Methylmercury Neurotoxicity: Exploring Potential Novel Targets

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Abstract

Mechanistic studies on the effects of MeHg in the central nervous system (CNS) have been limited to morphology, substrate uptake and macromolecular synthesis, differentiation, and changes in gene expression during development and adulthood, but its primary site of action has yet to be identified. Proper functioning of the nitric oxide synthase (NOS)-cyclic GMP and the cyclooxygenase (COX)-prostaglandin (PG) signaling pathways in the CNS depend on post-translational modifications of key enzymes by chaperone proteins. The ability of MeHg to alter or inhibit chaperone-client protein interactions is hitherto unexplored, and potentially *offers an upstream unifying mechanism for the plethora of MeHg effects,* ranging from reactive species generation (ROS) generation, mitochondrial dysfunction, changes in redox potential, macromolecule synthesis, and cell swelling. In view of the prominent function of astrocytes in the maintenance of the extracellular milieu and their critical role in mediating MeHg neurotoxicity, they afford a relevant and well-established experimental model. The present review is predicated on (a) the remarkable affinity of mercurials for the anionic form of sulfhydryl (-SH) groups, (b) the essential role of thiols in protein biochemistry, and (c) the role of molecular chaperone proteins, such as heat shock protein 90 (Hsp90) in the regulation of protein redox status by facilitating the formation and breakage of disulfide bridges. We offer potential sites where MeHg may interfere with cellular homeostasis and advance a novel mechanistic model for MeHg-induced neurotoxicity.

INTRODUCTION

Methylmercury: A Ubiquitous Environmental Threat

Mercury (Hg) is a global pollutant which knows no environmental boundaries. Even the most stringent control of man-made sources of mercury pollution will not eliminate human exposure to potentially toxic quantities, given its ubiquitous presence in the environment. Environmental exposure to mercury occurs primarily *via* the food chain due to accumulation of MeHg in fish. Latest statistics in the US indicate that 46 states have fish consumption advisories covering 40% of the nation's rivers, lakes and streams. In addition, mercury is a

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common pollutant in hazardous waste sites in the nation (EPA 2001) [1]. It is estimated that 3–4 million children live within one mile of at least one of the 1,300+ active hazardous waste sites in the USA [1].

The effects of prenatal MeHg exposure associated with maternal fish consumption on intellectual function in children have been investigated in two large, prospective, longitudinal studies; one in the Seychelles Islands [2] and the other in the Faroes Islands [3]. A National Academy of Sciences (NAS) expert panel reviewed the studies, concluding that the weight of the evidence supported MeHg's adverse health effects [4] and recommended that levels of mercury not exceed 5.0 μg/L in whole blood or 1.0 μg/g in hair, corresponding to a reference dose (RfD) of 0.1 μg/kg body weight/day.

Evidence that Astrocytes Mediate MeHg-Induced Neuro-Toxicity

Astrocytes are the most numerous non-neuronal cell type in the CNS. They make up ~50% of human brain volume [5]. Astrocytes perform several functions that are essential for normal neuronal activity, including glutamate uptake (80% of synaptic glutamate), glutamate release, K^+ and H^+ buffering, and water transport [6]. Interactions between axons and astrocytes (radial glia) furnish the cues for axon guidance of migrating neurons. Understanding of the molecular signals that regulate neuron-glia interactions has increased greatly with the advent of molecular and cellular biological techniques and genetically modified mice. Cell ablation studies using genetically targeted ectopic gene expression and gene knockout with single cell specificity have established the distinct roles played by astrocytes during development [7].

There is abundant evidence in support of the *pivotal* role of astrocytes in mediating neurotoxicity, establishing astrocytes as a unique and relevant experimental model for the assessment of mechanisms underlying MeHg-induced cytotoxicity.

1. Chronic exposure to MeHg in primates is associated with preferential accumulation of MeHg in astrocytes (and to some extent in microglia) [8–10].

2. MeHg inhibits astrocytic glutamate uptake and stimulates its efflux [11–15], increasing in vivo glutamate concentrations in the extra-cellular fluid (ECF) and sensitizing neurons to excitotoxic injury [16]. Central nervous system damage associated with MeHg correlates spatially with brain areas that have dense glutamatergic innervation [17]. The ionotropic glutamate receptor N-methyl-D-aspartate (NMDA) antagonist, dizocilpine (MK801), protects against neuronal damage induced by *in vivo* MeHg [17–19].

