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EFFECTS OF BROMINATED FLAME RETARDANTS ON ACTIVITY OF THE STEROIDOGENIC ENZYME AROMATASE (CYP19) IN H295R HUMAN ADRENOCORTICAL CARCINOMA CELLS IN CULTURE

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INTRODUCTION

Flame retardants are chemicals that are added to materials that inhibit or suppress combustion processes. These compounds are incorporated into the manufacture of electronic equipment, furniture, construction materials and textiles. Flame retardant compounds can be divided into different chemical classes : inorganic, nitrogen, phosphorous and halogened flame retardants.

Halogenated organic flame retardants are generally classified as either chlorinated or brominated flame retardants (BFRs). Polybrominated diphenyl ethers (PBDEs) were the first group of BFRs to be detected in the environment. In 1979, the presence of BDE-209 (deca-BDE) was found in soil and sludge samples collected from areas surrounding PBDE manufacturing facilities in the US (de Carlo, 1979). In addition to studies showing increasing PBDE levels in the tissues of wildlife species such as Lake trout and herring gull from the Great Lakes (Norstrom et al., 2002), it has been recently reported that over the last decade or so that PBDE levels have been rapidly increasing in human breast milk from European and North American women (Betts et al., 2002).

During the last decade there has been increasing concern about certain chemicalspresent in the environment, including BFRs and BFR metabolites, and their potential as endocrine disruptors (EDs) in humans and wildlife (Darnerud et al., 2001). Epidemiological information indicates that there are increased incidences of a number of adverse health problems, such as developmental effects, hormone dependent cancers and allergies with an unknown etiology (Cordle, 1984). Based on animal experiments with these EDs there is a possibility of altered sex differentiation, sexual behaviour, and immune function. Based on the multiplicity of mechanisms of action and large number of suspected compounds it is clear that endocrine disruption is a complex area to study. Consequently, it might be difficult to establish links between exposure to a suspected EDs and any endocrine effect in wildlife or humans. From an environmental point of view an increasingly important group of organohalogen compounds are the BFRs including PBDEs, tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCDD), which are extensively used as flame retardants at high production volume levels.

In the present study we focus on environmentally relevant BFRs and their possible effects on sex hormone synthesis and metabolism. To study the potential interactions of these chemicals (BFRs) with steroidogenic enzymes we use the human H295R adrenocortical carcinoma cell line to focus on the potential inhibitive effects on aromatase (CYP 19) enzyme activity (Sanderson et al., 2000). Aromatase is a steroidogenic enzyme that mediates the the conversion of androgens to estrogens.

MATERIALS AND METHODS

Cell culture conditions

H295R cells were obtained from the American Type Culture Collection (ATCC #CRL-2128) and grown in culture under conditions published previously (Sanderson et al., 2000). Wells (24-well plate) were seeded with 1 ml of cell suspension per well. The culture medium was changed 24h after seeding, during which time the cells attached to the plate and reached almost confluency Then the cells were exposed to the test

chemicals, which were added to the wells at various concentrations using 1 µl of stock solutions dissolved in DMSO.

As positive controls for CYP19 induction, cells were exposed to 30 µM of 8-bromo-cyclic adenosine monophosphate (8-br-cAMP, Sigma). And as positive controls for CYP19 inhibition, cell were exposed of 30 µM of 4-hydroxyandrostenedione (4-HA).

Chemicals

In this study, the cells were exposed to a selection of five BFRs (TBBPA (tetrabromobisphenol-A), 2,4,6-tribromophenol, 6OH-2,2',4,4' tetraBDE47 (6-hydroxy- tetrabrominated diphenyl ether), HBCDD (hexabromocyclododecane-D), FR-720 (commercial product available of tetrabromobisphenol-A, bis(2,3-dibromopropylether)). The compounds (except for FR-720) were synthesized by Ake Bergman (Stockholm University, Sweden). The compounds were purchased at 2.5mM and then differents concentrations were used (from 0.75µM up to 7.5µM) to test the inhibition or induction of aromatase (CYP19) in H295R cells

Aromatase assay

The catalytic activity of aromatase was determined based on the tritiated water-release method of Lephart and Simpson (1991). The specificity of the aromatase assay based on the release of tritiated water was verified by measuring the production of estrone that is the aromatization product of androstenedione, using a 125I-labeled double-antibody radioimmunoassay kit (DSL-8700; ICN,Costa Mesa, CA), and by using 4-HA, an irreversible inhibitor of the catalytic activity of aromatase, to block the formation of tritiated water from 1β-3H-androstenedione (Sanderson et al., 2001).

