Possible Mechanisms of Methylmercury Cytotoxicity

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Abstract

The objective of this review is to address the mechanisms of methylmercury (MeHg)-induced neuronal toxicity. Astrocytes play a key role in MeHg-induced excitotoxicity as manifested in the following terms: [1] MeHg preferentially accumulates in astrocytes. [2] MeHg potently and specifically inhibits glutamate uptake in astrocytes. [3] Neuronal dysfunction is secondary to disturbances in astrocytes. [4] Co-application of nontoxic concentrations of MeHg and glutamate leads to the typical appearance of neuronal lesions associated with excitotoxic stimulation. [5] MeHg induces swelling of astrocytes. Studies in our laboratory have addressed the osmoregulatory effects of MeHg, characterizing ion, electrolyte, and non-electrolyte transport changes in response to MeHg. The results are consistent with dysregulation of excitatory amino acid homeostasis and indicate that a glutamate-mediated excitotoxic mechanism is involved. Furthermore, these studies establish an important role for metallothioneins (MTs) in ameliorating the acute effects of MeHg on astrocyte function. The review will address the effects of MeHg on astrocytic swelling and ion fluxes, followed by a synopsis on the role of MTs in protecting these cells from MeHg-induced injury.

Environmental Pathways to Human Exposure to Methylmercury

As evidenced by tragic poisonings in Japan and Iraq, MeHg is a potent neurotoxin. In the 1950s, a chemical plant in Minamata Bay (Japan) discharged mercury into the Bay as part of waste sludge. Within the Bay’s sediment, inorganic mercury was methylated to MeHg, and as a result fish and shellfish became contaminated. Because the primary dietary source of proteins in the local population was largely derived from fish consumption, a MeHg epidemic erupted shortly thereafter (53, 54). Approximately a decade later, a second epidemic occurred in Iraq. Following a major drought in 1971, the local government opted to switch from its customary order of wheat to a more resilient variety. An order was placed with the Mexican government. Unfortunately, however, a single letter typographical error was made in the labeling of the fungicide the wheat was to be treated with. Consequently, instead of treatment with a relatively harmless mercury containing fungicide, the wheat was treated with the poisonous MeHg. To compound matters, the wheat had arrived in Iraq past the planting season. Being unaware of the significance of the packaging labeling (skull and crossbones poison designation) and the pink dye additive that was added to warn them of the poisonous nature of the wheat, the farmers proceeded to use the wheat for their customary baking of Pita bread. Within weeks of the wheat consumption, the effects of MeHg intoxication were widespread and devastating, leading to mass poisoning epidemic with approximately 450-deaths (15).

Considerable attention in the scientific and health policy fora continues to focus on the question of whether MeHg intake from a diet high in fish is associated with aberrant CNS function. A number of studies (27, 36) suggest that fetal exposure at levels attained by mothers eating fish regularly during pregnancy are associated with neurological as well as cardiovascular effects in their offspring (27, 28, 43, 51). Notably, this outcome has not been replicated in children exposed to MeHg in the Republic of the Seychelles (22, 23, 44).

Methylmercury (MeHg) and Astrocytic Volume

Astrocytes occupy ~25% of the CNS volume. The “foot” processes of astrocytes are closely associated with synapses, nodes of Ranvier, axonal tracts, and capillaries. Astrocytic functions include neurotrophic factor secretion, control of extracellular pH, inactivation of glutamate, as well as uptake and metabolism of neurotransmitters (35; Aschner and Kimelberg, 1996). Although not the only cell type to be adversely affected by MeHg (13), astrocytes play a key role in MeHg-induced excitotoxicity. [1] After chronic in vivo exposure in human and non-human primates, MeHg preferentially accumulates in astrocytes and specifically inhibits glutamate uptake in astrocytes (1), resulting in excessive concentrations of excitatory amino acids (EAAs) in the extracellular fluid (ECF). Other transport systems are 2-5 fold less sensitive to inhibition by MeHg (16). The effect of MeHg (CH2Hg)+ and Hg2+ on glutamate uptake is not mimicked by other divalent cations (6, 17, 42). [3] In the absence of glutamate, neurons are unaffected by acute exposure to mercury, suggesting that neuronal dysfunction is secondary to disturbances in astrocytes (17). [4] Co-application of nontoxic concentrations of MeHg and glutamate leads to the typical appearance of neuronal lesions associated with excitotoxic stimulation (39). [5] In human and non-human primates chronic in vivo exposure to MeHg is associated with swelling of astrocytes (19, 20, 45, 57). Swollen astrocytes undergo regulatory volume decrease (RVD) and re-establish volume by releasing intracellular ions and EAAs, such as glutamate and aspartate (32, 46), leading to neuronal injury according to the excitotoxic hypothesis (21).
Astrocytic swelling and EAA release are also associated with in vitro MeHg exposure (4, 5, 7, 11).

