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UP-REGULATION OF GLUTATHIONE SYNTHESIS IN RAT KIDNEY BY METHYL MERCURY

RELATIONSHIP TO MERCURY-INDUCED OXIDATIVE STRESS

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Abstract—Prolonged exposure of rats to methyl mercury hydroxide (MMH) results, during the initial phase of exposure, in the rapid accumulation of mercury as Hg^{2+} by kidney cortex and in a significant increase in oxidative stress, as characterized by the rate of formation of thiobarbituric acid reactive substances (TBARS) by renal mitochondria. These events are accompanied by a progressive increase in steady-state levels of the mRNA encoding γ-glutamylcysteine synthetase (GCS), the rate-limiting enzyme in glutathione (GSH) synthesis and a 2- to 3-fold elevation in renal cortical GSH levels. The present study showed that the increase in GSH content was accompanied by a concomitant decrease in the rate of TBARS formation. Subsequent to these initial phase events, continued MMH exposure was characterized by equilibration in the rate of renal Hg^{2+} accumulation, a sharp decrease in both the TBARS formation rate and GCS mRNA level, but sustained elevation of renal cortical GSH content. Depletion of GSH with buthionine sulfoximine subsequent to the decline in the rate of TBARS formation did not result in a rebound of the TBARS formation rate. These findings suggest that oxidative stress during the initial phase of MMH exposure is derived from the transformation of CH_3Hg^+ to Hg^{2+}, which, in turn, induces the synthesis of Hg^{2+}- and/or oxidant-scavenging GSH molecules via the up-regulation of renal GCS mRNA. The findings also suggest that resistance to Hg^{2+}-mediated oxidative stress may be more closely associated with the capacity for up-regulation of GSH synthesis than with elevated GSH levels *per se*.

Key words: glutathione; γ-glutamylcysteine synthetase; mRNA; methyl mercury; kidney; oxidative stress

GCS† is the first and rate-limiting enzyme in the synthesis of GSH, a principal endogenous antioxidant in mammalian tissues [1]. Depletion of cellular GSH, either by thiol conjugation or by inhibition of GCS, has been postulated to underlie oxidative tissue damage caused by metals and other chemical agents [2–7]. An increase in GSH levels in various tissues has been suggested to occur in response to chemical-induced oxidative stress [8–12]. The mechanism of this effect has not been determined as yet, although an oxidant stress-sensitive ARE has been identified recently in the 5′ flanking region of the human GCS gene [13], suggesting the role of reactive oxidants or other free radical species in this event.

The prooxidant properties of mercury are well established. Stacey and Kappas [14] reported induction of lipid peroxidation associated with Hg^{2+} treatment of isolated rat hepatocytes and suggested a causative role of oxidative stress in mercury cytotoxicity. Cantoni *et al.* [15, 16] subsequently demonstrated that Hg^{2+} elicits DNA strand breaks in cultured Chinese hamster ovary (CHO) cells and that this effect is associated with the production of oxygen free radicals and reduction of cellular GSH content.

In studies on the mechanisms of oxidative stress induced by mercury compounds in mammalian kidney [17–21], we have observed that, although mercury treatment initially results in a pronounced depletion of renal GSH, prolonged treatment with mercury as MMH via the drinking water, increases, rather than depletes, kidney GSH content. Elevation of renal GSH is accompanied by an early progressive increase in steady-state levels of GCS mRNA [22] and corresponds to a relative sparing of renal proximal tubule cells to oxidative tissue damage, assessed by either biochemical or ultrastructural techniques [23, 24]. Since Hg^{2+} is a potent prooxidant in mammalian kidney [17–21], this resistance to oxidative stress during prolonged MMH exposure may reflect the ability of kidney cortical cells to transform CH_3Hg^+ to Hg^{2+}, which, in turn, induces the synthesis of Hg^{2+}-scavenging GSH molecules via the genetic up-regulation of renal GCS mRNA.

