

Investigations of Methylmercury-Induced Alterations in Neurogenesis

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Methylmercury (MeHg) has been an environmental concern to public health and regulatory agencies for over 50 years because of its toxicity to the human nervous system. Its association with nervous system toxicity in adults and infants near Minamata Bay, Japan, in the 1950s initiated environmental health research inquiries that continue to this day. Observations of greater neurotoxicity with gestational compared with adult exposure suggest a unique susceptibility of the developing nervous system to MeHg. Despite extensive research conducted over the last half century, determination of definitive molecular mechanisms underlying the observed neurotoxic effects of MeHg have not been identified. This paper summarizes results of a series of experiments conducted to examine the effects of MeHg on neuroepithelial cell proliferation, a hypothesized mode of action for its selective effects on neurogenesis. Observed effects of MeHg on cell cycle entry and progression were associated with alterations in a variety of cell cycle regulatory molecules, including p21 signaling pathways. We place these studies in the context of other cellular responses involved in signal transduction, including oxidative stress, altered protein phosphorylation, and altered intracellular calcium homeostasis. Although existing information suggests that no single mechanism underlies the diverse array of effects associated with MeHg-induced developmental neurotoxicity, we demonstrate characteristic effects of MeHg on cell signaling that contribute to observed effects on cell proliferation. Experimentally derived cell cycle kinetic and cytotoxicity data allowed development of a biologically based dose–response model of MeHg-induced alterations in neurodevelopment, which can form the basis for information synthesis and hypothesis testing and for use in assessing risks from environmental exposures. *Key words:* cell cycle, children's health, developmental toxicology, methylmercury, neurogenesis, proliferation. *Environ Health Perspect* 110(suppl 5):859–864 (2002).

<http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/859-864faustman/abstract.html>

Methylmercury (MeHg) is an environmental organometal that acts as a neurotoxicant in humans, nonhuman primates, rodents, and other mammals, and the developing nervous system is an important target of its toxicity (Burbacher et al. 1990; Clarkson 1987; NRC 2000). Epidemiologic studies conducted worldwide since the 1950s have associated MeHg exposure with a range of effects ranging from mild behavioral alterations to death (reviewed in NRC 2000). Human exposure is associated primarily with consumption of fish and seafood containing trophically accumulated MeHg.

The effects of *in utero* MeHg exposure differ from effects observed after childhood or adult exposure (Harada 1968a, 1968b; Takeuchi 1968, 1977; Tokuomi 1968). Prenatal exposure to MeHg appears to result in a widespread pattern of adverse effects on brain development and organization compared with the relatively restricted damage observed when exposure occurs later in life (Choi et al. 1978; Hamada et al. 1993; Matsumoto et al. 1965; Takeuchi 1968). The widespread nature of damage that occurs in cases of fetal disease in both animal models and humans is characterized by *a*) decreased cellular abundance (hypoplasia) and microcephaly in which brain weight can be reduced

by as much as one-half to two-thirds or normal; *b*) altered cellular migration, which results in dysplasia and altered cortical cytoarchitecture; and *c*) gliosis (Choi 1986, 1989; Choi et al. 1978; Eto et al. 1992; Geelen et al. 1990; Matsumoto et al. 1965; Mottet 1974, 1989; Mottet and Ferm 1983; Takeuchi 1968, 1977).

The fetus appears to be more sensitive to the toxic effects of MeHg relative to the mother, and adverse neurodevelopmental effects have been reported in the offspring of women showing little or no overt toxicity (Clarkson et al. 1985; Harada 1977, 1978; Marsh 1987; Marsh et al. 1987). The mechanisms underlying the differences in sensitivity are unclear but can be attributed at least in part to interruption of the highly regulated processes associated with fetal growth and development that are not occurring in the mother. In particular, normal central nervous system (CNS) development requires a highly synchronized progression of events involving rapid and coordinated cell division, migration, differentiation, and selective cell loss (Herschkowitz 1988). Agents that interfere with any of these processes may interfere with downstream events (Faustman et al. 1996), and the developing CNS may not be able to compensate for damage that occurs during

particular stages in development (Bayer 1989; Rice and Barone 2000). This limited compensatory capacity likely underlies not only the relatively higher sensitivity of the fetus to MeHg exposure relative to the adult but also differences in neuropathology.

The biochemical rationale for MeHg-induced cellular toxicity is incompletely understood. As a general rule, the biochemical toxicity of MeHg and other mercurials is attributed to its extremely high affinity for protein sulfhydryl groups (Hughes 1957). Because of the common role for disulfide bond formation in stabilizing and maintaining protein tertiary structure, nonspecific changes in protein structure or enzyme function may occur following interaction between MeHg and cysteine sulfur groups. The nonspecific nature of interaction between MeHg and cellular macromolecules makes it unlikely that there is a single underlying event responsible for the myriad effects observed upon MeHg exposure.

