MECHANISMS OF CELL DEATH INDUCED BY THE ENVIRONMENTAL CONTAMINANTS METHYLMERCURY, PCB 153 AND PCB 126: *IN VITRO* STUDIES ON NEURAL AND ENDOCRINE CELLS.

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Introduction

The neurotoxic effects of the environmental contaminants methylmercury (MeHg) and polychlorinated biphenyls (PCBs) are well established in both humans and animals. A number of epidemiological and animal studies have shown that exposure to these toxicants impair cognition, i.e. learning and memory (Grandjean et al., 1997, Rice et al., 1996, Ribas-Fito et al., 2001, Tilson et al., 1990, Tilson and Kodavanti, 1998). In addition both MeHg and PCBs have endocrine disrupting properties and can interfere with hormones causing adverse effects not only on the reproductive system but also on the central nervous system during development and throughout life (Grady et al., 1978; Burton and Meikle, 1980, Arena et al., 2003; Winneke et al., 2002). Cell cultures have proven to be powerful experimental tools to investigate and elucidate the mechanisms of toxicity. However, the complexity of the nervous system requires the use of various cellular models representing specific *in vivo* targets.

In the present study we have used three different cell lines, including the mouse hippocampal neuronal cell line HT22, the pituitary cell line AtT20, and the neural stem cell line C17.2 to evaluate the cytotoxic effects of MeHg, PCB 153 and PCB 126. In addition, to further investigate the developmental toxicity of these chemicals, we used primary cultures of rat embryonic cortical neural stem cells (cNSCs).

We focused our attention on the mechanisms of cell death and examined whether cells exposed to the selected toxicants undergo apoptosis, an active cell death process requiring energy, or necrosis, a passive form of death. These two forms of cell death are characterized by various morphological and biochemical criteria. Morphologically, apoptosis is associated with cell shrinkage, nuclear and cytoplasmic condensation, externalization of phospahtidylserine (PS) at the plasma membrane level, and formation of apoptotic bodies. Release of mitochondrial factors , including cytochrome *c*, activation of specific proteases such as caspases and calpains, degradation of chromosomal DNA into large (300, 50 kb) and small (180 bp) fragments by endonucleases are the major biochemical features of apoptosis (Gorman et al. 1998; 2000). The plasma membrane integrity that is maintained in apoptotic cells, and the exposure of PS, which promotes the engulfment process by phagocytic cells, prevent the leakage of cytosolic components into the extracellular space and the subsequent inflammatory reaction. In contrast to apoptosis, necrosis is characterized by loss of membrane integrity, cellular swelling, damage to the organelles and cell lysis that may lead to tissue inflammation.

Since MeHg and PCBs can be accumulated in the same food sources, in particular fish and seafood, we also investigated the effects of simultaneous exposure to both types of contaminants to examine possible synergistic effects.

Material and methods

Cell cultures and treatment

The mouse hippocampal neuronal cell line HT22, and the AtT-20 cells, derived from a mouse pituitary tumor, were grown in medium containing 10% fetal calf serum. The C17.2 cell line, a clonal multipotent neural precursor cell line originally derived from the external germinal layer of neonatal mouse cerebellum (Snyder et al., 1992), was maintained in DMEM supplemented with 10% fetal calf serum and 5% horse serum. Primary cultures of embryonic cortical neural stem cells (cNSCs) obtained from E15 rat embryos were cultured in DMEM:F12 medium supplemented with N2 (Bottenstein and Sato, 1979). bFGF (10 ng/ml) was added every 24 hour, and the medium changed every other day (for more details see Tamm et al., 2006). All cells were cultured at 37°C in a 5% CO_2 atmosphere. All chemicals for cell culture were supplied by Life Technologies (Gibco BRL), except bFGF (R&D systems). Other chemicals were from: MeHg (Alfa), PCB153 (Sigma), PCB126

(AccuStandard), pepstatin (Roche), trolox (Sigma), E64d (Sigma), MnTBAP (Calbiochem), (Peptide Institute), PD150606 (Calbiochem).