In the present studies, we tested this hypothesis by measuring steady-state levels of renal GCS mRNA and GSH concentrations in relation to the renal metabolism of CH_3Hg^+ to Hg^{2+} and to TBARS formation as an index of Hg^{2+}-mediated oxidative stress potential. The findings support the view that up-regulation of renal GSH synthesis occurs as an early, rapid response to Hg^{2+}-induced oxidative stress in kidney cells.

MATERIALS AND METHODS

Materials

Male Fischer 344 rats (175–200 g) were obtained from Simonsen Laboratories, Gilroy, CA. MMH was purchased from Johnson Matthey Electronics, Ward Hill, MA. Monobromobimane was acquired from Cal-

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† Abbreviations: GCS, γ-glutamylcysteine synthetase; GSH, glutathione; MMH, methyl mercury hydroxide; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; BHA, 2,[3]-*tert*-butyl-4-hydroxyanisole); BCA, bicinchoninic acid; MDA, malondialdehyde; BSO, buthionine sulfoximine; and ARE, antioxidant response element.

biochem, La Jolla, CA. [^{32}P]ATP was obtained from ICN Radiochemicals, Irvine, CA. T_4-polynucleotide kinase was purchased from Fisher Scientific, Pittsburgh, PA. Random prime labeling kits were obtained from the Boehringer Mannheim Corp., Indianapolis, IN. The BCA protein reagent kit was acquired from the Pierce Chemical Co., Rockford, IL. GSH and guanidine thiocyanate were purchased from the Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were obtained from standard commercial sources and were of the highest purity available.

Animal treatment

Immediately upon receipt from the supplier, 36 rats were transferred to individual hanging, wire-bottom cages and permitted unlimited access to food and deionized water. Animal facilities were maintained at 22 ± 1° and on a 12-hr light/dark cycle. Following a 1-week acclimation period, rats were divided into two groups of 18 animals each and either continued on deionized water (controls) or given deionized water containing 10 ppm MMH, respectively. Water consumption was monitored daily. This concentration corresponds to an MMH dose of approximately 1.2 mg/kg/day, respectively, as previously described [24]. At weekly intervals, 3 animals from each group were killed, and renal cortical tissue was extracted for GSH, GCS mRNA, TBARS and mercury analyses. MMH treatment was continued for up to 6 weeks.

DNA probes

Detection of steady-state GCS mRNA levels in kidney cortex was accomplished as previously described [22], using a GCS cDNA probe [25] comprising a 390-bp fragment of the published sequence of the rat kidney GCS heavy subunit cDNA [26] and ^{32}P-labeled by random prime labeling (Decaprime). A 20-bp ribosomal 18S rRNA oligomer probe, provided by Dr. Curtis Omiecinski, Department of Environmental Health, was [^{32}P]ATP-labeled according to a standard 5′ end-labeling procedure [27], using T_4-polynucleotide kinase.

Assays

GSH assays were performed by HPLC according to the method of Hamel *et al.* [28], as previously described [22]. GSH content was quantitated spectrofluorometrically as the GS-bimane conjugate resulting from thiol derivatization with monobromobimane. Tissue mercury content and speciation was determined by atomic absorption by the method of Atallah and Kalman [29], as previously described [24]. Iron-dependent (20 μM Fe^{3+}: 100 μM ADP) lipid peroxidation was assayed spectrophotometrically at 535 nm as TBARS ($E = 1.56 \times 10^5$ M^{-1} cm^{-1}) formed after a 30-min incubation of mitochondria, according to Buege and Aust [30]. For assessment of lipid peroxidation in mitochondria from kidneys of MMH-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% 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 formation was linear for up to 30 min. TBARS were calculated from the change in absorbance at 535 nm versus 510 nm, following subtraction of absorbances of mitochondria incubated in the absence of TBA. TBARS (MDA) (nmol) were determined from a standard curve prepared using MDA bis(dimethyl acetal), as previously described [21]. Protein concentrations were determined according to Smith *et al.* [31], using BSA as a standard.

