Theriogenology 84 (2015) 927–939

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Analysis of porcine granulosa cell death signaling pathways induced by vinclozolin

Malgorzata Knet [a], Kamil Wartalski [a], Dorota Hoja-Lukowicz [b], Zbigniew Tabarowski [c], Maria Slomczynska [a], Malgorzata Duda [a,*]

[a] Department of Endocrinology, Institute of Zoology, Jagiellonian University, Krakow, Poland
[b] Department of Glycoconjugate Biochemistry, Institute of Zoology, Jagiellonian University, Krakow, Poland
[c] Department of Experimental Hematology, Institute of Zoology, Jagiellonian University, Krakow, Poland

ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 27 May 2015
Accepted 27 May 2015

Keywords:
Apoptosis
Caspase-3
Granulosa cell
Pig
Vinclozolin

ABSTRACT

Recent studies suggest that disturbing androgen-signaling pathways in porcine ovarian follicles may cause granulosa cell (GC) death. For this reason, we investigated which apoptotic pathway is initiated after GC exposure to an environmental antiandrogen, vinclozolin (Vnz), *in vitro*. Immunocytochemistry, Western blots, and fluorometric assays were used to quantify caspase-3 and -9 expression and activity. To elucidate the specific mechanism of Vnz action and toxicity, GCs were assessed for viability, cytotoxicity, and apoptotic activity using the ApoTox-Glo Triplex Assay. To further determine the mechanism of GC death induced by Vnz, we used the Apoptosis Antibody Array Kit. In response to Vnz stimulus, we found an increased level of caspase-3 protein expression ($P \leq 0.001$) and an increase in caspase-3 proteolytic activity ($P \leq 0.001$), confirming that Vnz is a potent proapoptotic factor. The strong immunoreaction of caspase-9 after Vnz treatment ($P \leq 0.001$) suggests that intrinsic mitochondrial apoptosis pathway was activated during GC death. On the other hand, caspase-8, being a part of the extrinsic receptor pathway, was also activated ($P \leq 0.001$). Therefore, it is possible that Vnz induces porcine granulosal apoptosis also through a parallel pathway. Activation of these two pathways was confirmed by the Apoptosis Antibody Array Kit. In conclusion, it is possible that the intrinsic signaling pathway may not act as an initial trigger for GC apoptosis but might contribute to the amplification and propagation of apoptotic cell death in the granulosa layer after treatment with this antiandrogen. Moreover, Vnz disturbs the physiological process of programmed cell death. Consequently, this could explain why atretic follicles are rapidly removed and suggests that normal function of the ovarian follicle may be destroyed.

1. Introduction

In recent years, the focus of research in reproductive toxicology has shifted away from industrial bulk chemicals, which often have nonspecific toxicity mechanisms, to micropollutants that have specific biological mechanisms; these pollutants include pharmaceuticals, which efficiently interfere with specific molecular targets and physiological systems [1]. Many of these substances interact with nuclear receptors, such as androgen or aryl hydrocarbon receptors, and can thereby disrupt endocrine system function. Because organisms evolved sensitivity to endogenous and exogenous endocrine-active substances as a means to respond to stimuli and to maintain internal homeostasis, they are vulnerable to inadvertent, endocrine-active

* Corresponding author. Tel.: +48 12 664 50 32; fax: +48 12 664 50 98.
E-mail address: maja.duda@uj.edu.pl (M. Duda).

0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.05.028

compounds in their environment, such as anthropogenic chemicals [2]. Endocrine-disrupting compounds, which interfere with the hormone system, may act as unintended exogenous signals. Because receptors are highly evolutionarily conserved [3], the receptor-mediated effects of endocrine-disrupting compounds are likely to occur in a broad variety of taxa. Such effects negatively influence the many processes occurring in the female reproductive tract.

Steroid hormones, androgens derived from the gonads, play essential roles in sexual maturation and differentiation in vertebrates and are key regulators in female reproductive physiology. By interacting with various factors and promoting granulosa cell (GC) differentiation, androgens are some of the most important agents influencing folliculogenesis [4]. On the other hand, they can antagonize follicular development by inducing apoptosis in GCs, promoting follicular atresia [5,6]. Androgens exert their biological effects by interacting with androgen receptors (ARs) in two distinct transduction pathways [7]. In the genomic or transcriptional pathway, ARs act as ligand-dependent transcription factors that regulate the expression of target genes containing the androgen response element in their promoters [4,8]. In the nongenomic pathway, a seven transmembrane G-protein–coupled receptor mediates the effect of androgen in cells lacking genomic ARs [9]. Nuclear ARs belong to a large superfamily of steroid hormone receptors that includes receptors for estrogen, progestin, and mineralocorticoids [10,11].