Several lines of evidence support the hypothesis that the reduced cell number observed upon MeHg exposure *in vivo* derives from inhibition of cell proliferation, primarily in mitosis (M), rather than cell necrosis. First, MeHg-induced inhibition of germinal cell proliferation demonstrates the capacity of MeHg to reduce the number of progenitor cells and indicates lasting effects from inhibition of cell proliferation during early CNS development (Choi 1991; Sager et al. 1984).

Second, histologic evidence has demonstrated reduced brain size and weight (microcephaly) and evidence of impaired cell cycle transition and mitotic inhibition, in the absence of focal necrosis (Choi 1989; Howard

This article is part of the monograph *Molecular Mechanisms of Metal Toxicity and Carcinogenicity*.

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This work was supported by National Institute of Environmental Health Sciences, National Institutes of Health grants P01-ES09601, P30-ES07033, R01-ES10613-01, and 2T-32ES0-7032, and U.S. Environmental Protection Agency grants R-826886-01-0 and R-825358-01-2. We express appreciation to A.J. Fornace Jr. and W. Harper for technical contributions, and to S.W. Hong for assistance in various aspects of this project.

Received 21 March 2002; accepted 4 June 2002.

and Mottet 1986; Matsumoto et al. 1965; Rodier et al. 1984). These findings suggest that impaired cell proliferation rather than necrotic damage underlies the observed microcephaly.

Third, examination of the cell cycle effects elicited by MeHg demonstrates mitotic inhibition both *in vivo* and *in vitro* (Miura et al. 1978; Ponce et al. 1994; Rodier et al. 1984; Sager 1988).

In this article we summarize results of a series of experiments conducted to characterize the biochemical, cellular, and molecular effects of MeHg on neuroepithelial cell proliferation. Observed effects demonstrate that MeHg can alter gene expression, intracellular redox balance, and intracellular calcium (Ca^{2+})_i homeostasis at levels of exposure also associated with altered cell cycle entry and progression. Results of these experiments were compiled in a biologically based dose–response model of MeHg-induced neurodevelopmental toxicity that is used to explore the role of alternative pathways on overall toxicity and generate hypotheses for further testing.

Materials and Methods

Primary rat embryo CNS cells and *in vivo* assessments were used to study the developmental toxicity of MeHg (Alfa Aesar, Ward Hill, MA, USA). The use of the *in vitro* cell culture system allowed well-controlled evaluation of the effects of MeHg on cell viability, differentiation, and cell cycling (Ou et al. 1999a; Ponce et al. 1994). As with other regions of the developing CNS, midbrain neuroepithelial cell proliferation appears to be sensitive to the effects of *in utero* MeHg exposure (Rodier et al. 1984). The primary neuroepithelial cell cultures derived from the gestation day 12 rat midbrain during the rapid phase of midbrain neuronal proliferation yield a mixed cell culture enriched in neuroblasts that undergo terminal differentiation without the addition of exogenous growth factors. As a result, these cells are an excellent model for investigating various aspects of normal brain development, including mechanisms of cell cycle regulation and terminal differentiation (Whittaker et al. 1993). Evaluation of these cultures demonstrates similarity in protein expression with the *in vivo* CNS midbrain (Whittaker et al. 1993). Because these cultures use primary cells rather than transformed or immortalized cell lines (e.g., neuroblastoma and glioma cell cultures), they serve as useful models for the evaluation of the mechanisms and effects of MeHg on developing CNS.

Methods used to investigate alterations in mRNA and protein levels of cell cycle regulatory genes [e.g., *p21*, growth arrest and DNA damage (*GADD*)45, *GADD153*] in developmentally exposed mice have been

previously described (Ou et al. 1997, 1999a). Briefly, these experiments involved prenatal MeHg exposure through drinking water (0, 3, 10 ppm) and harvest of fetal and adult neuronal tissues. Changes in gene expression and protein levels, examined by Northern blotting, Western blotting, and immunoprecipitation, were correlated with tissue MeHg content analysis.

The role of p21 in cell cycle alterations induced by MeHg was further examined using primary mouse embryonic fibroblasts (MEFs) of different p21 genotypes (wild type, heterozygous, and null) (Mendoza et al. 2002). Cells were harvested and isolated at day 14 of gestation and treated at passage 4–6 with either 0, 2, 4, or 6 mM MeHg or 50 nM colchicine (Sigma Chemicals, St. Louis, MO, USA) for 24 hr, with subsequent examination of cell cycle alterations by flow cytometry, as described below.

