Locally produced relaxin may affect testis and vas deferens function in rats

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Abstract

We have previously shown that the rat testis and vas deferens contain high levels of the relaxin receptor, RXFP1. The present study was undertaken to determine the expression of relaxin in these tissues, and the effect of exogenous relaxin on Sertoli cell proliferation and on the mRNA levels of some proteins that may contribute to epithelial secretion and tissue reorganization in the vas deferens. Relaxin mRNA levels in testis and vas deferens were much lower than in the prostate. Sertoli cells seem to be an important source of relaxin mRNA in testis. Relaxin immunoreactivity was detected in the seminiferous epithelium but not in the interstitial compartment. The relaxin precursor was expressed in the vas deferens, and relaxin immunoreactivity was detected in apical cells of the vas deferens. Castration, but not treatment with the anti-estrogen ICI 182,780, dramatically reduced relaxin mRNA levels in the prostate and vas deferens, and this effect was prevented by testosterone. *Rxfp1* mRNA levels in the vas deferens and prostate were not affected by castration or treatment with ICI 182,780. Exogenous relaxin increased the incorporation of ³H-thymidine in cultured Sertoli cells, and treatment of the vas deferens with 100 ng/ml relaxin increased the mRNA levels for the cystic fibrosis chloride channel (cystic fibrosis transmembrane regulator) about three times, and doubled mRNA levels for the inducible form of nitric oxide synthase and metalloproteinase 7. These results suggest that locally produced relaxin acts as an autocrine or paracrine agent in the testis and vas deferens to affect spermatogenesis and seminal fluid composition.

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Introduction

Relaxin is a 6 kDa peptide with a structure similar to insulin. Relaxin has a wide spectrum of actions (Sherwood 2004, Dschietzig *et al.* 2006). Its role in female reproduction is well known: relaxin is important during pregnancy and in preparation for parturition (Sherwood 2004), and it stimulates growth of the uterus in prepubertal animals (Bagnell *et al.* 1993, Yan *et al.* 2006, 2008). The main source of relaxin in females is the corpus luteum, but relaxin mRNA and/or protein have been detected also in preovulatory follicles (Bagnell *et al.* 1993, Einspanier *et al.* 1997, Ohleth *et al.* 1998), and it has been suggested that relaxin exerts a paracrine or autocrine role in the ovary, promoting follicular growth and ovulation.

Increasing evidence shows that relaxin is important also for male reproduction. In most species, the prostate is the main source of the hormone (reviewed in Sherwood (1994, 2004)). In humans, the relaxin gene and protein are expressed in the prostate (Sokol *et al.* 1989, Gunnersen *et al.* 1996, Garibay-Tupas *et al.* 2000), but relaxin immunoreactivity is also present in the

seminal vesicles and the ampular region of the vas deferens (Yki-Järvinen *et al.* 1983). In the boar, the seminal vesicle seems to be the main source of the hormone (Kohsaka *et al.* 1992), although a relaxin-like antigen may also be present in the testis (Dubois & Dacheux 1978). In the shark, the testis is a major source of relaxin (Steinetz *et al.* 1998). In mice, relaxin mRNA is present in the testis and prostate (Samuel *et al.* 2003a, 2003b). The sources of relaxin in the male rat are controversial. Immunohistochemical studies failed to demonstrate relaxin protein in the testis, prostate, seminal vesicle, and epididymis (Anderson *et al.* 1986), but relaxin mRNA was detected in the prostate and testis (Gunnersen *et al.* 1995).

Initially, it was thought that the relaxin produced in the male reproductive tract would be released exclusively in the seminal fluid to influence sperm motility (Sasaki *et al.* 2001, Kohsaka *et al.* 2003, Sherwood 2004). However, studies with relaxin gene knockout animals (M1RKO) show that relaxin also plays a role in growth and development of the male reproductive system (Samuel *et al.* 2003a). Male M1RKO show retarded growth of the testis, epididymis, prostate and seminal vesicle,

decreased sperm maturation, epithelial proliferation in the prostate, and increased apoptosis in testis and prostate (Samuel *et al.* 2003a). Deposition of collagen is increased in tissues of the male reproductive tract of M1RKO (Samuel *et al.* 2003a, 2005).

While relaxin gene knockout animals have provided a valuable demonstration that relaxin is important for the growth and development of the male reproductive tract, the role of relaxin in the adult remains unclear. We have previously shown that mRNA for the relaxin receptor RXFP1 is widely distributed in the reproductive tract of the male rat (Filonzi *et al.* 2007). Testis and vas deferens were the tissues with the highest levels of *Rxfp1* mRNA. In the testis, RXFP1 immunoreactivity was present in Sertoli cells and elongated spermatids; in the vas deferens, RXFP1 was detected in the smooth muscle layer and in the apical part of epithelial cells. However, relaxin produced in the prostate would have no access to relaxin receptors in the testis and vas deferens, and therefore the role of these receptors remains an enigma. Although relaxin receptors are present in the muscular layer of the vas deferens, they do not appear to affect contractile activity (Filonzi *et al.* 2007).

The present study was done on two tissues of the reproductive tract of the male rat that express high level of RXFP1 receptors: testis and vas deferens. Our study explored: 1) the expression of relaxin mRNA and protein in these tissues; 2) the role of relaxin on Sertoli cell proliferation and on expression of genes that may affect ion transport and tissue reorganization in the vas deferens; 3) the regulation of relaxin expression by maturation and sex steroids. Our results suggest that relaxin in the testis and vas deferens has an autocrine or paracrine role.

