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Selective Inhibition of Glutamate Uptake by Mercury in Cultured Mouse Astrocytes

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We studied the effects of organic and inorganic mercury (Hg) on the uptake of L-[^{3}H] glutamate (L-GLU) in cultured mouse astrocytes. Following exposure to mercuric chloride (MC) [0.2~5.0 μM], selective and dose-dependent inhibition of L-GLU uptake to 50% of control levels was observed, whereas 2-deoxyglucose (2-DG) uptake was not significantly affected. Methylmercuric chloride (MMC) also inhibited L-GLU uptake but 50% reduction was reached only at a concentration of 10 μM. Inhibition of L-GLU uptake by MMC appears to be closely linked to voltage-sensitive calcium channels as evidenced by the lack of L-GLU uptake inhibition by MMC in calcium-free medium or in the presence of the channel blocker verapamil. Exposure to a variety of divalent metallic ions, including $CuCl_2$, $FeCl_2$ and $ZnCl_2$, did not affect L-GLU uptake in astrocytes in vitro. Exposure to $PbCl_2$, however, resulted in a decline in L-GLU uptake, though to a much smaller degree than that observed with Hg compounds. Selective impairment of astroglial L-GLU transport may represent a critical early pathogenetic feature of Hg-induced neurotoxicity.

Key Words: Glutamate uptake, mercury, astrocytes, cultures

The sensitivity of the human central nervous system (CNS) to the toxic effects of mercurial compounds is well documented (Hunter and Russell, 1954; Takeuchi, 1968). Similar neurotoxic effects have also been successfully demonstrated in mammalian animal models (for review see Choi, 1983, 1989, 1992). However, the manner in which these compounds cause cellular injury has not been fully understood. Because mercury (Hg) reacts indiscriminately and with a high degree of affinity for sulfhydryl (SH) groups in all types of cells (Clarkson, 1987), methylmercury (MeHg) can induce metabolic perturbations in a variety of ways, including disruption of intracellular calcium homeostasis (Kauppinen *et al.* 1989; Komulainene and Bondy, 1987; Levesque and Atchison, 1991; Oyama *et al.* 1994), lipid peroxidation (Taylor *et al.* 1973; Yonaha *et al.* 1983), disturbances in protein synthesis (Cheung and Verity, 1985, Sarafian *et al.* 1983), disturbances in protein synthesis (Cheung and Veritыy, 1985, Sarafian *et al.* 1984) and induction of oxidative stress (LeBel *et al.* 1990; Oyama *et al.* 1994; Yee and Choi, 1994, 1995).

As a sequel to the widening belief that oxidative injury-inducing free radicals may be of importance in MeHg neurotoxicity, the concept has emerged that excessive activation of glutamate receptors can cause oxidative stress in target cells by increasing the rate of formation of reactive oxygen species (Coyle and Puttfarcken, 1993) and/or by depleting antioxidants (Coyle and Puttfarcken, 1993; Dykens *et al.* 1987). It has been shown that astrocytes possess high-affinity glutamate uptake mechanisms (Bridges *et al.* 1987; Hansson *et al.* 1985; Hertz *et al.* 1978; Waniewski and Martin, 1986), and that L-GLU

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levels are modulated by highly efficient astroglial uptake mechanisms (Yu *et al.* 1982). In addition, L-GLU receptors have been demonstrated in astrocytes (Backus *et al.* 1989; Gallo *et al.* 1989; Glaum *et al.* 1990). Therefore, any disturbance in astroglial L-GLU transport could theoretically give rise to excessive local accumulation of L-GLU, thereby triggering the development of excitotoxic cellular injury (Olney, 1969). The purpose of this study was to determine whether or not disturbances in astroglial L-GLU uptake might be involved in the pathogenesis of Hg-induced central neurotoxicity.