Investigations into the effects of MeHg on the cell cycle were performed using single- and dual-parameter flow cytometry. Single-parameter flow cytometric analysis of 4,6-diamidino-2-phenylindole (DAPI)-stained CNS cultures incubated with MeHg was used to quantify changes in DNA content as a means for examining changes in cell cycle phase distribution. Dual-parameter flow cytometric analysis of 5-bromo-2'-deoxyuridine (BrdU)-labeled cells, using two DNA fluorochromes, Hoechst 33258 and ethidium bromide (EB) was used to evaluate the progression of the primary CNS cells through the cell cycle (Rabinovitch et al. 1988). This method works well with asynchronous cell populations such as the primary cells used in experiments summarized here. DAPI, BrdU, Hoeschst 33258, and EB were purchased from Sigma.

The effects of MeHg on intracellular glutathione (GSH) and γ -glutamyl cysteine synthetase (γ -GCS) regulation in rat midbrain neuroepithelial cells was also examined (Ou et al. 1999b). These experiments used *N*-acetyl-L-cysteine and L-buthionine-(*S,R*)-sulfoximine (BSO) as control agents to induce and deplete, respectively, intracellular GSH. Relative changes in GSH content, γ -GCS activity, GSH levels, γ -GCS-heavy chain (γ -GCS-HC) mRNA expression, and γ -GCS-HC protein content were quantified over time in response to MeHg or control article treatment (Ou et al. 1999b).

Adherent cell analysis on the ACAS Ultima scanning laser cytometer (Meridian Instruments, Okemos, MI, USA) was used to examine the acute (17-min) effect of MeHg on indo-1 fluorescence as a measure of changes in intracellular calcium [Ca]_i. This instrument used a water-cooled Coherent Enterprise argon-ion laser (Coherent, Palo Alto, CA, USA) with excitation at 351–364 nm. Emission was split by a 445-nm longpass

dichroic and gathered independently by two photomultiplier tubes using either a 405/45-nm bandpass filter or a 530/30-nm bandpass filter. Ultraviolet (UV) laser output was maintained at 30 mW. Images were obtained through a 100 \times , 1.3 n.a., UV-corrected oil-immersion objective mounted on an Olympus IM-T-2 microscope. A determination of basal [Ca^{2+}]_i was performed on control cells (cells unexposed to MeHg) according to Grynkiewicz et al. (1985). Triplicate experiments were conducted to investigate the acute effects of MeHg exposure (17 min) on basal indo-1 fluorescence. Cells were incubated in 1 mL indo-1/AM solution for 45 min and then rinsed twice with either phosphate-buffered saline containing calcium and magnesium (Ca–PBS) or 0Ca–PBS (calcium- and magnesium-free PBS + 5 mM EGTA) (Gibco Life Technologies Inc., Grand Island, NY, USA). Cells were then incubated either in Ca–PBS or 0Ca–PBS and placed in a heated chamber (37°C) on the inverted microscope stage. After obtaining a 3-min baseline from a random field, MeHg was added to the chamber (0–10 μ M), and the indo-1 fluorescence was monitored for another 17 min. Scans were performed at 1-min intervals. Results are reported as percent change in the fluorescence ratio relative to the average basal fluorescence ratio without conversion to [Ca^{2+}]_i because of concerns raised by Hare et al. (1993) and Denny et al. (1993) that MeHg may be mobilizing divalent cations other than Ca^{2+} . Spectrofluorometric analysis of the indo-1 fluorescence spectrum showed no interaction between MeHg and indo-1. To investigate whether the addition of EGTA (Sigma) affected basal [Ca^{2+}]_i indo-1-loaded CNS cells were incubated in 0Ca–PBS and placed in a heated chamber (37°C) on the inverted microscope stage. After obtaining a 3-min baseline, EGTA (5 mM; Sigma) was added to the buffer, and indo-1 fluorescence was monitored for an additional 17 min.

Results

In vitro MeHg exposure resulted in primary neuroepithelial cell death in a time- and concentration-dependent manner (Ponce et al. 1994). For example, a 50% reduction in the number of live cells was observed following continuous exposure to approximately 2 μ M MeHg for 24 hr or to 0.25 μ M for 120 hr. This reduction in live cell number reflected both cell death and reduced cell production because of cell cycle inhibition.