Results

Relaxin gene expression in the testis, vas deferens, and prostate

The levels of relaxin transcript in testis and vas deferens from adult animals were similar and markedly lower than in the prostate (quantitative RT-PCR (qRT-PCR), Fig. 1, Newman-Keuls test, $P < 0.05$). Levels of relaxin transcripts were at least as high in primary cultures of Sertoli cells as in the whole testis, suggesting that Sertoli cells are an important source of relaxin.

We evaluated a relaxin antibody (N18, Santa Cruz Biotechnology, San Diego, CA, USA) by immunohistochemistry on two tissues known to produce large amounts of relaxin: prostate from adult rats and ovary from rats on the 21st day of pregnancy (Fig. 2A and B). We observed the expected labeling in the corpus luteum (Fig. 2A) and in the glandular epithelium of prostate (Fig. 2B), in accordance with previously described data (Golos *et al.* 1984, Sokol *et al.* 1989).

Figure 1 Real-time PCR analysis of the amount of relaxin transcript in testis, vas deferens, prostate, and primary culture of Sertoli cells. Values were normalized using β -actin as endogenous control, and are expressed as relative values using the vas deferens as a calibrator of each experiment (means $\pm$ S.E.M. of three independent experiments performed in triplicate). *Indicates a statistically significant difference from the other values ($P < 0.05$, ANOVA followed by Newman-Keuls).

Western blots with the N18 antibody showed a band with an apparent molecular mass of preprorelaxin (21 kDa) in the vas deferens, testis, and in tissues used as positive controls: ovary from pregnant rats and prostate (Fig. 2C and D). The ovary also contained a protein with apparent molecular mass of 42 kDa, which may be a preprorelaxin dimer that was first detected in arteries (Novak *et al.* 2006). In the vas deferens, a protein of about 16 kDa apparent molecular mass, probably related to a prorelaxin, was detected that predominated over the 21 kDa molecular form. The mature 6 kDa peptide was detected only in the prostate (Fig. 2C). Pre-incubation of the primary antibody with lysate of the ovary from a pregnant rat, a source rich in relaxin, completely eliminated the bands of 42, 21, and 16 kDa, and considerably reduced the 6 kDa form (Fig. 2D).

Localization of relaxin in the testis and effect of relaxin on Sertoli cell proliferation

To identify the cells that account for relaxin expression in the testes, immunohistochemistry with an anti-rat relaxin 1 antibody (Abcam, Cambridge, MA, USA) was performed. This antibody recognizes the relaxin precursor but not the mature peptide, and therefore indicates regions of hormone synthesis rather than regions of only storage of the fully processed hormone. In testis from immature 15-day-old animals, relaxin immunoreactivity was mainly detected in Sertoli cells, and no specific immunoreactivity was detected in the Leydig cells or other cells of the interstitium (Fig. 3A and B). In testis from adult 120-day-old rats, relaxin immunoreactivity was present in Sertoli cells and restricted to the seminiferous epithelium (Fig. 3C and D). Pachytene spermatocytes showed strong immunoreactivity, but immunolabeling was also detected in spermatids (Fig. 3C).

Figure 2 (A and B) Immunohistochemical determination of relaxin expression in the ovary of pregnant rat (A) and in the prostate (B) with N18 anti-relaxin antibody (1:100 dilution). Specific labeling (asterisk) was observed in luteal cells of the corpus luteum and in the apical portion of cells of the glandular epithelium of the prostate (arrows). Insets show negative controls incubated in the absence of primary antibody. Scale bars are 10 μ m. (C and D) Western blot analysis of relaxin expression in testis, vas deferens, and prostate with N20 antibody. Panel D shows nonspecific immunoreactions seen after pre-incubation of the primary antibody with a relaxin source (ovary of 21-day pregnant rat). Representative example from three to five independent experiments. Arrows indicate bands that correspond to the relaxin precursors or mature relaxin.

Since relaxin is known to stimulate cell proliferation in the female reproductive tract (Bagnell *et al.* 1993, Zhang & Bagnell 1993, Ohleth & Bagnell 1995, Lee *et al.* 2005, Masters *et al.* 2007), we tested the effect of relaxin on proliferation of Sertoli cells. Treatment with 100 and 200 ng/ml relaxin increased the incorporation of 3 H-thymidine by 33 ± 6 and $59 \pm 8\%$ respectively (Fig. 4). FSH, a classically known stimulator of Sertoli cell proliferation, increased 3 H-thymidine incorporation as expected (by $40 \pm 15\%$ with 50 ng/ml FSH). The effects of relaxin and FSH were not additive or synergistic (Fig. 4).

Expression of relaxin, metalloproteinase 7, and cystic fibrosis transmembrane regulator in the epithelial layer of the vas deferens and effects of relaxin on gene transcription in the rat vas deferens

The presence of relaxin receptors in the epithelial layer of the vas deferens (Filonzi *et al.* 2007) led us to investigate whether relaxin affects the mRNA levels of proteins that may contribute to epithelial secretion and tissue reorganization in the vas deferens. Immunohistochemistry with the anti-rat relaxin 1 (Abcam) showed immunoreactivity in the apical cells of the epithelium of the proximal region of the vas deferens (Fig. 5A).

Interestingly, most of the labeled apical cells had labeling near the basement membrane. No relaxin immunoreactivity was detected in the distal region of the vas deferens, where the apical cells are not present.

Potential targets for regulation by relaxin include metalloproteinase 7 (MMP7, see Filonzi *et al.* 2007) and the cystic fibrosis transmembrane regulator (CFTR), a multifunctional protein that is known to be a cAMP-activated Cl^- channel, and which is important for fluid transport in the excurrent ducts of the male tract (Leung *et al.* 2001, Ruz *et al.* 2004, Pietrement *et al.* 2008). MMP7 immunoreactivity was restricted to the supra-nuclear region of epithelial cells of the vas deferens (Fig. 5B). CFTR immunoreactivity was detected in the epithelial layer of the vas deferens, particularly in the apical region of the epithelial cells and in some microvilli (Fig. 5C).