3. MeHg selectively inhibits astrocytic (but not neuronal) uptake systems for cystine and cysteine transport [20–22] compromising glutathione (GSH) synthesis and the CNS redox potential [13]. Compared with astrocytes, neurons have lower levels of GSH and of a second putative antioxidant, metallothionein (MT) [23,24], making them more susceptible to the effects of increased ROS. Since GSH synthesis is dependent upon precursors derived from astrocytes [25, 26], MeHg-induced inhibition of cystine transport and astrocytic GSH

production would ultimately lead to decreased neuronal GSH levels and increased glutamate toxicity.

4. MeHg-induced ROS formation in astrocytes can be attenuated by antioxidants [27], reversing its functional effects on glutamate uptake inhibition [14]. Most recently, we have established MeHg-induced ROS generation using measurements of the sensitive lipid peroxidation biomarkers, isoprostanes [28].

5. Co-application of non-toxic concentrations of mercury with glutamate results in the appearance of typical neuronal lesions associated with excitotoxic stimulation [29].

6. In human and non-human primates chronic in vivo exposure to MeHg is associated with swelling of astrocytes [8, 9, 30, 31], a process associated with release of glutamate into the extracellular fluid [32].

7. In the absence of glutamate, neurons are unaffected by exposure to mercury, suggesting that neuronal dysfunction is secondary to disturbances in astrocytes [33].

8. Additional data invoke astrocytic cytosolic phospholipase A_2 ($cPLA_2$) as a target for MeHg toxicity, supporting the notion that cPLA2-stimulated hydrolysis and release of arachidonic acid (AA) play a role in MeHg-induced neurotoxicity [27].

MERCURY AND THIOLS

Thiol groups play fundamental structural and functional roles in protein chemistry, being located mainly within the active catalytic sites of many enzymes. Mercury compounds react specifically with active sulfhydryl (-SH) groups forming complexes of defined stoichiometry, -S-Hg-R. The high affinity of MeHg for the anionic form of -SH groups is responsible for the toxicological behavior of this compound [34, 35]. 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 15–23, whereas its affinity constants for oxygen-, chloride-, or nitrogen-containing ligands such as carboxyl or amino groups are about 10 orders of magnitude lower [36]. Indeed, wherever a MeHg compound has been identified in biological media, it has been complexed to -SH-containing ligands. Complexes with cysteine and GSH have been identified in blood [37, 38], and complexes with GSH have been identified in brain [39], liver [40], and bile [41]. Armed with this knowledge, a search for thiol-containing proteins present a number of intriguing molecular targets that might explain the vast array of cellular disruptions caused by MeHg exposure. The class of proteins known as molecular chaperones or heat shock proteins represents one attractive target.

Hsp90

The 90-kDa heat shock protein (Hsp90) is one of the most abundant proteins in cells, constituting 1–2% of total intracellular protein [42, 43]. It is constitutively and ubiquitously expressed and is the most abundant molecular chaperone of the eukaryotic cytoplasm. Chaperones help to achieve and maintain the conformational status of cellular proteins and

enzyme complexes. By influencing higher order protein structure, Hsp90 is involved in the conformational regulation of key proteins in multiple signaling pathways, including nitric oxide synthases (NOS), kinases, phosphatases and steroid hormone receptors.

Reactive cysteines are usually found in the vicinity of the ATP binding site of chaperone proteins [44]. Hsp90 regulates the redox status of other proteins by assisting in the formation and breakage of disulfide bridges. The cysteine groups participate in the binding of Hsp90 with its client proteins and with molybdate, a stabilizer of Hsp90-client protein interactions [44]. The high redox reactivity of its cysteines (cysteines 521 and 589/590) provides the mechanism by which this molecular chaperone helps maintain the redox status of the cytosol. Oxidizing conditions impair the chaperone activity of Hsp90, further proof of the active participation of sulfhydryl groups in the function of Hsp90 [44].

Hsp90 AND CYTOCHROME C

The primary function of the mitochondrial electron transport chain (ETC) is ATP synthesis. The ETC, present in the inner mitochondrial membrane, is grouped into four enzyme complexes. Damage to one or more of the ETC complexes may lead to impairment of cellular ATP synthesis [45]. The electron transport protein, cytochrome c (MW 12,000 kDa), serves as the electron carrier from complex III (ubiquinol:cytochrome c oxidoreductase) to complex IV (cytochrome c oxidase). Hsp90 reduces cytochrome c, an effect that is mediated by the -SH groups of Hsp90. The effects of Hsp90 can be blocked by the sulfhydryl reagents, arsenite and cadmium, indicating the involvement of the highly conserved vicinal cysteine pair, Cys589/590, in the reduction of cytochrome c [44]. The high reactivity of Hsp90 cysteine groups toward cytochrome c may indicate a role of this chaperone in modulation of the redox status of the cytosol in resting and apoptotic cells.

