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Hydroxylated polychlorinated biphenyls increase reactive oxygen species formation and induce cell death in cultured cerebellar granule cells

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Abstract

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that bioaccumulate in the body, however, they can be metabolized to more water-soluble products. Although they are more readily excreted than the parent compounds, some of the metabolites are still hydrophobic and may be more available to target tissues, such as the brain. They can also cross the placenta and reach a developing foetus. Much less is known about the toxicity of PCB metabolites than about the parent compounds. In the present study, we have investigated the effects of eight hydroxylated (OH) PCB congeners (2'-OH PCB 3, 4-OH PCB 14, 4-OH PCB 34, 4'-OH PCB 35, 4-OH PCB 36, 4'-OH PCB 36, 4-OH PCB 39, and 4'-OH PCB 68) on reactive oxygen species (ROS) formation and cell viability in rat cerebellar granule cells. We found that, similar to their parent compounds, OH-PCBs are potent ROS inducers with potency 4-OH PCB 14 < 4-OH PCB 36 < 4-OH PCB 34 < 4'-OH PCB 36 < 4'-OH PCB 68 < 4-OH PCB 39 < 4'-OH PCB 35. 4-OH PCB 36 was the most potent cell death inducer, and caused apoptotic or necrotic morphology depending on concentration. Inhibition of ERK1/2 kinase with U0126 reduced both cell death and ROS formation, suggesting that ERK1/2 activation is involved in OH-PCB toxicity. The results indicate that the hydroxylation of PCBs may not constitute a detoxification reaction. Since OH-PCBs like their parent compounds are retained in the body and may be more widely distributed to sensitive tissues, it is important that not only the levels of the parent compounds but also the levels of their metabolites are taken into account during risk assessment of PCBs and related compounds.

Keywords

PCBs; hydroxylated PCBs; reactive oxygen species; cell death; ERK1/2; Hydroxylated polychlorinated biphenyls; reactive oxygen species; cerebellar granule cells; cell toxicity; cell death

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Introduction

Polychlorinated biphenyls (PCBs) are persistent contaminants that are widely distributed in the environment. Their production was banned in the late 1970s, however, due to their persistence PCBs are still abundant in the environment and humans are still exposed, mainly through intake of contaminated food. Although PCBs can bioaccumulate, certain con-geners are metabolised, which can result in the formation of toxic, retained and/or persistent metabolites, e.g. methyl sulfonyl- (MeSO₂-) and hydroxylated- (OH-) PCBs (Letcher et al., 2002; McKinney et al., 2006; Verreault et al., 2005). Approximately 40 different OH-PCBs have been identified in human plasma (Hovander *et al.*, 2006) and the levels of these chemicals in human serum were found to constitute 10–30% of the total PCBs (Fangstrom et al., 2002; Sandau et al., 2000; Sjodin et al., 2000). Exposure to OH-PCBs has resulted in thyroid hormone disturbances, altered vitamin A levels, and inhibition of phase II sulfation and glucuronidation in experimental organisms (Brouwer and van den Berg, 1986; Ghisari and Bonefeld-Jorgensen, 2005; van den Hurk et al., 2002; Verreault et al., 2006). This suggests that biotransformation of PCBs potentially increases toxicity in exposed organisms.

The toxicity of PCBs is fairly well described, and includes alterations of neurotransmitter levels and reuptake, calcium homeostasis, intracellular signalling pathway activation, and for some congeners, cancer (Fonnum et al., 2006; Tilson and Kodavanti, 1998). The PCB metabolites are suspected to have many of the same effects as the parent compounds, and in addition they can alter thyroid hormone homeostasis and inhibit estrogen metabolism (Kester et al., 2000; Koopman-Esseboom et al., 1994; Schuur et al., 1998). Hydroxylation is generally the first step in the biotransformation process, and is followed by conjugation either with glucuronic acid or with sulfate moieties. Lower chlorinated PCBs are often more susceptible than highly chlorinated congeners to biotransformation to OH-PCBs in P450-catalyzed reactions (Liu et al., 2009). Biotransformation makes the compounds excretable, and is therefore considered a detoxification process. However, since OH-PCBs are more water soluble than their parent compounds they also have different distribution in the body; PCBs are mainly stored in adipose tissue, where they are sequestered away from sensitive organs such as the brain. OH-PCBs, on the other hand, are able to circulate and cross barriers such as the blood-placenta barrier at a higher rate than PCBs (Soechitram et al., 2004), and are therefore more likely to reach target organs. The OH-PCBs may therefore be more toxicologically relevant than the PCBs.

