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The effects of benzophenone-3 on apoptosis and the expression of sex hormone receptors in the frontal cortex and hippocampus of rats

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ABSTRACT

Benzophenone-3 (BP-3) is the most commonly used chemical UV filter. This compound can easily be absorbed through the skin and the gastrointestinal tract and can disturb sex hormone receptor function. BP-3 is lipophilic and should cross the blood-brain barrier and it may reduce the survival of neurons, although so far, its effects on nerve cells have been studied in only *in vitro* cultures.

The aim of the present study was to determine the effects of BP-3 on apoptosis and the expression of oestrogen, androgen and arylhydrocarbon receptors (AhR) in the rat frontal cortex and hippocampus. This compound was administered dermally to female rats during pregnancy and next to their male offspring through 6 and 7 weeks of age.

BP-3 in the frontal cortex induced the mitochondrial apoptosis pathway by increasing the active forms of caspase-3 and caspase-9, inducing the pro-apoptotic proteins Bax and Bak and increasing the number of cells with apoptotic DNA fragmentation. In the hippocampus, an increase in the caspase-9 level and a downward trend in the level of anti-apoptotic proteins were observed. In both brain regions, the contents of ERβ in the nuclear fraction and GPR30 in the membrane fraction were significantly reduced. BP-3 significantly increased AhR in the cytosol of the frontal cortex but had no effect on the content of this receptor in the hippocampus.

This is the first study showing that exposure to BP-3 induces the mitochondrial apoptosis pathway in the rat frontal cortex and this effect may result from a weakening of the neuroprotective effects of oestrogen and/or an intensification of AhR-mediated apoptosis.

1. Introduction

Benzophenone UV filters are commonly usedin various cosmetic products to minimize the harmful effects of UV radiation and as stabilizers in plastic-based packing materials. Among the chemical UV filters, BP-3 is the most widely used compound due to its broad range of applications. High concentrations of BP-3 have been detected in the waters of lakes and rivers as well as in wastewater and tissues of aquatic organisms, especially in fish fat (Fent et al., 2010). It is now known that this compound is not only the cause of irritation and allergic contact dermatitis but that it is also easily absorbed through the skin and the gastrointestinal tract and can disrupt numerous bodily functions. BP-3 may be present in cosmetic preparations in a maximum of 10%. For example, it has been demonstrated that after dermal application of a formulation containing 5% BP-3, the maximum concentration of these compound in human plasma was 200–300 μg/l, and after 24 h about 80–200 μg/l (Janjua et al., 2004; Tarazona et al., 2013). Similarly, Janjua et al. (2008) found that after a single application of the BP-3, its concentration in the serum in men was about 250 ng/ml (Janjua et al., 2008). In the current model, the concentration of BP-3 in the blood of rats was about 200 ng/ml. The main and thus far most examined action of the benzophenones, compounds belonging to the endocrine-disrupting chemical (EDC) family, is related to their effect on the sex hormone receptors, mainly the oestrogen receptors (ERs). BP-3 was shown to exert an agonist effect on human ERα and ERβ and an antagonistic effect on the androgen receptor (AR) and progesterone receptors (PRs) under *in vitro* conditions (Kunz and Fent, 2006; Ma et al., 2003; Schreurs et al., 2005). The oestrogenic effect of BP-3 and its main metabolite – benzophenone-1 – was also demonstrated in several *in vivo* studies (Schlecht et al., 2004; Suzuki et al., 2005). By affecting the function of steroid hormone receptors and influencing the synthesis of steroid hormones and the expression of their receptors, BP-3 may

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contribute to fertility reduction in both women and men as well as to the development of hormone-dependent tumours (Nashev et al., 2010; Schlumpf et al., 2001, 2004). Thus far, the effects of BP-3 on gonadal function have been examined, while other potentially adverse effects of this compound have been suggested but have not been well defined.

BP-3 is a lipophilic compound that can pass through the blood-brain barrier and affect the survival of nerve cells. Evidence confirming the possibility of BP-3's neurotoxic activity is currently mainly provided by *in vitro* research (Broniowska et al., 2016; Wnuk et al., 2017, 2018). Previously, we found that this compound evokes necrosis in high concentrations, while at a concentration of 10^{-8} M, it induces apoptosis in the human neuroblastoma SH-SY5Y cell line (Broniowska et al., 2016). Moreover, BP-3 activates the mitochondrial pathway of apoptosis in mouse neuronal primary cultures, and this effect is connected to changes in the expression of oestrogen receptors (Wnuk et al., 2017). Based on these results, prolonged exposure to BP-3, like other EDCs that affect the survival of nerve cells, may be an important risk factor for the development of neurodegenerative diseases in later life. However, since available *in vitro* studies cannot provide information about whether BP-3 truly crosses the blood-brain barrier and reaches the brain structures in concentrations sufficient to induce apoptosis, we recently performed *in vivo* studies to determine whether the described *in vitro* BP-3 effects also occur *in vivo*. Because people are exposed to BP-3 mainly through the skin, we administered this compound dermally to Sprague Dawley rats. In the case of the brain tissue, the neurotoxic effects of xenobiotics are especially dangerous during the prenatal period since the processes of progenitor cell proliferation, differentiation and neuron migration as well as synaptogenesis and myelination are most intense at that time (Li et al., 2014; Lien et al., 2015). Additionally, harmful compounds acting in the prenatal period increase the sensitivity of neurons to factors operating in later life. Moreover, BP-3 exerts a more potent effect on gonadal function when it acts during development than when it acts in adult animals. For these reasons, we administered BP-3 dermally to female rats during pregnancy and to their male offspring through 6 and 7 weeks of age.

