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Renal oxidant injury and oxidant response induced by mercury

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Renal oxidant injury and oxidant response induced by mercury. The role of oxidative stress in mercuric chloride ($HgCl_2$)-induced nephrotoxicity is uncertain and controversial. We demonstrate that LLC-PK_1 cells, exposed to $HgCl_2$, generate massive amounts of hydrogen peroxide, the latter completely quenched by the hydrogen peroxide scavenger, pyruvate. $HgCl_2$ exerts a dose-dependent cytotoxicity which is attenuated by pyruvate and catalase. Cellular generation of hydrogen peroxide arises, at least in part, from mitochondria since mitochondrial rates of generation of hydrogen peroxide increase in response to $HgCl_2$; $HgCl_2$ also provokes a shift in absorbance spectra in rhodamine 123 loaded-mitochondria and stimulates mitochondrial state 4 respiration. $HgCl_2$, applied for one hour, impairs cellular vitality as demonstrated by the MTT assay, an assay dependent in part on mitochondrial function. $HgCl_2$ impairs function in other organelles such as lysosomes that maintain a transmembrane proton gradient; these latter effects are partially attenuated by pyruvate. We complement these *in vitro* findings with *in vivo* evidence demonstrating that $HgCl_2$ stimulates renal generation of hydrogen peroxide. The functional significance of such generation of hydrogen peroxide was evaluated in rats deficient in selenium and vitamin E, a nutrient deficiency that impairs the scavenging of hydrogen peroxide and promotes the toxicity of this oxidant. In these rats serum creatinine values were significantly higher on sequential days following the administration of $HgCl_2$. To probe the renal response to oxidative stress induced by $HgCl_2$, we examined hydrogen peroxide-scavenging enzymes and redox-sensitive genes. Catalase activity was unaltered whereas glutathione peroxidase activity was decreased, effects that may contribute to the net renal generation of hydrogen peroxide. The redox sensitive enzyme, heme oxygenase, was markedly up-regulated in the kidney in response to $HgCl_2$. $HgCl_2$ also induced members of the *bcl* family, *bcl2* and *bclx*, genes that protect against apoptosis and oxidant injury. In another model of oxidant-induced renal injury, the glycerol model, *bcl2* mRNA was not induced at 6 and 24 hours after the administration of glycerol. In summary, we demonstrate that $HgCl_2$ potently stimulates renal generation of hydrogen peroxide *in vitro* and *in vivo* and such generation of peroxide contributes to renal dysfunction *in vitro* and *in vivo*. We also demonstrate that in response to $HgCl_2$, redox sensitive genes are expressed including heme oxygenase and members of the *bcl* family.

Well established and widely utilized as a model of acute renal failure is the administration of mercury-containing compounds such as mercuric chloride ($HgCl_2$) to experimental animals [1]. In studies of this model extending over many years, the origins of such injury are traced to diverse cellular and biochemical lesions; the latter encompasses impaired mitochondrial function [2], decreased oxidative phosphorylation [2, 3], calcium-dependent cell injury [4], impaired mitochondrial sequestration of calcium [5], primary derangements in the cytoskeleton [6], derangement in membrane phospholipids [7], liberation of unesterified free fatty acids from the lipid bilayer [8], complexation of critical thiol groups present in proteins and consumption of low molecular weight cellular thiols [9].

Oxidant-dependent mechanisms are also incriminated in the pathogenesis of $HgCl_2$-induced renal injury. However, the evidence for oxidant stress is conflicting, and based on the available literature, it is difficult to assign a significant role for this pathway of cell injury. Mercury diminishes renal content of glutathione [9, 10], one of the dominant antioxidants in the kidney [11]; these effects together with the exacerbation of mercury-induced nephrotoxicity by thiol depletion [9, 12] may be construed as evidence of oxidative stress. However, these findings do not persuasively provide support for an oxidative mechanism for renal injury since: (i) significant renal damage is already apparent *in vivo* at a time when renal glutathione content is still unaltered [10]; (ii) significant reduction in cytotoxicity may be effected without changes in glutathione content [9]; (iii) thiol-containing agents that protect against mercury-induced cytotoxicity [13, 14], may do so by potently binding mercury—a property uniformly shared by thiol-containing agents [15]—and thus the efficacy of these compounds may simply reflect reduced availability and/or delivery of mercury to the kidney. While some studies suggest increased peroxide formation in the kidney [13, 16], other studies fail to detect such evidence of peroxidation [10, 17], and, based on the administration of assorted antioxidants, the conclusion has been drawn that there is no convincing evidence for the involvement of oxygen free radicals in the pathogenesis of $HgCl_2$-induced acute renal failure [17]. Relevant to such considerations are studies of cytotoxicity of $HgCl_2$ on hepatocytes. Increased LDH release is accompanied by increased lipid peroxidation and consumption of cellular glutathione; however, antioxidants that potently reduce lipid peroxidation fail to protect against such cytotoxicity [18].

Given this lack of unanimity of opinion concerning oxidative injury in this disease model, we examined the involvement of oxidative stress in $HgCl_2$-induced renal injury, focusing in particular on hydrogen peroxide, an oxidant generated in increased amounts in a number of models of toxic nephropathy [19–21]. An added impetus for us to pursue these studies was provided by the analyses of Weinberg, Harding and Humes on the changes in oxygen consumption induced by mercury [2], observations that led us to infer that hydrogen peroxide could, conceivably, be generated in increased amount in response to mercury. We thus examined the generation of hydrogen peroxide *in vitro* and *in vivo* following the administration of $HgCl_2$, the potential sources of such oxidants, the functional significance of oxidant generation *in*

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vitro and *in vivo*, intracellular organelles that are potential targets for the effects of mercury, and finally, the anti-oxidant response elicited by $HgCl_2$.

Methods

Detection of hydrogen peroxide generated in LLC-PK1 cells exposed to $HgCl_2$

In all studies using cells in monolayer, LLC-PK_1 cells (American Type Culture Collection) were grown to confluence in DMEM containing 10% FBS maintained at 37°C in the presence of 95% air, 5% CO_2 as previously described [22]. To quantitate the amount of hydrogen peroxide generated in cells exposed to $HgCl_2$, we employed the probe, 2'7'-dichlorofluorescin diacetate, a hydrogen peroxide-sensitive fluorescent probe (Molecular Probes, Eugene, OR, USA) and ACAS 570 Interactive Laser Cytometer (Meridian Instruments Inc., Okemos, MI, USA) [22]. Cell monolayers were loaded with 2,7-dichlorofluorescin diacetate and then exposed to $HgCl_2$ or vehicle. To calibrate cellular fluorescence, we scanned confluent monolayers exposed to concentrations of hydrogen peroxide of 250 and 500 μM. We also employed the probe, dihydrorhodamine 123. This probe is nonfluorescent but its oxidized product, rhodamine 123, is fluorescent. Oxidation of dihydrorhodamine 123 to rhodamine 123 can be induced by several oxidants including hydrogen peroxide [23]. A stock of 10 mg dihydrorhodamine 123 (Molecular Probes) in 10 ml dimethylformamide (28.9 mM) was stored and protected from light at −20°C. This stock was diluted to 5 μM in HBSS immediately before use. Confluent monolayers were washed and loaded with dihydrorhodamine 123 for 30 minutes. The cells were washed and then scanned using the ACAS 570 laser cytometer.

Cytotoxicity determined by the ^{51}-Chromium release assay

Cytotoxicity was quantitated by the method of ^{51}-Chromium release [22] following exposure of the cell monolayer to $HgCl_2$ in the presence or absence of pyruvate (a nonenzymatic H_2O_2 scavenger) or catalase (an enzymatic H_2O_2 scavenger, 800 U/ml). LLC-PK_1 cells were radiolabeled overnight at 37°C by incubating each well with 5.0 μCi sodium [^{51}Cr] chromate (Amersham Corp., Arlington Heights, IL, USA) in DMEM containing 10% fetal calf serum. The following morning cell monlayers were washed and exposed to $HgCl_2$ for four hours.

Oxygen consumption by mitochondria

Oxygen consumption was performed using a Clark-type oxygen electrode and a microsensor (Chemical Microsensor, Diamond General, Ann Arbor, MI, USA) connected to a potentiometric recorder (Linear Instruments) [24]. The oxygen electrode was fitted to a 0.6 ml microchamber (5356 Model 600A, Oxygen Electrode Microsystem; Instech Laboratories). Mitochondria were isolated from the cortices of kidneys harvested from etherized male Sprague-Dawley rats. The cortex was homogenized on ice in a preparation buffer consisting of 210 mM mannitol, 70 mM sucrose, 0.5 mM EDTA, pH 7.4 with Tris base. Homogenization was performed with a Potter-Elvehjem homogenizer and a teflon pestle and completed using 4 strokes. The homogenate was centrifuged at 515 g for 10 minutes at 4°C. The supernatant was then centrifuged at 8240 g for 10 minutes at 4°C. The "fluffy" white layer was removed from above the pellet. The pellet was resuspended, taking care to avoid the dark layer at the base of the pellet, brought up to 5 ml in preparation buffer and centrifuged again at 8240 g for 10 minutes at 4°C; the pellet from this step was resuspended in 0.50 ml ice-cold buffer and kept on ice. The consumption buffer consisted of 210 mM sucrose, 10 mM KCl, 10 mM potassium phosphate buffer, 0.5 mM EDTA, pH 7.4 with Tris base and contained as substrate either 10 mM glutamate and 10 mM malate, or 10 mM succinate/5 μM rotenone; experiments were conducted at 33°C. A suspension of mitochondria was injected into the chamber and state 2 respiratory rate determined; ADP (200 μM) was added to initiate state 3, and following the utilization of ADP, state 4 respiratory rate was determined.

