Mercury is a potent neurotoxin that can delay neurological development in neonates, and has been proposed to be an environmental risk factor for several neurodegenerative conditions. The mechanisms by which environmental factors may influence the propagation of neurodegenerative diseases are not yet well delineated. However, it is known that neurons require trophic factor support for maintenance and survival following traumatic physical and toxic insults. We found that divalent mercury (HgCl₂) inhibited ciliary neurotrophic factor and interferon-γ receptor-mediated Janus tyrosine kinase (Jak)/signal transducers and activators of transcription (STAT) pathway activation in SK-N-BE(2)-C neuroblastoma cell cultures, but did not inhibit the fibroblast growth factor receptor tyrosine kinase. Results of dichlorofluorescein experiments showed increased levels of oxidative stress in HgCl₂-treated cells that was similar in magnitude to that caused by treatment with H₂O₂. The antioxidant agents glutathione, N-acetylcysteine, and sodium ascorbate each protected neurons against HgCl₂-induced inhibition of STAT activation. HgCl₂ also inhibited Jak-STAT signaling in cultures of chick retina neurons, but did not affect signaling in nonneuronal HepG2 cells and chick skeletal myotubes. The specific inhibition of growth factor–mediated Jak-STAT signaling pathways in neurons by HgCl₂-induced oxidative stress offers a new mechanism by which mercury may produce neurotoxic symptoms in the developing nervous system, promote neurodegeneration in mature neurons, and inhibit recovery following neurotrauma.

Key Words: ciliary neurotrophic factor; gp130; tyrosine kinase; cytokine; interferon-γ; transition metals; signal transduction; neurotoxicity.

Mercury is a neurotoxic metal pollutant found in the environment that may play a role in the development of some neurodegenerative diseases. It is a by-product of industrial processes, and bioaccumulates in the food chain (Krantz and Dorevitch, 2004). Mercury accumulates in the brain, especially after chronic exposures, resulting in degeneration and atrophy of the sensory cerebral cortex, paresthesia, ataxia, and hearing and visual impairment (Ninomiya et al., 1995). It also causes loss of Purkinje cells and the granule cell layer of the cerebellum, as well as neurons in the cerebral cortex, substantia nigra, and anterior horn of the spinal cord (Patrick, 2002).

At the cellular level, HgCl₂ interferes with mitochondrial respiration, causing oxidative stress (Konigsberg et al., 2001). Mercury exposure produces increased levels of reactive oxygen species (ROS) in the motor neurons of mice and in cultured astrocytes and in various regions of the brains of adult male Sprague-Dawley rats (Brawer et al., 1998; Hussain et al., 1997; Pamphlett et al., 1998). Oxidative stress is also implicated in the etiology of several neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS) (Beal, 2002; Chong et al., 2005). Thus, the acute and chronic neurotoxic effects of mercury may be enhanced through its ability to increase oxidative stress.

The nervous system is particularly susceptible to increased ROS levels. Neurons have a high density of mitochondria that generate ROS through the electron transport chain. Certain neuronal populations, such as motor neurons, also have diminished antioxidant capacities relative to other cell types (Halliwell, 1992; Philbert et al., 1991). Oxidative stress targets multiple cellular components in neurons, including DNA and membrane lipids, as well as structural proteins and enzymes (Maher and Schubert, 2000). It can also alter intracellular signaling pathways required for neuronal maintenance and survival (Dröge, 2002). The Janus tyrosine kinase (Jak)/signal transducers and activators of transcription (STAT) is one pathway that is sensitive to redox conditions (Druhé et al., 1998; Kaur et al., 2005). The Jak-STAT pathway is a rapid intracellular communication system used by many cytokines and growth factors to transduce signals from the plasma membrane to the nucleus in order to regulate cell proliferation and differentiation of most tissue types, including the vertebrate nervous system (Kisseleva et al., 2002; Leonard and Oshea, 1998). Cytokines bind to receptors, inducing Jak to phosphorylate and activate STAT transcription factors (Leonard and Oshea, 1998). In nonneuronal cells, such as fibroblasts, the Jak-STAT pathway is often activated by increased ROS levels (Carballo et al., 1999; Simon et al., 1998). However, in both neuronal cell lines and primary...
neurons, pro-oxidants have been found to inhibit receptor-mediated Jak phosphorylation of STATs (Kaur et al., 2005; Monroe and Halvorsen, 2006).