Astrocytic swelling in situ routinely occurs in pathological states, and it precedes neuronal damage (18, 49). Astrocytes swell more readily than neurons in response to lactacidosis and elevated K+, glutamate, and monoamine transmitters (33). In its exaggerated form this swelling is deleterious, and can be viewed as a pathological extension of more limited and controlled volume changes which are otherwise part of the normal homeostatic function of astrocytes. Mechanisms of astrocytic swelling have been recently reviewed (12, 35). Briefly they include: [1] The simultaneous operation of Cl-/HCO$_3$- and Na$^+$/H$^+$ exchange transporters, with H$^+$ and HCO$_3^-$ cycling from the intra- to extracellular spaces via membrane-permeant CO$_2$ when the increased intracellular NaCl cannot be pumped out. [2] Glutamate stimulated increase in the production of the metabolic products, CO$_2$ and H$^+$ (leading to swelling similar to the above). Glutamate also augments astrocytic cell swelling by interaction with metabotropic glutamate receptors. The Na$^+$/K$^+$/2Cl$^-$ co-transporter, Na$^+/K^+/ATPase$, and the Na$^+$-dependent electrogenic glutamate transporter are involved in this type of swelling (30). [3] High extracellular K$^+$ concentrations associated with stroke and head injury (50, 52) lead to swelling by uptake of KCl due to Donnan forces (58). In this condition uptake of ions is driven by electrochemical gradients and is common to cells that express requisite K$^+$ and Cl$^-$ channels. [4] Astrocytic swelling can result from non-specific breakdown of the selective permeability of the plasma membrane, as a result of free-radical generation (29). Upon swelling, astrocytes re-establish their volume, a process requiring intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) (40).

A series of studies conducted in our laboratory (5, 6, 8, 10) are consistent with the activation of electroneutral cotransport systems by MeHg (Figure 1). SITS (4-acetamido-4'-isothiocyanato stilbene-2,2'-disulfonic acid) and DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid), furosemide, or bumetanide were ineffective in reversing the MeHg-induced astrocytic swelling, suggesting no involvement of Cl-/HCO$_3$- anion exchange, or K$^+$/Na$^+$/2Cl$^-$ cotransport systems in this effect. It has been previously reported (31) that the stimulation of the initial unidirectional Na$^+$ influx by mercury was of the same magnitude even when all the cellular and extracellular Cl$^-$ was substituted with NO$_3^-$.

Figure 1. Schematic model representing the effects of MeHg on amino acid, and ion transport in hypotonic buffer. Insets represent the tested transport systems, and their specific inhibitors. MeHg specifically activated the Na$^+$/H$^+$ antipporter and its effects were partially or fully reversed by amiloride. For further details, please refer to text.
MeHg-treated astrocytes occurs exclusively via activation of an amiloride sensitive Na\(^+\)/H\(^+\) antiporter. (2) Amiloride can at least partially reverse the MeHg-induced inhibition of RVD in swollen astrocytes. The studies also establish that taurine and myoinositol release from astrocytes is differentially regulated. Thus, these two osmolytes may differ in their release pathways. Metallothioneins (MT) ameliorate MeHg-induced astrocytic dysfunction.

The metallothioneins (MTs), a class of cysteine-containing intracellular proteins, are characterized by a molecular mass of 6-7 kDa. These highly conserved proteins are expressed in all mammals and they have a wide organ and cellular distribution (2, 9, 24). MTs have been implicated as regulator molecules in gene expression, homeostatic control of cellular metabolism of metals, and cellular adaptation to stress (7, 24). Three MT isoforms (MT-I, MT-II and MT-III) have been identified within the mammalian central nervous system (CNS). MT-I and MT-II expression is abundant in astrocytes, whereas, MT-III is predominantly expressed in zinc-containing hippocampal neurons (6, 10, 38).

