INHIBITION OF VASOPRESSIN RELEASE IN THE RAT SUPRAOPTIC NUCLEUS BY EXPOSURE TO THE PBDE MIXTURE (DE-71) IN VITRO

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Introduction

Persistent organic pollutants (POPs) are long-lived toxic organic compounds and are of major concern for human and ecosystem health[1,2]. Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are examples of such chemicals. PBDEs and PCBs belong to a group of pollutants called polyhalogenated aromatic hydrocarbons and therefore, are structurally related. Owing to their persistence, the manufacture of PCBs was discontinued in 1977, however, PCBs are still found in significant quantities in the environment. PBDEs are currently being manufactured and used in large quantities as flame-retardant additives in polymers, especially in the manufacture of a variety of electrical appliances, building materials, foams and upholstery furnishings[3]. PBDEs in these materials are not chemically bound to plastic and foam, and therefore, easily leach out when items containing PBDEs decompose in landfills or are incompletely incinerated. Over time, these compounds become ubiquitous contaminants in the environment because of their high production, lipophilic characteristics, and persistence[4]. PBDEs are now found in air, water, fish, birds, marine mammals, and humans, and in many cases their concentrations are increasing over time[5]. In addition, PBDE levels found in several biological and environmental samples from the United States were several-fold higher than the levels found in European countries[4,5].

Our recent work showed that at environmentally relevant dosages, the commercial PCB mixture (Aroclor 1254) administered *in vivo* suppresses dehydration-induced dendritic release of vasopressin (VP) and exaggerates systemic release into the circulation[6]. In addition, when Aroclor 1254 (20 μM) was administered directly to tissue punches from supraoptic nucleus (SON) of dehydrated rats *in vitro*, dendritic VP release was abolished, suggesting that direct actions on magnocellular neuroendocrine cells (MNCs) are sufficient to inhibit VP release without the need for effects on other osmosensitive regions[6]. The objectives of the present study were to determine if *in vitro* exposure to PBDEs, (which are structurally similar to PCBs) induce comparable inhibition of dendritic release of VP.

Methods and Materials

Animals: Long-Evans hooded and Sprague Dawley male rats (300-400g) were obtained from Charles River Laboratory (Raleigh, NC) and housed in pairs in AAALAC approved animal facilities. Food and water were provided *ad libitum*. Temperature was maintained at 21± 2^0C and relative humidity at 50 ± 10% with a 12-h light/dark cycle. All experiments were approved by the institutional animal care and use committee of the National Health and Environmental Research Laboratory at U.S. EPA in compliance with NIH guidelines.

SON Punch Preparation: SON tissue punches were prepared as described[6]. Briefly, after decapitation, brains were quickly removed, and placed in cold oxygenated artificial cerebrospinal fluid (aCSF). The SON was dissected bilaterally from 1 mm coronal sections placed on an ice-cold slide. Each sample was transferred to an individual static well containing aCSF (pH 7.4, 37°C). Each well contained the bilateral SON from one rat brain in a total volume of 500 μl of incubation solution (analysate). Acutely-dissected samples were maintained in the wells with continuous oxygenation (95/5% O_2/CO_2). Control samples were incubated in normal aCSF (290 mOsm/L). SON punches were osmotically stimulated by incubation in hyperosmotic aCSF (350 mOsm/L).

In Vitro Toxicant Exposure: Tissue punches were exposed to either toxicant or DMSO vehicle for a 30-minute period at 37°C in 290 mOsm aCSF. Following the toxicant exposure, 290 mOsm aCSF was replaced with 500 µl of fresh 350 mOsm aCSF and incubated for an additional 10-min experimental period, after which aliquots of analysates were removed and frozen for subsequent VP and nitric oxide (NO) analysis. With the exception of the normosmotic control group, all tissues were incubated in 350 mOsm aCSF for the 10-minute experimental period to stimulate NO and VP release. Finally, SON punches were collected in cold buffer containing protease inhibitor cocktail, homogenized, and frozen for later protein determination using the bicinchoninic acid method (Pierce).
Quantification of VP: VP content in perfusate samples was measured using enzyme-immunoassay (arg8-vasopressin correlate EIA kit, Assay Designs) with a sensitivity of 3.39 pg/ml. VP values for each sample were normalized to the total protein present in each SON sample to control for variations in the size of the SON punches of origin and expressed as pg/ml/µg protein.
Quantification of NO: Aliquots of the analysate of each SON tissue punch were assayed for NO by the Griess Method (Oxford Biomedical, enzymatic kit) for detection of the oxygenation product of NO, nitrite. The reaction detects as little as 0.5 µM NO (using an 85 µl sample). The final product of the Griess reaction was then read at 540 nm on a Molecular Devices Spectra Max 190 plate reader. For the assay, 85 µl of incubation solution was analyzed to determine the concentration of NO (µM). The total NO produced by each punch (pmols) in the original 500 µl incubation volume was then normalized by dividing by total protein in the punch. Final NO values were expressed as pmol/µg protein.
Statistical Analysis: Data collected for this study were analyzed for main effects of toxicant or hydration state by separate one-way ANOVAs using Sigma Stat software. General linear, or repeated measures ANOVA was used where data met normal distribution/equal variance assumptions. Where overall significance of (p<0.05) was obtained, post hoc multiple comparisons were used to detect specific differences.

