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Lamprey GnRH-III Stimulates FSH Secretion in Barrows

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Contents

Although studies have indicated that follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release can be dissociated in the pig, the underlying mechanisms are still to be answered. Since it was demonstrated that lamprey gonadotropin-releasing hormone (l-GnRH-III) has preferential FSH-releasing potency in several mammalian species, we have investigated the gonadotropin-releasing activity of l-GnRH-III in barrows. Each of nine barrows (body weight: 85–90 kg; age: 207 days) received 2 ml saline (S-barrow), followed by 150 μg l-GnRH-III (1.6–1.7 μg/kg body weight) dissolved in 2 ml saline intramuscularly 7 days later. Three pre-treatment and 13 post-treatment blood samples were taken at intervals of 30 min to 8 h to assess basal and treatment-associated concentrations of FSH and LH, respectively, by radioimmunoassay. Animals were defined as having responded to treatment if, 2 h post-treatment, plasma FSH and/or LH levels were >3 SD of the respective basal concentrations. There was no treatment-associated FSH response after saline treatment, but a clear FSH response in all l-GnRH-III-injected barrows. On average, the maximum FSH level (205% of the basal concentration) was observed at 1 h post-treatment. Mean FSH values were elevated until 10 h post-treatment. There was no LH response either to saline or to l-GnRH-III. In conclusion, this study demonstrates a selective FSH-releasing activity of 150 μg l-GnRH-III in barrows. Further studies are needed to investigate whether this effect is ubiquitous in the pig and what the physiological relevance is.

Introduction

In the pig, mammalian luteinizing hormone-releasing hormone (LHRH) is assumed to be the only hypothalamic gonadotropin-releasing hormone (GnRH) that is equally and ultimately necessary for the control of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion (Lücking Jayes et al. 1997). However, there are several indicators for a division in the mechanism controlling LH and FSH secretion in the pig. In pre-pubertal gilts, feed restriction–re-alimentation altered LH but not FSH secretion (Booth et al. 1996). Active immunization of mature boars against LHRH lowered plasma LH concentration and pituitary LH content, but had no effect on plasma FSH (Awoniyi et al. 1988; Wagner and Claus 2004) and pituitary FSH content (Awoniyi et al. 1988). In the castrated mature boar and cross-bred gilt, the pulsatile release of LH and FSH can be dissociated (Liptrap et al. 1986; Flowers et al. 1991). An attempt was made to explain the differential regulation of FSH and LH in the pig by altering the LHRH pulse frequency (Lücking Jayes et al. 1997). However, when gilts were treated with the GnRH-I antagonist Antarelix, LH but not FSH was suppressed (Driancourt et al. 1995; Brüssow et al. 2001). This suggests that there might be a separate hypothalamic FSH-releasing component distinct from LHRH in the pig. However, investigations into such a component have not yet been carried out in the pig.

Previous studies primarily conducted in the rat and sheep indicate that there is a separate hypothalamic control of FSH release (McCann et al. 2001; Padmanabhan and McNeilly 2001; Padmanabhan et al. 2002). Until recently, numerous GnRH variants have been identified in many non-vertebrate and vertebrate species (Millar et al. 2004). Among those, lamprey GnRH-III (l-GnRH-III), a decapeptide that has 60% homology with the LHRH (Sower et al. 1993), has been demonstrated to exert a preferentially FSH-releasing action both *in vivo* and *in vitro* using the rat model (Yu et al. 1997, 2000, 2002). Similarly, l-GnRH-III administered intravenously during the luteal phase to cows resulted in a preferential FSH release and stimulated the growth of multiple follicles (Dees et al. 2001). Using immunostaining, l-GnRH-III-positive hypothalamic regions were revealed in different mammalian species including the rat, human and bovines (Dees et al. 1999; Chen et al. 2000; Hiney et al. 2002). Moreover, if rat hypothalami were gel filtrated on Sephadex G-25, the selective FSH-releasing activity of fractions containing l-GnRH as determined by radioimmunoassay (RIA) was neutralized using antiserum against l-GnRH (Yu et al. 2000). Collectively, data suggest that l-GnRH-III could be a putative candidate for a separate hypothalamic control of FSH release, i.e. it might be the long-sought-after FSH-releasing factor (McCann et al. 2001). Based on those data, this study was conducted to investigate the effect of l-GnRH-III on FSH and LH response in barrows, thereby proving l-GnRH-III to be a putative hypothalamic component involved in the control of FSH release in the pig.

