

## Influence of Compensatory Renal Growth on Susceptibility of Primary Cultures of Renal Cells to Chemically Induced Injury

Lawrence H. Lash,<sup>\*,1</sup> David A. Putt,<sup>\*</sup> and Rudolfs K. Zalups<sup>†</sup>

<sup>\*</sup>Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201; and <sup>†</sup>Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, Georgia 31207

Received June 30, 2006; accepted September 7, 2006

Primary cultures of rat renal proximal tubular (PT) and distal tubular (DT) cells from control and uninephrectomized (NPX) Sprague-Dawley rats were established to study whether the altered toxicological responses identified in freshly isolated cells are maintained in culture. Previous work showed that primary cultures of PT cells from hypertrophied rat kidneys maintained their differentiated properties, as evidenced by their high respiratory rate, active transport function, transport and metabolism of glutathione, and their hypertrophic phenotype. In the present study, primary cultures of PT cells from NPX rat kidneys, but to a much lesser extent DT cells, were more susceptible to cellular injury induced by either mercuric chloride, KCN, or *tert*-butyl hydroperoxide (tBH), than corresponding cells from normal rat kidneys. Direct comparisons of cytotoxicity and lipid peroxidation induced by tBH in freshly isolated renal cells showed that the primary cultures of cells from NPX rat kidneys retained their altered susceptibility relative to cells from control rats. These results show that primary cultures of PT cells from NPX rats are more sensitive to cellular injury induced by three mechanistically distinct toxicants, demonstrating their usefulness in the study of the molecular and biochemical basis for the altered phenotype of compensatory renal growth. This is the first report validating the use of a mammalian renal cell culture model to study the toxicological effects of compensatory renal cellular hypertrophy.

**Key Words:** compensatory renal growth; proximal tubular cells; primary cell culture; susceptibility; mercuric chloride; oxidative stress.

Reductions of functional renal mass can occur in humans as a consequence of renal disease, surgery, or aging. These reductions in renal mass induce compensatory changes in the remaining viable nephrons (especially along the proximal tubule) and are characterized primarily by profound morphological and functional changes, including increases in cell size, plasma membrane surface area, single-nephron glomerular filtration rate, rates of sodium transport, overall protein content,

mitochondrial respiration, and glutathione (GSH) transport and metabolism (Harris *et al.*, 1988; Lash and Zalups, 1994; Lash *et al.*, 2001a,b; Meyer *et al.*, 1996; Nath *et al.*, 1990; Zalups and Lash, 1990). Uninephrectomized (NPX) rats have been commonly used as a model to study compensatory renal growth. The acute hemodynamic, functional, and biochemical effects associated with reduced renal mass and compensatory growth are nearly complete in rats within 7–10 days post-nephrectomy and are maintained for at least 30 days thereafter (Meyer *et al.*, 1996; Shirley and Walter, 1991; Zalups *et al.*, 1987). Although varied changes occur throughout the nephron, the most prominent changes occur in the proximal tubular (PT) region (Meyer *et al.*, 1996; Shirley and Walter, 1991).

Compensatory renal cellular hypertrophy also has toxicological implications. Several studies have shown that NPX rats exhibit altered susceptibility (increased in most cases) to renal cellular dysfunction and injury induced by a variety of nephrotoxicants, including inorganic mercury ( $\text{Hg}^{2+}$ ) (Zalups, 1997, 2000; Zalups and Diamond, 1987; Zalups and Lash, 1994), analgesics (Henry *et al.*, 1983; Molland, 1976), and cadmium-metallothionein (Zalups *et al.*, 1992). Despite the wealth of literature showing associations between toxicity and various cellular processes that are altered by compensatory renal growth, detailed mechanisms explaining altered susceptibility to nephrotoxicants are lacking.

To study the biochemical and toxicological impact of uninephrectomy and compensatory renal growth in the rat kidney, we previously used suspensions of freshly isolated renal PT cells from NPX rats as an *in vitro* model system (Lash and Zalups, 1992, 1994). Suspensions of isolated distal tubular (DT) cells were also used as an alternative renal cell population that is not influenced by compensatory renal growth to the same degree as PT cells. Our previous findings show that PT cells from NPX rats retain their hypertrophied phenotype upon isolation and exhibit (relative to PT cells from control rats) increased cell size, GSH content, rates of GSH transport and metabolism, mitochondrial oxidative phosphorylation, and sensitivity to cytotoxicity induced by  $\text{Hg}^{2+}$  (Lash and Zalups, 1992, 1994). While freshly isolated cells are useful to measure acute cytotoxicity, metabolism, membrane transport, or other

<sup>1</sup> To whom correspondence should be addressed at Department of Pharmacology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201. Fax: (313)-577-6739. E-mail: l.h.lash@wayne.edu.

short-term processes, their usefulness is limited by their relatively short life span in suspension (i.e., up to 4 h) (Lash *et al.*, 1995). Consequently, we sought to develop an *in vitro* model system that would remain viable over an extended period of time during which the phenotypic changes induced by compensatory renal growth would be maintained.

When renal PT and DT cells from control and NPX rats were placed into primary culture using a serum-free, hormonally defined medium (Lash *et al.*, 2001b), they remained viable, grew to confluence in 5–6 days, and they expressed cytokeratins. In addition, only primary cultures of PT cells from NPX rats retained their altered morphology and exhibited increased rates of active sodium transport, GSH transport and metabolism, and mitochondrial function (Lash *et al.*, 2001b). These results support the hypothesis that primary cultures of PT cells could serve as a useful *in vitro* model in which to study processes occurring in response to compensatory growth that occur over longer time periods, relative to those that can be studied in suspensions of freshly isolated cells.

In the present study, our goal was to extend our application of primary cultures of PT and DT cells from NPX rats to investigate their susceptibility to selected, well-characterized toxicants. We hypothesize that PT cells from NPX rats in primary culture are more susceptible than PT cells from control rats in primary culture to a diverse array of cytotoxic and nephrotoxic chemicals. We chose to study the following three toxicants: *tert*-butyl hydroperoxide (tBH), a model oxidant (Lash and Tokarz, 1990), KCN, a mitochondrial toxicant (Lash *et al.*, 1996); and  $\text{Hg}^{2+}$ , a PT cell-selective toxicant *in vivo* (Zalups, 2000). As a first step toward establishing primary cultures of renal PT cells from NPX rats as a model, we studied acute cytotoxic responses so that direct comparisons can be made with suspensions of freshly isolated cells. Our findings demonstrate that primary cultures of renal PT cells from NPX rats develop more severe cellular injury and undergo cellular death at lower concentrations than PT cells from control rats. Having established that primary cultures of PT cells from NPX rats consistently reflect the altered toxicological susceptibility characteristic of the hypertrophied state, the model can be applied to a diverse array of mechanistic studies pertaining to chemically induced toxicity.

## METHODS

**Materials.** Percoll, collagenase (type I), powdered 1:1 mixture of Dulbecco's modified Eagle's medium:Ham's F12 (DMEM:F12), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), bovine serum albumin (fraction V), penicillin G, streptomycin sulfate, amphotericin B, insulin (from bovine pancreas), human transferrin, sodium selenite, hydrocortisone, 3,3',5'-triiodo-L-thyronine, and thyrocalcitonin (from bovine thyroid gland) were purchased from Sigma Chemical Co. (St Louis, MO). Epidermal growth factor was purchased from Upstate Biotechnology (Lake Placid, NY). Polystyrene tissue culture dishes were purchased from BD Falcon (Bedford, MA) or Corning (Corning, NY) and Teflon cell scrapers were purchased from Falcon.  $^{203}\text{HgCl}_2$

(specific activity 8–12 mCi/mg  $\text{Hg}^{2+}$ ) was produced by previously described methods (Aslamkhan *et al.*, 2003). Radioactivity of  $^{203}\text{Hg}^{2+}$  was determined by gamma spectroscopy at a counting efficiency of ~50%. All other chemicals were of the highest purity available and were purchased from commercial sources.

**Animals and surgical procedure.** Male Sprague-Dawley rats (175–200 g; Harlan, Indianapolis, IN) were allowed access to food and water *ad libitum* and were kept in a room on a 12-h light/dark cycle. Animals that underwent surgical nephrectomy (removal of right kidney) were allowed a minimum 10-day recovery period prior to isolation of renal PT and DT cells. For uninephrectomy, each rat was anesthetized with an ip injection of sodium pentobarbital (50 mg/kg body weight) before surgery. Uninephrectomy was performed by removal of the right kidney as described previously (Zalups and Lash, 1990). Control rats were surgically naïve, as previous studies have shown that findings from animals not treated surgically are not significantly different from those obtained from animals treated with a sham nephrectomy (Lash *et al.*, 1999; Zalups, 1995; Zalups and Lash, 1990). All animal procedures were approved by the institutional animal investigation committee and were in accordance with National Institutes of Health guidelines.