Staining for detection of apoptosis and necrosis

To detect the occurrence of necrotic cell death, trypsinized cells were mixed with 0.4% Trypan blue solution (Sigma) and counted under a phase contrast microscope. Cells with a damaged cell membrane (necrotic cells) stained blue, while cells with intact plasma membrane (healthy or apoptotic cells) remained unstained. The occurrence of apoptosis was evaluated on fixed or living cells. Cells grown on coverslips were fixed with ice-cold methanol/water (8/2=v/v), and stained with cell-impermeable propidium iodide (PI) to visualize nuclear condensation. Apoptotic cells were identified by the smaller size of the nucleus, irregular shape, and brighter intensity of the stained condensed chromatin. For vital stainings, cells grown on coverslips were incubated with a solution of Annexin V−FITC (0.5 μg/ml), which binds to phosphatidylserine, PI (1 μg/ml), and cell-permeable Hoechst 33358 (1 μg/ml) in a buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. Cells were analyzed with an Olympus BX60 fluorescence microscope equipped with a Hamamatsu digital camera ORCA II.

Immunoblotting and immunocytochemistry

Standard western blotting was used for the immunodetection of proteins (for details see Daré et al., 2002). The following primary antibodies were used: anti-Fodrin (Chemicon) and anti-cytochrome *c* (BD PharMingen). Immunocytochemistry was performed on cells, grown on coverslips, fixed with either 4% paraformaldehyde or 80% methanol and then washed with PBS. Primary antibodies were diluted in PBS with 0.3% Triton-X100 and 0.5% BSA (see Daré et al., 2002). The following primary antibodies were used: anti-Nestin (Chemicon),anti-Bax (BD PharMingen),anti-cytochrome c (BD PharMingen), anti-AIF (Santa Cruz), anti-P17 (kind gift from Dr.Momoi), anti-GFAP (Sigma), and anti βIII-tubulin (Biosite).

Analysis of mitochondrial function

The capacity of mitochondrial Ca^{2+} uptake was investigated in digitonin-permeabilized cells using a Ca^{2+} sensitive electrode (Orion Research, Beverly, MA, USA), as previously described (Ahlbom et al., 2000).

ATP determination

ATP concentrations were determined in a luminometric assay using the ATP dependency of the light-emitting luciferase-catalyzed oxidation of luciferin (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol.

Measurement of caspase activity

To evaluate the activity of class II caspases (2, 3 and 7), we measured the cleavage of the specific fluorogenic peptide substrate DEVD-AMC. The measurement was performed using a fluorometric assay (Nicholson et al., 1995), with some modifications (Gorman et al., 1999).

Detection of reactive oxygen species (ROS)

The Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes) was used, following the protocol provided by the manufacturer, to detect ROS in live cells.

Differentiation assay

To test whether the selected treatments may affect differentiation of cNSCs, we exposed embryonic cells to concentrations that are not cytotoxic and check the neuronal differentiation process. During normal culture conditions bFGF is added to the cNSC media to keep the stem cells in a proliferative and undifferentiated state. When the media is replaced and no further bFGF is added, the cells begin to spontaneously differentiate. This process takes approximately 7 days from the removal of bFGF to the appearance of a strong β-tubulin III (Tuj1) neuronal staining (see Tamm et al. 2006).

