The Effects of Methylmercury on Mitochondrial Function and Reactive Oxygen Species Formation in Rat Striatal Synaptosomes Are Age-Dependent

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Methylmercury (MeHg) is especially toxic to the developing central nervous system. In order to understand the reasons for this age-dependent vulnerability, we compared the effects of MeHg on formation of reactive oxygen species (ROS) and mitochondrial function in striatal synaptosomes obtained from rats of various ages. Basal ROS levels were greater, and basal mitochondrial function was lower, in synaptosomes from younger animals, compared to adult animals. MeHg induced ROS formation in synaptosomes from rats of all ages, although the increases were greatest in synaptosomes from the younger animals. MeHg also reduced mitochondrial metabolic function, as assessed by MTT reduction, as well as mitochondrial membrane potential; again, the greatest changes were seen in synaptosomes from early postnatal animals. These age-dependent differences in susceptibility to MeHg are most likely due to a less efficient ROS detoxifying system and lower activity of mitochondrial enzymes in tissue from young animals.

Key Words: methylmercury; synaptosomes; reactive oxygen species; mitochondria; development.

At present, the reasons for the greater sensitivity of the developing nervous system to MeHg are not well understood. Differences in toxicokinetics and toxicodynamics lead to greater accumulation of MeHg in fetal brain than in maternal brain. Even beyond these differences, however, the developing nervous system also appears to be more sensitive to MeHg, due to factors intrinsic to the brain (Choi, 1989). Elucidation of the mechanisms involved in MeHg toxicity would aid in understanding the greater sensitivity of the developing nervous system. Although such studies have been performed both in adult animals and in in vitro test systems, mechanisms of MeHg toxicity have not, to the best of our knowledge, been compared in developing and adult animals.

A number of mechanisms and molecular targets have been proposed to be involved in MeHg neurotoxicity, including alterations in calcium homeostasis (Komulainen and Bondy, 1987; Marty and Atchison, 1997), binding to sulfhydryl groups (Hughes, 1957), and apoptosis/necrosis (Kunimoto, 1994). However, in the recent years, several studies have implicated the formation of reactive oxygen species, or ROS, (Ali et al., 1992; LeBel et al., 1990; Sarafian and Verity, 1991; Yee and Choi, 1994, 1996) and disruption of mitochondrial function (Bondy and McKee, 1991; Hare and Atchison, 1992; Limke and Atchison, 2002) as two key mechanisms in MeHg-induced neuronal damage. We hypothesized that differences in ROS production and/or defense mechanisms, and mitochondrial enzyme activities contribute to the greater sensitivity of developing animals to MeHg, compared to adult animals. To test this hypothesis, we have compared ROS formation and mitochondrial function after MeHg exposure in synaptosomes from postnatal day 7, 14, or 21 rats to that of adult rats.

MATERIALS AND METHODS

Materials. Adult male and timed-pregnant Long Evans rats were obtained from Taconic Farms (Germantown, NY). Methylmercuric chloride (98%) was purchased from Sigma-Aldrich (St. Louis, MO), 2′,7′-Dichlorofluorescin-diacetate (DCFH-DA) and 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR). Pierce BCA protein assay
reagents were purchased from Pierce Biotechnology, Inc. (Rockford, IL). All other chemicals were analytical grade.

**Preparation of striatal synaptosomes.** All procedures involving animals were performed according to the protocols of the Wadsworth Center Institutional Animal Care and Use Committee. Striatal synaptosomes were prepared essentially as described previously (Andersen et al., 2003). Briefly, naive male Long Evans rats were sacrificed at an age of 10–14 weeks (adults) or on postnatal day (PND) 7, 14, or 21. For PND 7–21 rats, animals were collected from different litters. The brains were removed and placed on ice, and the striata were quickly dissected. The striata from the animals of the same age were pooled and homogenized on ice in 10 volumes of 0.32 M sucrose in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 4°C for 10 min at 600 × g in a Beckman TJ-6 centrifuge. The supernatant was then diluted 1:1 with 1.3 M sucrose, to obtain a suspension at a final concentration of 0.8 M sucrose. This suspension was further centrifuged at 20,000 × g for 30 min at 4°C, resulting in a myelin-rich supernatant and a pellet (P2) consisting of synaptosomes and free (extrasynaptosomal) mitochondria. The supernatant was discarded, and the pellet was resuspended in oxygenated HEPES buffer (pH 7.4). The synaptosomes were kept on ice until the experiments were performed, usually within 15–20 min.