**Isolation of Rat Renal PT and DT Cells.** Prior to surgery, all glassware and surgical tools were sterilized in an autoclave, and the abdomen of the rat was shaved and cleansed with 70% (vol/vol) ethanol. After surgical excision of the kidneys, renal cortical cells were obtained by collagenase perfusion (Jones *et al.*, 1979; Lash and Tokarz, 1989). Briefly, kidneys (or the remnant kidney in NPX rats) were (was) perfused first with EGTA-containing,  $\text{Ca}^{2+}$ -free Hanks' buffer at a flow rate of 8 ml/min for 10 min, followed by perfusion with Hanks' buffer containing 0.15% (wt/vol) collagenase (type I) and 2mM  $\text{CaCl}_2$  for 13–18 min at a flow rate of 5 ml/min. All buffers were continuously bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  and maintained at 37°C. The collagenase used was approximately 300 units/mg dry weight, with one unit defined by the supplier (Sigma Chemical Co.) as the amount of enzyme that will release peptides from native collagen and give the equivalent in ninhydrin color of 1.0  $\mu\text{mol}$  of L-leucine in 5 h at pH 7.4 and 37°C in the presence of  $\text{Ca}^{2+}$  ions. At the conclusion of collagenase perfusion, cells were released into Krebs-Henseleit buffer, pH 7.4, supplemented with 2.55mM  $\text{CaCl}_2$ , 10mM HEPES, and 2% (wt/vol) bovine serum albumin. Cell count and cell viability were estimated by mixing 0.1 ml of cells with 0.4 ml of 0.2% (wt/vol) trypan blue in saline and counting the total number of cells and the number of cells that took up the dye on a hemacytometer. The percentage of viable cells was calculated by subtracting the number of cells that took up the dye from the total number of cells, dividing this by the total number of cells, and multiplying by 100%. Typically, 85–95% of the cells from both control and NPX rats excluded the dye. When necessary, cell concentration was adjusted to between  $5 \times 10^6$  and  $8 \times 10^6$  cells/ml by dilution with Krebs-Henseleit buffer.

To obtain enriched fractions of renal PT and DT cells, cortical cells ( $5 \text{ ml}$ ,  $5 \times 10^6$  to  $8 \times 10^6$  cells/ml) were layered on 35 ml of an isosmotic 45% (vol/vol) Percoll solution in 50-ml polycarbonate centrifuge tubes and centrifuged for 30 min at  $20,000 \times g$  (Lash and Tokarz, 1989). The PT (upper layer) and DT (lower layer) cells were estimated to have purities of 97 and 88%, respectively, based on marker enzyme activities and cell type-specific respiratory responses (Lash, 1990; Lash and Tokarz, 1989). Based on enzymology and morphology (Lash, 1990; Lash and Tokarz, 1989), the renal PT cell preparation contains cells derived from both convoluted and straight segments of the proximal tubule; the renal DT cell preparation contains cells derived from the distal convoluted tubule, the cortical collecting duct, and the collecting tubule, but not from the medullary thick ascending limb, and is estimated to have less than 10% contamination from PT cells. Cell count and cell viability were estimated with trypan blue on a hemacytometer as described above. Cell viability (i.e., fraction of cells that excluded trypan blue) of both the PT and DT cells obtained after the Percoll separation from both control and NPX rats were typically 90 to 95%. Cell counts were not adjusted for viability before cells were plated for culture. For cell culture, freshly isolated PT and DT cells were suspended in 2 ml of Krebs-Henseleit buffer and diluted with an appropriate amount of culture medium before plating.

**Cell culture media.** Basal medium was a 1:1 mixture of DMEM:F12. Supplementation for both cell types included 15mM HEPES, pH 7.4, 20mM NaHCO<sub>3</sub>, 5 µg insulin/ml, 5 µg human transferrin/ml, 100 ng hydrocortisone/ml, 100 ng epidermal growth factor/ml, 30nM sodium selenite, and an antibiotic mixture containing 192 IU penicillin G/ml, 200 µg streptomycin sulfate/ml, and 2.5 µg amphotericin B/ml. Other supplements included 7.5 pg triiodothyronine/ml for PT cells and 5 ng thyrocalcitonin/ml for DT cells. Optimization of cell culture media for primary cultures of rat PT and DT cells was described previously (Lash *et al.*, 1995).

**Primary culture.** PT and DT cells were seeded at a density of  $0.2 \times 10^6$  cells/ml in media on 35-mm, polystyrene tissue culture dishes that had been coated with a 0.1 mg/ml collagen solution (Vitrogen 100; Collagen Corp., Palo Alto, CA). In cultures used for transport studies, the cells were plated onto 30-mm, 4-µm pore size Millicell-polycarbonate filter (PCF) culture plate inserts (Millipore, Billerica, MA). Cultures were grown at 37°C in a humidified incubator under an atmosphere of 95% air/5% CO<sub>2</sub>. Fresh media was added to the dishes after 24 h (day 1) and every 48 h thereafter.

**Cytotoxicity studies.** Cytotoxicity of KCN and tBH was assessed by measuring the release of lactate dehydrogenase (LDH) from cells as described previously (Lash, 1989). Briefly, appropriate concentrations of the toxicants were added to 35-mm culture dishes containing either freshly isolated or confluent primary cultures of PT or DT cells, and cells were incubated for various lengths of time at 37°C in either a Dubnoff metabolic shaking incubator (60 cycles/min) (freshly isolated cells) or a humidified incubator under an atmosphere of 95% air/5% CO<sub>2</sub> (cell cultures). For freshly isolated cells, cytotoxicity was determined by removing aliquots of cell suspensions at the appropriate time points, adding them to a cuvette with pyruvate and NADH, and measuring LDH activity by the decrease in A<sub>340</sub>; Triton X-100 was then added, and total LDH activity was measured. For primary cell cultures, the supernatant was removed at appropriate time points and assayed for LDH activity. This was followed by scraping the cells from the dishes and measuring total cellular LDH activity. For tBH and KCN, the fraction of necrotic cells was calculated as either the ratio of LDH activity  $\pm$  Triton X-100 for freshly isolated cells or as the ratio of LDH activity in the supernatant to the sum of activity in the supernatant and cells for primary cultures.

For cells exposed to mercuric chloride, the fraction of necrotic cells was calculated by determining inhibition of total LDH activity (supernatant + cells) because Hg<sup>2+</sup> directly inhibits LDH (Lash and Zalups, 1992). Data were expressed as percent inhibition of LDH activity by dividing total activities for each incubation by those in cells not exposed to mercuric chloride and multiplying by 100%. Results were expressed this way rather than as actual LDH activities because cells from NPX rats invariably exhibited higher LDH activities than corresponding cells from control rats; plotting actual values would obscure differences in percent inhibition of activity. Total activities in untreated cells from control or NPX rats are noted in the legend to Figure 7. We previously showed that inhibition of LDH activity correlates with other measures of cytotoxicity, such as trypan blue exclusion, and can thus be used to assess cell death in lieu of LDH release.

**Lipid peroxidation assay.** Malondialdehyde (MDA) content was measured as an index of lipid peroxidation in suspensions of freshly isolated cells and was quantitated as thiobarbituric acid-reactive material according to Stacey *et al.* (1980). Aliquots of freshly isolated cell suspensions (0.5 ml) were mixed with 0.5 ml of 10% (wt/vol) trichloroacetic acid and 1.0 ml of 0.76% (wt/vol) 2-thiobarbituric acid, and the mixtures were heated in a boiling water bath for 15 min. After cooling to room temperature, insoluble material was removed by centrifugation, and absorbance of supernatants was measured at 532 nm. Acid hydrolysates of 1,1,3,3-tetraethoxypropane were used as MDA standards. Standard curves were generated and were linear (regression line:  $y = 0.00280 + 0.05624x$ ;  $r^2 = 1.000$ ) between 0.2 and 10 nmol MDA per ml.

**Cytokeratin staining and confocal microscopy.** Cytokeratins were monitored as an epithelial cell marker of renal PT cells on day 6 of primary culture. Following fixation with 3.7% (vol/vol) formaldehyde and blocking with 0.2%

(wt/vol) bovine serum albumin, cells were incubated with a monoclonal antipan cytokeratin antibody conjugated to fluorescein isothiocyanate (FITC) in phosphate-buffered saline containing 0.1% (vol/vol) saponin. The stained cells were viewed and photographed with a Zeiss LSM 310 confocal laser scanning microscope.

