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THE EFFECT OF THE BROMINATED FLAME RETARDANT TETRABROMOBISPHENOL-A ON HUMAN GRANULOCYTES

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Introduction

The brominated flame retardants (BFRs) have attracted considerable concern the last two decades as a novel group of environmental contaminants. The BFRs primarily include the polybrominated diphenylethers (PBDEs) tetrabromobisphenol-A (TBBP-A), hexabromocyclododecane (HBCD) and the polybrominated biphenyls (PBB). Although it is claimed that most of the BFRs are non-toxic the observed increase in the environment is of major concern[1]. Previous investigation has shown that environmental contaminants such as the polychlorinated biphenyls (PCB) and methylmercury are immunotoxic[2-4].

Neutrophils, the predominant cell type among phagocytes, are of prime importance in many non-specific immune processes. In the course of their activity the phagocytes ingest invading microorganisms into phagocytic vacuoles and kill the prey by producing reactive oxidizing agents. This process is called “respiratory burst”, which also may cause harm to nearby tissue[5]. Respiratory burst may be induced in phagocytes by both calcium dependent- and independent pathways[6]. The activation of respiratory burst and inhibition of phagocytosis are suggested as biomarkers of immunotoxicity in wildlife species[7].

The aim of this study was to elucidate the effect of the major used BFRs on respiratory burst in human neutrophil granulocytes. BFRs are found in plasma samples and the general level in human blood seems to increase. This observation makes it important to study potential adverse effects on blood cells.

Materials and Methods

Chemicals
Pentabromodiphenylether (DE-71, Great Lakes), tetrabromobisphenol-A (BA-598, Great Lakes), hexabromocyclododecane (GD-788, Great Lakes) were purchased from Promochem (Sweden).

Isolation of human neutrophil granulocytes
Fresh blood samples were collected each morning from healthy male volunteers. The granulocytes were separated from EDTA blood by dextran sedimentation followed by a standard density-gradient centrifugation as previously described[8].

Assay for measuring reactive oxygen species
Formation of reactive oxygene species (ROS) was elucidated by the use of the fluorescent probe DCFH-DA[9]. 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[9]. 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.

Measurement of intracellular free calcium in granulocytes
Intracellular calcium was measured using the fluorescent Ca^{2+}-sensitive probe fura-2/AM by a method previously described[10]. The cells, preincubated with fura-2/AM, where incubated with BFRs in Hepes buffered HBSS with glucose.

Results and Discussion

TBBP-A made a potent concentration dependent increase in formation of ROS in granulocytes (Fig. 1). The flame retardants PBDE and HBCD had no effect (data not shown). The TBBP-A analog bisphenol-A also induced ROS formation, however the activation was much less indicating a structure activity relationship dependent on brom substitution.

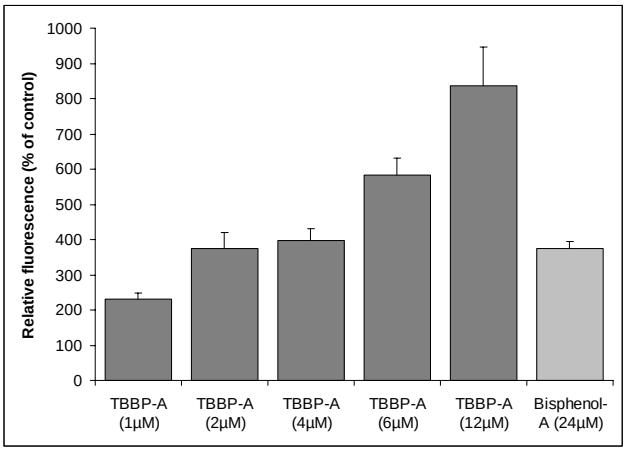

Fig. 1 Relative fluorescence as a measure for formation of ROS in human neutrophil granulocytes after exposure to increasing concentration of TBBP-A. All values are relative to the cell control (set to 100%). Values are mean ± SEM, five experiments in triplicate.

The ROS formation was attributed to an activation of the NADPH oxidase by the use of the NADPH-oxidase inhibitor DPI (Fig 2 and 3). The NADPH-oxidase was activated primarily by activation of the ERK1/2 and MEK in the MAP-kinase pathway as shown by the use of the ERK inhibitor UO126 (Fig 3). The TBBP-A induced activation of ERK1/2 was confirmed by Western blot (data not shown). The ERK/MEK-pathway is activated by a tyrosine kinase receptor. Incubation of the granulocytes with the tyrosine kinase inhibitor erbstatin analog (25 µM) reduced free radical production by TBBP-A exposed neutrophils (Fig. 3). These results strongly suggest

that TBBP-A induce activation of the NADPH-oxidase by the MAP-kinase pathway via stimulation of a tyrosine kinase receptor.

