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New Emerging Androgenic Actions in the Regulation of Sperm Production and Function

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Abstract

Androgenic actions are determinant for sperm production and function and, thus, for male fertility. Androgens exert their effects by interaction with the androgen receptor (AR), a transcription factor that modulates gene expression in target-cells and tissues. Variants of AR protein have been identified in the testis, revealing a new complexity in androgen signaling pathways. In addition, androgens may evoke responses by controlling intracellular calcium (Ca^{2+}) levels and/or activating Ca^{2+}-dependent pathways. However, until recently the knowledge about the role of androgens controlling testicular expression and activity of membrane and intracellular Ca^{2+} regulatory proteins was very limited or inexistent. Also the function of Ca^{2+} in sperm maturation in the epididymis only recently started to be known. This review describes recent advances identifying new AR isoforms in the testis, as well as the novel actions of androgens as modulators of Ca^{2+} homeostasis in reproductive tract discussing the consequent impact for male fertility.

Keywords: *androgens, sperm, testis, epididymis, androgen receptor variants, calcium*

Introduction

Androgens play a pivotal role in male reproductive and sexual function and are perfectly recognized as the main regulators of spermatogenesis promoting the expression of a myriad of paracrine factors that in turn will promote sperm production (Holdcraft and Braun, 2004a; b). The biological effects of androgens are mediated by interaction with their cognate intracellular receptor, the androgen receptor (AR), which acts as a transcription factor modulating gene expression in distinct cells and tissues (Aranda and Pascual, 2001; Kumar *et al*., 2004; Novac and Heinzel, 2005).

The AR belongs to the nuclear receptor superfamily which comprises a large number of proteins in species ranging from nematode to man, and represents the largest known family of transcription factors in eukaryotes (Gronemeyer and Laudet, 1995; Mangelsdorf *et al.*, 1995). In common with other members of the family, the AR has the following functional domains (Gronemeyer and Laudet, 1995): amino-terminal domain

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(NTD or A/B region), DNA-binding domain (DBD or C region), a hinge region (D region), and ligand-binding domain (LBD or E region). The NTD containing the transcription activation function AF-1 is a highly variable region (Tzukerman *et al.*, 1994; Lavery and McEwan, 2005). In contrast, the DBD is the best conserved domain, being characterized by the presence of two zinc-fingers, which are responsible for receptor binding to DNA at specific sequences called androgen response elements (AREs) (Schwabe *et al.*, 1990; Shaffer *et al.*, 2004). The poorly conserved hinge region, usually containing the nuclear localization signal, links the DBD to the LBD, and it has been suggested to have an inhibitory role over AR transactivation function (Haelens *et al.*, 2007). The LBD is responsible for ligand-binding, receptor dimerization and interaction with heat-shock proteins (HSPs) (Danielian *et al.*, 1992; Wurtz *et al.*, 1996), and contains the transactivation function AF-2 (Wang *et al.*, 2001). In the last years, cumulative evidences have been arising demonstrating the existence in different tissues and cells of AR variant proteins lacking NTD, DBD or LBD (Dehm and Tindall, 2011; Cavaco *et al.*, 2013), representing an increased complexity in androgen signaling pathways.

AR mediated actions represent the classical model of androgen signaling, but has also been shown that androgens may elicit cell effects through the activation of membrane-mediated responses or by the cross-talk with intracellular signaling pathways (Lange, 2004). Some of these effects are dependent of mobilization of calcium (Ca^{2+}), control of intracellular levels of this ion and activation of Ca^{2+}-dependent pathways (Gorczynska and Handelsman, 1995; Lyng *et al.*, 2000; Guo *et al.*, 2002; Loss *et al.*, 2011), which arouses the question about the androgenic actions controlling expression and activity of membrane and intracellular Ca^{2+} regulatory proteins.

Although the connection between Ca^{2+} and sperm functionality has been widely associated with the capacitation process that occurs in the female tract (Breitbart, 2002), experimental and clinical evidences have highlighted for the importance of this ion maintaining successful spermatogenesis (Benoff *et al.*, 1994; Hershlag *et al.*, 1995) and to the acquisition of sperm motility during transit through the epididymis (Weissgerber *et al.*, 2011; Weissgerber *et al.*, 2012; Correia *et al.*, 2013). Remarkably, new dimensions of androgens actions are constantly emerging, raising new hypothesis for their way of functioning with new hormonal roles being depicted. The purpose of this review is to provide an overview of the advances on this subject based on recent findings of our research group, including, the identification of AR isoforms in the testis and the action of androgens as modulators of Ca^{2+} homeostasis in the male reproductive tract.