Quantitation of mitochondrial generation of hydrogen peroxide by PHPA

We quantitated mitochondrial generation of hydrogen peroxide using the nonfluorescent compound, p-hydroxyphenylacetic acid (PHPA; Sigma Chemical Co.). PHPA is oxidized to a fluorescent product as hydrogen peroxide is reduced by horseradish peroxidase [25]. The generation of hydrogen peroxide by mitochondria was measured in a quartz crystal cuvette with a 1 cm path length in a Perkin-Elmer LS-5B luminescence spectrometer. While the method used to isolate mitochondria results in a high degree of purity of such preparations, a concern exists that there may be some degree of contamination with organelles such as peroxisomes and endoplasmic reticulum that may themselves generate hydrogen peroxide. Excitation was performed at 314 nm and emission was measured at 416 nm. Mitochondria were suspended in a solution containing 150 mM KCl, 10 mM tris-phosphate, 5 mM tris-HCl, 10 mM succinate, 133 μg/ml PHPA, 22 nM horseradish peroxidase, 0.2 μM antimycin A. After basal rates of generation of hydrogen peroxide were determined, $HgCl_2$ was added to achieve final concentrations of 1, 2 and 4 μM, and rates of generation of hydrogen peroxide again determined. A standard curve was generated using reagent hydrogen peroxide.

Effect of $HgCl_2$ on absorbance spectra of rhodamine 123-loaded mitochondria

Mitochondria were isolated from renal cortices employing a buffer consisting of 0.25 M sucrose, 2 mM Hepes, pH 7.4. Absorbance spectra were obtained using 2 ml of mitochondrial suspension (0.4 mg/ml) in a buffer containing 150 mM sucrose, 5 mM $MgCl_2$, 5 mM succinic acid, 5 mM KH_2PO_4, 20 mM Hepes, 2.5 μM rotenone and 2.62 μM rhodamine 123, pH 7.4 [26]. Absorbance spectra were obtained at 23°C from 440 to 540 nm using Beckman DU-640 spectrophotometer (Beckman Instruments, Fullerton CA, USA). Scans were repeated in the presence of $HgCl_2$ (5 μM) and $HgCl_2$ (5 μM)/pyruvate (5 mM).

MTT reduction by LLC-PK_1 cells

The MTT assay was performed by a modification of the previously described method [27]. LLC-PK_1 cells were grown to confluent monolayers in 96 well plates, and 1.25×10^4 cells were plated per well. The cell monolayer was gently washed with HBSS and exposed to $HgCl_2$ at concentrations of 1.25, 2.5, 5, and 10 μM for one hour. After washing the monolayer, 200 μl of a 1 mg/ml solution of MTT (Sigma Chemical Co.) in HBSS was added to each well. The cells were incubated one hour after which the overlying medium was removed and 100 μl of 2-isopropanol were added for 15 minutes. Absorbance at 540 nm was read for each

well using a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA). As described for this assay [27], data were expressed as a percentage of control, the latter performed in the absence of $HgCl_2$.

Assessment of lysosomal function using acridine orange

Acridine orange is retained within lysosomes. The acid pH of the lysosomes allows the visualization of lysosomes as red granules while the presence of the dye in the relatively more alkaline cytoplasm is associated with green fluorescence, and the ratio of green to red fluorescence provides an assessment of lysosomal function [28, 29]. A stock of acridine orange in absolute ethanol (250 μg/ml) was stored and protected from light at −20°C. This stock was diluted to 1.7 μg per ml in HBSS immediately before use, and 1 ml of this solution was applied for 15 minutes. Following washing, fluorescence of an area of cells was quantified using an ACAS 570 laser cytometer (Meridian Instruments, Inc., Okemos, MI, USA). Filters and a dichroic mirror were used to gather data at 530 nm and greater than 650 nm, and a given area of cells was scanned prior to and after the addition of $HgCl_2$. The ratios of green to red fluorescence of an area of cells were obtained using the Meridian analysis software.

Effect of $HgCl_2$ on renal function in vivo

In vivo nephrotoxicity of $HgCl_2$ (4 mg/kg body wt in distilled water injected subcutaneously) was assessed in male Sprague Dawley rats maintained on standard rat chow (Ralston Purina Co., St. Louis, MO, USA) and water *ad libitum*. Renal function was evaluated by daily serum creatinine determinations on a tail vein sample and measured by the Jaffe reaction (Creatinine Analyzer II, Beckman Instruments, Inc.) [30]. In studies in which the effect of a prooxidant state on the nephrotoxic actions of $HgCl_2$ was tested, male Sprague-Dawley weanling rats (3 weeks) were maintained a diet deficient in the antioxidants (selenium and vitamin E) or the control diet for an additional six weeks as described previously [30]. Rats in each dietary group were then subjected to $HgCl_2$ (2 mg/kg body wt, SQ) and daily serum creatinine measurements. In these studies we employed a lower dose of $HgCl_2$ since we hypothesized that $HgCl_2$ would exert increased toxicity in the antioxidant deficiency state. Rats on standard diets exhibit severe, and usually fatal, renal failure when treated with $HgCl_2$ at 4 mg/kg body wt. The deficient diet differed from the control diet only by its reduced content of selenium and vitamin E [30].

To address the functional significance of heme oxygenase induced in the kidney in response to $HgCl_2$, two complementary approaches were taken [31]. In the first study, heme oxygenase activity was inhibited by a specific competitive inhibitor, tin protoporphyrin, 20 μmol/kg body wt (Porphyrin products, Inc., Logan, UT, USA), administered subcutaneously three hours prior to treatment with $HgCl_2$ while control rats received vehicle. Identical doses of the inhibitor or vehicle were administered 8 and 24 hours after the first dose. In the second approach, heme oxygenase was induced in the kidney by rat hemoglobin, 30 mg/100 g body wt i.v., given 16 hours prior to the administration of $HgCl_2$ [31]. In these studies rats were maintained on standard purina rat chow and tap water *ad libitum*.

Measurement of generation of hydrogen peroxide in the kidney in vivo

Generation of hydrogen peroxide by the kidney *in vivo* was determined by quantitating % catalase inhibition in the presence of aminotriazole as described in detail in our previous study [32]. In this method the extent to which catalase is inhibited reflects the rate of generation of hydrogen peroxide. Catalase activity was expressed as k/mg protein as well as % of basal activity in rats treated with $HgCl_2$ or vehicle [32].

Extraction of RNA and Northern blot hybridization

RNA was extracted by the method of Chomczynski and Sacchi [33], and Northern blot hydridization was performed as previously described [31] using a human heme oxygenase cDNA (supplied by Dr. Rex Tyrrell, Epalinges, Switzerland) and a mouse *bcl2* cDNA (supplied by Dr. Stanley Korsmeyer, St. Louis, MO, USA) and a mouse *bclx* cDNA [34, 35]. Autoradiograms were quantified by video densitometry and standardized by the method of Correa Rotter, Mariash and Rosenberg [36]. This established method of standardization corrects for any variability due to loading and transfer, and factors the OD of the message for the given gene with the OD of the 18S rRNA, the latter obtained on a negative of the ethidium bromide stained nylon membrane. We used this method rather than the use of "housekeeping" genes (such as actin and GAPDH) for standardization since expression of "housekeeping" genes may be altered by the injurious insult [36].

Northern analyses for the expression of heme oxygenase were performed using RNA extracted from LLC-PK_1 cells exposed to $HgCl_2$ and from rats treated 6 and 16 hours previously with $HgCl_2$ (4 mg/kg body wt). RNA from kidneys harvested from rats treated with $HgCl_2$ (4 mg/kg body wt) *in vivo* was also probed for bcl2 and bclx at the 6 and 16 hours timepoints. To determine the specificity of expression of bcl2 induced by $HgCl_2$, we also studied the expression of bcl2 mRNA in the glycerol model of acute renal failure (10 ml/kg of 50% glycerol administered intramuscularly) 6 and 24 hours after the administration of glycerol [31].

Measurement of enzyme activities in vivo

As described in detail in our previous study [31], heme oxygenase activity was measured by the method of Pimstone et al [37], catalase by the method of Aebi [38] and glutathione peroxidase by the method of Lawrence and Burk [39].

Statistics

Data are expressed as means ± SEMs. One-way ANOVA was employed for comparisons involving more than two groups and the Student-Newman-Keuls test then applied. For single comparisons involving two groups, the unpaired Student's *t*-test was used. Comparisons are considered significant for $P < 0.05$.