Previously, results from our laboratory have shown that PCBs increase oxidative stress and cause cell death in cultured cerebellar granule cells *in vitro* (Mariussen et al., 2002; Mariussen and Fonnum, 2006). Similar investigations have not yet been carried out with hydroxylated PCBs. We hypothesised that the hydroxylated metabolites of PCBs could cause effects similar to their parent compounds in cerebellar granule cells. Thus, in the present study, we investigated the effect of eight lower chlorinated congeners of hydroxylated PCBs, each bearing one hydroxyl (in the *para*-position for 7 and in the *ortho*-position for one of the congeners, fig. 1) on reactive oxygen species formation and cell viability in rat cerebellar granule cells, and examined a possible mechanism for these events.

Methods

Chemicals and Reagents

Bovine serum albumin (BSA), cytosine- β -D-arabinofuranoside, deoxyribonuclease (DNase I), 2,7-dichlorofluorescein diacetate (DCFH-DA), L-glutamine, dimethyl sulfoxide (DMSO), poly-L-lysine, soya bean trypsin inhibitor, ($\pm$) α -tocopherol (vitamin E), and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). U0126 was purchased from AH Diagnostics AS (Norway). Fetal calf serum (heat inactivated), Basal medium Eagle (BME), penicillin/streptomycin, Hanks Balanced Salt Solution (HBSS) and HEPES buffer were purchased from

Gibco/Invitrogen (Norway). 2'-OH PCB 3, 4-OH PCB 14, 4-OH PCB 34, 4'-OH PCB 35, 4-OH PCB 36, 4'-OH PCB 36, 4-OH PCB 39, and 4'-OH PCB 68 were synthesized as described previously and had a purity >98 % (Lehmle and Robertson, 2001).

Preparation of Cerebellar Granule Cell Cultures

All procedures involving animals were performed according to protocols approved by the NDRE Institutional Animal Care and Use Committee. Rat CGC cultures were prepared from rat pups on postnatal day 7 (Gallo *et al.*, 1982), with slight modifications of the method as described earlier (Dreiem *et al.*, 2002). The cells were cultured in Basal Medium Eagle (BME) medium (cat. no. 21010, Invitrogen, Carlsbad, CA) adjusted to 25 mM KCl and supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The cells were plated in 12-well plates to a density of 1.125×10^6 per well for lactate dehydrogenase (LDH) assays, or in 50 mm cell culture dishes (4.5×10^6 cells per dish) for all other assays. Glial cell proliferation was prevented by adding cytosine- β -D-arabinofuranoside (final concentration 2.5 μ g/ml) 16–22 h after plating. Experiments were performed on day 7–9 in vitro.

Preparation of OH-PCB stocks and cell exposure—Stock solutions of OH-PCBs were prepared in DMSO. Aliquots of the stock solutions were added to the appropriate medium to yield the desired final concentration (final DMSO conc. $\leq 0.1\%$). Cells were exposed by replacing growth medium with freshly prepared buffer containing OH-PCBs. DMSO alone had no significant effect on ROS formation or cell death in any experiment.

Measurement of ROS formation

ROS was determined after a modification of the procedure described by (Myhre *et al.*, 2001). In brief, cerebellar granule cells were loaded with 5 μ M DCFH-DA (DCFH-DA stock solution 5 mM in methanol, stored at -20°C) directly in the cell culture medium for 20 min (at 37°C with 5% CO_2 and constant humidity). The medium with DCFH-DA was replaced with 1.5 ml incubation medium (HBSS with 20 mM HEPES and 10 mM glucose) containing the appropriate concentrations of OH-PCBs, and the cells were harvested with a cell scraper. Three aliquots of 250 μ l were taken from each cell culture dish and placed in the wells of a multiwell plate, and fluorescence was recorded every 3 min. in a Perkin-Elmer LS50B luminescence spectrometer (ex. wavelength 485 nm, em. wavelength 530 nm) for 3 h. In each experiment, two cell culture dishes were used for each OH-PCB concentration.