Increasing the complexity of AR signaling pathways in the testis

The classical mechanism of action of androgens (Fig. 1) involves interaction with AR and regulation of the transcription rate for a set of androgen target genes (Aranda and Pascual, 2001; DeFranco, 2002; Kumar *et al.*, 2004). After biosynthesis in endocrine tissues, androgens reach target cells via the blood stream passing the cell membrane by simple diffusion due to their lipophilic properties. In the cytoplasm, androgens bind transcriptionaly inactive AR, which is released from HSPs as a result of the conformational modifications induced by ligand-binding. Activated hormone-receptor complexes are translocated to the nucleus and upon binding to the AREs induce chromatin remodelling and interact with the transcription machinery, activating or repressing transcription of the target genes (Beato, 1988; Beato and Sánchez-Pacheco, 1996; DeFranco, 2002; Wiench *et al.*, 2011). In addition, full AR competence orchestrating the regulation of gene expression depends on the interplay between functional domains of receptor protein, particularly, DBD and LBD (Fig. 2).

The distinct functional domains of AR are encoded by the 8 separate exons of the *AR* gene, which has a structure conserved from fishes, amphibians and birds to mammals (Pinto *et al.*, 2013). Exons 2-3 and exons 4-8 encode, respectively, the DBD and LBD (Lubahn *et al.*, 1989), while NTD is almost entirely encoded by exon 1 (Fig. 2). However, the alternative use of exons or exon deletion by alternative splicing mechanisms, has been shown to generate a panoply of AR isoforms/variants (Dehm and Tindall, 2011; Cavaco *et al.*, 2013), some of which were identified in the testis of several species of vertebrates (Table 1) (Ahrens-Fath *et al.*, 2005; Laurentino *et al.*, 2012b).

Sequence analysis and *in vitro* approaches allowed predicting the functional role of these AR variants. Although devoid of NTD (Table 1), a variant named AR45 was shown to maintain the ability to bind androgen and translocate to the nucleus (Ahrens-Fath *et al.*, 2005). Moreover, this molecular variant seems to negatively regulate the action of classical AR, since it interacts with the NTD of full-length AR inhibiting its activity in a ligand- and DBD-dependent manner (Ahrens-Fath *et al.*, 2005).

In variants lacking the exon 2 of AR ($AR\Delta2^{stop}$ and $AR\Delta2^{23Stop}$), the exon deletion results in the introduction of a premature stop codon and thus, predicted proteins will be truncated presenting only the NTD (Laurentino *et al.*, 2012b). The physiological existence of both exon 2 deleted AR variants was previously demonstrated in androgen insensitivity and prostate cancer cases (Hellwinkel *et al.*, 1999; Jagla *et al.*, 2007). In the case of the $AR\Delta2^{23Stop}$ variant it was demonstrated that it is unable to translocate to the nucleus and do not mediate genomic actions (Jagla *et al.*, 2007). Nevertheless, it was shown their

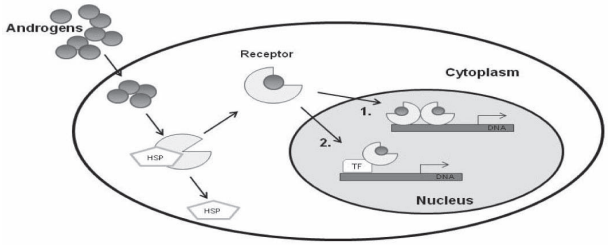

Fig. 1. Classical androgen receptor (AR) signaling mechanism. As lipophilic molecules androgens enter the cells and bind to cytoplasmatic AR. Hormone binding induces release of the chaperone heat-shock proteins (HSP) and a conformational change in the receptor allowing translocation to the nucleus of the hormone-receptor complex. (1) AR dimers interact directly with androgen-response elements in the DNA regulating the transcription of target genes. (2) Alternatively, hormone-bound receptors can interact with transcription factors (TF), which in turn bind to their responsive elements on the DNA, controlling gene expression.

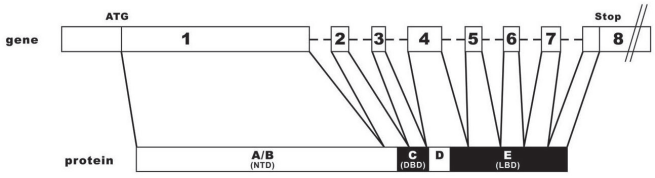

Fig. 2. Human androgen receptor gene (exons 1-8) and protein functional domains. A/B region represents the transactivation amino-terminal domain (NTD), C is the DNA-binding domain (DBD), D is the hinge region and E contains the ligand-binding domain (LBD). Shaded boxes indicate the most conserved domains, involved in DNA- and ligand-binding. ATG and Stop, in exons 1 and 8, indicate, respectively, the localization of the initiation of translation and stop codons. Dashed lines represent introns (not scaled).