We next investigated whether relaxin affects the transcription of *Cftr* and *Mmp7*, and two additional genes known to be affected by relaxin in other tissues, *Mmp2* and *Mmp9* (Lenhart *et al.* 2001, Mookerjee *et al.* 2005, Jeyabalan *et al.* 2006, 2007). As shown in Fig. 6A, relaxin increased the *Cftr* transcripts in the vas deferens threefold ($P < 0.05$; $N = 4$). We have previously detected by northern blot that mRNA levels for *Mmp7* were increased by incubation of the vas deferens with 1 μ g/ml relaxin (Filonzi *et al.* 2007). Here, we tested whether lower concentrations of relaxin are effective. There was

Figure 3 Immunohistochemical analysis of expression of relaxin in testes with the anti-rat relaxin 1 antibody (ab70803, Abcam). (A and B) Testes from immature, 15-day-old rats. Specific immunoreaction was observed in Sertoli cells (arrow heads); (C and D) Testes from adult, 120-day-old rats. Specific immunoreaction (asterisks) was observed in the seminiferous epithelium but not in the interstitial compartment (I). Representative example of three different analyses. Insets show negative controls incubated in the absence of the primary antibody. Scale bars are 10 μ m. ES, elongated spermatids; I, interstitium; L, Leydig cells; PS, pachytene spermatocytes; S, Sertoli cells.

Figure 4 Effect of relaxin on proliferation of Sertoli cells measured by incorporation of [methyl-³H] thymidine. Cells were incubated in the absence (control) or presence of FSH (50 ng/ml), relaxin (RLN, 100 and 200 ng/ml), or FSH+RLN (50 and 100 ng/ml respectively) for 24 h. Results were expressed as percentage of incorporation above control (mean $\pm$ S.E.M. of three independent experiments performed in triplicate). Incorporation of [methyl-³H] thymidine in the control was 1441 ± 164 d.p.m./well. * $P < 0.05$ compared to control, Student's *t*-test.

a significant increase in *Mmp7* transcripts after treatment with 100 ng/ml relaxin ($P < 0.02$; $N = 3$; Fig. 6A), but no significant changes were observed in *Mmp2* ($N = 4$) and *Mmp9* ($N = 5$), even with shorter (30 min) or longer (240 min) incubation periods, and higher relaxin concentration (500 ng/ml; not shown). As relaxin is known to increase nitric oxide (NO) production in several tissues (Dschiertzig *et al.* 2006), we tested its effect on the inducible nitric oxide synthase 2 (*Nos2*) of the vas deferens. Relaxin caused a significant increase in the mRNA levels for *Nos2* ($P < 0.05$, $N = 5$, Fig. 6A).

Relaxin affects expression of estrogen receptors in several tissues (Pillai *et al.* 2002, Siebel *et al.* 2003, Yan *et al.* 2008), but *in vitro* treatment of the vas deferens with 100 ng/ml relaxin did not significantly change the levels of transcripts for the estrogen receptors *Esr1*, *Esr2*, and *Gper* (Fig. 6B). Shorter and longer incubation periods (30 and 240 min) also failed to alter estrogen receptor transcript levels (not shown). Relaxin tended to down-regulate the mRNA level of its own receptor, but this effect was not statistically significant (Fig. 6B).

Regulation of the gene transcription for relaxin and its receptor (*Rxfp1*)

Although the mRNA levels for *Rxfp1* in testis were not different between 15- and 120-day-old rats, levels of relaxin transcripts in the testis from adult 120-day-old rats were much lower than in testis from immature 15-day-old rats (Fig. 7A). This prompted us to investigate the role of steroid hormones on the regulation of relaxin and relaxin receptor *Rxfp1* gene expression. As shown in Fig. 7B, 15 days after castration, expression of the relaxin gene in the prostate was abolished, and it was significantly reduced in the vas deferens. The inhibition of relaxin gene expression caused by castration was completely prevented by supplementation with testosterone. On the other hand, treatment of the animals with the estrogen receptor antagonist ICI 182,780 did not change the relaxin gene expression in the prostate (control = 0.8 ± 0.1 ; ICI = 0.7 ± 0.4 ; $N = 3$) or in the vas deferens (control = 1.0 ± 0.2 ; ICI = 0.9 ± 0.1 ; $N = 3$).

We also investigated whether castration, which drastically reduces the mRNA levels of relaxin, affects the mRNA levels of the relaxin receptor. Neither castration nor treatment with testosterone affected the *Rxfp1* mRNA levels (Fig. 7B). Blockade of the action of estrogen by treatment with the estrogen receptor antagonist ICI 182,780 did not cause any significant changes in *Rxfp1* gene transcription (not shown).

Discussion

The present study provides evidence that relaxin in the male reproductive tract of the rat is not only produced by the prostate, but low levels of the hormone are also synthesized by the testis and the vas deferens. The lower levels of the relaxin mRNA in testis and vas deferens compared with prostate contrast with the higher levels of the relaxin receptor *Rxfp1* mRNA previously found in those tissues (Filonzi *et al.* 2007). Locally produced relaxin may affect in an autocrine or paracrine way processes such as proliferation, fluid secretion, and NO synthesis.

