Opioidergic inhibition of luteinising hormone and prolactin release changes during pregnancy in pony mares

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Abstract

In equine species, luteinising hormone (LH) and prolactin (PRL) release are reduced throughout pregnancy but increase at foaling. The present experiments were designed to study a possible opioidergic regulation of LH and PRL release in pregnant Shetland mares (n=6). At various stages of pregnancy (days 26.4±0.6, 75.4±5.4, 171.8±2.4, 226.2±4.8, 282.7±3.4 and 319.8±2.1), mares were injected with the opioid antagonist naloxone (0.5 mg/kg body weight) and saline. The two treatments were always separated by 2 days, and mares served as their own controls. Two hours after being given naloxone and saline, mares were given the gonadotrophin-releasing hormone (GnRH) analogue buserelin (5 µg per animal). The naloxone experiment was repeated at 2 days after foaling. Blood for the determination of LH and PRL was withdrawn at 15 min intervals for 240 min, and naloxone or saline was injected after 60 min. Naloxone induced significant (P<0.05) LH release on days 172, 226 and 283 of pregnancy but not on days 26, 76 and 320 and 2 days after foaling. Buserelin caused a significant (P<0.05) increase in plasma LH concentrations on days 172, 226, 282 and 320 of pregnancy. The experiments indicate that endogenous opioids are involved in the inhibition of LH release during the second half of pregnancy in equine species. The deactivation of opioid effects on LH release might be a prerequisite for the onset of ovarian activity postpartum. Plasma PRL concentrations increased significantly (P<0.05) after naloxone administration on days 226, 282 and 320 of pregnancy. The naloxone-induced PRL release was most pronounced towards term, indicating an increase in the naloxone-releasable pool and/or the absence of other PRL-release inhibitory mechanisms.

Introduction

In equine species, as in other species, release of luteinising hormone (LH) and prolactin (PRL) from the anterior pituitary is affected by pregnancy. LH release is reduced in pregnant mares (Nett et al. 1975b) but increases after foaling (Nett et al. 1975a, Noden et al. 1978), leading to a fertile ovulation within 10 days postpartum in most mares. PRL release is low during most of pregnancy, increases shortly antepartum and is elevated for at least 2 weeks after foaling (Nett et al. 1975a, Lothrop et al. 1987, Worthy et al. 1987). The increase in PRL concentration is a prerequisite for normal galactogenesis (Ireland et al. 1991).

In non-pregnant mares, endogenous opioids inhibit LH release during the luteal, but not the follicular, phase (Behrens et al. 1993) and during the anovulatory season (Aurich et al. 1994). Opioids influence LH release via the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus (Parvizi & Ellendorff 1980, Alexander et al. 2000). The opioidergic inhibition of GnRH/LH release contributes to seasonal anoestrous in equine species, and transition into the breeding season coincides with a switch from a continuous opioidergic inhibition to an intermittent, cycle-dependent blockade of LH release (Aurich et al. 1994). In cyclic mares, the opioidergic inhibition of LH secretion is activated by a sequence of oestrogen and progesterone influence characteristic of the oestrous cycle. In seasonal anoestrous mares, the opioidergic inhibition of LH release can be stimulated by a prolonged period of oestrogen exposure or independently of ovarian factors (Aurich et al. 1994, 1995, 1997). Opioids inhibit PRL secretion in steroid-treated (Aurich et al. 1995) and melatonin-treated (Aurich et al. 1997) ovarietomised mares but not in non-pregnant, ovari-intact mares. In the non-pregnant mare, PRL release is primarily inhibited by dopamine (Aurich et al. 2000), and the physiological relevance of opioids for the regulation of PRL release remains to be established.