Although classically considered the cell powerhouse, mitochondria are also "gatekeepers" that ultimately determine whether a cell lives or dies [46]. Upon exposure to oxidizing species and thiol-reactive agents, mitochondria undergo a permeability transition whereby inner membrane proteins form a non-specific pore [47]. Pore opening causes a loss of the mitochondrial membrane potential and impairs ATP synthesis. Pore opening also results in an inability to sequester calcium. The resulting energy depletion and calcium overload are important factors in necrotic cell death. Pore opening also leads to release of cytochrome c from the outer surface of the inner mitochondrial membrane to the cytosol. Cytochrome c release is an important early event in apoptotic cell death [47].

Several heavy metals, including MeHg, induce neuronal apoptosis in whole animals and cultured cells [48]. Possible triggering mechanisms include calcium overload and generation of ROS [49]. Calcium binds directly to the divalent metal binding site on the matrix side of the mitochondrial permeability transition pore and induces its opening [47]. MeHg may also open the pore by oxidizing GSH and/or NADH resulting in the oxidation of the sensitive vicinal dithiol present in the S-site and P-site of the permeability transition pore, respectively, or by cross-linking the dithiol in the S-site [47]. It has been shown that exposure to mercurials partially inactivates cytochrome i oxidase [50]. It is notable that cytochrome c oxidase has 7 cysteine residues as sulfhydryl groups which are subject to

modification by hydrophobic mercurials, such as MeHg. It is also noteworthy that apoptosis and the stress response are highly interrelated and altered expression of heat shock proteins exerts great influence on the development of apoptosis [44]. Studies directed at the effect of MeHg on the ability of Hsp90 to reduce cytochrome c or inactive cytochrome c oxidase may shed light on the mechanisms of MeHg-induced cell death.

NITRIC OXIDE (·NO) IN THE CNS

·NO is a gaseous messenger molecule with diverse biological roles. In the brain, ·NO has a number of important biochemical and physiological functions, such as neurotransmission and learning, regulation of glycolytic enzymes, pain perception, immune function, and vascular regulation. ·NO is formed from L-arginine by NO synthase (NOS). In the brain, astrocytes may be a major source of ·NO, because these cells have the highest concentration of the ·NO precursor, Larginine [51, 52]. At least three isoforms of NOS have been identified, namely, neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). All three NOS isoforms use NADPH as an electron donor and employ five enzyme cofactors to catalyze oxidation of arginine to ·NO with stoichiometric formation of citrulline [53]. The calcium/calmodulin (CaM) complex is the common key cofactor that triggers NOS activation. Activation of NOS enzyme activity is ultimately determined by the binding affinity of CaM to NOS. At resting calcium levels, nNOS and eNOS are inactive. Agonists, such as glutamate, initiate nNOS or eNOS activation by raising intracellular Ca2+ concentrations sufficiently to maintain CaM binding; it is the high binding affinity of iNOS for CaM that renders iNOS fully active under basal calcium levels in quiescent cells. The production of ·NO must be modulated precisely because too much or too little ·NO formation will perturb cellular homeostasis. Each of the three major NOS isoforms is regulated at multiple levels, including transcriptional and post-translational steps (See Hsp90 and NOS below).

In the CNS, all three NOS isoforms are expressed constitutively or inducibly [52, 54]. nNOS is localized to discrete regions of the brain where constitutive expression is exclusive to neurons [55]. However, astrocytes express nNOS after induction by LPS and/or cytokines [56]. The expression of iNOS is below detectable levels in the normal brain, but is induced in neurons, astrocytes, and other cells in various pathological conditions [57–61]. For example, iNOS expression increases in astrocytes after transient global ischemia and after LPS and cytokine exposures [55]. eNOS is also induced in rat brain by ip injections of LPS. eNOS protein expression is localized to astrocytes of both grey and white matter as well as blood vessels [57]. Several groups have reported that eNOS is expressed preferentially in astrocytes rather than in neurons [62–65].

