Reduced tubulin tyrosination as an early marker of mercury toxicity in differentiating N2a cells

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Running title: mercury toxicity and tubulin tyrosination.

Abstract

The aims of this work were to compare the effects of methyl mercury chloride and Thimerosal on neurite/process outgrowth and microtubule proteins in differentiating mouse N2a neuroblastoma and rat C6 glioma cells. Exposure for 4 h to sublethal concentrations of both compounds inhibited neurite outgrowth to a similar extent in both cells lines compared to controls. In the case of N2a cells, this inhibitory effect by both compounds was associated with a fall in the reactivity of western blots of cell extracts with monoclonal antibody T1A2, which recognises C-terminally tyrosinated α-tubulin. By contrast, reactivity with monoclonal antibody B512 (which recognises total α-tubulin) was unaffected at the same time point. These findings suggest that decreased tubulin tyrosination represents a neuron-specific early marker of mercury toxicity associated with impaired neurite outgrowth.

Key words: Mercury, thimerosal, tubulin tyrosination, neurite outgrowth.

Introduction

Increased worldwide industrialisation has led to higher levels of pollution by potent neurotoxins such as methyl mercury. This compound has been linked with numerous toxic episodes in man, which were invariably associated with disturbed motor function and mental impairment, causing symptoms such as fever, tiredness, tremors and delusions in severe cases (Castoldi et al., 2001; Jacobson, 2001). Of particular concern is the fact that methyl mercury can cause congenital poisoning via transplacental transfer (Jacobsen, 2001), accounting for some of the reported cases of infant poisoning and raising awareness of its developmental toxicity.

The organic form of mercury is considered to be more toxic than inorganic mercury, presumably due to differences in its uptake and chemical reactivity (O'Kusky, 1992). However, although widespread exposure to methyl mercury is rare nowadays, aquatic microorganisms can convert inorganic mercury into organic mercury, which may then be ingested by larger species and eventually work its way up the human food chain, affecting both adults and children (Atchison, 2004; Counter and Buchanan, 2004). There is also concern over the use of ethyl mercury thiosalicylate (thimerosal) as a preservative in certain vaccines and topical medications, some of which are administered to infants, as the very young are believed to be more sensitive to mercury toxicity (Goldman and Shannon, 2001). Thus, the risk of mercury toxicity remains a cause for concern in today's society.

It has been suggested from cell culture studies that the neurotoxicity of methyl mercury is linked to its ability to inhibit axon outgrowth and to disrupt microtubules in developing neurons (Graff et al., 1997; Miura et al 1999; Heidemann et al., 2001; Parran et al., 2003). Indeed, subpopulations of dynamic microtubules enriched in C-terminally tyrosinated α-tubulin were shown to be more sensitive to disruption by methyl mercury (Graff et al., 1997). By contrast, the possible toxic effects of thimerosal on neurite outgrowth and the cytoskeleton are less well characterised and have not been compared directly with those of methyl mercury chloride in the same cellular system. However, both compounds have been associated with disturbed Ca^{2+} homeostasis, the generation of oxygen free radicals and glutathione depletion (Olivieri et al., 2000; Hajela et al., 2003; Ueha-Ishibashi et al., 2004; James et al., 2005), all of which are potential causes of microtubule disruption (Hargreaves,

1997). It is therefore possible that thimerosal affects the neuronal cytoskeleton in a similar manner to methyl mercury.

The aims of the present work were to determine the effects of sublethal concentrations of both compounds on the outgrowth of neurites by differentiating neuronal and glial cell lines and to establish whether any observed changes involved altered levels or tyrosination state of α-tubulin.

