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In Vitro Detection of Neuronal Stress Induced by Mercury Compounds in Cerebellar Granule Cells from hsp70/hGH Transgenic Mice

M. Bayo,[1] M.-Á. Serra,[2] L. A. Clerici[1]

[1] Physical and Chemical Exposure, Institute for Health and Consumer Protection, DG Joint Research Centre, I-21020 Ispra, Varese, Italy
[2] Area of Molecular and Cellular Biology, Faculty of Health Sciences, International University of Catalonia, E-08190 Sant Cugat del Vallès, Spain

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Mercury compounds are environmental pollutants long recognised as causing detrimental effects in the nervous system, inorganic mercury (Hg^{2+}) and, particularly, methylmercury (MeHg) are neurotoxic (Chang, 1990). Exposure to the metal causes cell death by the activation of apoptosis, either with Hg^{2+} (Rossi et al., 1997) or with MeHg (Nagashima et al., 1996). Environmental mercury contamination has increased very much in developed countries, thus increasing the risk of human exposure.

The use of *in vitro* systems allows the characterisation of the mode of action and molecular effects of toxic metals, thus giving further and/or complementary insight in their neurotoxic mechanism to classical *in vivo* studies. A general aim of these studies is also to develop biomarkers of exposure/effect of these metals (Manzo et al., 1996). On the other hand, the possibility of exploiting the heat shock response system as biomarker of stress caused by many physical and chemical agents has been previously recognised (González et al., 1989). Recently, it has also been suggested that heat-shock protein 70 (hsp70) overproduction can protect neurons against several types of injury (Sharp et al., 1999).

The developing central nervous system of fetuses and neonates and, particularly, the cerebellum is one of the main targets of mercury toxicity and a selective damage is produced in these cells (Choi, 1989). In particular, cerebellar granule cells (CGCs), which are characterized as glutamatergic neurons (i.e. they synthesize, store and release glutamate (Glu) and aspartate (Asp) upon stimulation), are sensitive to methyl mercury (Clarkson, 1987, Fonnum and Lock, 2000). Moreover, interference of Hg^{2+} with the glutamatergic pathway has been suggested as a potential mechanism underlying mercury neurotoxicity (Chang, 1990). Primary cultures of cerebellum neurons (cerebellar granule cells, CGCs) are thus a suitable model for *in vitro* neurotoxicity studies with mercury.

We have already shown that primary cultures of hepatocytes, embryonic fibroblasts, as well as kidney, lung and bone marrow cells, derived from our transgenic mouse model, in which a human growth hormone (hGH) gene has been put under the control of the heat shock protein 70 (hsp70) gene promoter, are quite sensitive to heavy metal insult, including MeHg (Sacco et al., 1997). Analogously, mouse NIH-3T3 fibroblasts

Correspondence to: L. A. Clerici

transfected with this chimeric gene have been also shown to respond to toxic metals, including Hg^{2+} (Fischbach et al., 1993). The detection of the reporter gene product (hGH) in the extracellular medium by specific immunoassays constituted a quite practical advantage.

The aim of the present work was to investigate the usefulness of primary CGCs derived from our transgenic mouse model to study neurotoxic effects of inorganic (Hg^{2+}) and organic (MeHg) mercury. A comprehensive strategy was proposed integrating cytotoxicity assays (MTT test) with the evaluation of the stress response (induction of hsp70). The possible use of the induction of hsp70 as biomarker of mercury-induced neurotoxicity is discussed.

MATERIALS AND METHODS

Mercuric chloride ($HgCl_2$, 99.5% purity min., Hg), N-methyl-D-aspartic acid (NMDA) and glycine (Gly) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) and methylmercury (II) hydroxyde (MeHgOH, MeHg) was purchased from Alfa (Karlsruhe, Germany) as a 1 M solution in H_2O. DNase I and soybean trypsin inhibitor (STI) were obtained from Roche (Mannheim, Germany), dizocilpine maleate (MK-801) was purchased from RBI (Natick, MS, U.S.A.). All chemicals used were of analytical grade. Aqueous solution of the studied compounds were added to the culture medium.