Flow cytometric cell cycle kinetic analysis of primary rat midbrain neuroepithelial cells was conducted upon exposure to MeHg or colchicine (Ponce et al. 1994). After 12 hr of MeHg exposure, a concentration-dependent increase in the number of cells in the DNA synthesis phase of the cell cycle (S phase) was

seen by DAPI staining and DNA content-based flow cytometry, and a dose-dependent increase in the number of cells in the gap 2 phase of the cell cycle (G_2/M) was observed after 24 hr. For example, at 30 hr of incubation, 2 and 4 μM MeHg significantly increased the proportion of cells in G_2/M when compared with control cells ($215 \pm 12\%$ and $266 \pm 66\%$, respectively). These investigations provided evidence that MeHg led to a time-dependent inhibition in S-phase and G_2/M in primary CNS cells. Mitotic inhibition was evident by BrdU-Hoechst bivariate flow cytometric analysis at 24-hr incubation in the presence of either colchicine or MeHg (Ponce et al. 1994). Examination of the dual parameter cytograms demonstrated that cycling cells appeared more sensitive to the cytotoxic effects of MeHg than noncycling cells.

An evaluation of the number of cells able to successfully complete one round of cell division demonstrated an inhibition of cell cycle transition following MeHg exposure at all time points and at all tested concentrations (Figure 1, Ponce et al. 1994). Following 48 hr of incubation, $57 \pm 6\%$ of control cells had progressed into a new gap 1 (G_1)-phase, whereas cells exposed to 1, 2, or 4 μM MeHg had only $40 \pm 4\%$, $7 \pm 2\%$ ($p \leq 0.05$), or $2 \pm 1\%$ ($p \leq 0.05$) progression into a new G_0/G_1 -phase. The cell cycle effects observed upon exposure to MeHg demonstrated concentration-dependent relationships: *a*) inhibition of cell cycle progression (1 μM MeHg); *b*) accumulation of cells in G_2/M (2 μM MeHg); and *c*) cessation of all

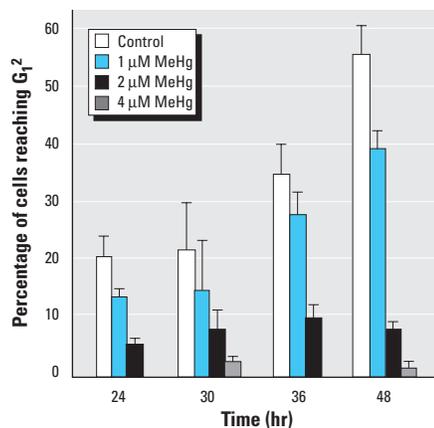


Figure 1. Proportion of CNS cells successfully completing one round of cell division and reaching second-round G_1 and (G_1^2) following 24-, 30-, 36-, and 48-hr incubation with BrdU and MeHg. MeHg exposure results in a concentration-dependent decrease in the ability of primary CNS cells to transition through the cell cycle, as demonstrated by BrdU-Hoechst flow cytometry. With higher exposures ($>4 \mu\text{M}$), MeHg blocks cell cycle transition. Cultures exposed to 2 and 4 μM MeHg showed a significant decline in the percentage of cells reaching a new G_0/G_1 following a successful M relative to controls at all time points ($p \leq 0.05$). Data from Ponce et al. (1994), with permission of Academic Press.

cycling activity (4 μM MeHg). Because the cells used in these investigations were derived from day 12 fetal rat midbrain, the results reported here support the hypothesis that MeHg can inhibit cell cycling during the rapid phase of CNS cell proliferation.

The effects of MeHg on the expression of a number of cell cycle regulatory genes in the primary rat midbrain neuroepithelial cell culture were examined, particularly genes known to be involved in the cell cycle control and growth arrest, including *GADD*, *p53*, and *p21* genes. We began by evaluating the constitutive expression level of *GADD* genes (*GADD45* and *GADD153*) during cell proliferation and commitment to differentiation. The *Gadd* genes are involved in growth arrest in response to stress and DNA damage and act at the G_1/S checkpoint where we have observed cell cycle inhibition following MeHg exposure. To evaluate the effect of MeHg on the expression of *GADD45* and *GADD153*, total RNA was isolated from cells treated with MeHg (0–2 μM , 24 hr). A concentration-related increase in the amount of *p21*, *GADD45*, and *GADD153* expression in the MeHg-exposed cells compared with the untreated cells was observed. A summary of the induction of *GADD45* and *GADD153* *in vivo* and *in vitro* is presented in Table 1. (Included in Table 1 are expression data on

γ -GCS, a gene involved in GSH synthesis implicated in response to oxidative stress.) Differences in the induction profiles of these two *GADD* genes have been observed (Zhan et al. 1994). However, to our knowledge, this was the first evaluation of the effects of MeHg on these genes. Because peak induction of *GADD45* has been reported to be maximal approximately 4 hr after exposure to an inducer, our measurement of *GADD45* expression at 24 hr postexposure may have underestimated peak induction (Fornace et al. 1989; Zhan et al. 1994).