The toxic effects of MeHg were investigated in a model of RVD. Pre-exposure of astrocytes to the potent MT inducers CdCl\(_2\) (1 \(\mu\)M) or ZnSO\(_4\) (100 \(\mu\)M) significantly increased MT expression both at the mRNA and protein levels (data not shown) and reversed the inhibitory effect of MeHg on RVD. The effect of CdCl\(_2\) was time-dependent, 120 hour pre-treatment resulting in the greatest reversal of the MeHg-induced inhibition of RVD. Given the ability of MT overexpressing cells to volume regulate in the presence of MeHg, we anticipated, and found that pre-treatment with CdCl\(_2\) will significantly increase the rate of \(^3\)H-taurine release (p<0.001) compared with cells treated with MeHg in the absence of CdCl\(_2\) (data not shown). Figure 2 depicts the correlation between the optical density of MT protein bands from western blots and the cell height at the termination of the hypotonic exposure (t = 45 on the x-axis). Each of the 4 groups was treated with hypotonic buffer plus MeHg, but pre-treatment with CdCl\(_2\) was varied (0-120 hours). An inverse correlation (r = 0.99) was found between cell height and the optical density of MT bands. These data strongly support the hypothesis that astrocytes induced to express high levels of MT proteins with CdCl\(_2\) are resistant to the acute inhibitory effect of MeHg on RVD.

Since MeHg activates the Na\(^+\)/H\(^+\) antiporter (See above and 10, 11), we determined whether attenuation of MeHg-induced swelling in astrocytes induced to express increased MT protein levels is correlated with reduced influx of Na\(^+\). Exposure to MeHg was associated with a significant increase in the initial uptake of \(^{22}\)Na\(^+\) (p<0.01 at 1 minute), corroborating earlier observations (10, 11, 31, 41, 48). This effect was abolished by 24-hour pre-treatment of the cells with 100 \(\mu\)M ZnSO\(_4\) (p<0.01). The uptake of \(^{22}\)Na\(^+\) at 5 minutes was not statistically different from controls. Likewise, at 10 minutes post exposure, there was no statistical difference between the 3 experimental groups. As expected (see above), swelling induced by MeHg also led to a significant increase in the release of \(^{51}\)Cr, and this effect was significantly (p<0.001) attenuated by MT overexpression (data not shown).

Given the possibility that Cd and Zn, may, in addition to MT induction cause cell damage that is undetected by our cellular toxicity assays, astrocytic sensitivity to MeHg was studied subsequent to transient MT gene transfection. We used a pGfa2-cLac plasmid as a vector to construct the pGFAP-MT-I plasmid. MT-I was expressed by pGFAP-MT-I plasmid transfection under the control of the glial fibrillary acidic protein (GFAP) promoter (51). In addition to MT-transfected cells, a plasmid control group, consisting of the same plasmid without target DNA, and the cell control group (without plasmid DNA) were transiently transfected. Transfection with the pGFAP-MT-I plasmid led to an increase in mRNA (x2.5 in astrocytes; x4.0 in astrocytomas) and MT-I proteins (x2.4 in astrocytes; x4.0 in astrocytomas) compared with controls (data not shown). \(^{51}\)Cr release in response to MeHg treatment was attenuated in cells overexpressing MT-I compared to controls.

Finally, in ongoing studies (60), MT-I proteins and the ability of MT-I over-expression to protect against MeHg toxicity were monitored in astrocytes isolated from MT-I/MT-II knockout mice ± MT-I cDNA transfection and astrocytes isolated from the wild-type mouse. Astrocyte MT proteins in wild type (WT), MT-I/MT-II knockout (MT-KO), and MT-I cDNA transfected MT-KO (MT+) were determined. No MT-I protein was detected in MT-KO astrocytes. MT-I cDNA transfected MT-KO (MT+) had significantly higher MT-I proteins compared to either WT or controls. An inherent difficulty with efflux measurements, and especially continuous perfusion methods is that detachment or lysis (death) of cells will also contribute to radioactivity in the perfusate. If this loss is limited and gradual, it will not be distinguishable from efflux. To distinguish actual release from cell lysis or sloughing, we have used the method advocated by Kimelberg et al. (1993) examining release of \(^{51}\)Cr from the cells. This technique...
allows for distinction between release of preloaded radioactive markers (such as \( ^3 \)H-D-aspartate or \( ^3 \)H-taurine) from swelling or MeHg-induced cell death or detachment. \( ^{51} \)Cr release (a cytotoxicity assay) in response to MeHg treatment in the 3 groups is shown in Figure 3. In response to MeHg treatment, \( ^{51} \)Cr release was significantly (p<0.05) higher in the MT-KO (MT-) astrocytes, and this effect was significantly (p<0.05) attenuated in MT-I cDNA transfected MT-KO (MT+*) cells. \( ^{51} \)Cr release in cells overexpressing MT-I was also significantly (p<0.05) reduced compared to astrocytes isolated from WT (Figure 3).

