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Review

Regulation of the reproductive cycle and early pregnancy by relaxin family peptides

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ABSTRACT

The relaxin family of peptide hormones are structurally closely related to one another sharing a heterodimeric A–B structure, like that of insulin. They may also be active as unprocessed B–C–A pro-forms. Relaxin has been shown to pay a key role within the ovary, being involved in follicle growth, and ovulation. Relaxin is produced in large amounts also by the corpus luteum where it acts as an endocrine hormone positively affecting implantation, placentation and vascularization during the all-important first trimester phase of pregnancy establishment. Relaxin exerts its functions via the receptor RXFP1. Insulin-like peptide 3 (INSL3) in contrast acts through the related receptor RXFP2, and plays an essential role in the production of androgens within growing antral follicles. INSL3 is also produced in large amounts by the male fetus shortly after sex determination, where it controls the first transabdominal phase of testicular descent. However, this fetal INSL3 is also able to influence placental and maternal physiology, indicating associations with later preeclampsia and/or fetal growth restriction. Other members of this relaxin-like family of peptides, such as INSL4, INSL5 and INSL6 are less well studied, though all suggest modulatory roles in ovarian and/or placental function.

Contents

1. Introduction

The relaxin family of peptide hormones, whilst structurally related to insulin and the IGFs, appears to have evolved as a separate branch of informational molecules already very early in evolution. Whilst there appear to be no members of the relaxin-like subfamily in insects and worms, several members have been characterized in vertebrates, and particularly in mammals. In deuterostomes, recent discoveries from starfish now suggest that an ancestral relaxin-3-like molecule was already present in this phylum and most importantly was playing a key role in the maturation and release of oocytes (Mita et al., 2009). Significantly, this

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molecule, called GSS (gonadal stimulating substance), is made within the radial nerves and appears to be performing a role much like the pituitary gonadotropins in other species.

Besides their cladistic similarity, the relaxin-like group of hormones – at least in mammals – is characterized by having receptors which belong to the G-protein coupled receptor (GPCR) family, unlike the insulin branch which all make use of receptor tyrosine kinases. The vertebrate ancestor of the relaxin-like family of peptides, called relaxin-3, is predominantly also a neurohormone, like GSS, and together with the related INSL5 (insulin-like peptide 5), both recognize GPCRs of class A, with small N-terminal extracellular domains (called RXFP3 and RXFP4) (Bathgate et al., 2006). In fish and amphibians, relaxin-3-like molecules are also involved in reproductive processes being highly expressed also in the gonads (Wilson et al., 2009). At some time prior to and concomitant with the emergence of mammals, with their very sophisticated system of viviparity which frees the reproductive process from the arbitrariness of the external environment, the relaxin family of peptide hormones underwent a further radiation. Thus in mammals, particularly in humans, we find altogether seven members of the relaxin hormone family: relaxin-3 and INSL5, predominantly associated with the brain and gut respectively; H1-relaxin, H2-relaxin, INSL3, INSL4 and INSL6, are all associated with reproductive functions specifically linked to viviparity. This has led to the coining of the term 'neohormone' for this group of peptide hormones (Anand-Ivell et al., 2013), which serve specifically mammalian physiologies, though others, such as hCG or interferon-tau, are also members. Significantly, H1-relaxin, H2-relaxin and INSL3 make use of different GPCRs (RXFP1 and RXFP2) from those used by relaxin-3 and INSL5 (INSL4 and INSL6 have as yet no known receptors). These GPCRs are affiliated to the class A, rhodopsin-like GPCRs and are distantly related to the receptors for the glycoproteohormones, LH, FSH and TSH, within subclass C of the LGR (leucine-rich repeat-containing GPCR) family (van Hiel et al., 2012). Thus, from an evolutionary and signaling viewpoint, the relaxin family of peptide hormones shares several features with the hormones of the HPG axis, though unlike these have evolved to accommodate additional functions related to viviparity and internal fertilization.

The present review explores the specific roles of relaxin family peptides in female physiology with emphasis on ovarian function, embryo formation and implantation, and early pregnancy up to the end of the first trimester. The role of these peptide hormones in later pregnancy, in lactation and in male reproductive function have been recently covered in other reviews (e.g. Bathgate et al., 2006; Parry and Vodstrcil, 2007; Ivell et al., 2011) and will not be further discussed here.