Results

Studies in vitro

Effect of $HgCl_2$ on cellular generation of hydrogen peroxide

The exposure of LLC-PK_1 cells to $HgCl_2$ led to marked generation of hydrogen peroxide. As demonstrated in a representative scan (Fig. 1), such cells exposed to $HgCl_2$ (5 μM) displayed striking DCF-dependent fluorescence as assessed by the laser cytometer. The concomitant presence of pyruvate, a nonezymatic

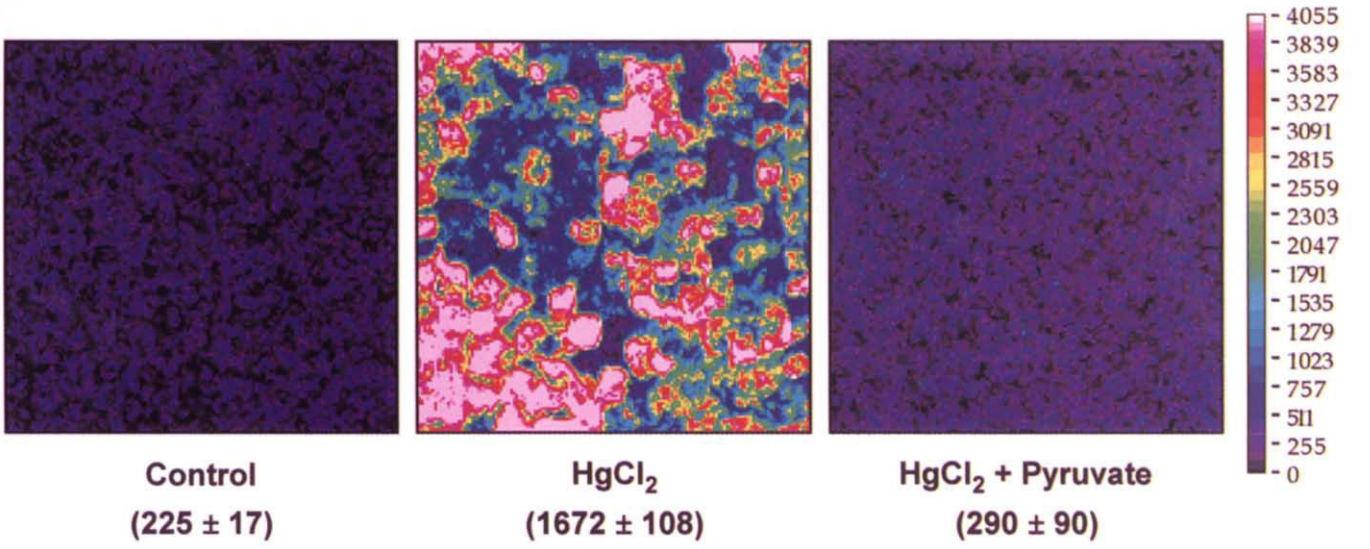

Fig. 1. *Effect of $HgCl_2$ on cellular generation of hydrogen peroxide.* LLC-PK_1 cells were loaded with DCF, exposed for approximately 1.5 hours to HBSS alone, HBSS containing $HgCl_2$ (5 μM) and HBSS containing $HgCl_2$ (5 μM)/pyruvate 5 (mM) and then scanned with the laser cytometer. For each condition the mean ± SEM are provided, $N = 8$ in each group.

Table 1. Fluorescence values in dihydrorhodamine 123-loaded LLC-PK_1 cells in response to $HgCl_2$ (5 μM) and $HgCl_2$ (5 μM) + pyruvate (5 mM)

	Control ($N = 5$)	$HgCl_2$ (5 μM) ($N = 5$)	$HgCl_2$ (5 μM) + Pyruvate (5 mM) ($N = 5$)
0	71 ± 10	71 ± 10	69 ± 9
2 hrs	91 ± 3	246 ± 110	83 ± 8
2.5 hrs	71 ± 10	158 ± 23[a]	82 ± 5

[a] $P < 0.05$ vs. control, vs. $HgCl_2$ + pyruvate, at 2.5 hours (ANOVA)

scavenger of hydrogen peroxide [22], prevented such increments in fluorescence. Quantitative analyses revealed that the fluorescence values of the $HgCl_2$-treated cells were significantly increased when compared to the vehicle-treated controls and to cells exposed to $HgCl_2$ + pyruvate, whereas there were no significant differences between vehicle-treated controls and cells exposed to $HgCl_2$+ pyruvate. As positive controls we exposed LLC-PK_1 cells to reagent hydrogen peroxide. The mean fluorescence values in cells exposed to hydrogen peroxide at concentrations of 250 μM and 500 μM were 773 ± 24 and 1903 ± 142 Fluorescence Units, respectively. Therefore, cellular fluorescence in cells exposed to $HgCl_2$ (5 μM) approached that induced by reagent hydrogen peroxide at a concentration of 500 μM. That treatment of cells with $HgCl_2$ stimulates cellular generation of hydrogen peroxide was corroborated using another oxidant-sensitive, less hydrogen peroxide-dependent, fluorescent probe, dihydrorhodamine 123. Dihydrorhodamine, a nonfluorescent compound is converted into a fluorescent moiety, rhodamine 123, after oxidation. As shown in Table 1, after two hours of exposure increased fluorescence was apparent in mercury-treated cells but not in mercury-treated cells concomitantly exposed to pyruvate.

Hydrogen peroxide-dependency of $HgCl_2$-induced cytotoxicity

To determine whether more prolonged exposure to mercury at these concentrations is cytotoxic, and to explore the role of hydrogen peroxide in such toxicity, we performed cytotoxicity studies using the chromium release assay. Cytotoxicity of $HgCl_2$ was barely detectable at 2.5 μM after four hours of exposure but quite pronounced at 5 μM (Fig. 2, left panel). The concomitant presence of pyruvate significantly reduced cell damage induced by both concentrations of $HgCl_2$ (Fig. 2, left panel). These protective effects of pyruvate against $HgCl_2$-induced cytoxicity were dose-dependent (Fig. 2, right panel). Catalase, the enzymatic scavenger of hydrogen peroxide, also reduced cell damage induced by $HgCl_2$ (Fig. 3).

Effect of $HgCl_2$ on mitochondrial function

To examine the mechanism underlying hydrogen peroxide-dependent cytotoxicity of $HgCl_2$, we undertook a series of studies on mitochondrial function. $HgCl_2$ (2 μM) stimulated state 4 respiration (28.1 ± 0.4 vs. 16.6 ± 0.8, nmol/mg prot/min, $P < 0.001$, $N = 5$, in the presence of glutamate/malate and 65.9 ± 1.3 vs. 45.5 ± 0.8, nmol/mg prot/min, $P < 0.001$, $N = 5$, in the presence of succinate and rotenone) but did not influence state 3 respiration in the presence of either fuel. $HgCl_2$ decreased mitochondrial respiratory control ratio when respiration was supported either by glutamate/malate (2.5 ± 0.2 vs. 4.3 ± 0.2, $P < 0.001$, $N = 5$) or by succinate and rotenone (2.0 ± 0.1 vs. 2.9 ± 0.1, $P < 0.001$, $N = 5$). These alterations in respiration, wherein state 4 is increased with mild or no reduction in state 3 (findings in agreement with the earlier observations by Weinberg et al [2]) raise the possibility that mercury stimulates mitochondrial generation of hydrogen peroxide. In studies of mitochondria harvested from the intact normal kidney, mitochondria display a dose-dependent increase in generation of hydrogen peroxide when exposed to increasing concentrations of mercury (Fig. 4). Thus increased generation of hydrogen peroxide by mitochondria may contribute to increased cellular production of hydrogen peroxide we observed.

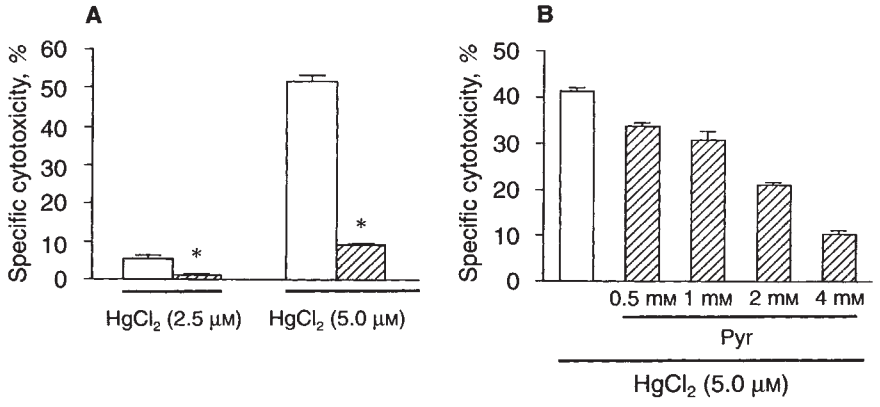

Fig. 2. *Protective effect of pyruvate against $HgCl_2$-induced cytotoxicity.* **A.** Cells were exposed to $HgCl_2$ (2.5 μM and 5 μM) for approximately four hours in the presence or absence of pyruvate (5 mM) and specific cytotoxicity determined by the chromium release assay. Each condition represents the mean ± SEM of 6 determinations, each determination performed in triplicate or quadruplicate. $^*P < 0.05$ in the comparisons between cytotoxicities at each concentration of $HgCl_2$ in the presence (▨) and absence (□) of pyruvate. **B.** Dose-dependent protective effects of pyruvate against $HgCl_2$-induced cytotoxicity, the latter determined by the chromium release assay. Each dose represents the mean ± SEM of 3 determinations.

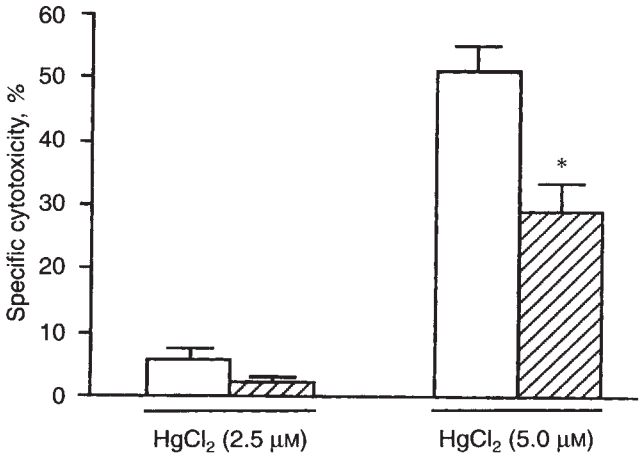

Fig. 3. *Protective effect of catalase against $HgCl_2$-induced cytotoxicity.* Cells were exposed to $HgCl_2$ (2.5 μM and 5 μM) for approximately four hours in the presence (▨) or absence (□) of catalase (800 U/ml) and specific cytotoxicity determined by the chromium release assay. Each condition represents the mean ± SEM of 6 determinations, each determination performed in triplicate or quadruplicate. $^*P < 0.05$ in the comparisons between cytotoxicities at each concentration of $HgCl_2$ in the presence and absence of catalase.