RNA preparation

Total RNA was extracted from renal cortical tissue by the guanidine thiocyanate method [32] and blotted directly onto a positively charged nylon filter (Boehringer Mannheim) under vacuum using a slot blot apparatus (Minifold II, Schleicher-Schuell, Keene, NH). After cross-linking and baking for 2 hr at 80°, the filter was prehybridized for 3 hr at 68° in hybridization solution containing 5 × SSC (1 × SSC = 0.15 M sodium chloride + 0.015 M sodium citrate), 1% blocking reagent, 0.1% *N*-laurylsarcosine and 0.02% SDS, and then incubated overnight at 68° in hybridization solution containing ^{32}P-labeled GCS probe. Following hybridization, the filter was washed and exposed to Kodak X-OMAT X-ray film at room temperature for 20–30 min. For 18S rRNA detection, the GCS mRNA probe was removed from the filter by rinsing in solution containing 50% formamide and 50% Tris-EDTA buffer, pH 8.0, for 2 hr at 68°. The filter was then rehybridized overnight in 50 mL oligomer-labeling buffer [27] at 52° using the ^{32}P-labeled rRNA probe. The intensities of the hybridization signals were quantitated by scanning densitometry (Hoefer Scientific Instruments). GCS mRNA levels were expressed relative to those of 18S rRNA.

Statistical analyses

Analysis of the significance of differences between groups was determined by Student's *t*-test. The level of significance was chosen at $P < 0.05$.

RESULTS

Methyl mercury (CH_3Hg^+) is rapidly taken up by the kidney cortex and converted to Hg^{2+} during the initial period of MMH exposure. As illustrated in Fig. 1, the total renal cortical mercury concentration increased progressively from 15 μg/g wet tissue (0.075 μmol/g) after week 1 to approximately 120 μg/g (0.6 μmol/g) by the end of week 6 of MMH exposure. In contrast, renal cortical CH_3Hg^+ levels declined to 74% of total renal cortical mercury content during week 1 of MMH treatment and to approximately 50% of total renal mercury content by week 3 of the treatment period. The decline in CH_3Hg^+ content was accompanied by a comparable increase in mercury as Hg^{2+}. The balance between organic and inorganic mercury species in the kidney cortex remained relatively constant between weeks 3 and 6 of the treatment period.

The first week of MMH treatment, during which CH_3Hg^+ was most rapidly converted to Hg^{2+}, was characterized by a rapid increase in the rate of TBARS formation, indicative of increased potential for oxidative stress and/or oxidative cell injury. As shown in Fig. 2, TBARS formation by renal mitochondria from MMH-treated rats increased to 2.6 times control levels during week 1 of MMH exposure and remained significantly elevated, although at somewhat lesser rates, during weeks 2 and 3 of MMH treatment, representing 1.89 and

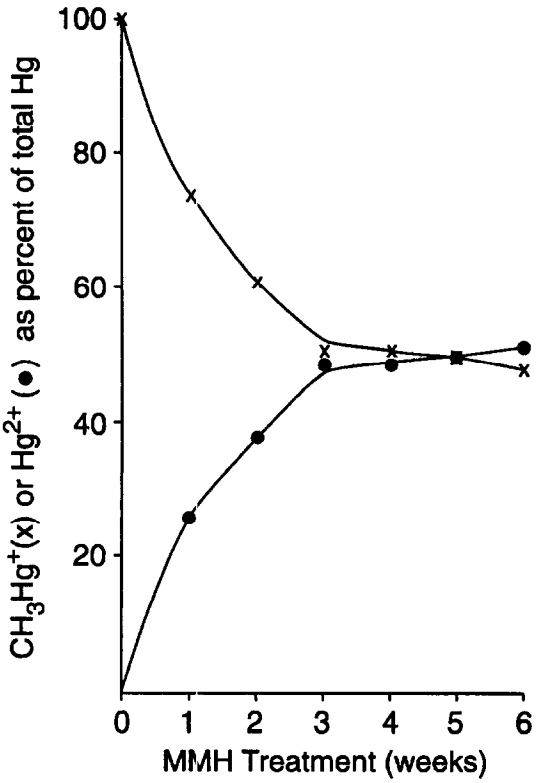

Fig. 1. Uptake and distribution of CH_3Hg^+ and Hg^{2+} by rat kidney cortex during a 6-week course of treatment with MMH (10 ppm) in drinking water. Mercury speciation analysis was performed as described in Materials and Methods. Values are the means of 3 determinations.

