Relaxin in the male reproductive system


Section of Experimental Endocrinology, Department of Pharmacology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil.

Abstract

Relaxin (RLN) belongs to a family of hormones structurally related to insulin and presents a broad spectrum of actions. Humans have three forms of RLN, encoded by three different genes (RLN1, RLN2 and RLN3), but nonprimate vertebrates have only two forms of relaxin (RLN1 and RLN3). RLN1 of these animals is encoded by Rln1, orthologous to the human RLN2 gene, and both genes, Rln1 and human RLN2, encode the major form of relaxin found in the male reproductive system. In the reproductive tract of human males, RLN is mainly produced by the prostate and secreted into the seminal fluid, where it seems to play a role in sperm function. RLN may also play a role in prostate cancer progression. The lack of RLN in animal models impairs male fertility, and RLN knockout mice display decreased sperm maturation. The precise role of RLN in the male reproductive system however is still far from clear. RLN action is due to its interaction with the G-protein coupled receptor RXFP1. Studies from our laboratory have shown that RLN and RXFP1 are expressed in rat Sertoli cells, and exogenous RLN stimulates Sertoli cell proliferation. RLN receptors can also be detected in the rat germ cells at different stages of development, suggesting that RLN may play a direct role in spermatogenesis. The RLN/RXFP1 distribution however appears to be species-dependent, because in the boar testis RLN production seems restricted to the Leydig cells, whereas RXFP1 is found in Leydig, Sertoli and germ cells. The co-expression of RLN and RXFP1 in several regions of the male reproductive system suggests that the peptide may act in an autocrine/paracrine fashion.

Keywords: germ cells, male reproductive system, relaxin, RXFP1, Sertoli cells, testis.

Introduction

Relaxin (RLN) was discovered in 1926 by Frederick Hisaw (Hisaw, 1926), who observed that injection of serum from pregnant guinea pigs or rabbits in non-pregnant female guinea pigs caused relaxation of the interpubic ligament. In 1930, Hisaw and coworkers verified that this effect could be mimicked by a crude extract from the corpus luteum of sows (Fevold et al., 1930). During the next 15-20 years RLN was found to promote growth of the mammary gland, inhibit uterine contractile activity and soften the uterine cervix (Hamolsky and Sparrow, 1945; Krantz et al., 1950; Graham and Dracy, 1953), and RLN therefore became considered a hormone of pregnancy and parturition. Since then, several other actions have been attributed to RLN, including actions in vascular and renal systems, brain, cancer metastasis and neoangiogenesis, and others (Sherwood, 2004; Bathgate et al., 2006). The antifibrotic action of RLN, which results from inhibition of collagen biosynthesis and promotion of collagen breakdown in several tissues, is among the most well established functions of RLN.

The knowledge about RLN function greatly improved after the discovery of the RLN receptor (Hsu et al., 2002). Although RLN is structurally similar to insulin, RLN interacts with RXFP1, a member of the G-protein coupled receptor (GPCR) family, and stimulates several intracellular pathways. The co-expression of RLN and its receptor in several tissues suggests that RLN may be more important as an autocrine or paracrine mediator than as an endocrine factor, especially in the male, where the levels of circulating relaxin are very low. This review intends to present an overview of structural and molecular aspects of the RLN-RXFP1 system, with special emphasis on the RLN role in the male reproductive system.

