# THE EFFECT OF TETRABROMOBISPHENOL-A ON RAT CEREBELLAR GRANULE CELLS

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## Introduction

Brominated flame retardants (BFR) have attracted considerable concern since they are persistent and widely distributed in the environment[1]. In contrast to the extensive research on the neurotoxic effects of polychlorinated biphenyls (PCB), there is little knowledge about the toxicity of BFRs, especially as neurotoxicity is concerned. Tetrabromobisphenol-A (TBBP-A) is the mostly used BFR in the world today and annual demand is estimated to 120 000 ton, representing approximately half the annual demand of BFRs[1]. TBBP-A is primarily used as a chemical bound flame retardant (approximately 90%) and large amounts are not expected to reach the environment. However, TBBP-A is found in significant amount in both sediments and biota[1, 2] and have therefore a potential of harmful effects, also neurotoxic.

In a previous proceeding we showed that exposure to TBBP-A induced death of cultured cerebellar granule cells[4]. The effect of TBBP-A was partly attributed to formation of oxidative stress. In this study we have elucidated the mechanisms for the formation of reactive oxygen species in cerebellar granule cells exposed to TBBP-A.

## Methods and Materials

*Preparation of cerebellar granule cells*

Primary cultured neurons from rat cerebellum were isolated mainly as previously described[5]. The cerebella from 6- to 8-day-old pups were dissected under sterile conditions and the cells were grown for 6-8 days in basal Eagle's medium containing fetal calf serum, penicillin/streptomycin, KCl and glutamine before exposure.

*Assay for measuring reactive oxygen species*

Formation of ROS was elucidated by the use of the fluorescent probe DCFH-DA[6, 7]. DFCH-DA is permeable across cell membranes and inside the cell the acetate moieties are cleaved by cellular esterases. DCFH readily reacts with ROS such as peroxynitrite ($ONOO^-$) and lipid peroxides to the fluorescent DCF[8]. The cells, preincubated with DCFH-DA, where incubated with BFRs in Hepes buffered HBSS with glucose. The incubated cells were then transferred to 96 wells microtiter plate reader. Phorbolester (PMA) was used as a positive control.

*Measurement of intracellular free calcium $[Ca^{2+}]_i$ in cerebellar granule cells*

$[Ca^{2+}]_i$ was measured in a plate reader using the fluorescent $Ca^{2+}$-sensitive probe fura-2/AM. The cells were cultured in chambers on coverslips (Ring et al., 2003, to appear). The cells, preincubated with fura-2/AM, where incubated with BFRs in standard saline solution Excitations

were obtained from filters at 340 nm and 380 nm and emission was at 510 nm. $[Ca^{2+}]_i$ was estimated using the equation previously described[9].

*Western blotting:*
Cells were stimulated with TBBP-A in different concentrations and time spans. Total proteins from the cells were separated on an SDS-PAGE gel and proteins were electrophoretically transferred to nitrocellulose membranes. Phospho-Erk was detected by western blotting with an anti phospho-Erk. Detection was by enhanced chemiluminescence.

## Results and Discussion

As reported earlier TBBP-A is a potent inducer of ROS formation in rat cerebellar granule cells[4] (Fig. 1). Previously we showed that TBBP-A induced cell death was inhibited by the addition of vitamin E and the NMDA receptor antagonist MK-801. Neither of these inhibitors had any convincing effect on the TBBP-A induced ROS formation (data not shown). This may be due to the time difference in exposure time between the cell death measurements and the DCF assay.

The activation of ROS was not attributed to activation of the NADPH-oxidase as observed in human granulocytes and which exist in small amount in granular cells (data not shown). However, the ERK1/2 inhibitor UO126 reduced the ROS formation by approximately 60% indicating that the TBBP-A induced ROS formation is attributed to activation of ERK1/2 in the MAP kinase pathway (Fig. 2). This was confirmed by Western blot (Fig. 3). ERK1/2 activation may be induced by tyrosine kinase receptor activation and the tyrosine kinase inhibitor erbstantin almost completely inhibited the ROS formation (Fig. 2), indicating the importance of this pathway.

