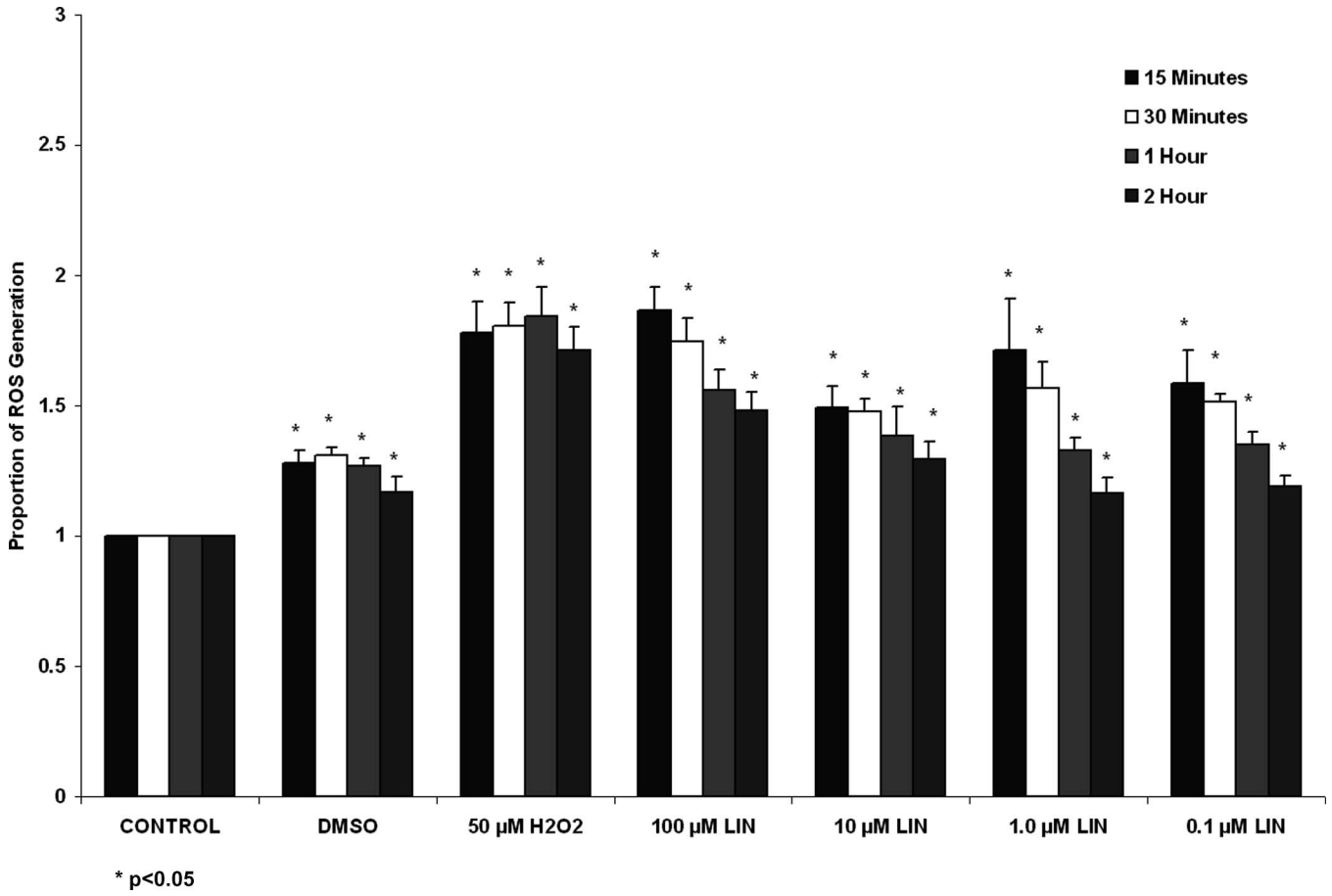

FIG. 2. Generation of reactive oxygen species (ROS) in MDCK cells after treatment with H_2O_2 and lindane. Asterisk indicates significant difference at $p < .05$.

