Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins

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The mechanisms of toxicity and, particularly, the potential carcinogenicity of inorganic mercury are still under debate. Results of mutagenicity and genotoxicity testing with mercury have been inconsistent; mercury induces DNA single-strand breaks at low concentrations in mammalian cells but has not proved mutagenic in several bacterial mutagenicity assays. We investigated the mutagenicity of subtoxic concentrations of inorganic mercury and the role of metal-binding proteins and free radicals in this process. A mutagenicity assay using NIH 3T3 cells, transfected with a vector containing lacZ as a reporter for mutational events, was applied. In this model, inorganic mercury significantly increased the mutation frequency in the lacZ gene, even at the lowest concentration tested. The mutation frequency was greatest at an Hg²⁺ concentration of 0.5 µM. To identify the mechanisms involved, different cellular responses to non-cytotoxic concentrations of HgCl₂ were measured. Hg²⁺ increased the intracellular amount of reactive oxygen species. This induction of oxidative stress was observed, although the intracellular glutathione (GSH) and metallothionein (MT) concentrations were increased significantly. Mercury-induced MT expression was even more pronounced after GSH depletion. Correspondingly, significant increase in the cellular GSH concentration (Lash and Zalups, 1996) and in induction of synthesis of MT in vitro (Aschner et al., 1997) and in vivo (Sugawara et al., 1998; Zalups et al., 1999). Generally, binding to GSH and MT is considered as a mechanism of cellular defence, as both GSH and MT sequester, transport and inactivate reactive metal ions including mercury (O’Brien and Salacinski, 1998; Miura et al., 1995). However, as the resulting metal-protein complexes also represent the transport form in the intact organism, these complexes may contribute to the accumulation of heavy metals in certain tissues, particularly the kidneys (Miura et al., 1995).

Hg²⁺ forms complexes with GSH, and moderate doses of HgCl₂ increase GSH concentration, for example in the renal cortex (Zalups and Lash, 1996). The elevated GSH levels appear to be the consequence of increased GSH synthesis, as a simultaneous increase in the activity of γ-glutamyl-cysteine synthetase, which catalyses the synthesis of GSH, has been observed (Lash and Zalups, 1996). However, at toxic doses of HgCl₂, depletion of GSH from the kidney occurs, resulting in the oxidation of reduced porphyrins, a typical feature of mercury toxicity (Gstaunthaler et al., 1983; Woods et al., 1990a,b). This GSH depletion is considered to contribute to the oxidative damage caused by metal ions, as GSH is an important scavenger of radicals (Fukino et al., 1986).

GSH participates in normal cell metabolism and acts as radical scavenger (Dekant et al., 1994). It is easily oxidized by a two-electron process, thereby protecting other cellular structures against oxidation (Munday, 1994). However, GSH could also act as a pro-oxidant under physiological conditions (Kachur et al., 1998), catalysing reactions that lead to a significant increase in H₂O₂ and OH production. If the oxidation of GSH involves a one-electron process, it leads to formation of the glutathione thyl radical (GS) (Munday, 1994). Under physiological conditions and in the presence of oxygen, these thyl radicals can form disulfide radical anions, superoxide radicals and hydrogen peroxide, contributing to cellular oxidative damage and thus DNA damage (Garcia de la Asuncion et al., 1996; Cadet et al., 1997; Cotgreave and Gerdes, 1998).

Mercury induces DNA single-strand breaks at low concentrations in mammalian cells but has given negative results in several bacterial mutagenicity assays (Rossmann, 1995). Bacterial mutagenicity assays may not indicate DNA damage resulting from free radical formation and oxidative stress (Ariza et al., 1994). As previous in vitro data are inconclusive (Schoey, 1996; Ariza and Williams, 1996), in contrast to in vivo data, which indicate that Hg²⁺ causes renal tumors in rodents, we re-evaluated the mutagenicity of Hg²⁺, using a

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mutagenicity assay with NIH 3T3 cells, transfected with a vector containing the lacZ gene as a reporter for mutagenicity (de Groene, 1995). NIH 3T3 cells have a consistent level of cellular GSH and produce MT in response to heavy metal exposure, so they were thought suitable for use in investigating the mutagenicity of subtoxic concentrations of inorganic mercury and the role of metal-binding proteins and free radicals in this process.

Materials and methods

Materials

Mercury chloride (HgCl$_2$·2H$_2$O) was obtained from Fluka Chemika (Bornem, The Netherlands) from which a stock solution (50 mM in double-distilled water) was prepared. L-Buthionine-[S,R]-sulfoximine (BSO) was purchased from Janssen Chimica (Beerse, Belgium). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, t-glutamine, penicillin, and streptomycin were all purchased from Gibco BRL–Life Technologies (Breda, The Netherlands).

