

# Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity

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## Abstract

This review addresses the mechanisms of methylmercury (MeHg)-induced neurotoxicity, specifically examining the role of oxidative stress in mediating neuronal damage. A number of critical findings point to a central role for astrocytes in mediating MeHg-induced neurotoxicity as evidenced by the following observations: a) MeHg preferentially accumulates in astrocytes; b) MeHg specifically inhibits glutamate uptake in astrocytes; c) neuronal dysfunction is secondary to disturbances in astrocytes. The generation of reactive oxygen species (ROS) by MeHg has been observed in various experimental paradigms. For example, MeHg enhances ROS formation both *in vivo* (rodent cerebellum) and *in vitro* (isolated rat brain synaptosomes), as well as in neuronal and mixed reaggregating cell cultures. Antioxidants, including selenocompounds, can rescue astrocytes from MeHg-induced cytotoxicity by reducing ROS formation. We emphasize that oxidative stress plays a significant role in mediating MeHg-induced neurotoxic damage with active involvement of the mitochondria in this process. Furthermore, we provide a mechanistic overview on oxidative stress induced by MeHg that is triggered by a series of molecular events such as activation of various kinases, stress proteins and other immediate early genes culminating in cell damage.

## Key words

- Methylmercury neurotoxicity
- Oxidative stress
- Glutamate and selenocompounds
- Reactive oxygen species
- Astrocytes

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## Introduction

Methylmercury (MeHg) is a major neurotoxicant that continues to pose appreciable risk to human health as evidenced by the tragic epidemics of MeHg poisoning in Japan and Iraq (1,2). All sources of environmental mer-

cury represent a potential risk for poisoning in humans through the methylation of inorganic mercury to MeHg in waterways, resulting in MeHg accumulation in the sea food chain and representing the most prevalent source for human consumption. Excessive MeHg ingestion from a diet high in fish is associated with

aberrant central nervous system (CNS) functions (3-5). Recent studies in human populations support the earlier findings that maternal exposure to mercury during pregnancy is associated with neurological as well as neuropsychological deficits detectable in the child at 6 to 7 years of age (6,7). Another recent study has pointed to the selective detrimental effects of MeHg on neurogenesis. However, despite these observations, the issue remains controversial, as exemplified by other studies (8,9) in which no association was noted between MeHg and neurodevelopmental outcomes in children at 66 months of age. Thus, the ultimate effects of MeHg in the human population remain unknown and clearly there is an urgent need to understand the mechanisms and consequences of MeHg exposure for CNS function. The current literature suggests that no single mechanism can explain the multitude of effects observed in MeHg-induced neurotoxicity.

### **The role of astrocytes in brain function**

Among various cell types, astrocytes represent a major cell type occupying approximately 50% of the CNS volume and their dysfunction following toxicant exposure has been implicated as a main cause of the observed neurotoxicity (10). The "foot" processes of these cells are known to be closely associated with synapses, axonal tracts, nodes of Ranvier, and capillaries. Astrocytes perform important duties and some of their functions include the maintenance of normal extracellular ion concentrations, the uptake of potassium ( $K^+$ ) and the control of extracellular pH. They express a pantheon of receptors and uptake systems for neurotransmitters, properties that were formerly thought to be exclusively neuronal. Thus, astrocytes can respond to and prevent the build-up of potentially dangerous levels of neurotransmitters (e.g., glutamate) in the extracellular fluid (11). Important roles of astrocytes dur-

ing early brain development include the synthesis and elaboration of cues for neuronal migration and the production of neurotrophic factors important for neuronal division and differentiation. Astrocytes also induce the high electrical resistance (tightness) of the blood-brain barrier that limits the transport of noxious substances entering the brain, and modulates optimal transport of nutrients and metabolites (12).

