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Review

The effects of flutamide on cell-cell junctions in the testis, epididymis, and prostate

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ABSTRACT

In this review, we summarize recent findings on the effect of the anti-androgen flutamide on cell-cell junctions in the male reproductive system. We outline developmental aspects of flutamide action on the testis, epididymis, and prostate, and describe changes in junction protein expression and organization of junctional complexes in the adult boar following prenatal and postnatal exposure. We also discuss findings on the mechanisms by which flutamide induces alterations in cell-cell junctions in reproductive tissues of adult males, with special emphasis on cytoplasmic effects. Based on the results from *in vivo* and *in vitro* studies in the rat, we propose that flutamide affects the expression of junction proteins and junction complex structure not only by inhibiting androgen receptor activity, but equally important by modulating protein kinase-dependent signaling in testicular cells. Additionally, results from studies on prostate cancer cell lines point to a role for the cellular molecular outfit in response to flutamide.

1. Androgens in male reproductive physiology

Androgens play a central role in the regulation of reproduction in males; they are essential for the development of reproductive organs such as the testis, epididymis, vas deferens, seminal vesicle, and prostate. Testosterone, the main testicular and the major circulating androgen, is produced by Leydig cells present in the testis interstitial space and subsequently diffuses into the seminiferous tubules [1]. During fetal development, testosterone is required for Wolffian duct development, and this action results in formation of the epididymis, vas deferens, and seminal vesicles. Furthermore, testosterone is required for prostate and external genitalia development; however, in these organs the hormone is metabolized by the converting enzyme 5α-reductase to dihydrotestosterone (DHT) [2]. In neonatal males, testosterone level is high for only a short time before production decreases and is maintained at a low level until puberty. Subsequently, the rising androgen level mediates growth and function of accessory sex glands, initiation of spermatogenesis, and development of secondary male sex characteristics [3,4].

In mature males, the maintenance of testosterone secretion, spermatogenic process, and male fertility are controlled by the hypothalamic-pituitary-gonadal axis and modulated by locally-produced factors [5]. Interestingly, quantitatively normal spermatogenesis is maintained only when testosterone levels greatly exceed the peripheral testosterone concentration [6]. Maddocks et al. [6] demonstrated that testosterone levels in the testes of men and rodents are 25- to 125-fold higher than that present in serum. This finding indicates that androgens could directly contribute to the maintenance of testicular cell function [7].

Based on both morphological appearance and differences in physiological function, the epididymis, a convoluted tubule localized between the efferent ducts and the vas deferens, is divided into the caput, corpus, and cauda. Each epididymal segment plays a significant role in sperm physiology, because spermatozoa are unable to fertilize an egg when they leave the testis [8]. In order to acquire fertilizing ability and forward motility properties, the male gamete has to transit the epididymis. The caput and corpus epididymidis are responsible for these physiological modifications and sperm transport, while the cauda epididymidis stores sperm [9]. These processes are androgen-dependent [10–12]. For example, Cooper [13] demonstrated that androgen withdrawal causes a gradual loss of the epididymal ability to sustain the sperm maturation process. Notably, throughout the length of the epididymis, communication systems are based on secretion of androgens and, in part, on the junctions between epididymal epithelial cells (see Section 3.3) that are important in regulating the appropriate luminal microenvironment within the epididymal duct for sperm maturation [14]. Finally, accessory glands, including prostate, are stimulated by androgen to produce necessary substances to allow sperm to fertilize eggs within the female reproductive tract [15].

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1.1. Mechanism of androgen action

Testosterone and its metabolite (DHT) are known to mediate their biological effects through binding to the intracellular androgen receptor (AR). This ligand-dependent transcription factor belongs to the superfamily of nuclear receptors and modulates target gene transcription [16]. In the testis, AR is expressed in Leydig cells between seminiferous tubules, in peritubular myoid cells that surround the seminiferous tubules, and in Sertoli cells within the tubules. Germ cells do not express AR [17]. Thus, Sertoli cells are the major cellular target and transducer of testosterone signals for developing germ cells. Testosterone signals can be translated directly into gene expression changes (the classical pathway), or testosterone can activate kinases that may regulate processes required to maintain spermatogenesis (the non-classical pathway). The classical, genomic action of steroids is generally detectable in hours to days, whereas non-genomic responses occur in seconds to minutes; this timing indicates a lack of transcription and translation of androgen-responsive genes.

In the classical pathway of androgen action, hormone binding to AR induces repositioning of ligand binding domain (LBD) helix 12 over the ligand–binding pocket. This structural change stabilizes the bound androgen and forms a platform for further interactions with coregulators onto promoter and enhancer sites. Androgen binding induces dissociation of chaperone proteins and exposure of the nuclear localization signal, followed by translocation to the nucleus and dimerization of the hormone-receptor complex [18]. In an AR dimer, two DNA-binding domains (DBD) recognize DNA sequences known as an androgen response element (ARE) that are located in or near promoter regions of target genes. Following DNA binding, AR regulates transcription of androgen-dependent genes [19]. Various coactivators, such as histone modifying enzymes or components of chromatin remodeling complexes, can modulate AR function through binding to the LBD or N-terminal domain. Interestingly, in its agonist-activated state, AR can also be regulated by corepressors such as silencing mediator of retinoid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR) through direct AR binding [20] (Fig. 1A).