In equine species, opioidergic regulation of LH and PRL secretion during pregnancy has not been investigated. The physiological duration of pregnancy in the mare is between 315 and 365 days. Progesterone and
oestrogen release, which could affect LH and PRL regulatory opioidergic pathways, change throughout pregnancy. Progesterone is secreted exclusively from the primary corpus luteum until day 40. Under the influence of equine chorionic gonadotrophin (eCG), secondary corpora lutea are formed after day 40 of pregnancy. After day 60, gestagens are also produced by the placenta, and from day 150 onwards, the placenta is the only source of gestagens in the pregnant mare (Holtan et al. 1991). High oestrogen concentrations in the blood of pregnant mares are found from the 4th to the 9th month of gestation (Nett et al. 1973).

The present experiment was designed to study a possible opioidergic regulation of LH and PRL release in pregnant pony mares. The effects of the opioid antagonist naloxone on LH and PRL concentrations in plasma at different stages of pregnancy were determined. In a preliminary study, the effects of repeated injection of the GnRH analogue buserelin, given at different intervals, on LH release were studied.

**Materials and Methods**

**Animals**

Experiments were performed according to Austrian federal animal welfare legislation. In the preliminary study, Shetland mares (n=15) were used. They were between 3 and 15 years of age (mean age 9 years) and weights were between 180 and 250 kg. The animals used in the main study were Shetland mares (n=7) aged 9·9 ± 2·6 years (s.d.) and weighing 165 ± 7 kg (s.d.). Ponies were kept as a single group in a spacious stable and had permanent access to pasture. In addition to having access to pasture, they were fed hay and mineral supplements; water was freely available. All mares were mated with a Shetland stallion every other day during oestrus. Oestrus was determined by daily checking for oestrous behaviour with the stallion and by daily determination of plasma progesterone concentrations. Because of the size of the animals, ovarian function was not determined by repeated transrectal palpation. The first day on which plasma progesterone concentrations exceeded 3·4 nmol/l was defined as day one after ovulation (i.e. day 1 of pregnancy). Six of the seven mares became pregnant between 23 June and 30 July. Pregnancy was confirmed by transrectal ultrasonography on days 18 and 45 after mating. The six pregnant mares were included in the study and foaled between 18 May and 14 June the following year. The average gestation length was 329·8 ± 8·5 days (s.d.).

**Experimental procedures**

In order to determine the interval between repetitive LH-releasing treatments, in a preliminary experiment 15 mares were injected twice with 5 µg of the GnRH agonist buserelin (Receptal; Hoechst, Unterschleissheim, Germany). The intervals between the two buserelin injections were 2 (∙n=5), 4 (∙n=5) and 6 days (∙n=5). Blood for the determination of LH concentrations was withdrawn at 15 min intervals from 45 min before to 60 min after the buserelin injections. Experiments were performed in February and all mares were seasonally acyclic.

The main experiments were performed on days 26·4 ± 0·6, 75·4 ± 5·4, 171·8 ± 2·4, 226·2 ± 4·8, 282·7 ± 3·4 and 319·8 ± 2·1 of pregnancy and 2 days after foaling. All experiments were begun between 0800 and 0900 h. During blood sampling, the ponies remained in their stable. An indwelling catheter (Braun, Melsungen, Germany) was placed in one jugular vein 15 min before the first blood sample was withdrawn. Blood for the determination of LH and PRL was withdrawn at 15 min intervals for 240 min. In all experiments during pregnancy, after 60 min of sampling, 80 mg naloxone-hydrochloride (Sigma Chemicals, Deisenhofen, Germany) per animal or 6 ml saline was injected intravascularly via the catheter. Naloxone was freshly dissolved in 6 ml saline and was filter-sterilized immediately before the injection. The dose of naloxone corresponded to 0·5 mg/kg body weight. Immediately after withdrawal, blood samples were centrifuged for 20 min at 1000 g, and plasma was frozen at −20 °C until hormone analysis. Two hours after the injection of naloxone or saline, 5 µg of the GnRH analogue buserelin was administered to enable investigation of pituitary responsiveness to GnRH. Blood samples were collected for another 60 min, i.e. until 180 min after naloxone or saline administration. Plasma progesterone concentrations were measured in the first blood sample taken on each day. In the experiment 2 days after foaling, blood samples were taken for 240 min, all animals received naloxone 120 min after the first blood sample was withdrawn, and no buserelin was given.