In our first study (unpublished data), we showed that after dermal BP-3 application, this compound was present in the hippocampus and the frontal cortex. It was present in these structures most susceptible to damage in concentrations of approximately 0.22 μM, which are sufficient to initiate the apoptosis process under *in vitro* conditions (Broniowska et al., 2016). The aim of the current research was to determine the effects of BP-3 on the selected markers of apoptosis, i.e., the expression of an executive enzyme of this process (caspase-3), the expression of the main proapoptotic (Bax, Bak) and antiapoptotic (Bcl-2 and Bcl-xL) proteins and the number of cells with apoptotic DNA fragmentation in the frontal cortex and the hippocampus. To distinguish whether the tested compound affects the intrinsic or extrinsic apoptosis pathways, the expression levels of active forms of caspase-9 and caspase-8 were also evaluated. Hippocampus and frontal cortex have been selected for research because they are the main structures involved in cognitive functions, disturbed mainly in neurodegenerative diseases but also in psychiatric diseases (depression).

Previous studies have shown that benzophenones mainly disturb the action of sex hormones, although some data have suggested that they also affect thyroid hormone function. Both sex hormones and thyroid hormones play an important role in maintaining the proper function and vitality of brain cells not only during development but also during adult life. The neuroprotective action of 17β-estradiol is relatively well established (Siddiqui et al., 2016). Oestrogens operate mainly via the classical nuclear receptors ERα and ERβ, which are ligand-dependent transcription factors; however, the neuroprotective effect of this hormone seems to also be exerted by rapid non-genomic responses via the activation of a membrane-associated, G protein-coupled oestrogen receptor (GPR30) (Liu et al., 2012). The influence of androgens on the process of apoptosis is not clear, as they have been shown to exert neuroprotective and neurotoxic effects depending on the conditions, e.g., age, sex (Zup et al., 2014). In the action of endocrine-disrupting chemicals, the arylhydrocarbon receptor (AhR), which binds many EDCs and mediates their neurodegenerative action, seems to be an important factor. Moreover, interaction between AhR and the oestrogen receptor signalling pathway seems to be important in the regulation of the brain apoptotic process (Kajta et al., 2009). Since the action of BP-3 on the expression of ER, AR and AhR mayby an important mechanism of pro-apoptotic action of this compound, its effects on the expression of ER (α and β), GPR30, AR and AhR were measured at the mRNA and protein levels in the hippocampus and frontal cortex. Because the levels of steroid hormones in the brain depend mainly on their production in the peripheral glands, the concentrations of testosterone, 17β-estradiol and progesterone were also measured in blood. The blood levels of prolactin were also assayed since enhanced levels of this hormone are a common cause of gonadotropin suppression and of a corresponding decrease in sex hormone synthesis. Chemical UV filters, in addition to the effects on the sex hormones, most often also disturb the function of the thyroid gland and therefore the plasma concentrations of free fractions of triiodothyronine (fT3) and thyroxin (fT4) as well as thyroid stimulating hormone (TSH) were determined (Schmutzler et al., 2007; Hofmann et al., 2009).

2. Materials and methods

2.1. Animals and treatment

Experiments were carried out on Sprague Dawley rats delivered from the Charles River Laboratories, Hamburg, Germany that initially weighed 200–250 g. Animals were kept in an animal house facility of Jagiellonian University Medical College in Cracow in air-conditioned rooms at a temperature of 22 +-2 °C in plastic cages with a 12-hour circadian cycle of light/night and constant access to water and pelleted feed. Vaginal smears were taken from the females daily to determine their oestrous cycle phases. On the pro-oestrous day, the females were placed with males for 12 h, after which their vaginal smears were subsequently examined for the presence of sperm. BP-3, obtained from Merck (Darmstsdt, Germany), was formulated every day in Essex cream (Schering-Plough, Brussels, Belgium) at a final concentration of 10% and administered from the first to the last day of pregnancy (approx. 22–23 days). For this purpose, an area of approximately 25 cm^2 (5 × 5 cm) on the backs of the animals was shaved, and cream with BP-3 was applied at a dose 100 mg/kg twice daily (at 8:00 and 17:00). Control female rats were treated with Essex cream without BP-3. After the birth, offspring were kept with the dam without any treatment. Twenty-one days after birth, male offspring were weaned and housed in groups of five per cage under standard conditions without any treatment. From 43 to 56 days of age, the animals whose mothers received BP-3 were administered dermal BP-3, whereas control animals were treated with Essex cream without BP-3. Body weights were recorded weekly, and animals were observed daily for any abnormalities. Twenty-four hours after the last BP-3 or vehicle application, the animals were killed by rapid decapitation, and their trunk blood was collected into tubes containing EDTA. The plasma was separated by centrifugation at 800 *g* at 2 °C for 15 min and stored at −20 °C until determination of hormones level in male offspring rats was performed. The brains were removed, and the brain structures (hippocampus and frontal cortex) were rapidly dissected on ice-cold glass plates, frozen on dry ice and stored at −80 °C.

2.2. Real-time PCR

After animals were sacrificed, their brains were isolated, dissected to remove specific structures (frontal cortex and hippocampus), immersed in RNA stabilization buffer (RNA Latter, Ambion, USA) for 24 h at 4 °C, and then stored at -80 °C. To extract the total RNA, the stabilized tissue was homogenized in TRI Reagent Solution (Invitrogen, USA), and