Biochemical Pharmacology, Vol. 45, No. 10, pp. 2017–2024, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

STUDIES ON Hg(II)-INDUCED H_2O_2 FORMATION AND OXIDATIVE STRESS *IN VIVO* AND *IN VITRO* IN RAT KIDNEY MITOCHONDRIA

BERT-OVE LUND,* DENNIS M. MILLER and JAMES S. WOODS†
Department of Environmental Health, University of Washington, Seattle, WA 98195, U.S.A.

(*Received* 17 *November* 1992; *accepted* 1 *February* 1993)

Abstract—Studies were undertaken to investigate the principal actions underlying mercury-induced oxidative stress in the kidney. Mitochondria from kidneys of rats treated with $HgCl_2$ (1.5 mg/kg i.p.) demonstrated a 2-fold increase in hydrogen peroxide (H_2O_2) formation for up to 6 hr following Hg(II) treatment using succinate as the electron transport chain substrate. No increase in H_2O_2 formation was observed when NAD-linked substrates (malate/glutamate) were used, suggesting that Hg(II) affects H_2O_2 formation principally at the ubiquinone-cytochrome *b* region of the mitochondrial respiratory chain *in vivo*. Together with increased H_2O_2 formation, mitochondrial glutathione (GSH) content was depleted by more than 50% following Hg(II) treatment, whereas formation of thiobarbiturate reactive substances (TBARS), indicative of mitochondrial lipid peroxidation, was increased by 68%. Studies *in vivo* revealed a significant concentration-related depolarization of the inner mitochondrial membrane following the addition of Hg(II) to mitochondria isolated from kidneys of untreated rats. This effect was accompanied by significantly increased H_2O_2 formation, GSH depletion and TBARS formation linked to both NADH dehydrogenase (rotenone-inhibited) and ubiquinone-cytochrome *b* (antimycin-inhibited) regions of the electron transport chain. Oxidation of pyridine nucleotides (NAD[P]H) was also observed in mitochondria incubated with Hg(II) *in vitro*. In further studies *in vitro*, the potential role of Ca^{2+} in Hg(II)-induced mitochondrial oxidative stress was investigated. Ca^{2+} alone (30–400 nmol/mg protein) produced no increase in H_2O_2 and only a slight increase in TBARS formation when incubated with kidney mitochondria isolated from untreated rats. However, Ca^{2+} significantly increased H_2O_2 and TBARS formation elicited by Hg(II) at the ubiquinone-cytochrome *b* region of the mitochondrial electron transport chain, whereas TBARS formation was decreased significantly when the Ca^{2+} uptake inhibitors, ruthenium red or [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), were included with Hg(II) in the reaction mixtures. These findings support the view that Hg(II) causes depolarization of the mitochondrial inner membrane with consequent increased H_2O_2 formation. These events, coupled with Hg(II)-mediated GSH depletion and pyridine nucleotide oxidation, create an oxidant stress condition characterized by increased susceptibility of mitochondrial membranes to iron-dependent lipid peroxidation (TBARS formation). Since increased H_2O_2 formation, GSH depletion and lipid peroxidation were also observed *in vivo* following Hg(II) treatment, these events may underlie oxidative tissue damage caused by mercury compounds. Moreover, Hg(II)-induced alterations in mitochondrial Ca^{2+} homeostasis may excerbate Hg(II)-induced oxidative stress in kidney cells.

Mercury(II) (Hg(II)) is an established nephrotoxicant, primarily causing injury to kidney proximal tubule cells [1–4]. Numerous studies *in vivo* and *in vitro* have demonstrated that renal proximal tubule cell mitochondria are a principal target of Hg(II) effects, as indicated by mitochondrial swelling [2, 3], impairment of oxidative phosphorylation [5–7], and ATP depletion [8]. Lipid peroxidation [9–11], DNA damage [12], porphyrinogen oxidation [13, 14] and depletion of reduced glutathione (GSH‡) [15, 16] by Hg(II) support an oxidative stress-like mechanism for Hg(II) toxicity.

The mitochondrial electron transport chain is the principal site of cellular production of reactive oxidants, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), with approximately 2–5% of the O_2 consumed in state 4 respiration resulting in H_2O_2 formation [17, 18]. H_2O_2 is metabolized by mitochondrial GSH peroxidase, leading to formation of oxidized glutathione (GSSG). It has been calculated that the normal rate of H_2O_2 formation and metabolism leads to a turnover of 10% of the mitochondrial GSH content per minute [19]. GSSG is recycled to GSH at the expense of NADPH by mitochondrial GSSG reductase. Thus, an increased generation of H_2O_2 may shift the normal ratios of GSH/GSSG and NADPH/$NADP^+$, a condition that in the presence of transition metals, such as iron, may cause oxidation of mitochondrial macromolecules, such as lipids or DNA [20].

In previous studies [9], we demonstrated that Hg(II) enhances H_2O_2 production from both the ubiquinone-cytochrome *b* (antimycin-inhibited) and the NADH dehydrogenase (rotenone-inhibited) regions of the rat kidney mitochondrial electron

* Present address: Department of Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Box 573, Biomedicum, S-571 23, Uppsala, Sweden.

† Corresponding author: Dr. James S. Woods, Battelle Seattle Research Center, 4000 NE 41st St., Seattle, WA 98105. Tel. (206) 528-3111; FAX (206) 528-3550.