**Transport studies.** Cellular transport of <sup>203</sup>Hg<sup>2+</sup> was measured in PT and DT cells that had been seeded in 30-mm diameter, 0.4-µm Millicell-PCF culture plate inserts. Medium containing the appropriate concentration of substrate was added to either the apical or basolateral cell surface to study transport across the brush-border membrane (BBM) or basolateral membrane (BLM), respectively. At indicated times, the polycarbonate membrane was removed from the insert, and the amount of radiolabel incorporated in the attached cells was quantitated with a Beckman 3000 gamma counter. Nonspecific binding of Hg<sup>2+</sup> to cells on culture dishes, which generally accounted for < 5% of total counts, was measured by first treating cultures on plates with 0.5 ml of 5% (wt/vol) trichloroacetic acid prior to addition of <sup>203</sup>Hg<sup>2+</sup>. Because a large portion of total cellular-associated Hg<sup>2+</sup> is due to binding to cellular plasma membranes (Lash *et al.*, 2005), measurements of cellular contents of Hg<sup>2+</sup> represent both binding and transport.

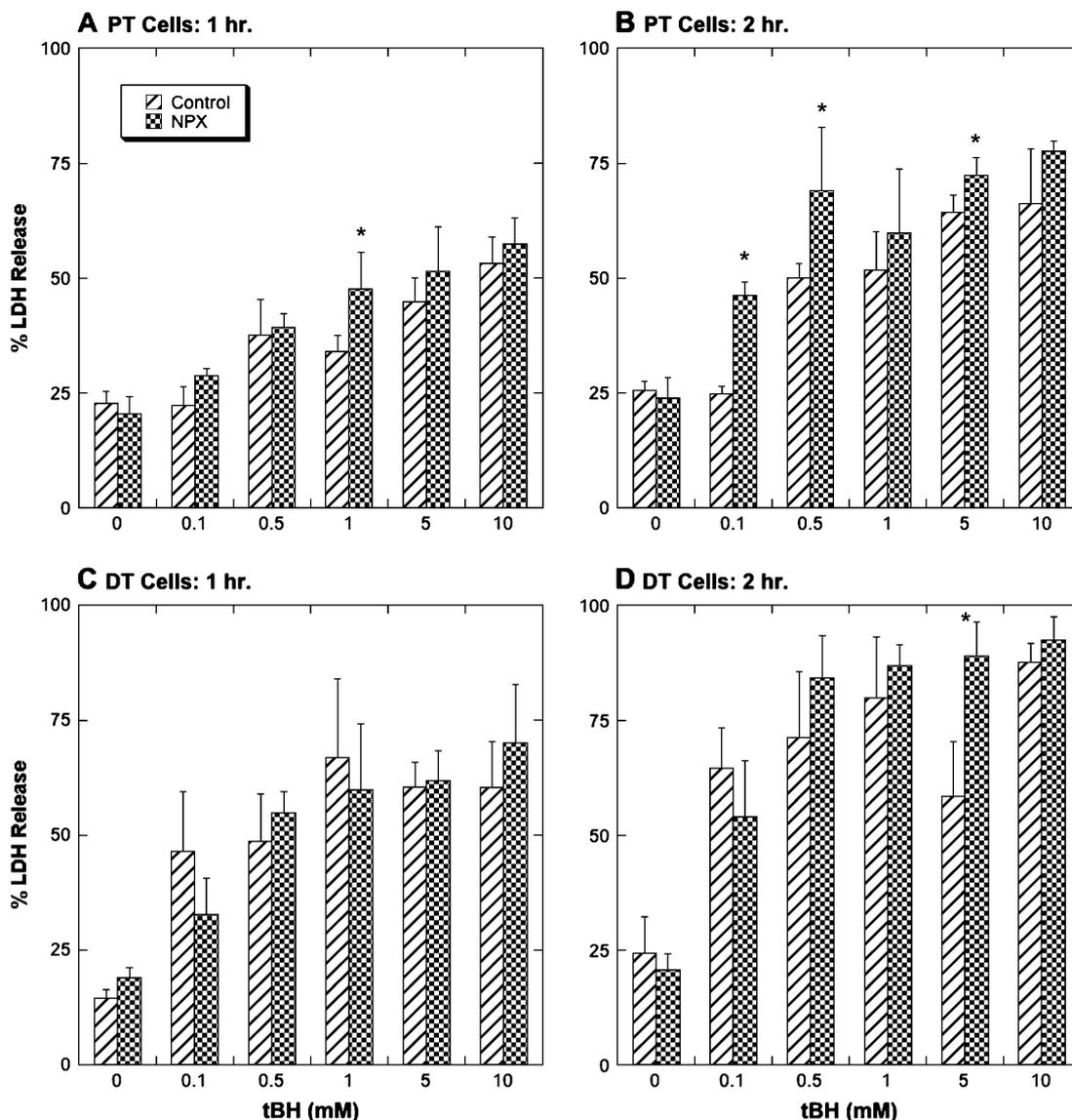
**Data analysis.** Results are expressed as means  $\pm$  SE of measurements from the indicated number of separate cell preparations. Significant differences among selected mean values were first assessed by a one- or two-way ANOVA. When significant *F* values were obtained with ANOVA, the Fisher's protected least significant difference *t* test was performed to determine which mean values were significantly different from one other with two-tailed *p* values < 0.05 considered significant.

## RESULTS

### *Cytotoxicity of tBH in Freshly Isolated Renal Cells*

Two initial experiments were performed in suspensions of freshly isolated PT and DT cells so that comparisons of cytotoxicity could be made directly in cell cultures. In the first experiment, PT and DT cells from control and NPX rats were incubated with three orders of magnitude range of concentrations of tBH for 1 or 2 h, and acute cellular necrosis was assessed by measurement of LDH release (Fig. 1). At the 1-h incubation time, only PT cells from NPX rats exposed to 1mM tBH exhibited significantly greater LDH release than control cells. In the 2-h incubations, however, PT cells from NPX rats exhibited significantly greater LDH release than PT cells from control rats when exposed to several concentrations of tBH. In contrast, there were no significant differences between LDH release elicited by tBH in DT cells from either NPX or control rats, with the exception of a single tBH concentration assessed at 2 h.

While there were significant differences in tBH-induced LDH release in PT cells from control and NPX rats, these were relatively modest in comparison with the differences in tBH-induced MDA formation (Fig. 2). Three key observations with regard to lipid peroxidation were made. First, even without incubating with tBH, both PT and DT cells from NPX rats exhibited markedly higher levels of MDA (4.5- and 2.2-fold higher, respectively) than the corresponding cells from control rats. This indicates that the basal level of oxidative stress is significantly higher in both PT and DT cells from NPX rats.



**FIG. 1.** Cytotoxicity of tBH in freshly isolated renal PT and DT cells from control and NPX rats. Suspensions of freshly isolated renal PT (A, B) and DT (C, D) cells from control and NPX rats ( $1 \times 10^6$  cells/ml) were incubated with the indicated concentrations of tBH for 1 or 2 h. At indicated times, aliquots were removed for determination of LDH release. Results are means  $\pm$  SE of measurements from four separate cell preparations. \*Significantly different ( $p < 0.05$ ) from the value in the corresponding control cell.

Second, the marked increases in MDA levels induced by tBH in both cell populations followed a similar pattern with increasing concentrations of tBH. Third, tBH-induced MDA formation was significantly higher in DT cells from NPX rats as compared with DT cells from control rats despite there being no difference in necrosis. Cellular contents of MDA were normalized to cell number because the cell is the basic functional unit of the model. However, protein content per cell increases as a consequence of compensatory hypertrophy and is a hallmark of the response in renal PT cells (Harris *et al.*, 1988; Lash and Zalups, 1992, 1994; Lash *et al.*, 2001b; Zalups and Lash, 1990). Even taking protein concentration ( $\text{mg}/10^6$  cells;  $n = 6$ )

into account (PT cells—control:  $0.36 \pm 0.03$ , NPX:  $0.56 \pm 0.04$ ; DT cells—control:  $0.56 \pm 0.06$ , NPX:  $0.75 \pm 0.08$ ), MDA content per  $10^6$  cells was still elevated, further supporting the conclusion that cells from NPX rats possess a higher state of oxidative stress.

