Naloxone Administration Does Not Relieve the Inhibition of Gonadotropin Release in Food-Restricted, Lactating Rats

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ABSTRACT Lactational anovulation is an important factor in determining birth spacing in women living in developing countries. Therefore, a more comprehensive understanding of the mechanisms involved in the relationships among lactation, nutrition and ovulation is important. This study was designed using the food-restricted, lactating rat to examine whether endogenous opioids might be involved in depressing gonadotropin release. Females were mated after 65 d of age and, beginning on d 42 of life, offered food in unrestricted amounts (control) or were food restricted to 50% of what the controls consumed. On d 15 of lactation, dams were injected with either naloxone hydrochloride (3 mg/kg body weight) or saline and killed 0, 15, 30 or 60 min later. Plasma was analyzed for luteinizing hormone, follicle-stimulating hormone and prolactin. Food restriction decreased plasma concentrations of luteinizing hormone and follicle-stimulating hormone (P < 0.005). Naloxone administration marginally influenced follicle stimulating hormone (P < 0.1), but not luteinizing hormone concentration regardless of diet group. The interaction among diet group, drug group and time of killing was significant for plasma prolactin concentration (P < 0.05). Food restriction lowered prolactin concentrations, but this effect was diminished with increasing time after injection of naloxone. Furthermore, the magnitude of the effect of food restriction was lessened and even reversed with treatment of naloxone. These results indicate that endogenous opioids are not the primary mechanism suppressing luteinizing hormone release in food-restricted lactating rats. J. Nutr. 126: 2113-2119, 1996.

INDEXING KEY WORDS:
• rats • lactation • nutrition • ovulation • opioids

The negative effect of lactation on ovarian function has been well documented in both human and nonhuman species (Delvoye and Robyn 1980, Díaz et al. 1988, Howie et al. 1982, Kennedy and Visness 1992, Peréz et al. 1972, Short et al. 1991, Smith and Neill 1977, Stern et al. 1986). In humans, this period of subfecundity may represent a woman’s only form of contraception. Therefore, extending the period of lactational subfecundity may be extremely important, especially in regions where repeated pregnancies occurring in close temporal proximity result in increased risks of maternal and infant morbidity and mortality.

Thus, although better nutritional status is undeniably beneficial for the mother and infant in the short term, improving nutritional status may also decrease birth spacing. This may have more long-term detrimental effects upon the mother and her entire family.

Although the physiologic mechanisms resulting in ovarian inactivity during lactation have been studied extensively and appear to involve the inhibition of gonadotropin release, the involvement of factors such as nutritional status is still unclear. The use of an animal model for studying these individual effects and interactions is currently warranted, because factors appearing to influence ovarian function in humans (such as nutritional status and suckling characteristics) are often both related to each other and confounded by other factors such as socioeconomic status.

We have been working with the rat as an animal model that displays reproductive responses to lactation and decreased food intake similar to those hypothesized for humans. Previous studies indicate that food restriction prolongs the lactational anovulatory period (McGuire et al. 1992), and that this is accompanied by both increased suckling stimulus and decreased gonadotropin release [McGuire et al. 1995]. Work by others has shown clearly that food restriction in the nonlactating rat inhibits gonadotropin release via the release of endogenous opioids (Dyer et al. 1985). Further, opioids also appear to influence gonadotropin release in the well-fed, lactating rat (Sirinathsinghji and Martini 1984, Taya and Sasamoto 1989). However, the mechanisms coordinating and resulting in decreased gonadotropin release in the food-restricted, lactating rat have not been studied. We sought to further our understanding of this animal model by studying whether opioids may be involved in the reproductive strategy used to inhibit gonadotropin release in the food-restricted, lactating dam.

We tested the hypotheses that 1) food restriction would inhibit release of gonadotropins and prolactin in the lactating rat and 2) inhibition of gonadotropin release by food restriction in the lactating rat would be overcome by blocking endogenous opioid pathways with naloxone.

**MATERIALS AND METHODS**

**Animal care and experimental design.** Female Sprague-Dawley rats (35 d old; n = 89) were purchased from Charles River Laboratories (Kingston, NY). During the initial portion of the study, all animals were housed individually in stainless steel, wire-bottomed cages with tap water available at all times in an environmentally controlled room (20–22°C, 45–50% humidity). Lighting was adjusted to provide 10 h of light beginning at 0700 h. Care of all animals was in compliance with applicable National Institutes of Health and local institutional guidelines.