**Assessment of ROS formation.** DCFH-DA, a nonfluorescent cell-permeable compound, diffuses passively across cell membranes. Once inside the cell, the acetate groups are cleaved by intracellular esterases, yielding 2′,7′-dichlorofluorescin (DCFH). DCFH can be oxidized by hydroxyl radicals, peroxynitrite, or H2O2 (in the presence of peroxidases) to a fluorescent compound, 2′,7′-dichlorofluorescin (DCF) (Myhre et al., 2003).

Synaptosomes were diluted 1:40 in HEPES buffer before loading with 10 μM DCFH-DA for 15 min at 37°C. DCFH-DA stock solutions (10 mM in dimethylformamide) were made fresh daily. After incubation, the synaptosomes were pelleted by centrifugation (1200 × g, for 7 min), and the buffer was replaced with fresh HEPES buffer only.

Working solutions of MeHg or H2O2 (the latter used as a positive control) were prepared daily by diluting stock solutions in HEPES buffer to 1.67 × the desired final concentration, and 150 μl of the working solutions were placed in each well of a 96-well microplate (Costar). The reaction was started by the addition of 100 μl of the synaptosome solution to each well (final reaction volume 250 μl). Four wells were used for each condition. Each plate was incubated in a shaker incubator at 37°C for 30 min before fluorescence was measured using a Perkin Elmer LS50 spectrometer (excitation wavelength 488 nm, emission wavelength 525 nm, band widths 5 nm). Blank values were obtained from wells containing buffer and synaptosomes that had not been loaded with DCFH-DA. Synaptosomal protein levels were determined with Pierce BCA reagents according to provided instructions. DCF fluorescence values were corrected for protein levels and autofluorescence of the samples, according to the formula

\[ F_{\text{blank}} = (F_{\text{obs}} - F_{\text{std}}) / \text{synaptosomal protein} \]

where \( F_{\text{obs}} \) is corrected fluorescence value, \( F_{\text{std}} \) is observed fluorescence in the sample, and \( F_{\text{blank}} \) is observed fluorescence in the blank.

**Assessment of mitochondrial metabolic function.** Mitochondrial metabolic function was assessed by the conversion of the dye methylthiazoletriazolium (MTT) to formazan. This assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase to metabolize MTT into formazan, a reaction that takes place only in functionally intact mitochondria. Working solutions of MeHg were prepared as described above. P2 synaptosomes were prepared as described for the ROS assay, but because protein contents were lower in the synaptosome fractions from the younger rats, the synaptosomes were diluted in HEPES buffer to 1:20 for PND 7 rats, 1:27 for PND 14 rats, and 1:40 for PND 21 and adult rats; these dilutions provided similar protein levels for samples from animals of differing ages. 150 μl of the working solutions were aliquoted into microtubes, and 100 μl of the synaptosome suspension and 25 μl of a 5.0 mg/ml solution of MTT in HEPES buffer were added to each tube. The samples were incubated for 30 min at 37°C. The purple formazan crystals were pelleted by centrifugation, and the supernatant discarded. The pellets were dissolved in DMSO and transferred to 96-well microplates (Falcon). The formation of formazan was quantitated spectrophotometrically at 570 nm using an EL808 Ultra Microplate Reader (Bio-Tek Instruments, Inc). Data were expressed as percentage of age-matched vehicle control.