Figure 5 Immunohistochemical localization of relaxin, metalloproteinase 7, and CFTR in the vas deferens of 120-day-old rats. (A) Relaxin-specific immunoreaction with the anti-rat relaxin 1 antibody (ab 70803, Abcam) was observed in the apical cells (arrows) of the epithelium of the proximal vas deferens and lower levels in the basal portion of the epithelial layer (arrow heads). (B) MMP7-specific immunoreactivity in the supranuclear region of the epithelial cells (arrows) in a cross section of the distal region of the vas deferens. (C) CFTR-specific immunoreactivity in the apical region of the epithelial cells and in microvilli (arrows), in a cross section of the distal vas deferens. Representative examples of three different analyses. Insets show negative controls incubated in the absence of the primary antibody. Scale bars are 10 μ m.

Figure 6 Real-time PCR analysis of the effect of relaxin on gene transcription in the vas deferens of 120-day-old rats. (A) Relative mRNA levels of *Ctr*, *Nos2*, *Mmp7*, *Mmp2*, and *Mmp9*; (B) relative mRNA levels of relaxin (*Rxfp1*) and estrogen receptors (*Esr1*, *Esr2*, and *Gper*). One vas deferens from each rat was incubated for 120 min at 35 °C with 100 ng/ml relaxin, and the contralateral was incubated in the absence of relaxin (control). Values were normalized with β -actin expression. Control value (=1) is indicated by dotted lines. Results are means $\pm$ S.E.M. of three to five independent experiments performed in triplicate. * P < 0.05 compared to control (Student's t -test).

Relaxin is a 6 kDa peptide that is produced in a way similar to insulin from a prehormone (for review, see Dschietzig *et al.* (2006)). There is evidence that the relaxin precursors are as active as the mature peptide (Soloff *et al.* 1992, Zarreh-Hoshyari-Khah *et al.* 2001). In the present study, molecular forms that may correspond to relaxin precursors were detected by western blot analysis in the ovary, prostate, testis, and vas deferens, but the mature relaxin peptide could be detected only in the prostate, suggesting levels of mature relaxin were below the detection limit in the other tissues. Novak *et al.* (2006) detected the mature peptide in the ovary of rats from late pregnancy. The difference with our result may be due to differences in the ability of the antibodies to detect low levels of the hormone or to differences in the stage of pregnancy. Levels of relaxin precursors are 30 times higher than that of the mature protein on day 20 of the pregnancy, but they decline sharply by the end of pregnancy (Crish *et al.* 1986, Soloff *et al.* 1992).

The variation in relaxin and relaxin precursor ratios in different tissues suggests that the mechanism or the rate of processing of preprorelaxin can vary. It remains to be determined whether the prominent 16 kDa band in the vas deferens corresponds to the 16 kDa

prorelaxin described by others (Soloff *et al.* 1992). If this is the case, the vas deferens would offer an opportunity for the study of the processing and function of this relaxin precursor.

The levels of relaxin gene expression in the various target tissues are always very low, and subjected to regulation at transcriptional and post-transcriptional levels (Garibay-Tupas *et al.* 2004). This tight regulation seems to be characteristic of many genes that encode hormones with autocrine and paracrine roles. The factors that regulate the relaxin gene are not completely clear. In the rat ovary, estrogen is a main regulator of relaxin gene expression and dihydrotestosterone does not have any effect (Crish *et al.* 1986), but the effect of estrogen may be indirect (Sherwood *et al.* 1986, Peters *et al.* 2000). Other steroid hormones, such as progesterone and glucocorticoids, seem to regulate relaxin gene expression via binding to a response element on the 5'-flanking region of the relaxin genes

Figure 7 Real-time PCR analysis of the regulation of relaxin and relaxin receptor *Rxfp1* gene expression. (A) Level of relaxin and *Rxfp1* transcripts in the testis of 15- and 120-day-old rats. Values were normalized with β -actin expression using vas deferens of 120-day-old animals as a calibrator of the experiment (means $\pm$ S.E.M. of three independent experiments performed in triplicate). (B) Effect of castration and supplementation with testosterone on expression of relaxin and *Rxfp1* in vas deferens and prostate of adult rats. Controls were sham operated; castrated rats were castrated 15 days before killing and immediately treated with either vehicle or testosterone. Values were normalized with β -actin expression, and are expressed as relative values using one of the controls of each tissue as a calibrator of the experiment (means $\pm$ S.E.M. of three independent experiments performed in triplicate). * P < 0.05 compared to other values (Newman-Keuls test).

(Garibay-Tupas *et al.* 2004). Relaxin also regulates its own gene expression through the binding of relaxin-activated glucocorticoid receptor to the promoter region of the human relaxin-2 gene (Dschieltzig *et al.* 2009).

Our finding that castration reduced relaxin gene expression in the prostate and vas deferens, and the reversal of these effects by administration of testosterone, suggests that testosterone is an important regulator of relaxin gene expression in the reproductive tract of the male rat. In accordance with our results, De Rienzo *et al.* (2006) found that testosterone increased the relaxin mRNA levels in frog Leydig cells. However, the effect of testosterone on relaxin gene expression seems cell type dependent. Dihydrotestosterone does not affect the production of relaxin mRNA in the rat ovary (Crish *et al.* 1986), and in the prostate cancer cell line LNCaP, androgens inhibit production of relaxin mRNA (Thompson *et al.* 2006). In another prostate cancer cell line, PC-3, androgens positively regulate the relaxin promoter (Brookes *et al.* 1998). Nevertheless, our observation that relaxin mRNA levels in the testis were much higher in immature than in mature rats corroborates the suggestion of De Rienzo *et al.* (2006) that relaxin gene expression depends on additional factors.