Results and Discussion

The degree to which PBDEs constitute a human health hazard is not yet clear and the potential ability of these substances to compromise neuroendocrine systems has not been fully investigated. To begin to address the potential deleterious effects of PBDEs on neuroendocrine activity, SON punches were removed from adult male rats and their VP release was measured. In agreement with previous studies[6,7], Figure 1 shows a significant compensatory increase in VP secreted within the SON of stimulated tissue relative to normosmotic controls (30.8±3.45 n=14 vs. 20.92±2.99 n=16 pg/ml/µg; p<0.05). Interestingly, the osmotically-induced rise in SON VP is significantly (p<0.001) reduced by exposure to either Aroclor 1254 or DE-71. As shown in Figure 1 panel A, exposure to 8.8 µg/0.5 ml and 5.0 µg/0.5ml of DE-71 and Aroclor 1254, respectively, inhibited osmotically-induced VP release by 64 and 60%, respectively. VP values for hyperosmotic, hyperosmotic plus DE-71 and hyperosmotic plus Aroclor 1254 groups are 30.8 ± 3.45, (n=14),11.22 ± 2.17 (n=10) and 12.31 ± 2.99 (n=10), respectively. Preliminary results in panel B show the effect of lower concentration of these mixtures (2.9 µg/ml and 1.65 µg/ml) of DE-71 and Aroclor 1254, respectively on VP release. Early data indicate that at these lower concentrations, VP release may be attenuated, but the values fail to meet statistical significance due to the limited numbers of animals used in the experiment.

The i*n vitro* effects of PBDEs on SON vasopressin release is noteworthy because this punch preparation is functionally isolated from possible actions of toxicants on osmoreceptors such as the subfornical organ or organum vasculosum of the lamina terminalis, which act pre-synaptically upon MNCs to influence plasma VP levels in the intact animal[8]. These data suggest that the toxicants within the mixtures are acting directly upon the MNCs.

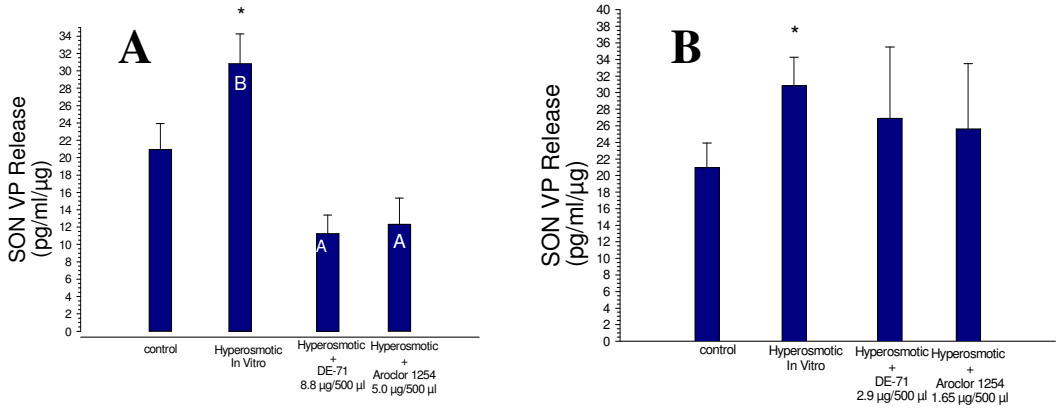

Figure 1. Panel A shows that *in vitro* application of Aroclor 1254 (5 µg/500 µl) and DE-71 (8.8 µg/500 µl) significantly reduces vasopressin release from osmotically stimulated SON punches (Kruskal-Wallis ANOVA on ranks, $p<0.001$), H = 16.3, df=2). The number of samples comprising each group are (from left to right): 16, 14, 10 and 10. The asterisk indicates a significant increase in VP in response to dehydration relative to corresponding normosmotic value (Student-Newman Keuls $p<0.05$). Bars with different letters are significantly different. Figure 1B reflects preliminary data showing that application of low doses of Aroclor 1254 (1.65 µg/500 µl) and DE-71 (2.9 µg/500 µl) may attenuate vasopressin release but the downward trend does not meet statistical significance. The number of samples comprising each group are (from left to right): 16, 14, 5 and 5.