Animal experimentation was performed in accordance with EC regulations for the care and use of laboratory animals (in particular, EC Directive 86/609). CD1 hsp70/hGH transgenic mice were kept in controlled conditions (10 h light: 14 h darkness, 22-24 °C) and with free access to food and water. Pregnant mice were checked daily; litter sizes varied from 8 to 10 pups per litter. Day of birth was assigned as day one of life. Cells were obtained from 7-8 day old pups cerebella by well-established procedures (Schousboe et al., 1989) when they are still capable of undergoing mitosis, whilst other types of cerebellar neurons have reached the differentiating stage. In each preparation cells obtained from the cerebella of one litter were pooled together. After the dissection of the cerebella and the removal of the meninges, the tissue was minced, the cells were dissociated with screens of 80 mesh (180 µm) and 100 mesh (150 µm) and recovered in 9.5 ml of an HIB solution (NaCl 120mM, KCl 5 mM, HEPES 25 mM and glucose 9.1 mM; pH 7.4) containing 0.5 % trypsin and 0.1 % DNase I, at 37°C for 15 min, with gentle shaking. Soybean trypsin inhibitor (STI) was then added to a final concentration of 0.1 mg/ml and cells were collected by short centrifugation at 200 x *g*. The pellet was resuspended in complete medium, then the cell suspension was triturated using a syringe with a long, narrow canula (13 gauge x 5 inches) and centrifuged at 200 x *g* for 15 min. Cells resuspended in complete medium (DMEM with 10% FBS, 25 mM KCl) were filtered through a nylon mesh (80 µm diameter), counted and seeded at an approximate density of $0.6\text{-}1 \cdot 10^6$ cells/cm^2 in 24-well plates (pre-coated with 50 µg/ml of poly-L-lysine) with 1.6 ml medium/well, and grown for up to 8 days at 37 ° C and 5% CO_2. Glial cell proliferation was prevented by adding cytosine arabinoside

(final concentration 10 μM) after 24 h. CGCs acquired differentiated morphology after 4-7 days in culture. At day 8 (day-in-vitro, DIV8) the cells were exposed to the different treatments, as described in the next section. The viability of the cells was assessed by the 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Kriegler et al., 1987; Wilmer et al., 1989); results were expressed as percentage of absorption with respect to control (set as 100% cell viability). The quantification of hsp70 expression (measurement of soluble hGH expression driven by the hsp70 promoter) was carried out using a hGH ELISA detection kit (Roche, Germany), as described elsewhere (Sacco et al., 1997); results were expressed as pg of hGH/million viable cells (as assessed with the MTT test described above). The significance of differences between mean values of different experimental treatments and controls was assessed by the Student's-*t* test.

RESULTS AND DISCUSSION

The responsiveness of our model was tested by inducing a standard heat-shock; CGC plates (DIV8) were placed in an incubator at 42 °C, with 5 % CO_2 for 1 h and then transferred back to the original incubator at 37 °C, with 5 % CO_2, for 24 h. An enhanced hsp70 expression was monitored up to 24 h, as reported in Fig. 1. Cell viability was not affected up to 24 h (data not shown). The figure is representative of three separate experiments. The effect of mercury compounds was then tested employing well-established *in vitro* treatments, which can also be found in the literature (Rossi et al., 1997; Castoldi et al., 2000; Daré et al., 2000).

CGCs were exposed for 24 h to 0.3, 1 and 3 μM of Hg or MeHg in DMEM with 10 % FBS (as described in Daré et al., 2000). The results are shown in Fig. 2. It can be seen that there is an increase in hsp70 expression with respect to control when increasing Hg and, particularly, MeHg concentrations were added to the media. A very good percentage of viable cells (more than 90%) is present at the end of the treatment for Hg throughout the range of concentrations studied and for MeHg up to 1 μM, whilst it drops dramatically for MeHg at 3 μM.

Short-term (1 h) exposure of cells to 0.1, 1 and 3 μM of Hg or to 0.1, 0.3 and 1 μM of MeHg was done in DMEM without serum (see e.g. Castoldi et al., 2000); the results are shown in Fig. 3. An increase in hsp70 expression for both mercury compounds is observed with increasing metal concentration; this increase was observed to be statistically significant at lower concentrations of MeHg as compared to Hg. Cell viability drops significantly only at 3 μM for Hg, whilst a clear decrease is evident for MeHg at concentrations al low as 0.1 μM, reaching values below 10 % at the highest concentration tested (3 μM).

