THE EFFECT OF HYDROXYLATED PCBs ON DCF-FLUORESCENCE AND CELL DEATH IN CULTURED RAT CEREBELLAR GRANULE CELLS

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Introduction

We have previously investigated the effects of polychlorinated biphenyls (PCBs) on free radical formation and cell death in cerebellar granule cells (1). PCBs may be metabolised to hydroxylated polychlorinated biphenyls (HO-PCBs) in the body. Therefore, we wanted to expand our study to include also the HO-PCBs. After hydroxylation, many of the PCBs are conjugated to either glucoronic acid or sulphate, which facilitates their excretion. Nevertheless, some of the HO-PCBs are retained in the body. The structures of some of the HO-PCBs show a certain similarity to the structure of the cathecholamines, and PCBs have been shown to affect the level of cathecholamines in the brain. Therefore, we compared the effect of some HO-PCBs with the effects of PCBs on some important physiological parameters in the brain. In the present communication we have compared the effects of PCB and HO-PCB on formation of DCF-fluorescence, which is used as a measure of reactive oxygen species (ROS) formation, and cell death in cultured rat cerebellar granule cells.

Materials and methods

Compounds

The compounds used was generally hydroxylated in the para position and is named according to Maervoet et al (2). p43, 4'-OH-3,3',5-TrClB; p44, 4-OH-3,5-DClB; p45, 4'-OH-2,3',5'-TrClB; p46, 4-OH-3,3',5-TrClB; p47, 4'-OH-2,3',4,5'-TeClB; p48, 4-OH-3,4',5,-TrClB; p50, 4-OH-3,3',4'-TrClB; p51, 2'-OH-4-MClB.

Cerebellar granule cell cultures

The cultures were prepared from male Wistar rat pups on postnatal day 7 essentially as described earlier (3,4). The cells were cultured in BME medium (Gibco) adjusted to 25 mM KCl and supplemented with 10% heat inactivated foetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin. The cells were plated in 12 well polystyrene Nunclon^{TM} Delta Multidishes to a density of 1.125 x 10^6 per well for lactate dehydrogenase assays, or in 60

mm polystyrene Nunclon™ Delta cell culture dishes (4.5 x 10^6 cells per dish) for measurement of reactive oxygen species formation. Glial cell proliferation was prevented by adding cytosine β-D-arabinofuranoside (final concentration 2.5ug/ml) 16-22 hr after plating. The experiments were carried out on day 7-9 *in vitro*.

Lactate dehydrogenase assay

After 7-9 days *in vitro* the cells were exposed to HO-PCBs for 18 hrs 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). Leakage of lactate dehydrogenase (LDH) to the medium was assessed as an index of cell injury (5). Aliquots of the incubation medium were collected and LDH activity was measured essentially as described by Ring and Tanso (6). The LDH activity in the media samples were determined by comparison to a standard curve of LDH from rabbit muscle (Sigma-Aldrich).

Dichlorofluorescein (DCFH) fluorescence.

The experiments were performed as described earlier (7). In brief, cells were incubated with 2μM dichlorofluorescin diacetate (DCFH-DA) in the growth medium for 20 min at 37°C. DCFH-DA is cell permeable and diffuses across the cell membrane, whereafter it is hydrolysed by intracellular esterases to yield dichlorofluorescin (DCFH), a nonfluorescent compound. DCFH can be oxidized by ROS to the fluorescent compound dichlorofluorescein (DCF). After loading with DCFH-DA, the medium was removed and replaced with fresh HBM containing the test substances. Thereafter, the cells were removed from the dishes by gentle scraping and transferred in triplicate to the wells of a 96 well microtiter plate. Fluorescence was recorded in a Perkin Elmer LS50B luminescence spectrophotometer (excitation wavelength 485 nm, emission wavelength 530 nm) for 1 hr.

Results and discussion

Formation of DCFH-fluorescence

The results show that the HO-PCBs increase DCFH-fluorescence in cerebellar granule cells to levels several fold higher than in the controls (Fig 1). In general, the ROS levels in cerebellar granule cells were much higher after exposure to HO-PCBs than what we have seen with any other group of compounds such as PCBs, brominated flame retardants, fluorinated hydrocarbons and hydrocarbon solvents (1, 4, 7, unpublished results). In fact, p44 gave a 10-fold higher response than the most active PCB (1). On the other hand, the monochlorinated congener p51, which is hydroxylated in the ortho-posistion, gave hardly any response. The most effective congeners were (in order of decreasing potency): p44 > p46 > p45 > p47 = p48 > p43 = p51.

As congeners p44 and p46 were the most active, they were chosen for mechanistic studies. The effects of p44 and p46 (50μM) was inhibited by 10 μM U0126, an inhibitor of Extracellular Signal Regulated Kinase (ERK) 1/2, and by the tyrosine kinase receptor inhibitor Erbstatin analogue (25 μM). Unlike the PCB-induced ROS-formation measured by an increase in DCF-fluorescence, which was dependent on NMDA-receptor-, NO synthase-, and phospholipase A2 activity (1), we did not find any effect of inhibitors of these pathways after HO-PCB exposure (data not shown).

This raises the question whether the increase in DCF-fluoresence is due to ROS formation or whether increase shows a catalytic role of the hydroxyl group in oxidising DCF. As controls we found that HO-PCB with the granular cells in the absence of DCF or HO-PCB with DCF in the absence of granular cells did not give any fluorescence.

