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The role of RFamide-related peptide 3 (RFRP3) in regulation of the neuroendocrine reproductive and growth axes of the boar☆

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ABSTRACT

RFamide-related peptide 3 (RFRP3) has been implicated in regulating reproduction and growth. This regulation appears to be dependent upon sex, species, physiological status, and developmental stage. The objective of the present study was to evaluate the effects of RFRP3 on circulating concentrations of luteinizing hormone (LH) and growth hormone (GH) in mature boars. The hypothesis was RFRP3 would reduce circulating concentrations of LH and increase concentrations of GH. Meishan boars (716.6 ± 2.8 days of age; 125.0 ± 12.4 kg BW) were randomly assigned to treatment: saline ($n = 4$) or RFRP3 (8.5 mg; $n = 5$). Plasma was collected at 15-min intervals during 3 periods: pre-treatment, treatment, and post-treatment. During the treatment period, saline or RFRP3 were administered at 15-min intervals. Treatment was administered as a loading dose of 5 mg RFRP3, followed by seven repeated injections of 0.5 mg RFRP3. Pulsatile secretion of LH and GH were not affected by saline treatment. Mean concentrations of LH in RFRP3-treated boars were greater ($P < 0.01$) in the pre-treatment period than in the treatment and post-treatment periods; however, the individual response to RFRP3 challenge was varied. RFRP3 suppressed ($P < 0.05$) mean concentrations of GH during the treatment period. It is concluded that RFRP3 can act to suppress LH secretion in some boars, but the minimal and varied response between animals does not strongly support the idea that RFRP3 is a potent hypophysiotropic hormone in the pig. Results indicate that RFRP3 may function in regulating the growth axis of swine.

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1. Introduction

Secretion of gonadotropin-releasing hormone (GnRH) and growth hormone-releasing hormone (GHRH) orchestrate the reproductive and growth axes, respectively. However, hypothalamic neuropeptides that function as central regulators of GnRH and GHRH are not well understood in the boar. A single member of the RFamide family of peptides, RFamide-related peptide 3 (RFRP3), has been implicated in regulating GnRH and GHRH neuronal expression and function (Ancel et al., 2012; Ducret et al., 2009; Johnson and Fraley, 2008) as well as secretion of luteinizing hormone (LH; Ancel et al., 2012; Johnson et al., 2007; Johnson and Fraley, 2008; Kadokawa et al., 2009) and growth hormone (GH; Johnson et al., 2007; Johnson and Fraley, 2008) in male mammals.

Responsiveness of the reproductive neuroendocrine axis to RFRP3 appears to be dependent upon sex, species, physiological status, and developmental stage. Recent evidence in the seasonally reproductive hamster suggests that the function of RFRP3 is sexually dimorphic. In the mature intact male hamster, central infusion of RFRP3 increased LH, FSH, testosterone, and GnRH neuronal c-Fos expression whereas peripheral injection of RFRP3 failed to alter LH secretion (Ancel et al., 2012). This contradicts findings from female hamsters, in which both central and peripheral treatment with RFRP3 inhibited LH secretion (Kriegsfeld et al., 2006). In non-seasonal species, such as rats or cattle, peripheral infusion of RFRP3 inhibited the gonadotropic axis of males (Johnson and Fraley, 2008; Kadokawa et al., 2009). The RFRP mRNA in the dorsomedial nucleus of the hypothalamus of mice decreased with maturity (Poling et al., 2012). Consequently, RFRP3 has been proposed to be involved in the onset of puberty; however, infusion of RFRP3 was unable to prevent the onset of puberty in the intact male rat (Johnson and Fraley, 2008). In spite of decreased expression during adulthood, RFRP3 suppressed concentrations of LH and sexual behavior and increased concentrations of GH and feed intake in the mature male rat (Johnson et al., 2007).

The role of RFRP3 in the boar is unknown. Therefore, the objective of the present study was to evaluate the effects of RFRP3 on circulating concentrations of LH and GH in mature, intact Chinese Meishan boars. It was hypothesized that RFRP3 would reduce circulating concentrations of LH, while increasing concentrations of GH.