GSH levels over time. The initial lindane-induced depletion of GSH corresponded with the onset of ROS generation noted after a 15-min incubation with lindane**.** Lindane-induced generation of ROS declined over time; similarly, a decrease in lindane-induced total GSH was detected over the same time course (Figure 4). Treatment with 100 µ*M* lindane produced a marked decline in total GSH as compared with lower concentrations of lindane. Depletion of GSH was measured in H_2O_2-treated MDCK cells at 15 min to 2 h after treatment. Total GSH levels in these cells were comparable with those in untreated control cells from 6 h onward. Treatment with the vehicle control (DMSO) produced a quantitative decrease in GSH levels corresponding with an increase in ROS production.

Measurement of Cytotoxicity

No evidence of LDH leakage from MDCK cells was found at any time point after treatment with lindane at both concentrations (10 and 100 µ*M*) (Figure 5). The mean leakage of LDH in lindane-treated cells was similar to LDH leakage observed in untreated control cells, DMSO-treated cells, and cells treated with H_2O_2. LDH leakage is indicative of necrotic injury. Our findings show that necrosis was not detected up to 48 h after treatment with lindane in MDCK cells. This finding correlated with our morphological analysis, which suggested lindane induces apoptosis rather than necrosis.

DISCUSSION

Oxidative damage and GSH depletion play a role in the mechanism of in vitro and in vivo toxicity of lindane in multiple organ systems. Studies showed adverse effects of lindane on the central nervous, cardiovascular, immune, and reproductive systems, as well as the liver and kidneys (Ananya et al., 2005; Agency for Toxic Substances and Disease Registry, 2005; Eldefrawi & Eldefrawi, 1987; Saradha et al., 2008; Videla et al., 1990, 1997, 2000). These experiments used detection of thiobarbituric acid-reactive substances (TBARS) as a biomarker for lipid peroxidation, a process induced by oxidative stress. To determine whether lindane exposure induces ROS generation and oxidative stress in renal distal tubule cells, the production of ROS was measured in live MDCK cells. Although many studies used alternative assays to detect oxidative stress (e.g., lipid peroxidation), our finding of a lindane-induced