‡ Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; TBARS, thiobarbiturate reactive substances; MDA, malondialdehyde.

transport chain *in vitro*, and have suggested that this effect may underlie Hg(II)-induced oxidative stress in kidney cells. In the present report, we describe enhanced renal mitochondrial H_2O_2 formation, GSH depletion and lipid peroxidation *in vivo* following treatment of rats with $HgCl_2$. We also describe studies *in vitro* aimed at characterizing the specific mechanisms by which Hg(II) increases mitochondrial H_2O_2 production and the relationship of this effect to Hg(II)-induced mitochondrial oxidative stress. Parameters chosen for evaluation include measures of mitochondrial membrane potential, GSH depletion, pyridine nucleotide oxidation and lipid peroxidation. Inasmuch as changes in mitochondrial redox status have been linked to disturbance of mitochondrial calcium homeostasis [21, 22], the possible role of Ca^{2+} in Hg(II)-induced mitochondrial oxidative stress reactions also was evaluated. Proposed events underlying Hg(II)-induced oxidative stress in kidney mitochondria are described.

MATERIALS AND METHODS

Materials. Antimycin, $HgCl_2$ (+99.999%), horseradish peroxidase (Type VI-A), NADH, rotenone, ruthenium red, scopoletine and thiobarbituric acid were obtained from the Sigma Chemical Co., St. Louis, MO. Monobromobimane and rhodamine 123 were purchased from Calbiochem, La Jolla, CA, and Molecular Probes, Inc., Eugene, OR, respectively. Other chemicals were reagent grade and were purchased from standard commercial sources. Solutions were prepared in doubly deionized water ($HgCl_2$, ruthenium red); 50 nM HEPES buffer, pH 7.5 (horseradish peroxidase, NADH); dimethyl sulfoxide (Me_2SO) (rotenone); or ethanol (antimycin, scopoletine, rhodamine 123).

Animal treatments. Male Sprague–Dawley rats (200–250 g) were acquired from Tyler Laboratories, Bellevue, WA, and were housed in the University of Washington vivarium in plastic cages (3/cage) with unlimited access to food (Wayne Rodent Blox) and deionized water. Animal facilities were maintained at 22 ± 1° and on a 12-hr light/dark cycle. For studies *in vivo* rats were fasted overnight, then injected intraperitoneally either with saline (controls) or $HgCl_2$ (1.5 or 2.25 mg/kg) (3/group) and killed by exsanguination 1–6 hr later, as indicated in figures and tables. These dosages have been determined from previous studies [23, 24] as sufficient to elicit mild or moderate oxidative stress in kidney cells without causing severe nephrotoxicity. Kidneys were immediately excised and placed in ice-cold 250 mM sucrose, 3 mM EDTA, 50 mM Tris buffer, pH 7.4. The renal cortex was dissected quickly for mitochondrial preparation. Renal mitochondria from fasted untreated rats were used for *in vitro* studies.

Mitochondrial preparation. Rat kidney cortical mitochondria were prepared by the method of Johnson and Lardy [25] with slight modifications, as previously described [9]. Final mitochondrial pellets were suspended in 10 mM Tris, 30 mM 4-morpholinepropanesulfonic acid (MOPS), 225 mM mannitol, 75 mM sucrose buffer, pH 7.5, except for mitochondria used to assess lipid peroxidation, which were suspended in 30 mM Tris, 155 mM KCl buffer, pH 7.5. $MgCl_2$ (5 mM) was included in the buffers as indicated in the tables and figures. In studies where electron transport inhibitors were employed, mitochondria were preincubated with the indicated transport inhibitor (rotenone or antimycin) for 1 min before $HgCl_2$ (0–30 nmol Hg/mg; 0–15 μM Hg) (in 1–5 μL distilled water) and substrate were added to incubation mixtures.

H_2O_2 assay. Mitochondrial H_2O_2 formation was measured spectrofluorometrically by the H_2O_2 and horseradish peroxidase-dependent oxidation of scopolectine at 450 nm following excitation at 365 nm, according to Boveris [26], as previously described [9]. This method continuously measures H_2O_2 that diffuses from the mitochondria to the surrounding incubation medium.

Lipid peroxidation assay. Iron-dependent (20 μM Fe^{3+}: 100 μM ADP) lipid peroxidation was assayed spectrophotometrically at 535 nm as thiobarbituric acid reactive substances (TBARS) ($E = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) formed after a 30-min incubation of mitochondria, according to Buege and Aust [27]. For assessment of lipid peroxidation in mitochondria from kidneys of mercury-treated rats, 3 mg of mitochondrial protein was washed twice with 1 mL of 1.15% KCl, 0.2% nicotinamide (to remove sucrose from isolated mitochondria), and 0.05% butylated hydroxyanisole (BHA) (to prevent artifactual oxidation). Mitochondria were then resuspended in 1 mL KCl, nicotinamide, BHA, mixed with 2 mL of TBA reagent (15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl), and heated at 70° for 45 min in a water bath. TBARS were calculated from the change in absorbance at 535 versus 510 nm, following subtraction of absorbances of mitochondria incubated in the absence of TBA. TBARS (malondialdehyde [MDA]) (nmol) were determined from a standard curve prepared using MDA bis(dimethyl acetal).

Glutathione assay. Mitochondria (2 mg/mL), prepared as above, were layered onto dibutylphthalate and centrifuged at 15,000 *g* into 5-sulfosalicylic acid, as described by Olafsdottir and Reed [28]. GSH was then derivatized with monobromobimane and analyzed by reverse phase HPLC, as previously described [9].

Other assays. Mitochondrial membrane potential was measured spectrofluorometrically at 527 nm using the potentiometric fluorescent dye, rhodamine 123 (1 μM), as a relative measure of mitochondrial membrane potential, following excitation at 503 nm, as described by Emaus *et al.* [29]. Pyridine nucleotide (NADPH) oxidation was measured spectrofluorometrically at 450 nm following excitation at 340 nm. Mitochondrial mercury concentrations (total mercury) were determined by cold vapor atomic absorption spectrometry, as described by Atallah and Kalman [30]. Protein concentrations were determined according to Smith *et al.* [31], using bovine serum albumin (BSA) as a standard.

Statistical analyses. Statistical analyses were conducted using a one-way analysis of variance (ANOVA) and by Dunnett's *t*-test. Data in Table 2 were analyzed using Student's *t*-test. $P < 0.05$ was chosen as the level of significance.