MATERIALS AND METHODS

Astroglial cell cultures were established from the cerebra of neonatal (postnatal day 1) C57BL/6J mice according to a modification of a previously published method (McCarthy and de Vellis, 1980). Briefly, the brains were removed and, after separation from the meninges, washed in saline solution (138 mM NaCl, 5.4 mM KCl, 1.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 22 mM glucose, 0.9 mM $CaCl_2$, pH 7.2). The separated cerebral cortex was finely minced and triturated, incubated in 0.25% trypsin for 25 minutes at 37℃ in saline solution in a rotary shaker, spun at 2000 rpm for 5 minutes and resuspended in modified Eagle's medium (MEM) supplemented by 15% fetal calf serum. Cell suspensions were consecutively filtered through Nitex 130 and 33 filters and plated onto poly-L-lysine-coated 75 cm^2 culture flasks at 37.5℃ in a water-saturated atmosphere of 5% CO_2/95% air. Cultures were fed twice a week until grown to confluence (approximately one week). The flasks were then placed overnight at 37℃ in a rotary shaker at 200 rpm. Floating cells (mostly oligodendrocytes) were removed and the remaining cells were thoroughly washed with MEM, dissociated with trypsin-EDTA in Hank's balanced salt solution, plated onto 9×30 mm poly-L-lysine-coated glass coverslips in multi-well culture chambers and grown to confluence (approximately 4 days). Lowry protein assays (Lowry *et al.* 1951) of representative cultures were performed on individual coverslips to allow standardization between cultures and to permit biochemical analysis of the L-GLU transport system. Immunocyto-chemical staining for glial fibrillary acidic protein (GFAP), fibronectin and factor 8 was also carried out in representative coverslips.

The L-GLU uptake assay

The uptake of L-[^{3}H] GLU was examined in monolayer cultures of mouse astrocytes following exposure to various concentrations (0～10 μM) of mercuric chloride ($HgCl_2$) and methylmercuric chloride (CH_3HgCl). In addition, the effects of divalent metallic ions, namely, copper chloride ($CuCl_2$), iron chloride ($FeCl_2$), lead chloride ($PbCl_2$) and zinc chloride ($ZnCl_2$), were similarly tested.

The coverslips were equilibrated for 10 min in Tris buffered salt solution (TBSS)[150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM dextrose, 20 mM Tris, Ph 7.3, 345 mosmol by sucrose] at 37℃, incubated in TBSS containing 0 to 10 μM of metallic solution ($HgCl_2$, CH_3HgCl, $CuCl_2$, $FeCl_2$, $PbCl_2$ and $ZnCl_2$) for 10 min at 37℃, transferred to TBSS containing 100 mM of L-GLU (approximate equilibrium concentration) containing L-[^{3}H] GLU (40～60 Ci/mmol, Dupont NEN) as a tracer for 4 min at 37℃, washed quickly in ice cold TBSS and transferred to vials of distilled water at room temperature for 10 minutes to lyse the cells. The ^{3}H content of the lysate was then analyzed in a liquid scintillation counter to determine the amount of L-GLU taken up by astrocytes.

The influence of extracellular calcium on L-[^{3}H] GLU uptake after CH_3HgCl exposure was also examined by the use of calcium-free culture medium and in the presence of the voltage-sensitive calcium channel blocker verapamil. Monolayer cultures of astrocytes were incubated for 30 min at 37℃ in calcium-free MEM and in the presence of 10 μM verapamil prior to equilibration in calcium-free and calcium-containing TBSS, respectively. L-[^{3}H] GLU uptake assays were then carried out as described above after exposures to 0 to 10 μM CH_3HgCl.

2-deoxy-D-glucose (DG) uptake assay

Following 10 min of equilibration in TBSS, the cover slips were exposed to 2-[^{3}H] DG (30～60 Ci /mmol, Dupont NEN) at a concentration of 20

nM (with a total 2-DG concentration of 5 mM) to measure 2-DG uptake at various incubation times (1~10 min) following exposure to various concentrations (0.2~10 μM) of CH_3HgCl and $HgCl_2$.