**Mitochondrial membrane potential.** Mitochondrial membrane potential (ΔΨm) was measured using the fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarboxyanine iodide). This dye exhibits a fluorescence shift from red to green, due to disassembly of dye aggregates, when the mitochondrial membrane potential decreases (Reers et al., 1991). Synaptosomes were prepared as described above and diluted 1:40 in HEPES buffer. The synaptosomes were then incubated with MeHg in HEPES buffer at 37°C for 30 min. The synaptosomes were pelleted by centrifugation (1500 × g, 4°C) for 5 min, the buffer was replaced with fresh buffer without MeHg, and JC-1 (stock solution 0.5 mM in DMSO) was added to a final concentration of 0.5 μM. After 20 min incubation at 37°C, fluorescence was measured in an EPICS’ Elite ESP flow cytometer (Coulter Corp.), using a 525 ± 10 nm standard filter for detection of green fluorescence and a custom-made 595 ± 10 nm filter (Omega Optical, Inc) for detection of orange fluorescence. For each sample, 10,000 events (synaptosomes) were analyzed. Due to day-to-day variations in fluorescence intensity, data were expressed as percentage of age-matched vehicle control.

**Statistical analysis.** Data were compared for significant differences by analysis of variance (ANOVA). When the overall test of significance lead to rejection of the null hypothesis, a post hoc test was performed to determine the source of the effects. Basal levels for ROS and MTT were compared by Bonferroni’s test, whereas the effects of MeHg on ROS, MTT, and ΔΨm were analyzed by Dunnett’s test. Differences were considered significant when \( p \leq 0.05 \).

## RESULTS

**ROS Formation**

ROS levels in nonexposed synaptosomes were dependent on the age of the donor rats (Fig. 1). The highest background levels were found in synaptosomes from young animals (5.2 ± 0.8, 6.3 ± 0.6, and 4.8 ± 0.5 fluorescence units per μg protein for PND 7, 14, and 21 animals, respectively), and the lowest levels in synaptosomes from adult animals (2.6 ± 0.1). ROS levels were significantly higher in synaptosomes from the young rats (PND 7, 14, and 21) than in synaptosomes from the adult; however, there were no significant differences in ROS levels among the younger animals.

MeHg-exposure increased ROS formation in synaptosomes from both adult and young animals (Fig. 2), with the highest ROS levels observed in synaptosomes from younger animals. The sensitivity to MeHg-induced increases in ROS was, in decreasing order: PND 7 > PND 14 > PND 21 > adult rat synaptosomes. In synaptosomes from PND 7 rats, MeHg concentrations as low as 1.0 μM gave a small, but significant, increase in ROS formation. For synaptosomes from PND 14 or older rats, 2.5 μM MeHg was needed to induce statistically significant increases in ROS. Univariate analysis of variance showed that there were significant effects of both age and MeHg on ROS levels (correlation coefficients 0.721 and 0.935,
respectively; \( p \leq 0.001 \), as well as a significant interaction between age and MeHg concentration (correlation coefficient 0.620; \( p \leq 0.001 \)).

It is possible that different properties of synaptosomes from young and adult rats could influence the uptake or deesterification of DCFH-DA, and that this could account for the differences in ROS formation that we observed among synaptosomes from rats of differing ages. To investigate this possibility, we used H\(_2\)O\(_2\) as a positive control. Because H\(_2\)O\(_2\) is cell permeable (Halliwell and Gutteridge, 1999), it crosses the synaptosomal membrane and oxidizes deesterified DCFH independently of intracellular ROS-generating mechanisms. We found that 100 \( \mu \)M H\(_2\)O\(_2\) increased ROS formation to the same extent (2–2.6 fluorescence units/\( \mu \)g protein) in preparations from rats of all ages (Table 1), demonstrating that differences in the effects of MeHg on ROS formation among the preparations were not due to differences in intrasynaptosomal DCFH concentration.

### Mitochondrial Metabolic Function

Basal levels for the reduction of MTT to formazan were dependent on the age of the donor rats (Fig. 3). The mean ± SE was 2.5 ± 0.3 for PND 7, 3.2 ± 0.2 for PND 14, 4.8 ± 0.2 for PND 21, and 4.1 ± 0.2 for adult rat synaptosomes (expressed as \( A_{570} \) corrected for protein contents of the samples). Because of these differences, results were expressed as percentage of age-matched vehicle (DMF) controls.