#### *Cytokeratin Staining and Cellular Morphology of Primary Cell Cultures of PT Cells*

Figure 3 demonstrates the morphology of confluent, primary cultures of PT cells from control and NPX rats, as shown by confocal microscopy and immunofluorescent staining for

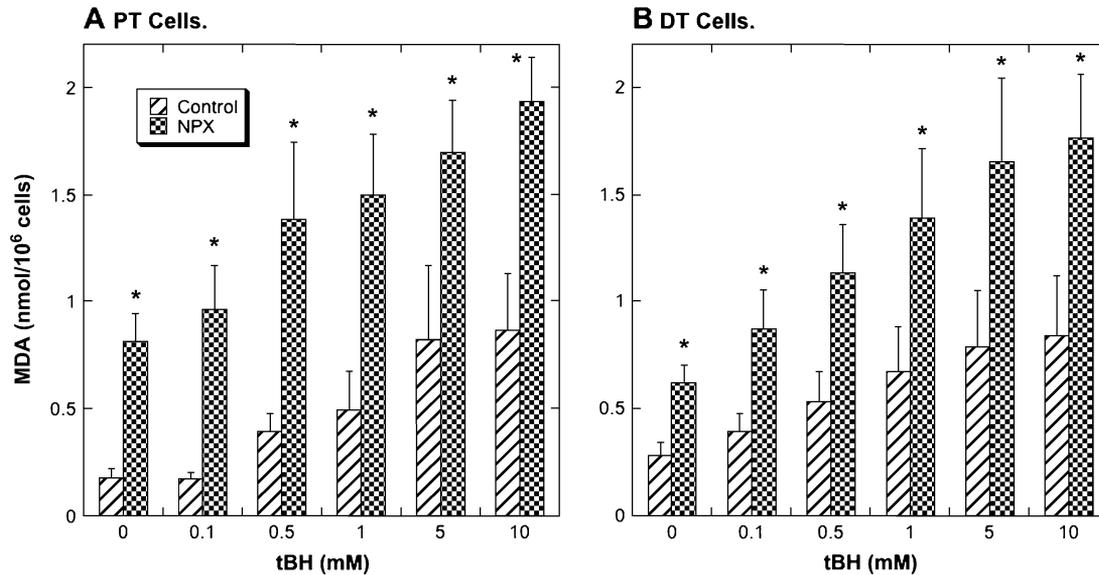


FIG. 2. tBH-induced lipid peroxidation in freshly isolated renal PT and DT cells from control and NPX rats. Suspensions of freshly isolated renal PT (A) and DT (B) cells from control and NPX rats ( $1 \times 10^6$  cells/ml) were incubated with the indicated concentrations of tBH for 2 h. Aliquots were removed for determination of MDA content. Results are means  $\pm$  SE of measurements from four separate cell preparations. \*Significantly different ( $p < 0.05$ ) from the value in the corresponding control cell.

cytokeratins, which is a marker for epithelial cells. PT cells from both control and NPX rats stained intensely for cytokeratins, and the larger size of the majority of PT cells from NPX rats relative to that of the PT cells from control rats was evident. These findings demonstrate that even after 6 days in culture, PT cells from NPX rats retain epithelial morphology and the hypertrophied phenotype that results from a reduction of renal mass and the accompanying compensatory renal growth.

#### Cytotoxicity of tBH and KCN in Primary Cultures of PT and DT Cells

Identical incubations with tBH as were done with freshly isolated cells were conducted in confluent primary cultures of PT and DT cells from both control and NPX rats (Fig. 4). While PT cells from NPX rats that were incubated for 1 h with tBH only exhibited significantly increased LDH release at the highest toxicant concentration, almost all the concentrations of tBH elicited significantly higher LDH release in PT cells from NPX rats incubated for 2 h. Furthermore, PT cells from NPX rats incubated with  $\geq 1$  mM tBH were completely dead (i.e., 100% LDH release), whereas PT cells from control rats exhibited between 64 and 77% LDH release. In contrast to these results, and similar to findings in freshly isolated cells, there was no consistent pattern of significantly elevated LDH release in cultured DT cells from NPX rats as compared to that from cultured DT cells from control rats.

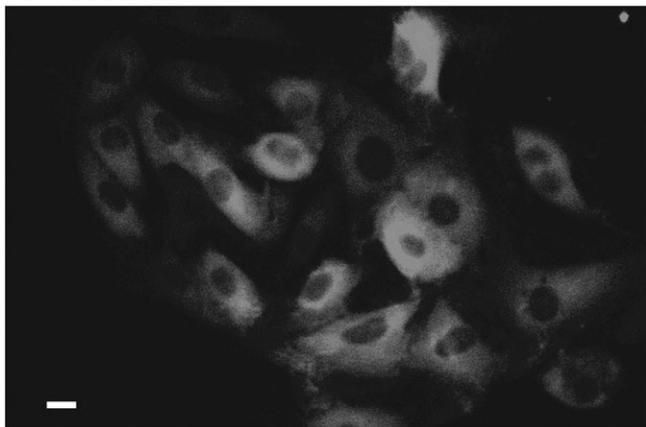
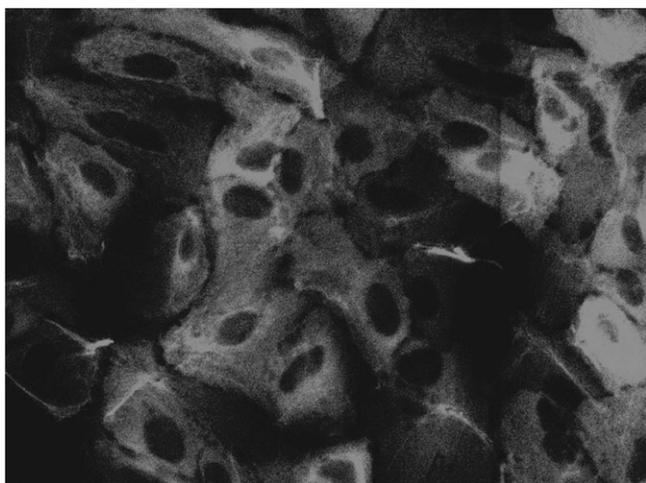
KCN is a potent mitochondrial inhibitor that causes greater cytotoxicity in DT cells than in PT cells (Lash *et al.*, 1996). Whereas freshly isolated DT cells exhibit increasing LDH release with increasing concentrations of KCN, little significant

cytotoxicity is evident in PT cells exposed to no more than 1 mM KCN. The primary cell cultures from both control and NPX rats also showed the same sensitivity pattern (Fig. 5). Although PT cells from control rats exhibited little LDH release when incubated for 1 or 2 h with up to 1 mM KCN, PT cells from NPX rats exhibited marked increases in LDH release that were significantly higher than those in PT cells from control rats after 2-h incubations with 0.5 and 1 mM KCN. As with tBH, primary cultures of DT cells from NPX rats exhibited no consistent pattern of differences in LDH release as compared to DT cells from control rats.

#### Transport and Cytotoxicity of Mercuric Chloride in Primary Cultures of PT and DT Cells

Important components of the mechanism of  $Hg^{2+}$ -induced nephrotoxicity and *in vitro* cytotoxicity in renal PT cells are transport into the cell and binding to plasma membranes, with total renal cellular contents of  $Hg^{2+}$  correlating with the extent of injury (reviewed in Lash *et al.*, 1998). Moreover, previous work of ours in isolated BLM vesicles from kidneys of control and NPX rats (Lash *et al.*, 2005) showed that the specific activity of uptake and binding for  $Hg^{2+}$ , both as the chloride salt and as a mercuric-thiol conjugate, was markedly increased after compensatory hypertrophy. Accordingly, we investigated whether the differences observed in transport and binding of  $Hg^{2+}$  that occurs after compensatory hypertrophy, as observed in freshly isolated renal PT cells and plasma membrane vesicles, can also be detected in primary cell cultures.

Using primary cell cultures grown on filter inserts, so that functional polarity was maintained and substrate had direct

**A Control Cells.****B NPX Cells.**

**FIG. 3.** Immunofluorescent staining for cyokeratins in primary cultures of renal PT cells from control and NPX rats. Freshly isolated PT cells from control (A) or NPX (B) rats were seeded at a density of  $0.5 \times 10^6$  to  $1.0 \times 10^6$  cells/ml and allowed to grow for 6 days. Cells were then washed twice with sterile phosphate-buffered saline, and cyokeratin expression was visualized using a monoclonal FITC-conjugated anti-mouse cyokeratin antibody. Note the enlarged size and modestly greater intensity of cyokeratin staining of the PT cells from the NPX rats relative to those of the PT cells from the control rats. Although growth appears to be greater in the PT cells from NPX rats, this does not reflect differences in growth rate. Fields on the confocal microscope were chosen to best illustrate the size difference in cells. Photomicrographs were taken at  $\times 100$  magnification on a Zeiss confocal laser microscope. Bar = 5  $\mu\text{m}$ .

access to either the BLM or BBM, both PT and DT cells from NPX rats exhibited significantly higher uptake of  $0.1\mu\text{M}$  or  $1\mu\text{M}$   $\text{Hg}^{2+}$  than the corresponding cells from control rats (Fig. 6). Even after taking into account the increase in cellular protein concentrations, PT cells from NPX rats still exhibited increased cellular accumulation of  $\text{Hg}^{2+}$  as compared to PT cells from control rats. After 60 min of incubation with either  $0.1$  or  $1\mu\text{M}$   $\text{HgCl}_2$ , cellular accumulation of  $\text{Hg}^{2+}$  was two- to fourfold greater in cells from NPX rats, was generally similar in PT and DT cells from a given surgical group, and was increased more when cells were exposed to  $\text{Hg}^{2+}$  at the apical

surface than at the basolateral surface. In contrast to these clear results, no consistent differences between  $\text{Hg}^{2+}$  uptake and cellular accumulation were observed in either cell type from either control or NPX rats, when cells were incubated with  $5\mu\text{M}$   $\text{Hg}^{2+}$  at the BBM or BLM. A likely explanation is that at this higher concentration of  $\text{Hg}^{2+}$ , cells from NPX rats experience greater cytotoxicity than cells from control rats, which would decrease recovery of accumulated  $\text{Hg}^{2+}$  due to cell lysis.