Using MEFs from normal, heterozygous, and null for *p21*, we examined the effects of MeHg on cell cycle progression. We observed no apparent effect of *p21* status on cell death or cell cycle phase distribution upon exposure to MeHg, although cells of all genotypes accumulated cells in G_2/M (Mendoza et al. 2002). Bivariate BrdU/Hoechst flow cytometric analyses demonstrated that *p21* partially regulated MeHg-induced inhibition of cell cycle progression, wherein a higher fraction of *p21*^(-/-) cells successfully completed one cell cycle round compared with both *p21*^(+/+) and *p21*^(+/-) cells (Figure 2). These results suggested a complex relationship between *p21* and other proteins involved in cell cycle regulation after exposure to MeHg, and suggested that a partial loss of the G_1 and

Table 1. Change in gene expression (mRNA levels) in control and treated rodent CNS cells *in vitro* and *in vivo* for four genes: *GADD45*, *GADD153*, γ -GCS-HC, and *p21*.^a

| | <i>GADD45</i> | <i>GADD153</i> | γ -GCS-HC | <i>p21</i> |
|---------------------------------|-----------------------------|-----------------|------------------|-----------------|
| Normal development | | | | |
| <i>In vitro</i> , embryonic CNS | 1.8 \times ↓ | BD | ND | 1.7 \times ↓ |
| MeHg exposed | | | | |
| <i>In vivo</i> , adult CNS | 1.6 \times ↑ 6.2 \times | 1.1 \times ↑ | 1.5 \times ↑ | 5.6 \times ↑* |
| <i>In vitro</i> , embryonic | 6.2 \times ↑* | 4.7 \times ↑* | 1.2 \times ↑ | 2 \times ↑* |

Abbreviations: ND, not determined; BD, below detectable levels; ↑, induction; ↓, inhibition/repression.

^aThe greatest induction was observed *in vitro* for *GADD45* and *GADD153* and for *p21* *in vivo* following 4 weeks of MeHg exposure. *GADD45* and *p21* expression decreased with CNS cell differentiation *in vitro*. Northern blots were carried out using brain tissue from adult mice chronically exposed to low levels of MeHg (10 ppm, 4 weeks) and from CNS cells exposed to 2 μM MeHg *in vitro* for 24 hr. Northern blots were also carried out using CNS cells to look for changes during normal neuronal differentiation. Arrows represent fold change after normalization to 18 S and control conditions. Table adapted from Ou et al. (1997, 1999a). *Significantly different from the expression levels of untreated controls ($p < 0.05$). Values shown are the means from at least three independent experiments.

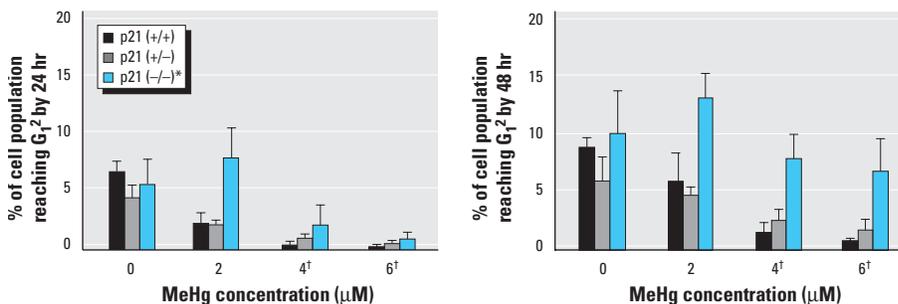


Figure 2. Effect of MeHg on cell cycle progression of MEFs of different *p21* genotypes. Asynchronous cells (passage 4–6) were treated with 0, 2, 4, and 6 μM MeHg and labeled with BrdU for (A) 24 hr and (B) 48 hr. Cells were harvested and then stained with Hoechst 33258 and EB. The fraction of cells ($n \geq 3$) reaching second-round G_1 (G_1^2) after 24 and 48 hr of MeHg treatment is shown (mean \pm SE). Statistically significant differences ($p \leq 0.05$) relative to control untreated cells were observed across both MeHg exposure groups (†) and *p21* genotype status (*). Data from Mendoza et al. (2002), with permission of Academic Press.

G₂/M checkpoints in p21^(-/-) cells contributed to the higher rate of progression of these cells through the cell cycle and out of mitotic inhibition. The lack of a complete abrogation of the G₁ or G₂ checkpoints in the MeHg response of p21^(-/-) cells may be attributed to redundant cell cycle regulatory pathways (Shackelford et al. 1999).