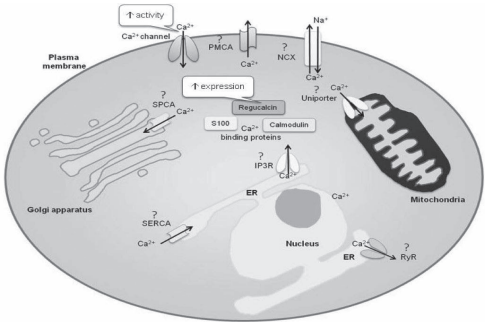

Fig. 3. Possible targets of androgenic regulation in the maintenance of intracellular Ca^{2+} homeostasis in testicular cells. Ca^{2+} homeostasis is achieved by the control of expression and/or activity of Ca^{2+} channels, pumps, exchangers and Ca^{2+} binding proteins.↑- demonstrated enhanced activity or expression in response to androgen stimulation; ? – unknown. PMCA, plasma membrane Ca^{2+} ATPase; NCX, Na^{+}/ Ca^{2+} exchanger antiporter; SPCA, Ca^{2+}/Mn^{2+} ATPase; SERCA, Ca^{2+} ATPase; IP3R, inositol 1,4,5-triphosphate receptor; RyR, ryanodine receptor.

Table 1: Functional variants of androgen receptor in the testis of vertebrates

	Variant name	Structural Features	Species*	References
Exon 1 deleted variant	AR45	Receptor without classical NTD domain	h, ch, o, m, mt, e, p, d	Ahrens-Fath et al., 2005 Weiss et al., 2007
Exon 2 deleted variants	ARΔ2^{Stop}	C-terminal truncated receptor without DBD and LBD, due to the introduction of a premature termination codon	h	Laurentino et al., 2013
	ARΔ2^{23Stop}	C-terminal truncated receptor without DBD and LBD, due to the introduction of a premature termination codon; differs from ARÄ2^{Stop} by insertion of new 23 amino acids	h, r, sb	Laurentino et al., 2013
Exon 3 deleted variant	ARΔ3	Receptor lacking the second zinc-finger in DBD	h, f	Laurentino et al., 2013
Exon 4 deleted variant	ARΔ4(120)	Receptor with an incomplete LBD.	h	Laurentino et al., 2013

* h, human (*Homo sapiens*); ch, chimpanzee (*Pan troglodytes*); o, orang-utan (*Pongo pygmaeus*); m, macaque (*Macaca mulatta*); mt, marmoset (*Callithrix jacchus*); e, elephant (*Loxondonta Africana*); p, pig (*Sus scrofa*); d, dog (*Canis familiaris*); r, rat *(Rattus norvegicus*); sb, seabream (*Sparus auratus*); f, frog (*Xenopus laevis*)

cytoplasmatic functions affecting the activity of transcription factors NF-kB and AP-1 (Jagla *et al.*, 2007).

Exon 3 of *AR* gene exclusively encodes the protein DBD, and thus, deletion of this specific exon predictably affects receptor interaction with DNA. An exon 3 deleted transcript (ARΔ3) identified in the human and frog testis (Table 1) (Laurentino *et al.*, 2012b) lacks the second zinc-finger of AR, which is responsible for receptor orientation and DNA-dependent dimerization (Umesono and Evans, 1989; Quigley *et al.*, 1992; Kaspar *et al.*, 1993). Identical variants were described in patients with androgen insensitivity (Quigley *et al.*, 1992) and in breast cancer tissues and cells (Zhu *et al.*, 1997). Genetically modified mice expressing a mutant AR with deletion of the second zinc-finger of the DBD were useful to demonstrate that these variants are inactive in DNA-binding and promotion of downstream signaling pathways (Notini *et al.*, 2005; Pang *et al.*, 2012).