### **Mechanisms of methylmercury-induced neurotoxicity and glutamate**

Although not the only cell type to be adversely affected by MeHg, a number of studies have established a key role for astrocytes in mediating MeHg neurotoxicity: a) astrocytes represent a preferential cellular site for MeHg accumulation (13-16); b) MeHg selectively inhibits astrocytic transport of cystine and cysteine (Figure 1), thereby adversely affecting their redox status and attenuating glutathione (GSH) content (17-21); c) MeHg inhibits astrocytic glutamate (and aspartate) uptake (Figure 1) and stimulates its efflux, thereby increasing glutamate concentrations in the extracellular fluid and sensitizing neurons to excitotoxic injury (17,18,22-24); d) MeHg-induced neuronal dysfunction is secondary to disturbances in astrocytes (25), and the *in vitro* co-application of non-toxic concentrations of mercury with glutamate results in the appearance of typical neuronal lesions found with excitotoxic stimulation (26); e) MeHg causes the activation of cytosolic phospholipase  $A_2$  leading to arachidonic acid release and further inhibition of the glutamate transporter (Figure 1), setting in motion an unimpeded cytotoxic cycle (27,28).

### **The role of reactive oxygen species in methylmercury-induced neurotoxicity**

Though free radicals are known to play a

physiological role in optimal cell function, excessive oxidative stress has been implicated in a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (29-31). Oxidative stress also plays an important role in other degenerative conditions such as autoimmune and inflammatory diseases (i.e., ischemia and rheumatoid arthritis), cancer, diabetes mellitus, and atherosclerosis (32-34), as well as in metal-induced toxicity (35,36). The balance between oxidative and reductive cellular processes is known to be adversely affected in these various disorders (37). Oxidative stress is associated with the accumulation of high

levels of toxic reactive species, such as reactive oxygen species (ROS), reactive nitrogen species, reactive nitrogen oxygen species, as well as unbound metal ions (38). Typical ROS include oxygen radicals such as superoxide radical, hydroxyl radical, as well as non-radical derivatives of oxygen including hydrogen peroxide ( $H_2O_2$ ). Reactive nitrogen species include nitric oxide radical, and reactive nitrogen oxygen species include the highly reactive oxidant species peroxynitrite, which is a product of reaction between nitric oxide and the superoxide radical. These reactive species are highly oxidizing and potentially damaging to cellular redox-sensitive proteins, enzymes