2. Relaxin

2.1. Relaxin and ovarian function

The term relaxin is used here to refer to the peptide called H2-relaxin in humans, relaxin-1 in rodents, and its homologs, and is thus distinct from the ancestral neurohormone relaxin-3 and its homologs, or from the recently evolved H1-relaxin found in humans and chimpanzees. Relaxin is the major relaxin-like peptide produced within the ovary of most mammalian species, and the hormone which was first extracted and shown to have relaxing-like properties on the term pubic symphysis in guinea-pigs and other rodents, hence its name. As this function suggests, relaxin is a major product of the corpus luteum of pregnancy, and has been identified in this structure in almost all mammals (except bovids) (Sherwood, 1994).

The corpus luteum develops in every estrous cycle from the mural cells of the ovulating follicle(s), and times its development accordingly from the day of ovulation within the normal cycle. In ruminants and probably also rodents the corpus luteum comprises cells of both the follicular granulosa as well as theca interna layers, following the dissolution of the follicle basement membrane and the LH surge-induced epithelial–mesenchymal-transition (EMT) that these cells then undergo. In humans and other primates, the corpus luteum appears to derive mostly from granulosa cells, with little contribution from the theca cell layer. This is important, because at least in humans and possibly in other species, relaxin in the corpus luteum appears to be made exclusively by the granulosa-derived luteal cells, beginning a few days after ovulation and the EMT. Consequently, in cultured human granulosa-lutein cells collected by aspiration at ovum pick-up following appropriate hormonal stimulation as part of an IVF program, relaxin production and secretion into culture medium occurs only after 6–10 days of culture and in vitro cell differentiation (Stewart and Vandevoort, 1997). In vivo this luteal expression of relaxin would normally be interrupted at luteolysis, and the commencement of a new cycle. If pregnancy occurs, then the corpus luteum is retained and relaxin continues to be produced and secreted by the corpus luteum throughout pregnancy, or as long as the corpus luteum functionally persists. It is luteal relaxin which appears to be the major contributor to circulating relaxin in female mammals, at least during the luteal phase of the cycle and in pregnancy. This is supported by the complete absence of circulating relaxin in ovum donor pregnancies in women with non-functioning or absent ovaries (Johnson et al., 1991).

Why this is important is that relaxin expression can also be detected in theca interna cells of antral follicles in both humans and pigs before the LH surge (Blankenship et al., 1994; Ohleth and Bagnell, 1999), as well as in both cell types after culture with luteinizing levels of the gonadotropin (Ohleth and Bagnell, 1999). Whilst this relaxin probably does not contribute to the circulation it likely is the major contributor to the relaxin detected in follicular fluid (Wathes et al., 1986). Thus within the ovary there appear to be two different sources of relaxin – the theca interna cells of follicles and the corpus luteum.

As mentioned above, relaxin acts primarily via the GPCR called RXFP1 (Bathgate et al., 2006). It may also activate the alternative receptor RXFP2 (which is specific for INSL3), but only in some species such as the human, and then only at highly supraphysiological concentrations. In transfected cells, and in some naturally receptor-expressing primary cells, such as human endometrial stromal cells (Bartsch et al., 2001, 2004; Ivell et al., 2007) or human myometrial cells (Heng et al., 2008), relaxin interacts with RXFP1 to activate G_s-mediated adenylyl cyclase causing an elevation of intracellular cAMP. It may also in some circumstances activate PI3-kinase in a $G_{i/o}$-dependent manner involving PKC-zeta (Nguyen and Dessauer, 2005). Within the ovary, RXFP1 relaxin receptors have been identified at the transcript or protein level on granulosa and cumulus cells of pig antral follicles (Feugang et al., 2011a), and possibly also on oocytes themselves (Feugang et al., 2011b). Moreover, treatment with relaxin of porcine cumulus–oocyte-complexes in vitro, whilst appearing to have little effect on oocyte maturation, did appear to positively influence the resulting embryos (Feugang et al., 2011a; Kim et al., 2010). RXFP1 is also expressed within the corpus luteum of monkeys and cats (Braun et al., 2012; Maseelall et al., 2009), though the precise cellular localization in these tissues has not been ascertained. One report also suggests the presence of RXFP1 in human granulosa cells of primordial, primary and secondary follicles (Shirota et al., 2005a), with relaxin treatment of ovarian cortical fragments leading to development of those follicles. Moreover the same authors show that relaxin treatment of cortical fragments can also cause

increased vascularization (Shirota et al., 2005b). Finally, in rats, treatment with relaxin targeted to the ovarian bursa in vivo appears to promote ovulation (Brännström and MacLennan, 1993), suggesting an additional role of relaxin in the proteolytic induction of follicle wall rupture (Hwang et al., 1996).