Results and discussion

Neuronal and endocrine cells

The hippocampal HT22- and the pituitary AtT20-cells were exposed to different concentrations of MeHg (from 0.5 to 4 μM), PCB153 (25-200 μM), and PCB 126 (12.5-100 μM) for 4-24 hours. The morphological changes induced by these substances indicated the occurrence of both apoptosis and necrosis, however apoptosis was less common in the AtT20 cells. The $Ca2^{+}$-dependent proteases calpains were activated in HT22 cells, as shown by the increase in the 150 kDa α-fodrin, a calpain breakdown product. Pre-treatment with the calpain inhibitor E64d exerted a partial protection in both HT22 and AtT20 cells against all the toxicants.There was no activation of caspases as further supported by the lack of protective effects of the pan-caspase inhibitor z-VAD-fmk. Lysosomal disruption was also observed, as visualized by decreased uptake of the lysomotrophic vital dye acridine orange in the HT22 exposed cells. In addition, the protective actions of the cathepsin D inhibitor Pepstatin confirmed the activation of lysosomal proteases in MeHg and PCB exposed cells. Furthermore, mitochondrial functions were impaired, as shown by the significant decrease in mitochondrial $Ca2^{+}$ uptake capacity and ATP levels. Presence of reactive oxygen species could be detected only in MeHg exposed cells, and in agreement, pre-treatment with antioxidants was only protective against cell death induced by MeHg, suggesting that oxidative stress does not play a major role in PCB toxicity in our experimental models. Analysis of data from simultaneous exposures to moderately cytotoxic doses of MeHg and PCB 153 or PCB 126, using the Bliss' independence criterion, suggested a competitive interaction between MeHg and PCBs in HT22 cells and a slightly synergistic or additive effects in AtT20 cells. Concomitant exposures to doses that were not cytotoxic did not induce significant additive or synergistic effects on apoptosis or necrosis. In summary, these data suggest that all three toxicants induce cell death in hippocampal and pituitary cells via activation of both calpains and lysosomal proteases, possibly through disruption of mitochondrial function and intracellular calcium signaling. In these models oxidative stress seems to play a role only in MeHg toxicity.

Neural stem cells

Both types of neural stem cells, cNSCs and C17.2, used in this study exhibited a higher susceptibility to the toxic effects of both MeHg and PCBs, as compared to neuronal and endocrine cells, especially the primary cultures of cNSC. Thus, the rat cNSCs and the murine-derived neural progenitor cell line C17.2 were exposed to lower concentrations of MeHg, (0.025-2 μM), PCB 153 (0.1-150 μM), and PCB 126 (1.25-25 μM). Apoptotic and necrotic morphology could be detected in C17.2 cells exposed to MeHg at 0.5 μM and ≥1 μM, respectively. In cNSCs, similar effects were induced by much lower concentrations (0.05 and ≥0.1 μM). Our results showed that NSCs undergo apoptotic cell death via Bax oligomerization, cytochrome *c* release from the mitochondria with subsequent activation of caspase-3. In addition to caspases, a caspase-independent calpain pathway was also activated during MeHg-induced cytotoxicity. The caspase and calpain pathways were activated in parallel in NSCs undergoing MeHg-induced apoptosis, as shown by the partial protection exerted by the caspases or calpains inhibitor alone, and the full protective effect when the inhibitors were combined (see Tamm et al. 2006). To study the mechanisms of PCB-induced apoptosis in NSC, C17.2 cells were exposed to 100 μM of PCB 153 and 25μM of PCB 126; cNSCs were exposed to 5 μM and 1μM , respectively. In contrast to MeHg, none of the PCB congeners investigated seemed to have an effect on mitochondria, and no impairment in mitochondrial Ca^{2+}-uptake capacity and ATP-levels could be detected. Bax oligomerization, cytochrome *c* release or caspase activity could not be observed after PCB exposure. Neither caspase- (zVAD-fmk), nor calpain-inhibitors (PD150606) showed protective effect against PCBs-induced cell death. However, lysosomal disruption was observed after exposure using acridine orange staining. In agreement, Pepstatin protected against cell death. The combined exposure to MeHg and PCB 153 or PCB 126 did not show additive or synergistic effects.

Interestingly, we observed that exposure to very low doses of MeHg (2.5-5 nM), significantly impaired neuronal spontaneous differentiation of cNSCs (see Tamm et al. 2006). Thus in light of our results, there seems to be a narrow margin of safety against risk of neurodevelopmental effects caused by MeHg.

In conclusion, these studies provide evidence for the activation of multiple cell death pathways in cells exposed to MeHg and PCBS. Different cell types are differently susceptible, with NSC being highly sensitive, and respond to the same toxic stimulus by activating diverse intracellular responses. The use of multiple *in vitro*

experimental models is a first choice approach to performe studies aimed at dissecting the molecular mechanisms of neurotoxic damage.

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