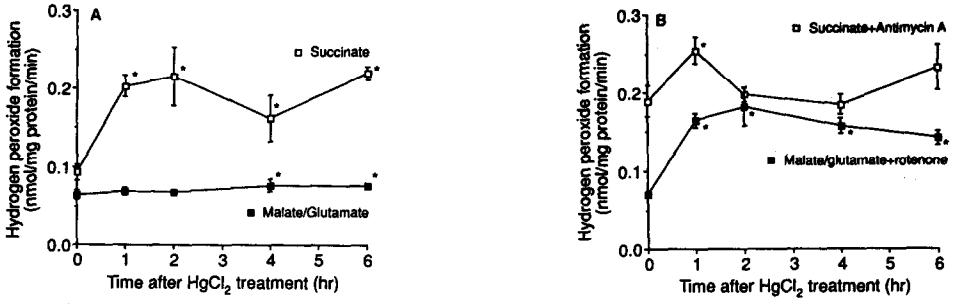

Fig. 1. Effects of Hg(II) treatment on H_2O_2 formation in isolated renal cortical mitochondria. Rats were treated with $HgCl_2$ (1.5 mg/kg, i.p.). Mitochondria were isolated at the indicated times following treatment and incubated (0.33 to 0.5 mg/mL) in Tris–MOPS–mannitol–sucrose–$MgCl_2$ buffer, pH 7.5, at 30° in the presence of respiratory substrates (6 mM succinate or 2.5/2.5 mM malate/glutamate) (A) or substrates and electron transport chain inhibitors (1 nmol/mg antimycin A or 3 nmol/mg rotenone) (B). H_2O_2 formation was measured spectrofluorometically using H_2O_2 and horseradish peroxidase-dependent scopoletine oxidation, as described in Materials and Methods. Control mitochondria were obtained from rats treated with vehicle (saline) 2 hr before they were killed. Values are means ± SD of 4 determinations. Key: * statistically different from control rates ($P < 0.05$).

RESULTS

The rates of H_2O_2 formation in kidney mitochondria isolated from rats treated with $HgCl_2$ (1.5 mg/kg i.p.) 1–6 hr before being killed are shown in Fig. 1. H_2O_2 formation increased 2-fold 1 hr after $HgCl_2$ injection and remained elevated for at least 6 hr following mercury treatment when succinate was used as the respiratory chain substrate (Fig. 1A). However, no apparent changes in the rate of H_2O_2 formation were observed when the NAD-linked substrates, malate/glutamate, were used. As shown in Fig. 1B, when antimycin A, an inhibitor of electron transport at the ubiquinone-cytochrome *b* region, was added to reaction mixtures, H_2O_2 formation by mitochondria from Hg(II)-treated rats was elevated significantly above that observed in zero time controls only at 1 hr following $HgCl_2$ injection, i.e. addition of antimycin A to the reaction mixture did not substantially increase the rate of mitochondrial H_2O_2 formation at the ubiquinone-cytochrome *b* region above that promoted by Hg(II) treatment. In contrast, when rotenone, an inhibitor of electron transport at the NADH dehydrogenase region, was employed, an almost 3-fold increase in the rate of H_2O_2 formation was observed throughout the entire 6 hr post-treatment assessment period.

As shown in Fig. 1A, a maximal rate of H_2O_2 formation of approximately 0.2 nmol/min/mg mitochondrial protein was observed 1–2 hr following $HgCl_2$ injection when succinate was used as the respiratory chain substrate. This effect occurred despite the fact that the mitochondrial mercury concentration did not achieve maximal levels of 0.57 ± 0.01 nmol/mg protein (mean ± SD; N = 4) until 4 hr following $HgCl_2$ treatment. Mitochondrial mercury content at 2 hr post-treatment was 0.40 ± 0.01 nmol/mg protein (N = 3).

Increased renal mitochondrial H_2O_2 production following $HgCl_2$ treatment was accompanied by a time- and dose-dependent decrease in mitochondrial GSH content. As shown in Table 1, the GSH concentration in renal mitochondria declined progressively to 82 and 73% of zero time control levels at 1 and 2 hr, respectively, following $HgCl_2$ treatment at 1.5 mg/kg. A more substantial decline in mitochondrial GSH content to 49% of zero time control levels was observed in kidney mitochondria from rats treated with $HgCl_2$ at 2.25 mg/kg at 2 hr following treatment.

Table 1. Effects of $HgCl_2$ treatment on renal mitochondrial GSH content *in vivo*

$HgCl_2$ (mg/kg)	GSH (nmol/mg protein) Time after treatment (hr) 0	1	2
1.5	6.04 ± 0.36	4.96 ± 1.41	4.42 ± 0.24
2.25	5.18 ± 0.96	4.35 ± 1.13	2.52 ± 0.82*

Kidney cortical mitochondria (2.0 mg/mL) were prepared from $HgCl_2$-treated rats at the times indicated. Controls (zero time) received saline injections only. GSH content was determined as described in Materials and Methods. Values are means ± SD of triplicate determinations (3 rats) in which mitochondrial GSH concentrations from each were measured in duplicate.

* Significantly different from control, $P < 0.05$.