Materials and methods

Cell culture and cell differentiation

Mouse N2a neuroblastoma and rat C6 glioma cell lines were obtained from ICN (Thane, UK) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % v/v foetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM glutamine as described previously (Flaskos et al., 1998). N2a and C6 cells were seeded at a density of 50,000 cells/ml in 24-well culture dishes (0.5 ml /well) or T25 cell culture flasks (10 ml) and incubated in growth medium for 24 h. They were then induced to differentiate in the presence and absence of methyl mercury chloride or thimerosal as indicated in Results, for 4 h in serum-free medium containing 0.3 mM cAMP or 2 mM sodium butyrate for N2a and C6 cells, respectively. The extent of cell differentiation was quantified by the outgrowth of axon-like neurites (greater than 2 cell body diameters in length) by N2a cells and total cellular process outgrowth in the case of C6 cells, in five randomly selected areas per culture well (Flaskos et al., 1998). Cell growth and viability were determined by the reduction of methyl blue tetrazolium (MTT) by cell monolayers, following the method of Denizot and Lang (1986).

Gel electrophoresis and western blotting analysis

Cell monolayers, incubated in T25 flasks in the presence and absence of mercury compounds as above, were gently rinsed with sterile phosphate-buffered saline (PBS), which had been pre-heated to 37 ºC. They were then lysed in PBS containing 0.5% sodium dodecyl sulphate (SDS), which had been pre-heated to 100 ºC, followed by immediate incubation at 100 ºC for 10 minutes in a heating block. Equal amounts of protein (20-30 μg), assayed by the method

of Lowry et al. (1951), were loaded into sample wells of polyacrylamide gels. Proteins were separated by gel electrophoresis (SDS-PAGE) on a 10 % polyacrylamide resolving gel overlaid with a 4 % stacking gel (Laemmli, 1970). Electrophoresed proteins were transferred electrophoretically onto nitrocellulose membrane filters by the method of Towbin et al., (1979), after which blots were blocked in 3 % w/v Marvel in PBS, then incubated overnight at 4 ºC with monoclonal antibodies that recognise total α-tubulin (clone B512) and α-tubulin tyrosinated in its carboxy terminal (clone T1A2), both of which were obtained from Sigma-Aldrich Company Ltd (Poole, UK). After six 10-min washes with PBS containing 0.05 % w/v Tween-20 (PBS/Tween) blots were incubated with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin diluted 1/1000 in blocking solution for up to 4 h at room temperature. After 6 further washes in PBS/Tween, antibody reactivity was detected in substrate buffer containing nitroblue tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate, as described previously (Flaskos et al., 1998). Reactivity was quantified densitometrically, using Quantiscan software (Biosoft; version 1.5).

Statistical analysis of data

Results for neurite outgrowth are expressed as mean ± SEM. Densitometric values are expressed as % of control ± SEM. Statistical significance of differences from controls were determined by ANOVA, using 95 % confidence limits.

Results

In order to determine the minimum lethal dose of mercury compounds, N2a and C6 cells were differentiated for 4 h in the absence and presence of a range of concentrations of methyl mercury chloride or thimerosal and cell growth and viability assessed by the ability of monolayers to reduce MTT. Neither compound at 1 μM or below affected MTT reduction by differentiating N2a or C6 cells (Table 1). However, both compounds inhibited MTT reduction in both differentiating cell lines at concentrations of 10 μM and above (not shown). The effects of sub-lethal concentrations of mercury compounds were then tested on the ability of differentiating N2a and C6 cells to produce neurites or cellular outgrowths, respectively. As shown in Table 1, both compounds at 1 μM caused significant inhibition of neurite/process outgrowth in both differentiating cell lines.

	Thimerosal		Methylmercury chloride	
	N2a	C6	N2a	C6
MTT Reduction (% control)	94.2 ± 8.3	91.1 ± 4.4	105.6 ± 6.4	98.0 ± 5.1
Neurite/process outgrowth (% control)	25.2 ± 7.7*	48.8 ± 7.9*	44.6 ± 7.1*	44.9 ± 4.6*

Table 1: Effects of exposure to thimerosal and methyl mercury chloride on MTT reduction and neurite outgrowth in differentiating N2a and C6 cells. N2a and C6 cells were induced to differentiate for 4 h in the presence or absence of 1 μM thimerosal or methyl mercury chloride, and assayed for MTT reduction and neurite outgrowth as described in Materials and methods. Values are expressed as a percentage of their corresponding control ± SEM for a minimum of 4 independent experiments. Asterisks indicate values that are significantly different from controls ($p < 0.05$).

