Relaxin in peripheral plasma of boars during development, copulation, after administration of hCG and after castration

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Seventy-two Yorkshire boars were used in five experiments to evaluate the temporal changes in relaxin concentrations in peripheral blood plasma during prepubertal development, copulation, after castration and after treatment with hCG. High concentrations of relaxin (484 ± 27 pg ml⁻¹) were detected at 11 weeks of age but there was no positive correlation with testicular development. Relaxin concentrations fluctuated in mature boars but the results do not suggest a diurnal rhythm, although there is the possibility of pulsatile secretion. A decrease ($P < 0.05$) in circulating relaxin was observed before and immediately after copulation. Castration of boars at 90, 115, 160 and 200 days of age did not significantly decrease relaxin concentrations within 48 h. Administration of hCG significantly depressed relaxin secretion at 90 days of age but not at 160 days of age. These studies suggest a non-gonadal source of boar relaxin that is not correlated with testicular growth or testosterone concentrations, is modulated by copulation and by hCG but only at specific stages of development.

Introduction

Relaxin, a 6 kDa peptide hormone, has been found primarily in reproductive tissues and circulating blood during pregnancy and parturition in females of several mammals but is also found in the male reproductive tract (Anderson, 1987; Weiss, 1989). Relaxin-like immunoactivity was detected in human and boar semen (Weiss *et al.*, 1986; Juang *et al.*, 1990). Porcine relaxin was shown to have a specific effect on improving the motility of human and boar spermatozoa (Lessing *et al.*, 1986; Juang *et al.*, 1989) and the fertilization capacity of human and mouse spermatozoa (Pupula *et al.*, 1986; Park *et al.*, 1988). The presence of relaxin in the male reproductive tract of rats, mice and humans is, however, still controversial (Kendall *et al.*, 1982; Yki-Jarvinen *et al.*, 1983; Anderson *et al.*, 1986). In boars, a relaxin-like immunocytological reaction was reported in interstitial and Sertoli cells of the testes (Dubois and Dacheux, 1978), and the presence of mRNA encoding relaxin was demonstrated in Leydig cells and in the prostate gland using reverse transcription and the polymerase chain reaction (Lobb and Porter, 1995; Lobb *et al.*, 1995). Kohasaka *et al.* (1992) found relaxin cytoplasmic immunoreactivity in boar seminal vesicles. There is little information about the physiological role

of relaxin in the male. In this study, the correlation between changes in relaxin concentrations in plasma and testicular development, and aspects of the physiological regulation of relaxin secretion were determined in the boar.

Materials and Methods

Animals and blood sample collection

Yorkshire boars were fitted with an indwelling cannula (i.d. 0.125 cm; o.d. 0.225 cm; Tygon microbore tubing, no. 14-170-5E, Fisher Scientific Co., Itasca, IL) inserted into the external jugular vein as described by Ford and Maurer (1978). Blood samples were collected into heparinized (143 U heparin) Vacutainer tubes (no. 02-685-3B, Fisher Scientific Co.) and centrifuged immediately at 2000 g for 15 min. Plasma was stored at -20°C until hormone assay. Twenty-six Yorkshire boars born within 6 weeks were weaned at 4 weeks of age and moved to an environmentally controlled nursery room in the pig research building (Animal Reproduction Laboratory, Iowa State University). After weaning, all boars remained in the nursery until 9 weeks of age; then they were moved to the finishing building and penned in the same stall. Boars were housed in the same building with same-age females throughout the study but were segregated by sex.

Experimental protocols

Experiment 1. Blood samples (3 ml) were obtained once a week from boars ($n = 26$) from birth to 3 weeks of age. At 5 to

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29 weeks of age, sequential blood samples were obtained at intervals of 2 weeks from six boars. Six other boars were used to obtain sequential blood samples at intervals of 2 weeks between 5 and 29 weeks after birth. Blood samples were obtained by venepuncture of the anterior vena cava.

Experiment 2 (sequential sampling over 24 h). Two days before the experiment, three sexually mature (> 1.5 years of age) and two prepubertal (115 days of age) boars were confined in individual crates in a controlled-climate room (26°C) with a 12 h light:12 h dark (lights on 07:00–19:00 h) lighting regimen. Boars were isolated from females during this period. Blood samples (7 ml) were collected every hour for 24 h except during 09:00–12:00 h and 21:00–23:00 h when blood was collected at intervals of 15 min.