Assessment of  $\text{Hg}^{2+}$ -induced cytotoxicity in primary cultures of PT and DT cells from control and NPX rat kidneys (Fig. 7) showed that both cell types from NPX rat kidneys exhibited greater amounts of inhibition of LDH activity as compared to that in the corresponding cells from control rat kidneys. With the exception of the 1-h time point, the increased toxicity in PT cells from NPX rat kidneys as compared to PT cells from control rat kidneys was greater than that between DT cells from NPX rat kidneys and DT cells from control rat kidneys. For example, in cells incubated for 2 h with  $10\mu\text{M}$   $\text{Hg}^{2+}$ , the percent control LDH activity in PT cells from control and NPX rats was 83.3 and 47.1%, respectively, whereas that in DT cells from control and NPX rats was 86.5 and 61.0%, respectively. Similar differences in percent control LDH activity were observed at the 4-h incubation time with  $10\mu\text{M}$   $\text{Hg}^{2+}$ . In cells incubated with  $50\mu\text{M}$   $\text{Hg}^{2+}$ , differences were even greater, again with the increased toxicity in PT cells from NPX rats relative to PT cells from control rats being much greater than the differences in toxicity between DT cells from NPX rats and DT cells from control rats.

## DISCUSSION

Results from the present work show that primary cultures of PT cells from NPX rats maintain a hypertrophied phenotype induced by compensatory renal growth. Besides maintaining enhanced cell size and increased activities of GSH-dependent and mitochondrial enzymes (Lash *et al.*, 2001b), primary cultures of PT cells from NPX rats exhibited increased oxidative stress and acute cellular necrosis induced by three mechanistically distinct toxicants relative to cultured PT cells from control rats. Although DT cells from NPX rats also exhibited some changes in susceptibility to toxicants relative to DT cells from control rats, the manifested changes in this mixed population of cells from the distal nephron were much more modest than those between the two populations of PT cells. This confirms that PT cells are the major population of tubular epithelial cells that exhibit the pronounced morphological and biochemical changes induced by uninephrectomy and compensatory renal growth.

Some of the experiments in the present study were first conducted in suspensions of freshly isolated PT and DT cells with the well-characterized oxidant tBH (cf, Figs. 1 and 2). Previous studies of ours (Lash, 1990; Lash and Tokarz, 1990; Lash *et al.*, 1995) have shown that DT cells are more sensitive

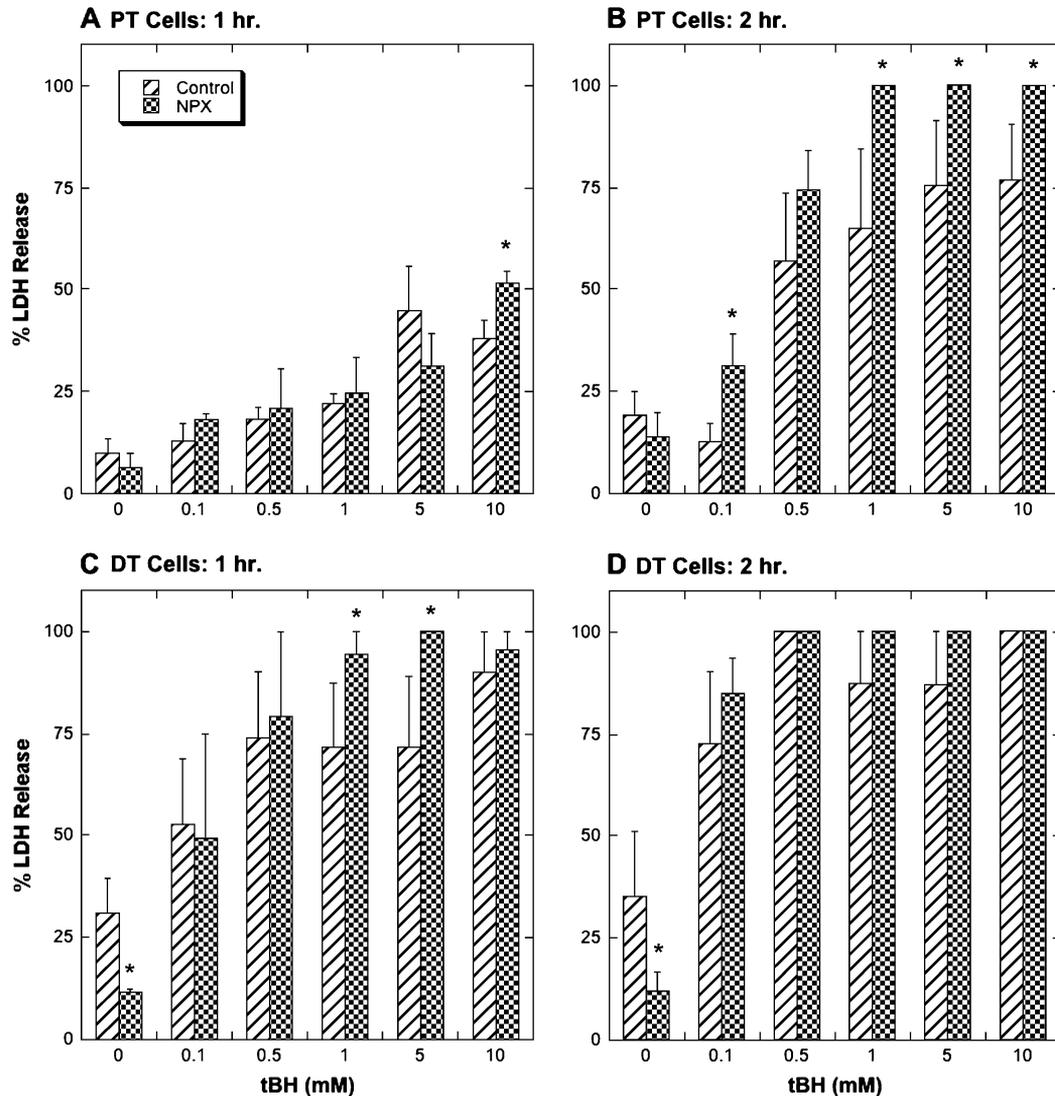
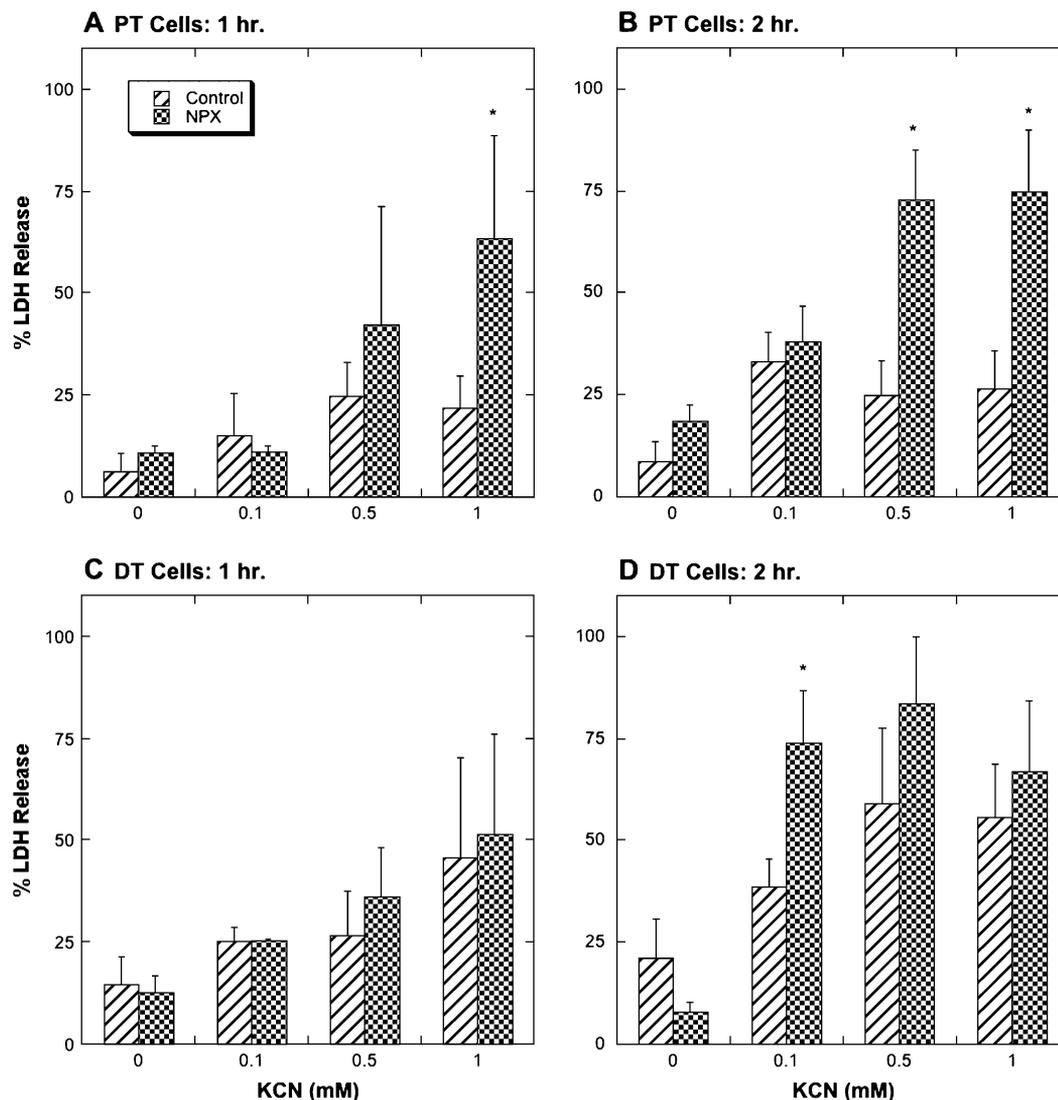