While it is now apparent that ·NO has many physiological roles, it is also clear that excessive ·NO generation is cytotoxic. Overactivation of glutamate receptors associated with cerebral ischemia and other excitotoxic processes results in massive release of ·NO [53]. ·NO mediates cellular toxicity by damaging critical metabolic enzymes and by reacting with O_2 to form an even more potent oxidant, per-oxynitrite [45]. High levels of ·NO are associated with inflammatory, neurodegenerative, and cardiovascular/ischemic pathologies. Several *in*

vitro studies show that ·NO produced by iNOS in astrocytes mediates neuronal cell death after excitotoxic injury [46, 66, 67].

·NO AND CYTOCHROME C OXIDASE

There is clear-cut evidence that ·NO controls cell respiration [68] and that the neurotoxicity of ·NO is mediated though mitochondrial dysfunction. Low nanomolar levels of ·NO rapidly and reversibly inhibit mitochondrial respiration by binding to the oxygen binding site of cytochrome c oxidase (complex IV). Such inhibition will lead to increased generation of O_2^- upstream of complex IV. Reversible inhibition of respiration by ·NO has been observed in intact cells, including astrocytes in culture [69]. This inhibition can be reversed by NOS inhibitors and by binding ·NO with hemoglobin [70]. Higher concentrations of ·NO can produce irreversible modifications of proteins and lipids and impairment of mitochondrial respiration. Cultured astrocytes activated to express iNOS produce up to 1 μM ·NO and strongly inhibit their own cellular respiration by inhibiting complex IV [71]. The generation of the extremely potent oxidizing agent, per-oxynitrite ($ONOO^-$), formed from the reaction of ·NO with $·O_2^-$, is a major mediator of these effects and is a strong inducer of the permeability transition pore [69]. Mitochondria are a major site of production of $·O_2^-$ under normal and pathological conditions and are a likely source of this anion for the generation of $ONOO^-$ [67]. These results suggest that any cell producing high levels of ·NO will inhibit its own respiration and that of surrounding cells. There is increasing evidence that defects in mitochondrial energy metabolism may underlie the pathology of neurodegenerative diseases such as Parkinson's disease or Alzheimer's disease [72].

HSP90 AND NOS

Beyond CaM, nNOS and eNOS are also regulated by various scaffolding proteins through protein-protein interactions. Several laboratories have shown that both eNOS and nNOS activity are upregulated by Hsp90 binding [73–75]. Bender *et al.* [74] demonstrated that nNOS exists as a molybdate-stabilized heterocomplex with Hsp90 in the cytosolic fraction of human embryonic kidney 293 cells stably transfected with rat nNOS. Furthermore, nNOS activity is reduced by the Hsp90 inhibitors, geldanamycin [73] and radicicol [76]. Song AJp *et al.* [74] showed that Hsp90 directly augments nNOS catalytic activity mediated, at least in part, by enhanced CaM binding. Billecke *et al.* [76] proposed that Hsp90 facilitates functional heme entry into apo-nNOS by opening the hydrophobic heme-binding cleft in the protein. Using electron paramagnetic resonance spectroscopy, Song *et al.* [75] directly measured ·NO signals from purified nNOS and demonstrated that Hsp90 augmented ·NO formation from nNOS in a concentration-dependent manner.

Besides ·NO, all NOS isoforms can also produce $·O_2^-$. Again, using electron paramagnetic resonance spectroscopy [77] showed that Hsp90 directly inhibits $·O_2^-$ formation from nNOS, particularly in the absence of L-arginine. This inhibition was not due to $·O_2^-$ scavenging because Hsp90 did not affect the $·O_2^-$ generated by xanthine oxidase. Significant $·O_2^-$ production was detected from nNOS even at normal intracellular levels of L-arginine, an effect that was prevented by the addition of Hsp90. These results support the hypothesis that normal coupling between Hsp90 and nNOS is important for normal nNOS function.

Disruption of this coupling may result in $\cdot O_2^-$ overproduction, which could in turn lead to nNOS dysfunction and CNS pathology. The importance of Hsp90/nNOS interactions in the promulgation of neurotoxicity is incompletely understood. Furthermore, (as has been shown for the glucocorticoid receptor), association of nNOS with Hsp90 opens the thiol moieties in the ligand binding domain to attack by thiol-derivatizing agents [73].