Cadmium radioisotope ($^{109}$CdCl$_2$, sp. act. 5 mCi/ml) was from Dupont (Mechelen, Belgium). Neutral Red (NR), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethylmethane sulfonate (EMS), NADPH, GSH, glutathione reductase (50 U/ml), 5,5’-dithio-bis-(2-nitrobenzoic acid) (DTNB), triethanolamine, sulfoalkylclic acid, Tris and hemoglobin were obtained from Sigma Chemical Co. (Zwijndrecht, The Netherlands). 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) was obtained from Molecular Probes (Leiden, The Netherlands). Ampicillin, X-Gal, Electric Max DH10B cells, HBS, calcium chloride, lysis buffer, proteinase K, chloroform:isoamyl alcohol, phenol:chloroform:isoamyl alcohol, LB agar, reagent D (Dw рестro enzyme) were from Gibco BRL–Life Technologies (Veennendaal, The Netherlands). Plasmid shuttle vector pSVSPORTI with a lacZ fragment was kindly provided by Dr E.de Groene (TNO Zeist, The Netherlands). Plasmid transformation was performed with an Escherichia coli Pulser and quartz cuvettes from Bio-Rad (Richmond, CA, USA).

Cell culture

NIH 3T3 cells (ATCC no. CRL 1658) were subcultured at 37°C in a 5% CO$_2$ atmosphere in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin.

Cytotoxicity tests

The effect of different concentrations of mercury chloride (0.1 µM–1 mM) was examined in NIH 3T3 cells using the NR uptake assay initially described by Nemes et al. (1979) and the MTT test according to Borenfreund et al. (1988). Cells were exposed to HgCl$_2$ for 24 h. Absorbance was measured spectrophotometrically at 540 nm for the NR test and 595 nm for the MTT assay.

Measurement of H$_2$O$_2$

Reactive oxygen species were measured essentially as described by Trayner et al. (1995) using H$_2$DCFDA as a fluorescent probe. Briefly, NIH 3T3 cells, cultured in 96-well plates, were pre-incubated with 10 µM H$_2$DCFDA in Krebs–Ringer–phosphate–glucose buffer (10 mM, pH 7.4) for 30 min. This solution was replaced by different concentrations of mercury (0.25–10 µM) in Krebs–Ringer–phosphate–glucose buffer. In order to deplete intracellular GSH, cells were cultured and incubated with medium additionally containing 5 µM BSO. After various incubation times (2–48 h), the fluorescence of the intracellular trapped probe oxidized by H$_2$O$_2$ was measured in a spectrophotofluorimeter (excitation 485 nm, emission 538 nm). Results were calculated as a percentage of the values in controls (which were incubated without Hg).

Determination of GSH

GSH levels were measured according to Griffith (1980). Briefly, per sample 4×10$^4$ cells were incubated with 10 nM DMEM containing 0.25, 0.5, 1.0, 5.0 or 10.0 µM Hg, with and without an additional pretreatment with 5 µM BSO. After 6 h, the cells were washed with PBS and collected by scraping in 0.5 ml PBS–EDTA–Triton. The lysed cells were incubated on ice for 5 min with 5 µl sulfoalkylcic acid and then centrifuged at 13 000 r.p.m. for 10 min. The supernatant was neutralized with 200 µl triethanolamine. To 25 µl of this sample, 100 µl of 6 mM DTNB and 825 µl of 0.25 mM NADPH (both dissolved in 100 mM phosphate–EDTA buffer, pH 7.5) were added. After incubation with 0.5 units of GSH reductase, the increase in extinction caused by the enzymatically induced reduction of the available GSH was measured spectrophotometrically at 412 nm. Total protein content was determined according to Lowry et al. (1951). Results were expressed as mmol GSH/g total protein.

Determination of MT

The cellular level of MT was determined by the $^{109}$Cd hemoglobin affinity assay (Eaton and Toal, 1982). Cells, cultured in Petri dishes (10 cm diameter), were scraped and suspended in 1 ml of 10 mM Tris–HCl (pH 7.4), lysed by sonication and heat treated (2 min, 100°C); 200 µl of the sample was saturated by incubation with 200 µl $^{109}$Cd solution (0.8 µCi/ml, 2.7 µg Cd/ml in Tris–HCl). Non-MT-bound $^{109}$Cd was centrifuged (10 min, 13 000 r.p.m.) after binding to 100 µl of 2% hemoglobin in Tris–HCl, followed by heat denaturation (2 min, 100°C). This step was repeated at least once before the radioactivity in 400 µl of the supernatant was determined. The Cd content of the sample allows direct calculation of its MT content (Onosaka and Cherian, 1982), which was related to the total protein content measured.