Ciliary neurotrophic factor (CNTF) is a member of the cytokine family of neurokines that includes leukemia-inhibitory factor (LIF), interleukin-6 (IL-6), cardiotrophin-1, interleukin-11, and oncostatin-M, all of which signal through the Jak-STAT pathway (Heinrich et al., 1998). CNTF has protective effects in several populations of neurons, including sympathetic, parasympathetic, sensory, motor, retinal, and embryonic hippocampal neurons (Arakawa et al., 1990; Finn et al., 1998; Ip et al., 1991; Manthorpe et al., 1985; Sentdner et al., 1990). Disruption of CNTF-mediated Jak-STAT signaling in adult mice results in deficient neuronal repair responses (Qiu et al., 2005). Gene ablation studies show that the loss of CNTF signaling is embryonic lethal (DeChiara et al., 2005). Gene ablation studies show that the loss of CNTF signaling in adult mice results in deficient neuronal repair responses (Qiu et al., 2005). Thus, CNTF signaling through the Jak-STAT pathway is critical both in early development and in the maintenance of the nervous system.

The effects of oxidative stress on susceptible cytokine signaling pathways are a newly described neurotoxic mechanism. We have investigated the effects of several heavy metals on Jak-STAT signaling and found that HgCl₂, like cadmium, can completely inhibit neuronal Jak-STAT activation. Metal-induced oxidative stress and its effects on neuronal Jak-STAT activation may represent a new paradigm by which heavy metals such as HgCl₂ are able to promote neuropathologic effects. Further, exposures to metals with the resulting loss of neurotrophic signaling offer a mechanism for the variable susceptibility seen among those at risk for developing neurodegenerative diseases.

**MATERIALS AND METHODS**

**Materials and reagents.** Human recombinant CNTF was provided by Regeneron (Tarrytown, NY), recombinant human interferon-γ (IFN-γ) and basic fibroblast growth factor (bFGF) were from Peprotech (Rocky Hill, NJ), and IL-6 was from R&D Systems (St Paul, MN). Cell culture media were purchased from Grand Island Biologicals (Grand Island, NY). Embryonated chick eggs (White Leghorn) were obtained from CBT Farms (Chestertown, MD).

For the immunoblot analyses, horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins, as well as the anti-phosphotyrosine-STAT (PY-STAT) antibodies were purchased from Cell Signaling Technology (Beverly, MA), while the HRP-conjugated anti-mouse immunoglobulins came from BD Transduction Laboratories (Lexington, KY) and the anti-Jak antibodies came from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PY-Jak antibodies were from Biosource International (Camarillo, CA).

**Cell culture and treatment.** The BE(2)-C human neuroblastoma cells (Ross et al., 1995) and HepG2 human hepatoma cells were derived from the American Type Culture Collection. All cell lines were grown in a 1:1 mixture of Ham’s F12 and Eagle’s Minimal Essential Medium supplemented with 10% (vol/vol) fetal calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin as previously described (Kaur et al., 2005; Malek and Halvorsen, 1997). Retinal neurons and skeletal muscle primary cultures were obtained from day 10–12 chick embryos as previously described (Wang and Halvorsen, 1998; Wishingrad et al., 1997) and grown in minimal essential media with 10% horse serum. Cultures (~70–80% confluent) were placed in serum-free media from 1 h prior to initiation of treatment protocols.

Cells were exposed to one of the several transition metals (HgCl₂, CuSO₄, ZnSO₄, FeCl₃, MnCl₂, or PbAc) for 5 h, rotenone (Calbiochem, LaJolla, CA) for 2 h, or H₂O₂ for 30 min as indicated before being challenged with CNTF (200pM), IFN-γ (1nM), or bFGF + heparin (10nM + 10 µg/ml) for 30 min. Some cells were also pretreated for 2 h with glutathione (GSH) before HgCl₂ treatment and challenged with CNTF or IFN-γ. Cell lysates from treated cultures were collected in SDS-polyacrylamide gel sample buffer and frozen at −20°C until immunoblot analysis. Cell survival was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometric assay as described previously (Monroe and Halvorsen, 2006).