MeHg exposure reduced mitochondrial metabolic function in synaptosomes from rats of all ages (Fig. 4), with significant reductions observed after exposure to MeHg at 2.5 \( \mu \)M or greater concentrations in synaptosomes from PND 7, PND 14, and PND 21 rats. Concentrations of MeHg ≥ 5 \( \mu \)M were needed to reduce mitochondrial function in adult rat synaptosomes. At 10 \( \mu \)M MeHg, MTT metabolism in all synaptosomal preparations was reduced to 25–35% of age-matched controls. Univariate analysis of variance showed that MTT reduction was highly dependent on MeHg concentration (correlation coefficient 0.917, \( p \leq 0.001 \)). There was also a significant
Mitochondrial Membrane Potential

MeHg reduced $\Delta \psi_m$ in synaptosomes from rats of all ages (Fig. 5); 0.1 $\mu$M MeHg reduced $\Delta \psi_m$ to $75 \pm 8$ and $73 \pm 6\%$ of control levels in synaptosomes from PND 7 and PND 21 rats, respectively. Small reductions were also observed at this exposure level in synaptosomes from PND 14 and adult rats, but these did not reach statistical significance. After exposure to 0.5 $\mu$M MeHg, large decreases in $\Delta \psi_m$ were observed in all preparations; however, the effects were much more pronounced in synaptosomes from PND 7 and PND 14 animals, for which $\Delta \psi_m$s were reduced to $17 \pm 4$ and $29 \pm 7\%$ of control levels, respectively. In synaptosomes from PND 21 and adult animals, 0.5 $\mu$M MeHg reduced $\Delta \psi_m$ to $57 \pm 10$ and $60 \pm 7\%$ of control levels. With higher MeHg exposures (1.0–2.5 $\mu$M) the $\Delta \psi_m$ was completely abolished in synaptosomes from rats of all ages. Univariate analysis of variance showed a strong correlation between MeHg concentration and reduction of $\Delta \psi_m$ (correlation coefficient 0.981; $p \leq 0.001$). There was also a significant correlation between age and reduction of $\Delta \psi_m$ and an interaction between age and MeHg concentration (correlation coefficients 0.448 and 0.716, respectively; $p = 0.007$ and $p \leq 0.001$).

**DISCUSSION**

**ROS Formation**

In the present study we have shown that basal ROS levels are higher in synaptosomes from young animals than in those from adults. It is well known that the fetus and the neonate have lower levels of antioxidants and enzymes that protect against ROS than do adult animals (Buonocore *et al.*, 2001). Mavelli and coworkers found that Mn-superoxide dismutase (MnSOD) activity was almost absent in the brains of 7-day-old rats, while the activity increased to about 90% of adult levels at 3 weeks of age (Mavelli *et al.*, 1982). Similarly, catalase and cytosolic Cu,ZnSOD levels also increased with age, although, in general, catalase levels in brain are very low, and the changes in Cu,ZnSOD levels were less prominent than with MnSOD (Mavelli *et al.*, 1982). Less efficient scavenging of ROS by antioxidants and enzymes such as SOD could therefore be a reason for the higher basal ROS levels found in synaptosomes from younger animals.

The finding that basal ROS levels are higher in synaptosomes from younger animals is in contrast to the observations of Driver *et al.* (2000), who found that striatal ROS levels were low during development and increased in the adult rat. This discrepancy could be due to the fact that Driver and coworkers used a crude homogenate preparation that contained glial fractions, as glia contains ROS scavengers, such as metallothionein and glutathione, and are important for maintenance of GSH levels in neurons (Dringen *et al.*, 2000; Kohler *et al.*, 2003).

Our results also show that MeHg exposure increases ROS formation in synaptosomes *in vitro*. These results are in agreement with previous findings that MeHg exposure increases oxidative stress, both *in vivo* in rodent brain (Ali *et al.*, 1992; Yee and Choi, 1994) and in several *in vitro* systems, including primary cultures of rat cerebellar granule cells, cerebral cortical neurons, astrocytes, and oligodendrocytes, and in synaptosomes from whole brain (except cerebellum) (Bondy and McKee, 1990; Shanker and Aschner, 2003; Yee and Choi, 1996). Although the exposure times in our experiments were considerably shorter than those used in cell-culture...
experiments, the MeHg concentrations required to induce ROS formation are in the same range (1–2.5 μM), demonstrating that striatal synaptosomes are highly sensitive to the toxic effects of MeHg.