MTT assay

Cell viability, as an indicator of cytotoxicity, was determined by measuring the capacity of H295R cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (Denizot and Lang, 1986). MTT is reduced to the blue-colored formazan by the mitochondrial enzyme succinate dehydrogenase, which is considered a sensitive measure of mitochondrial function. In short, the cells in each well on the 24-well plate were incubated for 30 min, at 37`C with 0.5 ml of MTT (1mg/ml) dissolved in culture medium without serum. Then, the MTT solution was removed, after which the cells were washed twice with PBS. The formazan formed in the cells was extracted by adding 1ml of isopropanol and incubation for 10 min at room temperature. The isopropanol fraction was measured spectrophotometricaly at 595 nm (FLUOstar Galaxy V4.30-0/ Stacker Control V1.02-0, BMG Labtechnologies).

Statistical analysis

All experiments were done in triplicate, per experiment each concentration was tested in quadruplicate. All results are presented as means with their standard deviations.

RESULTS AND DISSCUSION

Most of the compounds did not show any inhibition or induction of aromatase except with one major metabolite of BDE47. This metabolite, 6-hydroxy- tetrabrominated diphenyl ether (6OH-2,2',4,4'-tetraBDE47 (6-HO-BDE47)), is formed from the parent compound tetrabrominated diphenyl ether (2,2',4,4'-tetraBDE47) and has been found in human blood (Sjodin A et al.,2001) at median concentration (and range) of 1.3 (<0.8-49) pmol/g lipid weight.

After 24 hr exposure to 6OH-BDE the activity of aromatase in the H295R cells was concentration dependently decreased by almost 95 % at 7.5 µM and an IC_{50} value of 3.6µM , see figure 1.

Simultaneous analysis of MTT showed 40% cytotoxicity at the highest concentration (7.5 µM) indicating that the inhibition of aromatase may be partly due to the toxicity of the compound. Further experiments are needed to verify whether the inhibition of aromatase by 6-OH-BDE-47 is due to catalytic inhibition or cytotoxicity. The biological or toxicological environmentally relevance of this effect is presently unknown, but the common occurrence of BDE47 in human blood/milk (Meironyte et al.,1999) certainly warrants further examination. At present several other relevant hydroxylated metabolites of PBDEs are been studied for their inhibitory properties toward aromatase activity in H295R cells.

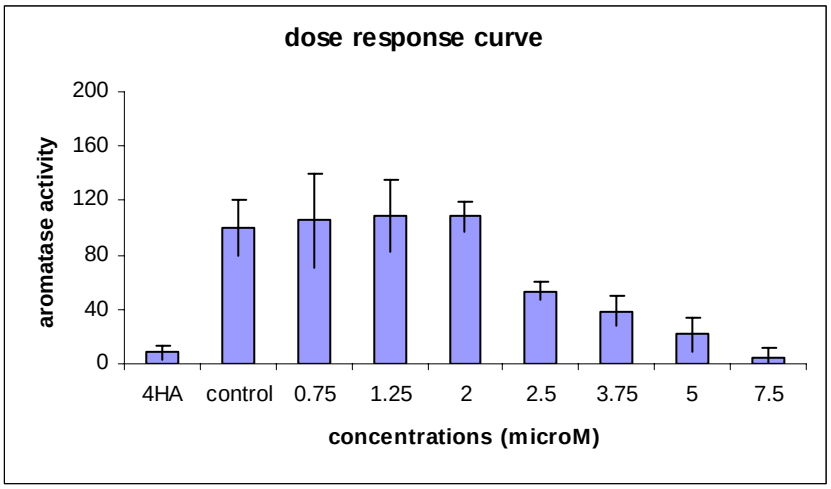

Figure 1. Differents concentrations of 6-OH-BDE47 caused inhibition of the aromatase partly due to cytotoxicity of the compound (data not showed).

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