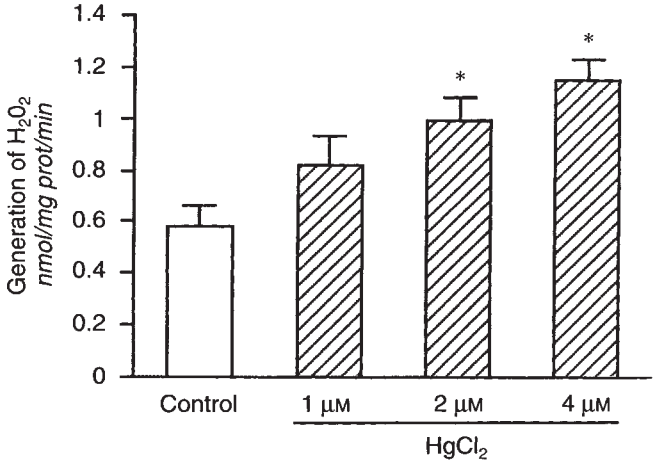

Fig. 4. *Effect of $HgCl_2$ on mitochondrial generation of hydrogen peroxide.* Generation of hydrogen peroxide by mitochondria was measured fluorimetrically by the PHPA assay. Each condition represents mean ± SEM of 4 determinations and data were analyzed by ANOVA and the Student-Newman-Keuls test. $^*P < 0.05$ versus Control.

To explore the mechanisms accounting for increased oxygen consumption and increased mitochondrial production of hydrogen peroxide, we examined the effect of $HgCl_2$ on absorbance spectra in rhodamine 123-loaded mitochondria. Rhodamine 123 is a cationic fluorescent probe that is retained within mitochondria based on mitochondrial membrane potential. The loss of the membrane potential, as induced by uncoupling agents, leads to the loss of this tracer from the mitochondrion, and shifts absorbance spectra to the left [26]. As shown in Figure 5, absorbance spectra in rhodamine 123-loaded mitochondria is shifted to the left upon exposure to mercury. Pyruvate (5 mM) had no effect on the spectral profile.

To examine whether mitochondrial injury occurred as part of the cytotoxic actions of mercury, we employed the MTT assay. This assay is critically dependent on the integrity of the mitochondrial enzyme, succinate dehydrogenase, in the reduction of MTT to formazan, a blue dye. Cells exposed for one hour to increasing concentrations of mercury were progressively impaired in their ability to reduce MTT (Fig. 6).

Effect of $HgCl_2$ on lysosomal function

We also examined the effect of mercury on lysosomal function by assessing lysosomal retention of the fluorescent probe, acridine orange. The acid pH of the lysosomes allows the dye in lysosomes to be visualized as red granules while the presence of the dye in the relatively more alkaline environment of the cytoplasm is associated with green fluorescence; the ratio of green to red fluorescent provides an index of lysosomal function. As shown in Figure 7, $HgCl_2$ led to a threefold increase in green/red fluorescence ratio, and such effects were attenuated, in part, by pyruvate. As a positive control, hydrogen peroxide (500 μM) markedly increased green/red fluorescence ratio: 2.2 ± 0.1 ($N = 7$) versus control values of 0.5 ± 0.1 ($N = 8$).

Effect of $HgCl_2$ on a redox-sensitive gene, heme oxygenase

To probe for added evidence of oxidative stress in cells exposed to $HgCl_2$, and to ascertain whether an antioxidant response occurs in cells so treated, we examined the capacity of mercury to induce heme oxygenase mRNA. This gene is induced, invariantly, in mammalian cells in response to oxidative stress and provides a biologic readout of such a change in the cellular redox state [40].

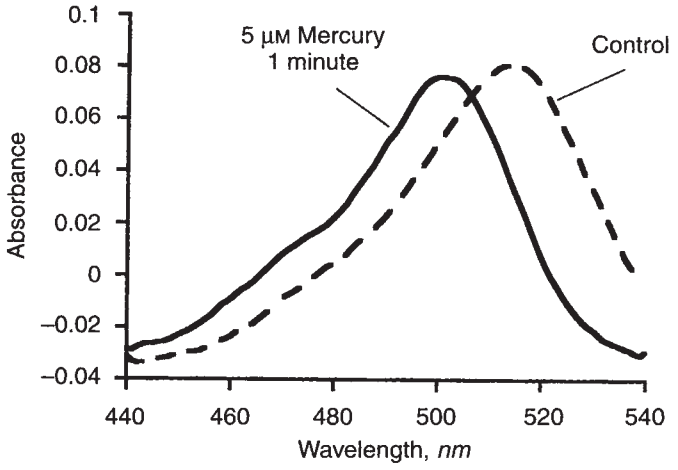

Fig. 5. *Effect of $HgCl_2$ on absorbance spectra in rhodamine 123-loaded mitochondria.* Spectrophotometric scans of a suspension of mitochondria loaded with rhodamine 123 in the absence and presence of $HgCl_2$ (5 μM) in the wavelength range 440 to 540 nm.

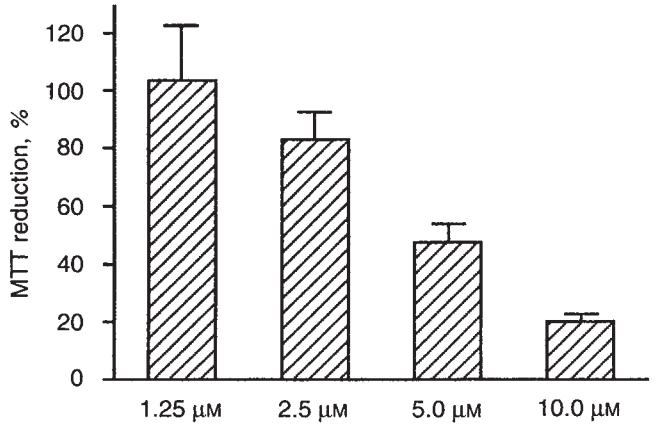

Fig. 6. *Effect of $HgCl_2$ on cellular reduction of MTT.* Cellular reduction of MTT (%) was studied in LLC-PK_1 cells exposed to increasing concentrations of $HgCl_2$ for one hour. MTT reduction at each concentration was significantly different from every other concentration except in the comparison between the concentrations of 1.25 and 2.5 μM.

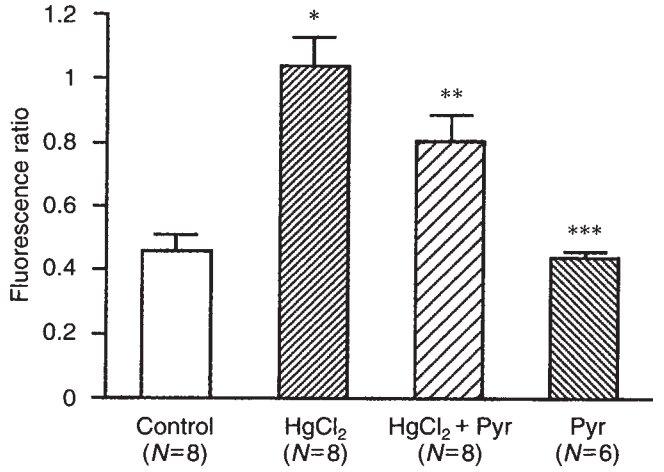

Fig. 7. *Effect of $HgCl_2$ on lysosomal function as assessed by acridine orange.* Ratio of green-to-red fluorescence in LLC-PK_1 cells loaded with acridine orange and exposed for approximately 1.5 hours to HBSS alone (Control), HBSS containing $HgCl_2$ (5 μM), HBSS containing $HgCl_2$ (5 μM) and pyruvate (5 mM), HBSS containing pyruvate (5 mM) alone. Data represent mean ± SEM and were analyzed by ANOVA and the Student-Newman-Keuls test. $^*P < 0.05$ versus Control, $^{**}P < 0.05$ versus $HgCl_2$ alone, $^{***}P < 0.05$ versus $HgCl_2$ + pyruvate.

As shown in the Northern analysis in Figure 8, the exposure of cells to mercury strongly induces heme oxygenase mRNA. Interestingly, pyruvate, which scavenges hydrogen peroxide generated in cells exposed to $HgCl_2$, and protects against cytotoxicity induced by $HgCl_2$, fails to inhibit the induction of heme oxygenase by mercury. As a positive control we demonstrate that hydrogen peroxide induces heme oxygenase mRNA while such induction is totally prevented by pyruvate (Fig. 9). Thus cells exposed to $HgCl_2$ express heme oxygenase, a gene readily induced by oxidative stress. Such induction of heme oxygenase mRNA, however, is independent of hydrogen peroxide.

Studies in vivo

Effect of $HgCl_2$ on renal generation of hydrogen peroxide

To determine whether $HgCl_2$ stimulates cellular generation of hydrogen peroxide *in vivo*, we utilized an established method for determining generation of hydrogen peroxide by organs *in vivo*, that is, inhibition of catalase in the presence of aminotriazole. In this method, inhibition of catalase in the presence of aminotriazole reflects generation of hydrogen peroxide: the greater the inhibition of catalase activity the greater the generation of hydrogen peroxide [19, 21, 32]. Basal catalase activity in the rats treated with vehicle or $HgCl_2$ in the absence of aminotriazole was not significantly different (0.40 ± 0.02 vs. 0.41 ± 0.02 k/mg protein, $N = 5$, P = NS); however, in the presence of aminotriazole, rats treated with $HgCl_2$ exhibited significantly lower absolute catalase activity (0.31 ± 0.03 vs. 0.23 ± 0.01 k/mg protein, $P = 0.04$, $N = 7$ in each group) as well as a significantly greater percent reduction in catalase activity when compared to basal values in the absence of aminotriazole (25.1 ± 6.6 vs. 43.2 ± 2.0%, $P = 0.02$, $N = 7$), findings that are indicative of greater generation of hydrogen peroxide in kidneys treated with $HgCl_2$.