Lactate Dehydrogenase Assay

Leakage of lactate dehydrogenase to the medium was assessed as an index of cell injury (Koh and Choi, 1987). The measurements were performed as described elsewhere (Dreiem *et al.*, 2005). In brief, the cell culture medium was removed and cells were incubated with the test substances for various times in Hepes buffered medium (HBM) (containing 1.26 mM CaCl_2 , 25 mM KCl, 0.44 mM KH_2PO_4 , 0.49 mM MgCl_2 , 0.41 mM MgSO_4 , 140 mM NaCl, 4.17 mM NaHCO_3 , 0.34 mM Na_2HPO_4 , 10.5 mM D-glucose, and 20 mM Hepes, pH 7.4). After incubation for the times indicated, a 50 μ l sample of the medium from each well was transferred to the wells of a custom made 48 well multiwell plate with glass bottom. 25 μ l of a 13.6 mM stock solution of pyruvate (final concentration 0.68 mM) was added and the volume was adjusted to 450 μ l with 0.1 M KPO_4 buffer (pH 7.5). The reactions were started by automated injection of 50 μ l of an 846 μ M stock solution of NADH (final concentration 84.6 μ M). The LDH activity was measured continuously at 28°C by monitoring the decrease in fluorescence emission at 460 nm using a BMG FLUOstar Optima fluorimeter (excitation wavelength 340 nm). The fluorescence decay rate was used to calculate the LDH activity in the samples by referring to a standard curve of rabbit LDH (Sigma–Aldrich)

Nuclear Morphology

Nuclear morphology indicative of apoptosis was assessed by Hoechst staining. Cells were exposed to test substances with or without inhibitors in HBM for 18 h and stained with Hoechst 33342 (Molecular Probes/Invitrogen; final concentration 2.5 µg/ml); necrotic cells were counterstained with propidium iodide (Molecular Probes/Invitrogen; final conc. 3.75 µM). The cells were immediately examined by fluorescence microscopy and photographed. Cells with condensed and fragmented nuclei were counted as apoptotic cells, and propidium iodide positive cells were counted as necrotic cells.

Assessment of cell death by trypan blue exclusion

Cell viability was assessed microscopically after staining with trypan blue (Sigma-Aldrich). Trypan blue is taken up into cells with a damaged cell membrane, but not into viable cells with an intact cell membrane. 100 µl of a 1% stock solution (w/v) of trypan blue was added to each cell culture dish (final concentration of trypan blue was 0.025%), and the dishes were left for 5 min at room temperature to allow uptake of the dye into the dead cells. Unstained (living) and stained (dead) cells were then counted (at 400×magnification).

Statistical analysis

Unless otherwise specified all results were evaluated by one-way ANOVA followed by comparison to unstimulated cell controls by Dunnett's test when appropriate.

Results

OH-PCB-induced ROS formation

All the OH-PCBs tested, except for 2'-OH PCB 3, induced ROS formation in the concentration range 5 – 50 µM (fig. 2a). 4-OH PCB 14 was the most potent, increasing ROS production more than fourfold at concentrations as low as 5 µM, and reaching approximately 25 × control levels at 20 µM. 4-OH PCB 34, 4-OH PCB 36, 4'-OH PCB 68 and 4-OH PCB 39 had intermediate potencies, increasing ROS production 8–12 fold at 20 µM. 4'-OH PCB 36 and 4'-OH PCB 35 only gave significant ROS increases when cells were exposed to high (50 µM) concentrations, whereas 2'-OH PCB 3 was inactive. Both the two *ortho*-chlorinated OH-PCBs (4'-OH-PCB 68 and 4-OH-PCB 34) and the non-*ortho*-chlorinated OH-PCBs induced cell death and ROS formation. Generally, ROS increased with time during the duration of the experiments (fig. 2b).