Experiment 3 (after castration). Boars were castrated at different developmental stages (90 days ($n = 4$), 115 days ($n = 2$), 160 days ($n = 4$) and 200 days ($n = 6$)). An indwelling cannula was implanted into the external jugular vein, as described previously, 5 days before castration. For castration, anaesthesia was induced and maintained with an i.v. injection of thiamylal sodium (0.3–0.6 g; Surital; Parke-Davis, Morris Plains, NJ). Testes and epididymides were removed surgically, trimmed of excess tissue and weighed separately. All animals were weighed before castration. Blood samples (7 ml) were obtained 1 h before (-1) and 0.5, 6, 24 and 48 h after castration, and the plasma was collected for hormone assay.

Experiment 4 (during copulation). Three mature Yorkshire boars (about 18 months old) were evaluated for the effect of copulation and ejaculation on the concentration of relaxin in peripheral plasma. A Tygon microbore external jugular cannula was implanted 2 days before the experiment for collection of blood before, during and after mating. Boars were confined in individual crates inside a controlled-climate room (26°C) with a 12 h light:12 h dark (lights on 07:00–19:00 h) lighting regimen. Boars were isolated from females during this period. The effect of male sexual behaviour and copulation on relaxin secretion was continuously monitored in these three boars. Oestrous gilts were penned separately in the same room with the boar for 1 h before mating, and three blood samples were obtained at intervals of 20 min from each boar during this period of aggressive sexual behaviour. During mating, an additional blood sample was collected. All gilts were removed immediately after mating, and sequential blood samples (7 ml) were withdrawn from the indwelling jugular catheters every 10 min for the first 1 h and every 20 min for the subsequent 3 h.

Experiment 5 (after administration of hCG). Sixteen Yorkshire boars were assigned randomly to one of four i.m. treatments: saline (3 ml) control administered on day 90 ($n = 4$); hCG (4000 iu; Follutein; Solvay Veterinary Inc., Princeton, NJ) administered on day 90 ($n = 4$); saline control administered on day 160 ($n = 4$) and hCG (4000 iu) administered on day 160 ($n = 4$). These animals were weaned at 4 weeks of age. The boars were moved to the finishing building and penned together after 9 weeks of age. During rearing, they were

maintained in all-male groups but were never isolated from possible audible and pheromonal stimuli of contemporary females.

The boars were moved to individual pens, and 2 days later they were fitted with an indwelling jugular cannula and they subsequently received hormone and saline treatments. Anaesthesia was induced by an injection of thiamylal sodium (0.3–0.6 g; Surital, Parke-Davis) into an ear vein and maintained by a closed-circuit system of halothane (3–10%, Ayerst Laboratories, Rouses Point, NY) and oxygen (400–1000 cc ml^{-1}) during surgery. The indwelling jugular catheter was implanted as described previously. Blood samples were collected every hour for 3 h before treatment and during the first 10 h after hormone or saline administration; thereafter, samples were collected at intervals of 2 h for up to 80 h after treatment.

Radioimmunoassay of relaxin in peripheral plasma

Relaxin concentrations in peripheral blood plasma of boars were quantified in duplicate aliquots of 200 μl by using a homologous radioimmunoassay for porcine relaxin as described by Li *et al.* (1989) and Juang *et al.* (1990). Mono-tyrosyl relaxin (3000 U mg^{-1}) was iodinated by a modified version of the chloramine-T procedure (Schwabe, 1983). Plasma samples (200 μl) were quantified by a double antibody radioimmunoassay. After addition of 300 μl 0.01 mol PBS l^{-1} –1% BSA (w/v) buffer, 100 μl anti-porcine relaxin antibody (Dual Rabbit R₆ from B. G. Steinetz, New York University, Tuxedo, NY) diluted 1:20 000 in 0.01 mol PBS l^{-1} –1% BSA containing 0.05 mol EDTA l^{-1} plus 6% normal rabbit serum was added to each tube. Tubes were incubated for 24 h at 4°C ; then, 100 μl ^{125}I -labelled porcine relaxin (9500–12 000 c.p.m. in 0.01 mol PBS l^{-1} –1% BSA) was added to all tubes. After incubation for 48 h at 4°C , 200 μl 1:10 goat antirabbit IgG (no. 0112-0081, Organon Teknika Co., West Chester, PA) was added to each tube and they were then incubated for another 18–24 h at 4°C . Three millilitres 0.01 mol PBS l^{-1} –1% BSA was then added to each tube and the tubes were centrifuged at 2200 g for 30 min; the supernatant was decanted, and the precipitate was counted in a γ -counter (Packard Cobra Autogamma 5003). Parallelism was established by testing different volumes of blood plasma. The maximum binding and nonspecific binding of the antibody were $35.5 \pm 3.4\%$ and $3.6 \pm 0.4\%$ ($n = 4$), respectively. The intra- and interassay variabilities were 6.5% and 6.7%, respectively. The sensitivity of the assay was 27 pg per tube.