To determine the functional significance of such enhanced cellular generation of hydrogen peroxide, we conducted studies employing rats that are impaired in their capacity to scavenge hydrogen peroxide and to limit the toxicity of hydrogen peroxide. We posited that such kidneys would exhibit greater functional impairment if the generation of hydrogen peroxide contributes to renal damage. Rats were maintained on a diet deficient in selenium and vitamin E. This dietary deficiency markedly impairs glutathione peroxidase activity (the major enzyme responsible for the degradation of hydrogen peroxide), while the concomitant deficiency of vitamin E heightens the toxicity of hydrogen peroxide so generated. As shown in Figure 10, basal renal function on Day 0, as reflected by serum creatinine values, prior to the administration of $HgCl_2$, was not significantly different. However, on Days 1 and 2 following the administration of $HgCl_2$, serum creatinine values were significantly greater in rats deficient in antioxidants. These findings would suggest that the generation of hydrogen peroxide by $HgCl_2$ is functionally significant since $HgCl_2$

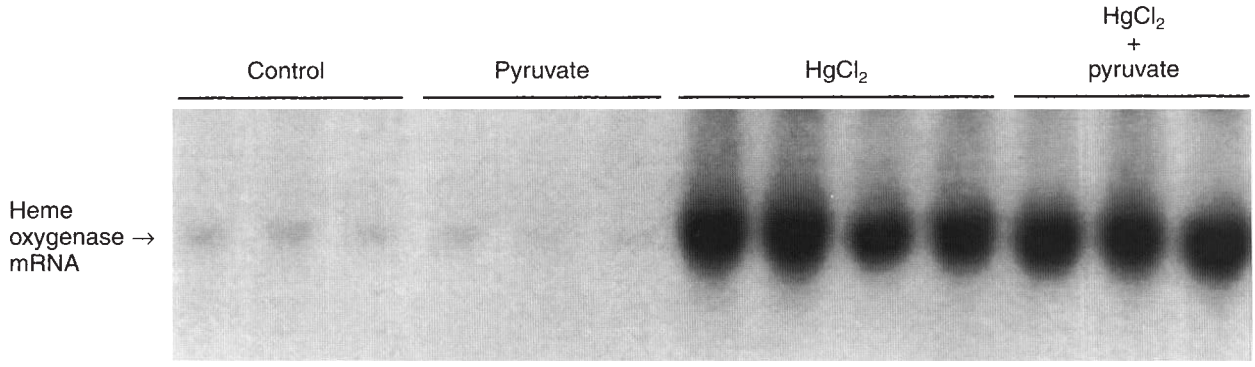

Fig. 8. *Effect of $HgCl_2$ on the induction of heme oxygenase mRNA in LLC-PK_1.* LLC-PK_1 cells were exposed to BME alone, BME containing pyruvate (5 mM), BME containing $HgCl_2$ (5 μM), BME containing $HgCl_2$ (5 μM) and pyruvate (5 mM) for two hours. These media were removed and all cells incubated for an additional four hours in DMEM alone, after which RNA was extracted and probed for heme oxygenase mRNA. The mean corrected OD units for heme oxygenase were 1.1 ± 0.1 (BME alone), 0.9 ± 0.1 (BME + pyruvate), 19.7 ± 0.6 (BME + $HgCl_2$), 27.4 ± 2.7 (BME + $HgCl_2$ + pyruvate).

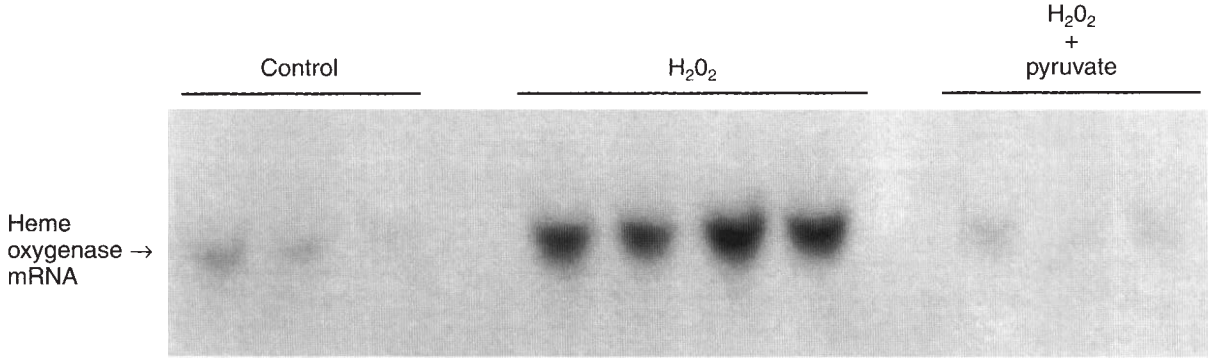

Fig. 9. *Effect of hydrogen peroxide in the absence and presence of pyruvate on the induction of heme oxygenase mRNA in LLC-PK_1 cells.* Cells were exposed to PBS alone, PBS containing hydrogen peroxide (200 μM), and PBS containing hydrogen peroxide (200 μM) and pyruvate (2 mM) for 0.5 hours. These media were removed, cells were incubated for an additional three hours in DMEM alone, after which RNA was extracted and probed for heme oxygenase mRNA. The mean OD units for heme oxygenase were 0.5 ± 0.4 (PBS alone), 12.2 ± 1.4 (PBS + H_2O_2), 2.0 ± 0.7 (PBS + H_2O_2 + pyruvate).

exerts heightened toxicity in rats impoverished in their capacity to degrade hydrogen peroxide and to protect against the toxicity resulting from hydrogen peroxide so produced.

Effect of mercury on renal expression of heme oxygenase

We tested whether the administration of $HgCl_2$ *in vivo* induced heme oxygenase. As demonstrated in Figure 11, there was a marked expression of heme oxygenase mRNA observable at six hours. Similar Northern analyses for heme oxygenase mRNA expression were observed at 16 hours. Such induction of heme oxygenase was also accompanied by increased enzyme activity, as reflected by measurements undertaken 16 hours after the administration of $HgCl_2$ (43.8 ± 5.4 vs. 81.8 ± 9.1 pmol/mg protein/hr, $P = 0.007$, $N = 5$ in each group). Thus, induction of heme oxygenase occurs *in vivo* as well as *in vitro* in response to $HgCl_2$. The relative specificity of heme oxygenase expression was underscored by the absence of induction of other antioxidant enzymes, in particular, the hydrogen peroxide scavenging enzymes. The administration of $HgCl_2$ failed to significantly alter catalase activity (0.40 ± 0.02 vs. 0.41 ± 0.02 k/mg protein, $N = 5$, P = NS),

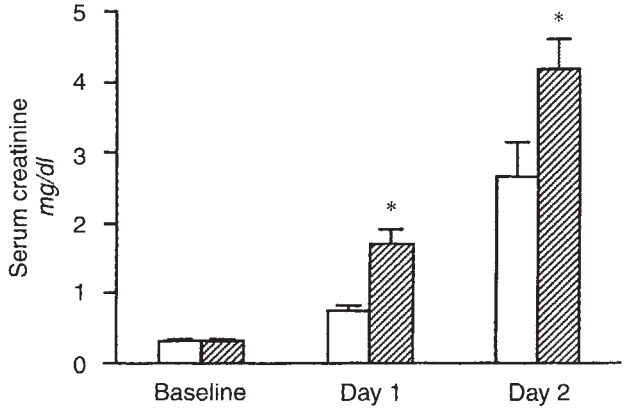

Fig. 10. *Effect of diets deficient in selenium and vitamin E on the course of $HgCl_2$ (2 mg/kg body wt)-induced acute renal failure.* Serum creatinine was determined prior to the administration of $HgCl_2$ (baseline) and on days 1 and 2 after the administration of $HgCl_2$ to rats maintained on diets deficient in selenium and vitamin E or the control diets for 5 to 6 weeks. *$P < 0.05$ in the comparisons between $HgCl_2$ treated-control (□, $N = 8$) and $HgCl_2$ treated-deficient (▨, $N = 7$) rats on respective days.

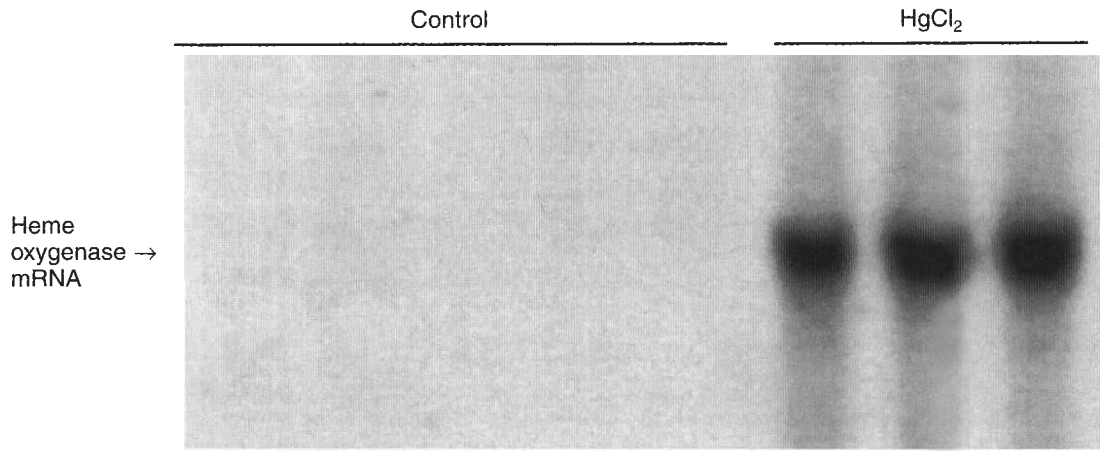

Fig. 11. *Effect of $HgCl_2$ on renal expression of heme oxygenase mRNA.* Northern analyses of kidneys six hours following administration of $HgCl_2$ (4 mg/kg body wt) or vehicle. Each lane represents RNA extracted from one kidney of an individual rat. The mean corrected OD units for heme oxygenase were 0.8 ± 0.2 (vehicle-treated) and 43.6 ± 4.2 ($HgCl_2$-treated).