**Immunoblot analysis.** Protein from whole cell lysates was separated on 7.5% gels by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride (Millipore Co, Bedford, MA) membrane as previously described (Koshlikova et al., 1996; Monroe and Halvorsen, 2006). Membranes were processed sequentially with the primary antibodies, anti-PY-STAT3 and anti-PY-STAT1, for assessment of STAT activation, followed by anti-STAT1 and anti-STAT3 for normalization. Each antibody was detected with the appropriate HRP-secondary antibody by enhanced chemiluminescence (ECL; Perkin Elmer Life Science, Boston, MA). Following analysis of each antibody, the membranes were incubated in stripping buffer (62.5mM Tris-HCl, 2% SDS, 100mM 2-mercaptoethanol, pH 6.7) for 30 min at 50°C, rinsed extensively, and then probed with the next primary antibody of interest. ECL results were digitized and quantified using an Epson 636 Professional Series scanner and the public domain program National Institutes of Health (NIH) Image.

**Oxidative stress and fluorescence microscopy.** Levels of oxidative stress were analyzed with 2′,7′-dichlorofluorescin diacetate (DCF; AnaSpec, Inc, San Jose, CA), a compound that fluoresces in the presence of hydroperoxides after intracellular esterases cleave the acetate groups (Lebel et al., 1992). Following exposure to 3µM HgCl₂ for 5 h or 1mM H₂O₂ for 30 min, cells were incubated at 37°C for 30 min with 10µM DCF, then rinsed with phenol red-free media and incubated for an additional 30 min. Images were captured using a Nikon FXA fluorescence microscope system with color digital and cooled, low light level CCD camera using a 60× objective. Captured images were processed and quantified in arbitrary units using NIH ImageJ.

**RESULTS**

**Mercury Selectively Inhibits Cytokine Receptor–Mediated Jak-STAT Activation.**

We exposed BE(2)-C human neuroblastoma cells to HgCl₂ and several other transition metals to determine their effect on cytokine-mediated STAT activation. As a measure of activation, we used phosphotyrosine (PY)-STAT1 and PY-STAT3 antibodies, which are specific for the active forms of these two STAT transcription factors. Exposure to HgCl₂, CuSO₄, or ZnSO₄ for 5 h each reduced the level of CNTF-induced phosphorylated STAT (Fig. 1). HgCl₂ inhibited STAT phosphorylation...
at a concentration several times lower than CuSO$_4$ and ZnSO$_4$, with 3 µM HgCl$_2$ completely inhibiting both STAT1 and STAT3 phosphorylation (Fig. 1A). We were unable to detect any effect on STAT phosphorylation from treatment with MnCl$_2$, PbAc, and FeCl$_2$ (Figs. 1A–C).

