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Primary and tumor mouse Leydig cells exposed to polychlorinated naphthalenes mixture: Effect on estrogen related-receptors expression, intracellular calcium level and sex hormones secretion

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ABSTRACT

We report the effects of polychlorinated napthalanes (PCNs) on the mRNA expression of estrogen-related receptors (ERRs) α, β and γ, calcium ($Ca2+$) concentration, and sex steroid secretion in mouse primary and tumor Leydig cells. The cells were exposed to a mixture of PCNs (10 nM) alone or in combination with one of sex steroid receptor antagonists; 182,780 (ICI; 10 μM); hydroxyflutamide (HF; 10^{-4} M) and G-coupled estrogen receptor antagonist (G15; 10 nM) respectively. The expression of mRNAs and protein for ERRα, β, and γ was detected in primary and tumor Leydig cells. The expression of ERRs was always lower in primary Leydig cells. Exposure of Leydig cells to PCNs significantly increased the expression of ERRs mRNA irrespective of the cell type. Concomitantly, an increased concentration of Ca2+ and sex steroids was revealed in exposed cells. After ICI, HF or G15 was added no changes in expression of ERRs was found. In Leydig cells changes in ERRs expression at mRNA level are clearly linked to changes in Ca2+ level and steroid secretion. Estrogen and androgen receptors are not involved in PCNs action in Leydig cells. The effect of PCNs on mouse Leydig cells is independent on the cell of origin (primary or tumor).

1. Introduction

Testicular dysgenesis syndrome (TDS) is associated with poor semen quality, testicular cancer, cryptorchidism, hypospadias and abnormal testicular differentiation that lead to male infertility (Skakkebaek et al., 2001). Testicular cancer comprises number of different neoplasms, depending on the cell of origin and the typical age at presentation (Woodward et al., 2002). While molecular mechanisms of germ cell tumors are currently extensively studied (Rajpert-De Meyts et al., 2013), only scarce data are available regarding hormonal and molecular mechanisms of development of testicular somatic cells (Leydig cells) tumor (Leydigioma). This tumor is diagnosed in both children and adults. In the latter ones, Leydigioma is always severe with a highly increased risk for second malignancies (Fragoso et al., 1998).

Leydig cells of testicular connective tissue are the main source of androgens that are prohormones in estrogen production. On the basis of current knowledge, it should be emphasized here that an absolute level of estrogens as well as an estrogen-androgen balance are fundamental for proper male fertility and reproductive tract function (Vaucher et al., 2009). The androgen receptor (AR) signaling plays a critical role in the development, function and homeostasis of male reproductive tissues and is well-described (Luccio-Camelo and Prins, 2011). Recently, androgen signaling *via* membrane AR or with the omission of AR, has been reported in tumor prostate cells (Walker and Cheng, 2005; Papadopoulou et al., 2009). On the other hand, estrogen signaling in male reproduction is not fully known. Physiological responses to estrogens, both genomic and rapid signaling, are initiated by nuclear receptors; estrogen receptor α (ERα) and estrogen receptor β (ERβ) as well as membrane G-coupled estrogen receptor (GPER). It was strongly confirmed that ERα plays an important role in maintaining an appropriate morphology and physiology of the rete testis and efferent ductules (Ki-Ho Lee et al., 2000) but up today the role of ERβ in testicular cells is unknown. Data regarding fertility of ERβ knock-

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outs are controversial (Krege et al., 1998; Antal et al., 2007). In contrast, GPER KO mice exhibit no reproduction defects but severe metabolic ones (Prossnitz and Hathaway, 2015).

In Leydig cells species-specific localization patterns of ERα, ERβ and GPER was reported in human and other mammals (Pelletier et al., 2000; Woodward et al., 2002; Kotula-Balak et al., 2012; Prossnitz et al., 2014; Vaucher et al., 2009; Mahmoud et al., 2015; Fietz et al., 2016). Need to add here that, in human Leydigioma significant alterations of expression patterns of these receptors were demonstrated (Bolyakov et al., 2007). ERα and ERβ mainly act as transcription factors but under pathological conditions e.g. tumor transformation, they are able to exert a fast effect *via* multiple signaling pathways as GPER (Scaling et al., 2014). In fact, last evidences confirm implication of estrogen signaling pathways in the development of various tumors of endocrine tissues (Bonkhoff and Berges, 2009).

Currently, it has become clear that estrogen action through ERs contributes only in a small part to the complexity of estrogen signaling. Estrogen-related receptors (ERRs) appeared to be involved directly and indirectly in estrogen signal transmission. These receptors show high degree of DNA sequence homology to ERs, and each of the three subtypes exhibits a considerable level of identity with ER in amino acid sequence in both highly conserved DNA-binding domain and a carboxyl-terminal ligand-binding domain (Giguere et al., 1988). ERRs can bind to functional estrogen response elements (EREs) in ER target genes, indicating an overlap between ERR and ER action (Huppunen and Aarnisalo, 2004). ERRs also bind to ERR-response element (ERRE), but as monomers. These receptors influence estrogen signaling by either synergizing and/or competing with ERs in regulation of multiple shared transcriptional targets through nongenomic signaling. Although no endogenous estrogens and ER ligands have been confirmed to bind to ERRs, evidence suggests that these receptors may be hormone-regulated. Vanacker et al. (1999) have observed that fetal calf serum contains factors that can markedly stimulate ERRs activity. Several natural phytoestrogens (isoflavones: genistein, daidzein, and biochanin A, and flavone: 6, 3, 4-trihydroxyflavone), as ERRs ligands with agonistic activities have been identified by structure-based virtual screening and biological functional assays (Roshan-Moniri et al., 2014). It should be noted here that, estrogen as well as many estrogenic compounds have been shown to influence the transcriptional activity of the ERRs (Giguère, 2002; Liu et al., 2003). In physiology and pathology of reproduction the role and regulation of ERRs is still not fully discovered. There are three ERR types described: ERRα, ERRß and ERRγ. Recent studies demonstrated that null mutation of ERRα has no reproductive consequences while there are strong indications of the involvement of ERRα in cancer initiation and progression (Jarzabek et al., 2009). Other studies revealed that ERRß knockouts are infertile due to anomalies in the fetal component of the placenta (Luo et al., 2014). In contrast, ERRγ seems to be an important regulator of vascular function (Horard and Vanacker, 2003), while its effect on reproduction remains to be still defined.