We used the androgens: testosterone (T), dihydrotestosterone (DHT), and vinclozolin (Vnz, a fungicide with antiandrogenic activity) to study the agonism and antagonism of the AR. Testosterone regulates a variety of ovarian functions by targeting AR or eliciting nongenomic mechanisms. Additionally, T is converted to estradiol or estrone *via* composite action of aromatase (P450arom) [12]. Testosterone can be also converted by 5α-reductase to DHT, which is thought to be more potent androgen than T because of its higher affinity for the AR. Furthermore, DHT cannot be aromatized to estradiol [13,14]. Vinclozolin is a commonly used dicarboximide fungicide registered in the United States and Europe to prevent fruits, vegetables, ornamental plants, and turf grasses from decaying. Two major ring-opened metabolites of Vnz (butenoic acid [M1] and enanilide [M2]) are found in rodent fluids and tissue extracts after *in vivo* exposure [15]. Vinclozolin possesses an antiandrogenic activity in mammals and fish [16,17]. Therefore, in our studies, Vnz was used alone and in combinations with T or DHT to check if its effects can be abolished by classic AR agonists. In mice, exposure to Vnz during the gonadal sex determination period promotes a transgenerational increase in pregnancy abnormalities and female adult-onset malformation of the reproductive organs [18,19]. In our previous study, we found that Vnz induces GC death in cultured porcine follicles [20]. Selective destruction of ovarian follicles appears to be a serious consequence of exposure to Vnz. Dicarboximide fungicides are a prime target in food analyses because of their presence in animal tissues [21] and their potential consequences for human health [22].

Programmed cell death (PCD), a genetically regulated process, controls cell numbers and eliminates unneeded cells and is, therefore, essential for tissue development and homeostasis [23]. Apoptosis, or type I cell death, is a well-characterized PCD pathway that affects single cells that are detached from neighboring cells and the basement membrane [24].

The two most common mechanisms of apoptosis are operating inside the cell either *via* an extrinsic pathway involving the activation of plasma membrane death receptors (Fas receptor/caspase-8 pathway) or *via* an intrinsic pathway which depends on mitochondrial release of cytochrome c (cytochrome c/caspase-9 pathway). Both apoptotic pathways lead to the activation of the executioner caspases 3, 6, and 7, which are the main proteases that degrade the cell [25–27].

Autophagy, a highly regulated catabolic process, is involved in the turnover of long-lived proteins and organelles and in removing damaged organelles [28,29]. In several conditions, autophagy is a stress-associated adaptation to avoid cell death, whereas in other settings, it is involved in an alternative pathway for cell death, known as autophagy-mediated, or type II, PCD [30]. Cathepsins, proteasomal proteins, are active primarily in type II PCD [31]. By contrast, the major effectors in type I PCD are caspases that activate Ca^{2+}/Mg^{2+}-dependent endonucleases, which cleave the DNA into fragments of 180 to 200 base pairs [24]. The crosstalk between apoptosis and autophagy is complex and involves the Bcl-2 family of proteins [23,29]. The prosurvival function of autophagy has been well characterized under nutrient and growth factor deprivation, but the precise role of this catabolic process in dying cells is uncertain [28,30].

In this study, we sought to unravel the potential impact and mechanisms of androgen and antiandrogen EDC interactions in porcine GCs during PCD to provide a sound basis for the risk assessment of Vnz in the reproduction of mammals. We treated GCs with Vnz and measured the effects on caspase-3 and -9 expression and activity. We assessed the involvement of Vnz in the apoptosis and autophagy pathways to identify the possible proapoptotic mechanisms triggered by this fungicide.

2. Materials and methods

2.1. Animals

Porcine ovaries were obtained from Polish Landrace sows at a local slaughterhouse. Ovaries were placed in cold PBS (pH 7.4, PAA: The Cell Culture Company, Piscataway, NJ, USA) containing antibiotic–antimycotic solution (5 mL/500 mL, PAA: The Cell Culture Company) and transported to the laboratory within 2 hours. Next, the experimental material was rinsed twice in sterile PBS supplemented with antibiotics. Twenty mature pig ovaries from 10 animals were selected for follicle isolation in each experiment. The phase of the estrus cycle was determined using the established morphologic criteria [32]. Medium follicles (diameter, 4–6 mm) were selected for cell cultures after their health was estimated based on morphometric criteria [33]. Briefly, follicles were dissected free from the ovarian stroma and separately classified under a microscope. Healthy follicles characterized by a well-vascularized