A decrease in the levels of phosphorylated STATs can result from either the selective inhibition of the Jak-STAT pathway or it could be attributable to a general decrease in cell viability. We tested viability in BE(2)-C cells treated with HgCl$_2$, CuSO$_4$, or ZnSO$_4$ under conditions that reduced STAT phosphorylation as seen in Figure 1D. HgCl$_2$ at concentrations up to 3 µM did not result in any significant decrease in cell viability (Fig. 2). However, cells incubated for 5 h in 300 µM CuSO$_4$ or 100 µM ZnSO$_4$ showed significant reductions in the MTT survival signal (Fig. 2). Therefore, a decrease in cell viability appears to explain the reduced PY-STAT signals.
produced by CuSO4 and ZnSO4 and we did not pursue further studies with these agents (Fig. 1D). As an additional test of the effects of HgCl2 on cell viability, cells were exposed to 3µM HgCl2 for 5 h in serum-free media. The media were then removed and replaced with media containing 10% serum, and the cells were returned to the culture incubator. Visible observation at 24 and 48 h revealed that cells exposed to HgCl2 were as abundant as control. Therefore, HgCl2 at concentrations less than 10µM inhibited CNTF-stimulated STAT phosphorylation in a concentration- and time-dependent manner. Complete inhibition of the CNTF-induced PY-STAT1 signal occurred with 3µM HgCl2 for 2 h (Fig. 3A). STAT3 phosphorylation appeared less sensitive and required 3µM Hg for 4 h for complete inhibition (Fig. 3A). The inhibition of the CNTF response was reversible upon removal of HgCl2. BE(2)-C cells exposed to HgCl2 for 5 h, and then allowed to recover in complete medium overnight, showed a robust restoration of CNTF-induced PY-STAT signaling (Fig. 3B). These results demonstrate that HgCl2-induced inhibition of Jak/STAT signaling is transient and reversible depending on the presence of HgCl2. In addition to CNTF, the Jak-STAT pathway can be activated by many other cytokines. In order to begin to detect the selectivity of HgCl2 action, we also tested the effect of HgCl2 on IFN-γ signaling, a cytokine that activates Jak-STAT1 signaling through a receptor distinct from that of CNTF (Boehm et al., 1997; Shuai et al., 1993). HgCl2 completely inhibited IFN-γ-induced PY-STAT1 in BE(2)-C cells after 4–5 h of exposure (Fig. 3C).

Since STAT is phosphorylated by a Jak that is associated with the CNTF receptor, we determined whether HgCl2 could inhibit Jak activity. BE(2)-C cells were treated with HgCl2 for 1 or 2 h and samples were assessed for Jak tyrosine auto-phosphorylation with antibodies specific for PY-Jak1 and PY-Jak2. After 1 h of HgCl2 exposure, PY-Jak1 and PY-Jak2 levels were each decreased (33 ± 13% and 57 ± 10% of control, respectively, n = 3, Fig. 4A). After 2 h of HgCl2 exposure, PY-Jak1 and PY-Jak2 were decreased nearly to background levels (5 ± 2% and 11 ± 6% of control, respectively). Total Jak1 levels, however, are not shown since they were not readily detectable in these BE(2)-C cells.

Since HgCl2 inhibited Jak-STAT activation by both CNTF and IFN-γ, we next determined whether HgCl2 affects kinases indiscriminately or shows selectivity for the Jak kinases. CNTF receptor–mediated activation of the MAP kinase pathway, which requires a Jak kinase, was inhibited (9% of control level) in BE(2)-C cells treated with HgCl2 (Fig. 4B). Fibroblast growth factor (FGF), however, activates the MAP kinase pathway in BE(2)-C cells using an integral receptor tyrosine kinase that is distinct from Jak (Monroe and Halvorsen, 2006). Incubation of BE(2)-C neuroblastoma cells with 3µM HgCl2 for 5 h did not reduce MAP kinase activity mediated by bFGF (Fig. 4C).

**Inhibition of STAT Signaling by Mercury Requires Increased Levels of Oxidative Stress**

We have previously shown that oxidative stress can inhibit Jak-STAT signaling in neurons (Kaur et al., 2005). Since mercury has also been shown to be a pro-oxidant compound in many cell types, we investigated whether HgCl2 increased ROS levels in BE(2)-C neuroblastoma cells under our treatment conditions. DCF, a compound that fluoresces in the presence of hydroperoxides, was used as a visual marker of oxidative stress (Oyama et al., 1994). Cells exposed to 3µM HgCl2 for 5 h had over twice the level of DCF fluorescence compared to control cells. The amount of ROS generated by HgCl2 was significantly higher than untreated cells, and was comparable to cells treated with 1mM H2O2 for 30 min (Table 1). Cells pretreated for 2 h with the antioxidant GSH reduced ROS levels in HgCl2-treated cells to near control levels (Table 1). We were unable to detect significantly increased DCF fluorescence in cells exposed to either 30µM CuSO4 or 10µM ZnSO4 for 5 h (Table 2).

