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.