OH-PCB-induced cell death

Cell death after 18 h exposure to OH-PCB was assessed by measuring LDH activity in the culture media, an indication of LDH leakage from the cytosol occurring during rupture of the cell membrane (fig. 3). In general, all OH-PCBs tested except 2'-OH PCB 3 greatly increased LDH leakage from the cells at the highest concentrations tested (50 µM). 4-OH PCB 36 and 4-OH PCB 14 were the most potent, producing significant increases in LDH release at 10 µM (4-OH PCB 36) and 20 µM (4-OH PCB 14). The increases in LDH release observed after exposure to 20 µM and 50 µM 4-OH-PCB 36 corresponded to ~35 % and 57 % necrotic cell death, respectively (Table 1). To further investigate OH-PCB-induced cell death, 4-OH PCB 36 was selected for microscopic assessment of cell death, counting PI positive cells as necrotic cells, and cells with condensed or fragmented nuclei as apoptotic cells. Exposure to 20 or 50 µM 4-OH PCB 36 for 18 h significantly increased cell death, to ~80% ($p = 0.002$) and 100% ($p < 0.001$), respectively (fig. 4). In unexposed cultures total cell death was $24.8 \pm 5.1\%$, of which $19.1 \pm 5.6\%$ were apoptotic cells. The percentage of apoptotic cells increased approximately 3-fold in cells exposed to 20 µM 4-OH PCB 36 compared to controls ($p = 0.047$), whereas necrosis was not significantly altered. In contrast, cells exposed to 50 µM 4-OH PCB

36 showed no increase in the percentage of cells undergoing apoptosis compared to control, while more than 75% of the cells ($p < 0.001$) were necrotic.

Inhibitory effect of U0126 and vitamin E on ROS after OH-PCB exposure

The MAP kinase ERK1/2 is activated and contributes to cell toxicity in cultured cerebellar granule cells and neutrophil granulocytes after exposure to toxicants such as brominated flame retardants and organic solvents (Dreiem et al., 2003; Reistad et al., 2007). Therefore, we examined whether ERK1/2 was involved in ROS formation after exposure to OH-PCBs. U0126, an inhibitor of ERK1/2 activation, reduced ROS formation after exposure to 4-OH PCB 36 or 4-OH-PCB 14 to levels that were undistinguishable from control (Fig. 5). U0126 alone did not have any significant effects of ROS formation. Vitamin E (α -tocopherol) is a potent antioxidant that ameliorates cell damage induced by PCBs and PBDEs (Mariussen et al., 2002; Sridevi et al., 2007), therefore, we investigated whether vitamin E could prevent ROS induced by 4-OH-PCB 36 and 4-OH-PCB 14. Vitamin E (50 μ M) reduced ROS after 4-OH-PCB 36 exposure by 30 % (Figure 6). Interestingly, vitamin E had no effect on ROS formation induced by 4-OH-PCB 14 (Figure 6).

Effect of U0126 on cell death after OH-PCB exposure

Cell death in 4-OH PCB 36-exposed cells (50 μ M) was reduced by 47% and 55% by co-treatment with 5 or 10 μ M U0126, respectively (Fig. 7). In cells exposed to 20 μ M 4-OH PCB 36, co-exposure with U0126 caused a trend towards reduced cell death compared to 20 μ M 4-OH PCB 36 alone, however, this reduction did not reach statistical significance. U0126 (10 μ M) also reduced cell death after exposure to 4-OH-PCB 14 by 50% (Fig. 7, insert). U0126 alone did not have any significant effects on ROS formation or cell death when compared to vehicle control (Bonferroni's test). Vitamin E did not reduce cell death after exposure to 4-OH PCB 36 (data not shown).