In all experiments, mares were used as their own controls and were treated with naloxone and saline. The interval between the two treatments in the same animal was 2 days. The order of treatments was randomised, with half of the animals receiving naloxone first and half receiving saline injections first. Two days after foaling, no control experiment was performed. LH and PRL release during the 2 h before and after injection of naloxone was compared. This procedure was chosen because of the rapidly changing hormone environments in postpartum mares. In early postpartum mares, data from control and naloxone experiments performed at 2 day intervals would not have been comparable.

**Hormone assays**

Concentrations of LH, PRL and progesterone in plasma were measured by radioimmunoassay (RIA) as described
previously (Behrens et al. 1993). The assay for LH utilised equine LH (Biogenesis, Poole, Dorset, UK) as the standard and for iodination, and an antibody raised in rabbits against equine LH (A 543; Biogenesis). The intra- and interassay coefficients of variation were 5-8 and 18-1% respectively. The minimal detectable concentration was 0.5 µg/l; the cross-reactivity with follicle-stimulating hormone was 2-8% and that with equine chorionic gonadotrophin was 23%.

The RIA for PRL was performed with an antisemur raised in rabbits against equine PRL (AFP 361687; Dr A F Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). The intra- and interassay coefficients of variation were 4-1% and 10-3% respectively, the minimal detectable concentration was 0.25 µg/l, and the cross-reactivity of the antibody with equine growth hormone (AFP 7424C, Dr A F Parlow) was <0-1% (Aurich et al. 1995). Progesterone was determined by RIA after extraction from plasma with n-hexane as described previously (Behrens et al. 1993). The intra- and interassay coefficients of variation were 4-5 and 7-9% respectively, and the minimal detectable concentration was 0.16 nmol/l.

**Statistical analysis**

In the preliminary experiment, basal LH concentrations were calculated as the means of the 4 pre-buserelin values. LH release in response to buserelin was calculated as the area under the curve (AUC; ng/ml per min) for the time period 0–60 min after the injection of buserelin \((\sum(x_n + x_{n+1})/2 \times 15)\) taking into account the 15 min sampling interval. Both basal LH concentrations and LH release after buserelin were compared by using the Wilcoxon matched pairs ranked sum test. In the main experiment, PRL and LH release in individual mares was calculated as the AUC (ng/ml per min) for the time period from 0–120 min after the injection of naloxone or saline. The pretreatment baseline, calculated as the mean of 5 values before the naloxone or saline injections, was subtracted from the value for each post-treatment sample. Statistical comparisons were made with the SPSS/PC+ statistics package (Norusis 1988). Because no assumption was made about the distribution of data, non-parametric tests were used. Naloxone and respective saline experiments in pregnant mares were compared by using the Wilcoxon matched pairs ranked sum test. Basal hormone concentrations in the same animals at different times, LH and PRL concentrations in mares 2 days postpartum before and after naloxone injections (the AUCs for 2 h before vs 2 h after naloxone injection) and LH and PRL concentrations before and after buserelin injections (the AUCs for 1 h before vs 1 h after buserelin injection) were compared by using Friedman’s two-way ANOVA, taking into account the sequential nature of the data. For comparisons of basal hormone concentrations, data from control experiments were used. The data presented are means ± s.e.m. values.

**Results**

**LH release after buserelin given at different intervals**

When mares were treated twice with buserelin at 2, 4 or 6 day intervals, basal LH concentrations before the first and the second treatments were not significantly different. LH release in response to the second injection did not differ significantly from the LH response to the first buserelin injection (see Fig. 1). LH release data (the AUC) for the time period 0–45 min after the first and second buserelin injection were as follows: 27.0 ± 4.1 and 26.8 ± 4.2 ng/ml per min for the 2 day interval; 28.1 ± 4.8 and 30.3 ± 3.2 ng/ml per min for the 4 day interval; and 26.6 ± 4.4 and 29.5 ± 3.8 ng/ml per min for the 6 day interval.