2. Materials and methods

2.1. Experimental design

This study was conducted using standard production and experimental practices that were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010) and were approved by the U.S. Meat Animal Research Center. Mature, Chinese Meishan boars (716.6 ± 2.8 days of age; 125.0 ± 12.4 kg BW) were fitted with indwelling jugular catheters (Ford and Maurer, 1978) at least 1 week prior to treatment. Boars were individually housed in 2 × 3 m pens with *ad libitum* access to water and were offered a fortified corn soybean-meal diet that met or exceeded nutrient requirements (NRC, 1998). Boars were assigned randomly to either saline ($n = 4$) or RFRP3 ($n = 5$) treatment. Blood was collected at 15-min intervals over three periods: pre-treatment (minute 0–75), treatment (minute 75–180), and post-treatment (minute 180–285). During the treatment period, 0.9% saline and RFRP3 (Val-Pro-Asn-Leu-Pro-Gln-Arg-Phe-NH2; GenScript Inc., Piscataway, NJ) suspended in 0.9% saline were administered at 15-min intervals immediately following blood collection. A loading dose of 5 mg of RFRP3 followed by repeated injections of 0.5 mg of RFRP3 every 15 min for 105 min resulted in a total infusion of 8.5 mg (approximately 0.07 mg RFRP3/kg BW) of peptide per animal. The dose was based from research with gilts (Heidorn et al., 2010). Blood was collected in heparinized syringes immediately before each 15-minute treatment infusion.

2.2. Hormone analyses

Plasma was harvested (2,000 × *g* for 20 min at 4 °C) and stored at −20 °C. Concentrations of LH and GH in plasma were determined by RIA as described previously (Kesner et al., 1987; Lents et al., 2008). The reference standards for LH (AFP-10506A) and GH (AFP-10864B) were provided by Dr. A.F. Parlow (Scientific Director of the NIH, NIDDK, National Hormone and Peptide Program). Pools of porcine plasma with LH concentrations that ranged from 0.22 to 3.75 ng/ml were included in each assay. The intra- and inter-assay CV were, 9.3 and 7.9%, respectively. Sensitivity of the LH assay (defined as 90% of Bo) was 0.1 ng/ml. Plasma concentrations of GH were determined in a single assay with an intra-assay CV of 12.0%. Sensitivity of the GH assay (90% of Bo) was 0.18 ng/ml. Plasma concentrations of testosterone were determined in duplicate according to Ford et al. (2001). The minimum sensitivity of the assay was 50 pg/ml and intra-assay CV was 13%.

2.3. Pulse analyses

Characterizations of LH pulses were adapted from previously described factors (Goodman et al., 2012): (1) the peak occurred within two samples of the previous nadir, (2) pulse amplitude exceeded assay sensitivity, and (3) the peak was two standard deviations above the preceding nadir (nadir concentrations within animal). Luteinizing hormone pulse frequency was quantified across all periods. Inter-pulse interval was quantified as the interval of time between pulse peaks from the pre-treatment to the post-treatment period and means calculated within animal for statistical analysis. Pulse amplitude was calculated across all period as the difference between the peak pulse concentration and the preceding nadir and means calculated within animal. One exception was when the pulse occurred within two samples of initiation of sampling, in these cases the following nadir was utilized.

2.4. Statistical analyses

Main effects of treatment on concentrations of LH and GH in plasma were determined using the MIXED