Discussion

In the present study we have shown that hydroxylated PCBs increase ROS levels and cell death in cerebellar granule cells in a manner similar to their parent compounds. The potency of the congeners varied from inactive (2'-OH PCB 3) to highly potent inducers of ROS and cell death (4-OH-PCB 14 and 4-OH-PCB 36). Previously it has been reported that 2'-OH PCB 3 is a weak inhibitor of gap junction intracellular communication in the rat liver epithelial cell WB-F344, and that OH-PCBs with the phenolic group in the *ortho* position are weak inhibitors of sulfotransferase (Machala et al., 2004; van den Hurk et al., 2002). However, the *ortho*-hydroxy-PCBs were 100 times more potent as inhibitors of glucuronosyltransferase than of sulfotransferase (van den Hurk et al., 2002), suggesting that the toxicity of hydroxylated PCBs may be dependent of the position of the hydroxyl group, but that the effects are end-point specific. Similarly, Kodavanti and coworkers found that the chlorination pattern of OH-PCBs influence activity; *ortho*-chlorinated OH-PCBs induced PKC translocation to the membrane, whereas both *ortho*- and non-*ortho*-chlorinated OH-PCBs inhibited microsomal calcium uptake (Kodavanti et al., 2003). In the present study we found that both *ortho*- and non-*ortho*-chlorinated OH-PCBs induced ROS formation and cell death.

Interestingly, the ROS levels observed after exposure to the most active OH-PCB were much greater than what has been reported for PCB congeners and Aroclor mixtures. The PCB congener 153 and the congener mixtures Aroclor 1254 and Aroclor 1242 increased ROS to levels 1.5 – 1.8-fold higher than controls (Mariussen et al., 2002), while in the present study we observed ROS levels more than 10 fold higher than control values after OH-PCB exposure. ROS-formation has not been assessed after exposure to the parent congeners of the OH-PCBs examined in the present study, preventing direct comparisons between parent and hydroxylated

PCBs. However, our results demonstrate that hydroxylated PCBs are potent inducers of oxidative stress, and suggest that hydroxylation reactions do not necessarily decrease PCB toxicity.

Congener 4-OH-PCB 36 was the most potent inducer of cell death, and the mode of cell death in CGCs was assessed after exposure to high and intermediate concentrations of this congener. Although cell death is now believed to be a continuum rather than two separate entities, it is commonly classified into apoptosis (active cell death), characterized by cell 'shut-down' with DNA degradation, externalization of phosphatidyl serine and formation of apoptotic bodies with no leakage of intracellular contents, and necrosis, which is passive, 'catastrophic' cell death characterized by rupture of the cell membrane, leakage of cytosolic contents and triggering of inflammation reactions. By assessing nuclear morphology we found that the mode of cell death after exposure to 4-OH PCB 36 was concentration-dependent: an intermediate concentration (20 μ M) caused ~80 % cell death which was mostly apoptotic. However, when the 4-OH-PCB 36 concentration was increased to 50 μ M, more than 75 % of the cells stained positive for PI and were therefore classified as necrotic. This is consistent with what is observed after exposure to several toxicants; a low dose damages the cell beyond repair, and apoptosis mechanisms are activated. However, if the dose is increased, cell damage is more rapid and severe, the cell cannot sustain the processes required for apoptosis and necrosis occurs.

Activation of the MAP kinase ERK1/2 is an important event in cell toxicity in cerebellar granule cells after exposure to PCBs and brominated flame retardants (BFRs) with structural similarities to PCB (Fan and Kodavanti, 2008; Reistad et al., 2007). Therefore, we investigated whether ERK1/2 was involved in cell toxicity after exposure to OH-PCBs. We found that U0126, an inhibitor of ERK1/2 activation, completely prevented ROS formation after exposure to 4-OH PCB 36 and 4-OH-PCB 14 (Figure 5), suggesting that ROS formation was mediated by ERK1/2 activation. Interestingly, the antioxidant vitamin E was not able to reduce ROS formation after exposure to 4-OH-PCB 14, and only caused a small reduction in ROS induced by 4-OH-PCB 36 without preventing cell death. Vitamin E has previously been shown to prevent ROS formation and cell death after exposure to Aroclor 1254 (Mariussen et al., 2002). Vitamin E is a powerful scavenger of lipid peroxyl radicals and hydroxyl radicals (Halliwell and Gutteridge, 1999), however, it is lipophilic and localizes mostly to membranes. DCFH primarily measures ROS in the cytosolic compartment (Reistad et al., 2007), therefore, it is likely that the rapid ROS increase seen after exposure to 4-OH-PCB 14 is localized to the cytosol, where ROS rapidly oxidize DCFH and cellular targets before they can be scavenged by vitamin E. In contrast, vitamin E gave a small reduction in ROS levels after co-exposure with 4-OH-PCB 36. This could be explained by the slightly lower rate of ROS formation by 4-OH-PCB 36, allowing more time for diffusion of ROS away from the site of production to membranes where they would be available for scavenging by vitamin E, or alternatively, differences in water solubility between the congeners could affect the site of ROS formation in the cells.