**Progesterone**

Plasma progesterone concentrations on days 26, 76, 172, 226, 283 and 320 of pregnancy were 44.4 ± 1.7, 36.7 ± 4.5, 32.3 ± 10.3, 14.4 ± 0.9, 17.2 ± 0.8 and 17.1 ± 1.1 nmol/l respectively.

**Basal LH and PRL concentrations**

Mean basal LH concentrations in plasma reached a maximum on day 76 of gestation (P<0.05 vs all other times) and were significantly (P<0.05) higher on day 172 of gestation than on days 26, 226, 283 and 320 of pregnancy and 2 days after foaling. Plasma PRL concentrations reached a nadir on days 172 and 226 of gestation (P<0.05 vs all other times) and increased significantly at the end of gestation and further after foaling (day 320 and 2 days postpartum vs all other times, P<0.05; see Table 1).

**LH release in response to naloxone and buserelin**

The opioid antagonist naloxone induced a significant (P<0.05 vs controls) LH release on days 172, 226 and 282 of pregnancy. On day 320, LH release after naloxone tended to increase but the difference with respect to the control experiment did not reach statistical significance. Treatment with naloxone did not influence LH release on days 26 and 76 of pregnancy and 2 days after foaling (see Figs 2 and 3). LH release in response to naloxone (the AUC) was 225.9 ± 94.0 ng/ml per min on day 172, 196.0 ± 37.0 ng/ml per min on day 226, 177.0 ± 41.1 ng/ml per min on day 282 and 108.9 ± 52.9 ng/ml per min on day 320 of pregnancy. The respective values for control experiments were 56.7 ± 46.5, 46.5 ± 8.7, 47.4 ± 23.3 and 56.4 ± 20.4 ng/ml per min. The GnRH agonist buserelin caused a significant (P<0.05) increase in plasma LH concentrations on days 172, 226, 282 and 320 but not on days 26 and 76 of pregnancy, irrespective of the pretreatment with either naloxone or saline (see Fig. 2).
PRL release in response to naloxone and buserelin

On days 226, 282 and 320 of pregnancy, PRL concentrations were elevated significantly after the injection of naloxone. The naloxone-induced PRL release increased from day 226 to day 282 and further to day 320 (day 226 vs day 320, \( P < 0.05 \); see Fig. 4). No further changes were found in response to buserelin. Neither naloxone nor buserelin caused any changes in plasma PRL concentrations on days 26, 76 and 172 of pregnancy. PRL release in response to naloxone (the AUC) was 237.9 ± 79.7 ng/ml per min on day 226, 575.5 ± 209.4 ng/ml per min on day 282 and 1498.0 ± 574.1 ng/ml per min on day 320 of pregnancy. The respective values for control experiments were 12.5 ± 12.6, 1.4 ± 31.7 and -47.7 ± 36.6 ng/ml per min. Two days after foaling, naloxone no longer affected plasma PRL concentrations (see Fig. 3).

Discussion

The present experiments indicate that endogenous opioids are involved in the inhibition of LH release in equine mares during the second half of pregnancy but not in early pregnant mares and not during the last days preceding parturition.

The preliminary study was performed to investigate if treatments could be performed at 2 day intervals without the first buserelin injection influencing the response to the second injection. The data showed that this interval is sufficient, at least when a low and sub-therapeutic buserelin dose is used, as in our study.