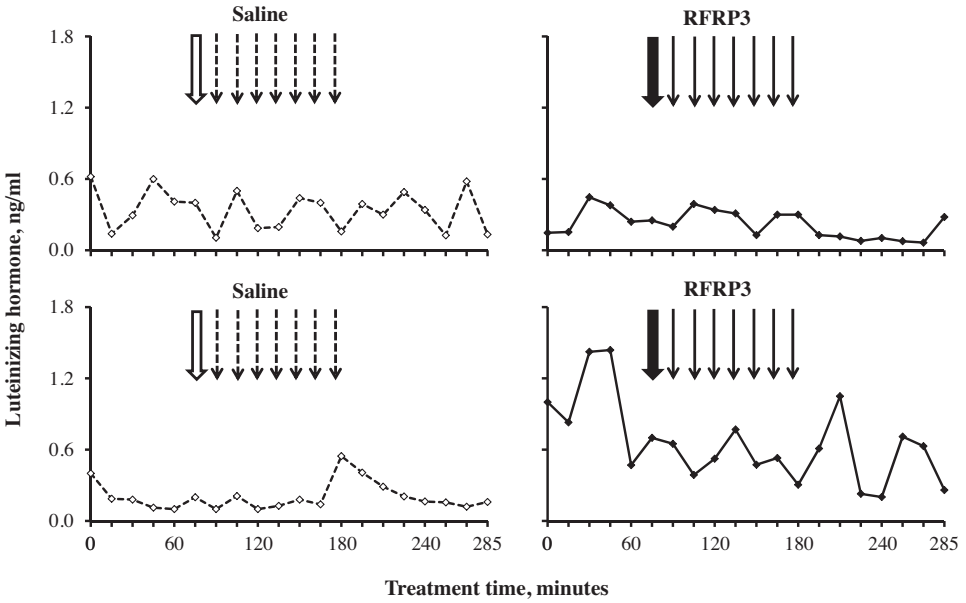

Fig. 1. Plasma concentrations of luteinizing hormone during the pre-treatment (minute 0–75), treatment (minute 75–180), and post-treatment (minute 180–285) periods in representative boars for each treatment group. Boars were treated with either saline (dashed line) or RFRP3 (8.5 mg; solid line) during the treatment period. The large arrow indicates administration of the loading dose (5 mg RFRP3/boar), while the small arrows indicate the repeated injections (0.5 mg RFRP3/boar).

procedure of SAS (SAS Institute Inc., Cary, NC). The model included treatment, period, and treatment × period interaction. Compound symmetry was used to model the covariance structure for the repeated measure. Quantification of differences in mean pulse frequency between treatments was analyzed using the Kruskal–Wallis test for nonparametric, noncontinuous data. Mean inter-pulse interval and pulse amplitude were analyzed by the GLM procedure of SAS with treatment included as the source of variation. Data are reported as Least Squares means ± SEM, unless stated otherwise. Effects were considered significant when $P \leq 0.05$.

3. Results

3.1. Effects of RFRP3 on secretion of LH

Individual profiles of LH in plasma of representative boars for each treatment group are presented in Fig. 1. Animal to animal variation in the secretory pattern of LH can be observed. There was a treatment × period interaction ($P < 0.04$) for mean concentrations of LH (Fig. 2). Mean concentrations of LH in plasma did not differ ($P > 0.52$) between periods for saline-treated animals; however, mean concentrations of LH were greater ($P < 0.01$) in the pre-treatment period compared with treatment and post-treatment periods in RFRP3-treated boars. There was no difference between treatments in number of LH pulses (4.3 ± 1.3 compared with 4.4 ± 0.5 pulses; $P = 0.90$), LH pulse amplitude (0.38 ± 0.16 compared with 0.37 ± 0.08; $P = 0.95$), or inter-pulse interval ($P = 0.27$) between saline- and RFRP3-treated boars, respectively, over the entire sampling time. Plasma concentrations of testosterone averaged 6.2 ± 2.0 ng/ml, and were not different ($P > 0.30$) between saline and RFRP3-treated boars.

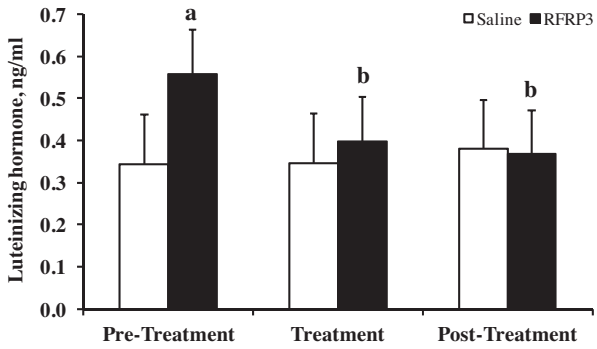

Fig. 2. Least squares means (±SEM) for plasma concentrations of luteinizing hormone during the pre-treatment (minute 0–75), treatment (minute 75–180), and post-treatment (minute 180–285) periods. Boars were treated with either saline ($n = 4$; no fill) or 8.5 mg RFRP3 ($n = 5$; solid fill) during the treatment period. Treatment × Period, $P < 0.04$. [a,b]Within treatment, means are different ($P < 0.01$).

