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Interplay between estrogen-related receptors and steroidogenesis-controlling molecules in adrenals. *In vivo* and *in vitro* study

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ABSTRACT

Estrogen-related receptors (ERRs) α, β and γ appear to be novel molecules implicated in estrogen signaling. We blocked and activated ERRs in mouse (C57BL/6) adrenals and adrenocortical cells (H295R) using pharmacological agents XCT 790 (ERRα antagonist) and DY131 (ERRβ/γ agonist), respectively. Mice were injected with XCT 790 or DY131 (5 μg/kg bw) while cells were exposed to XCT 790 or DY131 (0.5 μg/L). Irrespectively of the agent used, changes in adrenocortical cell morphology along with changes in lutropin, cholesterol levels and estrogen production were found. Diverse and complex ERRs regulation of multilevel-acting steroidogenic proteins (perilipin; PLIN, cytochrome P450 side-chain cleavage; P450scc, translocator protein; TSPO, steroidogenic acute regulatory protein; StAR, hormone sensitive lipase; HSL and HMG-CoA reductase; HMGCR) was revealed. Blockage of ERRα decreased P450scc, StAR and TSPO expressions. Activation of ERRβ/γ increased P450scc, StAR and HMGCR while decreased HSL expressions. PLIN expression increased either after XCT 790 or DY131 treatment. Additionally, treatment with both XCT 790 or DY131 decreased activity of Ras/Raf, Erk and Akt indicating their involvement in control of morphology and steroidogenic function of cortex cells. ERRs are important in maintaining morpho-function of cortex cells through action in specific, opposite, or common manner on steroidogenic molecules.

1. Introduction

The cortex of adrenal gland synthesizes catecholamines that facilitate the acute stress response, the so called “fight-or-flight” response. The cortex, synthesizes steroid hormones that mediate energetic homeostasis of the organism including chronic stress response. The cortex is organized into three concentric zones, each responsible for the production of different steroid hormones. The zones are; zona glomerulosa (ZG) which synthesizes mineralocorticoids, zona fasciculata (ZF) that synthesizes glucocorticoids, and zona reticularis (ZR) synthesizing a subset of sex steroid precursors, including dehydroepiandrosterone sulfate (DHEA-S) (Arnold, 1866). In some rodents, ZR is absent and instead the fetal/X-zone is present (Kim et al., 2009), while in mature rodents ZF and ZR are not clearly distinguishable (Xing et al., 2015). Due to dynamic DHEA-S passage, fetal adrenal cortex along with placenta and maternal adrenal cortex, form a unique fetomaternal endocrine system regulating estrogen production during fetus development (Kaludjerovic and Ward, 2012).

Estrogen receptors are ubiquitously expressed in adrenals of mammals (de Cremoux et al., 2008, Trejter et al., 2015). Recently, it was confirmed that estradiol synthesis is dependent on an external source of estrogen precursors (*e.g.* gonadal) and the level of estrogen synthase expression (Barakat et al., 2016). It should be added here that testis is an important source of estrogen which maintains normal spermatogenesis (Hess, 2003). What is more, testicular estrogens are regulators of pancreas and bone physiology too (Hannemann et al., 2013). Estrogens exert a multidirectional effect on function of the hypothalamus-pituitary-adrenals axis, affecting primarily hypothalamus and pituitary hormone synthesis and/or secretion (Malendowicz, 1994; Goel et al., 2014; Handa and Weiser, 2014). Additionally, estrogens control cortex function. These effects may be mediated either *via* canonical estrogen receptors (ERs), G-coupled membrane estrogen receptor (GPER) or be a result of their interference with adrenocorticotropin (ACTH)-sensitive intracellular pathways, particularly those associated with