Materials and Methods

Animals

All experimental procedures involving animals in this study were approved by the Authorities of the Land of Brandenburg, Germany. For the experiment, nine barrows [(Large White ♀ × German Landrace ♂) ♀ × Pietrain ♂; body weight (mean ± SD): 87.4 ± 1.7 kg; range: 85–90 kg; age: 217 days (animals belonged to three litters born on the same day)] were used. The animals were kept in separate pens with partially slatted floors. They were fed a total of 2.7 kg of a standard ration for barrows (13 MJ per kg feed) per day and had *ad libitum* access to water. Light was provided for 15 h/day.

Experimental design

Each barrow was used to assess the effect of both saline (S-barrow) and of l-GnRH-III (l-GnRH-III-barrow) on FSH and LH secretion. The substances were tested at an interval of 7 days (saline: day 0; l-GnRH-III: day 7). The experimental design, i.e. the procedures used for the application of the substances and for bleeding, including the bleeding intervals, was identical for S- and l-GnRH-III-barrows. Saline (2 ml) and l-GnRH-III (150 μg diluted in 2 ml saline) were injected intramuscularly into the base of the ear. Blood was collected by puncture of the anterior vena cava with sterile disposable 18-G needles of 15 cm in length. Blood sampling was performed by two skilled people who were able to perform the sampling procedure within a minimum period of time (approximately 1 min). The bleeding intervals were chosen according to the results of a study conducted to investigate the effects of l-GnRH-III in cows (Dees et al. 2001). Prior to the application of saline and l-GnRH-III, three blood samples (5 ml each) were drawn at intervals of 0.5 h to assess basal concentrations of FSH and LH. Immediately after the third sample, saline or l-GnRH-III was injected. Thirteen blood samples were then taken as follows: each 30 min up to 3 h, and at 4, 6, 8, 10, 12, 16 and 24 h. After sampling, the blood was centrifuged at $3000 \times g$ for 20 min, and serum was recovered and stored at −20°C until FSH and LH analysis.

Peptide

The l-GnRH-III decapeptide (amino acid sequence: pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) was synthesized as a pure chemical-synthetic preparation at BFC BioPept-Feinchemie GmbH (Grabe, Germany). For this study, the peptide was dissolved in saline at a concentration of 75 μg/ml and the solution was adjusted to a pH value of 6.5–7.0. Purity of l-GnRH-III in the solution was 96.08% as tested by HPLC and mass spectrometry.

Hormone assays

Each blood serum sample was analysed for FSH and LH by RIA in duplicates using double-antibody technique in a blind trial. Unlabelled porcine FSH and LH (pFSH and pLH, respectively; both from the Diagnostic Product Co., Los Angeles, CA, USA) were used as standards. The standards were compared with standard curves generated using respective reference preparations supplied by the National Institute of Health (NIH, Bethesda, USA). ^{125}I-radioiodinated pFSH and pLH (both Diagnostic Product Co.; radioiodinated with Chloramin T method using $Na^{125}I$), respectively, were used as tracers (activity in tubes of approximately 10 000 cpm). Analysis of FSH and LH was performed in 400 μl blood serum. Initially, to the serum samples, 100 μl antiserum against the respective gonadotropin were given and mixed for 1 min. As antiserum, lyophilized polyclonal rabbit anti-porcine FSH and anti-porcine LH (Diagnostic Products Cooperation) diluted in 10 ml aqua bidestillata, respectively, was used. After incubation at 37°C in a water bath for 1 (LH) or 3 (FSH) h, 100 μl of the respective tracer solutions were added, the resulting aliquots again mixed and incubated at room temperature for 2 (LH) or 3 (FSH) h. To separate free from antibody-bounded hormone 1000 μl of a precipitation reagent (pure polyethylene glycol with goat anti-rabbit-IgG; Diagnostic Product Co.) were added to each of the aliquots, mixed for 1 min and then centrifuged at $3000 \times g$ at 24°C for 20 min. The supernatants were decanted and the resulting pellets measured for pFSH or pLH, using a 12-channel gamma-counter (STRATEC Electronic GmbH, Birkenfeld, Germany) for 1 min. The sensitivity of the assays was 0.07 mIU/ml for FSH and 0.1 mIU/ml for LH. Intra- and interassay coefficients of variation were 9.2% and 12.4% for FSH, and 8.5% and 11.5% for LH respectively. There was a cross-reactivity between pFSH and pLH of $< 1.5\%$. Both pFSH and pLH cross-reacted with porcine thyroid-stimulating hormone (TSH), prolactin and growth hormone (STH) at $< 1\%$ (respective standards were supplied by the NIH).