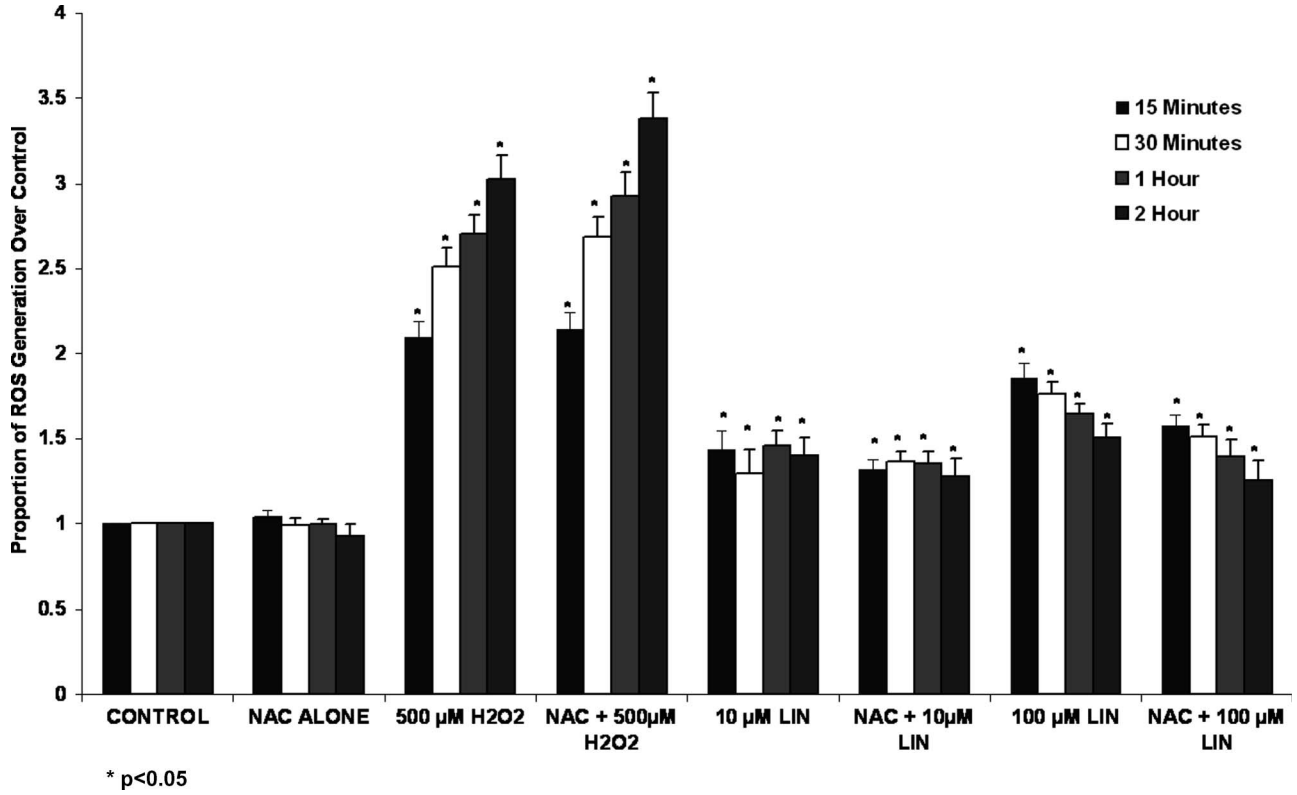

FIG. 3. Attenuation of reactive oxygen species (ROS) generation in MDCK cells after pretreatment with *N*-acetylcysteine (NAC). Asterisk indicates significant difference at $p < .05$.

increase in ROS production in MDCK cells using a fluorescent microassay are consistent with reported results using a rat liver cell model showing a similar rise in oxidative stress using the TBARS assay (Junqueira et al., 1988; Videla et al., 2000). Investigators also noted lindane-induced lipid peroxidation in the rat cerebral cortex and CHO-K1 cells (Sahoo & Chainy, 1998; Banerjee et al., 1999; Sahoo et al., 2000; Garcia-Fernandez et al., 2002; Srivastava & Shivanandappa, 2005).

To further evaluate lindane-induced cellular oxidative stress, MDCK cells were pretreated with NAC to assess the effect of exogenous thiol groups on modulation of oxidative damage. Cao et al. (2000) showed that NAC reduces oxidant-induced ceramide production associated with oxalate exposure in MDCK cells. The exogenous thiol source for GSH enables cells to further quench generation of ROS in addition to endogenous antioxidant induction, or it can directly quench free radicals upon exposure (Cao et al., 2000; Tylicki et al., 2003). For this reason, NAC was removed after pretreatment, and the cells were washed prior to lindane treatment to prevent direct quenching of ROS by NAC in the cell media. In the present study, pretreatment with NAC did not protect MDCK cells treated with H_2O_2 or lindane (10 µ*M*) against oxidative stress. However, pretreatment with NAC did decrease ROS generation in cells treated with lindane (100 µ*M*) at all time points. NAC induced a quantitative attenuation in ROS generation at higher concentrations of lindane, which may be attributed to a threshold of free radical generation in our test system. The lack of ROS generation observed in H_2O_2-treated cells after pretreatment with NAC may be attributed to the concentration of NAC used in our experimental model.

Barros et al. (1988) noted depletion of GSH in hepatocytes 4 h after treatment with lindane in rats, which was consistent with later findings using similar in vivo models (Videla et al., 2000). Studies also reported lindane-induced depletion of GSH and GSH enzymes for in vivo rat brain experiments (Sahoo et al., 2000). MDCK cells and renal proximal tubule cells respond in a similar manner under conditions of oxidative stress, resulting in decreased total GSH levels after exposure to oxalate and calcium oxalate (COM) crystals (Byer & Khan, 2005). Our findings are consistent with these in vitro and in vivo study results. Total cellular GSH levels were measured to determine the protective capacity of GSH against oxidative stress in MDCK cells. Exposure of MDCK cells to lindane modulated the levels of the endogenous antioxidant, GSH, in a concentration-dependent manner. A decline in GSH levels was noted that correlated with a decline in ROS generation over time. This decline in ROS generation may be attributed to the quenching of free radicals by GSH, thereby depleting the cellular stores of this antioxidant in MDCK cells.