In conclusion, therefore, within the ovary relaxin produced by theca and/or granulosa cells of the follicle may act in an autocrine/paracrine fashion to influence granulosa/cumulus cell function, though the precise localization of RXFP1 receptors and the mechanisms of relaxin action still remains relatively obscure. Indirect evidence suggest that relaxin may be involved in oocyte maturation and quality, since in addition to the pre-implantation studies described above, there is a positive association between the subsequent capacity of cultured human granulosa-lutein cells obtained at IVF to generate relaxin and the long-term success of the resulting pregnancy (Stewart and Vandevoort, 1999). Whether relaxin has an autocrine/paracrine role also within the corpus luteum remains to be shown. At this stage nothing is known about the possible signaling mechanisms that may be involved in any of these possible actions of relaxin.

2.2. Relaxin and the early embryo

As mentioned above, exposure of oocytes, cumulus-oocyte-complexes, and early embryos to relaxin appears to be moderately beneficial, in terms of rate of development to blastocyst stage and number of cells in the inner cell mass. This has been shown for porcine (Feugang et al., 2011a) and primate embryos (Vandevoort et al., 2011), and most recently also for cryopreserved cat oocytes (Luu et al., 2013). The natural source of relaxin for such effects in vivo would either be from the ruptured follicle or the low circulating levels deriving from the new corpus luteum, though both sources would not be considerable. A further source is possibly from the oviduct itself (Tang and Chegini, 1995). The blastocyst is also expressing the relaxin-receptor, RXFP1, or shows specific relaxin-binding, in both trophoblast and inner cell mass (Einspanier et al., 2001; Vandevoort et al., 2011). Whether relaxin is acting directly on the embryo and/or trophoblast to aid implantation is not known, though certainly relaxin does appear to influence the receptive endometrium. In addition to these effects on the embryo, it should not be forgotten that relaxin may also have a positive effect directly on spermatozoa to help fertilization (Ferlin et al., 2012).

2.3. Relaxin and the endometrium in early pregnancy

Assessing circulating relaxin profiles in different species shows marked differences. Most such relaxin is derived from the corpus luteum of pregnancy, supplemented in some species later by the placenta, and in rodents and pigs there is a steady increase in relaxin from the first days of pregnancy until term, with maximum levels approaching 100–200 ng/ml. In contrast, in primates and particularly in humans, from being virtually undetectable in the cycle, circulating relaxin tends to peak in the first trimester, with a maximum in women of only about 1 ng/ml. What is the role of this first trimester relaxin? In addition to effects on the implanting embryo itself (see above), the uterus appears to be a major relaxin target, with RXFP1 relaxin-receptors expressed on uterine epithelial cells, on endometrial stromal cells, as well as in the myometrium (Ivell et al., 2007; Parry and Vodstrcil, 2007; Heng et al., 2008). Within the endometrium, the stromal cells appear to be the principal endocrine drivers dictating both the epithelial response as well as uterine remodeling essential for placentation (Ramathal et al., 2010). In vivo during the luteal phase of the cycle and in early pregnancy ovarian hormones such as progesterone, together with estradiol, induce the endometrial stromal compartment first to proliferate and then to differentiate in the process known as decidualisation. This decidualisation is essential to create a receptive uterus into which a blastocyst can implant, and generally takes about 6–10 days to provide the so-called 'window-of-implantation'. In vivo in primates, including women, ovaries can be removed or absent and pregnancy can still occur more or less normally only with additional progesterone supplementation. Endometrial stromal cells derived at hysterectomy from relatively normal cycling women can be induced in vitro to decidualise under progesterone and estradiol influence with a similar time-kinetic of about 6–10 days (Gellersen et al., 2007). However, a similar decidualisation response can also be induced by relaxin alone, acting through RXFP1, but within a much shorter time-frame of only 2–3 days (Ivell et al., 2007). Moreover, studies using monkeys have shown that supplementary relaxin in vivo is able to specifically induce a thickening of the endometrium, improved vascularization, and reduced pregnancy loss (Goldsmith et al., 2004; Hayes et al., 2004; Einspanier et al., 2009), consistent with a very positive impact on implantation and placentation. Also in women, it has been shown that there is a good association between mid-pregnancy levels of circulating relaxin, reduced spontaneous abortion, and increased blood flow (Jauniaux et al., 1994; Anumba et al., 2009).