Statistical analysis

Statistical analysis was performed in SPSS (SPSS GmbH, Munich, Germany). Basal concentrations of FSH and LH were expressed as the mean ($\pm$ SD) of the pre-treatment values ($n = 3$ for each hormone and barrow) of the S- and l-GnRH-III-barrows, respectively, and compared using the *t*-test for independent samples. Comparison between respective FSH and LH values of S- and l-GnRH-III-barrows was performed using ANOVA. Repeated measurement ANOVA and a subsequent paired Student's *t*-test were used to investigate whether there were differences between FSH and LH values, respectively, within the sampling period. However, animals were defined as displaying a response to treatment if after 2h post-treatment plasma levels of either FSH or LH or both gonadotropins were > 3 SD the respective basal concentrations before treatment. Significance was expressed as $p < 0.05$.

Results

Mean basal FSH concentrations were almost identical in S- and l-GnRH-III-barrows (1.06 ± 0.12 mIU/ml vs 1.07 ± 0.08 mIU/ml). In S-barrows, there was no treatment-associated FSH response during the sampling period (Fig. 1). In contrast, l-GnRH-III elicited a clear FSH response, which was uniformly obtained in all nine l-GnRH-III-treated barrows as evident from the low coefficient of variations (2.8–6.5%) estimated for the FSH concentrations in blood samples obtained between 0.5 and 10 h post-l-GnRH-III application. The maximum mean FSH value reached 205% of the mean basal concentration and was observed at 1 h after l-GnRH-III treatment (between 1 and 1.5 h for individual boars). Mean FSH values then decreased slowly reaching basal FSH concentrations at 12 h after l-GnRH-III application. Mean basal LH concentrations were similar in S- and l-GnRH-III-barrows (3.3 ± 0.3 mIU/ml vs 3.1 ± 0.5 mIU/ml). Although the mean LH values estimated in blood serum samples drawn from

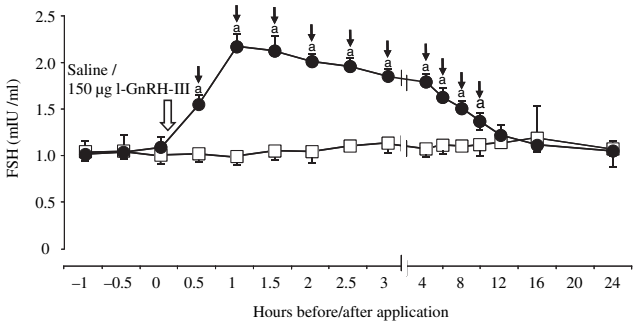

Fig. 1. Mean (±SD) FSH concentration in blood serum of barrows before and after application of saline (□; n = 9) or 150 µg l-GnRH-III (●; n = 9). Letter (a) indicates that values of saline- and l-GnRH-III-treated barrows were significantly different ($p < 0.05$). Black arrows indicate values that were > 3 SD above the basal concentration before treatment. There was a clear FSH response after l-GnRH-III application. No response was observed in barrows treated with saline

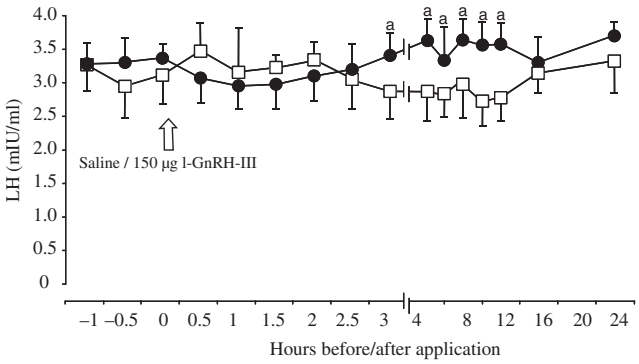

Fig. 2. Mean (±SD) LH concentration in blood serum of barrows before and after application of saline (□; n = 9) or 150 µg l-GnRH-III (●; n = 9). Letter (a) indicates that values of saline- and l-GnRH-III-treated barrows were significantly different ($p < 0.05$). There was no LH response after saline or l-GnRH-III application

l-GnRH-III-barrows between 3 and 12 h after treatment were higher ($p < 0.05$) than the corresponding values estimated for saline-treated barrows, there was no LH response either to saline or to l-GnRH-III (Fig. 2).