RESULTS

Monolayer cultures of astroglial cells such as that illustrated in Fig. 1, showing strong immunofluorescence of GFAP within the cytoplasm, were used throughout these experiments. Since preliminary studies showed the L-GLU equilibrium concentration of astrocytes to be 90 ~100 mM and to display simple first-order kinetics, this concentration was used for each of the metals to determine the initial rate of uptake (i.e., over 4 minutes). Exposure to $HgCl_2$(0~10 μM) produced the most dramatic and immediate drop in L-GLU uptake, in a dose-dependent manner. During the initial 4 min of incubation, No significant changes in L-GLU uptake were observed. However, after 14 min of incubation, L-GLU uptake declined approximately 50% at concentrations below 2 μM, and continued to drop steadily to 14.2% of its original level at 10 μM (Fig. 2). Exposure to CH_3HgCl for 14 min at 0 to 10 μM concentrations also resulted in a significant decline, although it was less than that ovserved with $HgCl_2$ (Fig. 2). A 50% decrease in L-GLU uptake occurred at 10 μM as opposed to 2 μM for $HgCl_2$. No significant change in the uptake of L-GLU was observed when cells were

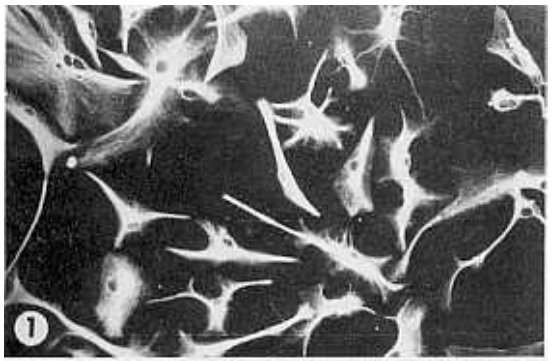

Fig. 1. Photomicrograph showing monolayer of astrocytes used in this study. Note strong fibrillary GFAP immunofluorescence within the cytoplasm of astrocytes. Indirect immunofluorescence ×200.

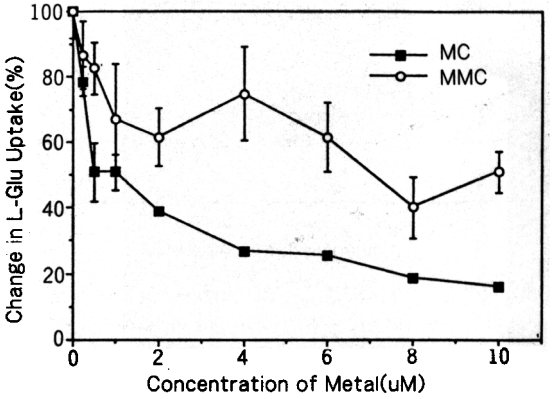

Fig. 2. Effects of $HgCl_2$ and CH_3HgCl on L-GLU uptake in cultured astrocytes. Note immediate drop of uptake in a dose-dependent manner following exposures to $HgCl_2$. After 14 min of incubation the uptake declined approximately 50% at concentrations below 2 μM and to 14.2% of its original level at 10 μM. Following exposure to MMC, 50% decline in L-GLU uptake occurred at 10 μM.

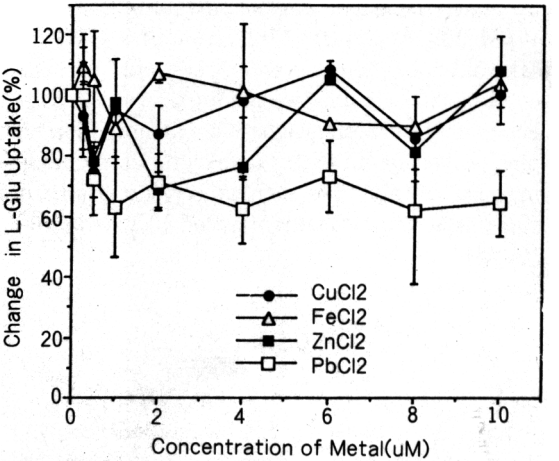

Fig. 3. L-GLU uptakes following exposure to $CuCl_2$, $FeCl_2$, $ZnCl_2$ and $PbCl_2$, respectively. No significant change in L-GLU uptake was noted.