OXIDANTS AND ANTIOXIDANTS IN ASTROCYTES

The generation of ROS begins with univalent transfer of an unpaired electron to O_2, yielding $\cdot O_2^-$. Some ROS, including $\cdot O_2^-$, H_2O_2, NO, and $ONOO^-$ are important signaling molecules that regulate cell function. There is also a growing body of evidence to implicate excessive or inappropriate generation of ROS in neuropathology [44]. Potential sources of $\cdot O_2^-$ in the CNS include NOS, cox, lipoxygenase, NAD(P)H oxidase, xanthine oxidase, and mitochondria [78]. NOS-dependent O_2^- formation has been implicated in multiple neuropathological conditions. The dominant effect of increased ROS production by astrocytes appears to be an attenuation of $\cdot NO$ signaling by $\cdot O_2^-$. Studies suggest that inactivation of $\cdot NO$ by $\cdot O_2^-$ contributes to impaired cell function and that $\cdot O_2^-$ scavengers are effective in restoring optimal cell function [79–81]. Local cellular levels of $\cdot O_2^-$ reflect both the rate of $\cdot O_2^-$ formation and the rate of its removal by endogenous antioxidants (primarily superoxide dismutases (SODs) and GSH). GSH is an important intracellular antioxidant in astrocytes. Reduced GSH can react with reactive nitrogen species (RNS) and hence can limit mitochondrial damage. The reactions of RNS with GSH provide a mechanism to explain the loss of cellular GSH that can occur following $\cdot NO$ exposure. Furthermore, these reactions will divert RNS away from critical cellular targets, such as the ETC, thereby explaining why GSH status appears to be so critical in dictating susceptibility to $\cdot NO$ and $ONOO^-$. Loss of GSH may be an important factor in the neurotoxicity associated with excessive $\cdot NO$ formation and mitochondrial damage [54, 82].

While MeHg is known to induce oxidative stress, it has yet to be determined whether this is due to a change in the balance of $\cdot NO$ and other ROS. Studies on molecular teratogenic mechanisms of MeHg in early developing rat embryos have noted significant induction of iNOS mRNA and protein as potential mechanisms of aberrant neurulation [83]. In mature rats, MeHg treatment results in significant increases in nNOS activities in the cerebrum and cerebellum, notably two areas that are preferentially affected in the adult animal [84].

MeHg causes ROS generation in nerve cells [85, 86] and MeHg exposure *in vivo* elevates ROS in brain regions sensitive to MeHg, but not in regions less sensitive to MeHg [87, 88]. Consistent with the oxidative stress findings above, studies in cultured primary rat astrocytes suggest that a multitude of MeHg effects (cell swelling, glutamate release, inhibition of glutamate uptake) can be attenuated by antioxidants [14, 89], whilst astrocytes depleted of GSH or those isolated from metallothionein knockout mice (MT–/–) are significantly more sensitive to the effects MeHg [90, 91]. The impact of MeHg on Hsp90 chaperone function, NOS isoform expression and activity, NOS uncoupling and region-specific ROS formation should prove to be a profitable area for future study.

ROS AND GLUTAMATE NEUROTOXICITY

The excitatory neurotransmitter glutamate has been shown to play a crucial role in neuronal damage associated with trauma, ischemia, toxins and chronic neurodegenerative disorders. Glutamate neurotoxicity (GNT) takes place as a result of glutamate binding to the *N*-methyl-D-aspartic acid (NMDA) receptor and to a minor extent to other receptor subtypes. GNT is marked by acute neuronal swelling and depends on extracellular Na^+ and Cl^- uptake by the cell that causes plasma membrane depolarization. This causes Ca^{2+} channel opening that triggers massive influx of extracellular Ca^{2+} and release from intracellular Ca^{2+} stores, resulting in calcium overload that initiates a cascade-like effect leading to cell death [92]. A causal relationship between GNT and ROS production was first proposed by Dykens [93] who observed that neuronal damage caused by the excitotoxin, kainate, is prevented by SOD. Conversely, the depletion of the antioxidant GSH was found to exacerbate GNT [94]. Electron paramagnetic resonance spectroscopy provided direct evidence that NMDA receptor activation leads to the generation of $\cdot O_2^-$ [19]. Glutamate stimulates production of $\cdot O_2^-$ *via* both NOS- and AA-dependent mechanisms [95]. Schulz *et al.* [96] providing the first *in vivo* evidence that excitotoxic neuronal injury is linked to free radical generation from mitochondria.