1.97 times control values, respectively. Subsequently, the rate of TBARS formation declined to values that remained not significantly different from control levels during the remainder of the exposure period.

The increase in renal Hg^{2+} content and TBARS formation during the first 3 weeks of MMH exposure was accompanied by a significant elevation in steady-state GCS mRNA levels. As shown in Fig. 3, GCS mRNA levels corrected for renal cortical 18S rRNA content was elevated 2.2, 3.3, and 5.2 times control values at weeks 1, 2, and 3 following initiation of MMH treatment, respectively. After week 3, GCS mRNA content declined to values that were not significantly different from those of control kidneys and remained unchanged during the remainder of the MMH exposure period.

The increase in GCS mRNA levels during the first 3 weeks of MMH treatment was accompanied by a 2- to 3-fold increase in renal cortical GSH content. As shown in Fig. 4, GSH increased concomitantly with the increase in GCS mRNA levels during the initial 3 weeks of MMH treatment and remained elevated even following the decline in GCS mRNA levels during weeks 4–6 of MMH exposure. The sustained elevation in cortical GSH content between weeks 4 and 6 of MMH treatment predominated over the decline in TBARS formation, which decreased from approximately twice control values at 3 weeks to 1.29 times control values by week 4 of MMH exposure (Fig. 4). TBARS formation remained at levels that were not significantly different from those of controls throughout the remainder of the treatment period.

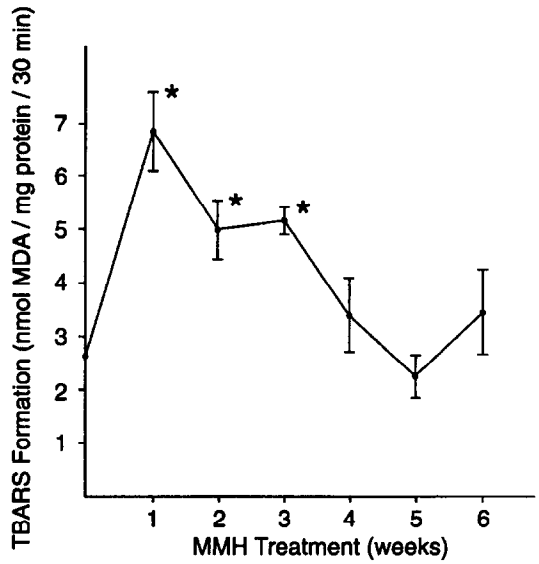

Fig. 2. Course of change in oxidative stress in rat kidney as measured by the rate of TBARS formation by renal cortical mitochondria during MMH (10 ppm) exposure in drinking water for up to 6 weeks. TBARS concentrations were measured after a 3-min incubation, as described in Materials and Methods. Values are means ± SD of 3 determinations. Key: (*) significantly different from MMH-untreated control value, $P < 0.05$.

Further studies were conducted to ascertain if the decline in TBARS formation was functionally linked to the elevation in GSH levels. In these studies, rats were treated with MMH as above for 4 weeks and then were given BSO (1 g/kg, i.p.), a selective inhibitor of GCS [33] and an effective depletor of both cytosolic and mitochondrial GSH levels [34]. Rats were then killed 12 hr later for the assessment of renal GSH content and