Radioimmunoassay of testosterone in peripheral plasma

Testosterone concentrations in peripheral blood were determined in duplicate 10–200 μl aliquots of plasma by radioimmunoassay as described by Ford *et al.* (1980). A rabbit antiserum prepared against testosterone-7 α -BSA (no. 61-315, Lot TT-2, Miles-Yeda Ltd, Naperville, IL) was used in this assay and had less than 17% crossreactivity with 5 α -dihydrotestosterone. Plasma samples were extracted twice by mixing with ethyl ether for 15 min, freezing and then decanting the ethyl ether. Recovery was on average greater

Table 1. The average body mass and testes and epididymides masses during development in Yorkshire boars

Parameter	Age (days)			
	90 (n = 8)	115 (n = 2)	160 (n = 7)	200 (n = 6)
Body (kg)	33 ± 3.3	41 ± 11.4	93 ± 7.7	116 ± 6.3
Testes (g)	35 ± 5.8	50 ± 12.8	262 ± 22.6	322 ± 31.5
Epididymides (g)	16 ± 1.1	28 ± 4.6	100 ± 15.7	91 ± 22.7

Values are means ± SEM.

Fig. 1. Peripheral plasma concentrations of relaxin during prepubertal development and puberty in Yorkshire boars ($n = 26$). Blood samples were obtained at weekly intervals for 3 weeks after birth and at intervals of 2 weeks between 5 and 29 weeks. Values are means ± SEM. **Fig. 2.** Sequential concentrations of relaxin in peripheral plasma of boars during 24 h. (○) Prepubertal Yorkshire boars 115 days of age ($n = 2$) and (●) sexually mature Yorkshire boars > 1.5 years of age ($n = 3$). Samples were taken every hour for 24 h except during 09:00–12:00 h and 21:00–23:00 h when samples were collected at intervals of 15 min. Values are means ± SEM.

than 90%, and the samples were not corrected for procedural losses. Parallelism was established by testing different volumes of plasma. Sensitivity of the assay was 5 pg per tube. A pool of boar plasma was included in each of the five assays and had a mean of 1.7 ng ml^{-1} , with an interassay coefficient of variance of 7.1%. The intra-assay coefficient of variance was 8.6%.

Statistical analyses

Experimental units in this study were the individual boars, and they were assigned to treatment groups at random. Data were analysed by using a one-way ANOVA and Student's *t* test was used for comparison between treatment groups (Snedecor and Cochran, 1980; Spector *et al.*, 1985).

Results

From day 115, body mass and testicular and epididymides masses showed marked increases up to 200 days of age (Table 1). During this period, the average concentration of relaxin in peripheral blood plasma ranged from 268 to 484 pg ml^{-1} (Fig. 1). Relaxin concentrations decreased ($P < 0.05$) from the first to third weeks of age but increased thereafter. Relaxin concentrations were at a maximum ($P < 0.05$) between 63 and 77 days of age and decreased thereafter up to 105 days of age. After 175 days, a slight but not significant increase in relaxin was observed and relaxin remained fairly constant thereafter.

The data do not support a significant positive correlation between relaxin secretion and testicular development before day 28, except that there is a negative relationship between these two parameters. The ratios of testicular mass to body mass were 1.06, 1.22, 2.82 and 2.78 g kg^{-1} body mass at days 90, 115, 160 and 200, respectively. The negative relationship between plasma relaxin and testicular mass persists even after compensating for changes in testis mass relative to body mass.