We also examined the effects of MeHg on regulation of Ca²⁺_i homeostasis and GSH as potential biochemical bases for the altered signal transduction and cell cycling observed in the primary rat midbrain neuroepithelial cells. The role of GSH in MeHg-mediated cell cycle alterations was examined in our laboratory with the use of GSH-modulating agents. We observed that GSH depletion by BSO did not significantly alter MeHg-induced cytotoxicity in embryonic CNS cells (Ou et al. 1999b). In contrast, acute exposure of primary neuroepithelial cells to MeHg caused an immediate increase in the indo-1 fluorescence ratio, demonstrating an increase in free [Ca²⁺]_i (and possibly other divalent cations) at MeHg concentrations observed to

affect cell cycling (Figure 3). The elevation in the basal fluorescence ratio was a strong function of both the MeHg exposure concentration and the presence of extracellular calcium (Ca²⁺_e). Cells exposed to MeHg (1, 2, 4 μM) in the presence of Ca²⁺_e demonstrated an increase in indo-1 fluorescence ratio that rose consistently over the 17 min of observation (Figure 3). The fluorescence ratio for cultures exposed to MeHg in the absence of Ca²⁺_e demonstrated a markedly lower percentage change above baseline when compared with cultures exposed to MeHg in the presence of Ca²⁺_e (Figure 3). In the absence of Ca²⁺_e, a lag of several minutes was observed before the fluorescence ratio increased following MeHg exposure. These results demonstrate that as the MeHg exposure concentration increased, the time to intracellular divalent cation mobilization decreased, and the relative contribution of intracellular divalent cations to the total indo-1 response increased.

The dose–response information on cell cycle transition rates, cell viability and differentiation in primary rat midbrain neuroepithelial

cells exposed *in vitro* was used to develop a biologically based dose–response model of MeHg-mediated developmental neurotoxicity (Leroux et al. 1996). This model incorporates MeHg-dependent rates to estimate the number of cells in the fetal midbrain over time based on transition probabilities for cell growth, death, and transformation (Figure 4). This model views development as a process involving replication of uncommitted cells (e.g., neuroepithelial cells), differentiation of uncommitted cells to cells committed to differentiation (e.g., neuronal cells), and cell death, wherein each of these transitional processes occurs with a given probability. Transition probabilities were modeled as Poisson processes. Kolmogorov forward equations were used to estimate the mean and variance of the number of cells in each of the possible states at any given time. In this way, the probability of malformation could be predicted as a function of the number of normal, committed cells by a given time point (Leroux et al. 1996).

Discussion

Results from experiments summarized here suggest that the effects of MeHg on cell cycle alterations likely underlie the neuronal hypoplasia observed after gestational exposure and the enhanced susceptibility of the fetus to MeHg exposure relative to that of the adult. Moreover, these experiments suggest a multifactorial relationship between exposure and effect involving both timing and degree of exposure, genetic makeup, and other factors. Biochemically, the effects of MeHg on GSH may not be sufficient to explain the observed effects of MeHg on cell cycle progression (Ou et al. 1999b). In contrast, we have observed strong effects of MeHg on intracellular divalent cation regulation, presumably associated with changes in Ca²⁺_i, and support a role for the loss of Ca²⁺_i homeostasis in the cell cycle alterations observed in the primary CNS cell cultures after MeHg exposure. MeHg interferes with the maintenance of normal Ca²⁺_i in multiple ways (Atchison and Hare 1994), and immature neurons may be particularly susceptible to MeHg-mediated alterations in Ca²⁺_i (Mundy and Freudenrich 2000). This interference reflects the myriad pathways through which cells regulate Ca²⁺_i. Results from experiments described here support a model wherein MeHg exposure led to a sustained elevation in [Ca²⁺]_i through alterations in both the plasma membrane permeability toward Ca²⁺_e (or which required the presence of Ca²⁺_e) and through mobilization of Ca²⁺_i stores. It is likely that the sustained elevation of [Ca²⁺]_i influences multiple processes and has a wide range of outcomes. Among these processes are *a*) the activation of cytolytic, Ca²⁺-dependent enzymes such as DNases, proteases and lipases; *b*) the alteration of Ca²⁺-dependent protein