Mutation analysis

NIH 3T3 cells were transfected with pSVSPORTI-lacZ using the calcium phosphate co-precipitation method (Trottier et al., 1992). Six hours after plating, the cells were washed with PBS and incubated for 18 h at 37°C and 5% CO$_2$ in supplemented DMEM. Thereafter, different Hg concentrations were applied, with and without BSO. EMS (250 µg/ml), a direct mutagen, served as a positive control (de Groene et al., 1995). After 48 h incubation, the shuttle vector was extracted from the cells using the Hirt extraction protocol (Hirt, 1967). Plasmids were used to transform DH10B bacteria, which were then plated on LB agar containing 40 µg X-gal/ml. The mutation frequency was determined by the ratio of mutant (white) colonies to the total amount of colonies formed.

Data analysis

Statistical analysis included analysis of variance (ANOVA) followed by Student’s t-test. The concentrations that resulted in 50% inhibition of cell culture viability (IC$_{50}$) and their correlation coefficients (r) in the toxicity assays were calculated using a non-linear regression model for the sigmoid curve with variable slope (GraphPad Prism V2.0a). P values of <0.05 were considered statistically different.

Results

Cytotoxicity

To determine the cytotoxicity of mercury chloride in NIH 3T3 cells, a wide concentration range (0.1 µM–10 mM Hg) was tested. Two different endpoints were assessed: cellular membrane integrity (assessed by means of the NR uptake assay) and loss of succinate dehydrogenase activity (assessed using the MTT assay). As shown in Figure 1, incubation of NIH 3T3 cells with inorganic mercury resulted in a dose-dependent response, which differed depending on the endpoint of toxicity. From these data the IC$_{50}$ was calculated: it was 97.9 µM Hg for the NR test (r = 0.99) and 149.1 µM Hg for the MTT test (r = 0.99). The IC$_{5}$ was 6.5 µM Hg (r = 0.72).
and 5.8 µM Hg ($r = 0.71$) for the NR and MTT tests, respectively.

At a concentration of 5 µM Hg, a cell viability rate exceeding 95% could be expected. Considering this finding, the concentration range used in all following assays was selected.

**Intracellular GSH concentration and the influence of BSO**
In NIH 3T3 cells incubated with 0.5 and 1.0 µM Hg, the intracellular GSH concentrations increased (Figure 2). Higher concentrations, such as 5.0 and 10.0 µM of inorganic mercury, decreased the GSH content.

To deplete the cellular GSH concentration, cells were incubated with 5 µM BSO, an inhibitor of glutathione synthesis. This BSO concentration had been evaluated in an initial cytotoxicity experiment (data not shown), which indicated that 5 µM BSO decreased cell viability by <30%. The effects of Hg/BSO co-incubations on cellular GSH concentrations are shown in Figure 3. As expected, BSO decreased the intracellular GSH levels significantly at the concentrations tested. It also prevented GSH induction by low levels of Hg.

**Intracellular MT concentration and the influence of GSH depletion**
The effect of different Hg$^{2+}$ concentrations on the intracellular MT content was measured in NIH 3T3 cells. As shown in Figure 2, inorganic mercury induced MT synthesis. A gradual dose-dependent increase in MT concentration was observed up to an Hg$^{2+}$ concentration of 5 µM, followed by a decline at 10 µM, a concentration at which Hg$^{2+}$ was cytotoxic (see Figure 1). The additional incubation with BSO resulted in an additional increase in intracellular MT (Figure 4), particularly at low doses. This increase was significant in comparison with non-Hg-treated controls, as well as with the respective cell cultures incubated with Hg$^{2+}$ alone.

**Radical formation**
The formation of reactive oxygen intermediates was monitored with a fluorescence probe allowing continuous measurements. The results, presented in Table I, indicate a rapid increase in radical formation within the first 2 h.

**Mutagenicity of mercury chloride**
Exposure of NIH 3T3 cells to inorganic mercury resulted in a significant increase in the mutation frequency of the lacZ reporter gene (Figure 5). At Hg$^{2+}$ concentrations of 0.25, 0.5 and 1.0 µM the mutation frequency was increased by a factor of 8.7, 9.7 and 8.3, respectively. Co-incubation of the cells with Hg$^{2+}$ and BSO resulted in a further increase in the mutation frequency, with a positive dose–response correlation in the concentration range tested (0.0–1.0 µM Hg$^{2+}$) (Figure 6). At the two higher concentrations, 0.5 and 1.0 µM Hg$^{2+}$, the mutation frequency was equal to that of positive control, 500 µM EMS, which resulted in a mutation rate of ~3.6×10$^{-4}$.