Following from work by Linda Tseng and others (Bell et al., 1991; Guo et al., 1994), we have explored the signaling pathways used by relaxin in human endometrial stromal cells from the cycle (hESC cells). Whilst superficially relaxin simply causes an elevation of cAMP because of the G_s-mediated stimulation of adenylyl cyclase, as can be seen also in RXFP1-transfected over-expressing HEK293T cells (Bartsch et al., 2001; Hsu et al., 2002; Halls et al., 2006; Ivell et al., 2007; Heng et al., 2008), relaxin's impact in the naturally receptor-expressing cells is more complex. Firstly, it additionally causes a modest induction of type 4 phosphodiesterases (Bartsch et al., 2004), in part to act as a feedback mechanism to regulate intracellular cAMP. More importantly, however, relaxin-stimulated activation of adenylyl cyclase in these cells is absolutely dependent on a specific tyrosine-kinase activity (Bartsch et al., 2001; Ivell et al., 2007), which is unlike the activity of any other GPCR. In a recent microarray analysis on hESC cells stimulated by relaxin for 3 days (Ivell et al., unpublished) we have shown that relaxin not only activates cAMP and downstream protein-kinase A targets, but also specifically activates the Wnt5a/TWIST morphogenetic pathway with concomitant down-regulation of the Wnt inhibitor dikkopf-1. At the same time it down-regulates a series of cell cycle genes, thereby switching the hESC cells from a proliferative to a non-proliferative phenotype. Thirdly, relaxin activates a series of cytokine-dependent genes, such as CXCL12, at the same time suppressing genes otherwise regulated by PI3-kinase. Finally, relaxin action induces a series of TGFß-dependent genes. Altogether, this pattern of gene regulation is reminiscent of what happens during an EMT (epithelial-mesenchymal transition), is largely in concordance with what occurs during decidualisation, and cannot be explained simply by an elevation of intracellular cAMP. Importantly, this is occurring in the absence of progesterone.

In the myometrium, it is long established that in some species, such as rat and pig, relaxin acts directly on smooth muscle cells to induce quiescence, effectively blocking both spontaneous and oxytocin-induced contractions (MacLennan et al., 1986). Whilst this is generally considered in the context of the pre-term uterus where preemptive labor is deleterious, it is also reported to be a property of the early pregnant uterus. Here it is thought that, by inhibiting myometrial contraction, relaxin may have a positive effect on implantation. Additionally, in the rat it has been shown that relaxin acting on the myometrium may be involved in spacing of embryos in multiparous species (Rogers et al., 1983). In the human, relaxin does not appear to have any effect to inhibit spontaneous

or oxytocin-induced contractility (MacLennan et al., 1986), although we have recently shown that human myometrial cells obtained at routine hysterectomy do indeed respond via RXFP1 to relaxin, and just like hESC cultures generate cAMP in a tyrosine kinase-dependent manner (Heng et al., 2008).

2.4. Relaxin and the cardiovascular system

Pregnancy represents a major physiological disruption from the point of view of fluid and osmolyte control in the body. In order to cope with this new situation set-points for vasopressin secretion, heart stroke volume, vasodilation, and arterial compliance are readjusted. Largely based on experiments in rats, this shift appears to be achieved uniquely by circulating relaxin (Conrad, 2011). It is now established that relaxin can directly act on both arterial smooth muscle cells as well as on endothelial cells. The former slow response involves the production of metalloproteinases, and the generation of endothelin, which then acts via endothelin receptors to activate cGMP and NO (Conrad, 2011). Additionally, there is a rapid relaxin response in these vessels involving RXFP1 coupling to PI3-kinase and the phosphorylation/activation of eNOS (McGuane et al., 2011).

Besides the cardiovascular properties of relaxin discussed above, relaxin is also known as an inducer of angiogenesis in the context of wound-healing (Unemori et al., 2000; Segal et al., 2012), as well as in other tissues subject to growth stimulus such as the endometrium during early pregnancy (e.g. Goldsmith et al., 2004). It appears to achieve this by a local induction of vascular endothelial growth factor, VEGF, for example by endometrial stromal cells (Unemori et al., 1999). Moreover, the property of relaxin to encourage extracellular matrix turnover, and thus to be a permissive factor in the invasion of blood vessels, has led to a discussion as to whether relaxin is a positive or rather a negative factor in regard to tumor expression of relaxin, for example in the context of breast or prostate cancer and their metastasis (Klonisch et al., 2007).