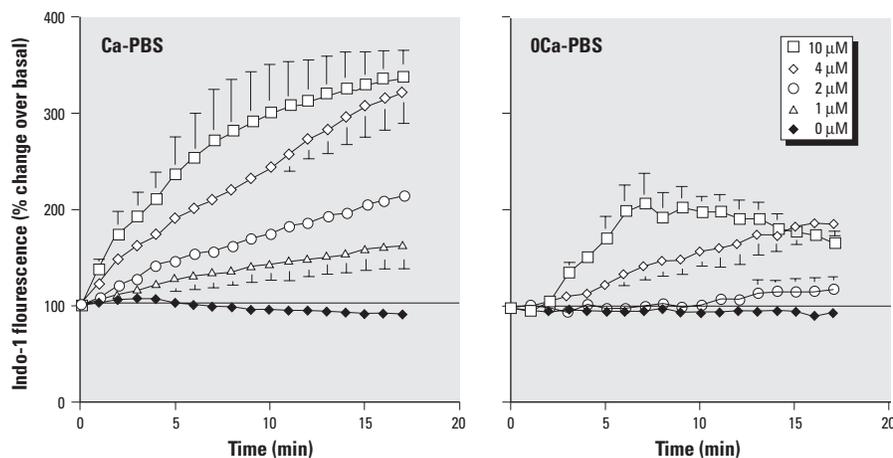


Figure 3. Effect of MeHg exposure on the 405 nm/530 nm fluorescence ratio of indo-1 over time in primary rat neuroepithelial cells following *in vitro* MeHg exposure. Data were obtained from cells exposed to MeHg both in the presence (Ca–PBS) and absence (0Ca–PBS) of Ca²⁺_e. Data are presented as mean ± SE (*n* = 3) change in Indo-1 fluorescence ratio relative to basal. Cell death was negligible over the exposure duration. No statistical analysis was performed on these data.

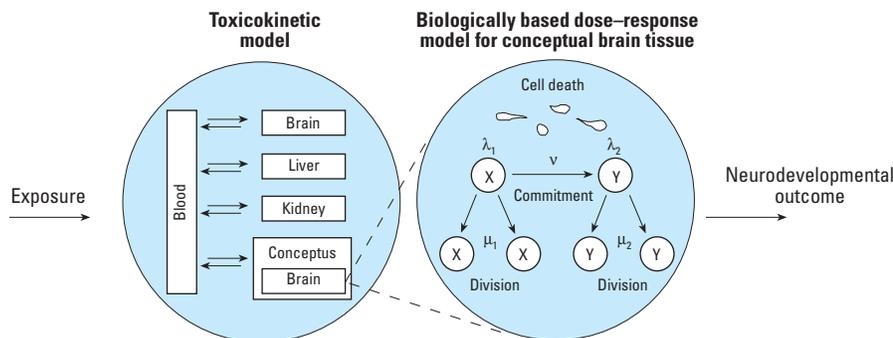


Figure 4. Neurodevelopmental risk model incorporating a toxicokinetic exposure model and a biologically based dose–response model for MeHg-induced neurodevelopmental toxicity. Figure adapted from Faustman et al. (1999).

kinase function; and c) the dysfunction of other Ca^{2+} -dependent proteins.

Microtubule polymerization is sensitive to elevated $[\text{Ca}^{2+}]_i$, and the phosphorylation of microtubule-associated proteins are, in some instances, under the control of Ca^{2+} -dependent protein kinases. Thus, MeHg-induced alterations in $[\text{Ca}^{2+}]_i$ may affect microtubule dynamics independent of direct interactions of MeHg on microtubule polymerization (Nicotera et al. 1992; Onfelt 1986) and thus alter neuronal migration and the formation of the mitotic spindle. Ca^{2+} also plays a central role in cell cycle regulation independent of its effects on microtubule polymerization (Berridge 1995). Thus, the appearance of mitotic figures and neuronal ectopia, described in humans and animals exposed to MeHg *in utero*, may have as a common underlying cause the loss of Ca^{2+} regulation.

The effects of altered Ca^{2+} can be directly associated with altered cell cycle regulation at the level of the mRNA expression and protein production/degradation. MeHg-induced changes in gene expression have been observed at low levels of exposure in other systems. For example, marked alterations in gene expression have been observed in neuroblastoma and glioma cells exposed *in vitro* to 0.05–0.2 μM MeHg for 6–8 weeks (Ramanujam and Prasad 1979). In adult rat nervous system (both central and peripheral), the alterations in expression of 120 genes on 2-dimensional gels, measured as protein level, were not uniform following *in vivo* exposure (10 mg/kg) but changed as a function of both protein species and period of exposure (Omata et al. 1991). Because a general effect on protein synthesis would result in a uniform decline in protein level over time, it was proposed that differential effects at the mRNA level were occurring (e.g. DNA transcription, mRNA processing, or mRNA alterations by MeHg). However, because the cellular content of mRNA and protein is established by a balance between synthesis and degradation, impaired degradation by MeHg in these studies cannot be excluded as a possible underlying mechanism.

Our experiments have shown that MeHg increases the intracellular mRNA and protein content of key cell cycle regulatory molecules under exposure conditions associated with impaired cell proliferation. p21 (Waf1, Cip1) is a negative regulator of several cyclin-dependent kinases. By inhibiting the activity of G_1 cyclin-dependent kinases, p21 is believed to inhibit cell cycle transition at the G_1 -S margin by blocking retinoblastoma protein phosphorylation (Harper et al. 1993). In addition to its role in the G_1 transition, p21 also inhibits DNA replication by suppressing the ability of proliferating cell nuclear antigen (PCNA) to activate DNA polymerase δ (Waga et al.