The expression of the relaxin receptor *Rxfp1* gene in prostate and vas deferens does not seem to be regulated either by testosterone or by estrogen, because castration and treatment with the anti-estrogen ICI 182,780 did not affect *Rxfp1* expression. Furthermore, whereas others described an increase in uterine *Rxfp1* mRNA levels after estrogen treatment (Yan *et al.* 2008), we did not observe any changes in mRNA levels for relaxin or *Rxfp1* after ICI 182,780 treatment. One might consider that estrogen could regulate the relaxin or *Rxfp1* mRNA levels through a mechanism involving the more recently described estrogen G-protein coupled receptor, GPER (Filardo 2002, Revankar *et al.* 2005). This does not seem to happen because ICI 182,780 acts as an agonist on GPER (Thomas *et al.* 2005), and we should therefore expect an increase in relaxin and *Rxfp1* mRNA levels.

Although relaxin regulates *Esr1* and *Esr2* expression in the female tract, it failed to affect expression of *Esr1* and *Esr2* and the G protein-coupled estrogen receptor GPR30 (*Gper*) in the vas deferens. Relaxin is also known to reduce mRNA levels of the *Rxfp1* receptor in the cervix (Yan *et al.* 2008), and our results show a similar trend in the vas deferens.

We have previously shown that the relaxin receptor RXFP1 is expressed in Sertoli cells (Filonzi *et al.* 2007). In the present study, we demonstrated that Sertoli cells represent an important source of relaxin gene expression in the testis, suggesting an autocrine role of relaxin in Sertoli cells. The high levels of relaxin mRNA and protein in the testis of 15-day-old animals suggest that relaxin plays a role in early phases of development. The immunohistochemical studies, showing relaxin immunoreactivity in Sertoli cells of immature animals,

and the proliferative effect of relaxin on these cells corroborate this idea. The demonstration that relaxin stimulates proliferation in cultured Sertoli cells is in line with previous reports that relaxin stimulates cell proliferation in granulosa and theca cells (Bagnell *et al.* 1993, Zhang & Bagnell 1993, Ohleth & Bagnell 1995), endometrium (Masters *et al.* 2007), and cervix (Lee *et al.* 2005). The number of Sertoli cells in the adult testis determines testis size and daily sperm production. Whereas relaxin may indirectly affect spermatogenesis in the adult through stimulation of Sertoli cell proliferation, relaxin may also directly affect spermatogenesis, because relaxin was present in pachytene spermatocytes, suggesting a role in meiosis. Relaxin was also present in spermatids, and may act on relaxin receptors in elongating spermatids (Filonzi *et al.* 2007). In conclusion, relaxin may play a more comprehensive role in spermatogenesis and spermiogenesis.

Although relaxin receptors are present in the muscular layer of the vas deferens, relaxin does not alter the contractility of the organ (Filonzi *et al.* 2007). Relaxin receptors were also detected in the epithelial layer of the vas deferens. It is particularly interesting to note that relaxin is expressed in the apical cells of the vas deferens epithelium, suggesting a role in secretion and composition of the seminal plasma. The apical cells in the vas deferens are a distinct population of epithelial cells that co-express high levels of H⁺-ATPase on the luminal plasma membrane and carbonic anhydrase II in the cytoplasm (Brown *et al.* 1992, Breton *et al.* 1996, 1999). These two proteins are important for transepithelial proton translocation (Brown & Breton 2000). Furthermore, relaxin caused a discrete increase in the mRNA levels for *Cftr*. CFTR allows Cl⁻ efflux to the lumen and modulates fluid transport, but also affects bicarbonate secretion, and therefore affects sperm-fertilizing capacity and male fertility (Chan *et al.* 2006, Xu *et al.* 2007). Mutations in this gene not only cause the cystic fibrosis syndrome (Quinton 1990, Van der Ven *et al.* 1996, Rowe *et al.* 2005), but also the bilateral absence of the vas deferens. However, the presence of CFTR in the vas deferens has been controversial. Using *in situ* hybridization, Trezise *et al.* (1993) did not detect *Cftr* mRNA in the rat vas deferens, whereas Patrizio & Salameh (1998) reported a strong signal in the human vas deferens. Pietrement *et al.* (2008) reported that CFTR co-localized with aquaporin 9 and NHERF1 in the apical membrane of rat epididymis and vas deferens, but they only show results for the epididymis. In the present study, we detected immunoreactivity to CFTR in the epithelial layer of the vas deferens, especially close to the apical membrane and in some microvilli.

The role of relaxin in the regulation of metalloproteinase expression and activity is well known, and it is thought that the fibrosis induced by relaxin knockout is due to changes in metalloproteinase activity (Samuel *et al.* 2003b, 2005). We found previously that relaxin

stimulates *Mmp7* gene expression in the vas deferens (Filonzi *et al.* 2007), and we now show that this effect is selective to *Mmp7*, because the mRNA levels for *Mmp2* and *Mmp9* remained unchanged after relaxin treatment. MMP7 presents a broad spectrum of actions (reviewed in Li *et al.* (2006)), including a role in apoptosis, cell proliferation, and release of growth factors. In the male reproductive tract, it has been demonstrated that MMP7 is important for testicular integrity and fertility (Rudolph-Owen *et al.* 1998), and it is present in human semen (Riccioli *et al.* 2005). Our immunohistochemical studies show that MMP7 expression is expressed only in epithelial cells of the vas deferens. Therefore, it seems likely that MMP7 produced in the epithelium of the vas deferens is released to the lumen to affect spermatozoa. Whether MMP7 is also released to the basolateral compartment to participate in tissue remodeling is not clear.

