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The effects of the environmental antiandrogen vinclozolin on the induction of granulosa cell apoptosis during follicular atresia in pigs

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ABSTRACT

The aim of this study was to investigate whether the androgens testosterone and dihydrotestosterone (DHT) and the antiandrogenic fungicide vinclozolin (Vnz) exert proapoptotic effects on porcine granulosa cells (GCs), and to examine the roles of these compounds in follicular atresia. Granulosa cells isolated from pig follicles were cultured for 24 hours, and then exposed to 0.1 μM testosterone, 0.1 μM DHT, 14 μM Vnz, or the equivalent concentrations of testosterone and Vnz or DHT and Vnz for a further 24 hours. Apoptosis and necrosis of the GCs were determined via Hoechst staining and flow cytometry analyses of annexin V-stained cells. Whole porcine follicles were also exposed to the same compounds and combinations of compounds for 24 hours. The sections were stained with hematoxylin and eosin for morphologic assessments, and a Terminal deoxynucleotidyl Transferase Biotyn-dUTP Nick-End Labeling (TUNEL) assay was performed to determine the number of apoptotic cells. The progesterone and estradiol concentrations secreted into the culture media by isolated GCs and follicles were also measured. Exposure to the androgens resulted in an increased number of apoptotic GCs both *in vitro* and in the organotypic model. Vinclozolin exposure increased and decreased the number of necrotic and apoptotic GCs, respectively. Furthermore, compared with control follicles, those exposed to testosterone, DHT, or Vnz displayed enhanced atresia, and coadministration of Vnz attenuated the promotive effect of these androgens on atresia. Estradiol secretion was stimulated by the combination of testosterone and Vnz, whereas exposure to Vnz alone reduced it. Progesterone production declined after the combined addition of androgens and the antiandrogen. In summary, Vnz caused massive necrosis of GCs *in vitro* and induced apoptosis of GCs in whole follicles. The androgens testosterone and DHT enhanced these effects. The results presented here suggest that selective destruction of porcine follicles is a serious consequence of exposure to Vnz, and may lead to premature ovarian failure in affected animals.

1. Introduction

Androgens were originally regarded as hormones that influence physiological processes in males primarily. This perception has now changed because numerous studies have demonstrated the effects of androgens such as testosterone and dihydrotestosterone (DHT) on female physiology [1]; in fact, androgens are important regulators of folliculogenesis [2–6]. Androgens exert their biological effects primarily by binding to the nuclear androgen receptor (AR), a transcription factor that regulates gene

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expression, and modulate follicular function by interacting with various factors. The promotion of granulosa cell (GC) differentiation is the major AR-mediated effect of androgens in the follicle [7]. The porcine AR is expressed at high levels in ovarian follicles *in vivo* [8] and in cell and organotypic cultures [9]. In the ovary, androgens are produced primarily by theca cells in response to luteinizing hormone stimulation, and serve as substrates for the synthesis of estrogenic compounds. Testosterone and androstenedione are converted to estradiol or estrone by cytochrome P450 aromatase [10–12]. In addition, 5α-reductase can convert testosterone to DHT, which is thought to be a more potent androgen than testosterone because of its higher affinity for the AR. Dihydrotestosterone cannot be aromatized to estradiol, and was originally thought to act exclusively through the AR; however, recent studies have demonstrated that although DHT binds with high affinity to the AR, its metabolites (3α- and 3β-diol) bind preferentially to both types of estrogen receptor [13,14]. Although studies of many species have suggested that androgens have favorable effects on follicular growth and health, they can also antagonize follicular development by inducing apoptosis in GCs, thereby promoting follicular atresia, when their production exceeds a certain level [15–18]. Atresia is an active and strictly regulated process by which follicles that are not selected for ovulation are eliminated from the ovary. The balance between the growth of healthy follicles and their loss by atresia maintains a constant cell mass and homeostasis of the ovary throughout the reproductive life of an organism [19,20]. A previous study demonstrated that the death of GCs triggers atresia of the follicles in rats [21], which has since been reported in cows [22], ewes [23], and pigs [24,25].

