BIOTRANFORMATION OF THE BROMINATED FLAME RETARDANT TETRABROMOBISPHENOL-A BY HUMAN NEUTROPHIL GRANULOCYTES

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Introduction

Tetrabromobisphenol A (TBBPA) is industrially the most important individual brominated flame retardant used with an annual demand of approximately 120 000 metric tons[1]. TBBPA is primarily used as a chemically bound flame retardant, which is supposed to limit its spread in the environment. However, several reports have shown that this compound is present in the environment, and that it may leak from treated material[2]. Significant amounts of TBBPA have also been found in human plasma samples, even in non-occupational exposed people[3]. Concerns have been raised about the possible interference of TBBPA with thyroid hormone function[4] and about its potential immunotoxic effects[5].

Little is known about the fate of TBBPA in animal models and in human. TBBPA was shown to have a relatively short elimination half-life[6]. In rodents, this BFR forms several conjugates which are readily excreted in bile[7]. In vitro models (human and rat liver sub-cellular fractions: microsomes, S9 fractions) confirm that conjugation (to glucuronic acid) is a major metabolic pathway for TBBPA. However, in vitro, P-450 dependent oxidations produce the largest part of TBBPA metabolites, leading, among other, to the oxidative cleavage of the molecule. Moreover, the results of these experiments indicate that reactive intermediates may be formed through oxidative metabolic pathways[8].

Neutrophils, the predominant cell type among phagocytes, are involved in non-specific immune processes. In response to a variety of agents, neutrophils release large quantities of superoxide anion ($O_2^{\bullet-}$) in a phenomenon known as respiratory burst. Previously it was reported that TBBPA potently induces respiratory burst in granulocytes[9]. The effect was attributed to activation of ERK followed by activation of the NADPH oxidase. Inappropriate activation of respiratory burst is associated with tissue injury and impairment of the ability to clear invading microorganisms[10].

The aim of this study was to elucidate the metabolic fate of TBBPA in human neutrophil granulocytes, in order to increase our knowledge about the potential of this BFR to induce adverse effects, and to investigate the possible connection with previous findings[5,8,9] using a cell model of prime importance in many non-specific immune processes.

Materials and Methods

Chemicals

[^{14}C]-TBBPA was synthesised from ring-[^{14}C]-BPA (Moraveck biochemicals, USA) and purified as described elsewhere[8]. Its structure was confirmed by MS and NMR. It was purified by HPLC to reach *ca.* 100% purity. Unlabelled TBBPA was obtained from Merck Eurolab (VWR international, France).

Isolation and exposure of human neutrophil granulocytes

Fresh blood samples were collected each morning from healthy male volunteers (n=3). The granulocytes were separated from EDTA blood by dextran sedimentation followed by a standard density-gradient centrifugation as previously described[9]. [^{14}C]-TBBPA, fortified when necessary with unlabelled TBBPA (concentrations: 1, 5, 10, 20, 50 µM) was mixed with Hepes buffered HBSS with glucose followed by the addition of granulocytes (final concentration 2x10^6/ml) and exposed at 37ºC for one hour. Additional incubations were carried out at 10

µM in the same conditions in the presence of 4 µM diphenylene-iodonium (DPI), which is a NADPH oxidase inhibitor. The samples were then centrifuged to separate the incubation media from the cells, and the supernatants were transferred to new vials. Control incubations were carried out in the same conditions, with no cells, and 10 µM [^{14}C]-TBBPA.

Radioactivity repartition and qualitative analysis of TBBPA metabolites.
The radioactivity present in supernatants was determined by direct counting using a Packard liquid scintillation counter. Cells were extracted with acetonitrile/water 50:50 v/v prior to radioactivity determination. Remaining pellets were solubilized with Packard Soluene-350 to determine the amount of non extractable radioactivity. All supernatants and cell extracts were analysed by HPLC using a Nucleodur C8 column (Macherey-Nagel, France) connected for radioactivity detection to a Radiomatic Flo-One/ß A500 instrument (Radiomatic, France). Analytical conditions were adapted from Zalko et al.[8]. Isolation of metabolites was carried out by combining HPLC separation and C18 Chromabond glass cartridge (Macherey Nagel) SPE.
Structural characterisations were carried out on a Finnigan LCQ decaXP quadrupole ion trap mass spectrometer (Thermo Electron, France) fitted with an electrospray ionisation source operated in the negative ionisation mode.

Results and Discussion
Radioactivity repartition in supernatants and in cells
Depending on the TBBPA concentration, the proportion of radioactivity recovered in supernatants varied between 15 and 30% of the radioactivity put in the incubations (Fig 1; left). Most of the radioactivity remained in the cells and decreased from 85 to 70%, depending on the TBBPA concentration put in incubations (Fig 1; right).
The proportions of metabolites in each compartment appeared to increase with TBBPA concentration, in the incubation media as well as in the cells. In the presence of diphenylene-iodonium (DPI), the metabolites production was inhibited in both compartments (Fig 1).