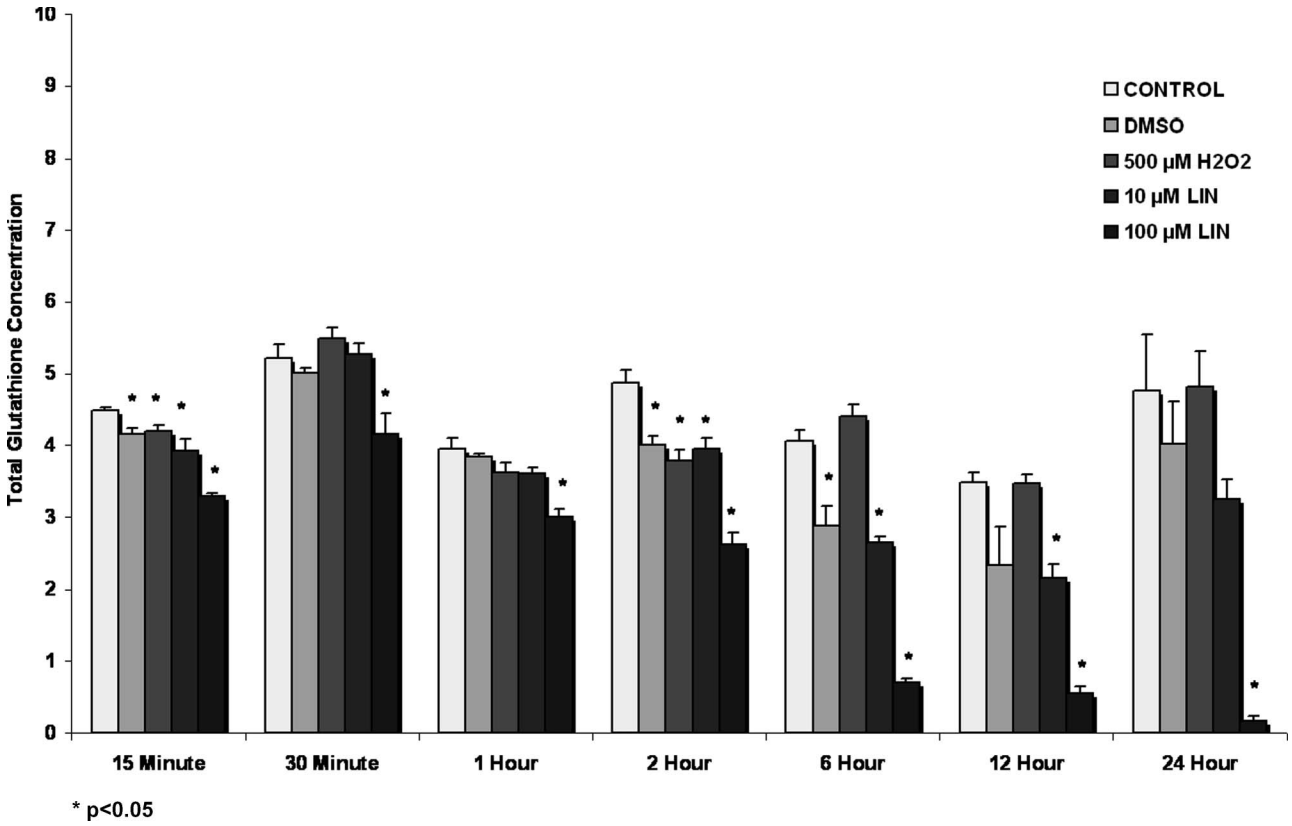

FIG. 4. Total glutathione measured in MDCK cells after treatment with lindane. Asterisk indicates significant difference at $p < .05$.