Since HgCl2 increased levels of oxidative stress in a time frame comparable to its ability to inhibit CNTF activity, we investigated whether protecting against oxidative stress would also protect CNTF-induced STAT activation. Pretreatment of cells for 2 h with GSH protected STAT phosphorylation from the inhibitory effects of HgCl2 (Fig. 5). GSH pretreatment also prevented HgCl2 from inhibiting IFN-γ–induced STAT1 phosphorylation and CNTF-induced ERK phosphorylation (Figs. 3C and 4B). Other antioxidants, such as 20mM N-acetylcysteine (NAC) and 1mM sodium ascorbate (Asc), also protected STAT signaling against inhibition by HgCl2 (Fig. 5).
FIG. 3. Time and concentration analysis of mercury inhibition of cytokine receptor–mediated STAT phosphorylation. (A) BE(2)-C cells were treated with the indicated concentrations of HgCl₂ (0.3–3.0 μM) for 1–4 h and then challenged with 200pM CNTF for 30 min. Cell lysates were collected, processed for immunoblotting and serially probed with antibodies for PY-STAT1, PY-STAT3, STAT1, and STAT3. (B) BE(2)-C cells were treated with 1 or 3 μM HgCl₂ for 5 h as indicated. Some cells (acute) were then challenged with 200pM CNTF for 30 min and cell lysates collected and frozen. Other cells (24-h washout) were rinsed three times in media with serum, and then allowed to recover for 24 h. After 24 h, these cells were also challenged with 200pM CNTF for 30 min and cell lysates collected and frozen. All cell lysates were then processed for immunoblotting and serially probed with antibodies as in “A”. (C) BE(2)-C cells were pretreated for 2–5 h with 3μM HgCl₂ as indicated, then challenged with 1nM IFN-γ for 30 min. Total cell lysates were processed for immunoblotting and serially probed with antibodies as in “A”. Some cells were pretreated first with 15mM GSH for 2 h as indicated. All results were confirmed in at least three independent experiments.

FIG. 4. Selective inhibition of Jak by mercury. (A) BE(2)-C cells were treated with 3μM HgCl₂ for 1–2 h and then challenged with 200pM CNTF for 30 min. Cell lysates were collected and processed for immunoblotting using antibodies for PY-Jak1, PY-Jak2, Jak2, PY-STAT1, PY-STAT3, STAT1, and STAT3. (B) BE(2)-C cells were exposed to 3 μM HgCl₂ for 5 h, then challenged with 200pM CNTF for 30 min. Total cell lysates were processed for immunoblotting for phospho-ERK1 and 2 (P-ERK1/2) and total ERKs 1, 2, and 3 (pan-ERK) levels using the corresponding antibodies. (C) BE(2)-C cells were exposed to 3μM HgCl₂ for 5 h, then challenged with 10nM bFGF + 10 mg/ml heparin (bFGF) for 30 min. Total cell lysates were processed for immunoblotting for phospho-ERK1 and 2 (P-ERK1/2) and total ERKs 1, 2, and 3 (pan-ERK) levels as in “B”. All results were confirmed in at least three independent experiments.
The Inhibitory Effects of Mercury Are Neuron-Specific

Jak-STAT signaling in neurons, compared with nonneuronal cells, is selectively inhibited by oxidative stress (Kaur et al., 2005). Therefore, we determined whether HgCl2 demonstrated a similar selectivity of action for neurons. Primary retinal neurons and skeletal myocytes were cultured from E10 chick embryos and exposed to HgCl2. Retinal cultures pretreated with 3lM Hg for 5 h or 1mM H2O2 for 30 min showed dramatic inhibition of PY-STAT3 signaling (STAT1 was not activated by CNTF in these primary cells). Further, the inhibitory effect of HgCl2 was partially protected against by pretreatment with GSH (Fig. 6A). Retinal cells exposed to GSH-alone (Fig. 6A) had slight reductions in PY-STAT3 levels (GSH levels were 92 ± 7% of control values); however, this reduction was not significantly different over three determinations. Skeletal myocyte cultures were also incubated with 3lM HgCl2 for 3 and 5 h, as well as 1mM H2O2 for 30 min, and 10lM rotenone for 2 h. None of these pro-oxidants, however, produced any apparent inhibition of CNTF-induced STAT3 activation in cultured muscle cells (Fig. 6B).