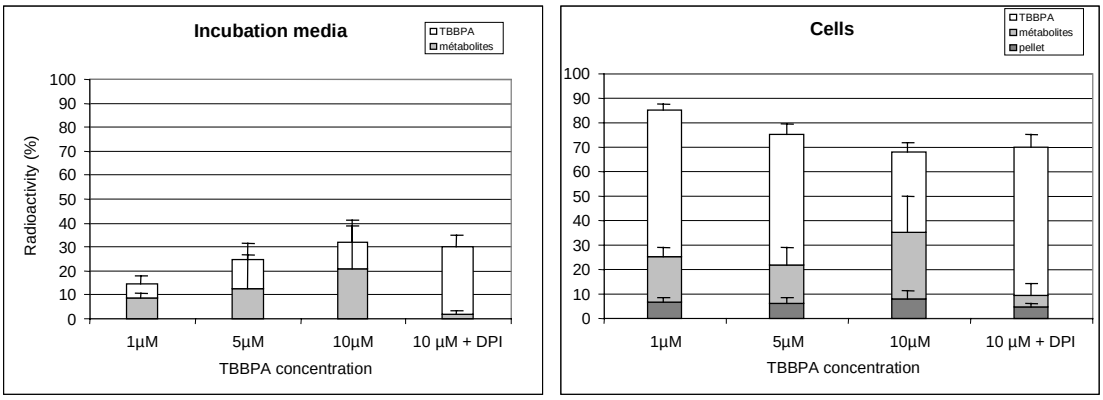


Fig 1. Radioactivity repartition (%) in the incubation media (left) and in the intracellular fraction (right)
Mean ±SD for granulocytes incubations from 3 human male volunteers

Radio-HPLC profiling
The radio-chromatographic profiling of [^{14}C]-TBBPA incubated with no cells showed that no degradation of TBBPA occurred under our incubation conditions, nor during storage before analysis. A single peak was observed in radio-HPLC, with a retention time of 18,2 min, corresponding to unchanged TBBPA.
At 10 µM, the radioactivity present in incubation media, *e.g.* in the extra-cellular compartment, accounted for one third of the radioactivity put in incubations. Whatever the TBBPA concentration, one major metabolite was detected. Its retention time was 11,3 min (Fig. 2; metabolite A). Another metabolite was eluted before TBBPA, but was formed in smaller quantity (Fig. 2; metabolite B). Most of the radioactivity put in incubation remained

in the cell pellet, and was extracted with acetonitrile/water. The corresponding analysis (Fig. 2, left) showed the presence of unchanged TBBPA, as well as metabolites exhibiting retention times of 37 and 38 min, respectively (Fig. 2; C).

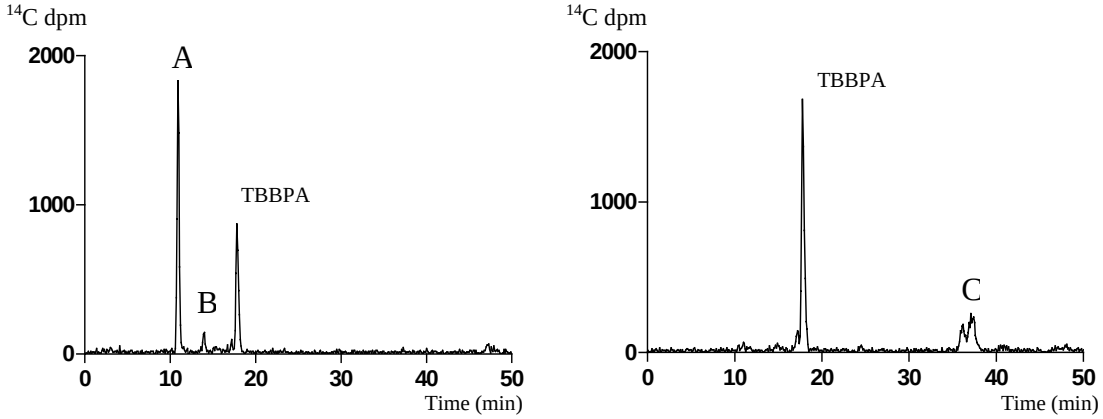


Fig 2. TBBPA biotransformation by human granulocytes [10 µM ^{14}C-TBBPA, 1 hr, 37°C]
Radio-HPLC analyses of the incubation media (left), and the intra-cellular fraction extract (right)

In incubations of [^{14}C]-TBBPA carried out with granulocytes in the presence of DPI, the biotransformation of TBBPA was dramatically reduced (Fig. 1). Only low amounts of metabolites A and C could be detected, indicating that DPI, a NADPH oxidase inhibitor, is an efficient inhibitor of TBBPA metabolites formation in this *in vitro* system.

