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Endocrine disrupting compounds modulates adiponectin secretion, expression of its receptors and action on steroidogenesis in ovarian follicle

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ABSTRACT

We determined the effect of dioxin-like polychlorinated naphtalenes (PCN) (Halowax 1051) (100 pg/ml), benzo(a)pyrene [B(a)P] (2.5 ng/ml), hexachlorobenzene (HCBz) (0.2 ng/ml) and non-dioxin like polybrominated diphenyl ether (BDE-47) (25 ng/ml) and bisphenol A (BPA) (20 ng/ml) on ovarian adiponectin secretion (ELISA) and its receptors expression (Western blot). Ovarian cells co-culture was used to examine action of endocrine disruptors (EDCs) on adiponectin (10 μg/ml) stimulated steroidogenesis. B(a)P, HCBz, testosterone (T) decreased adiponectin secretion and receptors expression, BPA, BDE-47, estradiol (E2) had the opposite effect, while PCN had inhibitory effect only on adiponectin secretion. In adiponectin stimulated cells dioxin-like compounds decreased E2 and except of PCN had no effect on T, while non-dioxin like increased E2 and decreased T secretion. Results indicated to modulatory role of EDCs on adiponectin and its receptor and its action on ovarian steroidogenesis, suggest that dioxin- like compounds may contribute to the ovarian dysfunction in obesity-related disorders.

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1. Introduction

There is emerging evidence that exposure to various endocrine disrupting chemicals (EDCs) may be an important contributor to the obesity epidemic [1,2]. Although the study of obesogens is less than 10 years old, about 15 chemicals and classes of chemicals have already been shown to result in weight gain due to developmental exposure in animal models. These include bisphenol A (BPA), tributyl tin, some flame retardants (PBDEs), perchlorinated biphenyls (PCBs), and some organochlorine pesticides (DDT metabolites, oxychlordane, parathion, chlorpyrifos), triflumizole (fungicide), high fructose corn syrup, nicotine, benzo(a)pyrene (B(a)P), some phthalates, some surfactants (PFOAs), diethylstilbestrol, and genistein; the list continues to grow. Among them, persistent organic pollutants (POPs) may be particularly interesting because low dose 8 organochlorine (OC) pesticides or PCBs have been strongly linked to type 2 diabetes, insulin resistance, and metabolic syndrome; obesity is believed to play a critical role in all of these disorders [3–5]. Earlier toxicological studies showed that some pesticides like hexachlorobenzene (HCBz) cause obesity in animals [6,7]. BPA has also been found to alter lipid metabolism, promote the development of adipocytes (fat cells), and increase the accumulation of fat tissue [8,9]. Recent evidence from many laboratories has shown that a variety of environmental EDCs can influence adipogenesis and obesity. Obesogens can be defined functionally as chemical agents that inappropriately regulate and promote lipid accumulation and adipogenesis [10].

Adipose tissue has emerged as a source of endocrine regulators that act on numerous tissues and processes. Adiponectin, which in contrast to leptin decreases during obesity, exerts multiple effects on target tissues, including increased sensitivity to insulin, vasodilation, antiatherogenic activity, abrogation of adhesion molecule expression, and inhibition of cellular responses to epidermal and platelet-derived growth factors [11]. Circulating adiponectin is significantly decreased in women with polycystic ovarian syndrome (PCOS), independently of obesity [12]. PCOS is characterized by insulin resistance at the ovarian level, elevated androgen synthesis, insufficient FSH to induce aromatization of androgens, and the consequent arrest of follicular growth and anovulation [13]. Commim et al. [14] provided evidence for a direct link between fat cell metabolism and ovarian steroidogenesis, suggesting that disruption of adiponectin and/or its receptors plays a key role in pathogenesis of hyperandrogenism in PCOS. Studies on the level and expression of adiponectin and their receptors (AdipoR1 and

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AdipoR2) in the ovary are sparse. Lord et al. [15] were the first to demonstrate that pig ovaries and ovarian follicles express AdipoR1 and AdipoR2. Subsequent studies confirmed the expression of these receptors in the human ovary [16]. The various isoforms of adiponectin: trimer, hexamer, and high molecular weight (HMW) are present in both porcine and human follicular fluid at concentrations equivalent to serum concentrations [17,18]. Additionally, using granulosa cells aspirated from medium-sized (3–5 mm) follicles of ovaries collected from prepubertal gilts, Ledoux et al. [17] showed that adiponectin is present in porcine follicular fluid at concentrations similar to those found in serum. These authors demonstrated that adiponectin, at physiologically relevant levels (10–25 μg/ml), induces the expression of genes associated with periovulatory remodeling of the ovarian follicle. Moreover, using isolated corpora lutea (CL) cells, granulosa cells, and theca cells from cycling pigs, Maleszka et al. [19] showed that the expression of adiponectin is significantly higher in porcine CL during all investigated days of the luteal phase than in theca cells isolated on days 17–19 of the cycle. These authors, in *in vitro* study, showed that exposure to adiponectin at doses 1–10 μg/ml decrease progesterone (P4) secretion by CL cells, increased estradiol secretion by granulosa cells, and had no effect on testosterone (T) secretion by theca cells [19].