In tumor cells not only cell signaling is disturbed leading to the increase of cell proliferation, but also there is a modulation of cellular behavior and function including disturbed lipid homeostasis characteristics (Schlegel et al., 2010). It is well known that hormonal stimulation of Leydig cells by lutropin (LH)/human chorionic gonadotropin (hCG) entails increased intracellular calcium ($Ca2+$) levels and steroid production. Signaling *via* Ca2+ induces transcription cascade with the involvement of steroidogenesis-regulating and −relating factors that control steroidogenesis in Leydig cells (Abdou et al., 2012).

Polychlorinated naphthalenes (PCNs), a family of chlorinated polycyclic aromatic hydrocarbons, consist of 75 possible congeners. In the past, congener mixtures of PCNs were commercially produced in several countries under the trade names of Halowax, Nibren and Seekay waxes and Cerifal Materials (Falandysz, 1998). Recently, the composition of PCN congeners in technical Halowax mixtures has been reported (Falandysz et al., 2000). The mixture of these compounds was mainly used in the electrical industry with ideal chemical properties and thermal stability in the 20th century. PCNs have been formally restricted in the majority of countries, but these compounds are still released into the environment, for example, as a result of thermal processing of plastics containing polychlorinated biphenyls, in which PCNs also occur as trace contaminants (Falandysz, 1998; IPCS, 2001; Taniyasu et al., 2003; Falandysz et al., 2014). Environmental studies show that complex mixtures of PCNs contaminate fish as well as food of animal origin (IPCS, 2001). It is presumed that these are the major sources of human exposure, making a 90% daily intake of PCNs per person (Kilanowicz et al., 2009).

Exposure to PCNs has been linked to malignant neoplasms of connective tissue as well as liver, lymphatic and hematopoietic organs in factory workers (Howe et al., 2001). Studies reported various properties of PCNs depending on an examined tissue (Howe et al., 2001; Kilanowicz et al., 2009; Barc and Gregoraszczuk, 2016). Only sporadic studies of the effect of PCNs on fertility and endocrine disruption have been reported to date (IPCS, 2001). In pregnant rats treated with hexachloronaphthalene acceleration onset of spermatogenesis in male offspring was found (Omura et al., 2000). Villeneuve et al. (2002) confirmed that individual polycyclic aromatic hydrocarbons are able to induce both dioxinlike and estrogenic responses in desert topminnow, *Poeciliopsis lucida,* hepatoma cells (PLHC-1 cells), rat hepatoma cells (H4IIE-luc cells) and human breast carcinoma cells (MCF-7). However, individual PCNs cannot bind to calf uterine ER as was shown by Kramer and Giesy (1999). An interest also exists in involvement of aryl hydrocarbon receptor in PCNs action in female reproductive tissues (Barc and Gregoraszczuk, 2014).

Until now, biology and role of ERRs as well as their interactions with endogenous and exogenous substances and molecules in cells of the male reproductive system were not discovered. It will be interesting to investigate the implication of ERRs and Ca2+ signaling network in steroid production and modulation of these signaling in physiological and pathological Leydig cell conditions. In the light of above facts the present study was undertaken to examine if and how the mixture of PCNs effects on ERR (α, β and γ) mRNA expression, Ca2+ concentration and sex steroid hormones level in primary and tumor mouse Leydig cells.

2. Materials and methods

2.1. Chemicals

Mixture of PCNs—Halowax 1051 (H; Koppers Co., Pittsburg, PA, USA) consists of 0.0037 mg/g MonoCNs, 0.0016 mg/g DiCNs, 0.014 mg/g TriCNs, 0.43 mg/g TetraCNs, 0.39 mg/g PentaCNs, 1.6 mg/g, 83 mg/g HeptaCNs and 870 mg/g OctaCN with a total content of CNs 955 mg/g as described by Falandysz et al. (2006). A stock solution (1 M) was dissolved in dimethyl sulfoxide (DMSO). The Halowax 1051 dose (10 μM) was based on studies by Gregoraszczuk et al. (2011) and Barć and Gregoraszczuk (2015). As the Halowax 1051 interacts with both ER and AR in steroidogenic cells of female tissues (Barć and Gregoraszczuk, 2015), specific ER and AR antagonists have been used. ER antagonist ICI 182,780 (ICI; Faslodex, Sigma–Aldrich, St. Louis, MO, USA) was dissolved in ethanol (stock conc. 10 μM), aliquoted and stored at −20 °C. Cells were treated with 1uM ICI as previously described (Kotula-Balak et al., 2013). Hydroxyflutamide (HF, Sigma-Aldrich) an AR antagonist were dissolved in DMSO (stock conc. 2.5×10^{-6} M). Cells were treated with 10^{-4} M HF as previously reported (Chojnacka