Relaxin structure and biosynthesis

The structure of RLN was described in the late 1970’s: a peptide structurally similar to insulin, with two chains (A and B) linked by disulfide bonds (Schwabe et al., 1978). The receptor binding site is located in the middle of the B-chain (Bullesbach and Schwabe, 2005a). The A-chain ensures the correct three-dimensional conformation of B-chain and permits it to interact with and activate the receptor (Bathgate et al., 2006).
The RLN family of peptides structurally related to insulin also includes the insulin-like growth factors IGF1 and 2, the insulin-like peptide of Leydig cell (INSL3), the placental insulin-like peptide (INSL4) and the insulin-like peptides INSL5 and INSL6 (Sherwood, 2004; Ivell et al., 2011). Insulin is considered the ancestor of the family, and a RLN3-like ancestor seems to have originated the entire RLN peptide family (Wilkinson et al., 2005; Ivell et al., 2011). The human RLN family encompasses three members, H1-RLN, H2-RLN and H3-RLN, encoded by genes H1, H2 and H3, respectively (Hudson et al., 1983, 1984). The H1 gene is the result of ancestral duplication of the H2 gene. The H3 gene encodes the highly conserved neuropeptide H3-RLN (Hsu; 2003; Wilkinson et al., 2005; Bathgate et al., 2006; Kong et al., 2010). The H1 and H2 genes are expressed in decidua, placental trophoblast, prostate, atrium, ventricle, arteries and veins. Only the H2 gene is expressed in the corpus luteum, endometrium and mammary gland (Hansell et al., 1991; Dschietzig et al., 2003, 2006). The H3 gene is expressed in spleen, thymus, leukocytes, lymph nodes, testes, and brain, where H3-RLN plays a role as a neuropeptide (Bathgate et al., 2002). Only the H2-RLN seems to be secreted into the bloodstream and semen (Bathgate et al., 2006). A gene orthologous to human H1 has been described in monkeys, but is not expressed in rats and mice (Hansell et al., 1991, Sherwood, 2004). Rats and mice have a gene orthologous to the human H2 gene, which encodes the RLN1 of these animals. A gene orthologous to the human H3 gene has also been described in rat, mouse and pig, and it encodes the RLN3 of these animals (Bathgate et al., 2006).

Similar to insulin, RLN is synthesized as a 21 kDa pre-pro-hormone, with an aminoterminal signal sequence, and B, C and A domains, respectively. The signal peptide is cleaved in the interior of the endoplasmic reticulum to originate pro-RLN, which is transported via Golgi to the secretory granules (Ivell et al., 2011). The mature peptide (6 kDa) is produced after a double cleavage that releases the C peptide from the pro-RLN: a prohormone convertase-1 cleaves between the B and C domains, and subsequently furin-like convertases cleave the mature peptide (6 kDa) is produced after a double cleavage that releases the C peptide from the pro-RLN: a prohormone convertase-1 cleaves between the B and C domains, and subsequently furin-like convertases cleave between the C and A domains, releasing the C domain (Marriot et al., 1992). Upon specific stimulation, equal amounts of RLN and pro-RLN are released, and pro-RLN, unlike pro-insulin, seems to be biologically active (Vu et al., 1993; Zarreh-Hoshyari-Khah et al., 2001). Several agents are able to stimulate RLN secretion, including luteinizing hormone, chorionic gonadotropin, basic fibroblast growth factor, progesterone and glucocorticoids (Dschietzig et al., 2006).

**Relaxin receptors**

The RLN receptor remained unknown until 2002, when Hsu and colleagues found that RLN was able to bind and activate the still orphan receptors LGR7 and LGR8, which belong to the LGR (leucine-rich repeat containing GPCRs) family of GPCRs. All members of the LGR family present a large extracellular aminoterminal domain rich in leucine repeats (LRRs; Hsu et al., 2002). The LGR family originated during the early evolution of metazoans and comprises three subfamilies (A, B and C), each one presenting a unique LRR domain (Hsu, 2003). Type A LGRs include the follicle-stimulating hormone receptor (FSHR), the luteinizing hormone receptor (LHR) and the thyroid-stimulating hormone receptor (TSHR). In mammals, the type B LGRs (LGRs 4-6) remain orphans. There are only two type C LGRs: LGR7 and LGR8 (Hsu et al., 2002). According to the recommendation of the nomenclature committee of IUPHAR (International Union of Pharmacology) LGR7 and LGR8 are now called RXFP1 and RXFP2 (RLN family peptide receptors 1 and 2; Bathgate et al., 2006). RXFP1 and RXFP2 contain 10 leucine-rich repeats in the aminoterminal region and are the only LGRs to present a low density lipoprotein (LDL) receptor class A module at the end of the N-terminal region. The LDL-A module is essential for hormone binding, cAMP production, receptor maturation and cell surface delivery (Hsu, 2003; Halls et al., 2007b; Kern et al., 2007).