3.2. Effects of RFRP3 on secretion of GH

Individual profiles of GH in plasma of representative boars for each treatment group are presented in Fig. 3. There was a treatment × period interaction ($P < 0.01$) for mean concentrations of GH (Fig. 4). Mean concentrations of GH in RFRP3-treated boars were reduced ($P < 0.04$) during the treatment period compared with pre- and post-treatment periods. Mean concentrations of GH did not differ ($P > 0.39$) between saline- and RFRP3-treated boars during the pre- and post-treatment period.

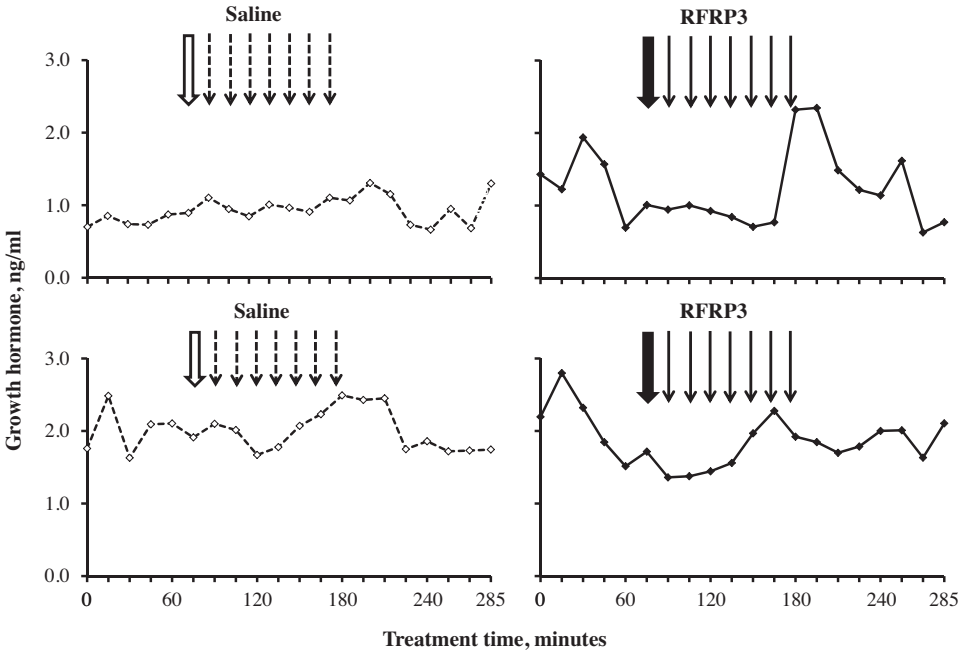

Fig. 3. Plasma concentrations of growth hormone during the pre-treatment (minute 0–75), treatment (minute 75–180), and post-treatment (minute 180–285) periods in representative boars for each treatment group. Boars were treated with either saline (dashed line) or RFRP3 (8.5 mg; solid line) during the treatment period. The large arrow indicates administration of the loading dose (5 mg RFRP3/boar), while the small arrows indicate the repeated injections (0.5 mg RFRP3/boar).

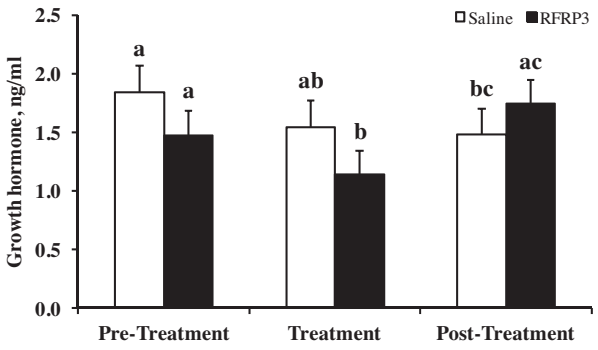

Fig. 4. Least squares means (±SEM) for plasma concentrations of growth hormone during the pre-treatment (minute 0–75), treatment (minute 75–180) and post-treatment (minute 180–285) periods. Boars were treated with either saline ($n = 4$; no fill) or 8.5 mg RFRP3 ($n = 5$; solid fill) during the treatment period. Treatment × Period, $P < 0.01$. [a,b,c]Within treatment, means are different ($P < 0.05$).