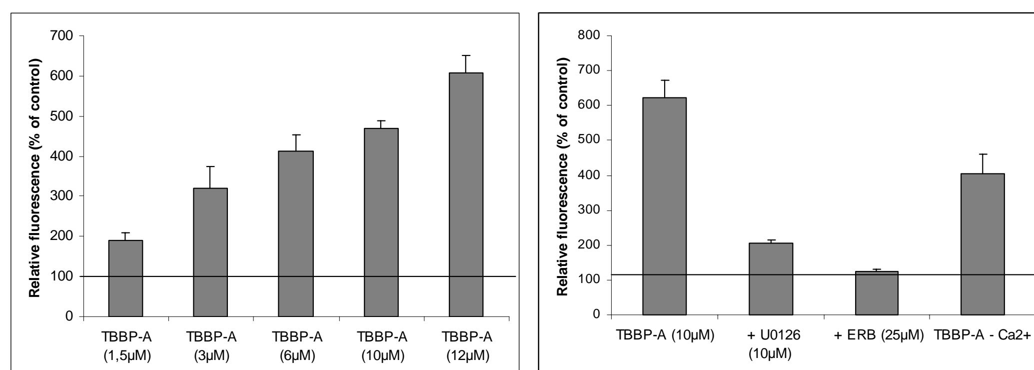


Fig. 1 and 2. Relative fluorescence as a measure for formation of ROS in cerebellar granule cells after exposure to increasing concentration of TBBP-A and in combination with the, MEK 1/2 inhibitor U0126 (10 µM), the tyrosine kinase inhibitor Erbstatin analog (25 µM) and in calcium free medium. All values are relative to the cell control (set to 100%). Values are mean ± SEM, five experiments in triplicate.

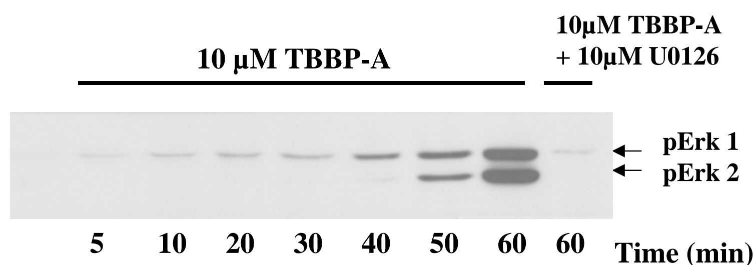


Fig. 3. Phosphorylation of Erk 1/2 kinases in cerebellar granule cells exposed to TBBP-A as a function of time.The TBBP-A exposed cells were also tested in combination with the Erk 1/2 inhibitor U0126.

Increased $[Ca^{2+}]_i$ may be involved in formation of reactive oxygen species[10]. Exposing granular cells to TBBP-A in a calcium free medium reduced the ROS formation, but less than observed by the use of ERB and UO126. However, measurement of $[Ca^{2+}]_i$ showed that in cerebellar granule cells TBBP-A also induced a concentration dependent increase in $[Ca^{2+}]_i$ (Fig. 4). $[Ca^{2+}]_i$ is an important mediator of the activation and regulation of enzymatic activity[11]. Many toxic agents cause abnormal activity of cell membrane ion pathways that directly or indirectly give rise to intracellular release of or influx of calcium into the cell. Monitoring the $[Ca^{2+}]_i$ is therefore an important parameter in the characterization of a toxic agent.

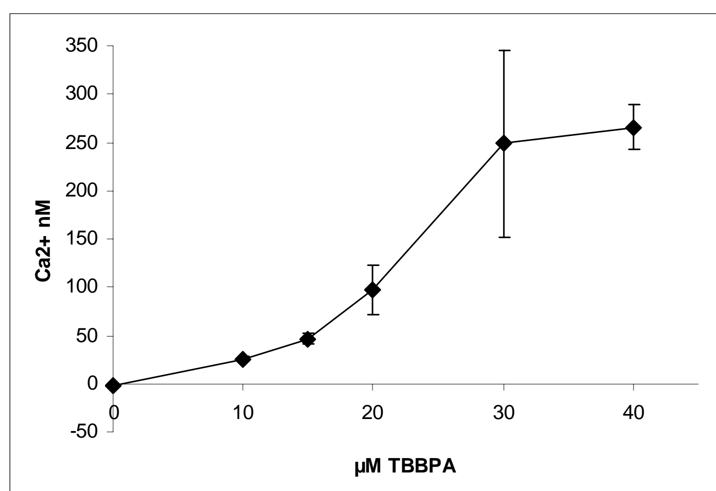


Fig. 4. The figure shows the rise in calcium after 15 min exposure to TPBBPA at the concentrations indicated. Error bars indicate standard deviations from 6 parallel samples albeit from the same batch of cells.

The present work shows that TBBP-A is a potent inducer of ROS formation. The effect is much more potent than previously found with PCBs[7]. The effect was attributed to an activation of tyrosine kinase receptors and induction of ERK1/2 in the MAP Kinase pathway. The consequences of this activation of cell preparations are not known. TBBP-A was also shown to increase $[Ca^{2+}]_i$. An abnormal rise in $[Ca^{2+}]_i$ can cause disruption of the cellular homeostasis, but it can also be a secondary consequence of abnormality in the cellular regulation[11].

TBBP-A is a so-called reactive BFR, but it is shown that residual unreacted TBBP-A may leak from TBBP-A treated products. The component is detected in both human plasma and mothers' milk and may therefore have adverse effects.

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