TBARS formation, an indicator of lipid peroxidation, was measured in renal mitochondria isolated from rats treated with $HgCl_2$ at 1.5 mg/kg. The rate of TBARS formation in mitochondria from zero time controls was 0.094 ± 0.015 nmol MDA/mg protein, whereas rates from rats at 1 and 2 hr following mercury treatment were 0.114 ± 0.049 and 0.158 ± 0.025 nmol MDA/mg protein, respectively, suggestive of increased lipid peroxidation due to Hg(II) treatment. These increases in TBARS

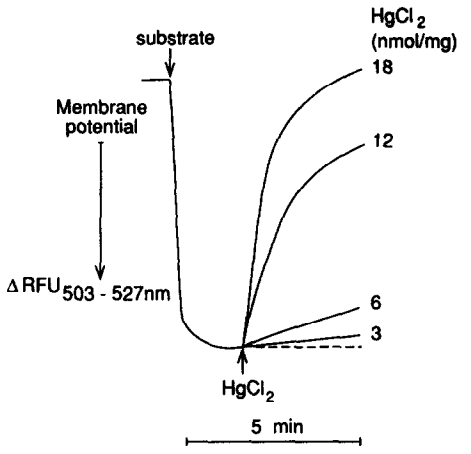

Fig. 2. Effects of Hg(II) on kidney mitochondrial membrane potential. Mitochondria (0.4 mg/mL) were incubated in Tris–MOPS–mannitol–sucrose buffer, pH 7.5, at 30° and relative membrane potential was measured spectrofluorometrically using rhodamine 123. The figure shows concentration-dependent effects of $HgCl_2$ on mitochondrial membrane potential.

formation in mitochondria from $HgCl_2$-treated rats, however, were not statistically significant ($P < 0.05$).

Studies *in vitro* were conducted to investigate the potential mechanisms underlying the observed increase in H_2O_2 formation following mercury treatment and to relate this effect to mercury-induced oxidative stress. H_2O_2 formation is known to be increased by agents that disrupt the ionic flux across the mitochondrial inner membrane [32]. Initial studies were conducted, therefore, to measure the effect of Hg(II) on the mitochondrial membrane potential. Mitochondrial incorporation of the cationic fluorescent dye, rhodamine 123, is dependent on the maintenance of an electrochemical potential across the inner mitochondrial membrane [33]; hence, dissipation of electrochemical potential is indicated by a decrease in fluorescence of this dye [34]. Figure 2 shows that Hg(II) causes a concentration-dependent decrease in rhodamine fluorescence (membrane potential) when added to suspensions of rat kidney mitochondria *in vitro*. At concentrations of 3–18 nmol/mg protein, Hg(II) produced a decrease in fluorescence compared with controls, consistent with a dissipation of electrochemical potential in mitochondria using either succinate (6 mM) or malate/glutamate (2.5/2.5 mM) as substrate. Hg(II) did not affect the fluorescence of rhodamine in the absence of mitochondria in the reaction mixture.

The increased rate of mitochondrial H_2O_2 formation and loss of membrane potential following Hg(II) addition were accompanied by depletion of mitochondrial GSH content and increased formation of TBARS linked to specific sites of the mitochondrial electron transport chain. As shown in Table 2, a 50–60% increase in H_2O_2 formation was observed over a 10-min incubation period from both NADH dehydrogenase (rotenone-inhibited) and ubiquinone-cytochrome *b* (antimycin-inhibited) regions of the electron transport chain following addition of Hg(II) (18 nmol/mg protein) to reaction mixtures. Also, as shown in Table 2, a significant depletion of mitochondrial GSH content, by greater than 50%, and increased lipid peroxidation, assessed by formation of TBARS, were observed at both regions of the electron transport chain following incubation of mitochondria with Hg(II) (12 nmol/mg protein) for 30 min. Mitochondrial GSH concentrations prior to initiation of the 30-min incubation period were comparable to those reported in Table 1.

A decrease in pyridine nucleotide redox status is a well-established cause of increased oxidant stress [35, 36]. Therefore, the redox state of pyridine nucleotides in electron transport-inhibited mitochondria exposed to Hg(II) was evaluated. As shown in Fig. 3, Hg(II), at concentrations greater than 12 nmol/mg mitochondrial protein, caused a concentration-dependent increase in the rate of NAD(P)H oxidation when added to kidney mito-

Table 2. Effects of $HgCl_2$ on mitochondrial H_2O_2 formation, GSH content and TBARS formation *in vitro* at specific sites of the mitochondrial electron transport chain

Addition	H_2O_2 (nmol/mg/min)	GSH (nmol/mg)	TBARS* (nmol MDA/mg/30 min)
Succinate + antimycin (Control 1)	0.23 ± 0.01	1.15 ± 0.14	6.71 ± 0.03
Control 1 + Hg(II)	0.37 ± 0.05†	0.43 ± 0.05†	7.83 ± 0.12†
Malate/glutamate + rotenone (Control 2)	0.06 ± 0.01	1.37 ± 0.17	3.46 ± 0.23
Control 2 + Hg(II)	0.09 ± 0.01‡	0.64 ± 0.05‡	15.70 ± 0.38‡

Mitochondria (0.33 to 0.5 mg/mL) were prepared from rat kidney cortex and incubated with succinate (6 mM) + antimycin (1 nmol/mg) or malate/glutamate (2.5/2.5 mM) + rotenone (3 nmol/mg) at 30°, as described in Materials and Methods. $HgCl_2$ was dissolved in doubly distilled water and added where indicated at 18 nmol/mg for H_2O_2 assay and 12 nmol/mg for GSH and TBARS assays. H_2O_2 formation was followed for 10 min, whereas GSH and TBARS concentrations were determined after 30-min incubations, as described in Materials and Methods. GSH concentrations at zero time were 4.60 ± 0.56 and 5.48 ± 0.68 nmol/mg in Control 1 and Control 2 mitochondrial preparations, respectively. Values are means ± SD of 3 or 4 separate determinations. TBARS formation was linear for 30 min.

* Fe:ADP (20:100 μM) present in reaction mixtures.

† Significantly different from Control 1, $P < 0.05$.

‡ Significantly different from Control 2, $P < 0.05$.