When the effect of mercury compounds and the neurotransmitter NMDA was investigated (see e.g. Rossi et al., 1997), CGCs were exposed for 24 h in DMEM devoid of serum, to 3 μM Hg or 3 μM MeHg or 50 μM NMDA, or their combinations. Cell viability and stress response were evaluated at the end of the exposure time (results in Table 1). Viable cells decrease with exposure time when

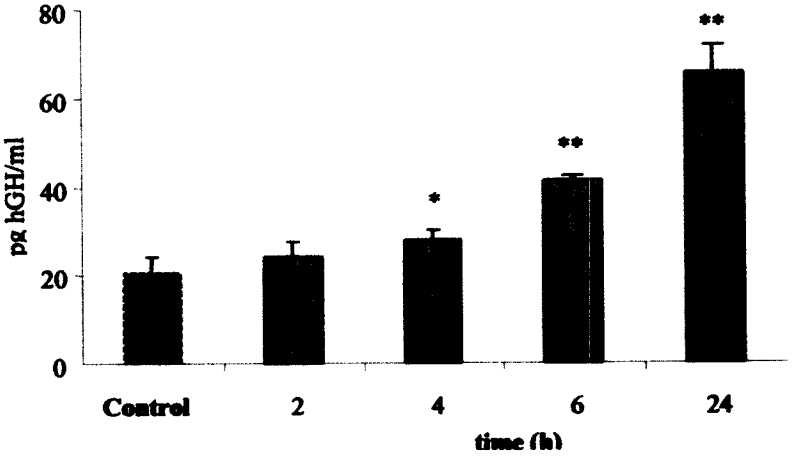

Figure 1. Results of the hsp70-driven hGH expression of CGCs after 1h heat-shock at 42°C and subsequent maintenance at 37°C for 24 h. Aliquots of culture media (200mL) were then withdrawn at fixed time intervals, as indicated (see also Materials and Methods). Values are representative of three separate experiments (n=3) and are compared with the control (*p<0.05, **p<0.01, Student's t test).

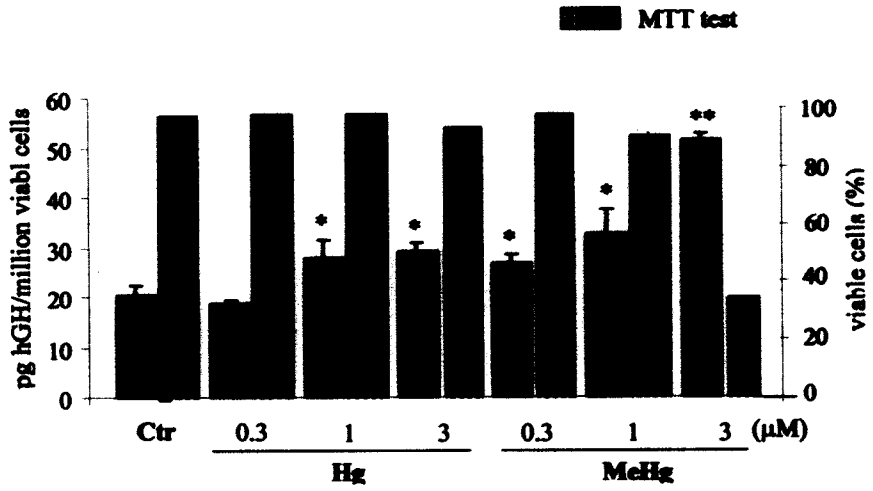

Figure 2. Results of the hsp70-driven hGH expression of CGCs exposed for 24 h to Hg or MeHg (0.3, 1 or 3 μM) in complete medium (DMEM with 10% FBS). For details see Materials and Methods. Values are representative of two separate experiments (n=2) and are compared with the control (*p<0.05, **p<0.01, Student's *t* test).

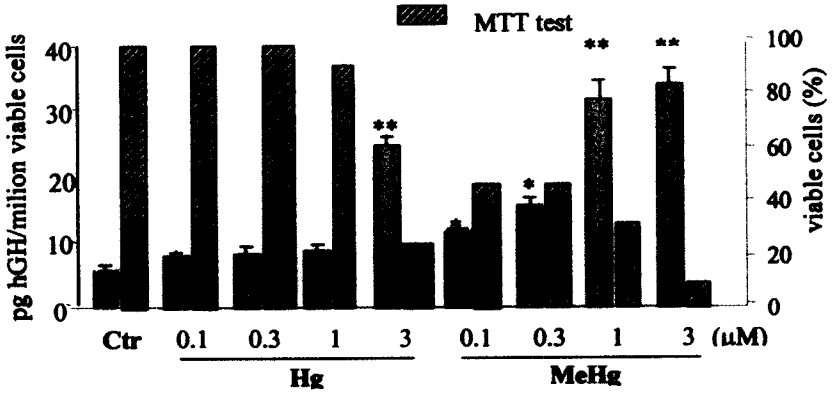

Figure 3. Results of the hsp-70 driven hGH expression of CGCs after 1 h exposure to Hg or to MeHg in DMEM without serum and 24 h recovery in the original culture medium (DMEM with 10% FBS). For details see Materials and Methods. Values are representative of two separate experiments (n=2) and are compared with the control (*$p<0.05$, **$p<0.01$, Student's *t*-test).

mercury compounds are present; MeHg is more toxic than Hg and their combination with NMDA enhanced only Hg toxicity. The amount of reporter gene product (hGH) significantly increases with respect to control for MeHg; when NMDA is added, this is also true for Hg, whilst for MeHg remains the same. It must be underlined in this latter case that the cell viability is very poor (7.5%) even without the addition of the NMDA, suggesting that the organic mercury induces in any case a dramatic cellular stress with a prompt and massive production of hsp70 before a sudden death of the cells.