In addition to completely preventing ROS formation, U0126 also reduced cell death after exposure to 4-OH-PCB 36 and 4-OH-PCB 14, suggesting that activation of ERK1/2 is involved in cell death after exposure to these congeners. However, the protection was not complete; U0126 only reduced cell death by approximately 50%, suggesting that additional mechanisms are involved in cell death after exposure to 4-OH-PCB 36 and 4-OH-PCB 14. One possible additional mechanism for OH-PCB induced cell death could be calcium-induced death: Kodavanti and coworkers have shown that 4-OH-PCB 14 (and other OH-PCBs not investigated in the present study) inhibit calcium uptake into cerebellar microsomes (Kodavanti et al., 2003), thereby impairing calcium buffering and increasing cytosolic calcium levels. Increased intracellular calcium levels were also suggested to be involved in increased c-Jun expression after exposure to 4(OH)-2',3,3',4',5'-penta chlorobiphenyl in PC12 cells (Shimokawa et al.,

2006). Increased cytosolic calcium can not only activate ERK1/2 via activation of Ras and the MAP kinase pathway (Rosen et al., 1994), thereby providing a potential mechanism for the activation of ERK1/2 by OH-PCBs; calcium also activates calcium-dependent proteases, lipases and nucleases, and causes excessive mitochondrial calcium uptake leading to energy failure, opening of the mitochondrial permeability pore and induction of apoptosis (Fonnum and Lock, 2004).

ERK1/2 activation has been demonstrated in cerebellar granule cells and in non-neuronal cells after exposure to PCBs and PCB-like BFRs, causing increased oxidative stress and cell death (Fan and Kodavanti, 2008; Reistad et al., 2005; Reistad et al., 2007). Therefore, ERK1/2 activation has been suggested as a common mode of action for polyhalogenated biphenyls and structurally related chemicals (Fan and Kodavanti, 2008); in the present study we have shown that the toxicity of hydroxylated PCBs also is dependent, at least partly, on this mechanism. Although ERK1/2 also can promote neuronal survival in some situations, a role for ERK1/2 in neuronal toxicity is supported by data showing that ERK1/2 is implicated in neuronal injury after insults such as cerebral ischemia, brain trauma and neurodegenerative diseases (Chu *et al.*, 2004).

In conclusion, we have shown that 4-OH-PCBs, like their parent compounds, are able to induce ROS formation and cell death in cerebellar granule cells in culture. The effects are at least partly mediated by activation of the MAP kinase ERK1/2. These observations suggest that the hydroxylation of PCBs does not by itself constitute a detoxification reaction, and that PCB metabolites such as hydroxylated PCBs may contribute to the neurotoxicity of PCBs.

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Fig. 1.
Structures of the examined OH-PCBs.

Fig. 2. OH-PCBs increase DCF fluorescence in cerebellar granule cells

a) Cerebellar granule cells were loaded with 5 μ M DCFH-DA and exposed to different concentrations of 2'-OH PCB 3, 4-OH PCB 14, 4-OH PCB 34, 4'-OH PCB 35, 4-OH PCB 36, 4'-OH PCB 36, 4-OH PCB 39, and 4'-OH PCB 68. ROS formation was assessed by measurement of DCF fluorescence every 3 min. in a BMG FLUOstar Optima fluorimeter for 3 h. Points represent mean and bars represent SE of 3 to 5 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA, Dunnett's test). b) Time curve showing the increase in DCF fluorescence after exposure to 20 μ M of 2'-OH PCB 3, 4-OH PCB 14, 4-OH PCB 34, 4'-OH PCB 35, 4-OH PCB 36, 4'-OH PCB 36, 4-OH PCB 39, and 4'-OH PCB 68. The figure shows one representative experiment.