The non-classical AR pathway originates at the plasma membrane or in the cytoplasm and triggers the release of intracellular Ca^{2+} and activation of protein kinases such as mitogen-activated protein kinase (MAPK). One of the first reports of rapid testosterone action was described in adult male rats. Yamada et al. [21] demonstrated increased hypothalamic neuron firing with seconds of testosterone application, while Gorczynska and Handelsman [22] showed that testosterone application rapidly increases the cytosolic Ca^{2+} concentration in Sertoli cells. Next, evidence showed that non-classical testosterone effects are independent of AR binding to DNA [23]. Such signaling pathways may be initiated when testosterone binds to classical AR, which subsequently interacts with and causes the phosphorylation of tyrosine Src kinase. Activated Src causes phosphorylation and stimulation of epidermal growth factor receptor (EGFR) *via* an intracellular pathway that leads to further activation of Raf, MEK and ERK kinases (the MAPK pathway). As a result, expression of cAMP response element binding protein (CREB)-regulated genes is induced [23–25]. Testosterone-mediated activation of EGFR also contributes to the activation of 3-kinase-phosphatidylinositol (PI3K) [26]. One of the downstream targets of PI3K is the serine-threonine kinase AKT. Activation of AKT by PI3K results in AKT translocation to the plasma membrane and dual phosphorylation that fully activates its kinase activity [27,28]. Interestingly, the Raf/MEK/ERK and PI3K/AKT signaling pathways can cross-talk, since Raf-1 activity is reduced by AKT, as was shown in the MCF-7 line [29] and non-malignant cells [30] (Fig. 1B).

In 2014, a novel membrane androgen receptor was identified by Berg et al. [31].This receptor was seven-transmembrane protein that had high sequence similarity with zinc transporter ZIP9 (SLC39A9) subfamily members. Androgens activated a Gs protein coupled to ZIP9, followed by increased cAMP in ovarian cells. ZIP9 expression was also detected in prostate and breast cancer cell lines, where the receptor mediated testosterone-induced apoptosis through MAPK and zinc dependent pathways [32] (Fig.1B). Furthermore, the role of ZIP9 in mediating non-classical androgen signaling in spermatogonia and Sertoli cells were evidenced *in vitro* [33,34]. Notably, testosterone binding to ZIP9 in a Sertoli cell line that lacked AR resulted in rapid activation of ERK1/2 kinase, phosphorylation of transcription factors CREB and ATF-1, and, in turn, increased claudin-1 and -5 expression and tight junction (TJ) formation [34].

2. Anti-androgens

Anti-androgens are chemical compounds that target androgen signaling. Pharmacological anti-androgens are used to treat androgen-dependent diseases such as prostate cancer, precocious puberty, and scalp hair loss in men or hirsutism in women. They interfere with androgen biosynthesis or metabolism to impair androgen function, or interact with AR to inhibit ligand-dependent transcription. Anti-androgens are therefore classified as androgen synthesis inhibitors (*e.g.*, abiraterone acetate - an irreversible inhibitor of CYP17A1) or AR antagonists [35]. The latter group includes both steroidal (cyproterone acetate) and nonsteroidal compounds (*e.g.*, flutamide, bicalutamide, and enzalutamide) that compete with androgen for the LBD of AR. Antagonist binding induces inactive LBD conformation in which helix 12 is displaced from its hormone-bound configuration [36,37]. This change prevents the binding of coactivators and/or enhances recruitment of transcriptional corepressors such as SMRT and N-CoR [38].

Flutamide was the first nonsteroidal AR antagonist approved by the U.S. Food and Drug Administration (FDA) for the treatment of prostate cancer, and it became the structural basis for other nonsteroidal anti-androgens [39,40]. Other first-generation anti-androgens include nilutamide and bicalutamide. Due to its better tolerability, rare hepatotoxicity (see below), and longer half-life when compared with flutamide and nilutamide, bicalutamide is at present the most widely used non-steroidal anti-androgen in clinical practice [41,42]. Enzalutamide is a second-generation, competitive AR antagonist that has several times higher affinity to AR than bicalutamide. In contrast to previously mentioned drugs, enzalutamide inhibits not only androgen binding to AR, but also AR nuclear translocation, its ability to bind DNA, and interactions with coactivators. Thus, it is indicated for the treatment of metastatic castration-resistant prostate cancer in which increased androgen signaling activity despite low androgen levels is frequently observed [43]. In 2018, a new second-generation AR antagonist structurally related to enzalutamide (called apalutamide) was approved by the FDA for the treatment of non-metastatic castration-resistant prostate cancer. Currently, new drugs that exhibit improved therapeutic efficacy and fewer side effect are under development [44].

It should be noted that not only pharmaceuticals, but also a variety of chemicals used as pesticides and insecticides (*i.e.*, vinclozolin, procymidone, linuron, and p,p'-DDE) as well as compounds used in the plastics industry and in consumer products (phthalates and paraben esters) exhibit anti-androgenic properties and actions [45]. These substances are classified as environmental endocrine disruptors. It is widely accepted that exposure to these chemicals during development interferes with endogenous hormonal axes in animals and humans and results in developmental defects and diseases later in life [46].

2.1. Flutamide

Flutamide (2-methyl-*N*-[4-nitro-3-(trifluoromethyl)-phenyl]propamide) is a pure anti-androgen reported to block binding of androgen to the AR ligand-binding pocket [47] (Fig. 2A). Flutamide does not prevent AR nuclear localization [48], but it decreases the association of AR with various coactivators, including STAT3, GRIP1, and TIF2 [49–51]. In androgen-dependent prostate cancer cells, flutamide did not inhibit AR binding to the prostate-specific antigen regulatory region, but it