Table 2. Effect of tin protoporphyrin (Tin Pro, an inhibitor of heme oxygenase) or vehicle on daily serum creatinine values in $HgCl_2$-induced acute renal insufficiency (Study 1) and effect of prior administration of hemoglobin (Hgb, an inducer of heme oxygenase) or vehicle on daily serum creatinine values in $HgCl_2$-induced acute renal insufficiency (Study 2)

		Serum creatinine *mg/dl*	
	N	Day 1	Day 2
Study 1			
Vehicle-treated	7	2.5 ± 0.1	5.6 ± 0.2
Tin Pro-treated	7	2.5 ± 0.1	5.6 ± 0.2
Study 2			
Vehicle-treated	5	2.9 ± 0.2	5.9 ± 0.7
Hgb-treated	6	3.1 ± 0.1	6.3 ± 0.1

while such treatment reduced glutathione peroxidase activity (125.2 ± 29.1 vs. 84.2 ± 13.5 nmoles/mg protein/min, $P = 0.02$, $N = 5$).

To determine the functional significance of such induction of heme oxygenase, we studied the effects of manipulations that either inhibit heme oxygenase activity, or conversely, manipulations that recruit heme oxygenase activity in the kidney prior to the administration of mercury. As demonstrated in Table 2, the administration of tin protoporphyrin, a specific competitive inhibitor of heme oxygenase failed to influence renal dysfunction as measured by serum creatinine. Additionally, the recruitment of heme oxygenase in the kidney by the administration of hemoglobin prior to the administration of $HgCl_2$ did not protect against the renal dysfunction that resulted from $HgCl_2$.

Effect of $HgCl_2$ on renal expression of bcl2 and bclx

Members of the family of *bcl* genes, specifically, *bcl2* and *bclx*, are considered cytoprotective agents that prevent apoptotic cell death, and in some instances, display antioxidant properties. In the light of our findings demonstrating oxidative stress exerted by mercury, we studied the expression of these genes following the administration of $HgCl_2$ *in vivo*. As indicated by the northern analyses at 16 hours, *bcl2* and to a lesser degree *bclx* were up-regulated in the kidney (Fig. 12). Thus in response to $HgCl_2$-mediated renal injury, members of the *bcl* family, *bcl2* and *bclx*, are induced in the kidney.

To determine the specificity of induction of *bcl2* in kidneys of rats treated with $HgCl_2$, we studied the renal expression of *bcl2* mRNA in the glycerol model. Neither at 6 or at 24 hours after the administration of intramuscular glycerol was *bcl2* induced in the kidney (Northern blot not shown), findings that are in contrast with the prominent expression of heme oxygenase at these timepoints in the glycerol model [31] and *bcl2* mRNA expression in the $HgCl_2$-induced renal injury (Fig. 12).

Discussion

These studies demonstrate that renal epithelial cells generate copious amounts of hydrogen peroxide when exposed to relatively modest concentrations of $HgCl_2$. Hydrogen peroxide contributes to the cytotoxicity of $HgCl_2$ since the concomitant presence of a non-enzymatic scavenger for hydrogen peroxide (pyruvate) or an enzymatic scavenger (catalase) reduced cell injury. We complement these *in vitro* findings with *in vivo* studies demonstrating that $HgCl_2$ stimulates generation of hydrogen peroxide by the rat kidney *in vivo*. That such generation of hydrogen peroxide contributes to renal damage *in vivo* was substantiated by the exaggeration of the nephrotoxic actions of $HgCl_2$ in rats impaired in their capacity to scavenge hydrogen peroxide and to limit the toxicity of this oxidant. In aggregate, we provide *in vitro* and *in vivo* evidence demonstrating that $HgCl_2$ stimulates renal generation of hydrogen peroxide, and that hydrogen peroxide contributes to mercury-induced nephrotoxicity.

To determine the mechanisms accounting for increased cellular generation of hydrogen peroxide, we examined the effects of mercury on the mitochondrion. Mercury stimulated state 4 mitochondrial respiration and decreased respiratory control ratios, findings consistent with the original observations of Weinberg et al [2]. These findings intimated to us that generation of hydrogen peroxide by mitochondria may be increased by mercury since increased basal oxygen consumption by mitochondria is often attended by increased production of hydrogen peroxide [41]. We demonstrate that mitochondria exposed to mercury generate increased amounts of hydrogen peroxide in conjunction with

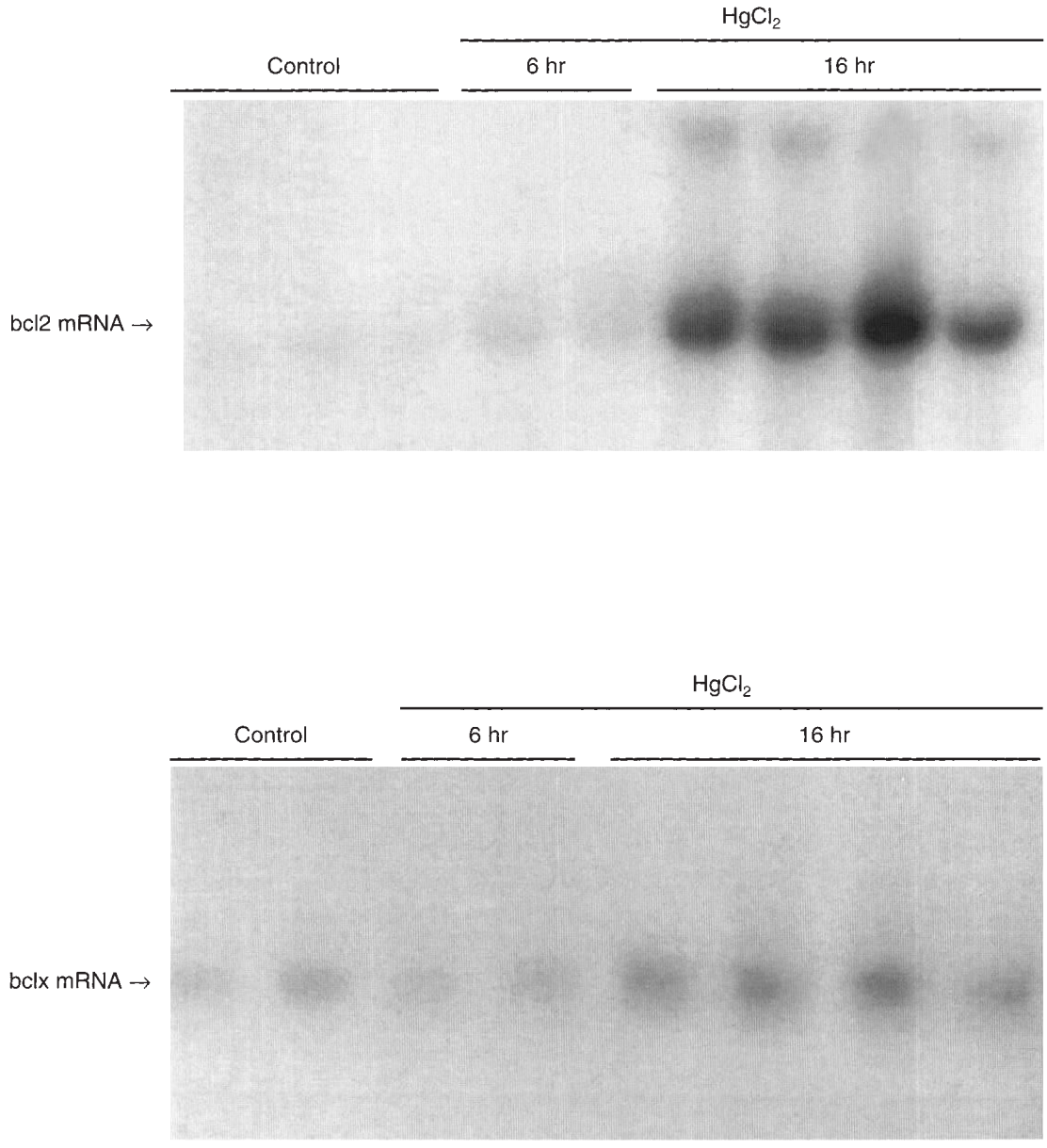

Fig. 12. *Effect of $HgCl_2$ on renal expression of bcl2 and bclx mRNA.* Northern analyses of kidneys 6 and 16 hours following administration of $HgCl_2$ (4 mg/kg body wt) representing expression of *bcl2* (upper panel) and *bclx* (lower panel). Each lane represents RNA extracted from one kidney of an individual rat. The mean corrected OD units for *bcl2* were 0.01 ± 0.01 (vehicle-treated), 0.08 ± 0.03 ($HgCl_2$-treated, 6 hr) and 7.73 ± 1.25 ($HgCl_2$-treated, 16 hr). The mean corrected OD units for *bclx* were 2.7 ± 0.6 (vehicle), 1.8 ± 0.1 ($HgCl_2$-treated, 6 hr) and 6.1 ± 0.4 ($HgCl_2$-treated, 16 hr).