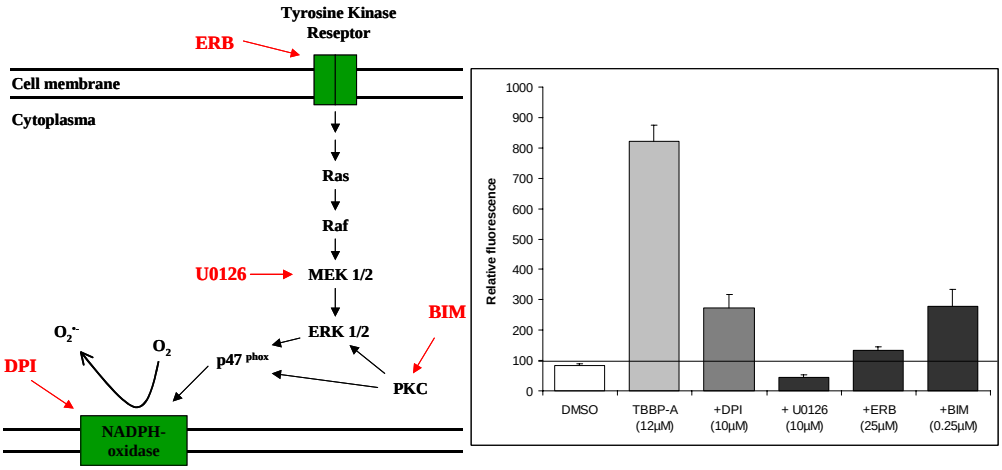

Fig. 2 and 3. Signaling pathways involved in ROS formation in humane neutrophil granulocytes stimulated with TBBP-A. Relative fluorescence as a measure for formation of ROS in cerebellar granule cells after exposure toTBBP-A in combination with the, the NADPH-oxidase inhibitor DPI (10µM), MEK 1/2 inhibitor U0126 (10 µM), the tyrosine kinase inhibitor erbstatin analog (ERB, 25 µM) and the proteine kinase C (PKC) inhibitor BIM (0.25µM). All values are relative to the cell control (set to 100%). Values are mean ± SEM, five experiments in triplicate.

Also the PKC inhibitor BIM reduced TBBP-A induced ROS formation (Fig. 3). PKC is an important activator of the NADPH-oxidase directly via $p47^{phox}$ or ERK1/2, and might be activated by calcium dependent mechanisms. The use of the calcium sensitive probe Fura-2/AM showed that TBBP-A also induced a concentration dependent increase in calcium influx into the granulocytes (Fig 4). The cytoplasmic calcium concentration is an important mediator of the activation and regulation of enzymatic activity. An abnormal rise in intracellular calcium can cause disruption of the cellular homeostasis but it can also be a secondary consequence of abnormality in the cellular regulation. The elevation of intracellular calcium may therefore have importance for the observed ROS formation in TBBP-A exposed granulocytes, e.g. activation of PKC.

Previously it has been shown that the environmental contaminant PCB both activates the NADPH-oxidase and induces calcium influx in human granulocytes[3, 11]. Since all these compounds are found in plasma samples we postulate that our findings may have importance for proper function of the granulocytes, which are of prime importance in many non-specific immune responses.

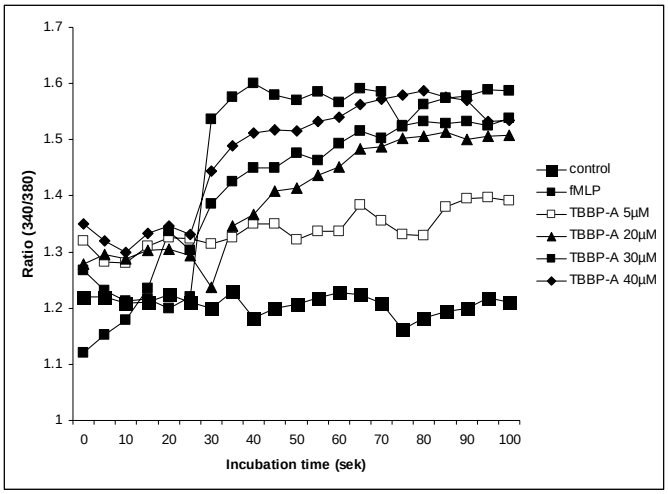

Fig. 4. The figure shows changes in intracelluar calcium in human neutrophile granulocytes after exposure to TBBP-A. Fura-2 AM was used and the fluorescence emission ratio (excitation 340 and 380 nm) increases markedly at administration of TBBP-A indicating significant changes in cytoplasmic calcium. The NADPH-oxidase activator fMLP was used as positive control.

Acknowledgement

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