Most importantly, the increases in ROS seen after MeHg exposure were dependent on the age of the donor rats, with the highest ROS levels seen in synaptosomes from the youngest animals. To the best of our knowledge, age-related differences in ROS formation after MeHg exposure in rat tissue have not been reported previously. There are several possible explanations for our observations. First, as already mentioned, young animals have lower levels of antioxidants and less active ROS detoxifying enzymes than do adults, which may result in a reduced capability to clear cells of toxic ROS once they are formed. Furthermore, in a recent study it was shown that prenatal exposure to MeHg decreased antioxidant enzyme activity (Vicente et al., 2004). Second, it is possible that ROS generating systems are more active in tissue from young animals, resulting in higher ROS levels. Although the route of MeHg-induced ROS formation is presently unknown, mitochondria are believed to be key targets for MeHg (Hare and Atchison, 1992) and are important sites of ROS formation (Halliwell and Gutteridge, 1999). An increased capacity for ROS formation in the mitochondria of the young animals could render the system more vulnerable to the effects of MeHg. Furthermore, these mechanisms are not exclusive, so a combination of increased ROS formation and less ROS detoxification could synergistically cause the large increases in ROS levels in young animals.

**Mitochondrial Metabolic Function**

Basal levels of MTT reduction were significantly lower in synaptosomes from the youngest rats, compared to preparations from older rats. This age-dependent difference is most likely due to lower activity of succinate dehydrogenase (SDH), the enzyme primarily responsible for reduction of MTT, in the mitochondria of young rats (Pollak and Duck-Chong, 1973; Potter et al., 1945). The activity of SDH in brain is constant with essential sulfhydryl groups. It is possible that the low SDH activity needed to maintain normal mitochondrial function, then a lower basal SDH activity in the young animals may cause reduced tolerance to toxicants that further decrease SDH activity. The low basal SDH activity in tissue from young rats may therefore contribute to the higher sensitivity of the developing brain to MeHg.

Our finding that MeHg reduces mitochondrial metabolic function in the MTT assay is in agreement with results of a previous study in primary cerebellar granule cells, where exposure to 2.5 μM MeHg for 1 h reduced MTT metabolism to approximately 45% of control levels (Castoldi et al., 2000). In the adult brain, the cerebellar granule cell is the cell type with the highest sensitivity to MeHg; these cells die at concentrations at which surrounding cells like astrocytes and Purkinje cells are spared (Hunter and Russel, 1954). However, after developmental MeHg exposure, damage is more widely distributed, and severe degeneration and atrophy can be present throughout the cerebellum and the cerebral hemispheres (Eccles and Annau, 1987). Furthermore, previous experiments in our laboratory have shown reduced levels of dopamine in striatal synaptosomes after in vitro exposure to MeHg (Shan and Seegal, unpublished data), and there is evidence that methylmercury affects the activity of the plasma membrane dopamine transporter in rat striatal tissue (Faro et al., 2002). Therefore, we chose to investigate effects of MeHg on synaptosomes from the striatum. Our results indicate that striatal synaptic terminals also are highly sensitive to MeHg exposure.