1994). Although p21 appears to be the most pleiotropic mediator of p53-dependent cell cycle arrest, both p21 and p53 may act independently of the other. For example, p21 is not required for p53-dependent apoptosis (Brugarolas et al. 1995), and p21 may act independently of p53 in cell senescence and in cell cycle withdrawal upon terminal differentiation (Parker et al. 1995). p21 is activated after oxidative stress (Russo et al. 1995), and as with p53, p21 is also a substrate for ubiquitin-mediated protein degradation (Maki and Howley 1997). In addition to their critical role in the G_1 -S transition, p53 and p21 may be involved in G_2 -M transition control. For example, elevation of p53 protein can arrest cells not only in G_1 but also at G_2/M (Guillouf et al. 1995). In cells lacking p21, the uncoupling of S- and M-phase transition via a G_2 checkpoint by exposure to anticancer drugs results in many grossly deformed polyploid nuclei (Waldman et al. 1996). As with p21, the role of p53 and other factors on regulated cell cycle checkpoint control following MeHg exposure can be investigated using transgenic systems (Caelles et al. 1994; Schreiber et al. 1999; Shaulian and Karin 2001).

GADD45 and *GADD153* (Chop 10) are members of a family of GADD genes (Fornace et al. 1989) that were originally identified from subtractive hybridization experiments performed with cells exposed to UV radiation or the mutagen methyl methanesulfonate (Fornace et al. 1988). The expression of *GADD45* and *GADD153* is induced by DNA-damaging agents, serum deprivation, and oxidative stress (Chen et al. 1992). Both are nuclear proteins implicated in controlling the G_1 -S transition. *GADD45* is activated by p53 and competes with p21 for binding to PCNA (El-Deiry et al. 1993). In the ML-1 human myeloid leukemia cell line, overexpression of *GADD45* results in growth inhibition, as evidenced by a delayed S-phase entry (Smith et al. 1994). *GADD153* acts as a dominant-negative inhibitor of other C/EBP transcription factors and is induced upon differentiation of fibroblasts to adipocytes (Ron and Habener 1992). Unlike other members of the C/EBP family, *GADD153* works at or around the G_1 -S checkpoint, as microinjection of *GADD153* protein into synchronized NIH-3T3 cells can arrest the cell cycle at the G_1 -S boundary (Barone et al. 1994).

The intracellular content of these cell cycle regulatory molecules is controlled by both their selective synthesis and degradation. For example, inhibition of proteasome activity leads to an increase in p21 protein levels

(Adams et al. 1999), and inhibition of E1 enzyme function results in increased p53 abundance (Larota et al. 1999). There is reasonable evidence to suggest a role for MeHg in interfering with regulated protein degradation leading to altered cell proliferation. Specifically, because the thiol proteinases, including the ubiquitin/proteasomes and calpains, absolutely require the activity of a conserved cysteine for normal function, it is plausible to propose that MeHg may directly interfere with their activity. The activities of the calpains and ubiquitin/proteasomes are regulated by intracellular redox status and free Ca^{2+} ; (Berleth and Pickart 1996; Hochstrasser 1996), both of which are sensitive to MeHg exposure (Atchison and Hare 1994; Sarafian and Verity 1991). Regulated protein degradation also involves protein phosphorylation-mediated cell signaling, and MeHg alters intracellular protein phosphorylation.

We have demonstrated the use of a probabilistic model of midbrain neurogenesis to examine the neurodevelopmental toxicity of MeHg. Although the model relies on some data generated in *in vitro* systems, the model allows for parameter refinement as *in vivo* data become available. Such models necessarily simplify the processes involved in regulating CNS ontogeny; however, we have found such models to provide a framework for synthesizing information obtained at the biochemical, cellular, and organ level. With regard to MeHg, for example, a model can serve as a platform for incorporating changes in biochemical processes (e.g., Ca^{2+} homeostasis), with molecular effects (e.g., signal transduction, gene expression, protein level), cellular effects (e.g., cell cycling), and ultimately, on the organ system (e.g., hypoplasia). From a research perspective, development of such models can serve as the basis for systematic and rational hypothesis generation and testing. By comparing predictive results from the model against experimental observations and by linking the biological model of effects with pharmacokinetic models, we also hope to refine our understanding of the sensitive drivers of exposure and effect and thus refine risk assessment. Finally, such models can be used to examine the relative utility of *in vitro* systems as models of *in vivo* effects (Faustman et al. 1999).

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