We also investigated the Jak-STAT response of HepG2 human hepatoma cells exposed to HgCl2 using IFN-γ and IL-6 to stimulate STAT phosphorylation. IL-6 is a member of the CNTF cytokine family that also acts through the shared gp130 receptor subunit and which activates Jak-STAT signaling more readily in HepG2 cells than does CNTF (Jiao et al., 2003). Neither 3lM HgCl2 for 5 h nor 1mM H2O2 for 30 min generated an inhibitory effect on PY-STAT levels stimulated by either cytokine in HepG2 cells (Figs. 6C and 6D). However, HgCl2 and H2O2 each increased oxidative stress in HepG2 hepatoma cells to levels similar to those seen in BE(2)-C cells (Table 1).

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>BE(2)-C cells</th>
<th>HepG2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>111 ± 6</td>
<td>116 ± 13</td>
</tr>
<tr>
<td>H2O2</td>
<td>238 ± 2*</td>
<td>198 ± 14*</td>
</tr>
<tr>
<td>HgCl2</td>
<td>234 ± 2*</td>
<td>202 ± 16*</td>
</tr>
<tr>
<td>GSH + HgCl2</td>
<td>116 ± 6**</td>
<td>121 ± 5**</td>
</tr>
</tbody>
</table>

*Note. BE(2)-C or HepG2 cells were treated with either control medium, with media containing 15mM GSH for 7 h, with 1mM H2O2 for 30 min, or with 3lM HgCl2 for 5 h. Some cultures were also pretreated with GSH for 2 h prior to addition of HgCl2. All plates were then processed for DCF fluorescence and the results quantified. Results are means normalized to untreated controls, ± SE, from n = 4 and n = 3 independent determinations for BE(2)-C cells and HepG2 cells, respectively.

* p < 0.01 compared to control cells (Student’s t-test).

**TABLE 2**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Oxidative Stress Index (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O2</td>
<td>224 ± 11*</td>
</tr>
<tr>
<td>CuSO4</td>
<td>140 ± 15</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>134 ± 12</td>
</tr>
</tbody>
</table>

*Note. BE(2)-C cells were treated with either control medium, with media containing 1mM H2O2 for 30 min, or with 3lM CuSO4 or 10lM ZnSO4 for 5 h. All plates were then processed for DCF fluorescence and the results quantified. Results are means normalized to untreated controls, ± SE, from n = 3 independent determinations.

* p < 0.01 compared to control cells (Student’s t-test).

**FIG. 5.** Antioxidants protect against mercury-induced Jak-STAT inhibition. BE(2)-C cells were pretreated for 2 h with 15mM GSH or 20mM NAC and cells were washed 3× with PBS. Cells were then treated with 3lM HgCl2 for 5 h. Some cells were co-treated with 1mM sodium Asc along with 3lM HgCl2 for 5 h. All cells were then challenged with 200pM CNTF for 30 min and lysates processed for immunoblotting with antibodies against PY-STAT1, PY-STAT3, STAT1, and STAT3. Results were confirmed in at least three independent experiments.

**DISCUSSION**

Sublethal concentrations of HgCl2 (< 10μM) increased oxidative stress and selectively inhibited cytokine-mediated Jak-STAT activation without inducing cell death. These inhibitory effects were seen only in neuronal cells (BE(2)-C neuroblastoma cells and retinal neurons), and could be prevented by pretreating cells with antioxidants. HgCl2 inhibited Jaks, but not FGF receptor tyrosine kinase activity. We have shown previously that oxidative stress is capable of inhibiting Jak-STAT signaling in nerve cells (Kaur et al., 2005), and that CdCl2 produces similar effects (Monroe and Halvorsen, 2006). The inhibitory effects of mercury and cadmium on
Jak-STAT signal transduction may play a role in the etiology of chronic metal neurotoxicity, since disruption of Jak-STAT signaling results in neuropathology (Alonzi et al., 2001).