**Mitochondrial Membrane Potential (ΔΨₘ)**

The results of the present study demonstrate that MeHg exposure leads to a reduction in ΔΨₘ in mitochondria, with the greatest effects seen in tissue from the youngest animals. It is known that the activity of several mitochondrial enzymes is low at birth and increases with age in rats; these enzymes include (in addition to SDH) glutamate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, cytochrome oxidase, and ATP phosphohydrolase (Pollak and Duck-Chong, 1973; Potter et al., 1945). A lower basal activity of such enzymes in the neonate, relative to adults, could mean that the added insult of MeHg will have a greater effect on mitochondria from younger animals. For example, cytochrome oxidase (complex IV) is an enzyme of the mitochondrial electron transport chain that contributes to the electrochemical gradient across the inner mitochondrial membrane. This enzyme is inhibited by methyl- and ethylmercury (Bickar et al., 1984; Mann and Auer, 1980; Usuki et al., 1998), possibly due to interactions of mercury with essential sulfhydryl groups. It is possible that the low basal activity of this enzyme in young animals renders mitochondria more vulnerable to MeHg-induced inhibition of cytochrome oxidase, contributing to age-dependent differences in ΔΨₘ.

Reductions in ΔΨₘ may influence neuronal survival, as cells induced to undergo apoptosis show an early reduction in ΔΨₘ prior to exhibiting other apoptotic markers, such as DNA fragmentation or externalization of phosphatidylserine. Complete disruption of ΔΨₘ is only found in cells that are destined to die by apoptosis or necrosis (Kroemer et al., 1997). The complete loss of ΔΨₘ in our experiments, therefore, indicates that MeHg exposure, even at very low concentrations, could be sufficient to induce neuronal death in young animals. Although
apoptosis is a cellular phenomenon and thus cannot be studied entirely in synaptosomes, this observation is in agreement with findings of previous studies, in which 0.1–3.0 μM MeHg was shown to induce apoptosis in various cell types in culture, including cerebellar granule cells from rats and mice, neuroblastoma cells, and PC12 cells (Bulleit and Cui, 1998; Castoldi et al., 2000; Kunimoto, 1994; Miura et al., 1999).

Our results also demonstrate that ΔΨm is a very sensitive endpoint for MeHg toxicity, since significant reductions were observed even after exposure to MeHg concentrations as low as 0.1 μM. These results are in agreement with an earlier study by Limke and Atchison (2002), who found that mitochondria in primary cultures of rat cerebellar granule cells depolarize irreversibly after 50-min exposure to 0.5 μM MeHg. In contrast, Castoldi and coworkers did not observe effects of 1 μM MeHg on ΔΨm for up to 4–6 h (Castoldi et al., 2000). Differences between these studies may be due to methodology, since in the study by Castoldi and coworkers, the loss of membrane potential was not assessed quantitatively. Nevertheless, our results clearly indicate that the striatal synaptosome preparation is highly sensitive to MeHg-induced effects.

In addition to the mechanisms investigated in the present study, several other mechanisms have been suggested for MeHg-induced brain damage. These include inhibition of glutamate uptake in astrocytes, with subsequent rise in extracellular glutamate and excitotoxicity, and alterations in intracellular calcium levels (for review see Costa et al., 2003). In vivo, such mechanisms may operate in concert to cause the symptoms of MeHg poisoning. For example, increased cytosolic calcium can lead to calcium accumulation in the mitochondria and depolarization of the inner mitochondrial membrane, leading to loss of the proton gradient and opening of the mitochondrial permeability pore. Increased intracellular calcium can also lead to formation of ROS by activation of enzymes such as nitric oxide synthase, leading to formation of nitric oxide (Halliwell and Gutteridge, 1999). Further research is necessary to elucidate the role of these mechanisms in MeHg-induced developmental neurotoxicity.

In conclusion, we have shown that ROS are produced, and that mitochondrial function and ΔΨm are reduced, in striatal synaptosomes from rats after exposure to MeHg. The use of synaptosomes as a model system allows the comparison of preparations made from rats at different developmental stages, and although the effects are present in tissue from both young and adult rats, they are most prominent in preparations from the youngest animals. This demonstrates that factors intrinsic to the brain, and not toxicokinetics alone, contribute to the high sensitivity of the developing brain to MeHg. From the present study it cannot be determined whether MeHg-induced increases in ROS levels are a cause or a consequence of mitochondrial damage; further experiments are currently being undertaken to elucidate this. In any case, the lower basal activities in ROS-detoxifying mechanisms and mitochondrial enzymes, either alone or in combination, are likely to contribute to the observed age-related differences in sensitivity to MeHg.

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