To determine if toxicant effects on VP release are strain specific, pilot work has been conducted on another common strain of laboratory rat, the hooded Long-Evans rat. In Long-Evans rats, osmotically-induced VP release was inhibited by 62% and 52% ($p<0.05$). VP values for hyperosmotic, hyperosmotic plus DE-71 and hyperosmotic

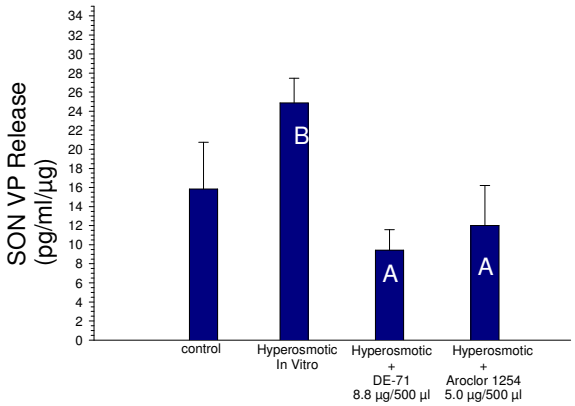

Figure 2 Panel A shows that *in vitro* application of Aroclor 1254 (5 µg/500 µl) and DE-71 (8.8 µg/500 µl) significantly reduces vasopressin release from osmotically stimulated SON punches obtained from Long-Evans (Student-Newman Keuls $p<0.05$). The number of samples comprising each group are (from left to right): 6, 4, 5 and 6.

plus Aroclor 1254 groups are 24.88 ± 2.57 (n=4), 9.4 ± 2.18 (n=5), and 12.0 ± 4.2 (n=6), respectively, after exposure to 8.8 µg/0.5ml DE-71 and 5.0 µg/0.5ml Aroclor 1254, respectively (Figure 2). Inhibition of VP release by these toxicants was similar in the two rat strains tested indicating that the effects were not strain-specific. The concentrations of toxicants that caused significant effects on SON VP release were very low and biologically

relevant. At similar concentrations, we have demonstrated effects of these toxicants on intracellular signaling mechanisms including protein kinase C translocation and calcium homeostasis[9].

Sharma and Kodavanti[10] have shown that cytosolic NOS in hypothalamic homogenates is highly sensitive to ortho-substituted PCB congeners. Therefore we further investigated the potential role of toxicant effects on nitric oxide synthase (NOS) activity in osmotically stimulated SON punches. It is known that NO is vital to normal VP output into the circulation and has an important function in the regulation of body fluid homeostasis[11]. In addition, our previous results indicated the NO-dependence of stimulated dendritic VP release[12]. In the brain, the levels of NOS are among the highest in MNCs of the supraoptic and paraventricular nuclei and NOS activity is upregulated by dehydration in rat SON[13]. In a set of experiments designed to test the effect of Aroclor 1254 and DE-71 on nitric oxide release from SON punches, it was observed that NO production increased markedly in the analysate of osmotically stimulated tissue (11.08 ± 2.1 vs 40.1± 4.14, n=13; $p<0.001$). Further, NO production is apparently dampened after exposure to Aroclor 1254 and less markedly after exposure to DE-71 (data not shown). Therefore, it is plausible that Aroclor 1254 may disrupt central VP release by altering NOS activity and NO signaling within the SON. Since central VP release may regulate output of VP hormone into the systemic circulation[14], our findings implicate PCBs and PBDEs as potential disruptors of endocrine function, osmoregulation and blood pressure control. Furthermore, although the osmoregulatory and cardiovascular role of VP is fairly well understood, the function of the central vasopressenergic system extends beyond the SON to cortical regions where the peptide acts as a neurotransmitter/neuromodulator that can influence learning and memory, social behavior, circadian rythmicity and, neurotrophism[15]. Therefore, because central vasopressin release is responsible not only for endocrine regulation of body fluid and cardiovascular function but also plays a role in cognitive functions such as learning and memory, our findings should apply broadly to other brain regions and higher brain function.

Acknowledgments

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