FIG. 4. Cytotoxicity of tBH in primary cultures of renal PT and DT cells from control and NPX rats. Primary cultures of renal PT (A, B) and DT (C, D) cells from control and NPX rats were incubated with the indicated concentrations of tBH for 1 or 2 h. At indicated times, LDH release was determined. Results are means  $\pm$  SE of measurements from four separate cell cultures. \*Significantly different ( $p < 0.05$ ) from the value in the corresponding control cell.

than PT cells to the toxicological effects of tBH. We confirmed that the cytotoxic effects of tBH were greater in DT cells from control rats than in PT cells from control rats. MDA formation was also measured in the present study as an index of lipid peroxidation and oxidative stress. The most dramatic finding was that the basal level of MDA (i.e., without stimulation by toxicant exposure) was markedly greater (4.5- and 2.2-fold in PT and DT cells, respectively) in cells derived from NPX rat kidneys relative to that in corresponding cells from control rat kidneys. This is consistent with previous findings showing that compensatory renal growth induces a hypermetabolic state in the remnant kidney, primarily in the proximal tubule (Harris *et al.*, 1988; Lash *et al.*, 2001a; Nath *et al.*, 1990). The other interesting finding is that tBH-induced MDA formation was markedly increased to similar extents in both PT and DT cells

from NPX rats, despite the fact that differences in LDH release were primarily observed only in PT cells. This suggests that factors other than oxidative stress contribute to the altered susceptibility of PT cells from NPX rat kidneys to tBH-induced cell death.

Primary cell cultures of PT and DT cells from control and NPX rat kidneys were exposed to tBH under conditions identical to those in the suspensions of freshly isolated cells (cf, Fig. 4). The findings from the cultured cells confirm that DT cells from control rats are more sensitive than corresponding PT cells to the cytotoxic effects of tBH. In primary cultures from NPX rats, however, the cytotoxic effects of tBH were greater in PT cells, although little changes in the cytotoxic effects of tBH were detected between the two populations of DT cells. In the final analysis, the sensitivity of primary cultures of PT and DT



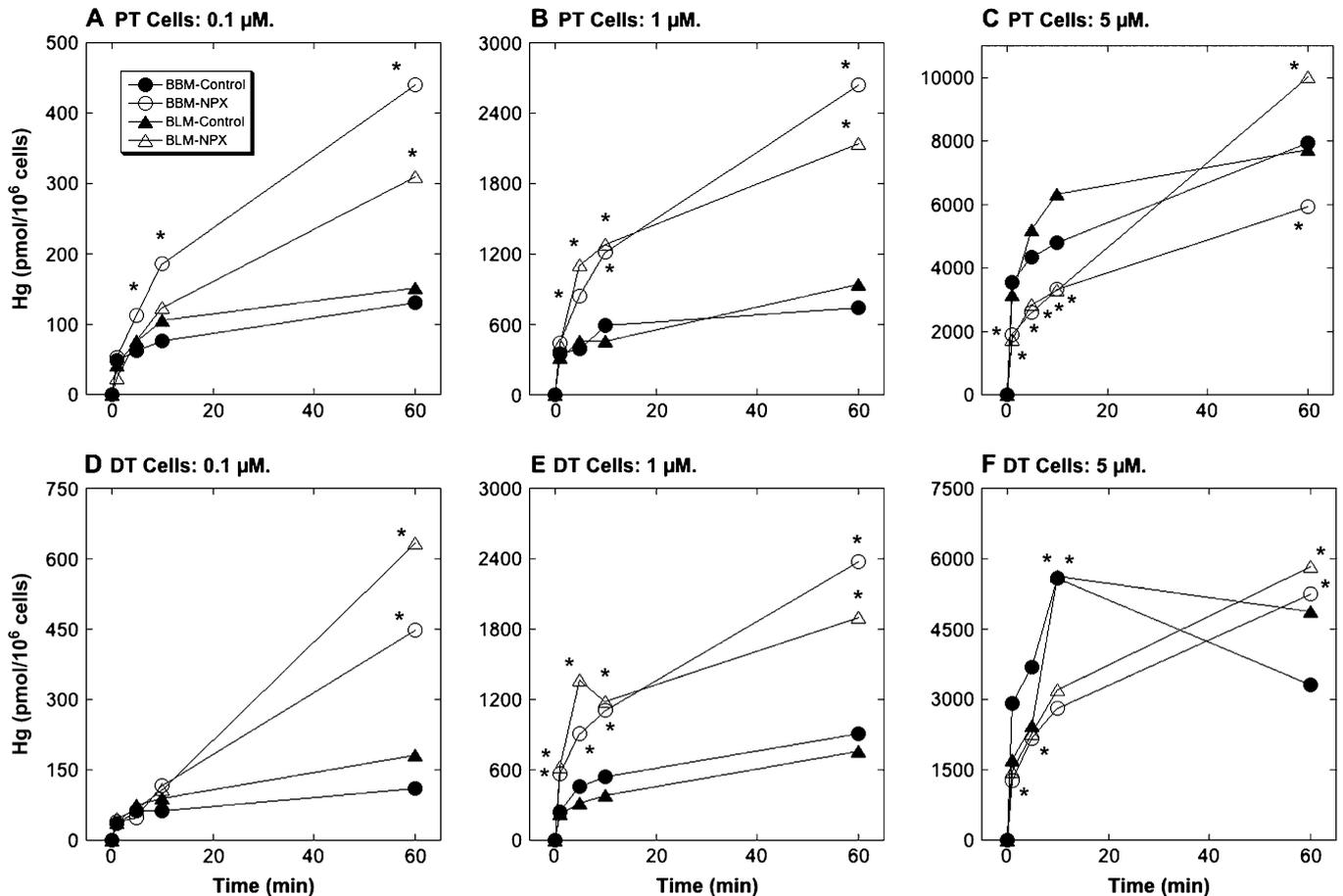
**FIG. 5.** Cytotoxicity of KCN in primary cultures of renal PT and DT cells from control and NPX rats. Primary cultures of renal PT (A, B) and DT (C, D) cells from control and NPX rats were incubated with the indicated concentrations of KCN for 1 or 2 h. At indicated times, LDH release was determined. Results are means  $\pm$  SE of measurements from four separate cell preparations. \*Significantly different ( $p < 0.05$ ) from the value in the corresponding control cell.

cells from NPX rats prove to be similar. PT cells from NPX rats exhibited significantly higher levels of LDH release than those from control rats, especially after 2 h of incubation with most concentrations of tBH tested. In the primary cultures, as in the freshly isolated cells, there were no consistent, significant differences in cytotoxicity between DT cells derived from NPX rats and DT cells derived from control rats.

We extended our studies with the mitochondrial toxicant, KCN; which has been shown to be more toxic in DT cells than in PT cells (Lash *et al.*, 1996). Indeed, in primary cell cultures, PT cells from control rats incubated with up to 1mM KCN for 1 or 2 h exhibited very modest increases in LDH release (maximal percent LDH release  $\sim$ 27%). In primary cultures of PT cells isolated from NPX rats, however, percent LDH release increased to  $\sim$ 75% by the end of a 2-h incubation with either

0.5mM or 1mM KCN. In contrast, no consistent differences in the cytotoxic effects of KCN were detected between primary cultures of DT cells from NPX rats and those from control rats. This is consistent with the findings of previous studies (Lash *et al.*, 2001b), which demonstrate that mitochondrial enzymes increase as a consequence of compensatory renal growth primarily in PT cells but not in DT cells.