4. Discussion

This is the first investigation into the effects of RFRP3 on *in vivo* secretion of LH and GH in boars. Chinese Meishan boars have greater concentrations of LH and gonadal steroids than cross-bred boars of European breeds (Wise et al., 1996; Zanella et al., 1996). It was thus reasoned that the greater LH concentration of Meishan boars made them a more desirable choice than white composite boars to evaluate the potential inhibition of RFRP3 on LH in male pigs. The suppressive effects of peripheral infusion of RFRP3 on secretion of LH in these boars were slight and inconsistent between animals. A role of RFRP3 in regulation of the growth axis of the boar appears possible. These results further classify the functional role of RFRP3 outside of the reproductive axis and demonstrate the variation in function of this peptide across mammalian species.

Present results indicate that RFRP3, when administered intravenously, can act to suppress mean concentrations of LH in mature boars. Kadokawa et al. (2009) and Caraty et al. (2012) employing a similar approach observed that repeated intravenous infusion of RFRP3 did not reduce mean concentration of LH in gonadectomized male cattle or ovariectomized ewes, respectively. Additionally, repeated peripheral infusion of RFRP3 failed to suppress LH secretion in cyclic mares during the breeding season (Thorson et al., 2014). In rodents, RFRP3 suppressed (Johnson et al., 2007; Pineda et al., 2010a), had no effect (Anderson et al., 2009; Murakami et al., 2008), or increased (Ancel et al., 2012) *in vivo* secretion of LH. There are similar conflicting reports on the *in vivo* effects of RFRP3 on LH secretion in sheep (Caraty et al., 2012; Clarke et al., 2008). These inconsistent results have lead to controversy about the functional role of RFRP3 as a hypophysiotropic hormone in mammals.

In estrous cyclic mares (Thorson et al., 2014) and ovariectomized ewes (Caraty et al., 2012), RFRP3 did not affect variables of LH pulses. Kadokawa et al. (2009) observed that intravenous infusion of RFRP3 suppressed frequency of LH pulses but did not affect LH pulse amplitude in gonadectomized male cattle. Conversely, RFRP3 suppressed LH pulse amplitude but not LH pulse frequency in ovariectomized ewes (Clarke et al., 2008). When individual LH profiles were examined, LH secretion in some but not all boars appeared to be suppressed during the RFRP3

treatment period. Other boars appeared to have a latent response to the suppressive effects of RFRP3. The later response is difficult to explain given the relatively short half-life of RFRP3 in general circulation (Caraty et al., 2012; Pineda et al., 2010b; Smith et al., 2012). The modest effect of RFRP3 to suppress LH pulses in steers and ewes stopped immediately upon cessation of repeated RFRP3 injection. In ewes, nerve terminals containing RFRP3 are located in the external zone of the median eminence (Clarke et al., 2008) suggesting a pituitary site of action. In accordance with this finding, it has been reported that RFRP3 is secreted in a pulsatile fashion into the hypophyseal portal circulation of the mature ovary-intact ewe (Smith et al., 2012). These previous data, together with the recent observation that RFRP3 suppressed GnRH-induced secretion of LH from primary cultures of anterior pituitary cells of gilts (Li et al., 2013), are strong justification for the approach utilized in the current study in which a large loading dose was followed by repeated injection every 15 min into the peripheral circulation.