RLNs from some species appear to bind and activate both RXFP1 and RXFP2. This has allowed the establishment of a rank order of affinity (Halls et al., 2007). With human RXFP1, the rank order is H2-RLN = rhesus monkey RLN > porcine RLN > H1-RLN > H3-RLN > rat RLN >> INSL3 (no binding). With RXFP2, the order is INSL3 > H1-RLN = H2-RLN > porcine RLN = rhesus monkey RLN >> H3-RLN = rat RLN (no binding). Therefore, in contrast to human RLN, rat RLN does not activate RXFP2.

Although human RLN can bind to both RXFP1 and RXFP2, the phenotype of the RXFP1 knockout mice (Kamat et al., 2004; Krajnc-Franken et al., 2004) is very similar to that of RLN knockout mice (Zhao et al., 1999, Samuel et al., 2003a, b), while the phenotype of the RXFP2 knockout (Overbeek et al., 2001; Gorlov et al., 2002) is similar to the INSL3 knockout (Zimmermann et al., 1999), suggesting that RLN is the endogenous ligand of RXFP1 and INSL3 is the endogenous ligand of RXFP2. Furthermore in vivo pharmacological studies confirmed that INSL3 and RXFP2 represent an exclusive hormone-receptor pair (Bogatcheva et al., 2003). RXFP1 and RXFP2 have two RLN binding sites: a high affinity site, present in the LRR region of the amino-terminal ectodomain, and a secondary low affinity binding site, located in the transmembrane loops (Sudo et al., 2003; Halls et al., 2005). The presence of the unique LDL-A module at N-terminus of these receptors is essential for activation of the cAMP signaling pathway. Its absence allows normal ligand-receptor binding, but signaling is absent (Scott et al., 2006). In fact, the LDL-A module competitively inhibits RXFP1 function and might be used to
supress RLN signaling. RXFP1-LDL-A expression in the prostate cancer cell line PC3 cells inhibited AKT phosphorylation and metalloproteinase 2 activation, and led to the down-regulation of several genes previously implicated in tumorigenesis (Feng and Agoulnik, 2011).

Both A- and B-domains of RLN are required for RXFP1 and RXFP2 activation. The B-domain is responsible for primary ligand binding, while the A-domain is responsible for secondary ligand binding. Arginine residues at positions 13 and 17 and an isoleucine or valine at position 20 within the B-domain of RLN are required to bind to specific residues in the LRR domain of RXFP1 receptor, forming a “RLN binding cassette” (Arg-X-X-Arg-X-X-Ile/Val; Bullesbach and Schwabe, 2000, 2005b). The first eight amino acids at the N-terminus of the A-domain of RLN are important for RXFP1 binding affinity and cAMP signaling activation (Hossain et al., 2008).

Splice variants of RXFP1 and other members of the LGR family have been described, which can be retained in the endoplasmic reticulum or secreted, and may function as dominant-negative counterparts of the wild-type receptor (Scott et al., 2005, 2006; Halls et al., 2007; Kern et al., 2008).

Relaxin-activated signaling pathways

RLN binding to RXFP1 induces activation of multiple intracellular effectors (Kong et al., 2010). In the human embryonic kidney cell line HEK293T overexpressing RXFP1, RLN induces a biphasic increase of cAMP, caused by an initial activation of Gs and inhibition of Goβ, followed by an increase in cAMP that involves βγ subunits of Gi3 (Halls et al., 2006, 2007a, b). βγ subunits of Gi3 activate the phosphatidyl inositol 3 kinase (PI3K), which, in turn, activates the atypical isofom of protein kinase C, PKCζ that activates adenylyl cyclase V/VI (Halls et al., 2006, 2007a, b). The last 10 amino acids of the RXFP1 C-terminus, specifically reside Arg752 and partly Ser755, represent an absolute requirement for coupling to the Gαβγ-PI3K-PKCζ pathway, and the coupling of the receptor to this pathway is dependent upon the localization in membrane raft microdomains (Halls et al., 2009). In other cell lines, such as human breast cell line T-47D, the increase in cAMP level is monophasic and is completely due to activation of Gαs (Halls et al., 2009).