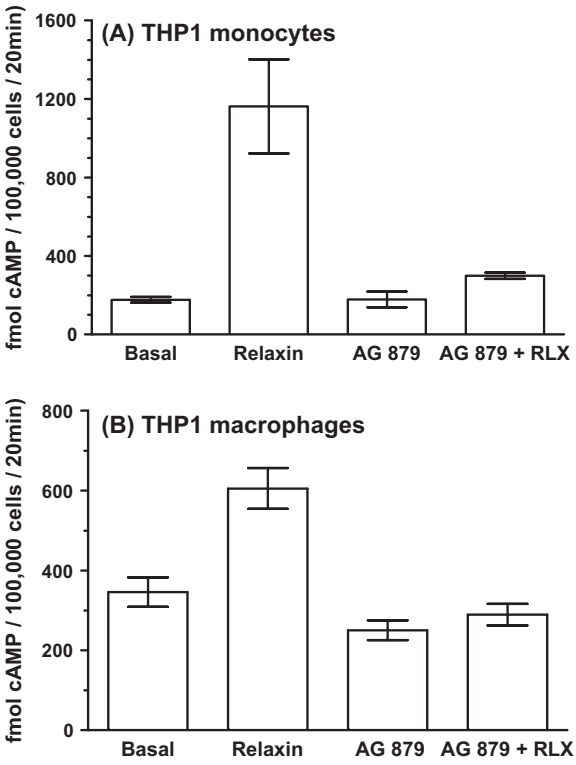

Fig. 1. Relaxin (100 ng/ml) stimulates cAMP production in THP1 monocytes (A) as well as in THP1 macrophages, obtained from the former by treatment with 100 nM phorbol myristate acetate. Moreover, relaxin-dependent cAMP production is completely inhibited in both cell types by incubation with the tyrosine kinase inhibitor tyrphostin AG879 (10 μM).

2.5. Relaxin and the immune system

One of the best used cell models to explore relaxin signaling in cells, where the RXFP1 receptor is naturally expressed, is the human monocyte cell-line THP1 (Anand-Ivell et al., 2007). These cells appear to show a biphasic relaxin-dependent signaling mechanism. Within minutes, relaxin first appears to engage the classical Gs-dependent pathway with induction of adenylyl cyclase (Anand-Ivell et al., 2007; Ivell et al., 2007), though very quickly it subsequently shifts to the PI3-kinase pathway, and PKC-zeta translocation (Nguyen and Dessauer, 2005; Halls et al., 2007). As for human endometrial stromal cells, also this activation of adenylyl cyclase is absolutely dependent on a very specific tyrosine kinase activity (Bartsch et al., 2001; Anand-Ivell et al., 2007). Not only these monocytes are relaxin-responsive, but in vitro induction of their differentiation into macrophages by phorbol esters, also leads to a relaxin-responsive cell type (Fig. 1; Anand-Ivell et al., unpublished). It should therefore be no surprise that relaxin is able to stimulate a number of immune cell types, particularly in the endometrium (Piccinni et al., 1999, 2006). It has been shown that relaxin encourages the activation of resident T cells into Th1-like effectors producing IFNgamma (Piccinni et al., 1999), which in turn has been shown to assist in the remodeling of spiral arteries (Monk et al., 2005). Recently, we have been able to show that relaxin in the uterine lumen of mice is specifically able to induce a number of pro-inflammatory cytokines, including CXCL1 and CXCL10 (Glynn, Ivell et al., unpublished), though the precise mechanisms involved are not yet clear. Importantly, however, together these results show that relaxin in the endometrium is a potent modulator of the pro-inflammatory cytokine network, which is important for appropriate implantation and placentation (Granot et al., 2012).