Our transgenic mice model seems to be a sensitive model in the study of the neurotoxicity of mercury compounds. In particular, it has been proved useful for highlighting an increased stress response, other than to heat-shock, also when CGCs were exposed to mercury compounds, either an organic compound (MeHg) or to the inorganic form (Hg). In addition, an increased toxicity induced by the former compound as compared with the latter could also be seen. A confirmation of the role of NMDA receptors in the neurotoxic mechanism of inorganic mercury (Hg^{2+}) has also been obtained.

The overall meaning of these results is the responsiveness of CGCs from transgenic mice cultured *in vitro* in complete medium to physical (heat shock) and chemical (inorganic and organic mercury) *stimuli* with an increased expression of the reporter gene product (soluble hGH) driven by the stress protein hsp70 promoter (Fig. 1 and

Table 1. Cerebellar granule cells viability and stress response after 24-h exposure to mercury compounds.

Experimental conditions	OD_{570} [mean (± SD)]	Viable cells (%)	pg hGH/10^6 viable cells [mean (± SD)]
Control	8.824 (± 0.068)	100	5.3 (± 4.4)
3 μM Hg	0.645 (± 0.039)	78.3	7.8 (± 4.8)
3 μM MeHg	0.062 (± 0.001)*	7.5	26.5 (± 7.9)*
50 μM NMDA	0.870 (± 0.013)	100	7.6 (± 1.0)
3 μM Hg + 50 μM NMDA	0.484 (± 0.241)*	58.7	24.1 (± 7.2) *
3 μM MeHg + 50 μM NMDA	0.063 (± 0.001)*	7.6	25.3 (± 7.5) *

CGCs viability and stress response after 24-h exposure to mercury compounds as measured by the MTT test (absorbance at 570 nm) and by the expression of hsp70 (measured as pg of reporter gene hGH per million viable cells), respectively.
Means are the result of two different experiments, with two replicates each (n=2); the statistical significance was established using the Student-*t* test (* $p<0.05$). The proportion of viable cells is indicated with respect to the control (set as 100% viability). For details, see Materials and Methods.

Fig. 2). This fact has not been yet reported in the literature and confirms *in vivo* data of an induction of hsp70 in CGCs after hyperthermic stress in rats (Blake et al., 1990; Marini et al., 1990) and rabbits (Masing and Brown, 1989; Manzerra and Brown, 1992). These cells could thus be used as a suitable model to investigate this molecular endpoint as biomarker in metal-induced neurotoxicity.

However, as the complete medium contains also serum, which may introduce unidentified compounds that could interfere or prevent the toxic effect of Hg and/or MeHg, the expression of hsp70 by the cells with respect to metal concentration was thus studied for a short exposure time (1 h) in serum-deprived medium followed by 24-h recovery in complete medium. MeHg and, to a less extent, Hg, produced an increase in hsp70 expression correlated with a decrease in cell viability (Fig. 3). This effect was also observed when longer exposure times were studied (Table 1), MeHg decreases cell viability more than Hg does, both at concentrations capable of inducing apoptosis, e.g. up to 1 μM for MeHg and up to 3 μM for Hg) (Marty and Atchinson, 1998).

On the other hand, inorganic Hg-induced toxicity (assessed by the MTT test) is enhanced when NMDA is added to the exposure medium, as described elsewhere (Rossi et al. 1997) (Table 1). Similarly, hsp70 expression is enhanced when Hg and NMDA are combined with respect to 3 μM Hg alone (Table 1), an affect that was confirmed by addition of the well-known NMDA antagonist MK-801 to the medium, which abolished this enhancement for Hg and NMDA (data not shown). It can be concluded that ionotropic NMDA receptors could play a role in inorganic Hg-induced neurotoxicity in CGCs, as proposed by many authors (see Klaassen, 1996). The role of NMDA receptors on MeHg toxicity could not be assessed due to its high toxicity at the concentration tested (92.5 % cell death at 3 μM MeHg).

The use of a neural *in vitro* model derived from transgenic mice described in this work, shows that the expression of hsp70 as biomarker of mercury neurotoxicity remains a suitable possibility, which merits further research.

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