The opioid antagonist naloxone did not affect LH release on days 26 and 76 after ovulation in pregnant mares. This is in contrast to cyclic mares, in which opioids inhibit LH release during the luteal phase (Behrens et al. 1993). In pregnant mares less than 2 weeks later, naloxone no longer releases LH, although the oestrogen–progesterone environment is similar to that of dioestrus. The disappearance of naloxone-induced LH release could be explained either by deactivation of the opioidergic system or by a reduced pituitary responsiveness to GnRH in early pregnancy. The later interpretation is supported by the facts that opioids affect LH release indirectly by inhibiting GnRH release (Parvizi & Ellendorff 1980,

Table 1 Basal plasma LH and PRL concentrations (ng/ml) in mares at different times of pregnancy and at 2 days after foaling; values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Day (month)</th>
<th>LH</th>
<th>PRL</th>
</tr>
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<tbody>
<tr>
<td>26.4 ± 0.6 (Aug.)</td>
<td>3.1 ± 0.6a</td>
<td>2.0 ± 0.4a</td>
</tr>
<tr>
<td>75.4 ± 5.4 (Sept./Oct.)</td>
<td>63.3 ± 8.6b</td>
<td>1.6 ± 0.5a</td>
</tr>
<tr>
<td>171.8 ± 2.4 (Dec./Jan.)</td>
<td>4.1 ± 0.5c</td>
<td>0.4 ± 0.1b</td>
</tr>
<tr>
<td>226.2 ± 4.8 (Feb./Mar.)</td>
<td>2.1 ± 0.5a</td>
<td>0.5 ± 0.1b</td>
</tr>
<tr>
<td>282.7 ± 3.4 (Mar./Apr.)</td>
<td>1.8 ± 0.3a</td>
<td>1.4 ± 0.3a</td>
</tr>
<tr>
<td>319.8 ± 2.1 (May/Jun.)</td>
<td>1.7 ± 0.3a</td>
<td>4.1 ± 2.0a</td>
</tr>
<tr>
<td>Day 2 postpartum (May/Jun.)</td>
<td>2.4 ± 0.5a</td>
<td>8.4 ± 3.4a</td>
</tr>
</tbody>
</table>

* Values with different superscript letters in the same column are significantly different.
Alexander et al. (2000) and that buserelin did not cause any LH release on day 26 of pregnancy.

High LH concentrations around day 76 of pregnancy consist mainly of eCG, which reaches maximal concentrations in plasma towards day 60 (Rowlands 1949, Allen 1975). Equine CG has evolved by expression of the LH gene in the placenta; however, LH and CG are considered as different hormones (Stewart & Allen 1995). The

Figure 2 Concentrations of LH in plasma of pregnant mares (n=6) before and after injection of naloxone (●) or saline (○; time, 0 min) and buserelin (time, 120 min) (arrows); values are means ± S.E.M. a There is a significant difference between naloxone treatment and control treatment (P<0.05; AUC for the time period from 0 to 120 min after naloxone or saline injections). b LH values before and after the injection of buserelin are significantly different (P<0.05).
release in these species (Whisnant et al. 1986). LH regulatory opioidergic pathways are influenced by gonadal steroids. In the pig, progesterone is secreted from the corpus luteum throughout pregnancy, whereas in the horse during the second half of pregnancy, gestagens in the maternal blood originate in the placenta. Different gestagen patterns may explain, in part, why opioid effects on LH release differ in pigs and equine species.

Plasma PRL concentrations were low throughout pregnancy. A further reduction during winter must be considered as a seasonal variation, which has been described previously in non-pregnant mares (Johnson 1986). An opioidergic inhibition of PRL release was not detectable before day 226 of pregnancy. The amount of PRL released in response to naloxone increased continuously thereafter. An opioid-mediated inhibition of PRL release at the end of pregnancy has also been demonstrated in rats. The stimulatory effect of naloxone on PRL release was negatively correlated with progesterone levels and reached a maximum after prepartum luteolysis. In addition to a fall in progesterone levels, an increase in circulating oestrogens may contribute to the activation of a PRL-inhibitory opioidergic pathway (Soaje & Deis 1994, 1997). Progesterone stimulates dopamine release into hypophysal portal blood (Cramer et al. 1979). In the prepartum rat, opioids may thus limit the increase in PRL induced by the fall in progesterone (Soaje & Deis 1994, 1997). Although, in equine species, naloxone induces a release of PRL in the presence of still-high gestagen concentrations, it can be speculated that changes in the oestrogen and gestagen profiles in mares during pregnancy cause a decrease in the dopaminergic inhibition of PRL release, and that opioids prevent a release of PRL after reduction of the dopaminergic blockade.