Effect of TBBPA concentration on metabolite A production

The formation rate of metabolite A (retention time 11,3min) was determined for all incubations and expressed in nmoles of metabolised TBBPA per million of cells and per hour (Fig. 3). This metabolite was present in small amount in cells, but was the major metabolite detected in incubation media. In the incubation media, its production curve appeared to be an enzymatic curve with a saturation occurring around 10µM TBBPA.

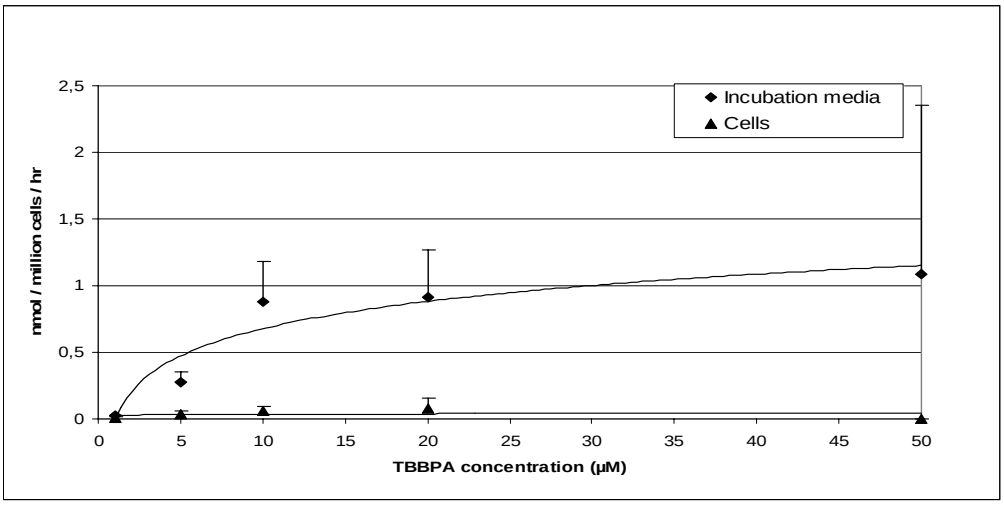
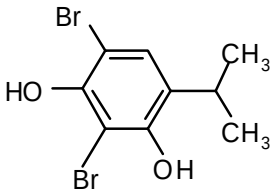


Fig 3. Effect of TBBPA concentration on metabolite A production in the incubation media and in the intracellular fractions, respectively, for incubations carried out with granulocytes from 3 human male volunteers (Mean ±SD).

Metabolites structure

Metabolite A was purified from supernatants and was analysed by ESI-MS. The $[M-H]^-$ quasi-molecular ion was detected at *m/z* 309, with an isotopic pattern consistent with that of a molecule bearing 2 bromine atoms. MS^2 analysis demonstrated the loss of a molecule of water. Based on this analysis, we conclude that metabolite A is hydroxylated 2,6-dibromo-4-isopropyl-phenol. The comparison of the results with previously isolated metabolites of TBBPA[8] strongly suggests that the hydroxylation site is located on the aromatic ring (Fig. 4). Metabolites B and C structures are under investigation. Based on the HPLC retention time, it is postulated that the group of metabolites C may correspond to hexa- and hepta-brominated compounds formed following a first-step oxidation of TBBPA.

Fig. 4. Metabolite A was identified as hydroxylated 2,6-dibromo-4-isopropyl-phenol by ESI-MS. 3 isomers could correspond to this structure. Based on its pattern of fragmentation and on a comparison with TBBPA metabolites standards, metabolite A should correspond to 2,6-dibromo-4-isopropyl-3-hydroxy-phenol



Discussion

Current data indicate that TBBPA bio-accumulation potential is low, compared to other BFR. Though the largest sell BFR currently marketed, and because it is not considered as a POP, TBBPA has raised fewer concerns regarding its possible effects on human health than PBDEs, for instance. However, human exposure to TBBPA has been demonstrated[3,11] in various countries, suggesting a continuous exposure to low doses of this compound. The immunotoxicity of TBBPA was previously questioned following the results of *in vitro* studies carried out with mice splenocytes[5]. Moreover, it was shown that TBBPA activates NADPH oxidase and enhances the formation of reactive oxygen species in human neutrophil granulocytes[9]. The major metabolite present in incubation supernatants was characterized as hydroxy-dibromo-isopropylphenol, a breakdown molecule also produced by P450 dependent pathways by rat and human sub-cellular fractions[8]. The release of superoxide anion, mediated by NADPH oxidase, appears to trigger TBBPA oxidation by human neutrophil granulocytes. Leukocytes are able to produce oxidised metabolites of several drugs[12], one of the main pathways being the involvement of the NADPH oxidase, through the production of superoxide anion, later converted to hydrogen peroxide and used by myeloperoxidase. The present study demonstrates that TBBPA is extensively biotransformed by human granulocytes. The oxidative breakdown of TBBPA is inhibited by DPI, a NADPH oxidase inhibitor, suggesting the ultimate involvement of myeloperoxidase in the cleavage of this BFR.

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