RLN can activate the synthesis of cAMP in several systems, such as human sperm (Ferlin et al., 2011), the pubic symphysis of mice (Braddon, 1978), rat uterus, where it inhibits the spontaneous contractile activity (Sanborn et al., 1980), cultured rat myometrial cells (Hsu et al., 1985), human endometrial cells (Chen et al., 1988; Fei et al., 1990), breast cancer cells (Bigazzi et al., 1992), and monocyte/macrophage cell line THP-1, where RLN-induced cAMP production promotes THP-1 adhesion and migration (Figueiredo et al., 2006). Relaxin-stimulated cAMP signaling pathway may also occur in signalosomes (Halls and Cooper, 2010), which are macromolecular receptor-linked protein complexes that facilitate the activation of downstream targets. In this case, RXFP1 is pre-coupled to adenylyl cyclase 2 (AC2) through the A-kinase-anchoring protein (AKAP) 79. Stimulation of RXFP1 with RLN activates AC2, leading to cAMP production. This signalosome is negatively regulated by β-arrestin-2, which binds to Ser704 of RXFP1 and recruits the phosphodiesterase PDE4D3 and protein kinase A (PKA) to the protein complex. This non-classic mechanism of cAMP production may explain why circulating relaxin can affect physiological targets where there is minimal production of the hormone.

The stimulation of recombinant or endogenous RXFP1 by RLN can also activate the MAPK pathway. A rapid RLN-induced ERK1/2 activation within 5 min was seen in human endometrial stromal cells, in THP-1 monocyctic cells and in smooth muscle cells from coronary artery (Zhang et al., 2002). However, this effect may be cell type dependent, because the RLN-induced ERK1/2 activation in rat myofibroblasts involved both a rapid (2 min) and a smaller but sustained (50 min) component (Mookerjee et al., 2009), and in endothelial cells of umbilical vein and in HELA epithelial cells, RLN stimulated the ERK1/2 pathway only after much longer periods of stimulation (45-90 min; Dschietzig et al., 2003).

RLN stimulates NO production to induce vasodilation. This may involve a PI3K/AKT-mediated activation of endothelial nitric oxide synthase 3 (NOS3), or a PKA-mediated phosphorylation and inhibition of IkB, causing activation of nuclear factor kB (NFκB) and an increase of nitric oxide synthase 2 (NOS2) gene transcription (Nistri and Bani, 2003). In addition, NO production may be involved in the RLN-mediated inhibition of differentiation of renal myofibroblasts (Mookerjee et al., 2009).

Relaxin in male reproduction

The role of RLN in male reproduction is still unclear (Ivell et al., 2011). It was initially thought that RLN was mainly produced by the prostate and released to the seminal fluid to affect sperm motility (Sasaki et al., 2001; Kohsaka et al., 2003; Sherwood, 2004). For example, antiserum against RLN reduced sperm motility (Sarosi et al., 1983), RLN stimulated sperm motility and attenuated the decline in the percentage of motile spermatozoa (Essig et al., 1982), helped restore decreased sperm motility (Lessing et al., 1986), and improved penetration of spermatozoa.
into cervical mucus (Brenner et al., 1984). Finally, Carrell et al. (1995) demonstrated that recombinant RLN binds to sperm with high affinity. On the other hand, Jockenhövel et al. (1990) and Newinger et al. (1990) failed to find an effect of RLN on sperm function. However, it has been found recently that RXFP1 is expressed in human spermatozoa and that RLN stimulates sperm motility, mitochondrial function, apoptosis, capacitation, acrosome reaction (Gianesello et al., 2009; Ferlin et al., 2011; Miah et al., 2011), providing additional evidence that RLN is important for fertilizing ability and preservation of sperm functionality.

With the recent availability of knockout animals for RLN or its receptors it has been possible to establish the physiological importance of this hormone. RLN has an antifibrotic effect in several tissues, and the RLN knockout mouse is a model of fibrosis (Samuel et al., 2003a, b, 2005). RLN interferes with collagen metabolism and increases the expression and activity of metalloproteinases (MMPs) in uterine, cardiac, vascular and renal tissues (Lenhart et al., 2001; Jeyabalan et al., 2003, 2007; Mookerjee et al., 2005).