Microtubules are known to be important in the regulation of neurite outgrowth in differentiating cells (Cambray-Deakin, 1999) and microtubule disruption is a known effect of methyl mercury exposure in cultured neurons (Graff et al., 1997; Miura et al 1999; Heidemann et al., 2001; Parran et al., 2003). Given that microtubules enriched in tyrosinated tubulin (i.e. labile subpopulations) are particularly sensitive to rapid disassembly following methyl mercury exposure (Graff et al., 1997), it was of interest to determine whether the observed inhibition of neurite outgrowth was associated with altered levels or tyrosination state of α-tubulin. For this, Western blots of lysates from cells induced to differentiate for 4 h in the absence and presence of 1 μM thimerosal or methyl mercury chloride, were probed with antibodies that recognise total α-tubulin (B512) and tyrosinated α-tubulin (T1A2). Densitometric analysis of tubulin-reactive bands (50 kDa) revealed a significant decrease in reactivity with monoclonal antibody T1A2 compared to controls on blots of thimerosal- and methyl mercury chloride-treated N2a but not C6 cell extracts (Fig. 1). By contrast, there was no significant fall in reactivity with monoclonal antibody B512 on blots of any mercury compound-treated cell extract compared to controls (Fig. 1).

Discussion

The results presented in this paper indicate that 4 h exposure to sublethal concentrations of both thimerosal and methyl mercury chloride leads to inhibition of neurite/process outgrowth in differentiating N2a and C6 cells. The fact that this inhibition occurs sublethally is supported by the lack of effect of this concentration of both compounds on the ability of cells to reduce MTT under the same experimental conditions (Table 1). The ability of methyl mercury chloride to inhibit neurite outgrowth is in good agreement with other workers using this compound on neuronal primary cultures or cell lines (Miura et al 1999; Heidemann et al., 2001; Parran et al., 2003), although with one exception (Heidemann et al., 2001) these studies used cytolethal concentrations of mercury. The neurite inhibitory concentrations reported by Heidemann et al. (2001) in chick brain primary neuron cultures are only slightly lower than those used in the current work with established cell lines. In their study, cells were exposed to methyl mercury for 2 h then assessed for axon outgrowth up to 2 days later, showing approximately 50% inhibition compared to untreated controls. Thus the two types of cellular systems are comparable in terms of morphological effects of sublethal concentrations of methyl mercury. To the best of our knowledge, this paper represents the first report of inhibition of neurite outgrowth by sublethal concentrations of thimerosal, suggesting that

thimerosal is capable of inhibiting neurite outgrowth to a similar extent as methyl mercury chloride.