Almeida *et al.* [97] showed that GNT is associated with $\cdot$NO-mediated mitochondrial dysfunction and GSH depletion. $\cdot$NO is synthesized *via* activation of Ca^{2+}-dependent nNOS after glutamate receptor stimulation. Furthermore, glutamate-receptor stimulation in neurons causes a burst of $\cdot O_2^-$ formation, which can combine with $\cdot$NO to form $ONOO^-$. In rat neurons in primary culture, glutamate caused a dose-dependent increase in cGMP, delayed neurotoxicity and ATP depletion. GNT was prevented by NOS inhibition and by NMDA receptor antagonism [97, 98]. In striatal astrocytes from mouse embryos, glutamate evokes release of AA [99]. Released AA increases the accumulation of extracellular glutamate by blocking its reuptake and in turn glutamate acts on glutamate receptors to increase neuronal intracellular calcium and release additional AA [99]. Thus, AA inhibits glutamate reuptake by astrocytes and potentiates NMDA- evoked current in neurons. Interestingly AA and ROS were found to inhibit glutamate uptake in astrocytes *via* two distinct and additive mechanisms [100]. Ciani *et al.* [101] demonstrated that $cPLA_2$ and NOS activation give rise to ROS, whose deleterious action can be counteracted either by inhibiting these enzymes or by scavenging the excess of free radicals produced by them. More recently it has been also demonstrated that $cPLA_2$-stimulated hydrolysis and release of AA are potential mediators of MeHg-induced neurotoxicity [102].

DIFFERENTIAL SUSCEPTIBILITIES OF NEURONS AND ASTROCYTES

Within the CNS, the susceptibility of different brain cell types to $\cdot$NO and $ONOO^-$ exposure may be dependent on the intracellular reduced GSH concentration and an ability to increase glycolytic flux in the face of mitochondrial damage [44]. Acute exposure to $ONOO^-$ selectively damages neurons, whereas astrocytes remain unaffected [103]. $ONOO^-$ reacts with thiol-containing compounds and multiple lines of evidence support a key role for GSH in dictating cellular susceptibility to $ONOO^-$. In particular, astrocytes have high reduced GSH concentrations [23, 104]. $ONOO^-$ causes a pronounced loss of neuronal GSH

concentrations, without an effect on GSH levels in astrocytes [82]. This apparent preservation of GSH may reflect the greater activity, in astrocytes, of γ-glutamylcysteine synthetase, which is the rate limiting enzyme for GSH synthesis [105]. GSH depletion in neurons may be due to $\cdot$NO-dependent inhibition of cysteine uptake or from the direct reaction of $\cdot$NO/ONOO$^-$ with intracellular GSH. The importance of GSH in protecting the ETC from oxidizing species such as ONOO$^-$ is further illustrated by experiments with GSH depleted astrocytes. Under such conditions marked damage to the ETC and cell death occurs following ONOO$^-$ exposure [106]. Conversely, the apparent increase in resistance of neurons to $\cdot$NO when co-cultured with astrocytes can be explained by up-regulation of neuronal GSH as a result of the astrocytic release of GSH followed by cleavage, via γ-glutamyltranspeptidase (GABA), to dipeptides that can be utilized by the neuron for GSH synthesis [25, 104].

Susceptibility of different brain regions or cell types to MeHg [107] may also be dependent on factors such as the intracellular reduced GSH concentration and the ability to increase glycolytic flux in the face of mitochondrial damage. These observations are consistent with morphological observations in which astrocytes that accumulate MeHg appear normal, whilst neurons that are found in their proximity and are void of MeHg undergo cell death [10]. In view of the importance of antioxidants, in particular GSH, it is possible that agents that are capable of increasing cellular concentrations of GSH may prove to be of therapeutic importance.

PGES/p23

Prostaglandin (PG) biosynthesis can be divided into two kinetically distinct responses, the immediate and delayed responses, involving recruitment of distinct biosynthetic enzymes whose expression and activation are differentially regulated [107]. The immediate response occurs within minutes after stimulation by an agonist that increases cytoplasmic calcium. cPLA$_2$ is a prerequisite for supplying AA to constitutive COX-1 [108]. Delayed PG synthesis proceeds gradually over a long period after a proinflammatory stimulus and is accompanied by *de novo* induction of COX-2. The preference for COX-2 over COX-1 in the delayed response is partially explained by the ability of COX-2 to metabolize lower levels of AA to PGH$_2$ than is required for COX-1- mediated catalysis [108].

cPGES is a terminal enzyme of the COX-1-mediated PGE$_2$ biosynthetic pathway. Peptide microsequencing of purified enzyme revealed that it is identical to p23, a putative chaperone molecule that binds to the ATP-dependent conformation of Hsp90 and acts as a cofactor in the molecular chaperone function of Hsp90 [109]. cPGES/p23 is expressed ubiquitously and in abundance in a wide variety of tissues and cell types. Its expression is constitutive and not affected by proinflammatory stimuli, with the exception of rat brain, where LPS treatment increases cPGES levels several fold. cPGES/p23 is functionally linked with COX-1 in marked preference to COX-2 to produce PGE2 from exogenous and endogenous AA. The cPLA$_2$/COX-1/cPGES/p23 cascade is crucial for the production of PGE$_2$ that is required for maintenance of tissue homeostasis [109]. cPGES/p23 is highly conserved among animal species (>95%) and requires GSH for optimal activity. Enzyme activity is also stimulated by other -SH reducing agents, such as dithiothreitol (DTT) and 2-mercaptoethanol [110].

cPGES may be regulated by Hsp90 in that the Hsp90-bound form is functionally active [108].