**Mercury Inhibits Jak-STAT Activation in Neurons through Oxidative Stress**

HgCl₂ increased oxidative stress and inhibited receptor-stimulated Jak-STAT signaling in nerve cells over the same concentration and time range. Experiments using H₂O₂ and rotenone, which also generate oxidative stress, produced similar reductions in signaling as HgCl₂ in retinal cell cultures (Fig. 6A) (Kaur et al., 2005). Antioxidants such as GSH, sodium Asc, and NAC protected both CNTF- and IFN-γ-induced Jak-STAT activation from blockade by HgCl₂ (Figs. 3C, 4B and 5). These results suggest that the inhibition of CNTF receptor–mediated Jak-STAT phosphorylation by HgCl₂ requires ROS generation. Therefore, we propose that HgCl₂ inhibits Jak-STAT signaling through increased intracellular oxidative stress.

CuSO₄ and ZnSO₄ reduced STAT phosphorylation levels, however, they also decreased cell viability in a manner that could account for the effect (compare Fig. 1D with Fig. 2). Also, we did not observe an increased DCF signal at concentrations of CuSO₄ (30µM) and ZnSO₄ (10µM) that did not significantly reduce cell viability (Table 2). Copper and zinc have been shown to produce significant cytotoxicity in other neuroblastoma cell lines at concentrations comparable to those that were toxic in our studies (Arciello et al., 2005; Daniels et al., 2004). Therefore, we suspect that CuSO₄ and ZnSO₄ may affect other cellular functions (i.e., disrupt lysosomal integrity), resulting in cytotoxic events, before significant intracellular ROS can form (Pourahmad et al., 2001).

The effects of oxidative stress on Jak-STAT activation depend on the particular cell type. Previous studies have shown that increased ROS levels increase STAT activity in lymphocytes and fibroblasts (Carballo et al., 1999; Simon et al., 1998). Kaur et al. (2005) demonstrated a neuronal-specific inhibition following oxidative stress in both cell lines (SH-SY5Y and HMN-1) and embryonic chick neuron cultures (retina and...
Ciliary ganglia). CdCl_2 also selectively inhibits neuronal Jak-STAT activation (Monroe and Halvorsen, 2006). HgCl_2 inhibited Jak-STAT activation in both Be(2)-C neuroblastoma cells (Figs. 1A and 1D, 4 and 5), which have neuronal characteristics (Biedler et al., 1978), and chick retinal cultures (Fig. 6A). However, the Jak-STAT pathway could still be activated by cytokines in human HepG2 cells and chick skeletal muscle cultures following HgCl_2 exposure (Fig. 6B–D). These results suggest that, like CdCl_2 and H_2O_2, HgCl_2 is an agent that selectively inhibits Jak-STAT signaling in neural-derived cells. The inhibitory effect of HgCl_2 is more specific for Jak-STAT signaling in neurons than nonneuronal cells; however, this selectively was not due to a reduced production of oxidative stress in nonneuronal cells. Since increased ROS production is necessary but not sufficient to inhibit Jak/STAT activation, the exact mechanism that leads to selective neuronal inhibition is still unknown.

Mercury Appears to Target Jak Kinase

Several observations suggest that Jak kinases are the target of inhibition by HgCl_2 on cytokine-mediated STAT activation. The cytokines CNTF and IFN-γ, which utilize different receptors to activate Jak-STAT signaling (Darnell et al., 1994; Stahl et al., 1994), were used to determine whether HgCl_2-induced inhibition was independent of a particular receptor. IFN-γ binds to its α- and β-receptor subunits, which then autoactivate their associated Jaks (Boehm et al., 1997; Shuai et al., 1993), while CNTF assembles the gp130 and LIF receptor β subunits through binding to its α subunit (Heinrich et al., 1998). Since HgCl_2 inhibits both CNTF- and IFN-γ-mediated Jak-STAT activation (Figs. 3A and 3C), HgCl_2 likely inhibits Jak-STAT signaling at a step independent of receptor activation.