In case of deletions of exon 4, Socorro S group (Laurentino *et al.*, 2012b) described an AR variant (ARΔ4(120)) in which the sequence alteration is expected to result in a protein lacking part of the LBD, but retaining complete DBD. Although compromising or altering ligand binding, this modification seems do not impair receptor transactivation. Several alternatively spliced AR variants lacking LBD and maintaining the capacity to activate transcription were detected in hormone-refractory prostate cancer (Guo *et al.*, 2009; Dehm and Tindall, 2011). It is predictable that ARÄ4(120) variant might be implicated in the ligand-independent activation of AR or in the regulation of prototype AR activity, as has been found for estrogen receptor (ER) variants lacking LBD, which are dominant negative regulators or enhancers of prototype ERs (Desai *et al.*, 1997; Chaidarun and Alexander, 1998).

The discovery of AR variants in the testis of evolutionarily distant vertebrate species (Table 1) strongly supports their functional relevance, highlighting for an increased complexity of AR signaling pathways this tissue. In a near future, the role of the AR variants mediating androgenic actions in the testis should be addressed, which will improve our understanding of normal and abnormal spermatogenesis, since some variants seem to be negative modulators of AR actions. Nevertheless, these findings indicate that a "Brave New World" of androgen signaling has yet to be discovered.

Androgenic Effects in Calcium Homeostasis and Signaling in the Testis

Among the roles of androgens governing the spermatogenic output it has been shown that testosterone is required for maintenance of blood-testis barrier (Meng *et al.*, 2005; Wang *et al.*, 2006; Willems *et al.*, 2010; Meng *et al.*, 2011; Hejmej *et al.*, 2012), Sertoli-spermatid adhesion (Wong *et al.*, 2005; Wang *et*

al., 2006), inhibition of apoptosis of germ cells (Troiano *et al.*, 1994; Woolveridge *et al.*, 1998; Tesarik *et al.*, 2002; Bakalska *et al.*, 2004), progression of spermatogenesis at meiotic stages (Chang *et al.*, 2004) and release of mature sperm (Holdcraft and Braun, 2004a; Shupe *et al.*, 2011).

Within the seminiferous epithelium, Sertoli cells are responsible for transducing and integrate the molecular mechanisms underlying the androgenic support of spermatogenesis to the germ cells (Walker and Cheng, 2005; Walker, 2009). Many aspects of the physiological actions of testosterone in Sertoli cells seem to involve rapid effects including transient rapid influxes of Ca^{2+} (Gorczynska and Handelsman, 1995; Lyng *et al.*, 2000; Loss *et al.*, 2011; de Castro *et al.*, 2012) and activation of Ca^{2+}-dependent intracellular signaling pathways (Gorczynska and Handelsman, 1991; Ree *et al.*, 1999; Loss *et al.*, 2011)

In 1995, Handelsman group (Gorczynska and Handelsman, 1995) demonstrated that testosterone increases intracellular Ca^{2+} in Sertoli cells through direct activation of a transmembrane influx of extracellular Ca^{2+} across the Sertoli cell plasma membrane, and also through an AR-mediated process involving the classical slower and long term genomic effect as transcription factor. Although it has been shown that testosterone actions mediated by the AR regulate gene expression levels of several Ca^{2+} homeostasis regulators, namely Ca^{2+} channels (Golden *et al.*, 2002; Bodding *et al.*, 2003; Bowles *et al.*, 2004; Golden *et al.*, 2004; Bidaux *et al.*, 2005; Nudler *et al.*, 2005; Hsu *et al.*, 2010), Ca^{2+}pumps (Foradori *et al.*, 2007; Liu *et al.*, 2008; Hsu *et al.*, 2010), Ca^{2+}exchangers (Golden *et al.*, 2002; 2004), Ca^{2+} sensor proteins (Berry *et al.*, 2011) and Ca^{2+}-binding proteins (Haarbo *et al.*, 1991; Furuya and Isaacs, 1993; Averboukh *et al.*, 1996; Zhu *et al.*, 1998; Steele *et al.*, 2006; Hsu *et al.*, 2010) in different cell types, in the testis the downstream effectors of androgenic genomic control of Ca^{2+} homeostasis are almost totally unknown (Fig. 3). This question started to be addressed by the recent report of (Laurentino *et al.* 2011) which showed that androgens regulate the expression of Ca^{2+}-binding protein regucalcin (RGN) in rat seminiferous tubules cultured *ex vivo*. RGN is a protein that does not contain the typical EF-hand as Ca^{2+}-binding motif (Yamaguchi and Yamamoto, 1978) and plays an important role in intracellular Ca^{2+} homeostasis by modulating the activity of enzymes regulating Ca^{2+} concentration, and enhancing Ca^{2+}-pumping activity through the plasma membrane, endoplasmic reticulum and mitochondria of several cell types (Marques *et al.*; Yamaguchi, 2005). Thus, the control of RGN expression may be one of the genomic mechanisms by which androgens contribute to maintain intracellular Ca^{2+} concentrations in testicular cells.