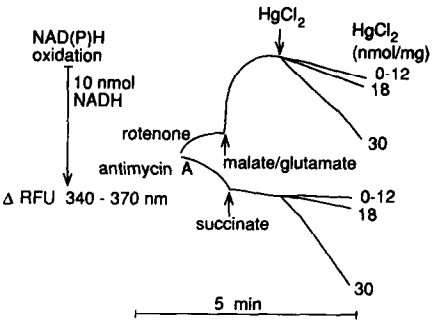

Fig. 3. Effects of Hg(II) on pyridine nucleotide oxidation in electron transport-inhibited mitochondria. Mitochondria (0.5 mg/mL) were incubated in Tris–MOPS–mannitol–sucrose buffer, pH 7.5, at 30°, and pyridine-nucleotide oxidation was followed spectrofluorometrically. Antimycin (1 nmol/mg protein) or rotenone (3 nmol/mg protein) was preincubated with mitochondria before addition of substrate (6 mM succinate or 2.5/2.5 mM malate/glutamate, first arrow) and 0–30 nmol/mg $HgCl_2$ (second arrow).

Table 3. Effects of Hg(II) and Ca^{2+} on H_2O_2 formation at specific sites of the mitochondrial electron transport chain

Addition	H_2O_2 formation (nmol/mg protein/min) + Rotenone	+ Antimycin
Substrate* (control)	0.07 ± 0.01	0.24 ± 0.01
Hg(II)	0.16 ± 0.01†	0.53 ± 0.07†
Ca^{2+}	0.08 ± 0.01	0.28 ± 0.01
Hg(II) + Ca^{2+}	0.22 ± 0.02†	0.32 ± 0.02†

Mitochondria (0.33 to 0.5 mg/mL) were incubated in the presence of either rotenone (3 nmol/mg) or antimycin (1 nmol/mg) and the appropriate substrate, indicated below. $HgCl_2$ (18 nmol/mg) or $CaCl_2$ (400 nmol/mg) was added where indicated. $MgCl_2$ (5 mM) was present in all reaction mixtures. H_2O_2 formation was determined by the H_2O_2 and horseradish peroxidase-dependent oxidation of scopoletine, as indicated in Materials and Methods. Values are means ± SD of triplicate determinations.

* Substrates employed were malate/glutamate (2.5/2.5 mM) with rotenone and succinate (6 mM) with antimycin.

† Significantly different from control, $P < 0.05$.

chondria supplemented with either malate/glutamate and rotenone or succinate and antimycin.

Finally, the possible role of calcium (Ca^{2+}) in Hg(II)-induced H_2O_2 formation and lipid peroxidation was investigated. Mitochondrial Ca^{2+} homeostasis is sustained through processes dependent upon maintenance of the mitochondrial inner membrane potential as well as the reduced status of GSH [37] and pyridine nucleotides [36]. Ca^{2+} cycling resulting from Hg(II)-induced disruption of these parameters could therefore contribute to membrane damage, uncoupling of oxidative phosphorylation, and increased reactive oxidant formation. To test this possibility, the effects of Ca^{2+} and the calcium uptake inhibitors, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA) and ruthenium red, on Hg(II)-induced H_2O_2 formation and lipid peroxidation (TBARS formation) by renal mitochondria were evaluated. As shown in Table 3, Hg(II) produced a substantial increase in H_2O_2 formation from both NADH dehydrogenase (rotenone-inhibited) and ubiquinone-cytochrome *b* (antimycin-inhibited) sites of the electron transport chain. In contrast, Ca^{2+} alone had no influence on H_2O_2 formation from either region. However, Ca^{2+} appeared to increase the rate of Hg(II)-promoted H_2O_2 formation from the NADH dehydrogenase site. Similar observations were made with respect to Hg(II)-promoted lipid peroxidation. As shown in Table 4, renal mitochondria incubated with either malate/glutamate and rotenone or succinate and antimycin (control) demonstrated moderate levels of TBARS formation in the absence of either Hg(II) or Ca^{2+}. Addition of Hg(II) promoted a 4.8-fold increase in TBARS formation linked to the NADH dehydrogenase site, although not from the antimycin-inhibited region. Ca^{2+} (30 nmol/mg protein) alone did not increase TBARS formation above control levels at either site. However, Ca^{2+} increased the rate of TBARS formed at both sites when added with Hg(II). Ruthenium red, which prevents mitochondrial Ca^{2+} uptake, significantly reduced TBARS formation in control mitochondria. When added, following addition of Hg(II), ruthenium red significantly decreased (2 nmol/mg) or prevented (5 nmol/mg) TBARS formation observed in the presence of Hg(II) alone. Finally, EGTA (50 μM) a calcium chelator, also prevented Hg(II)-induced TBARS formation.

DISCUSSION

The present studies support the view that oxidative injury to kidney cortical cell mitochondria may underlie nephrotoxicity associated with mercury exposure. Previous studies from this laboratory [3, 9] and others [6, 7, 38] have demonstrated that the principal toxic effects of Hg(II) arise from alterations in the structural integrity of the mitochondria inner membrane, resulting in loss of the normal cation selectivity which permits it to participate effectively in oxidative metabolism. In this respect, Hg(II) markedly increases the normally low permeablity of the mitochondrial inner membrane to K^+ and Mg^{2+}, resulting in acceleration of the energy-dependent efflux of these cations at the expense of efficient oxidative phosphorylation [6]. Recent studies [39] suggest that Hg(II) may also mediate electroneutral exchange of Cl^-/OH^- across lipid membranes, further contributing to the collapse of ionic and pH gradients. Previous studies from this laboratory [9] have shown that the action of Hg(II) to perturb mitochondrial inner membrane function is accompanied by depletion of mitochondrial reduced glutathione content and increased formation of H_2O_2 by the mitochondrial electron transport chain *in vitro*. The present studies suggest that such reactions occur *in vivo* following treatment with $HgCl_2$, and, moreover, that increased H_2O_2 formation may

Table 4. Effects of Hg(II), Ca^{2+} and Ca^{2+} antagonists on iron-dependent lipid peroxidation at specific sites of the mitochondrial electron transport chain