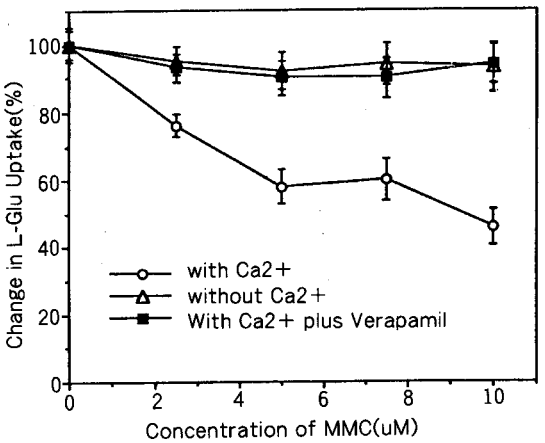

Fig. 4. *The pattern of L-GLU uptake in calcium-free medium and in the presence of voltage-sensitive calcium channel blocker verpamil under MMC exposure. Note absence of L-GLU inhibition following preincubation in 10 μM verapamil and in calcium-free medium, whereas MMC inhibition was still notable in the presence of calcium in the culture medium.*

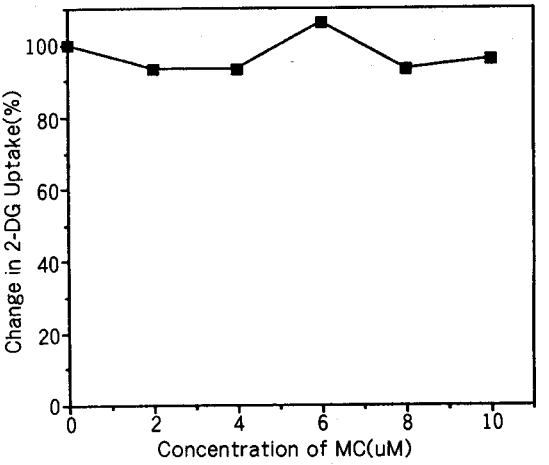

Fig. 5. *2-DG uptake following exposure to MC. No signficicant change in 2-DG uptake was noted at concentrations of metal associated with significant disturbance in L-GLU uptake.*

exposed to $CuCl_2$, $FeCl_2$ or $ZnCl_2$ (Fig. 3). On the other hand, exposure to $PbCl_2$ resulted in a sharp drop in L-GLU uptake below 2 μM that leveled off at approximately 63% of the original level, but the effect was not further enhanced at 10 μM (Fig. 3). CH_3HgCl inhibition of L-GLU uptake did not take place when cells were incubated either in calcium-free medium or in the presence of the voltage-sensitive calcium channel blocker verapamil (Fig. 4). No significant change in 2 DG uptake was seen at concentrations of $HgCl_2$ that dramatically affected L-GLU uptake (Fig. 5).

DISCUSSION

The results of this study clearly indicate that mercurial compounds cause significant and selective inhibition of L-GLU uptake in cultured mouse astrocytes at levels that do not affect the uptake of 2-DG, a sensitive indicator of certain other vital cellular functions (Brookes, 1988). The lack of a dose-dependent L-GLU uptake response following exposure to other divalent metal ions, such as $CuCl_2$, $FeCl_2$ and $ZnCl_2$, further suggests that such inhibition is relatively specific for mercurial compounds. At concentrations below 2 μM, exposure to $HgCl_2$ resulted in a saturation type of uptake pattern, whereas at higher concentrations (up to 10 μM) a more gradual decrease in L-GLU uptake was demonstrated. Although $PbCl_2$ showed similar uptake saturation initially, its magnitude was much less than that observed with $HgCl_2$. Exposure to 0 to 10 μM CH_3HgCl, on the other hand, caused a direct and gradual decrease in L-GLU uptake without uptake saturation.