3. INSL3

3.1. INSL3 in the ovary during the cycle and early pregnancy

INSL3 (insulin-like peptide 3) is structurally very closely related to relaxin, and for this reason was originally referred to as the relaxin-like factor (RLF; Büllesbach et al., 1999; Ivell, 1997). It was originally identified as a major product of the testicular Leydig cells in the male by independent differential cloning strategies (Adham et al., 1993; Pusch et al., 1996). In the female, it is produced in much lower amounts than in the male, but in the equivalent cells to those in the male, namely in the theca interna cells of ovarian antral follicles from cows, monkeys and humans (Bathgate et al., 1996; Bamberger et al., 1999; Hanna et al., 2010), as also in the corpus luteum (Bathgate et al., 1996; Balvers et al., 1998). Although no information is available for females, in males it appears to circulate as both the heterodimeric A–B peptide, as also the larger unprocessed B–C–A pro-form retaining the C (connecting) peptide (Minagawa et al., 2012; Ivell et al., 2013; Siqin et al., 2013). Both forms appear to be fully bioactive. Immunohistochemical studies suggest that INSL3 is expressed almost exclusively by the theca cells of growing healthy antral follicles. In the cow, no staining is observed in secondary follicles, and there is a negative correlation with signs of atresia (Irving-Rodgers et al., 2002). In bovine theca cell primary cultures INSL3 appears to be stimulated by low concentrations of LH but is inhibited by high luteinizing concentrations of the gonadotropin (Bathgate et al.,

1999), supporting studies at the mRNA level which suggest that INSL3 gene expression is inhibited in vivo by the LH surge (Bathgate et al., 1996, 1999). However, more recent studies suggest no effect of low LH concentrations (Glister et al., 2013). In vivo INSL3 mRNA appears to recover expression in the corpus luteum, at least in the cow (Bathgate et al., 1996, 1999).

An INSL3 knockout mouse has been described by two independent groups, and indicates a significant female reproductive phenotype, with reduced numbers of antral follicles, fewer corpora lutea, and smaller litter sizes, together implying an importance for INSL3 in promoting the numbers of growing antral follicles and resulting ovulations (Nef and Parada, 1999; Spanel-Borowski et al., 2001). Together with the immunohistochemical studies on bovine follicles (Irving-Rodgers et al., 2002), as well as some studies in the male (Amory et al., 2007; Kawamura et al., 2004), the implication is that INSL3 acts in an anti-apoptotic/pro-survival role within growing antral follicles.

INSL3 interacts with a Class A GPCR with a large extracellular domain, referred to as RXFP2 (Bathgate et al., 2006). This receptor, though very similar to the relaxin-receptor RXFP1, cannot be activated in vivo by any other known peptide, not even relaxin (Bogatcheva et al., 2003), making the INSL3-RXFP2 ligand-receptor pair unique. Human RXFP2 may respond to relaxin in vitro, but only at very high concentrations not found in vivo. Within the ovary, RXFP2 has been identified at the mRNA level by RT-PCR in oocytes as well as in the theca interna cells themselves (Glister et al., 2013). It is absent from granulosa or cumulus cells. Functionally, it appears that INSL3 is involved in an autocrine/paracrine short-loop feedback system regulating the production of androgens, particularly androstenedione, by theca cells (Glister et al., 2013). Down-regulation of RXFP2 expression in theca cells using antisense RNA suppresses androgen production (Glister et al., 2013). Moreover BMPs generated within the antrum from granulosa cells and/or the oocyte also suppress theca cell INSL3 expression and thus help to regulate follicular androgen production (Fig. 2). It should be remembered that theca cell androgen production is a major rate-limiting step in ovarian steroidogenesis during the follicular phase, since estrogens can only be made within the follicles by aromatase in the granulosa cells converting imported androgens.

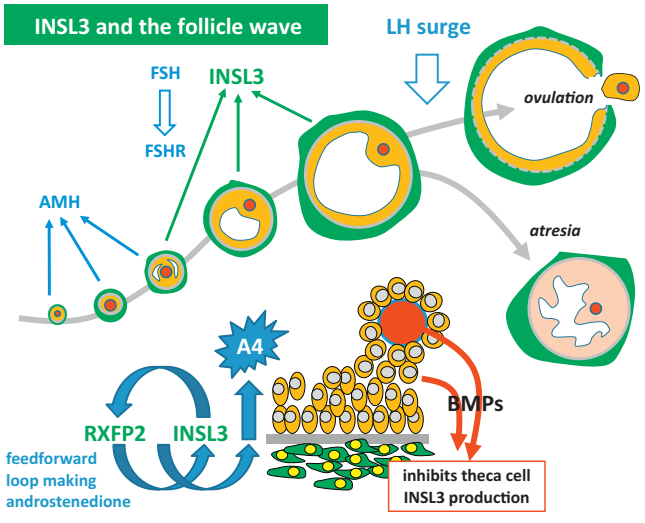

Fig. 2. The role of INSL3 within the growing antral follicle. INSL3 is produced by the follicular theca interna cells prior to the LH surge and following the production by these follicles of anti-mullerian hormone, AMH. Within the antral follicle Bone Morphogenetic Proteins (BMPs) from the granulosa cells (yellow) and/or the oocyte (red) inhibit INSL3 production, which itself via its receptor RXFP2 stimulates the production of the main androgen, androstenedione (A4) by the theca cells (green).