We also found that CNTF-stimulated Jak activation of ERK was inhibited by HgCl_2 (Fig. 4B), but FGF tyrosine receptor kinase activity was not affected (Fig. 4C). This suggests that HgCl_2-induced oxidative stress does not indiscriminately inhibit all tyrosine kinases. We showed that HgCl_2 inhibited PY-Jak1 and PY-Jak2 within 1–2 h, which was before PY-STAT3 inhibition and at about the same time as PY-STAT1 inhibition (Figs. 3A and 4A). These results suggest that HgCl_2 inhibits CNTF activation of Jak and support the idea that Jak kinase inhibition is responsible for reduced STAT phosphorylation.

These current results, together with our previous findings using nitric oxide, rotenone and H_2O_2 (Kaur et al., 2005), and CdCl_2 (Monroe and Halvorsen, 2006) support our conclusion that oxidative stress inhibits Jak kinase activity, resulting in lowered STAT phosphorylation and decreased activity in neurons. Others have reported that the intracellular redox environment can influence Jak kinase activity, with oxidized forms reversibly inhibited in both in vitro and cellular assays (Duhé et al., 1998). However, Jak may not necessarily be the only affected protein in this pathway. Other proteins that are common to CNTF- and IFN-γ receptor subunits and play important roles in signal transduction may also be sensitive to oxidative stress. For example, protein tyrosine phosphatases, which have a redox–sensitive active site cysteine (Finkel, 2000; Krejza and Schieven, 1998), may be required for Jak-STAT activation (Jiao et al., 2003). Further research is needed to investigate whether oxidized forms of Jak or other proteins required for signaling are produced in HgCl_2-treated neurons, and how they might result in the observed neuron-specific inhibition.

Mercury Neurotoxicity and Neurodegenerative Disease

Mercury is highly neurotoxic, even at sublethal exposures in neonates, and it can cause severe neurologic developmental delays (Crump et al., 1998; Grandjean et al., 2003). Neonatal rats exposed to HgCl_2 showed a dose-dependent inhibition of brain growth, as well as altered neuronal development (Bartolome et al., 1984). Brain protein content and acetylcholinesterase were significantly decreased in rats fed 2 mg/kg HgCl_2, while lactate dehydrogenase and lipid peroxidation products were increased (El-Dermeddash, 2001). There was also an elevation of Mn-SOD, a mitochondrial protein that protects against overproduction of superoxide by pro-oxidant compounds, in the brains of mice exposed to HgCl_2 (Kumagai et al., 1997). Behavioral and developmental deficiencies have been reported in animals exposed to heavy metals and it has been proposed that these pathologies are the result of neurotrophic factor signal disruption and altered neural development (Chen and Shi, 2002; Leonard et al., 2004). In addition to its direct neurotoxic effects, mercury has also been implicated as an environmental factor associated with neurodegenerative diseases.

There are several shared characteristics between mercury neurotoxicity and some neurodegenerative diseases, including increased intracellular levels of oxidative stress and dysfunctions in neurotrophic signaling pathways. The finding that Jak-STAT signaling is specifically inhibited in neurons by several pro-oxidant compounds, including heavy metals, suggests that this pathway could be a common denominator explaining the shared pathologies of metal neurotoxicity and diseases such as Alzheimer’s and ALS. The presence of environmental toxins generating oxidative conditions in neurons would help to explain why treatment modalities utilizing exogenous neurotrophic factors that function through the Jak-STAT pathway have been effective in animal models but not in human disease (ALS CNTF Treatment Study Group, 1996). It also suggests that the addition of antioxidant agents may be beneficial in treatment regimens for these nervous system disorders. Further research is needed to determine the relationship of heavy metals to disease state progression in vivo, as well as effects of metal toxicity in the presence of conditions that predispose individuals to neurodegenerative diseases such as Alzheimer’s, ALS, and Parkinson’s disease.
CONCLUSIONS

Mercury is a potent neurotoxin that can disrupt neonatal neurological development and produce significant neuropathology in adults. In this report, we have shown that HgCl₂, at sublethal concentrations, is capable of suppressing neuronal Jak-STAT signaling, an important pathway for neuronal development and repair, through oxidative stress. The conditions that make neurons more susceptible are currently unknown. Inhibition of cytokine signaling by oxidative stress may play an important role in the etiology and treatment of metal neurotoxicity and perhaps other neurodegenerative diseases.

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REFERENCES