Agonist- or antagonist-bound steroid hormone receptors, such as the AR, regulate gene expression by binding to regulatory elements in the promoter regions of their target genes, often in a tissue-dependent manner [26]. Our group has previously devised a model system for agonism and antagonism of the AR [16,17]. Using this model, we hypothesized that exposure of porcine GCs or whole follicles to the antiandrogen 2-hydroxyflutamide (2-Hf) would block the effects of excessive concentrations of testosterone on the expression of androgen-regulated genes, and showed that coadministration of 2-Hf and high levels of testosterone to cultured porcine GC or whole follicles *in vitro* upregulates the expression of anti-apoptotic proteins, thereby promoting survival [16]. Moreover, we showed that atresia of porcine follicles is initiated primarily by apoptosis of GCs [17].

Over the past decade, increasing incidences of reproductive disorders have been reported in several animal species [27], including humans. Endocrine disrupting chemicals (EDCs), including certain industrial pollutants, pesticides, and fungicides present in the environment, have the potential to alter sexual development and reproductive processes in animal and human populations by acting as antiandrogens [28]. Chemicals that act as AR agonists or antagonists disturb fetal steroidogenesis, and can induce reproductive malformations in humans and laboratory animals [29]. In this study, we focused on vinclozolin (Vnz), a commonly used fungicide that functions as an antiandrogenic agent in mammals and fish [30,31]. In mice, exposure to Vnz during gonadal sex determination promotes a transgenerational increase in pregnancy abnormalities and adult-onset malformations in the reproductive organs of females [32,33]. Although 2-Hf affects ovarian apoptosis and steroidogenesis [16,17], 2-Hf and Vnz have distinct effects on the activity of the AR [34]; therefore, Vnz may have a unique effect on AR expression. Considering the multiple possible mechanisms of action of an antiandrogen compound, the aim of this study was to determine the ability of Vnz to influence apoptosis of GCs and to compare it with that of 2-Hf.

2. Materials and methods

2.1. Animals

Porcine ovaries were obtained from Polish Landrace sows at a local slaughterhouse. The ovaries were placed in cold PBS (pH 7.4; PAA Laboratories, Dartmouth, MA, USA) containing 10 μL/mL antibiotic/antimycotic solution (AAS; PAA Laboratories), and transported to the laboratory within 1 hour of collection. The ovaries were then rinsed twice with sterile PBS supplemented with antibiotics. Approximately 20 mature pig ovaries from 10 animals were selected for follicle isolation in each experiment. Each ovary yielded three to five follicles; therefore, the total number of follicles collected varied from 60 to 100. The phase of the estrous cycle was determined according to the established morphologic criteria [35]. Medium-sized follicles (4–6 mm in diameter) classified as healthy by morphometric criteria [36] were selected for organ cultures. Briefly, the follicles were dissected from the ovarian stroma, and classified under a microscope. Healthy follicles were characterized by a well-vascularized follicular wall and clarity of the follicular fluid. Early atretic follicles were traversed by few or no blood vessels, and the surfaces of the follicles were opaque with the progression of atresia. This procedure was chosen to minimize the variability between tissues and animals.

2.2. Granulosa cell preparation and culture

Granulosa cells were isolated according to a technique developed in our laboratory [37]. The GCs were scraped from the follicular wall using round-tipped ophthalmologic tweezers, washed several times in sterile PBS, and then recovered by low speed centrifugation at 90× *g* for 10 minutes. Erythrocytes were removed from the samples using red blood cell Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA). The viabilities of the GCs were estimated using the trypan blue exclusion test (mean ± standard deviation, 92 ± 3%). The cells were seeded into six-well culture plates (Nunc, Kalmstrup, Denmark) at a density of 8×10^5 cells per mL, and were cultured in McCoy's 5A medium (HyClone Laboratories Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (PAA Laboratories) and AAS (5 μL/mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 24 hours. After attachment of the cells to the culture plates, the culture medium was replaced and supplemented with testosterone (0.1 μM), DHT (0.1 μM), Vnz (14 μM), or