Although INSL3 in circulating blood is much lower in women than in men, it is nevertheless mostly measurable (ca. 0–100 pg/ml) and appears to reflect the growth of antral follicles (Anand-Ivell et al., 2013). In cows, blood concentrations are a little higher (Anand-Ivell et al., 2011; Satchell et al., 2013), and in in heifers whose ovarian cycle has been synchronized by a luteolytic dose of prostaglandin F2alpha, INSL3 rises to a coordinated peak reflecting the growth of an antral follicle wave (Satchell et al., 2013). Such experiments also confirm that antral follicles are indeed the major, if not exclusive, source of circulating INSL3 within the non-pregnant female mammal. Because INSL3 is produced largely by growing antral follicles, circulating levels are influenced by the number of such follicles in the ovary. Thus circulating INSL3 is significantly elevated in women with diagnosed PCOS (polycystic ovarian syndrome) (Havelock et al., 2005; Gambineri et al., 2007; Anand-Ivell et al., 2013), and is reduced in women with low ovarian reserve (Anand-Ivell et al., 2013), or who are peri- or post-menopausal (Ivell and Anand-Ivell, 2009). We are currently following up such studies to determine whether INSL3 can become an important new clinical parameter to assess follicle growth and development in women of reproductive age.

Although, functions for INSL3 in the female mammal are currently focused on ovarian physiology, circulating concentrations are still sufficiently high to be able to activate RXFP2 receptors in distant organs. In this context it is important to observe that recently INSL3 by acting on osteoclasts has been shown to have a significant role in the context of bone turnover and metabolism (Ferlin et al., 2008). Indeed, where the RXFP2 receptor (human subjects with a homozygous RXFP2 mutation) or its ligand INSL3 (INSL3 knockout mice) are dysfunctional, then there is a markedly increased osteopaenia (Ferlin et al., 2008). Thus in addition to estradiol, INSL3 is a further player in women of reproductive age contributing to the maintenance of healthy bone metabolism, and whose loss following the menopause may contribute to the increased prevalence of osteoporosis in older women.

3.2. INSL3 in the early fetus and amniotic fluid

We know very little about any role for INSL3 in the early pre-implantation embryo, or indeed in the endometrium or myometrium at any stage of reproductive life. Human myometrial cells do not appear to respond to INSL3, although they do possess full-length RXFP2 receptor mRNA (Heng et al., 2008). However, it is becoming very clear that the male fetus is a major producer of INSL3 already by mid-gestation. INSL3 is made by the fetal-type Leydig cells (FLC) of the embryonic testis very shortly after SRY-dependent sex determination. In humans, this INSL3 can be detected in amniotic fluid already at 12 weeks of gestation (Anand-Ivell et al., 2008), and the timing of INSL3 production in the fetus coincides with the first transabdominal phase of testicular descent (Anand-Ivell et al., 2008; Hughes and Acerini, 2008). In fact the failure of the testes to descend (cryptorchidism) is the sentinel phenotype of INSL3- as well as RXFP2-knockout mice (Nef and Parada, 1999; Zimmermann et al., 1999; Kamat et al., 2004).

The early first–second trimester fetus has very permeable skin, such that hormones produced within the fetus early in gestation are clearly measurable also in amniotic fluid. In human amniotic fluid obtained at routine amniocentesis we can measure quite considerable amounts of INSL3 of fetal testis origin (it is not detectable in amniotic fluid from female fetuses) between weeks 12 and 18; thereafter it appears to decline to baseline (Anand-Ivell et al., 2008). We can also detect INSL3 in amniotic fluids from rats and ruminants (Anand-Ivell et al., unpublished). Why this is important is that we were able to show for male fetuses a significant relationship between the amniotic fluid level of INSL3 and later onset of preeclampsia and/or reduced corrected birthweights (Anand-Ivell

et al., 2008). Such findings imply an interaction between fetal INSL3 and placental function in the mother early in gestation at a time when the placental bed is being established. More recently, in a study using pregnant cows, it was possible to show that INSL3 of fetal origin was definitely able to cross the placenta to enter the maternal circulation (Anand-Ivell et al., 2011), again implying that at least in the first half of gestation fetal INSL3 is potentially able to influence maternal and/or placental physiology. We are currently looking at possible ways that INSL3 can gender-specifically influence this important establishment phase of pregnancy, which has been shown to be decisive for the health and wellbeing of all offspring (McMillen and Robinson, 2005).