In contrast, membrane-associated PGES (mPGES) is a microsomal GST-like 1 (MGST-L1) enzyme in the MAPEG (<u>m</u>embrane-<u>a</u>ssociated <u>p</u>roteins involved in <u>e</u>icosanoid and <u>G</u>SH metabolism) superfamily [108]. mPGES is an inducible perinuclear enzyme preferentially coupled with inducible COX-2 to promote delayed PGE2 generation. It is strongly induced following exposure to interleukin-1 and LPS in several cell types. The main PG species produced during the "delayed response" is PGE_2 associated with COX-2 expression. COX-2 inhibitors reduce PGE2 more profoundly than other PGs. Whether cofactors assist the functional linkage between COX-2 and mPGES remains unclear. The small size and lipophilic properties of PGE2 allow it to pass across the blood-brain barrier (BBB) and to diffuse into CNS neurons.

Astrocytes release glutamate through a calcium dependent process mediated by PGs [111, 112]. Pharmacological inhibition of PG synthesis prevents glutamate release, whereas application of PGE2 promotes calcium-dependent glutamate release from cultured astrocytes and brain slices [111]. As shown by our lab, glutamate is released as part of regulatory volume decrease (RVD) and 80% of the released glutamate is taken up by astrocytes [6]. MeHg induces astrocytic swelling and inhibits RVD in part by inhibiting reuptake of glutamate [32]. It is not known if the mechanism by which MeHg inhibits glutamate uptake in astrocytes is mediated by PGE_2. Distinct distribution of different receptor subtypes for PGE2 (of which there are 4) may account for the regional specificity of the cellular response triggered by MeHg in discrete brain structures, such as calcarine cortex, and the cerebellum [107].

Tying together the remarkable affinity of MeHg for -SH groups, the essential role of thiols in chaperone protein/client interactions, the crucial role of redox status and GSH sufficiency in cellular homeostasis and susceptibility to MeHg toxicity, we have identified Hsp90, cytochrome c, NOS and PGE_2 as potential upstream molecular targets of MeHg's cytotoxic effects. Fig. (1) depicts a working model where MeHg is posited to affect the cross talk between astrocytes and neurons. Panel **A** represents the normal interrelationship between astrocytes and neurons and Panel **B** depicts the alterations in astrocytic and neuronal cell signaling that occur with MeHg toxicity. Please refer to the figure legend for details.

CONCLUSIONS

In summary, we point to novel sites that are likely sensitive to interference by MeHg. Our theory is predicated upon a number of observation: (a) the remarkable affinity of mercurials for the anionic form of sulfhydryl (-SH) groups, (b) the essential role of thiols in protein biochemistry, and (c) the role of molecular chaperone proteins, such as heat shock protein 90 (Hsp90), in the regulation of protein redox status by facilitating the formation and breakage of disulfide bridges. Molecular chaperones help to achieve and maintain the conformational homeostasis of cellular proteins. The 90-kDa heat shock protein, Hsp90, is the most abundant molecular chaperone in the eukaryotic cytoplasm, helping to organize efficient multiprotein complexes for the execution of essential cellular processes. It has been shown

that cysteine groups of Hsp90 participate in its interactions with client proteins and that oxidizing conditions impair the chaperone function of Hsp90. This appears to be a fruitful area that merits future research.