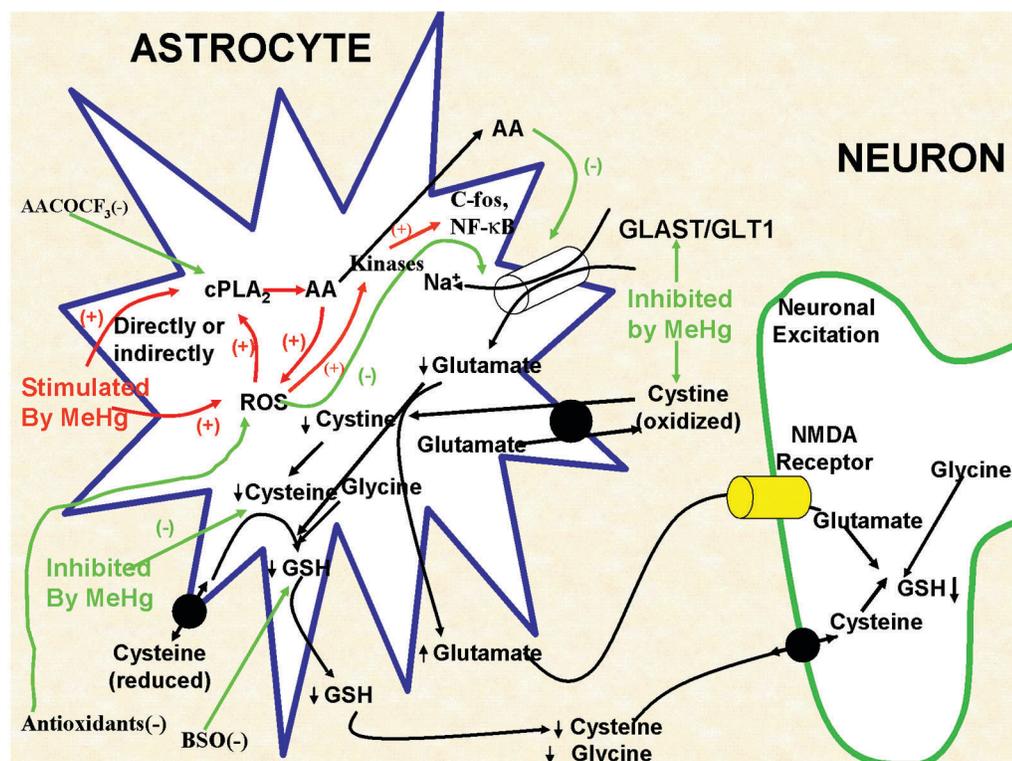


Figure 1. A schematic model of some of the currently proposed as well as some of our previously studied processes resulting in methylmercury (MeHg)-dependent neurotoxicity. Red lines and text indicate processes where MeHg stimulates cellular processes, whereas green lines and text indicate targets for MeHg-induced inhibition. For example, MeHg is known to increase the release of arachidonic acid (AA) from astrocytes and to stimulate glutamate release, increasing its synaptic levels. At the same time MeHg also inhibits glutamate uptake, as well as a number of the amino acids that are associated with the synthesis of astrocytic glutathione (GSH). Combined, these effects lead to a reduction in intracellular GSH levels and increased synaptic glutamate levels, which in turn activate NMDA receptors on adjacent neurons, leading to excitotoxicity. cPLA<sub>2</sub> = cytosolic phospholipase A<sub>2</sub>; ROS = reactive oxygen species; NF-κB = nuclear factor kappa B; BSO = L-buthionine-[S,R]-sulfoximine; NMDA = N-methyl D-aspartate; GLAST = glutamate aspartate transporter; GLT1 = glutamate transporter 1.

and DNA, and they also cause peroxidation of membranes.

ROS are known to mediate MeHg-induced neurotoxicity in multiple experimental models. For example, MeHg induces ROS formation *in vivo* (rodent cerebellum), and *in vitro* (isolated rat brain synaptosomes) (39), as well as in cerebellar neuronal cultures, a hypothalamic neuronal cell line and in mixed reaggregating cell cultures (40-42). In addition, an increase in ROS has been observed in a) mitochondria isolated from MeHg-injected rat brains (36), b) mitochondria isolated *in vitro* from rat brain and then exposed to MeHg (43), and c) mitochondria from Hg- and glutamate-exposed astrocytes and neurons (44,45). MeHg-induced ROS production and MeHg-induced glutamate dyshomeostasis are connected phenomena affecting each other. In fact, MeHg-induced inhibition of astrocyte glutamate transporters leads to increased glutamate concentrations in the extracellular fluid, causing hyperactivation of N-methyl D-aspartate type glutamate receptors and leading to an increase in Na<sup>+</sup> and Ca<sup>2+</sup> influx (46). Increased intracellular Ca<sup>2+</sup> levels are associated with the generation of ROS (47). On the other hand, MeHg-induced ROS (mainly H<sub>2</sub>O<sub>2</sub>) production appears to directly inhibit astrocyte glutamate transporters, leading to increased glutamate concentrations in the extracellular fluid (17,18). This ROS formation resulting from MeHg- and glutamate-induced oxidative stress contributes to mitochondrial dysfunction. Overproduction of ROS is mediated, at least in part, by glutamate, since this toxicity can be attenuated by N-methyl D-aspartate receptor antagonists. The source of glutamate is likely to be astrocytic, given the effect of MeHg on glutamate uptake and release (Figure 1) from astrocytes (17,18,48). Therefore, although the toxic damage caused by MeHg might be most prevalent in neurons, a large body of literature suggests that neuronal damage in response to MeHg most likely represents aberrant control of the extracellular milieu by astrocytes.