Taking into consideration results of data of Commin et al. [14] suggesting that disruption of adiponectin and/or its receptors plays a key role in pathogenesis of hyperandrogenism in PCOS we evaluated the effect of selected EDCs: dioxin-like [Halowax 1051, HCBz and B(a)P] and non-dioxin-like (BDE-47 and BPA) compounds on adiponectin secretion and the protein expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in porcine ovarian follicles. We selected EDCs based on previously study confirmed its activation of different receptors: aryl hydrocarbon receptor (AhR) by dioxin-like componds [20–22] and estradiol receptors (ER) by non-dioxin-like compounds [23]. In addition, based on the previous data showing that, in the ovary, Halowax 1051 [24], HCBz [25] and B(a)P had stimulatory effect on T secretion, while BDE-47 [26] and BPA on estradiol (E2) secretion, using a co-culture model of both theca and granulosa cells, we examined the possible modulatory effect of selected EDCs on adiponectin stimulated steroid secretion (T and E2) and the protein expression of steroidogenic enzymes (17βHSD and CYP19).

2. Material and methods

2.1. Reagents and antibodies

M199 medium, fetal bovine serum (FBS, heat inactivated), phosphate buffered saline (PBS), and a penicillin/streptomycin solution (penicillin 10000 units/ml, streptomycin 10 mg/ml) were purchased from GmbH (Bienenweg, Germany). Tris, Na-deoxycholate, Nonidet NP-40, sodium dodecyl sulfate (SDS), protease inhibitors (EDTA-free), dithiothreitol (DTT), Tween 20, bromophenol blue, 1 bromo-3-chloro-propane, trypsin, synthetic steroids including T (cat. # 86500) and E2 (cat. # E2257), and Western blotting luminol reagent (cat. #sc-2048) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human adiponectin (cat. #1065-AP-050) from R&D Systems Inc. (Minneapolis, MN, USA) was utilized in this experiment because porcine adiponectin was not readily available at the onset of the experiment. Sequences of porcine adiponectin, AdipoR1, and AdipoR2 found to be highly homologous to those of the human [27]. A Bradford protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Merck Millipore (Darmstadt, Germany).

The PCN mixture (Halowax 1051) was obtained from Koppers Co. (Pittsburg, PA, USA) [28] and dissolved in DMSO. B(a)P was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in $CHCl_3$. The reference standard HCBz was purchased from the US EPA Environmental Monitoring System Laboratory (Research and Developmental Analytical Reference Standards; Las Vegas, NV, USA) and dissolved in DMSO. BDE-47 was purchased from Chiron AS (Trondheim, Norway) and dissolved in isooctane (Maliincrodt Baker B.V., Deventer, the Netherlands). BPA was obtained from AccuStandard Inc. (New Haven, CT, USA) and dissolved in absolute ethanol. All solvents, which we used, at the concentration < 0.1% had no effect on all measured parameters, based on dose dependent preliminary study (data not shown).

Antibodies against adiponectin (cat. # sc-26496), AdipoR1 (cat. # sc-46749), AdipoR2 (cat. # sc-46751), 17βHSD (cat. # sc-66415), and CYP19 (cat. # sc-14244) as well as a horseradish peroxidase-conjugated antibody (cat. # sc-2020) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin antibody (cat. #A5316) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample collection and ovarian follicle incubation

Porcine ovaries were collected from mature (7–8 months of age) crossbred gilts (Large White and Polish Landrace) at a local abattoir. Ovaries were collected in a bottle filled with sterilized ice-cold saline containing antibiotic-antimycotic solution and were transported to the laboratory. Approximately 1 h elapsed from slaughter to delivery to the laboratory. Medium sized follicles (4–5 mm) were obtained from animals in estrous on days 10–12 [29]. Estrous was designated as day 0. After isolation, ovarian follicles were cut using small scissors to facilitate the penetration of the compounds into the tissue. Follicular walls, including theca and granulosa cells and excluding oocytes and follicular fluids, were individually placed in 24-well plates in M199 medium (without phenol red) supplemented with 5% dextran-coated, charcoal-treated FBS (5% DC-FBS) to exclude any estrogenic effects caused by the medium and selected EDCs: Halowax 1051 at 100 pg/ml, B(a)P at 2.5 ng/ml, HCBz at 0.2 ng/ml, BPA at 20 ng/ml, BDE-47 at 25 ng/ml, and the steroids E2 at 10 ng/ml and T at 10^7 M. Endogenous hormones were added as a control for the endocrine activity of EDCs at doses used in previously study [30]. The follicles were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. The doses of all selected EDCs were chosen based on previous observations [24–26] and preliminary dose response experiments. After incubation, 250 μl of medium was separated from the follicles and stored at −20 °C for adiponectin secretion analysis. Ovarian follicles were homogenized twice in ice-cold lysis buffer. Lysates were cleared by centrifugation at 15,000*g* at 4 °C for 30 min, and the protein content was determined by a protein assay using bovine serum albumin as the standard. All samples were stored at −20 °C until adiponectin, AdipoR1, and AdipoR2 protein expression analysis. The total number of ovarian follicles in this experiment was 108. Each treatment was conducted in four wells, and each experiment was repeated three times (n = 3).

2.3. Cell culture

Granulosa cells (Gc) and theca interna cells (Tc) from medium sized follicles (4–5 mm) obtained from the ovaries of pigs on days 10–12 of the estrous cycle were subsequently prepared according to the technique described by Stoklosowa et al. [31]. After isolation, Gc and Tc were collected and resuspended in M199 supplemented with 10% FBS. For co-culture experiments, the viability of granulosa and theca cells was determined using the trypan blue exclusion test. Cells were subsequently plated in 96-well tissue culture plates at a

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