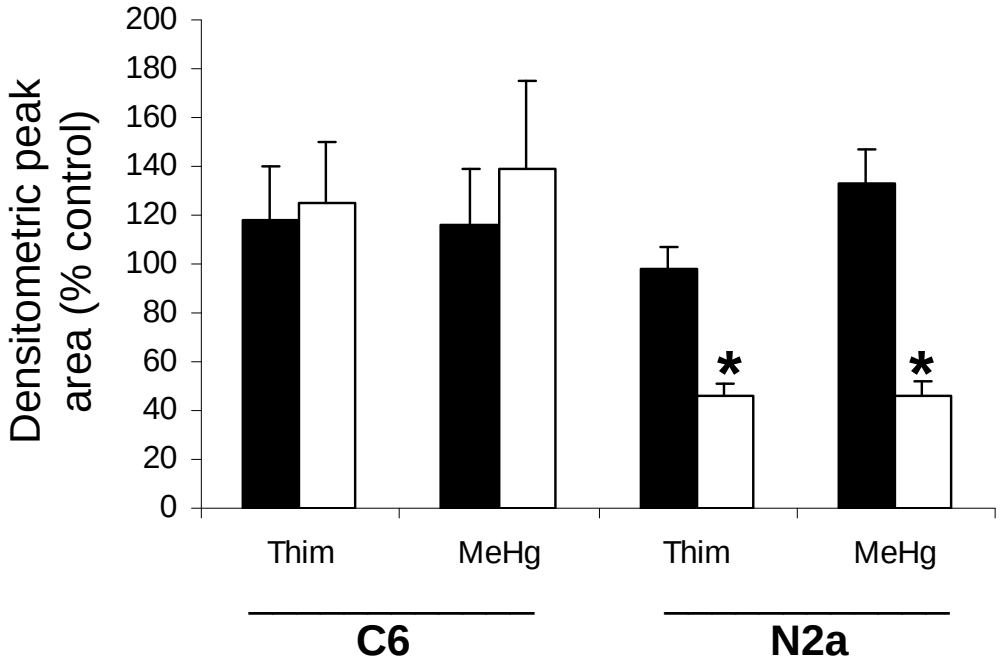

Figure 1: **Densitometric analysis of Western blots probed with antibodies against total and tyrosinated forms of α-tubulin**. N2a and C6 cells were induced to differentiate in the presence and absence of 1 μM thimerosal or methyl mercury chloride, lysed and separated by SDS-PAGE and Western blotting, after which the resultant blots were probed with monoclonal antibody B512 (anti-total α-tubulin; filled bars) or T1A2 (anti-tyrosinated α-tubulin; unfilled bars), as described in Materials and methods. Values are expressed as a percentage of their corresponding control ± SEM for a minimum of 5 independent experiments. Asterisks indicate values that are significantly different from controls ($p < 0.05$).

Previous work by Graff et al. (1997) indicated that subpopulations of microtubules recognised by an antibody against tyrosinated α-tubulin, which are known to be relatively dynamic or labile (Webster et al., 1987), are rapidly lost when cells are stained by indirect immunofluorescence. As the authors suggested, this could indicate a preferential disassembly of these microtubules (Graff et al., 1997) compared to more stable (i.e. mercury-resistant) populations. However, it is also possible that some of the tyrosinated tubulin may have become detyrosinated and/or its retyrosination may have been impaired during differentiation in the presence of mercury compounds and that this may have contributed at least partly to

the reduced immunofluorescence staining. Indeed, our data suggest that there is a reduction in the amount of tyrosinated α-tubulin compared to controls in differentiating N2a cells, indicating that there is a significant early reduction in tubulin tyrosination or loss of C-terminal tyrosine compared to controls after 4 h exposure to both methyl mercury and thimerosal.

The observed reduction of tubulin tyrosination under conditions that inhibit neuronal cell differentiation in vitro is consistent with the proposed vital role of tubulin: tyrosine ligase in neuronal development in chick brain (Erck et al., 2005). However, it is unclear as to whether this alteration in the tyrosination state of α-tubulin reflects a direct effect on tubulin:tyrosine ligase or tubulin:tyrosine carboxypeptidase, the enzymes responsible for the regulation of tubulin tyrosination (Barra et al., 1988), as tubulin tyrosination in differentiating glial cells appears to be unaffected by the same treatment. As glial cells are thought to have a more effective glutathione system than neuronal cells for protection against oxidative damage (Dringen et al., 1999), it may be that oxidative stress can influence α-tubulin tyrosination to a greater extent in differentiating N2a cells.

In conclusion, the results from this work indicate that reduced α-tubulin tyrosination represents an early neuron-specific marker of mercury toxicity in vitro and that sublethal concentrations of thimerosal are as effective as methyl mercury chloride at inducing this molecular change.

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