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steroidogenesis (Gallo-Payet et al., 2017). Liver is also important in the physiological control of the adrenal axis (Kharb et al., 2013). This organ is critical for the regulation of corticosteroid binding globulin and lipoproteins and metabolizes circulating corticosteroids under local estrogen control. Estrogens stimulate adrenal cortex growth during development by promoting cell proliferation and enhancing steroidogenic activity through increasing activity of steroidogenic regulatory genes and proteins (Xing et al., 2015). In the fetal adrenals, estradiol and ACTH form a positive regulatory loop in which estradiol increases ACTH secretion and ACTH increases estradiol in the ovary (Rehman et al., 2007). ACTH inhibition of testosterone secretion in guinea pig was showed by Fenske (1997) but O'Shaughnessy et al. (2003) reported ACTH-testosterone stimulation in fetal mouse gonad. No measurements of estradiol secretion or concentration were performed.

It is worth noting here, that steroidogenesis is multi-level controlled process (Miller, 2013). It requires the coordinated expression of number of genes, proteins of various function (enzymes *e.g.* P450 side-chain cleavage; P450scc; transporters *e.g.* translocator protein; TSPO, steroidogenic acute regulatory protein; StAR; regulators and receptors), signaling molecules and their regulators in response to ACTH stimulation. Moreover, for cell steroidogenic function global lipid homeostasis is crucial. Perilipin (PLIN), hormone sensitive lipase (HSL) and HMG-CoA reductase (HMGCR) are members of cell enzymatic machinery controlling lipid homeostasis (Liu et al., 2012). In the light of above, not much is known about proteins maintaining lipid balance and their effect on downstream effectors under estrogen imbalance conditions.

Estrogen-related receptors (ERRs) are nuclear hormone receptors that control throughout life multiple homeostatic processes such as energy metabolism of lipids (Alaynick et al., 2007). The three different Err genes, α, β and γ, have highly conserved ligand and DNA binding domains and thus, their control is based on compensation. The homology between genes of ERRs and ERs is 36% in the ligand binding domain and 68% in the DNA binding domain. ERRs bind both ERR response elements (ERREs) and the closely related estrogen response elements (EREs) embedded within an ERRE sequence on DNA to modulate transcription of target genes (Byerly et al., 2013). Whether ERRs bind estradiol like they bind their pharmacological agent (tamoxifen) or other estrogen-like compounds is not a resolved issue (Vanacker et al., 1999; Roshan-Moniri et al., 2014). Recently, ERRs are of great interest for their potential involvement in metabolic dysregulation in diseases such as diabetes and heart failure (Huss et al., 2015). ERR implication in muscle differentiation and hypertrophy, innate immune function, thermogenesis, and bone remodeling but also in regulation of tumor and microenvironment metabolism, cell cycle and proliferation, epithelial–mesenchymal transition, and metastatic mechanisms was provided too. Therefore, their usage as potential therapeutic targets is considered. Studies with the use of microarrays confirmed the expression of ERRs in rat and human adrenals as well as their involvement in both development and tumorigenesis of adrenals (Felizola et al., 2013; Trejter et al., 2015). It is interesting to note here, that upregulation of vimentin (major cell cytoskeletal component) expression, resulted in tissue cytoarchitecture organization (fibrosis, cancer progression) in various organs and is well-known effect of estradiol stimulation consistent with epithelial-mesenchymal transition (Sumida et al., 2016).

In the present study, mouse adrenals and adrenocortical cells (H295R), with pharmacologically blocked ERRα or activated ERRβ/γ, were used to gain insight into the molecular mechanisms of ERR regulation of multilevel-acting molecules in lipid homeostasis including steroidogenesis.

To this end, *in vivo* and *in vitro* systems were used. We analyzed cell morphology by light microscopy and phase-contrast microscopy. Tissue/cell lysates were used either for steroidogenic protein immunoexpression analyses or for cholesterol content measurement. Lutropin level was estimated in the serum while in tissue lysates or culture media estradiol level was measured.