In the reproductive tract of female mice, the disruption of the Rln or the Rxfp1 gene causes the same abnormalities: an absence of the relaxation and elongation of the interpubic ligament and impaired nipple development (Zimmermann et al., 1999; Kamat et al., 2004). In the male reproductive system, however, studies with knockouts of Rln (Rln−/−) or Rxfp1 (Rxfp1−/−) genes are controversial (Ivell et al., 2011). Studies from Samuel et al. (2003a, b, 2005, 2007) with Rln−/− suggest that RLN plays a major role in growth and development of the male reproductive system. These authors show that the Rln−/− mice have smaller testis, epididymis, prostate, seminal vesicle, decreased sperm maturation, and decreased epithelial proliferation in the prostate (Samuel et al., 2003a, b, 2005). They also report notable changes in the extracellular matrix of the testis and prostate in Rln−/− mice, and increased apoptosis. Another Rln knockout developed by Ganesan et al. (2009) however failed to show similar effects. The consequences of the disruption of the Rxfp1 gene are not clear either. Kamat et al. (2004) did not find abnormalities in the testes and prostate of Rxfp1−/− mice, whereas Krajen-Franken et al. (2004), using a different strain of knockout mice, observed impaired spermatogenesis, leading to azoosperma and reduced fertility in animals from the first generations, even though the following generations or older animals had normal fertility.

Although most RLN production in the male reproductive system occurs in the prostate, studies from ours and other laboratories have demonstrated that testes are also a source of RLN (Gunnersen et al., 1995; Kohsaka et al., 2009; Cardoso et al., 2010; Kato et al., 2010). It seems that the main site of relaxin expression in the testes varies depending on the species. While RLN in the rat is mainly found in the seminiferous epithelium and is absent in the interstitial compartment, (Cardoso et al., 2010; Fig. 1A and 1B), in the boar testis RLN expression is only found in Leydig cells (Kato et al., 2010).

Figure 1. Immunolocalization of relaxin in testicular cells using anti-rat relaxin antibody (ab70803, Abcam; 1:100 dilution). (A) Testis section from immature, 15-day old rats; (B) Testis section from adult, 120-day old rats. RLN immunoreactivity (asterisk) was observed in the seminiferous epithelium but not in the interstitial compartment. (C) Co-culture of Sertoli and germ cells from 7-day old animals, after 7 days in culture. RLN immunoreactivity was observed in Sertoli cells (asterisk), and in germ cells (circle). (D) to (F) Primary culture of Sertoli cells from 15-day old animals, after 4 days in culture. (D) Nuclei were stained with DAPI (blue); (E) Relaxin immunofluorescence (red) was detected with Alexa Fluor 594-labeled IgG; (F) Merged image. Bars = 10 μm. Insets show negative controls incubated in the absence of the primary antibody. (A) and (B) were reproduced from Cardoso et al. (2010). RLN mRNA levels are higher in testis of immature than adult rats (Cardoso et al., 2010). Sertoli cells of immature 15-day-old rats represent an important source of Rln mRNA, and relaxin precursor is expressed in cultured Sertoli cells from 15-day-old rats (Fig. 1D-1F). Furthermore RLN induces proliferation of cultured rat Sertoli cells, suggesting an autocrine/paracrine role for RLN in testis (Filonzi et al., 2007; Cardoso et al., 2010). We have recently investigated the signaling pathways involved in the proliferative effect of RLN in a primary culture of...
Sertoli cells from immature rats. In this system, the mitogenic effect of RLN involved activation of both the MEK/ERK1/2 and the PI3K/AKT pathways (unpublished data). Furthermore, since RLN stimulates NO production in several systems by a PKA-mediated phosphorylation and inhibition of IkB, causing activation of nuclear factor κB (NFκB) and an increase of gene transcription of inducible NOS (Nos2; Nistri and Bani, 2003), we investigated the effect of RLN on the mRNA levels of Nos2 in the primary culture of rat Sertoli cells. Preliminary experiments of real time RT-PCR have shown that RLN tended to increase the expression of Nos2 (Fig. 2). This result is intriguing because studies with Nos2−/− mice have demonstrated that NOS2 plays an important role in the regulation of somatic cell number in the testis, with an impact on survival of germ cells (Auharek et al., 2011).