Addition	TBARS formation (nmol MDA/mg protein/30 min)	
	+ Rotenone	+ Antimycin
Substrate* (control)	3.19 ± 0.18	2.04 ± 0.33
Hg(II)	15.31 ± 1.12†	2.81 ± 0.84
Ca^{2+}	4.23 ± 0.72	3.25 ± 1.07
Hg(II) + Ca^{2+}	17.92 ± 1.54†	4.60 ± 0.09†
Substrate* + RR (2 nmol/mg)	0.27 ± 0.07†	0.08 ± 0.13†
Hg(II) + RR (2 nmol/mg)	8.04 ± 1.65†	0.38 ± 0.13†
Hg(II) + RR (5 nmol/mg)	0.00†	0.00†
Hg(II) + EGTA	0.37 + 0.13†	0.00†

Mitochondria (0.33 to 0.5 mg/mL) were incubated in the presence of either rotenone (3 nmol/mg) or antimycin (1 nmol/mg) and the appropriate substrate, indicated below. $HgCl_2$ (12 nmol/mg), $CaCl_2$ (30 nmol/mg), ruthenium red (RR), and EGTA (50 μM) were added where indicated. Fe:ADP (20:100 μM) was present in all reaction mixtures. TBARS formation was determined spectrophotometrically at 535 nm, as described in Materials and Methods. Values are means ± SD of triplicate determinations.

* Substrates employed were as described in Table 3.

† Significantly different from control, $P < 0.05$.

be accompanied by increased peroxidation of mitochondrial lipids, consistent with an oxidative stress-like condition. The rapid increase in H_2O_2 production observed 1 hr following the injection of $HgCl_2$ further implies that this effect of Hg(II) may be involved in the generation of nephrotoxicity. The mitochondrial concentration of Hg(II) found in the present studies following $HgCl_2$ treatment (0.4 to 0.6 nmol/mg protein) was similar to that reported by Weinberg *et al.* [7], showing impaired oxidative phosphorylation 1 hr after exposure to Hg(II) as the earliest sign of toxicity.

The GSH content of renal mitochondria from untreated rats observed in this study (5–6 nmol/mg protein) is comparable to that reported by other investigators [40]. Several studies *in vitro* [28, 40–42] using preparations from various tissues have suggested that depletion of mitochondrial, rather than cytosolic, GSH is more critically related to development of chemical-induced cell injury. Whether the modest decrease in renal mitochondria GSH content observed in the present studies after treatment of rats with 1.5 mg/kg $HgCl_2$ is consistent with the production of nephrotoxicity is difficult to assess without knowing the relative contribution of all events that underlie Hg(II)-induced kidney cell damage. However, GSH depletion has been shown to contribute to altered mitochondrial membrane permeability, impaired antioxidant defense mechanisms and loss of Ca^{2+}, pyridine nucleotide and protein thiol homeostases [43]. It is possible, therefore, that even modest depletion of mitochondrial GSH content could exacerbate the direct prooxidant actions of Hg(II) observed in the present studies, contributing to oxidant stress and the development of neurotoxicity during mercury exposure. In this respect, mitochondrial GSH depletion would be viewed as a contributing, rather than sufficient, cause of mercury-induced nephrotoxicity. Further studies are required to quantitate the specific contribution of mitochondrial GSH depletion to this effect.

A significant increase in mitochondrial H_2O_2 formation following injection of rats with $HgCl_2$ was detected when succinate was included in the mitochondrial reaction mixture (Fig. 1A), supporting the view that the ubiquinone-cytochrome *b* region is the principal site of mitochondrial H_2O_2 formation *in vivo* [32]. The significantly increased H_2O_2 formation observed (Fig. 1A) when mitochondria from $HgCl_2$-treated rats were incubated in the absence of an electron transport chain inhibitor (antimycin) suggests that Hg(II) interacts directly with the succinate dehydrogenase/ubiquinone-cytochrome *b* region to promote H_2O_2 formation. This view is further supported by the observation that the addition of antimycin to the reaction mixture did little to increase further H_2O_2 formation at that site (Fig. 1B). Telkkä and Mustakallio [44] also have shown histochemically that $HgCl_2$ inhibits succinate dehydrogenase in rat kidney tubule cells, supporting the hypothesis that Hg(II) directly increases the H_2O_2 formation by interacting with the ubiquinone-cytochrome *b* region of the mitochondrial electron transport chain. In contrast, $HgCl_2$ treatment had little effect on mitochondrial H_2O_2 formation at the NADH dehydrogenase region (Fig. 1A), unless rotenone, an inhibitor of electron transport at that site, was also present in the reaction mixture (Fig.1B). As Hg(II) exposure has been shown to increase the cellular oxidation of NAD(P)H, one might speculate that a lowered NADH concentration could increase the utilization of succinate as a primary substrate for the electron transport chain, further increasing the potential for mitochondrial H_2O_2 formation during Hg(II) exposure.

A role for Ca^{2+} in Hg(II)-induced oxidative stress is suggested from the present studies. The mitochondrial inner membrane possesses a uniport carrier that allows mitochondria to sequester Ca^{2+} in relatively high concentrations [45]. Collapse of the membrane potential, such as occurs with Hg(II) exposure, allows Ca^{2+} to rapidly exit mitochondria via the uniport [21, 46]. Moreover, Ca^{2+} efflux from mitochondria through an alternative antiport has been shown to be promoted by oxidation of mitochondrial pyridine nucleotides [36, 47] or GSH [37]. The existence of different Ca^{2+} uptake and release pathways in mitochondria provides the basis for Ca^{2+} cycling [48], leading to membrane damage, impaired respiration and increased reactive oxidant formation [36, 47, 49]. The present results suggest that Hg(II) may stimulate this process by causing a collapse of the inner mitochondrial membrane potential and changes in mitochondrial redox balance. The impairment of Hg(II)-induced TBARS formation by the Ca^{2+} uptake inhibitors ruthenium red and EGTA might, therefore, be interpreted in light of the property of these agents to prevent Ca^{2+} reuptake by mitochondria, hence, amelioration of Ca^{2+} cycling and the asssociated toxicologic consequences. Alternatively, EGTA or ruthenium red could have effects on iron-dependent lipid peroxidation, such as iron chelation or perturbation of iron binding, independently of effects on Ca^{2+} homeostasis.