4. Other insulin/relaxin-like peptides and female reproduction

Although INSL3 and relaxin are the most studied members of this relaxin-like family of peptides, at least two other members are also thought to be of physiological importance for reproduction, namely INSL4 and INSL6. For neither peptide has a receptor yet been identified, though both have been shown to possess specific functionality.

INSL4 (previously called EPIL or early placental insulin-like) is made in the human placenta and evidently evolved as a paralogue from the relaxin gene with which it remains syntenous (Ivell and Grutzner, 2009; Arroyo et al., 2012). INSL4 is made in large amounts by the syncytiotrophoblast and maternal decidua (Laurent et al., 1998; Millar et al., 2005), and experiments on amniotic epithelial cells suggest that unlike relaxin it has a pro-apoptotic role (Millar et al., 2005), a view which is strengthened by its increased expression in placentae of growth-restricted fetuses (Millar et al., 2005). INSL6 is also a paralogue of relaxin and in the male is expressed in substantial amounts by meiotic and post-meiotic germ cells (Lok et al., 2000; Ivell et al., 2011). Indeed a recent INSL6-knockout mouse shows a marked male infertility phenotype (Burnicka-Turek et al., 2009). Much less is known about INSL6 in the female. The INSL6 knockout mouse indicates only a male reproductive phenotype, with no impairment of female fertility (Burnicka-Turek et al., 2009). Although it is expressed largely by germ cells in the testis, for the female gonad, GEO microarray records suggest expression of INSL6 rather in the somatic cell compartment. However, a targeted and systematic analysis of INSL6 expression and function in the female is lacking.

Although we know least about its physiology, H1-relaxin is a paralogue of ovarian relaxin found in the placenta only in women and higher apes (Hansell et al., 1991). Because of its very close structural similarity to relaxin, not only can it activate RXFP1, and thus behave like ovarian relaxin, most immunoassays cross-react, thus preventing an understanding of any discrete functionality.

For the remaining members of the relaxin-like family of peptides, INSL5 and relaxin-3, their main physiological roles appear to be predominantly as gut or brain hormones, respectively. However, the INSL5 knockout mouse displays impaired fertility in both males and females (Burnicka-Turek et al., 2012). In the female there appear to be no significant differences from wild type in regard to numbers of oocytes ovulated, follicle number or number of corpora lutea. Instead there is a markedly perturbed ovarian cyclicity suggesting a systemic impairment of ovarian control, possibly linked to the marked distortion of glucose homeostasis in these genetically modified mice. The relaxin-3 knockout mice show no impairment of either male or female fertility (Smith et al., 2009). However, there is a suggestion that relaxin-3 can modulate the HPG axis, presumably acting at the hypothalamus (McGowan et al., 2008). Moreover, there is a single report, using an unvalidated assay, suggesting circulating levels of relaxin-3 in women as high as 100–200 ng/ml (Ghattas et al., 2013), with a slight increase associated with metabolic syndrome. Why this is significant is that at this concentration, which is markedly higher than that for ovarian relaxin in women (maximally 1 ng/ml), circulating relaxin-3 is likely to be able to activate peripheral RXFP1 receptors, for which it is a cognate ligand (Hossain et al., 2011). There is no information about the source of this circulating relaxin-3, though highest relaxin-3 gene expression occurs in the brain. There may also be some local expression of relaxin-3 in organs of the female reproductive system, particularly in the ovary, since it has been shown to be expressed by Leydig cells in the testes (Silvertown et al., 2010), and in fish relaxin-3 has a major source within the ovary (Wilson et al., 2009). More research is clearly needed on this important topic.

5. Conclusions

Relaxin-like peptides have evolved very much in association with reproductive function, particularly in mammals, where they can be considered as neohormones (Anand-Ivell et al., 2013), and where in both males and females they play key roles in the modulation of reproductive physiology associated with viviparity. In this review, we have highlighted the importance of both relaxin and INSL3 in female reproduction, and drawn attention to substantial gaps in our knowledge of these hormones and of the other members of this peptide family. With increasing availability of new molecular tools, assays and antibodies, specific for these peptides, we should expect to see substantial new advances made in our understanding of their roles and importance, particularly in regard to female reproductive physiology.

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