The mechanism of relaxin action in the testis and vas deferens remains to be determined. Relaxin has been shown to up-regulate the inducible form of *Nos2* in MCF-7 breast adenocarcinoma cells (Bani 1997) and in endothelial cells from human umbilical vein (Quattrone *et al.* 2004). On the other hand, relaxin up-regulates the endothelial-type NOS but not *Nos2* in mouse myometrium (Bani *et al.* 1999). In the present study, we found that relaxin increased the *Nos2* mRNA in the vas deferens, suggesting that NO may play a role in the relaxin action in this organ, and that relaxin may contribute to the NOS activity found in seminal plasma. In the male reproductive tract, NO probably plays an important role in several aspects of testicular function, including spermatogenesis and sperm maturation (Zini *et al.* 1996). NOS has been localized in the reproductive organs of the male rat, and the highest levels of NOS activity were present in the caudal segment of the epididymis and in the vas deferens (Burnett *et al.* 1995, Zini *et al.* 1996). NOS activity has also been detected in human seminal plasma, it may affect sperm function, and it is interesting to note that men without detectable NOS in sperm presented bilateral absence of the vas deferens (Zini *et al.* 2001).

In conclusion, we have shown that relaxin is produced by the testis and vas deferens, where it may affect proliferation of Sertoli cells and gene expression. Both tissues co-express relaxin and its receptor, suggesting that relaxin plays an autocrine/paracrine role in the male reproductive tract.

Materials and Methods

Hormones

Porcine relaxin and human FSH (hFSH) were obtained from Dr A F Parlow, from the National Hormone Peptide Program (Torrance, CA, USA).

Animals

Wistar rats were housed in the Animal Facility of the Instituto Nacional de Farmacologia e Biologia Molecular, UNIFESP-EPM, and maintained on a 12 h light:12 h darkness schedule, at 23 °C, with food and water freely available. All procedures were approved by the Institutional Research Ethics Committee (protocol 1657/07).

In vivo treatments

Anti-estrogen treatment

Animals were treated as previously described (Oliveira *et al.* 2002, Yasuhara *et al.* 2008). Rats were treated once a week for 2 months with corn oil (control group) or ICI 182,780 (10 mg/rat, s.c., AstraZeneca), starting at an age of 30 days. This treatment does not alter plasma estradiol and testosterone (Yasuhara *et al.* 2008).

Castration

Ninety-day-old rats were anesthetized with sodium pentobarbital (30 mg/kg) and the testes were removed. Some castrated rats were given weekly s.c. injections of 25 mg testosterone (Durateston 250, Organon, São Paulo, Brazil), starting on the day of castration. Fifteen days after castration, animals were killed by decapitation and the prostate and vas deferens were removed.

In vitro treatments with relaxin

To analyze the effects of relaxin on gene expression, the vas deferens was removed from 120-day-old rats, dissected free from fat and connective tissues, and rinsed with PBS (137 mM; NaCl, 2.68 mM; KCl, 6.03 mM; Na₂HPO₄, 1.47 mM; KH₂PO₄, pH 7.4). To increase contact of the hormone with the lumen, the vas deferens was opened and cut into four pieces. The tissues were transferred to a 6-well plate containing phenol red-free Ham's F12/DMEM 1:1 (Gibco, Invitrogen), pH 7.2–7.4, and incubated in a humidified atmosphere of 5% CO₂–95% air at 35 °C. After equilibration for 15 min, one duct was incubated with 100 ng/ml relaxin, and the contralateral duct with PBS, for 30, 60, or 240 min. Stimulation was stopped by cooling to 0 °C, and the total RNA was extracted from the tissues.

Primary cultures of Sertoli cells

The testes from 15-day-old rats were removed and decapsulated, and Sertoli cells were isolated (Lucas *et al.* 2004, 2008). Aliquots of 1 ml of the cell suspension were plated in 12-well plates at a density of $\sim 4 \times 10^4$ cells/well (about 10 000 cells/cm²) in phenol red-free F12/DMEM containing 0.02 g/l gentamicin (Sigma Chemical Co.), pH 7.2–7.4, supplemented with 10 µg/ml insulin, 10 µg/ml transferrin, 10 ng/ml sodium selenite, and 10 ng/ml epidermal growth factor (Sigma). The cells were grown for 48 h in a humidified atmosphere of 5% CO₂–95% air at 35 °C, treated with 20 mM Tris–HCl (pH 7.4) to lyse residual germ cells (Galdieri *et al.* 1981), and allowed to grow for another 24 h. The culture medium was replaced by

one without supplements 20 h before the experiments. At this stage, the cells were 50–60% confluent, and viability, as determined by trypan blue exclusion, was more than 90%. The characterization of the culture was described elsewhere (Lucas *et al.* 2008).

Immunohistochemistry

Tissues from immature (15-day-old) or from adult rats (120-day-old males or females around the 21st day of pregnancy) were removed, fixed in Bouin, and embedded in paraffin. Sections of 5–7 µm thickness were cut and mounted on silanized slides (Dako, Carpinteria, CA, USA). Immunoperoxidase reaction was performed as previously described (Yasuhara *et al.* 2008). Briefly, tissue sections were incubated for 5 min at room temperature with 0.1 M glycine in Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, pH 7.4). Nonspecific antibody binding was minimized by incubation with blocking solution: TBS containing 5–10% BSA (for relaxin and CFTR) or 10% rabbit serum (for MMP7) for 15 min at 37 °C in a humidified chamber. Slides were then incubated with the appropriate primary antibody: 1) an overnight incubation at 4 °C with 1:50–1:500 dilution of a goat polyclonal antibody raised against the N-terminal region of human relaxin (N-18, Santa Cruz Biotechnology), which recognizes the relaxin mature peptide and its precursor; 2) 2 h incubation at 37 °C with 1:100 dilution of a rabbit polyclonal antibody against a region surrounding amino acid 130 of the rat relaxin 1 (ab70803, Abcam), which recognizes only relaxin precursors but not the mature peptide; 3) 2 h incubation at 35–37 °C with a 1:100 dilution of a goat polyclonal antibody raised against a peptide mapping near the N-terminal region of human CFTR (N-20, Santa Cruz Biotechnology; 4) 2 h incubation at 37 °C with 1:80 dilution of a goat polyclonal antibody raised against a peptide mapping near the C-terminus of human MMP7 (C-17, Santa Cruz Biotechnology). The sections were washed with TBS containing 0.1% Tween 20 (TBS-T), incubated with blocking solution for another 15 min, and then incubated with the appropriate secondary antibody, conjugated to HRP (1:250 rabbit anti-goat, Jackson ImmunoResearch, West Grove, PA, USA or 1:100 donkey anti-rabbit, GE, Piscataway, NJ, USA) for 30 min at 37 °C. Negative control slides were developed in the absence of the primary antibody. The stained sections were visualized with a Nikon E800 microscope (Nikon, Melville, NY, USA). Images were captured with a CoolSNAP-Pro CCD digital camera and processed with Image-Pro Express Software (Media Cybernetics, Silver Spring, MD, USA).