The relevance of the present findings to effects of Hg(II) *in vivo* seems likely in light of studies demonstrating the toxic effects of Hg(II) on mitochondria of different cellular origin. Thus, while the effects of Hg(II) *in vivo* are localized primarily to cells of the SIII segment of the renal proximal tubule, studies *in vitro* have documented mercury-induced oxidative damage to mitochondria from hepatocytes [8], heart [38], and other tissues [50, 51]. These findings suggest that mitochondria, regardless of cellular origin, respond similarly to Hg(II) *in vitro*, despite the fact that the toxicokinetics of mercury may predispose only specific portions of the nephron susceptible to Hg(II) toxicity in the intact organism. Thus, while the present studies employed mitochondria from renal cortical preparations containing cells from various segments of the nephron (although predominantly from the proximal tubule), we view the findings as representative of the action of Hg(II) in the kidney *in vivo*. Further studies, however, are required to substantiate the target cell selectivity of Hg(II) in this tissue.

In summary, Hg(II) perturbs the inner membrane structure and bioenergetics of kidney mitochondria with associated increased H_2O_2 production, GSH depletion, oxidation of pyridine nucleotides and lipid peroxidation. Inasmuch as increased H_2O_2 formation, GSH depletion and lipid peroxidation are also observed in renal cortical mitochondria following Hg(II) treatment, these events may underlie oxidative tissue damage and nephrotoxicity caused by mercury compounds.

Acknowledgements—We thank Ms. Cherie Luckhurst for performing mitochondrial mercury analyses. This research was supported by Grants ES03628, ES04696 and T23-ES07032 from the National Institutes of Health. B. L. was partially supported by the Swedish Institute.

REFERENCES

1. Nicholson JK, Timbrell JA and Sadler PJ, Proton NMR spectra of urine as indicators of renal damage. Mercury-induced nephrotoxicity in rats. *Mol Pharmacol* **27**: 644–651, 1985.
2. Goyer RA and Rhyne BC, Toxic changes in mitochondrial membranes and mitochondrial function. In: *Pathobiology of Cell Membranes* (Eds. Trump BF and Arstila AU), Vol. 1, pp. 383–428. Academic Press, New York, 1975.
3. Fowler BA and Woods JS, Ultrastructural and biochemical changes in renal mitochondria following chronic oral methyl mercury exposure: The relationship to renal function. *Exp Mol Pathol* **27**: 403–412, 1977.
4. Woods JS, Mechanisms of metal-induced alterations of cellular heme metabolism. *Comments Toxicol* **3**: 3–25, 1989.
5. Southard JH and Nitisewojo P, Loss of oxidative phosphorylation in mitochondria isolated from kidneys or mercury poisoned rats. *Biochem Biophys Res Commun* **52**: 921–927, 1973.
6. Weinberg JM, Harding PG and 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.
7. Weinberg JM, Harding PG and Humes HD, Mitochondrial bioenergetics during the initiation of mercuric chloride-induced renal injury. II. Functional alterations of renal cortical mitochondria isolated after mercuric chloride treatment. *J Biol Chem* **257**: 68–74, 1982.
8. Nieminen A-L, Gores GJ, Dawson TL, Herman B and Lemasters JJ, Toxic injury from mercuric chloride in rat hepatocytes. *J Biol Chem* **265**: 2399–2408, 1990.
9. Lund B-O, Miller DM and Woods JS, Mercury-induced H_2O_2 production and lipid oxidation *in vitro* in rat kidney mitochondria. *Biochem Pharmacol* **42**: S181–S187, 1991.
10. Yonaha M, Itoh E, Ohbayashi Y and Uchiyama M, Induction of lipid peroxidation in rats by mercuric chloride. *Res Commun Chem. Pathol Pharmacol* **28**: 105–112, 1980.
11. Benov LC, Benchev IC and Monovich OH, Thiol antidotes effect on lipid peroxidation in mercury-poisoned rats. *Chem Biol Interact* **76**: 321–332, 1990.
12. Cantoni O, Evans RM and Costa M, Similarity in the acute cytotoxic response of mammalian cells to mercury(II) and X-rays: DNA damage and glutathione depletion. *Biochem Biophys Res Commun* **108**: 614–619, 1982.
13. Woods JS, Calas CA, Aicher LD, Robinson BH and Mailer C, Stimulation of porphyrinogen oxidation by mercuric ion. I. Evidence of free radical formation in the presence of thiols and hydrogen peroxide. *Mol Pharmacol* **38**: 253–260, 1990.
14. Woods JS, Calas Ca and Aicher LD, Stimulation of porphyrinogen oxidation by mercuric ion. II. Promotion of oxidation from the interaction of mercuric ion, glutathione, and mitochondria-generated hydrogen peroxide. *Mol Pharmacol* **38**: 261–266, 1990.
15. Gstraunthaler G, Pfaller W and Kotanko P, Glutathione depletion and *in vitro* lipid peroxidation in mercury or maleate induced acute renal failure. *Biochem Pharmacol* **32**: 2969–2972, 1983.
16. Chung A-S, Maines MD and Reynolds WA, Inhibition of the enzymes of glutathione metabolism by mercuric chloride in the rat kidney: Reversal by selenium. *Biochem Pharmacol* **31**: 3093–3100, 1982.
17. Boveris A, Oshino N and Chance B, The cellular