increased oxygen consumption. One possible mechanism underlying such alterations in mitochondrial function relate to the effects of mercury on mitochondrial transmembrane potential. We demonstrate that, within seconds, mercury shifts the absorbance spectrum of rhodamine 123-loaded mitochondria to the left. Prior studies by Emaus et al demonstrate that a similar left shift in rhodamine 123-loaded mitochondria is induced by uncoupling agents, and such shift in the absorbance spectrum is due to the alteration of mitochondrial membrane potential and the attendant loss of rhodamine 123. We speculate that a similar mechanism may underlie the spectral changes in mitochondria exposed to $HgCl_2$. However, we point out that we did not measure mitochondrial content of rhodamine 123 and that other explanations may account for the observed changes. The toxicity of mercury on the mitochondrion was highlighted by studies using the MTT assay, one that is dependent, in part, on mitochondrial function. In these studies we demonstrate a pronounced dose-dependent toxicity of mercury. Thus, mercury has several effects on the mitochondrion: mercury possibly promotes leakiness of mitochondrial membranes and increases mitochondrial oxygen consumption; mercury increases mitochondrial production of hydrogen peroxide *pari passu* with increased oxygen consumption;

and mercury impairs an index of cellular vitality that is dependent on the mitochondrial enzyme, succinate dehydrogenase.

In light of these effects of mercury on the mitochondrial transmembrane potential, we examined the effects of mercury on another organelle in which a transmembrane proton gradient exists, namely, the lysosome. Lysosomes, the repository for hydrolytic enzymes, are capable of maintaining an acid pH within their compartment as a consequence of the pumping of protons across the lysosomal membrane; such a proton gradient activates lysosomal enzymes. It should be emphasized, however, that the mechanisms for generating proton gradients in lysosomes and mitochondria are different. Lysosomal function may be studied by the fluorescent dye, acridine orange. This dye accumulates in lysosomes, and within the acidic lysosomal environment, acridine orange marks the lysosomes as red granules when viewed under blue light; acridine orange in the extralysosomal compartment displays green fluorescence. The ratio of green to red fluorescence provides an index of the extent to which acridine orange is retained in the lysosomes and/or the capacity for this organelle to reduce its pH. Recent evidence indicates that hydrogen peroxide and other oxidants increase the ratio of green to red fluorescence, findings that we confirmed in our study and ones that indicate that oxidants redistribute acridine orange into the extralysosomal compartment and/or impairs proton pumping [28, 29, 42]. We provide the novel observation that $HgCl_2$ also increases the ratio of green to red fluorescence, an effect attenuated in part by pyruvate. Therefore, in lysosomes mercury may influence the proton secreting capability directly and/or increase the permeability of the membrane. In this regard, it is of interest that mercury influences ion transport or transfer across plasma or cellular membranes in a number of settings. For example, in neurons, mercury increases permeability of the plasma membrane to calcium [43]; mercury increases the permeability of colonic epithelia and, in particular, chloride secretion across these cells [44, 45]; mercury increases membrane potassium conductance in cultured epithelial cells [46]; finally, in liposomes mercury directly effects chloride/hydroxyl ion exchange [47].

The exposure of tissues to oxidative stress often instigates an anti-oxidant response that defends against or limits the severity of oxidative injury [40]. Interestingly, it has long been known that bacteria, resistant to the toxic effects of environmental mercury, possess *mer* genes that transport mercury into their cytoplasm after which Hg^{2+} is reduced to the volatile nontoxic moiety, Hg^0 [48]. We, thus, surveyed anti-oxidant enzymes relevant to renal generation of hydrogen peroxide. Mercury did not influence basal catalase activity but did reduce the activity of glutathione peroxidase, the latter effects possibly arising from direct effects of $HgCl_2$ on the enzyme. Such reduction in the activity of glutathione peroxidase activity, one of the key hydrogen peroxide-degrading enzymes, may contribute to increase renal generation of hydrogen peroxide. In contrast to these enzymes, the gene heme oxygenase was markedly induced in the kidney at a relatively early timepoint. Induction of heme-oxygenase is a protective response in certain settings [31, 40]. For example, in the glycerol model of heme protein-associated renal injury, there is fulminant induction of heme oxygenase and such induction is protective [31, 40]. However, in $HgCl_2$ nephrotoxicity, neither did the inhibition of heme oxygenase activity nor its induction prior to the administration of $HgCl_2$ modulate renal function, the latter assessed by serum creatinine measurements. While the functional significance of the induction of heme oxygenase by mercury awaits elucidation, its prompt and prominent induction by $HgCl_2$ is noteworthy for two main reasons. First, such induction of heme oxygenase underscores the ability of $HgCl_2$ to recruit redox-sensitive genes. Second, this induction of heme oxygenase may be germane to acquired resistance to acute renal failure [49]: the prior administration of $HgCl_2$ renders animals resistant to glycerol-induced renal failure [49], while the prior administration of glycerol-induced renal failure induces resistance to $HgCl_2$ [49]; such acquired resistance to renal failure has never been explained. The induction of heme oxygenase by mercury cannot account for the resistance to mercury-induced renal injury conferred by prior induction of the glycerol model. We, however, speculate that the induction of heme oxygenase by mercury may contribute to resistance to renal injury displayed upon subsequent exposure to glycerol induced acute renal failure.

As a possible antioxidant response, we also explored the expression of members of *bcl* genes *in vivo* in the kidney following the administration of mercury. The *bcl2* gene prevents apoptotic cell death induced by a wide variety of insults and in particular, is efficacious against oxidant induced cell damage [50, 51]. For example, expression of *bcl2* in GT1-7 to seven neural cell lines prevents cell death induced by glutathione depletion and the attendant oxidant stress and lipid peroxidation that occur in these cells [52]. Enhanced expression of *bcl2* in IL-3-dependent murine pro-B lymphocytes protects these cells against oxidant-mediated cell death and suppresses lipid peroxidation [53]. That the induction of *bcl2* in the mercury-treated kidney is not simply a nonspecific effect of renal injury or oxidative injury is suggested by our data demonstrating that bcl2 is not induced in the glycerol model, this latter model representing another model of oxidative stress [21]. Interestingly, *bclx* is also induced at 16 hours in conjunction with *bcl2*. Like *bcl2*, *bclx* protects against apoptotic death [54], and *bclx* deficient mice exhibit massive cell death of immature hematopoietic cells and neurons [55]; in lymphocytes *bclx* protects against oxidant-induced cell death [35]. It is of particular interest that the cellular site of expression of these *bcl* members (*bcl2* mainly, and *bclx* exclusively) is the mitochondrion; this organelle, as we demonstrate, is significantly altered by mercury. We speculate that the induction of *bcl2* and *bclx* may represent a cytoprotective response that may be particularly salutary to the mitochondrion, the latter representing a cellular locus greatly perturbed by mercury.

In summary, we demonstrate that mercury stimulates generation of oxidants, specifically hydrogen peroxide, in the kidney *in vitro* and *in vivo*, and that such generation of hydrogen peroxide contributes to renal injury. We also demonstrate that there are discrete functional effects of mercury at organelles such as the mitochondrion and the lysosomes that may be relevant to the mechanisms by which kidney damage arises. Finally, we demonstrate that in response to such oxidative stress, the kidney summons a series of oxidant-responsive genes, including heme oxygenase and members of the *bcl* family (*bcl2* and *bclx*), genes recognized as cytoprotective in other settings of oxidant stress.

Acknowledgments

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Appendix

Abbreviations used in this paper are: PBS, phosphate buffered saline; HBSS, Hank's balanced salt solution; BME, basal medium Eagle; DMEM, Dulbecco's modified Eagle's medium.