With the present inconsistency in understanding of the role of RFRP3 in regulating LH secretion in mammals, it remains unclear where the site of RFRP3 action lies. Based on reports that RFRP3 inhibited GnRH-induced secretion of LH from primary cultures of anterior pituitary cells (Clarke et al., 2008; Kadokawa et al., 2009; Li et al., 2013), in the present study RFRP3 was administered into the peripheral circulation of boars. However, RFRP3 immunoreactive nerve terminals have been observed in close proximity to GnRH cell bodies in rats (Kriegsfeld et al., 2006) and sheep (Smith et al., 2008); and RFRP3 was found to stimulate GnRH neurons, as measured by expression of c-Fos, in rats (Ancel et al., 2012). Moreover, RFRP3 reduced the firing rate of GnRH neurons in sections of mouse brain in culture (Ducret et al., 2009). The putative RFRP3 receptor, GPR147, was found in the hypothalamus of the gilt and RFRP3 suppressed secretion of GnRH from cultured hypothalamic explants obtained from gilts (Li et al., 2012, 2013). Whether RFRP3 functions centrally to inhibit the secretion of LH in the pig requires further investigation.

It should be noted that the dose of RFRP3 used in the present study was greater than that in our preliminary study (Heidorn et al., 2010) in which doses of RFRP3 that were comparable to those reported to suppress LH pulses in sheep (Clarke et al., 2008) or steers (Kadokawa et al., 2009) were ineffective at suppressing LH secretion in ovariectomized prepubertal gilts. As previously described, the inhibitory effects of RFRP3 on LH secretion are inconsistent; even when used at similar doses in the same species. As discussed by Thorson et al. (2014), the lack of biological activity between different preparations of RFRP3 seems an implausible explanation for the inconsistent results reported for RFRP3 in the literature. The last four C-terminal amino acids and amidation signal are required for binding and activation of GPR147 (Findeisen et al., 2011; Yoshida et al., 2003) and these are well conserved among species, including the pig (Li et al., 2012). Expression of the GPR147 gene in the hypothalamus and adenohypophysis of pigs was observed to fluctuate with stage of the estrous cycle (Li et al., 2012); thus, subtle differences in the amount of GPR147 available for binding RFRP3 likely represent a more reasonable explanation for these discrepancies, and may explain the individual response to RFRP3 observed in the current study.

The RFRP3 neuropeptide is not solely selective to the reproductive axis. There was no difference in mean circulating concentrations of GH between saline-treated and RFRP3-treated boars during the treatment period; however, examination of individual secretory patterns indicates that GH was suppressed during the early treatment period in RFRP3-treated boars (see Fig. 3). After RFRP3 treatment ceased, GH secretion was restored. As a consequence, mean concentrations of GH in RFRP3-treated boars were greater in the post-treatment period. In contrast to the present results in the boar, circulating concentrations of GH rapidly increased following intracerebroventricular infusion of RFRP3 in prepubertal (Johnson and Fraley, 2008) and mature (Johnson et al., 2007) intact, male rats. The stimulatory effect of RFRP3 on GH secretion in the rat was evident at lesser doses, but not at greater doses (Johnson et al., 2007). The decreased GH secretion may be attributable to the relatively large dose of RFRP3 used in boars in the present study. Central infusion of RFRP3 increased GHRH mRNA in accordance with the subsequent increase in plasma concentrations of GH (Johnson and Fraley, 2008) indicating that the site of GH regulation in rats lies within the hypothalamus. Whether RFRP3 may function through its receptor in the hypothalamus (Li et al., 2012) to regulate GHRH neuronal function within the arcuate and ventromedial nuclei of boars (Leshin et al., 1994) remains to be elucidated.

In conclusion, the present study represents the first investigation into the effects of RFRP3 on *in vivo* secretion of LH and GH in the boar. It was recently reported that RFRP3 inhibited GnRH-induced secretion of LH from primary cultures of porcine anterior pituitary cells collected from gilts (Li et al., 2013). The current results indicate that RFRP3 can suppress mean concentrations of LH in sexually mature boars. The secretory response of LH to RFRP3 was minimal and varied between animals, which does not strongly support the idea that RFRP3 is a potent hypophysiotropic hormone in the pig. The role of RFRP3 in regulating the gonadotropic axis of mammals remains controversial due to inconsistent results reported in the literature for a number of species. The current data, unfortunately, do not clarify the role of RFRP3 in regulating *in vivo* secretion of LH in the pig; however, alterations in secretion of GH may suggest a functional role of RFRP3 in the neuroendocrine growth axis of male pigs. Further study into these mechanisms is required to understand the role that RFRP3 plays in regulating swine physiology.

Conflict of interest

The authors declare no conflict of interest.

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