The concentrations of relaxin determined from sampling every hour were in the same range as those in samples taken once a week ($P > 0.02$, Fig. 2). Relative to within boar mean 24 h concentrations of relaxin, low relaxin concentrations were observed at 09:00, 13:00 and 18:00 h. These data suggest that relaxin could be secreted in a pulsatile manner at this time in the boar's development. Relaxin concentrations were determined in boars after castration in four age groups. The results (Table 2) show that, independent of age at time of castration, peripheral blood concentrations of relaxin measured during the 48 h after castration were unaffected by castration. Concentrations of relaxin in peripheral blood decreased (325 versus 430 pg ml^{-1} ; $P < 0.05$) in boars when exposed to oestrous sows. Concentrations of relaxin returned to the pre-exposure concentrations during the act of copulation and then declined and remained significantly ($P < 0.05$) lower up to 180 min after mating but had increased 240 min after mating (Fig. 3).

Administration of hCG to boars aged 90 days resulted in a significant ($P < 0.05$) decrease in relaxin concentrations from 10 h after hormone treatment (Fig. 4). In contrast, when hCG was administered to older boars (aged 160 days), peripheral

Table 2. Peripheral plasma concentrations of relaxin (pg ml⁻¹) in Yorkshire boars before and after castration

Time (h)*	Age (days)			
	90 (n = 4)	115 (n = 2)	160 (n = 7)	200 (n = 6)
- 12	318 ± 24	284 ± 34	285 ± 39	267 ± 12
- 1	291 ± 41	254 ± 54	281 ± 38	298 ± 17
+ 1	283 ± 46	270 ± 11	269 ± 49	288 ± 18
+ 6	252 ± 41	288 ± 61	274 ± 56	238 ± 21
+ 24	340 ± 33	224 ± 46	273 ± 40	249 ± 23
+ 48	383 ± 65	258 ± 11	230 ± 45	237 ± 18

Values are means ± SEM.

*Time before and after castration.

Fig. 3. Relaxin concentrations in peripheral plasma of Yorkshire boars ($n = 3$) before, during and after copulation with sows in oestrus. The arrowhead indicates the time of copulation. Values are means ± SEM.

relaxin concentrations were unaffected ($P > 0.41$) in comparison with the vehicle-treated controls. The magnitude and duration of endogenous testosterone release in response to exogenously administered hCG was age-dependent. In the 90-day-old boars, testosterone concentrations increased from 3 ng ml⁻¹ to a peak of 18 ng ml⁻¹ 14 h after hCG treatment and in 160-day-old boars, testosterone increased from 3.5 ng ml⁻¹ to a peak of 32 ng ml⁻¹ 14 h after hCG injection. In contrast to relaxin concentrations, testosterone concentrations increased ($P < 0.05$) soon after the hCG injection and remained consistently higher during the 48 h after treatment compared with those of diluent-treated controls.

Discussion

The present study shows that there is an increase in concentrations of relaxin in boar peripheral plasma from 9 to 11 weeks of age compared with earlier and later stages of development. Development of the reproductive tract in these boars was normal and similar to that reported by Allrich *et al.* (1982). In comparison with previous reports, circulating relaxin concen-

trations in this investigation showed no correlation with the development of the testes, seminiferous tubules, Leydig cells and Sertoli cells (Allrich *et al.*, 1982; Colenbrander *et al.*, 1982; van Vorstenbosch *et al.*, 1984; Lunstra *et al.*, 1986). In addition, there was no correlation in the secretion pattern of relaxin with concentrations of testosterone, LH, FSH and oestradiol in peripheral plasma (Allrich *et al.*, 1982; Ford, 1983; Vandlem *et al.*, 1986).

The study reported here indicates that endogenous relaxin in mature boars does not support a diurnal rhythm although it is possible that secretion is of a pulsatile nature. In boars, LH, testosterone and prolactin are secreted into blood in a pulsatile, rather than continuous, manner (Lapwood and Florcurz, 1978; Klindt and Stone, 1984). Anticipation of sexual activity and copulation affects relaxin concentrations. However, after exposure of boars to sows in oestrus, relaxin concentrations in peripheral blood decreased and remained significantly lower for 4 h after mating. A sharp increase in corticosteroid, testosterone and LH blood concentrations after copulation in aggressive boars was reported by Ellendorff *et al.* (1975) and Liptrap and Raeside (1978).