2. Materials and methods

2.1. Animals and treatments

Mature male mice (C57BL/6) (n = 30) (8–10 week-old; approx. 24.3 ± 1.53 g of weight) were obtained from Department of Genetics and Evolution, Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków. Animals were maintained in 12 h dark-light (250 lx at cages level) cycle with stable temperature condition (22 °C), relative humidity of 55 ± 5% and free access to water and standard pelleted diet (LSM diet, Agropol, Motycz, Poland). Animals were killed by cervical dislocation. The use of the animals was approved by the National Commission of Bioethics at the Jagiellonian University in Krakow, Poland (No. 151/2015).

Mice were allotted into experimental groups (each group including 10 animals); control (Cont.) and treated receiving selective ERRα antagonist 3-[4-(2,4-Bis-trifluoromethylbenzyloxy)-3-methoxyphenyl]-2-cyano-*N*-(5-trifluoromethyl-1,3,4-thiadiazol-2-yl)acrylamide (XCT 790) (Tocris Bioscience, Bristol, UK) or selective ERRβ/ERRγ agonist *N*-(4-(Diethylaminobenzylidenyl)-*N*'-(4-hydroxybenzoyl)-hydrazine (DY131) (Tocris Bioscience). Both XCT 790 and DY131 were dissolved in dimethyl sulfoxide (DMSO) and the stock solutions were kept at −20 °C. Animals from the experimental groups were injected subcutaneously with freshly prepared solutions of XCT 790 (5 μg/kg bw) or DY131 (5 μg/kg bw) in phosphate buffered saline (six injections were performed; one injection every second day). Mice from control groups received vehicle only (DMSO f.c. 0.09 mL/kg). Dose, frequency and time of XCT 790 and DY131 administration were based on literature data (Yu and Forman, 2005; Hu et al., 2015) and it was finally selected upon our study in bank voles (Pawlicki et al., 2017) as well as preliminary *in vivo* study (doses range 5, 50, 100 μg/kg bw). Total time of experiment was 12 days. Mice were sacrificed on the next day after last injection.

Both adrenals of each individual of control and treated mice were surgically removed. For immunohistochemistry, one adrenal was fixed in 10% formalin and embedded in paraplast. For protein expression and intraadrenal cholesterol and estrogen concentration analyses second adrenal was immediately frozen in a liquid nitrogen and stored at −80 °C. Blood was collected from liver vein and used for lutropin level measurements.

2.2. Cell culture and treatments

Human adrenocortical carcinoma cells (NCI-H295R) (ATCC® CRL-2128™) were cultured in RPMI-1640 (Gibco Invitrogen) supplemented with 2% fetal calf serum, 0.1% selenium/insulin/transferrin (Gibco Invitrogen), and penicillin/streptomycin according Rodriguez et al., (1997). Middle passages of H295R cells were used for the study. Cells were plated overnight at a density of 1×10^5 cells/ml per well. Cell viability was measured using the trypan blue exclusion test and was determined to be ~ 93–95%. Twenty-four hours before the experiments, the medium was removed and replaced with medium without phenol red supplemented with 5% dextran-coated, charcoal-treated FBS (5% DC-FBS) to exclude estrogenic effects of medium. Next, cells were treated with selective ERRα antagonist (XCT 790) (Tocris Bioscience, Bristol, UK) or selective ERRβ/ERRγ agonist (DY131) (Tocris Bioscience). Both XCT 790 and DY131 were dissolved in dimethyl sulfoxide (DMSO) and the stock solutions were kept at −20 °C. Based on literature data (Yu and Forman, 2005; Lanvin et al., 2007; He et al., 2008; Hu et al., 2015), our study (Pawlicki et al., 2017) and preliminary study (dose range 0.5, 1, 1.5, 2 μg/L) cells were treated with freshly prepared solutions of XCT 790 (0.5 μg/L) and DY131 (0.5 μg/L) in phosphate buffered saline for 24 h. Control cells were treated with DMSO (f. c. less than 0.1%). Cell viability after treatment was ~93–95%.

Adrenals *in vivo* and adrenal cells *in vitro* both control and treated