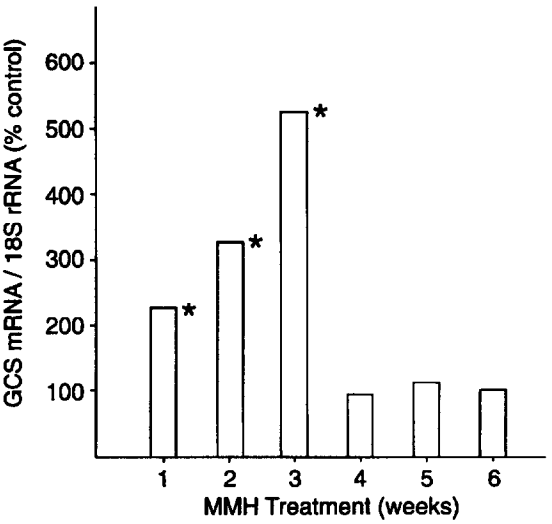

Fig. 3. Course of change in GCS mRNA steady-state level in rat kidney cortex during MMH (10 ppm) exposure in drinking water for up to 6 weeks. GCS mRNA and 18S rRNA levels were quantitated as described in Materials and Methods. Values are means of 3 determinations. Key: (*) significantly different from MMH-untreated control value, $P < 0.05$.

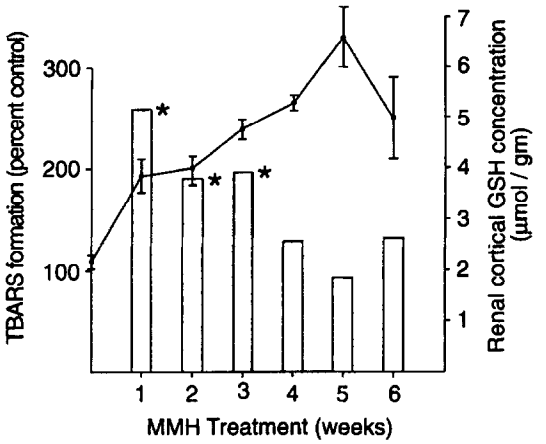

Fig. 4. Course of change in rat renal cortical GSH (●) concentration in relation to the rate of TBARS formation (bars) during MMH (10 ppm) exposure in drinking water for up to 6 weeks. GSH concentrations and mitochondrial TBARS formation rates were determined as described in Materials and Methods. GSH levels in kidneys of MMH-treated rats were significantly different ($P < 0.05$) from those of controls at all time points. TBARS formation rates in kidneys of MMH-treated rats were significantly different from those of untreated controls at the points indicated by an asterisk (*), $P < 0.05$. Values are means ± SD of 3 determinations.

TBARS formation. As indicated in Table 1, BSO treatment resulted in a sharp reduction in GSH levels in kidneys of MMH-exposed rats, to 56% of BSO non-treated levels. In contrast, the rate of TBARS formation was not elevated significantly above the level observed in MMH-exposed rats that did not receive BSO treatment.

DISCUSSION

In previous studies [17–21], we have demonstrated that prooxidant properties of Hg^{2+} in rat kidney. Central to this effect is a Hg^{2+}-mediated depolarization of the mitochondrial inner membrane, followed by a dose-dependent increase in H_2O_2 production linked to both complex I and III regions of the mitochondrial electron transport chain and a concomitant increase in iron-dependent lipid peroxidation [21]. The present studies demonstrate the likelihood that comparable effects occur *in vivo* during prolonged MMH exposure and suggest further that mercury-mediated oxidative stress, as measured by TBARS formation, occurs as a function of the rate of accumulation of Hg^{2+} by renal cortical cells following conversion from CH_3Hg^+. The conversion of organic to inorganic mercury by the kidney appears to follow first-order kinetics, indicating that the rate of Hg^{2+} formation is greatest immediately following initiation of MMH treatment. Saturation of this process is observed when the total mercury concentration reaches between 50 and 60 μg/g of kidney tissue. The present findings indicate that oxidative stress, as measured by the rate of TBARS formation, follows a comparable process, linked to the rate of Hg^{2+} accumulation by renal cortex during the initial period of MMH exposure. Similar findings have been reported previously with regard to renal porphyrinogen oxidation and excretion during prolonged MMH treatment [24].