Fig. 3. OH-PCBs increase cell death (measured by LDH release) in cerebellar granule cells in vitro Cerebellar granule cells were exposed to different concentrations of 2'-OH PCB 3, 4-OH PCB 14, 4-OH PCB 34, 4'-OH PCB 35, 4-OH PCB 36, 4'-OH PCB 36, 4-OH PCB 39, and 4'-OH PCB 68 for 18 h. LDH activity in the media were assessed by monitoring of NADH fluorescence decay rate in a BMG FLUOstar Optima fluorimeter. Points represent mean and bars represent SE of 5 to 6 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA, Dunnett's test).

Fig. 4. 4-OH PCB 36 induces apoptosis or necrosis in cerebellar granule cells dependent on concentration

Cerebellar granule cells were exposed to 4-OH PCB 36 in HBM for 18 h before staining with Hoechst 33342 and propidium iodide and examination by fluorescence microscopy. PI positive cells were counted as necrotic cells, and PI negative cells showing condensed and/or fragmented nuclei were counted as apoptotic cells. Columns represent mean and bars represent SE of three independent experiments.

Fig. 5. U0126 reduces OH-PCB-induced ROS formation in cerebellar granule cells

Cerebellar granule cells were loaded with 5 μM DCFH-DA and exposed to 20 or 50 μM 4-OH PCB 36 in the presence or absence of the MEK1/2 inhibitor U0126. ROS formation was assessed by measurement of DCF fluorescence every 3 min. in a BMG FLUOstar Optima fluorimeter for 3 h. The insert shows the effect of U0126 on ROS induced by 50 μM 4-OH-PCB 14. Columns represent mean and bars represent SE of 3 independent experiments. **: $p < 0.01$ and *** $p < 0.001$ compared to vehicle (no OH-PCB), a: $p < 0.001$ compared to 20 μM OH-PCB without U0126, b: $p < 0.05$ compared to 50 μM OH-PCB without U0126 (ANOVA, Bonferroni's test).

Figure 6.

Vitamin E reduces ROS levels after exposure to 4-OH PCB 36.

Cerebellar granule cells were loaded with 5 μ M DCFH-DA and exposed to 4-OH PCB 14 or 4-OH PCB 36 (50 μ M) in the presence or absence of 50 μ M vitamin E (vE). ROS formation was assessed by measurement of DCF fluorescence every 3 min. in a BMG FLUOstar Optima fluorimeter for 3 h. Columns represent mean and bars represent SE of 3 independent experiments. * $p < 0.05$ compared to 4-OH-PCB 36 without vitamin E, (paired t-test).

Fig. 7. U0126 prevents cell death in cerebellar granule cells exposed to 4-OH PCB 36

Cerebellar granule cells exposed to 20 or 50 μM 4-OH PCB 36 in the presence or absence of the MEK1/2 inhibitor U0126. Cell death was assessed by trypan blue staining. Columns represent mean and bars represent SE of 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared to vehicle (no 4-OH-PCB 36), a: $p < 0.01$ and b: $p < 0.001$ compared to 50 μM 4-OH PCB 36 without U0126 (ANOVA, Bonferroni's test). The insert shows the effect of 10 μM U0126 on cell death after exposure to 50 μM 4-OH-PCB 14 (columns represent means and bars represent SE of three wells in one experiment). a: $p < 0.01$ compared to 50 μM 4-OH-PCB 14 without U0126.

Table 1

Necrotic cell death assessed by LDH leakage and trypan blue exclusion assay.

<i>Conc. 4-OH-PCB 36 (μM)</i>	0 (Vehicle)	20	50
<i>LDH leakage (10^{-3}U)</i>	1.1 $\pm$ 0.2	8.0 $\pm$ 1.6	10.8 $\pm$ 1.3
<i>Trypan blue (% cell death)</i>	13.6 $\pm$ 3.3	35.7 $\pm$ 3.1	56.5 $\pm$ 1.2