Figure 2. Relative mRNA level of Nos2 in primary culture of Sertoli cells from 15-day old rats. Cells were treated with recombinant human relaxin (Phoenix Pharmaceuticals) for 2 and 4 hours. The level of Nos2 mRNA was analyzed by quantitative RT-PCR as previously described (Cardoso et al., 2010). Results are expressed as means ± S.E.M. of 6 independent experiments (6 different cultures).

We have previously shown that mRNA for RXFP1 is widely distributed in the reproductive system of the male rat, with testes and vas deferens having the highest levels (Filonzi et al., 2007). In the testis, immunohistochemistry showed expression of RXFP1 in Sertoli and post-meiotic germ cells (elongated spermatids). In addition, Kato et al. (2010) characterized by semi-quantitative RT-PCR and immunohistochemistry the expression of RXFP1 in Leydig and Sertoli cells of the boar testis during postnatal development. RXFP1 transcripts have also been detected in the rhesus monkey testis (Silvertown et al., 2010). In the vas deferens RXFP1 immunoreaction was detected in the smooth muscle layer and apical part of epithelial cells, suggesting a role in secretion and composition of the seminal plasma (Filonzi et al., 2007). After incubation with vas deferens slices, RLN increased the tissue level of matrix metalloproteinase 7 mRNA (Filonzi et al., 2007), suggesting that RLN may be involved in collagen and matrix remodeling and/or apoptosis. This suggestion seems consistent with the general role of RLN as a regulator of collagen biosynthesis in several tissues.

The role of RLN receptors in the adult testis remains to be determined. Since RXFP1 receptors are expressed in germ cells during specific stages of the development, one may speculate that RLN participates in the spermatogenic process. The localization of RXFP1 receptors in Sertoli cells further supports the idea that the hormone plays a role in spermatogenesis. To investigate the role of RLN at specific stages of spermatogenesis we developed a co-culture system of Sertoli and germ cells from 7-day old rats. After 7 days of culture, we observed the differentiation of both Sertoli and germ cells, and the expression of RLN (Fig. 1C) and RXFP1 (not shown). This co-culture provides an attractive tool to characterize the role of RLN in spermatogenesis.

RLN seems to play a role in prostate cancer progression (Thompson et al., 2006; Feng et al., 2007). H1-RLN was upregulated in tumors compared to normal prostate tissue (Welsh et al., 2003). The transcriptional regulation of RLN in the prostate has not been elucidated yet. The RLN promoter is positively regulated by androgens in the PC3 prostate cancer cell line transfected with the androgen receptor, but not in LNCaP and non-prostate cells such as liver, kidney, bladder, lung, breast and ovarian cells (Brookes et al., 1998). In addition, castration of rats drastically inhibits the Rh mRNA levels in the prostate, and this is recovered by treatment of the castrated animals with testosterone (Cardoso et al., 2010).
Concluding remarks

Although insight in the role of RLN and RXFP1 has improved significantly in several systems, relatively little is known about the role of RLN in male reproduction. RLN is released in the seminal plasma and affects semen function. More recent studies show that relaxin is present throughout the male reproductive tract, and its colocalization with RXFP1 suggests that RLN may act as an autocrine or paracrine factor. The control of RLN expression is poorly understood, and the interplay between RLN and other local factors and hormones such as testosterone and FSH is not yet clear. Advances in the molecular characterization of RXFP1 structure and signaling might provide important tools for the treatment of reproductive disorders in the male. The better understanding of the role of RLN in progression of prostate cancer may reveal its utility for development of new therapeutic agents.

Acknowledgments

Research in the authors’ laboratory is supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant number 2006/60406-7, 2008/57239-7 and 2010/10274-2 to M.F.M.L.). We thank Espedita M. J. Silva Santos for technical assistance, and Dr. Guus Schoorlemmer for critical reading of the manuscript. C.S.P. and M.F.M.L. were supported by Research fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). A.R.N. and M.T.P were supported by Doctoral fellowships, and T.F.G.L. by a Postdoctoral fellowship from FAPESP.

References

Male rat.


Halls ML, Cooper DM. 2010. Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, b-arrestin 2, PDE4D3 complex. EMBO J, 29:2772-2787.