Similar findings were reported by Brookes (1988, 1992) who demonstrated selective inhibition of L-GLU transport in astrocytes by $HgCl_2$ at a fraction of the concentration needed to inhibit 2-DG transport or to produce cytotoxic effects. Metallic Hg (Hg°) is highly volatile, and toxic damage generally results from inhalation of Hg vapor. Hg vapor which readily crosses the blood-brain barrier (BBB), is oxidized to Hg^{2+} and probably exerts its toxic effects in this form. A contrast between the effects of $HgCl_2$ and CH_3HgCl on L-GLU transport and on pro-

tein synthesis in astrocytes was also noted by Brookes and Kristt (1989).

Although the precise mechanism for the differential effects of organic and inorganic Hg is not clear, the bifunctional nature of Hg^{2+} as opposed to the monofunctional character of CH_3Hg^+ in forming Hg-sulfide bridges between cysteine residues in proteins has been suggested as the reason for the difference (Brookes and Kristt, 1989). Cantoni *et al.* (1984) also demonstrated significant inhibition of astroglial L-GLU transport at a fraction of the concentration of $HgCl_2$ needed to produce cytotoxic change.

In previous work (Choi and Lapham, 1981; Choi *et al.* 1980, 1981; Choi and Kim, 1984; Choi and Simpkins, 1986) we have shown that cytotoxic changes in cultured neurons and astrocytes become evident by light microscopy at much higher Hg concentrations and at longer exposure times. The most apparent ultrastructural findings following exposure to cytotoxic levels are stripping of the cell membranes and the appearance of electron-dense coagulative changes within them (Choi and Lapham, 1981). However, following a brief exposure (5 min) to 10 μM methylmercuric chloride (MMC), a marked shift in the distribution of anionic groups along the astrocytic surface membrane was observed in the absence of any discernible ultrastructural alteration (Peckham and Choi, 1986). It was hypothesized that such alterations in surface charge might trigger a cascade of pathological responses on the part of cytoplasmic organelles, including depolymerization of microtubules (Abe *et al.* 1975; Choi, 1991; Choi and Lapham, 1981; Miura *et al.* 1978; Sager *et al.* 1983), as a result of rapid influx of extracellular Ca^{2+} through the damaged cell membranes.

It is important to note that L-GLU uptake inhibition by MMC did not occur in the absence of extracellular calcium or in the presence of the voltage-sensitive calcium channel blocker verapamil, at least within the limits of the experimental paradigm employed in this study (Fig. 4). It appears, therefore, that the L-GLU uptake mechanism in astrocytes is closely linked to the verapamil-sensitive calcium channels, and that MeHg probably affected calcium entry through this channel. A significant increase in intrasynaptosomal and intracytoplasmic free Ca^{2+} following exposure to MeHg has been reported (Glaum, 1990; Komulainen and Bondy, 1987) and is thought to be related to nonspecific damage to the plasma membrane by MeHg, rendering it leaky to Ca^{2+} (Komulainen and Bondy, 1987). It is not certain whether or not a similar L-GLU uptake pattern in astrocytes would exist when damage to the plasma membrane is severe enough to be leaky to Ca^{2+}. Nonetheless, it is clear that impairment of L-GLU transport in astrocytes following exposure to Hg at a fraction of the concentration neeed to produce cytotoxic damage is potentially significant and may represent a critical feature of Hg-induced neurotoxicity.

Although our findings support the hypothesis that Hg-induced disturbances in astroglial L-GLU transport may be of importance in the development of neurotoxic cellular injury, whether or not the same mechanisms are operative in vivo remains speculative. Previous studies in our laboratory (Choi, 1989) showing significant modifications in EAA receptor densities in selected regions of the cerebrum following prolonged MeHg intoxication in young adult mice suggest the possibility of excitotoxic damage and/or alterations in plasticity in selected neuronal groups. It has been reported that L-GLU transport in cultured astrocytes differs greatly depending upon site of origin (Hansson *et al.* 1985; Shousboe and Divac, 1979). It is possible, therefore, that differences in astroglial function in different regions of the brain with regard to L-GLU transport may also contribute to selective vulnerability to Hg toxicity. More precise information relating to the distribution and concentration of Hg within brain tissue and to the quantitative contribution of astroglia toward inactivation of L-GLU in vivo would help to clarify some of these questions.

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