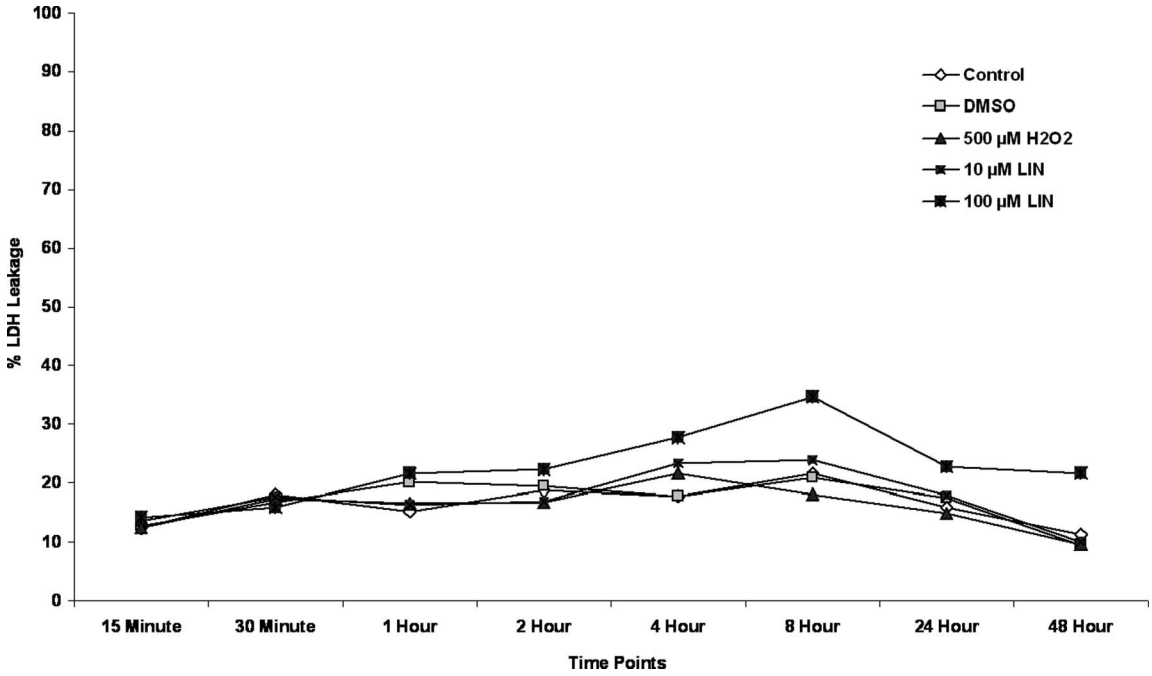

FIG. 5. Percent LDH leakage from MDCK cells after treatment with lindane.

Studies reported lindane-induced LDH leakage in perfused rat livers and CHO-K1 cells (Videla et al., 1997; Garcia-Fernandez et al., 2002). LDH leakage from the cell indicates membrane damage and cytotoxicity characteristic of necrosis. Inhibition of apoptosis and interrupted autophagy was found to result in lindane-induced necrosis in cultured rat hepatocytes autophagic and apoptotic pathway (Zucchini-Pascal, 2009). In our study, lindane exposure did not result in a statistically significant rise in percent MDCK cells with LDH leakage at the concentrations used for these experiments. Our findings are consistent with those of our morphological analysis, indicating an apoptotic rather than necrotic response to treatment with lindane. In addition to observing overt changes in cellular response to lindane treatment, the morphological response was used to determine the time course for measuring oxidative stress and GSH levels in renal cells. Our conflicting findings regarding response to treatment with lindane may be attributed to inherent differences in the study models used, as well as use of lindane at varying concentrations between our experiments and those used by others.

Most studies of renal toxicity focus on markers of proximal tubule injury. Lindane is a known GABA antagonist. One study showed that exposure to the GABA antagonist fenvalerate (in combination with the pesticide DEET) resulted in renal toxicity targeted at the distal nephron (Dorman et al., 1990). Our objective was to evaluate whether toxicity of lindane occurred specifically in the renal distal tubule.

The kidneys function to regulate the volume and composition of body fluids to maintain homeostasis, as well as participating in detoxification and excretory function (Goldstein & Schnellmann, 1996). Given this role, the kidneys are subject to exposure to chemical agents and their metabolites. Data showed that lindane exposure results in oxidative damage and GSH depletion in renal distal tubule cells. Generation of free radicals in MDCK cells did not produce necrosis, but rather morphological changes indicative of apoptosis. Additional investigations identifying the pathway of lindane-induced nephrotoxicity are required for identification of biomarkers of exposure and effect associated with lindane exposure under normal conditions.

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