Table 1. Effects of GSH depletion by BSO on TBARS formation in rat kidney mitochondria

Treatment	GSH (μmol/g)	TBARS (nmol MDA/mg protein/30 min)
Control	5.25 ± 0.63	2.63 ± 0.51
BSO	2.94 ± 0.74*	2.79 ± 0.27

Rats were exposed to MMH in the drinking water for 4 weeks, and then were given distilled water (control) or BSO (1 g/kg) by i.p injection 12 hr prior to being killed. Renal cortical GSH and mitochondrial TBARS formation were measured as described in Materials and Methods. Values are means ± SD of 3 determinations.

* $P < 0.05$.

A rapid increase in steady-state GCS mRNA levels was observed concomitantly with the increase in tissue Hg^{2+} content and TBARS formation during the initial 3-week period of MMH exposure. In contrast, both GCS mRNA levels and TBARS formation declined dramatically after week 4 of MMH exposure, following attainment of equilibrium between organic and inorganic mercury species in kidney cells. These observations are consistent with the hypothesis [22] that up-regulation of the GCS gene is linked mechanistically to oxidative stress-sensitive events mediated by Hg^{2+} in kidney cells and, moreover, that the rate of GCS up-regulation occurs as a function of the rate of Hg^{2+}-induced oxidant formation. Mulcahy and Gipp [13] have reported recently the presence of an ARE, a *cis*-acting regulatory element that is responsive to oxidative stress [35], in the 5′ flanking region of the human GCS gene, supporting this view. Although not yet demonstrated with respect to the ARE of the GCS gene, the AREs found in the regulatory domains of genes encoding other antioxidant enzymes, e.g. type 1 NAD(P)H:quinone oxidoreductase [36] and GSH *S*-transferase-Ya [37], are known to be regulated via the transcriptional proteins, Fos and Jun, whose binding to the ARE is regulated by the reduced or oxidized status of cysteine residues in their DNA binding domain [38]. Hg^{2+} promotes increased production of reactive oxygen species via deregulation of mitochondrial electron transport [18, 20, 21] as well as through its interaction with reduced GSH [17], possibly directly or indirectly promoting GCS up-regulation via such modulation of ARE-binding capability. Further studies are in progress to define the nature of the specific molecular events involved in this process.

GSH is considered to play an important role in the regulation of intracellular levels of reactive oxygen species by direct reaction, scavanging, and via the glutathione peroxidase system [1]. In the present studies, the functional relationship between cellular GSH content and mercury-mediated oxidative stress during chronic mercury exposure is questioned by the finding that the rate of TBARS formation was attenuated only in concert with the rapid increase in GSH levels during the first 3 weeks of MMH exposure, but was not elevated substantially following depletion of steady-state GSH levels by BSO. If the dramatically lowered TBARS levels observed after 4 weeks of MMH treatment were attributable solely to elevated GSH, a notable increase in TBARS formation subsequent to BSO treatment would

have been anticipated. That no such elevation in TBARS formation was observed suggests that the GSH concentration *per se* may be less important in terms of protecting cell constituents from oxidative injury than the overall capacity to increase GSH levels as an initial response to oxidative stress. Findings from other studies that elevated GSH levels did not afford resistance to drug- [39] or radiation- [40] mediated oxidative stress support this view. Should this be the case, these findings would be consistent with the argument [41] that GSH serves more importantly in the immediate and early, rather than sustained, protection of cellular constituents from oxidative insult derived from a variety of sources including that associated with mercury compounds.

In conclusion, the present studies describe the rapid and early up-regulation of GSH synthesis at the level of GCS mRNA associated with prolonged Hg^{2+} accumulation by rat kidney. A corresponding increase in oxidant stress levels was attenuated in association with the rapid increase in the cellular GSH levels during the initial rate of Hg^{2+} accumulation. However, the prolonged elevation in renal GSH content did not appear to be associated directly with the sustained attenuation of oxidant stress formation that accompanies equilibration of Hg^{2+} content following the initial phase of chronic MMH exposure. The findings suggest that CH_3Hg^+ is converted rapidly to Hg^{2+}, which, in turn, promotes up-regulation of GSH synthesis as an initial adaptive response to Hg^{2+}-mediated oxidative stress in kidney cells.

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