Discussion

This study has demonstrated that l-GnRH-III releases FSH in barrows when given intramuscularly at a dose of 150 µg per animal (corresponds to 1.7–1.8 µg/kg body weight), and that such a release did not occur when saline was administered. Moreover, using 150 µg per animal, the l-GnRH-releasing activity on FSH was rather selective, as no concurrent increase in the LH concentrations was observed. Whether the higher level of LH in l-GnRH-III-treated barrows between 3–12 h post-treatment compared to controls was due to l-GnRH-III remains to be answered. However, in this study it does not fulfil the requirements defined for a response to l-GnRH-III (i.e. an increase by 3 SD within 2 h of application). Moreover, an immediate increase in LH within a few minutes, as a response to l-GnRH-III, would have been expected and as demonstrated in a study where barrows were treated with different doses of GnRH-I agonists Buserelin and D-Phe^6-LHRH (Möller-Holtkamp et al. 1995). However, further investigations including dose response studies are needed to test whether a selective effect of l-GnRH-III on FSH secretion is ubiquitous in the pig. Nonetheless, the results of this study are in accordance with previous reports demonstrating a preferential FSH-releasing activity of l-GnRH-III *in vitro* using hemipituitaries of male rats (Yu et al. 1997, 2000, 2002) or *in vivo* after administration to ovariectomized, oestrogen/progesterone-blocked rats (Yu et al. 1997) and intact adult cows (Dees et al. 2001). However, they are in contrast to the results of two recent studies in which l-GnRH-III failed to stimulate a preferential release of FSH when administered to ovariectomized, oestradiol/progesterone-treated rats (Kovacs et al. 2002) and to cattle (Amstalden et al. 2004).

The results of this study support the suggestion of previous studies that there might be a separate mechanism different from the LHRH that is involved in the control of FSH release in the pig (Awoniyi et al. 1988; Driancourt et al. 1995; Brüssow et al. 2001). Several factors including gonadal steroids and peptides like activin, follistatin and inhibin have been shown to affect gonadotropin secretion (Padmanabhan and McNeilly 2001; Nett et al. 2002; Padmanabhan et al. 2002). However, LHRH is assumed to be the only hypothalamic releasing factor responsible for both LH and FSH synthesis and release (Lücking Jayes et al. 1997). In fact, the administration of LHRH or of its analogues (Buserelin; Triptorelin; D-Phe^6-LHRH; D-Ala^6, des-Gly^{10}-LHRH) to intact or castrated boars and to female pigs stimulates the release of LH, but also of FSH albeit with a lower amount (Traywick and Esbenshade 1988; Möller-Holtkamp et al. 1995; Brüssow et al. 1996; Wise et al. 2000). These results indicate that LHRH and its analogues trigger the release of both gonadotropins, but are more potent in stimulating LH than FSH release. Using ovariectomized gilts immunized against LHRH, Lücking Jayes et al. (1997) tried to explain the relative autonomy of FSH secretion in the pig by alteration in the LHRH pulse frequency. While a higher frequency was more effective in releasing LH, a lower frequency stimulated preferentially FSH release. Moreover, they concluded that LHRH is ultimately necessary for a continued FSH synthesis and release. Recently, a functional GnRH-type II receptor has been demonstrated in the pig (Neill et al. 2002, 2004; Morgan et al. 2003) indicating that GnRH-II is present in the pig. Although the physiological function of GnRH-II is generally unclear, studies in several species including musk shrews, monkeys and sheep suggest that GnRH-II might be involved in the control of certain reproductive processes including gonadotropin secretion (Millar 2003; Neill et al. 2004). In swine, if boars or gilts were treated with the GnRH-I antagonists Cetrorelix and Antarelix, LH, but not FSH, was effectively suppressed (Driancourt et al. 1995; Wise et al. 2000; Zanella et al. 2000; Brüssow et al. 2001). As both Cetrorelix and Antarelix are ineffective in blocking the GnRH-type II receptor (Neill 2002), the failed alteration of the FSH release in GnRH-I antagonist-treated pigs might be

explained by interactions of GnRH-II and its receptor, as it is recently suggested for the ewe (Padmanabhan et al. 2003) and discussed elsewhere (Millar 2003). However, the results of the present study indicate that there could be an additional or another hypothalamic mechanism controlling FSH release in the pig, and that this mechanism could involve l-GnRH-III or a related peptide. As l-GnRH-III does not stimulate [^{3}H]-inositol phosphate production of COS-1 cells transfected with human GnRH-type I receptor and shows only weak potency on COS-1 cells transfected with the monkey GnRH-type II receptor (Neill 2002), the selective effect of l-GnRH-III on FSH release observed in barrows in this study might be mediated via a separate receptor, as it is suggested for the rat (Yu et al. 2002).

Conclusion

In conclusion, this study demonstrates a selective FSH-releasing activity of l-GnRH-III given intramuscularly at a dose of 150 μg to barrows. Dose–response studies are required to prove whether this selective effect is ubiquitous in the pig. The physiological relevance of GnRH-III in the pig needs to be investigated.

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