Experimental studies have investigated

the potential protective effects of antioxidant molecules (i.e., GSH precursors and antioxidant selenocompounds) against MeHg-induced neurotoxicity (49,50). Since hydrogen peroxide has been shown to be an important hazardous molecule involved in MeHg toxicity (17,18,50), such protective effects appear to be related, at least in part, to the ability of these compounds to mitigate the deleterious effects of hydrogen peroxide. MeHg exhibits a direct inhibitory effect on the activity of glutathione peroxidase in mouse CNS, leading to increased lipid peroxidation and decreased glutamate uptake into cerebrocortical slices (50,51). Organoseleno compounds with thiol peroxidase activity show protective effects that appear to be related to the maintenance of H<sub>2</sub>O<sub>2</sub> status at low physiological levels in MeHg-exposed systems (50,52). Although the GSH precursor N-acetylcysteine can contribute to the maintenance of GSH intracellular homeostasis, which is crucial for the detoxification of hydrogen peroxide by glutathione peroxidase, part of the beneficial effects elicited by N-acetylcysteine under *in vivo* conditions is also related to its ability to accelerate urinary MeHg excretion in poisoned animals (53). However, even though antioxidant molecules have been showing protective effects against MeHg-induced neurotoxicity under experimental conditions (21,50), their use as possible therapeutic agents in MeHg poisoning is far from becoming a reality. Currently, the only way to prevent or ameliorate toxicity in MeHg poisoning is to accelerate its elimination from the body. Strategies for removing MeHg include hemodialysis, exchange transfusion, and chelation therapy (54,55).

### Signaling pathways mediating methylmercury-induced neurotoxicity

A number of reports indicate oxidative stress-induced activation of some signaling molecules (i.e., distinct kinases/transcription factors (nuclear factor kappa B, NF-κB),

(activator protein-1, etc.)/early response genes (c-fos, c-jun, etc.)). This activation leads to induction of various target genes (i.e., inducible nitric oxide synthase, cyclooxygenase II, manganese-superoxide dismutase, inducible form (HSP-72), cytokines, etc.), which contribute to cell damage (56-58). Studies have also shown that distinct kinases mediate the metal/toxicant-induced toxicity both downstream and upstream of generated ROS (43). For example, the ROS-generating nicotinamide-adenine dinucleotide phosphate oxidase enzyme is stimulated by zinc in astrocytes and neurons in a protein kinase C (PKC)-dependent manner. Furthermore, the upstream involvement of tyrosine kinase, PKC, and mitogen-activated protein kinase (MAPK) pathway kinases in MeHg-induced generation of ROS in synaptosomes has been demonstrated with selective inhibitors (43). The generated ROS resulting from oxidative stress (induced by a variety of agents like copper, arsenic, chromium, H<sub>2</sub>O<sub>2</sub>, angiotensin II, and from degenerative conditions) causes the downstream activation of a variety of kinases (p38MAPK, ERK and JNK), as well as transcription factors such as NF- $\kappa$ B, leading to a cytotoxic response (59,60).

There are no detailed and systematic studies examining how MeHg-induced ROS formation in astrocytes ultimately leads to cytotoxicity, and what, if any, is the role of various signal transduction pathways in this process. Figure 2 (see legend for a detailed description) depicts various targets that may be affected by MeHg. Future studies with astrocytes should be designed to answer these issues and to determine if the neurotoxic effects of MeHg are, at least in part, due to the generation of ROS and may activate signaling pathways involving distinct kinases (i.e., PKC, tyrosine protein kinase, p38MAPK, and ERK MAPK), phospholipase A<sub>2</sub>, as well as immediate early genes (c-fos) and transcription factor NF- $\kappa$ B (please refer to Figure 2). Since mitochondria are known mediators

of ROS generation, future studies on the efficacy of mitochondrial permeability transition pore inhibitors and mitochondrial cytochrome C release in attenuating MeHg-induced cellular damage should be profitable. Another consequence of increased oxidative stress is the induction of the mitochondrial permeability transition, a Ca<sup>2+</sup>-dependent process characterized by the opening of the permeability transition pore in the inner mitochondrial membrane. This causes increased permeability to protons, ions and other solutes  $\leq$ 1500 Da, in turn, leading to a