Naloxone-inducible PRL release does not exist in intact, non-pregnant mares but can be activated by oestradiol–progesterone and melatonin treatment in ovariectomised mares (Aurich et al. 1994, 1997). Thus, pregnancy is the only reproductive state in which opioids inhibit PRL secretion in non-pretreated mares. The dopaminergic inhibition is possibly not strong enough to overcome the naloxone-induced stimulation of PRL release. Moreover, the naloxone-induced PRL release increases markedly towards term, indicating a diminishing dopaminergic inhibition and an increase in the naloxone-releasable PRL pool. Two days after foaling, plasma PRL concentrations were no longer inhibited by opioids and were significantly higher than at any stage of pregnancy. The loss of an opioidergic inhibition of PRL release with the end of pregnancy could explain this marked increase in plasma PRL concentrations after foaling.

In conclusion, the results of this study provide new insights into the neuromodulatory regulation of LH and PRL release in pregnant mares. Opioids inhibit GnRH/LH release during the second half of pregnancy but not during early pregnancy in the mare. A deactivation

antiserum used in our study did not sufficiently differentiate between LH and CG, and LH concentrations at day 76 of pregnancy have to be interpreted as LH immuno-reactivity. The CG concentrations were not affected by naloxone. Because eCG release is not regulated via GnRH (Thompson et al. 1982), a GnRH-mediated effect of naloxone on pituitary LH release was not to be expected. In addition, there is no direct opioidergic regulation of CG release from the endometrial cups. An effect of naloxone on pituitary LH release at that stage of pregnancy cannot be excluded and could have been masked by the high concentrations of eCG.

After the eCG phase, opioids inhibit LH release, indicating that opioidergic mechanisms contribute to the reduction in LH secretion during pregnancy. The seasonal increase in mean plasma LH concentrations that has been demonstrated in cyclic mares during spring and summer (Turner et al. 1979) did not occur in pregnant animals. The opioid inhibition of LH release was markedly reduced during the last days before foaling, and no opioidergic inhibition of LH release existed 2 days after foaling. This might be a prerequisite for the early onset of ovarian activity after parturition in the mare. In pigs and beef cattle, lactation is accompanied by a period of anoestrus. Endogenous opioids, released in response to the suckling stimulus, inhibit gonadotrophin release in these species (Whisnant et al. 1986, Armstrong et al. 1988). In contrast, in the mare, lactational anoestrus as a physiological condition does not exist and suckling does not induce an opioid-mediated suppression of LH release.

The opioidergic regulation of GnRH/LH release during pregnancy in equine species differs from that in the pig. In sows, naloxone treatment induces a short-term LH release on day 40 but not on day 70 of pregnancy (Szafranska et al. 1994). LH regulatory opioidergic pathways are influenced by gonadal steroids. In the pig, progesterone is secreted from the corpus luteum throughout pregnancy, whereas in the horse during the second half of pregnancy, gestagens in the maternal blood originate in the placenta. Different gestagen patterns may explain, in part, why opioid effects on LH release differ in pigs and equine species.

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In conclusion, the results of this study provide new insights into the neuromodulatory regulation of LH and PRL release in pregnant mares. Opioids inhibit GnRH/LH release during the second half of pregnancy but not during early pregnancy in the mare. A deactivation

Figure 3 Concentrations of LH (●) and prolactin (■) in the plasma of mares 2 days after foaling, before and after the injection of naloxone (arrow); values are means ± S.E.M. Naloxone had no significant effect on LH and PRL release.
of the opioidergic inhibition of LH release shortly before foaling might be a prerequisite for the early onset of follicular growth postpartum. In addition to LH release, PRL release is inhibited during the later stages of pregnancy, and the amount of PRL released in response to the opioid antagonist naloxone increases towards term.

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