production of hydrogen peroxide. *Biochem J* **128**: 617–630, 1972.
18. Turrens JF and Boveris A, Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* **191**: 421–427, 1980.
19. Sies H and Moss KM, A role of mitochondrial glutathione peroxidase in modulating mitochondrial oxidation in liver. *Eur J Biochem* **84**: 377–383, 1978.
20. Chance B, Sies H and Boveris A, Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59**: 527–605, 1979.
21. Reed DJ, Review of the current status of calcium and thiols in cellular injury. *Chem Res Toxicol* **3**: 495–502, 1990.
22. Nicotera P, Bellomo G and Orrenius S, The role of Ca^{2+} in cell killing. *Chem Res Toxicol* **3**: 484–494, 1990.
23. Woods JS and Fowler BA, Renal porphyrinuria during chronic methyl mercury exposure. *J Lab Clin Med* **90**: 266–272, 1977.
24. Woods JS and Southern MR, Studies on the etiology of trace metal-induced porphyria: Effects of porphyrinogenic metals on coproporphyrinogen oxidase in rat liver and kidney. *Toxicol Appl Pharmacol* **97**: 183–190, 1990.
25. Johnson D and Lardy H, Isolation of liver or kidney mitochondria. In: *Methods in Enzymology* (Eds. Estabrook RW and Pullman ME), Vol. 10, pp. 94–96. Academic Press, New York, 1967.
26. Boveris A, Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 429–435. Academic Press, Orlando, 1984.
27. Buege JA and Aust SD, Microsomal lipid peroxidation. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 302–310. Academic Press, New York, 1977.
28. Olafsdottir K and Reed DJ, Retention of oxidized glutathione by isolated rat liver mitochondria during hydroperoxide treatment. *Biochim Biophys Acta* **964**: 377–382, 1988.
29. Emaus RK, Grunwald R and 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.
30. Atallah RH and Kalman DA, Selective determination of inorganic and methyl mercury in tissues by continuous flow and cold vapor atomic absorption spectrometry. *J Anal Toxicol* **17**: 87–92, 1993.
31. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto GK, Goere NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
32. Cadenas E and Boveris A, Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* **188**: 1–37, 1980.
33. Ehrenberg B, Montana V, Wei M-D, Wuskell JP and Loew LM, Membrane potential can be determined in individual cells from the Nernstian distribution of cationic dyes. *Biophys J* **53**: 785–794, 1988.
34. Bunting JR, Phan TV, Kamali E and Cowben RM, Fluorescent cationic probes of mitochondria. Metrics and mechanism of interaction. *Biophys J* **56**: 979–993, 1989.
35. Scott MD, Zuo L, Lubin BH and Chiu DT-Y, NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes. *Blood* **77**: 2059–2064, 1991.
36. Lehninger AL, Vercesi A and Bababunmi EA, Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides. *Proc Natl Acad Sci USA* **75**: 1690–1694, 1978.
37. Beatrice MC, Stiers DL and Pfeiffer DR, The role of glutathione in the retention of Ca^{2+} by liver mitochondria *J Biol Chem* **259**: 1279–1287, 1984.
38. Southard JH, Penniston JT and Green DE, Induction of transmembrane proton transfer by mercurials in mitochondria. I. Ion movements accompanying transmembrane proton transfer. *J Biol Chem* **248**: 3546–3550, 1973.
39. 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.
40. McKernan TB, Woods EB and Lash LH, Uptake of glutathione by renal cortical mitochondria. *Arch Biochem Biophys* **288**: 653–663, 1991.
41. Romero FJ and Roma J, Careful consideration of the effects induced by glutathione depletion in rat liver and heart. The involvement of cytosolic and mitochondrial glutathione pools. *Chem Biol Interact* **70**: 29–37, 1989.
42. Lash LH and Anders MW, Mechanism of *S*-(1,2-dichlorovinyl)-L-cysteine- and *S*-(1,2-dichlorovinyl)-homocysteine-induced renal mitochondrial toxicity. *Mol Pharmacol* **32**: 549–556, 1987.
43. Reed DJ, Glutathione: Toxicological implications. *Annu Rev Pharmacol Toxicol* **30**: 603–631, 1990.
44. Telkkä A and Mustakallio KK, Proximal or distal mercurial inhibition of succinic dehydrogenase in the kidney tubules of rat. *Science* **121**: 146, 1955.
45. Scarpa A and Azzone GF, The mechanism of ion translocation in mitochondria. 4. Coupling of K^{+} efflux with Ca^{2+} uptake. *Eur J Biochem* **12**: 328–335, 1970.
46. Chavez E and Holguin JA, Mitochondrial calcium release as induced by Hg^{2+}. *J Biol Chem* **263**: 3582–3587, 1988.
47. Bellomo G, Martino A, Richelmi P, Moore GA, Jewell SA and Orrenius S, Pyridine-nucleotide oxidation, Ca^{2+} cycling and membrane damage during *tert*-butyl hydroperoxide metabolism by rat-liver mitochondria. *Eur J Biochem* **140**: 1–6, 1984.
48. Gunter TE and Pfeiffer DR, Mechanisms by which mitochondria transport calcium. *Am J Physiol* **258**: C755–C786, 1990.
49. Masini A, Ceccarelli D, Trenti T, Corongui FP and Muscatello U, Perturbation of liver mitochondrial Ca^{2+} homeostasis in experimental iron overload: A possible factor in cell injury. *Biochim Biophys Acta* **1014**: 133–140, 1989.
50. Christie NT and Costa M, *In vitro* assessment of the toxicity of metal compounds. IV. Disposition of metals in cells: Interactions with membranes, glutathione, metallothionein, and DNA. *Biol Trace Element Res* **6**: 139–158, 1984.
51. Miura K and Imura N, Mechanisms of methylmercury cytotoxicity. *CRC Crit Rev Toxicol* **18**: 161–188, 1987.