As a final assessment of altered susceptibility to toxicants in primary cultures derived from NPX rats, we determined the effects of uninephrectomy and compensatory renal growth on the cellular accumulation and acute cytotoxic effects of  $\text{Hg}^{2+}$ . Although  $\text{Hg}^{2+}$  is selectively toxic in proximal tubules *in vivo* (Zalups, 1997; Zalups and Diamond, 1987; Zalups and Lash, 1990, 1994), we also studied its transport and acute cytotoxicity in primary cultures of DT cells. Previous studies showed

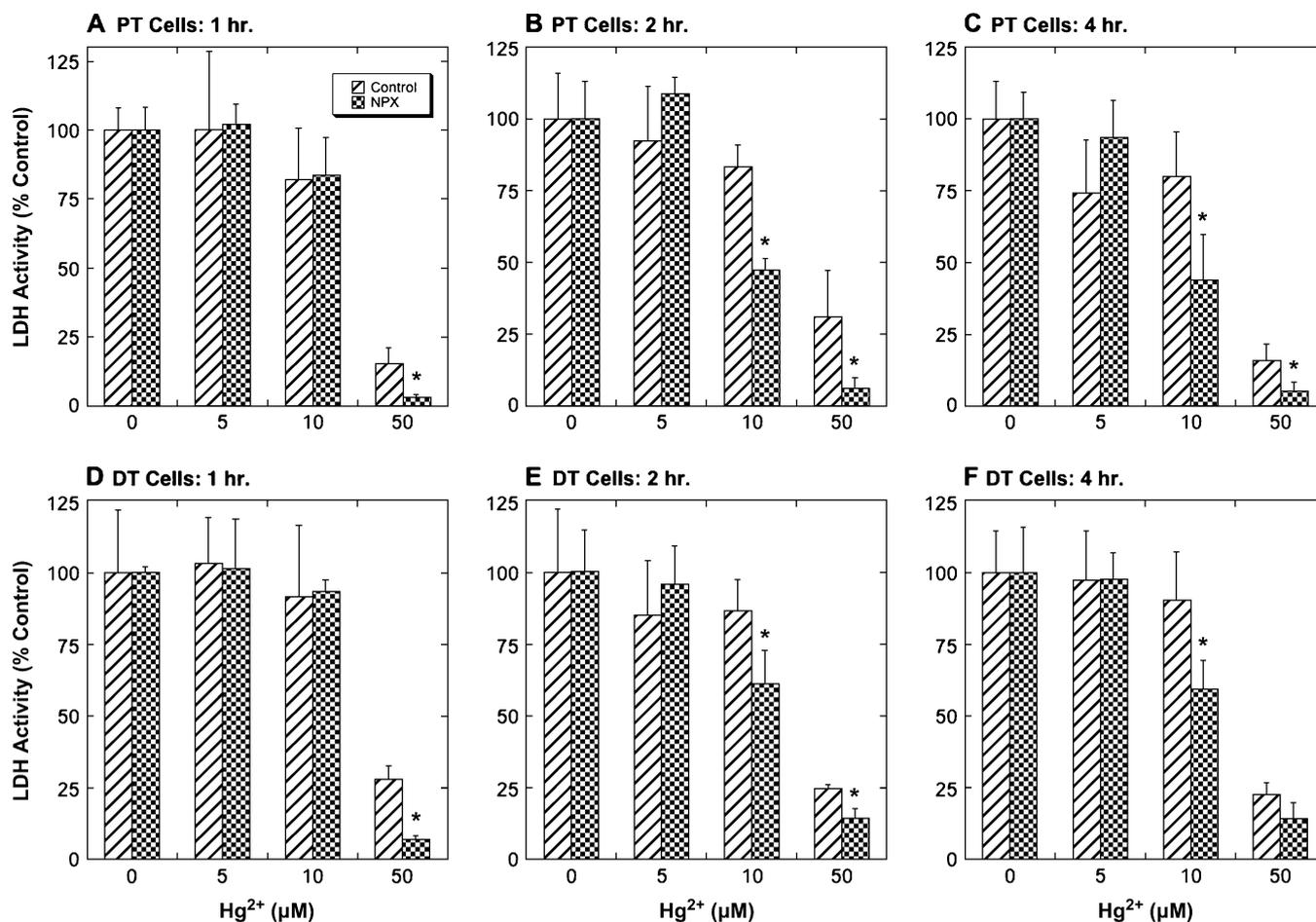


**FIG. 6.** Brush border and basolateral accumulation of mercuric chloride in primary cultures of renal PT and DT cells from control and NPX rats. Net cellular accumulation of  $\text{Hg}^{2+}$  was measured in primary cultures of PT (A, B, and C) and DT (D, E, and F) cells grown on Millicell filter inserts. Cells were incubated for up to 60 min with 0.1, 1, or  $5\mu\text{M}$   $\text{HgCl}_2$ , containing  $^{203}\text{Hg}^{2+}$ , added to either the lower compartment (BLM uptake) or upper compartment (BBM uptake). At the indicated times, filters were washed, and incorporation of radiolabel was determined by gamma counting. Results are means of measurements from four separate cell cultures. SE values, which were omitted for clarity, were between 5 and 25% of the mean values. \*Significantly different ( $p < 0.05$ ) from the corresponding sample from NPX rats.

that this cell population is highly sensitive to the cytotoxic effects of  $\text{Hg}^{2+}$  *in vitro* (Lash and Zalups, 1992; Lash *et al.*, 1999). Although the proximal tubule is the primary region of the nephron, affected most profoundly by compensatory renal growth, net cellular uptake and accumulation of  $\text{Hg}^{2+}$  from either the BBM or BLM were markedly and equally increased in primary cultures of both PT and DT cells from NPX rats (relative to those in corresponding types of cells from control rats) (cf, Fig. 6). These effects were observed only at the two lower concentrations of  $\text{Hg}^{2+}$  tested (i.e., 0.1 and  $1\mu\text{M}$ ), whereas at  $5\mu\text{M}$   $\text{Hg}^{2+}$ , no differences in accumulation were observed. In terms of the cytotoxic effects, however, increases in cellular death, as assessed by inhibition of LDH activity, were greater in PT cells than in DT cells.

Mechanisms by which compensatory renal growth enhances susceptibility of PT cells to the cytotoxic effects of  $\text{Hg}^{2+}$  likely include changes in the intracellular metabolism of thiols (Houser and Berndt, 1986, 1988; Lash and Zalups, 1994;

Zalups and Lash, 1990). The role of thiols, in particular GSH, in the renal disposition and nephropathy induced by  $\text{Hg}^{2+}$  is somewhat paradoxical. Under some circumstances, increases in GSH are associated with protection from the cytotoxic effects of  $\text{Hg}^{2+}$ , whereas under other circumstances a direct correlation is observed between sensitivity to  $\text{Hg}^{2+}$ -induced cellular injury and GSH levels. As an example of the first circumstance, addition of exogenous GSH to suspensions of renal PT cells protects from the cytotoxic effects of  $\text{Hg}^{2+}$  (Lash *et al.*, 1999), which is as one might expect from a  $\text{Hg}^{2+}$ -binding agent. Similarly, mercuric conjugates of GSH are transported into PT cells more slowly than  $\text{HgCl}_2$ , thereby leading to lower levels of intracellular accumulation of  $\text{Hg}^{2+}$  and decreased cytotoxicity (Lash *et al.*, 1998). In contrast to these results, both *in vivo* and *in vitro* studies of the influence of uninephrectomy and compensatory renal growth on renal GSH status and susceptibility to renal cellular injury induced by  $\text{Hg}^{2+}$  demonstrate an association between increased renal GSH



**FIG. 7.** Cytotoxicity of mercuric chloride in primary cultures of renal PT and DT cells from control and NPX rats. Primary cultures of renal PT (A, B, and C) and DT (D, E, and F) cells from control and NPX rats were incubated with the indicated concentrations of HgCl<sub>2</sub> for 1, 2, or 4 h. At indicated times, total LDH activity was determined. Results are expressed as percent control LDH activity and are means  $\pm$  SE of measurements from four separate cell preparations. 100% control activities (mU/10<sup>6</sup> cells; mean  $\pm$  SE; control/NPX) were as follows: PT cells/1 h—123  $\pm$  10/169  $\pm$  14, PT cells/2 h—132  $\pm$  21/159  $\pm$  21, PT cells/4 h—120  $\pm$  16/195  $\pm$  18; DT cells/1 h—133  $\pm$  29/155  $\pm$  3, DT cells/2 h—126  $\pm$  28/166  $\pm$  25, DT cells/4 h—110  $\pm$  16/156  $\pm$  25. \*Significantly different ( $p < 0.05$ ) from the value in the corresponding control cell.

contents and increased susceptibility to the nephropathy or renal cellular injury induced by Hg<sup>2+</sup> (Lash and Zalups, 1992; Lash *et al.*, 2001b; Zalups and Diamond, 1987; Zalups and Lash, 1990; Zalups *et al.*, 1987). Along these same lines, activities of GSH synthesis and other GSH-dependent enzymes are also increased in primary cultures of renal PT cells from NPX rats relative to PT cells from control rats (Lash *et al.*, 2001b), suggesting that the increased cytotoxic effects induced by Hg<sup>2+</sup> are also associated with increased intracellular contents of GSH.