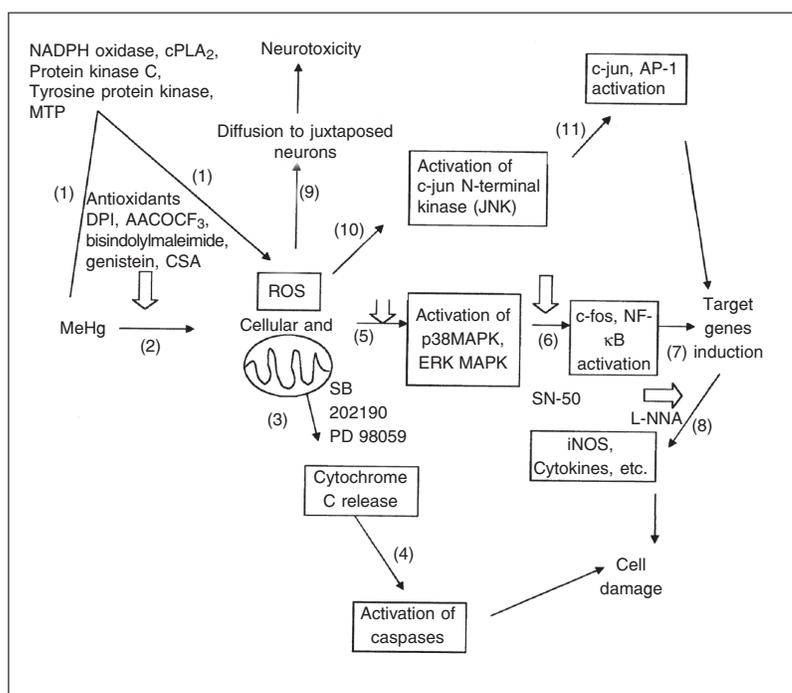


Figure 2. A model depicting the role of oxidative stress in methylmercury (MeHg) neurotoxicity: 1) by involvement of various enzymes; 2) increased reactive oxygen species (ROS) can potentially be prevented with a) antioxidants, b) nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase inhibitor (DPI), c) cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) inhibitor (AACOCF<sub>3</sub>), d) protein kinase C inhibitor (bisindolylmaleimide), e) tyrosine protein kinase inhibitor (genistein), and e) microsomal triglyceride transfer protein (MTP) inhibitor (CSA); 3,4) release of cytochrome C into cytosol from mitochondria; 5) reactive oxygen species (ROS) formed may lead to the activation of p38 mitogen-activated protein kinase (p38MAPK) and extracellular-signal regulated kinase (ERK) MAPK, which may be inhibited by pretreatment with inhibitors SB 202190 and PD 98059; 6) stimulated kinases may mediate the activation of c-fos and nuclear factor kappa B (NF- $\kappa$ B); 7,8) possible induction of target proteins like inducible nitric oxide (iNOS) may be blocked with nitro-L-arginine (L-NNA), an iNOS inhibitor; 9) the diffusion of ROS from astrocytes to adjacent neurons can lead to possible mitochondrial damage in neurons; 10,11) there may be involvement of other signaling (i.e., JNK, c-jun, and AP-1) pathways in modulating MeHg cytotoxicity. SN-50 = inhibitory peptide derived from the p50 protein.

collapse of the mitochondrial inner membrane potential ( $\Delta\Psi_m$ ). Loss of the  $\Delta\Psi_m$  results in colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis, and further generation of ROS. The concentration-dependent deleterious effects of MeHg on mitochondrial  $\Delta\Psi_m$  in cultured astrocytes suggest that  $\Delta\Psi_m$  is a very sensitive endpoint for MeHg toxicity, and these effects are consistent with increased ( $\text{Ca}^{2+}$ ); triggering ROS formation and in-

creased oxidative stress. What has yet to be established is the temporal sequence of events reported here and whether changes in membrane potential precede the changes in oxidative stress. This would be consistent with early reports from our laboratory showing that MeHg increases cellular permeability to ions such as  $\text{Na}^+$  (and  $\text{K}^+$ ), and that an increase in  $\text{Na}^+$  permeability via  $\text{Na}^+/\text{H}^+$  exchange, offsetting  $\text{K}^+$  loss, is the primary mechanism in its inhibition of regulatory volume decrease in astrocytes.

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