During prepubertal development, sequential blood sampling revealed that plasma relaxin concentrations are low and fluctuate in a manner similar to that seen in mature boars. Furthermore, frequent blood sampling during 24 h revealed no consistent pattern of pulsatile or diurnal secretion. There was a lack of association of relaxin immunoreactivity to growth of the reproductive tract in boars. Although hCG treatment abruptly increased testosterone concentrations in peripheral plasma, relaxin remained at basal concentrations for 54 h. Low circulating concentrations of immunoreactive relaxin in boars, as determined by the R6 antibody, may represent specific relaxin binding or binding of an unidentified ligand. These studies revealed no striking hormone-dependent effects on relaxin production and secretion into peripheral blood of boars. The role of relaxin in boars appears to be more complex than predicted. It is emphasized that relaxin concentrations are three to four times higher in the seminal plasma than in blood plasma of mature boars (Juang *et al.*, 1990); thus, changes in hormone concentrations in peripheral blood are not as descriptive in males as in pregnant, or hysterectomized, females.

The source of relaxin in the male reproductive tract remains controversial. Relaxin immunoactivity was reported in the prostate gland of armadillos, humans and rats (Cameron *et al.*, 1982; Kendall *et al.*, 1982; Yki-Jarvinen *et al.*, 1983). Lobb and Porter (1995) reported finding mRNA encoding relaxin in boars and immunoactivity for relaxin was reported in boar seminal vesicles by Kohasaha *et al.* (1992). Although Leydig cells of boar testes contain mRNA encoding relaxin (Lobb *et al.*, 1995), hCG treatment of intact boars in the study reported here did not increase relaxin secretion but did sustain an increased testosterone secretion. Thus, gonadotrophin treatment appears to have little effect on expression of the relaxin gene by Leydig cells in immature and mature boars. In sows, the corpora lutea of the ovaries are a major source of relaxin during the oestrous cycle and pregnancy (Anderson *et al.*, 1973). Concentrations of relaxin in blood remain low (< 1 ng ml⁻¹) during the oestrous cycle but increase steadily to peak concentrations (> 60 ng ml⁻¹) during late pregnancy, but without a distinct pattern of pulsatile change in hormone secretion (Sherwood and

Fig. 4. Concentrations of (a, c) testosterone and (b, d) relaxin in peripheral plasma of Yorkshire boars given an i.m. injection of (Δ) 4000 iu hCG ($n = 4$) or ($\blacktriangle$) 3 ml PBS ($n = 4$) at (a, b) 90 days of age or (c, d) 160 days of age. Values are means $\pm$ SEM. The arrows indicate the time of administration of hCG or PBS.

Rutherford, 1981; Felder *et al.*, 1986). In contrast to the lack of effect of gonadotrophin treatment on relaxin secretion in the boars in the present study, LH and prolactin increase relaxin production by ageing porcine corpora lutea *in vitro* (Huang *et al.*, 1992), and *in vivo* prolactin maintains relaxin secretion for 10 days in hypophysectomized-hysterectomized gilts (Li *et al.*, 1989). In addition, expression of the relaxin gene in corpus luteum of sows appears during the oestrous cycle, pregnancy and lactation (Bagnell *et al.*, 1990). In the uterine endometrium of sows, expression of the relaxin gene and relaxin immunostaining have been detected between days 10 and 20 of the oestrous cycle in pregnant and nonpregnant animals (Knox *et al.*, 1994). Thus, there are marked differences in the expression of the relaxin gene and relaxin production during the different reproductive states in boars and sows. Relaxin concentrations were similar between intact and recently castrated boars in the present study, supporting the hypothesis that the testis might be a target tissue rather than a source of relaxin (Yamamoto and Bryant-Greenwood, 1981).

The results of the present study indicate that peripheral blood concentrations of relaxin in boars remain low (< 500 pg ml $^{-1}$) before puberty and are generally unrelated to pre-pubertal development and sexual maturity, and are three to four times lower than those found in seminal plasma. Age-dependent increases in gonadotrophin-induced testosterone secretion were without effect on relaxin secretion.

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