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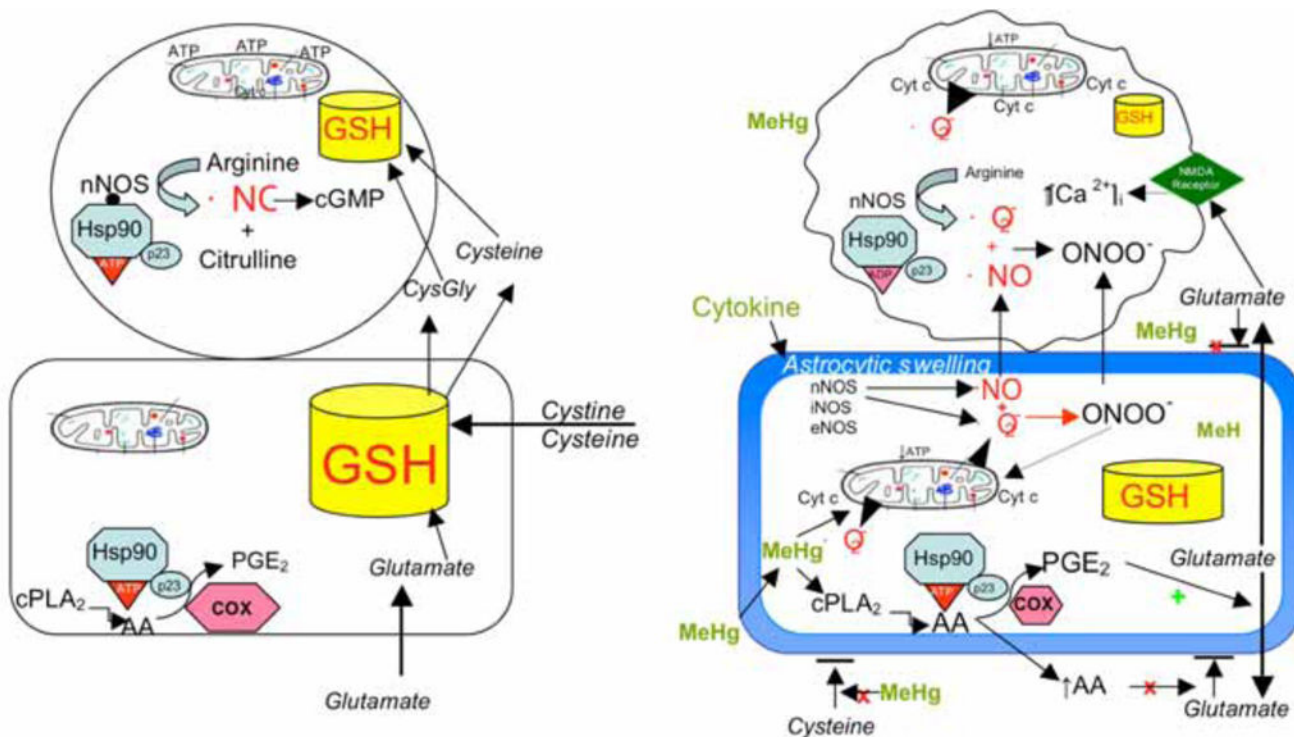
Fig. (1).

Panel **A** represents the **normal** interrelationship between astrocytes and neurons, in which astrocytes contain a larger pool of GSH (and other antioxidants) than is found in neurons. Astrocytes synthesize GSH from the uptake of cystine, cysteine and glutamate. Neuronal GSH concentrations are dependent largely on release of precursors from astrocytes. Healthy neurons express nNOS which, when bound to Hsp90 in the proper ATP conformation, produces ·NO which activates guanylate cyclase to produce cGMP for maintenance of important biological functions. Astrocytes do not express NOS but do produce PGE$_2$, which is important for normal tissue homeostasis. Intact mitochondrial function and intracellular GSH levels in both cell types are important for normal ATP production and cell bioenergetics. Panel **B** depicts the alterations in astrocytic and neuronal cell signaling that occur with **MeHg toxicity**. MeHg induces astrocytic swelling triggering glutamate release, and inhibits uptake of cystine and cysteine, reducing astrocytic ability to synthesize GSH. MeHg also stimulates cPLA$_2$ and AA release which stimulates increased production of PGE$_2$ *via* PGES/p23 bound to Hsp90. PGE$_2$ stimulates glutamate release and AA blocks reuptake, resulting in markedly increased levels of extracellular glutamate. Glutamate stimulates NMDA receptors on neurons increasing $[Ca^{2+}]_I$ which causes activation of nNOS and mitochondrial dysfunction. MeHg (perhaps by induction of cytokines) induces astrocytes to express nNOS (and other NOS isoforms) which produce both ·NO and ·O$_2^-$. These ROS combine to form ONOO$^-$, which together with NO diffuse to adjacent neurons increasing oxidant stress. This will markedly reduce intracellular GSH, especially in neurons. Increased ·NO, ONOO$^-$ and Ca^{2+} overload damages the mitochondrial ETC,

resulting in reduced ATP formation, increased $\cdot O_2^-$ formation, and cyt c release, all of which initiate the cascades leading to neuronal death.