Western blot for detection of relaxin

Testis, vas deferens, and prostate from 120-day-old rats and ovaries from rats in the 21st day of pregnancy were isolated, frozen immediately in liquid nitrogen, and pulverized in a tissue grinder. The pulverized tissues were homogenized with a Polytron homogenizer in ice-cold lysis buffer (50 mM Hepes, pH 7.5, 5 M NaCl, 10% glycerol, 1% Triton X-100, and 10 mM EGTA), in the presence of protease inhibitors (1 µg/ml leupeptine, 1 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 11 200 g for 30 min at 4 °C.

Protein content of the supernatant was determined with the Bio-Rad protein assay and a BSA standard (Bio-Rad Laboratories).

Proteins from supernatant (100 µg/lane) were incubated with sample buffer containing dithiothreitol and β-mercaptoethanol, and subjected to 15% SDS/PAGE. Proteins were electrotransferred overnight (20 V at 4 °C) to PVDF membranes (0.45 µm pore size, ImmobilonP, Millipore, Bedford, MA, USA). Membranes were blocked for 2 h at room temperature in TBS (10 mM Tris, 150 mM NaCl, pH 8.0) containing 0.2% Tween 20 and 5% nonfat dry milk. The membranes were incubated with anti-relaxin antibody N18 (Santa Cruz Biotechnologies) diluted 1:100 in blocking solution for 16 h at 4 °C. This antibody was chosen after immunohistochemical studies showed its ability to detect immunoreactivity in the ovary of pregnant rats and in the rat prostate (see Results). Membranes were washed in TBS-T, incubated for 1 h at room temperature with a 1:20 000 dilution of secondary antibody (rabbit anti-goat HRP-conjugated anti-IgG, Jackson ImmunoResearch Lab.), and washed again with TBS-T. Immunoreactive bands were visualized on preflashed Biomax XAR film (Eastman Kodak Co.) by ECL reagent (Luminol, PerkinElmer, Boston, MA, USA). In negative controls, primary antibody was pre-incubated for 16 h at 4 °C with the lysate from ovaries of 21-day pregnant rats, and processed as described above. Apparent molecular weights of protein bands were determined from molecular weight standards (prestained protein marker, broad range 6–175 kDa, New England Biolabs, Boston, MA, USA).

[Methyl-³H] thymidine incorporation assays

Incorporation of [methyl-³H] thymidine into cell DNA was measured as described by Guizzetti *et al.* (1996). Previous studies in our laboratory indicated that incorporation of [methyl-³H] thymidine (2 µCi/ml, specific activity 79.0 Ci/mmol, Amersham Biosciences) in cultured Sertoli cells increased linearly between 2 and 10 h of incubation. All studies were performed using 6 h of [methyl-³H] thymidine incubation (Lucas *et al.* 2004).

Primary Sertoli cell cultures were initially incubated with 2 µCi/ml [methyl-³H] thymidine for 6 h at 35 °C. Incubation was continued in the absence (control incorporation) and presence of relaxin (100 and 200 ng/ml), hFSH (50 ng/ml), or relaxin and hFSH (100 and 50 ng/ml respectively) for 24 h at 35 °C. The reaction was stopped by cooling the cells at 0 °C. The medium was aspirated, and the cells were rinsed twice with ice-cold PBS and 5% trichloroacetic acid (Sigma). The cells were then solubilized with 0.5 N NaOH, collected with cotton swabs, and transferred to 5 ml OptiPhase HiSafe 3 scintillation liquid (PerkinElmer Life Science Products). Bound radioactivity was determined in scintillation β counter (LS 6000 IC, Beckman Coulter Inc., Palo Alto, CA, USA). Results were expressed as percentage of [methyl-³H] thymidine incorporation above control.

Real-time qRT-PCR

The mRNA levels for the following genes were analyzed by quantitative real-time PCR: relaxin (*Rln1*); the cystic fibrosis chloride channel (*Cftr*); *Mmp2*, *Mmp7*, and

Table 1 Oligonucleotides used in the real-time PCR.