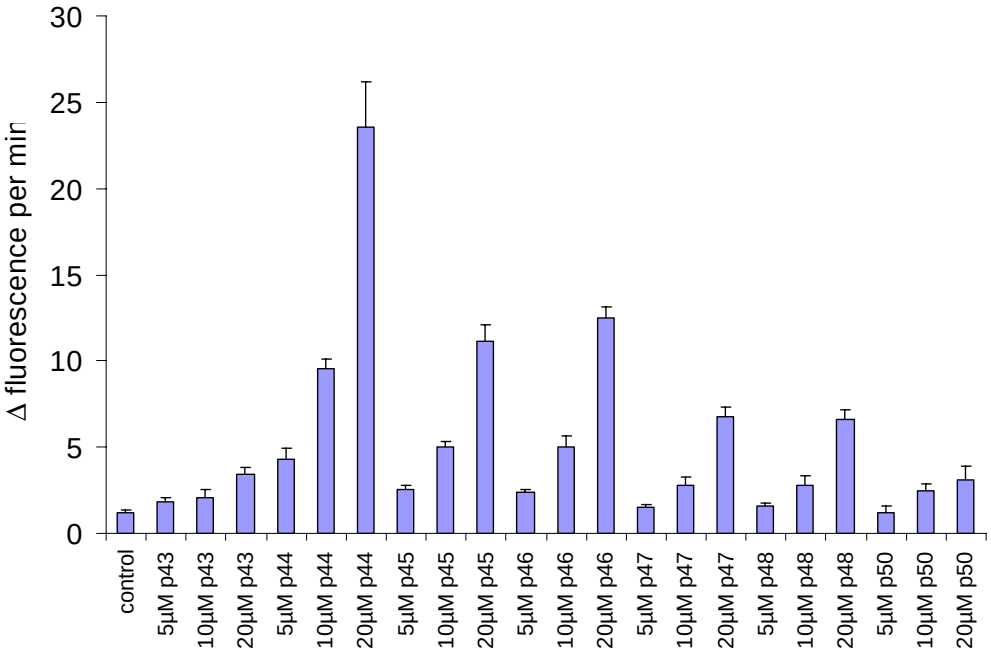

Fig.1 ROS formation expressed as change in DCF fluorescence per min in rat cerebellar granule cells exposed to increasing concentrations of different HO-PCB congeners. Values are mean + SEM of 5 independent experiments.

Cell death

Cerebellar granule cells were exposed to the HO-PCB congeners (5-50μM) for 18 hrs and cell death was assessed by measurements of LDH released to the medium (Fig 2). The congeners that were most potent in the ROS assay, p44 and p46, were also the most potent inducers of cell death. At 20 μM both p44 and p46 lead to release of high levels of LDH. At 50 μM all the compounds induced cell death, except for congener p51, which did not induce cell death or ROS formation. In comparison, Aroclor 1254, which is a commercial PCB mixture, has been shown to induce cell death at 12.5 μM under similar conditions (1). The same study (1) showed that Aroclor 1254-induced cell death was ameliorated by the NMDA receptor inhibitor MK 801, vitamin E, and the NO synthase inhibitor L-NAME, i.e. inhibitors of pathways leading to free radical formation.

These results demonstrate that HO-PCBs induce both DCF-fluorescence and cell death in rat cerebellar granule cells in culture. The mechanism of DCF-fluorescence and cell death remains to be elucidated, however, the results suggest that there is a correlation between ROS formation and cell death.

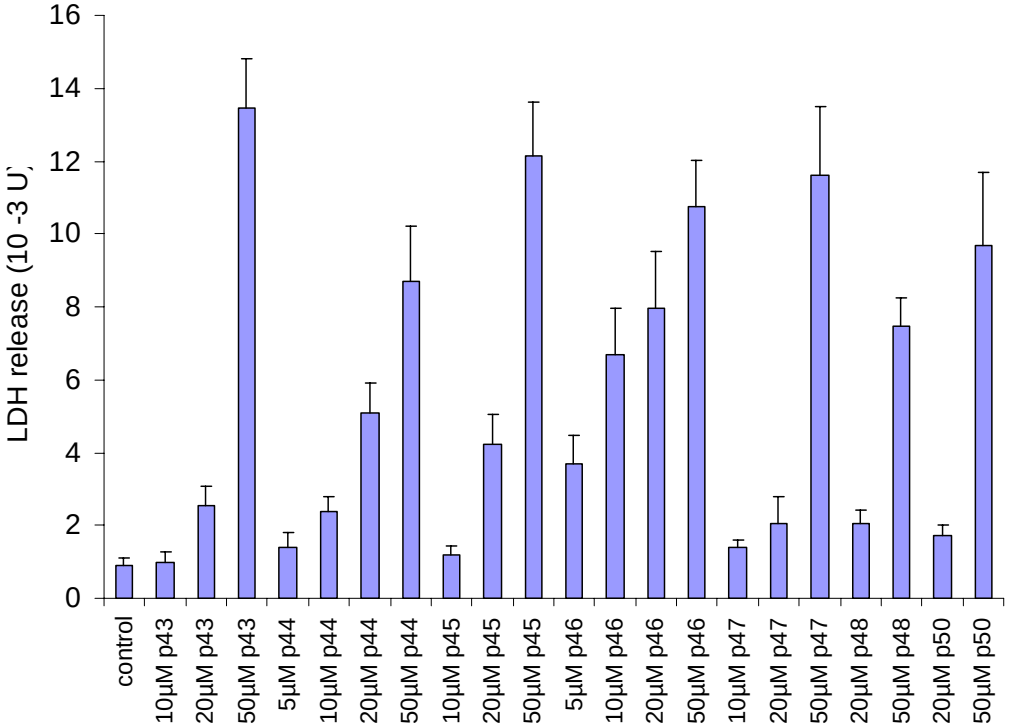

Fig. 2 Cell death expressed as LDH release in cerebellar granule cells exposed to increasing concentrations of different HO-PCB congeners. Values are mean + SEM of 5 independent experiments.

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