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Altered expression of genes involved in programmed cell death in primary cultured rat cerebellar granule cells acutely challenged with tetrabromobisphenol A

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ABSTRACT

In the present study, primary cultures of rat cerebellar granule cells (CGC) and the RT^2 Profiler PCR array were used to examine the effect of acutely applied brominated flame retardant tetrabromobisphenol A (TBBPA) on the expression of 84 genes related to the main modes of programmed cell death. CGC, at the 7th day of culture, were exposed to 10 or 25 μM TBBPA for 30 min. Then, 3, 6, and 24 h later, the viability of the cells was examined by the staining with propidium iodide (PI) or using the calcein/ethidium homodimer (CA/ET) live/dead kit, and RNA was extracted for the evaluation of gene expression by RT-PCR. At 3, 6 and 24 h after the treatment, the number of viable neurons decreased, according to the PI staining method, to 75%, 58% and 41%, respectively, and with the CA/ET method to 65%, 58% and 28%, respectively. In CGC analyzed 3 h after the treatment with 25 μM TBBPA or 6 h after 10 μM TBBPA, the only change in the gene expression was a reduction in the expression of *Tnf*, which is associated with autophagy and may activate some pro-apoptotic proteins. Six hours after 25 μM TBBPA, only 2 genes were over-expressed, a pro-apoptotic *Tnfrsf10b* and *Irgm,* which is related to autophagy, and the genes that were suppressed included the anti-apoptotic gene *Xiap*, the necrosis-related *Commd4*, pro-apoptotic *Abl1*, 5 genes involved in autophagy (*App, Atg3, Mapk8, Pten,* and *Snca*) and 2 genes that participate in two metabolic pathways: *Atp6v1g2* (pro-apoptotic and necrosis) and *Tnf* (pro-apoptotic, autophagy). Autophagy-related *Snca* and *Tnf* remained under-expressed 24 h after treatment with 25 μM TBBPA, which was accompanied by the over-expression of the pro-apoptotic *Casp6*, the anti-apoptotic *Birc3,* 2 genes related to autophagy (*Htt* and *Irgm*) and 2 genes (*Fas* and *Tp53*) that are involved in both apoptosis (pro-apoptotic) and autophagy. These results show a complex pattern of TBBPA-evoked changes in the expression of the genes involved in the programmed neuronal death, indicating no induction of programmed necrosis, an early suppression of the autophagy and anti-apoptotic genes, followed by a delayed activation of genes associated with apoptosis.

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1. Introduction

Tetrabromobisphenol A (TBBPA) which belongs to brominated flame retardants is produced in mass quantities and used as additive to the industrial products of daily use in order to reduce their flammability (Alaee and Wenning, 2002; de Wit, 2002). Although TBBPA has been considered a weak environmental toxin because of the short half-life and absence of biomagnification (Darnerud, 2003), this substance has been detected in samples of fresh waters, seafood and also of human body fluids (Qu et al., 2011; Birnbaum and Staskal, 2004; Thomsen et al., 2002; Abdallah and Harrad, 2011). High bioconcentration factor of TBBPA caused by lipophilicity may enhance its accumulation during chronic exposure of humans to this substance at relatively low concentrations (Hendriks et al., 2012). There are reports describing selective accumulation of TBBPA in the striatum of mice acutely exposed to this substance with accompanying behavioral disturbances (Nakajima et al., 2009). Neurodevelopmental effects of TBBPA observed in some studies (Lilienthal et al., 2008) have not been confirmed by others (Williams and DeSesso, 2010). Nevertheless, the potential neurotoxicity of TBBPA is disturbing because its degradation products are also toxic and there are indications pointing to enhanced TBBPA toxicity in combination with other neurotoxins (Reistad et al., 2007; Ziemińska et al., 2012).

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TBBPA shows *in vitro* neurotoxic effects of a complex mechanism (Hendriks et al., 2012; Reistad et al., 2007; Zieminska et al., 2017). Using fluorescent probes, TBBPA is shown to disturb calcium homeostasis and induce oxidative stress in the cell lines and primary cultures of neurons (Ogunbayo et al., 2008; Ogunbayo and Michelangeli, 2007; Reistad et al., 2007). In the primary cultures of rat cerebellar granule cells (CGC) challenged with TBBPA, increases in the intracellular calcium concentration ($[Ca^{2+}]_i$), which are mediated by NMDA (N-methyl-D-aspartic acid) glutamatergic receptors and dysfunctional ryanodine receptors (Zieminska et al., 2014, 2015, 2017), appear to be the primary events inducing oxidative stress, mitochondrial dysfunction and cell death. Moreover, recent data indicate that the direct induction of the production of reactive oxygen species (ROS) by TBBPA in the cells is also possible (Szychowski et al., 2016; Zieminska et al., 2017 Zieminska et al., 2017)