Transcript (accession number)	Orientation	Sequence (5' → 3')	Nucleotide position
<i>Actb</i>	Forward	GTA GCC ATC CAG GCT GTG TT	481–500
NM_031144.2	Reverse	CCC TCA TAG ATG GGC ACA GT	584–565
<i>Rln1</i> (relaxin)	Forward	AGG AGT GGA TGG ACC AAG TC	120–139
NM_013413.1	Reverse	ATG GCA CAA CTT CTG CAG TG	269–250
<i>Mmp7</i>	Forward	CAC GGA GAC AAC TTC CCA TT	515–534
NM_012864.2	Reverse	CAC CAT CCG TCC AGT ACT CA	632–613
<i>Cftr</i>	Forward	TGG AGG TGA AAT GGT TGT CA	3743–3762
NM_031506.1	Reverse	ACC CTC TGG CCA GGA CTT AT	3844–3825
<i>Esr1</i>	Forward	CCA TAT CCG GCA CAT GAG TA	1760–1779
NM_012689.1	Reverse	TGA AGA CGA TGA GCA TCC AG	1873–1854
<i>Esr2</i>	Forward	CTC ACG TCA GGC ACA TCA GT	1669–1688
NM_012754.1	Reverse	TGT GAG CAT TCA GCA TCT CC	1776–1757
<i>Gper</i>	Forward	CCC TTG ACA GGC CAC ATA GT	952–971
NM_133573.1	Reverse	CTC CGT GCT GTC TGG TAT GA	1140–1121
<i>Rxfp1</i>	Forward	TGA GGC AGA AAC TTC CGA AT	264–283
NM_201417.1	Reverse	TCC ATT GCA GGG ACA TTA CA	406–387
<i>Nos2</i>	Forward	CCT GTG TTC CAC CAG GAG AT	1601–1620
NM_012611.3	Reverse	CGC TTT CAC CAA GAC TGT GA	1744–1725

Mmp9; the inducible form of *Nos2*; the estrogen receptors α (*Esr1*), β (*Esr2*), and GPR30 (*Gper*); and the relaxin receptor *Rxfp1*.

RNA extraction and cDNA synthesis

Total RNA was extracted with the TRIzol reagent (Invitrogen) according to the standard protocol (Chomczynski & Sacchi 1987). RNA was further treated with RNase-free DNase I (Qiagen) to eliminate genomic DNA contamination, and purified using the RNeasy Mini Kit (Qiagen) according to instructions of the manufacturer. RNA concentration was measured by u.v. spectrophotometry, and OD 260:280 nm ratios between 1.8 and 2.1 were obtained for all RNA samples. Ribosomal RNA integrity was checked on agarose gel electrophoresis, and a sharp and clear 2:1 ratio of ethidium bromide-stained 28S:18S rRNA bands was observed for all samples.

Two micrograms total RNA were used to synthesize the first strand cDNA at 50 °C with the Superscript III first strand synthesis Supermix (Invitrogen) and the oligo-dT primer supplied with this kit.

Quantitative PCR

For the qPCR, we used the SYBR Green system (Applied Biosystems, Foster City, CA, USA). The primers (Invitrogen) were designed with the Primer3 program (Rozen & Skaletsky 2000) and spanned exon–exon boundaries whenever possible (Table 1). β -Actin (*Actb*) was used as an endogenous control and to normalize cDNA amount and PCR efficiency. Primers for all target genes and for β -actin were designed to have approximately the same amplification efficiency ($=1$). Efficiency was calculated based on the amplification curves obtained with five cDNA concentrations (0.01, 0.1, 1, 10, and 100 ng/ml). The slope of the amplification curves $C_t \times \log$ (cDNA) was calculated. The efficiency value was calculated from the equation:

$$E = 10^{(-1/\text{slope})} - 1.$$

Controls without cDNA or without primers were included in each assay. Samples were run in an ABI PRISM 7500 Sequence

Detection System (Applied Biosystems) using default conditions of amplification (50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min). The dissociation curves were obtained at the end of the amplification to confirm specificity of the amplification. Each sample was run in triplicate. The average cycle threshold (C_t) was determined with Applied Biosystems software. The mean $\pm$ S.E.M. of C_t values for the endogenous control, β -actin, was 15.9 ± 0.1 ($N=40$). The C_t values for the targets ($N=3-6$) were as follows: *Rln1* in prostate, 27.2 ± 0.2 ; *Rln1* in testis of 120-day-old animals, 31.6 ± 0.3 ; *Rln1* in vas deferens, 31.3 ± 0.5 ; *Rln1* in testis of 15-day-old animals, 28.1 ± 0.2 ; *Rln1* in Sertoli cells, 28.2 ± 0.6 ; *Cftr*, 30.0 ± 0.7 ; *Mmp7*, 24.5 ± 0.6 ; *Mmp2*, 18.0 ± 0.1 ; *Mmp9*, 15.1 ± 0.1 ; *Esr1*, 23.8 ± 0.4 ; *Esr2*, 28.3 ± 0.1 ; *Gper*, 26.6 ± 0.1 ; *Rxfp1*, 29.1 ± 0.4 , and *Nos2*, 27.7 ± 0.9 . Data were analyzed by the comparative $\Delta\Delta C_t$ method (ABI PRISM User Bulletin #2, Applied Biosystems). The control values were always used as a calibrator in each experiment, except in the experiments to compare expression levels of relaxin in different tissues, and relaxin expression in testis of 15- and 120-day-old rats, where the calibrator was the value obtained with the vas deferens. Data are expressed as mean $\pm$ S.E.M. of the $2^{-\Delta\Delta C_t}$ from three to five different cDNAs (from three to five different animals). At the end of the experiments, samples were run in 2% agarose gel electrophoresis to further confirm the absence of nonspecific amplification. The size of the expected products was compared to a DNA ladder (100 bp ladder, Invitrogen). Identity of the PCR products was further confirmed by automated DNA sequencing with the DYEnamic ET Terminator Sequencing Kit (Amersham Biosciences).

Statistical analyses

Data were expressed as mean $\pm$ S.E.M. Statistical analysis was carried out by ANOVA followed by Newman–Keuls test for multiple comparisons or by Student's *t*-test to compare the differences between two data. *P* values <0.05 were accepted as significant.

Declaration of interest

The authors declared that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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