**Interaction of MeHg With the Glutamate Transporter**

As previously mentioned, glutamate is the predominant excitatory neurotransmitter; it is also a potent neurotoxin. Following its release, and the activation of a variety of ionotropic and metabotropic glutamate receptors, glutamate is removed from the synapse. This is achieved by active uptake by transporters located pre- and post-synaptically, or by diffusion out of the synapse and uptake by astrocytes. The uptake of glutamate is coupled by the co-transport of 2-3 Na\(^+\) ions, the counter-transport of K\(^+\), and either co-transport of 1 H\(^+\) or counter-transport of 1 OH\(^-\) (14).

cDNAs encoding five glutamate transporters have been cloned (reviewed in 26). The cDNA sequence of the glutamate transporters is consistent with 8-9 putative transmembrane alpha-helices domains. Recent evidence also suggests that glutamate transporters are vulnerable to the action of biological oxidants that can result in reduced uptake function. For example, Trotti et al. (1998) identified specific 'redox-sensing' elements, consisting of cysteine residues in the structures of at least three transporter subtypes [GLT1 and GLAST (astrocyte-specific), and EAAC1 (neuron-specific)]. These 'redox-sensing' elements have been shown to regulate transport rate via thiol-disulfide redox interconversion. The redox modulation of glutamate uptake was examined by measuring transporter-mediated electrical currents and amino acid influx in voltage-clamped Xenopus oocytes expressing the human neuronal glutamate transporter EAAC1 (55). Up and down changes of the glutamate uptake currents were measured in response to treatment with dithiothreitol and 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). The redox interconversion of cysteines induced by dithiothreitol/DTNB influenced the \( V_{max} \) of transport, while the apparent affinity for glutamate was not affected. Formation or breakdown of disulfide groups did not affect the pre-steady-state currents, suggesting that these manipulations do not interfere with the Na\(^+\) binding/unbinding and/or the charge distribution on the transporter molecule.

The mechanisms associated with the inhibitory effect of MeHg on glutamate uptake are presently unknown. GLT1 and GLAST contain nine and three cysteine residues in their sequence, respectively (47). Since many transporter proteins are sensitive to redox states, including the glutamate transporter (55, 56), it is possible to invoke cysteine residues within the transporter as potential targets for MeHg binding and disruption of transporter function. The inter-molecular interactions of MeHg with the glutamate transporter and its effect on glutamate transporter gene expression have not as yet been explored.

**Summary**

The above studies establish the sensitivity of astrocytes to MeHg. The studies also show that MT-I protein could be overexpressed in MT-I and MT-II null astrocytes by transient transfection of the MT-I gene. Exposure of MT null astrocytes to MeHg promoted efflux of Na\(^{2+}\)\( ^{51} \)CrO\(_4^−\), whereas MT enrichment in MT null astrocytes by MT transient transfection reduced Na\(^{2+}\)\( ^{51} \)CrO\(_4^−\) release. The studies also support the notion that the protective effect of MTs was directly correlated to the cellular MT protein levels, such that astrocytes with the highest protein level were the most
resistant to MeHg cytotoxicity. Variations in the distribution and effects of MeHg seem to be principally dependent upon this reaction. The affinity of MeHg for the anionic form of -SH groups (log K, where K is the association constant) is extremely high, on the order of 10^{10-15} - 23. Indeed, the formation of a MeHg-cysteine complex is associated with selective transport of MeHg across the blood-brain barrier (3), as well as astrocytes (5). Given that about 20 of the 61-62 amino acids of MT-I and MT-II, respectively, are cysteines, and the extraordinary affinity of MeHg for -SH groups, MeHg would be expected to rapidly bind to intracellular MTs, diminishing its ability to bind to other functionally critical -SH groups. It is well established that MTs on cysteine residues are highly sensitive, and in many transporter proteins, they account for functional changes that represent regulatory mechanisms. The ability of MTs to bind MeHg may, therefore, prevent functional changes in various transporters. Moreover, the studies establish that MT-null astrocytes present a useful model to examine the role of MT in detoxification in the CNS, and it can be profitably used in future studies to decipher regulatory mechanisms of MT gene expression.

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