The molecular pathways mediating TBBPA-induced neuronal cell death are not entirely clear. The role of necrosis in TBBPA-induced cytotoxicity seems obvious. The development of focal necrosis in the liver of rats chronically exposed to high doses of TBBPA was detected, and this effect was accompanied by the induction of apoptosis-related genes (Chen et al., 2016; Tada et al., 2006, 2007). The involvement of necrosis in TBBPA-induced neurotoxicity was assumed based on the release of lactate dehydrogenase to the culture media of primary neocortical and hippocampal neurons incubated for 6 h in the presence of TBBPA at a low (50 μM) concentration (Szychowski and Wójtowicz, 2016; Wojtowicz et al., 2014). The role of apoptosis in TBBPA-induced cytotoxicity was repeatedly demonstrated *in vitro* in mouse TM4 Sertoli cells, SH-SY5Y neuroblastoma cells and in primary mouse cortical and hippocampal neurons (Al-Mousa and Michelangeli, 2012; Ogunbayo et al., 2008; Szychowski and Wójtowicz, 2016). However, it was initially suggested that TBBPA in the CGC cultures induces caspase-independent cell death (Reistad et al., 2007). It is unclear whether these early findings can be attributed to a programmed necrosis called necroptosis, which is a caspase-independent cell death pathway (Degterev et al., 2005; Duprez et al., 2009). Data show the induction by TBBPA of the signaling mechanisms that can lead to necrosis in macrophages and in mice and human lung cells (Han et al., 2009; Koike et al., 2016; Watanabe et al., 2010). Thus, although recent data demonstrated the role of caspases in the TBBPA-induced apoptotic death of the neuronal cell models (Al-Mousa and Michelangeli, 2012; Ogunbayo et al., 2008; Szychowski and Wójtowicz, 2016), it seems reasonable to consider the possibility that, in addition to apoptosis and sporadic uncontrolled necrosis, programmed necrosis may also participate in the mechanism of TBBPA neurotoxicity. In contrast to apoptosis, to our knowledge, there is no information in the literature on the impact of TBBPA on the process of autophagy in neurons. Additionally, the temporal relation between the up- or down-regulation of the genes related to major cell death pathways, i.e., programmed necrosis, apoptosis and autophagy (Duprez et al., 2009; Fink and Cookson, 2005; Nikoletopoulou et al., 2013), in neurons challenged with TBBPA is unclear.

The modes of neurotoxicity can be conveniently inferred from the differential expression of genes involved in a particular type of cell death. The primary culture of rat CGC is a model of glutamatergic neurons widely used in studies concerning excitotoxicity and apoptosis (Gallo et al., 1987; Contestabile, 2002). Therefore it has been useful in the previous TBBPA toxicity studies (Reistad et al., 2007; Zieminska et al., 2014, 2015, 2017). In the present study, continuing the previous research on TBBPA cytotoxicity we used primary cultures of rat CGC acutely challenged with TBBPA as a model. The RT^2 Profiler PCR array was utilized to examine the differential expression of genes related to apoptosis, programmed necrosis and autophagy at 3, 6 and 24 h after this treatment.

2. Materials and methods

2.1. Materials

TBBPA (99.8% purity) was synthesized and delivered commercially by the Institute of Industrial Organic Chemistry, Analytical Department, in Warsaw, Poland. Dimethyl sulfoxide (DMSO), fetal calf serum and other materials for cell culture were purchased from Sigma Chemical Poland (Poznan, Poland). All other chemicals were of analytical grade.

2.2. Animals

Seven-day-old Wistar rats of the outbred stock CmD:(WI)WU were used to isolate cerebellar granule cells for culturing. The rats were bred in the animal house of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw. The animals were maintained at room temperature with a constant humidity of approximately 60% on a 12:12 h dark-light cycle. The dams were fed and given access to water *ad libitum*.

2.3. Primary cultures of cerebellar granule cells

A standard method of CGC isolation and culturing was used exactly as previously described (Zieminska et al., 2014, 2015, 2017). The rat pups were decapitated, the cerebella were collected and chapped into 400 μm cubes. The method consisted of tripsinization and trituration of the cerebellar slices, followed by suspending the cells in the growth medium containing the basal Eagle medium supplemented with 10% fetal calf serum, 25 mM KCl, 4 mM glutamine and antibiotics. The cell suspensions were sieved on poly-L-lysine coated plates at a cell density of 2×10^6 per well on 12-well plates for the viability tests or at a density of 3×10^7 cells per cell culture flask for examination of changes in gene expression. To reduce contamination with non-neuronal cells, 7.5 μM cytosine arabinofuranoside was applied. The experiments were performed after 7 days of *in vitro* culture. CGC at 7th day in vitro achieve expression of active NMDA receptors (Marini et al., 1999), which are involved in TBBPA neurotoxicity (Zieminska et al., 2015).

2.4. Modeling and evaluation of the acute TBBPA cytotoxicity

Our present study investigated the effects of acute 30 min exposure of primary CGC cultures on 25 μM TBBPA. Previously published data have shown that under these conditions, 10 and 25 μM TBBPA induce concentration-dependently highly expressed neuronal death that develops over the next 24 h (Zieminska et al., 2015, 2017). However, only 25 μM concentration of TBBPA was selected for present experiments because the RT^2 Profiler PCR array showed that 10 μM TBBPA induces only negligible changes in gene expression (Figs. 1–3).

After replacing the growth medium with Locke 25 buffer containing 134 mM NaCl, 25 mM KCl, 2.3 mM $CaCl_2$, 4 mM $NaHCO_3$, 5 mM HEPES (pH 7.4), 5 mM glucose, and freshly prepared 25 μM solutions of TBBPA in 0.5% DMSO or vehicle, the CGC were incubated for 30 min at 37 °C. Then, after two gentle washes with Locke 25 buffer, the original growth medium was restored and the CGC were cultured for additional periods of 1.5, 3, 6, 12 and 24 h to evaluate TBBPA cytotoxicity, and of 3, 6 and 24 h for the RT^2 Profiler PCR array, in order to relate changes in gene expression to development of toxic changes in CGC.

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