In conclusion, findings from the present study are the first to characterize the use of primary cell culture as an experimental model to study the toxicology of compensatory renal growth. Advantages of the use of primary cell culture to study the toxicological implications of compensatory renal growth include the abilities to precisely define and control incubation conditions and to manipulate cellular biochemistry to charac-

terize mechanisms of action. As in the *in vivo* state, when PT cells from NPX rats are placed in primary culture under serum-free, hormonally-defined conditions, they retain their altered phenotype and enhanced sensitivity to several chemical toxicants. Maintenance of the hypertrophied phenotype in primary culture also suggests that the hypertrophic response is not determined by humoral factors alone but that genetic or other factors are involved. Having now established that the primary cell culture model exhibits the same enhanced sensitivity to toxicants as freshly isolated cells, future studies can use the model to explore underlying mechanisms that determine the sensitivity.

#### ACKNOWLEDGMENTS

This work was funded by National Institute of Environmental Health Sciences (NIEHS) grants R01-ES05157, ES05980, and ES11288 (to R.K.Z.).

National Institute of Diabetes and Digestive and Kidney Diseases grant R01-DK40725 (to L.H.L.), and the NIEHS Center for Molecular Toxicology with Human Applications (grant P30-ES06639) at Wayne State University.

## REFERENCES

- Aslamkhan, A. G., Han, Y.-H., Yang, X.-P., Zalups, R. K., and Pritchard, J. B. (2003). Human renal organic anion transporter 1-dependent uptake and toxicity of mercuric-thiol conjugates in Madin-Darby canine kidney cells. *Mol. Pharmacol.* **63**, 590–596.
- Harris, D. C. H., Chan, L., and Schrier, R. W. (1988). Remnant kidney hypermetabolism and progression of chronic renal failure. *Am. J. Physiol.* **254**, F267–F276.
- Henry, M. A., Sweet, R. S., and Tange, J. D. (1983). A new reproducible experimental model of analgesic nephropathy. *J. Pathol.* **139**, 23–32.
- Houser, M. T., and Berndt, W. O. (1986). The effect of unilateral nephrectomy on the nephrotoxicity of mercuric chloride in the rat. *Toxicol. Appl. Pharmacol.* **83**, 506–515.
- Houser, M. T., and Berndt, W. O. (1988). Unilateral nephrectomy in the rat: Effects on mercury handling and renal cortical subcellular distribution. *Toxicol. Appl. Pharmacol.* **93**, 187–194.
- Jones, D. P., Sundby, G.-B., Ormstad, K., and Orrenius, S. (1979). Use of isolated kidney cells for study of drug metabolism. *Biochem. Pharmacol.* **28**, 929–935.
- Lash, L. H. (1989). Isolated kidney cells in the study of chemical toxicity. In *In Vitro Toxicology: Model Systems and Methods* (C. A. McQueen, Eds.), pp. 231–262. The Telford Press, Caldwell, NJ.
- Lash, L. H. (1990). Susceptibility to toxic injury in different nephron cell populations. *Toxicol. Lett.* **53**, 97–104.
- Lash, L. H., Hueni, S. E., Putt, D. A., and Zalups, R. K. (2005). Role of organic anion and amino acid carriers in transport of inorganic mercury in rat renal basolateral membrane vesicles: Influence of compensatory renal growth. *Toxicol. Sci.* **88**, 630–644.
- Lash, L. H., Putt, D. A., Horky, S. J., III, and Zalups, R. K. (2001a). Functional and toxicological characteristics of isolated renal mitochondria: Impact of compensatory renal growth. *Biochem. Pharmacol.* **62**, 383–395.
- Lash, L. H., Putt, D. A., and Zalups, R. K. (1998). Role of extracellular thiols in uptake and distribution of inorganic mercury in rat renal proximal and distal tubular cells. *J. Pharmacol. Exp. Ther.* **285**, 1039–1050.
- Lash, L. H., Putt, D. A., and Zalups, R. K. (1999). Influence of exogenous thiols on mercury-induced cellular injury in isolated renal proximal tubular and distal tubular cells from normal and uninephrectomized rats. *J. Pharmacol. Exp. Ther.* **291**, 492–502.
- Lash, L. H., Putt, D. A., and Zalups, R. K. (2001b). Biochemical and functional characteristics of cultured renal epithelial cells from uninephrectomized rats: Factors influencing nephrotoxicity. *J. Pharmacol. Exp. Ther.* **296**, 243–251.
- Lash, L. H., and Tokarz, J. J. (1989). Isolation of two distinct populations of cells from rat kidney cortex and their use in the study of chemical-induced toxicity. *Anal. Biochem.* **182**, 271–279.
- Lash, L. H., and Tokarz, J. J. (1990). Oxidative stress in isolated rat renal proximal and distal tubular cells. *Am. J. Physiol.* **259**, F338–F347.
- Lash, L. H., Tokarz, J. J., Chen, Z., Pedrosi, B. M., and Woods, E. B. (1996). ATP depletion by iodoacetate and cyanide in renal distal tubular cells. *J. Pharmacol. Exp. Ther.* **276**, 194–205.
- Lash, L. H., Tokarz, J. J., and Pegouske, D. M. (1995). Susceptibility of primary cultures of proximal tubular and distal tubular cells from rat kidney to chemically induced toxicity. *Toxicology* **103**, 85–103.
- Lash, L. H., and Zalups, R. K. (1992). Mercuric chloride-induced cytotoxicity and compensatory hypertrophy in rat kidney proximal tubular cells. *J. Pharmacol. Exp. Ther.* **261**, 819–829.
- Lash, L. H., and Zalups, R. K. (1994). Activities of enzymes involved in renal cellular glutathione metabolism after uninephrectomy in the rat. *Arch. Biochem. Biophys.* **309**, 129–138.
- Meyer, T. W., Baboolal, K., and Brenner, B. M. (1996). Nephron adaptation to renal injury. In *The Kidney*, 5th ed. (B. M. Brenner, Ed.), pp. 2011–2048. W.B. Saunders Company, Philadelphia, PA.
- Molland, E. A. (1976). Aspirin damage in the rat kidney in the intact animal and after unilateral nephrectomy. *J. Pathol.* **120**, 43–48.
- Nath, K. A., Croatt, A. J., and Hostetter, T. H. (1990). Oxygen consumption and oxidant stress in surviving nephrons. *Am. J. Physiol.* **258**, F1354–F1362.
- Shirley, D. G., and Walter, S. J. (1991). Acute and chronic changes in renal function following unilateral nephrectomy. *Kidney Int.* **40**, 62–68.
- Stacey, N. H., Cantilena, J. R., Jr, and Klaassen, C. D. (1980). Cadmium toxicity and lipid peroxidation in isolated hepatocytes. *Toxicol. Appl. Pharmacol.* **53**, 470–480.
- Zalups, R. K. (1995). Progressive losses of renal mass and the renal and hepatic disposition of administered inorganic mercury. *Toxicol. Appl. Pharmacol.* **130**, 121–131.
- Zalups, R. K. (1997). Reductions in renal mass and the nephropathy induced by mercury. *Toxicol. Appl. Pharmacol.* **143**, 366–379.
- Zalups, R. K. (2000). Molecular interactions with mercury in the kidney. *Pharmacol. Rev.* **52**, 113–143.
- Zalups, R. K., and Diamond, G. L. (1987). Mercuric chloride-induced nephrotoxicity in the rat following unilateral nephrectomy and compensatory renal growth. *Virchows Arch. B Cell. Pathol. Incl. Mol. Pathol.* **53**, 336–346.
- Zalups, R. K., Gelein, R. M., and Cherian, M. G. (1992). Shifts in the dose-effect relationship for the nephropathy induced by cadmium-metallothionein in rats after a reduction in renal mass. *J. Pharmacol. Exp. Ther.* **262**, 1256–1266.
- Zalups, R. K., Klotzbach, J. M., and Diamond, G. L. (1987). Enhanced accumulation of injected inorganic mercury in renal outer medulla after unilateral nephrectomy. *Toxicol. Appl. Pharmacol.* **89**, 226–236.
- Zalups, R. K., and Lash, L. H. (1990). Effects of uninephrectomy and mercuric chloride on renal glutathione homeostasis. *J. Pharmacol. Exp. Ther.* **254**, 962–970.
- Zalups, R. K., and Lash, L. H. (1994). Recent advances in understanding the renal transport and toxicity of mercury. *J. Toxicol. Environ. Health* **42**, 1–44.

Copyright of Toxicological Sciences is the property of Oxford University Press / UK and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.