References

1. WEINBERG JM: The cellular basis of nephrotoxicity (Chapt 39), in *Diseases of the Kidney*, (5th ed, vol II), edited by SCHRIER RW, GOTTSCHALK CW, Boston, Little, Brown and Company, 1993, pp 1031–1098
2. WEINBERG JM, HARDING PG, HUMES HD: Mitochondrial bioenergetics during the initiation of mercuric chloride-induced renal injury. I. Direct effects of *in vitro* mercuric chloride on renal cortical mitochondrial function. *J Biol Chem* 257:60–67, 1982
3. SOUTHARD J, NITISEWOJO P, GREEN DE: Mercurial toxicity and the perturbation of the mitochondrial control system. *Fed Proc* 33:2147–2153, 1974
4. AMBUDKAR IS, SMITH MW, PHELPS PC, REGEC AL, TRUMP BF: Extracellular Ca^{2+}-dependent elevation in cytosolic Ca^{2+} potentiates $HgCl_2$-induced renal proximal tubular cell damage. *Toxicol Ind Health* 4:107–123, 1988
5. CHAVEZ E, HOLGUIN JA: Mitochondrial calcium release as induced by Hg^{2+}. *J Biol Chem* 263:3582–3587, 1988
6. ELLIGET KA, PHELPS PC, TRUMP BF: $HgCl_2$-induced alteration of actin filaments in cultured primary rat proximal tubule epithelial cells labelled with fluorescein phalloidin. *Cell Biol Toxicol* 7:263–280, 1991
7. MORRISON AR, PASCOE N: Modification of renal cortical subcellular membrane phospholipids induced by mercuric chloride. *Kidney Int* 29:496–501, 1986
8. TROYER DA, KREISBERG JI, VENKATACHALAM MA: Lipid alterations in LLC-PK_1 cells exposed to mercuric chloride. *Kidney Int* 29:530–538, 1986
9. BOHETS HH, VAN THIELEN MN, VAN DER BIEST I, VAN LANDEGHEM GF, D'HAESE PC, NOUWEN EJ, DEBROE ME, DIERICKX PJ: Cytotoxicity of mercury compounds in LLC-PK_1, MDCK and human proximal tubular cells. *Kidney Int* 47:395–403, 1995
10. GSTRAUNTHALER G, PFALLER W, KOTANKO P: Glutathione depletion and *in vitro* lipid peroxidation in mercury or maleate induced acute renal failure. *Biochem Pharm* 32:2969–2972, 1983
11. MEISTER A, ANDERSON ME: Glutathione. *Annu Rev Biochem* 52:711–760, 1994
12. GIRARDI G, ADRIANA TM, MONICA EM: The implication of renal glutathione levels in mercuric chloride nephrotoxicity. *Toxicology* 58:187–195, 1989
13. GIRARDI G, ELIAS MM: Effectiveness of N-acetylcysteine in protecting against mercuric chloride-induced nephrotoxicity. *Toxicology* 67:155–164, 1991
14. KLEINMAN JG, MCNEIL JS, SCHWARTZ JH, HAMBURGER RJ, FLAMENBAUM W: Effect of dithiothreitol on mercuric chloride- and uranyl nitrate-induced acute renal failure in the rat. *Kidney Int* 12:115–121, 1977
15. VALLEE BL, ULMER DD: Biochemical effects of mercury, cadmium and lead. *Ann Rev Biochem* 41:91–128, 1972
16. LUND BO, MILLER DM, WOODS JS: Mercury-induced H_20_2 production and lipid peroxidation *in vitro* in rat kidney mitochondria. *Biochem Pharm* 42:S181–S187, 1991
17. PALLER, MS: Free radical scavengers in mercuric chloride-induced acute renal failure in the rat. *J Lab Clin Med* 105:459–463, 1985
18. STACEY NH, KAPPUS H: Cellular toxicity and lipid peroxidation in response to mercury. *Toxicol Appl Pharmacol* 63:29–35, 1982
19. GUIDET BR, SHAH SV: *In vivo* generation of hydrogen peroxide by rat kidney cortex and glomeruli. *Am J Physiol* 256(Renal Fluid Electrol Physiol 25):F158–F164, 1989
20. WALKER PD, SHAH SV: Gentamicin enhanced production of hydrogen peroxide by renal cortical mitochondria. *Am J Physiol* 253(Cell Physiol 22):C495–C499, 1987
21. GUIDET BR, SHAH SV: Enhanced *in vivo* H_2O_2 generation by rat kidney in glycerol-induced renal failure. *Am J Physiol* 257(Renal Fluid Electrol Physiol 26):F440–F445, 1989
22. NATH KA, ENRIGHT H, NUTTER LM, FISCHEREDER MF, ZOU JN, HEBBEL RP: The effect of pyruvate on oxidant injury to isolated and cellular DNA. *Kidney Int* 45:166–176, 1994
23. BANATI RB, ROTHE G, VALET G, KREUTZBERG GW: Respiratory burst activity in brain macrophages: A flow cytometric study on cultured rat microglia. *Neuropathol Applied Neurobiol* 17:223–230, 1991
24. LEMMI CAE, PELIKAN PCD, SIKKA SC, HIRSCHBERG R, GEESAMAN B, MILLER RL, PARK KS, LIU SC, KOYLE M, RAJFER J: Cyclosporine augments renal mitochondrial function in vivo and reduces renal blood flow. *Am J Physiol* 257(Renal Fluid Electrol Physiol 26):F837–F841, 1989
25. HYSLOP PA, SKLAR LA: A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: Its use in the simultaneous fluorimetric assay of cellular activation processes. *Anal Biochem* 141:280–286, 1984
26. EMAUS RK, GRUNWALD R, LEMASTERS JJ: Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: Spectral and metabolic properties. *Biochim Biophys Acta* 850:436–448, 1986
27. DENIZOT F, LANG R: Rapid colorimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Meth* 89:271–277, 1986
28. ABOK K, HIRTH T, ERICSSON JLE, BRUNK U: Effect of iron on the stability of macrophage lysosomes. *Virchows Arch* 43:85–101, 1983
29. ZDOLSEK J, ZHANG H, ROBERG K, BRUNK U: H_2O_2-mediated damage to lysosomal membranes of J-774 cells. *Free Rad Res Comms* 18:71–85, 1993
30. NATH KA, PALLER MS: Dietary deficiency of antioxidants exacerbate ischemia reperfusion injury in the rat. *Kidney Int* 38:1109–1117, 1990
31. NATH KA, BALLA G, VERCELLOTTI GM, BALLA J, JACOB HS, LEVITT MD, ROSENBERG ME: Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J Clin Invest* 90:267–270, 1992
32. NATH KA, NGO EO, HEBBEL RP, CROATT AJ, ZHOU B, NUTTER LM: Alpha-keto acids scavenge hydrogen peroxide *in vitro* and *in vivo* and reduce menadione-induced DNA injury and cytotoxicity. *Am J Physiol* 268(Cell Physiol 37):C227–C236, 1995
33. CHOMCZYNSKI P, SACCHI N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
34. FANG W, RIVARD JJ, MUELLER DL, BEHRENS TW: Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. *J Immunol* 153:4388–4391, 1994
35. FANG W, RIVARD JJ, GANSER JA, LEBIEN TW, NATH KA, MUELLER DL, BEHRENS TW: Bcl-x_L rescues WEHI 231 B lymphocytes from oxidant-mediated death following diverse apoptotic stimuli. *J Immunol* 155:66–75, 1995
36. CORREA-ROTTER R, MARIASH CN, ROSENBERG ME: Loading and transfer control for Northern hybridization. *Biotechniques* 12:154–158, 1992
37. PIMSTONE NR, ENGEL P, TENHUNEN R, SEITZ PT, MARVER HS, SCHMID R: Inducible heme oxygenase in the kidney: A model for the homeostatic control of hemoglobin catabolism. *J Clin Invest* 50:2042–2050, 1971
38. AEBI H: Catalase, in *Methods of enzymatic analysis* (vol 2) edited by BERGMEYER HU, New York, Academic Press, 1974, pp 673–684
39. LAWRENCE RA, BURK RF: Glutathione peroxidase activity in selenium deficient rat liver. *Biochem Biophys Res Commun* 95:351–358, 1976
40. NATH KA, AGARWAL A, VOGT B: Functional consequences of induction of heme oxygenase (chapt 5), in *Contemporary Issues in Nephrology. "Acute renal failure: emerging concepts and therapeutic strategies,"*

edited by GOLIGORSKY MS, series editor, STEIN J, New York, Churchill Livingstone, 1995, pp 97–118
41. CADENAS E, BOVERIS A: Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* 188:31–37, 1980
42. ZDOLSEK JM, SVENSSON I: Effect of reactive oxygen species on lysosomal membrane integrity. A study on a lysosomal fraction. *Virchows Arch* 64:401–406, 1993
43. ATCHISON WD, HARE MF: Mechanisms of methylmercury-induced neurotoxicity. *FASEB J* 8:622–629, 1994
44. BOHME M, DIENER M, RUMMEL W: Chloride secretion induced by mercury and cadmium: Action sites and mechanisms. *Toxicol Appl Pharmacol* 114:295–301, 1992
45. BOHME M, DIENER M, MESTRES P, RUMMEL W: Direct and indirect actions of $HgCl_2$ and methyl mercury chloride on permeability and chloride secretion across the rat colonic mucosa. *Toxicol Appl Pharmacol* 114:285–294, 1992
46. JUNGWIRTH A, RITTER M, PAULMICHL M, LANG F: Activation of cell membrane potassium conductance by mercury in cultured renal epitheloid (MDCK) cells. *J Cell Physiol* 146:25–33, 1991
47. KARNISKI LP: Hg^{2+} and Cu^{+} are ionophores, mediating Cl^{-}/OH^{-} exchange in liposomes and rabbit renal brush border membranes. *J Biol Chem* 267:19218–19225, 1992
48. SUMMERS AO: Organization, expression, and evolution of genes for mercury resistance. *Ann Rev Microbiol* 40:607–634, 1986
49. OKEN DE, MENDE CW, TARABA I, FLAMENBAUM W: Resistance to acute renal failure afforded by prior renal failure: Examination of the role of renal renin content. *Nephron* 15:131–142, 1975
50. UEDA N, SHAH SV: Apoptosis. *J Lab Clin Med* 124:169–77, 1994
51. VEIS DJ, SORENSON CM, SHUTTER JR, KORSMEYER SJ: Bcl-2 deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. *Cell* 75:229–240, 1993
52. KANE DJ, SARAFIAN TA, ANTON R, HAHN H, GRALLA EB, VALENTINE JS, ORD T, BREDESEN DE: Bcl-2 inhibition of neural death: Decreased generation of reactive oxygen species. *Science* 262:1274–1277, 1993
53. HOCKENBERY DM, OLTVAI ZN, YIN X-M, MILLIMAN CL, KORSMEYER SJ: Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75:241–251, 1993
54. BOISE LH, GONZALEZ-GARCIA M, POSTEMA CE, DING L, LINDSTEN T, TURKA LA, MAO X, NUNEZ G, THOMPSON CB: Bcl-x. a Bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74:597–608, 1993
55. MOTOYAMA N, WANG F, ROTH KA, SAWA H, NAKAYAMA K, NAKAYAMA K, NEGISHI I, SENJU S, ZHANG Q, FUJII S, LOH D: Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* 267:1506–1510, 1995