On the other hand, there are an amount of studies highlighting for the importance of Ca^{2+} in preservation of

spermatogenesis. Ca^{2+} is essential for the maintenance of Sertoli cell tight junctions in the blood-testis barrier (Grima *et al.*, 1998) and modulates the activity of enzymes interfering in Sertoli cell architecture (Franchi and Camatini, 1985). The tight regulation of Ca^{2+} influx and efflux maintaining intracellular Ca^{2+} homeostasis also seems to be essential for Leydig cells steroidogenesis, for example by controlling the expression of steroidogenic acute regulatory protein (Manna *et al.*, 1999; Pandey *et al.*, 2010). Moreover, treatment with Ca^{2+} channel blockers to relieve hypertension causes male reversible infertility (Benoff *et al.*, 1994; Hershlag *et al.*, 1995), and abnormal Ca^{2+} currents, through conformational defective L-type voltage-dependent Ca^{2+} channels, have been observed in infertile, but not fertile, men (Ma and Shi, 1999). In rodents, treatment with L-type and T-type Ca^{2+} channel blockers, or Ca^{2+} antagonists, induces testicular regression (Latif *et al.*, 2008; Latif *et al.*, 2009), with spermatogenic arrest at elongated spermatid stage (Lee *et al.*, 2006) and significant reduction in sperm density, amount of mature spermatids and Sertoli cells (Almeida *et al.*, 2000). In this aspect, it is also noteworthy the finding that an altered expression of RGN was found in human testis with abnormal phenotypes of spermatogenesis (Laurentino *et al.*, 2012).

Also it is widely accepted the importance of Ca^{2+} mediated mechanisms ensuring sperm ability to move progressively and to fertilize, namely, in the capacitation process (Breitbart, 2002). However, the role of Ca^{2+} in sperm maturation occurring during transit in the epididymis has been much less studied. Ca^{2+} concentrations in the epididymal fluid are quite low in comparison with those of other ions such as sodium, potassium, chloride, ammonium, and magnesium (Wales *et al.*, 1966) and probably for this reason the role of this ion rendering sperm released from the testis functional gametes has remained hidden for decades.

The sperm maturation in the epididymis involves a series of morphological, biochemical and physiological changes (Cornwall, 2009), some of which not yet totally understood. Nevertheless, it is established that epididymis functions promoting sperm maturation rely on the sperm interaction with the complex microenvironment of epididymal lumen, which contains a set of proteins secreted by epididymis epithelial cells (Guyonnet *et al.*, 2011), in a manner highly dependent of the androgens actions controlling gene expression (Robaire and Hamzeh, 2011). Moreover, it has been suggested that proteins with marked expression differences among caput, corpus and cauda regions of the epididymis have a relevant role in epidydimal physiology and sperm function (Jervis and Robaire, 2001). Recently we found that the Ca^{2+}-binding protein RGN is an androgen regulated gene (Maia *et al.*, 2009; Laurentino *et al.*, 2011) presenting a 2-fold higher expression in the corpus relatively to caput and cauda regions of epididymis,

being also detected in the epididymal fluid (Correia *et al.*, 2013). These facts suggested a role for RGN in sperm maturation and in fact, transgenic animals overexpressing RGN display an altered function of epididymis characterized by a diminished influx of Ca^{2+} that seems to be associated with increased concentrations of Ca^{2+} in epididymal fluid and diminished sperm motility (Correia *et al.*, 2013). Thus, androgenic actions maintaining Ca^{2+} levels in the epididymal lumen through regulation of RGN expression (Fig. 3) may be an aspect of utmost importance for sperm maturation.

Final remarks

Adequate sperm production, maturation and function involve complex cellular and biochemical processes tightly controlled by androgenic actions in the testis and epididymis. The findings on the identification of new AR variants in the testis have demonstrated an unexplored complexity in the actions of androgens in this tissue, opening new lines of research to unravel the AR transcriptome, as well as new signaling pathways. Also, disclosing the Ca^{2+}-regulatory mechanisms dependent of androgens in testicular cells and epididymis, will add a deeper understanding of the spermatogenic process help refining strategies for male infertility treatment and/or contraception.

Finally, the androgenic regulation of spermatogenesis and sperm functionality, despite being an "old" question in reproductive biology studies, still offers new and exciting perspectives of research in a fascinating field, moving forward, and with several parts of the puzzle to be solved.

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