**Discussion**
The mechanisms of toxicity and particularly the potential carcinogenicity of inorganic mercury are still under debate, as the induction of renal tumors has only been observed in rats, and mutagenicity and genotoxicity testing *in vitro* have given inconsistent results. HgCl$_2$ was not mutagenic in the Ames test with different *Salmonella typhimurium* type strains, but was found to be genotoxic in the Mutatox and SOS tests (Codina *et al*., 1995). The failure to detect mutagenicity in...
bacterial test systems may be due to inefficient uptake of mercury from the culture medium, so that the concentrations to which the bacterial DNA is exposed are too low to induce frameshift mutations and/or base-pair substitutions. This hypothesis was supported by the finding that HgCl₂ can bind to substances in the culture medium for the bacterial tester strains, preventing uptake by bacteria and hindering reliable mutagenicity testing (Codina et al., 1995). Subsequently, it has been shown that mutagenicity assays with eukaryotic cell systems seem to be superior for mutagenicity testing of inorganic mercury and other heavy metals (Ariza et al., 1994; Ariza and Williams, 1996). The mutagenicity assay introduced here is based on eukaryotic cells, which are transfected with the lacZ gene as a reporter for mutational events. The obvious advantage of this system is the combination of cellular uptake and cellular defence mechanisms in eukaryotic cells with a target for mutations. The reporter gene is incorporated in the nucleus, at the site of nuclear DNA. Thus, it can be extracted easily after exposure to the compound being tested (De Groene et al., 1995). As illustrated by our results, inorganic mercury had a mutagenic effect in this model, significantly increasing the mutation frequency in the lacZ gene even at the lowest concentration tested. The maximum mutation frequency was observed at a Hg²⁺ concentration of 0.5 µM.

These data are comparable to results of Ariza and Williams (1996), who found Hg²⁺ to be mutagenic in a Chinese hamster ovary (CHO) cell line at concentrations as low as 0.1 µM using the E.coli xanthine–guanine phosphoribosyltransferase (gpt) gene as a marker. In the same cell line, Howard et al. (1991) detected chromosomal aberrations and sister chromatid exchanges. DNA single-strand breaks have been reported in rat and mouse fibroblasts exposed to Hg²⁺ (Zashukina et al., 1983). Finally, Anwar and Gabal (1991) found a correlation between the exposure of a human population to Hg²⁺ and the appearance of micronuclei and chromosomal aberrations in human lymphocytes; however, for both changes the duration of exposure was important, suggesting the existence of a certain threshold dose.

In order to identify the mechanisms involved in the
observed increase in the mutation frequency of the lacZ gene induced by Hg2+, different cellular responses to HgCl2 exposure were measured. Initial cytotoxicity assays were used to select appropriate concentrations of Hg2+ that were devoid of significant immediate cytotoxicity. Subsequent assays were conducted at these concentrations, but even at these low concentrations, Hg2+ increased the amount of reactive oxygen intracellularly. This observed induction of oxidative stress occurred, although control measures demonstrated that, upon exposure to Hg2+, the intracellular GSH concentration was increased above control values. An increase in cellular GSH content following mercury exposure was also observed in hepatoma cells (Prestera et al., 1993), rat kidney mitochondria (Lund et al., 1993) and proximal and distal tubules of rat kidney (Lash and Zalups, 1996).

Hg2+ also significantly increased the cellular MT content. This mercury-induced MT expression was even more pronounced after GSH depletion following co-incubation with BSO. This points towards the co-regulation of GSH and MT synthesis (Foulkes, 1993). Correspondingly, radical formation, which increased after mercury exposure, was also more pronounced in the presence of BSO. Taken together, these findings suggest that the observed mutations may be a consequence of oxidative processes, rather than indicating a direct interaction of mercury with nuclear DNA. The results also indicate that the auto-induction of MT by Hg2+ fails to prevent this mutational event. The latter might be explained by the fact that up-regulation of MT requires transcriptional activity, and so occurs with delay. This hypothesis is supported by the finding that measurable radical formation, which increases significantly in the initial phase of mercury exposure, decreases with time, commencing 6–12 h after onset of Hg2+ treatment.

In conclusion, the results presented here confirm the general properties of mercury in eukaryotic cells. Hg2+ increases cellular GSH levels at low concentrations, but complexes and depletes GSH at higher concentration. MT synthesis is induced, but induction occurs with delay, thus failing to prevent immediate effects of Hg2+ in vitro. As a result of the compromised cellular defence mechanisms, Hg2+ induces oxidative stress particularly at the onset of exposure, which declines in time, when MT synthesis increases. The initial high radical formation may induce DNA damage and mutational alterations, as demonstrated by the increased mutation rate of the lacZ gene, used in these experiments as a reporter gene. These results support the assumption that inorganic mercury has a mutagenic potential which is based on its ability to induce the production of reactive oxygen species.

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