During a 7-d adjustment period, all rats were allowed free access to a purified diet, AIN-76A™ (Dyets, Bethlehem, PA; AIN 1977 and 1980). Immediately after this period, animals were assigned randomly to one of two dietary treatment groups: control [n = 42] or food-restricted [n = 47]. Throughout the remainder of the study, control rats were allowed free access to diet AIN-76A™, and food-restricted rats were fed a modified version of diet AIN-76A™ that contained 50% more of the usual percentages of vitamins and minerals [Kliewer and Rasmussen 1987]. Food-restricted dams were fed in the morning and offered an amount equal to 50% (by weight) of the amount consumed by a randomly selected group of control rats. During pregnancy and lactation, food-restricted rats were fed amounts equal to 50% of what this group of controls had consumed on the particular day of pregnancy or lactation.

Beginning on d 65 of life, dams were bred with males of the same strain purchased from the same supplier. On d 18 of pregnancy, dams were transferred to polycarbonate cages. The first day that pups were observed with the dam was designated as d 0 of lactation. At this time, dams were assigned systematically, within dietary treatment group, to experimental drug treatment group (naloxone or saline) and time of killing (0, 15, 30 or 60 min after administration of drug or placebo). On d 1 of lactation, litters were culled to five pups.

**Drug administration and sample collection.** On d 15 of lactation between 1100 and 1200 h, dams and pups were separated, and dams were anesthetized briefly by inhalation of diethyl ether and injected (intramuscular) with either naloxone hydrochloride (Sigma Chemical, St. Louis, MO; 3 mg/kg body weight, diluted in saline at 2.5 g/L) or an appropriate volume of physiologic saline (0.2–0.3 mL). Animals assigned to the 0-min subgroup were then killed by cardiac puncture; those assigned to the remaining groups were reanesthetized immediately before their assigned time of killing and subsequently killed by cardiac puncture. Whole blood (~5 mL) was collected in heparinized syringes and centrifuged. Plasma was removed, aliquoted and stored at −20°C until analyzed for concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin.

**Hormone analyses.** Plasma LH, FSH and prolactin concentrations were measured in duplicate (100 µL plasma/tube) using appropriate RIA (Amerlex™ assay systems, Amersham, Arlington Heights, VA).

**Statistical analyses.** All data analyses were conducted using SYSTAT (Version 5, Evanston, IL: SYSTAT, Inc. 1992). Data concerning weights at randomization were analyzed by one-way ANOVA with dietary treatment group in the model. All other data were analyzed by three-way ANOVA with dietary treatment group, drug group and time of sample collection as well.
as the appropriate interactions in the model. Pre-
planned comparisons between individual means were
analyzed by Student's t test. For interactions, $P < 0.10$
was considered significant, whereas for main effects
and individual comparisons between means, $P < 0.05$
was considered. Only if interactions were not found to
be significant are main effects discussed in this report.
Values presented in this report represent means ± SEM.

**RESULTS**

**Adequacy of randomization and disposition of final
rats.** At randomization (d 42 of life), there was no dif-
ference between control and food-restricted groups in
body weight (150.5 ± 2.7 and 144.2 ± 2.0 g, respec-
tively). Of the 89 rats, 42 and 47 were randomized into
the control and food-restricted groups, respectively. Of
these, four control and seven food-restricted rats did
not become pregnant, and six control and four food-
restricted dams were excluded from the study for other
reasons (predominately sampling difficulties). There-
fore, unless stated otherwise, the data presented in the
following sections are those representing the remaining
rats: $n = 32$ and $n = 36$ for control and food restricted
groups, respectively.

**Dam and litter weights.** Throughout lactation, con-
trol dams weighed significantly ($P < 0.0001$) more than
did food-restricted dams (Fig. 1). Similarly, there was a
significant effect of diet group on total number of pups
and litter weight on d 1 of life, such that litters of
control dams contained more pups and weighed more
than did those nursed by food-restricted animals (13.4
± 0.6 pups, 91.8 ± 5.1 g, and 11.1 ± 0.3 pups, 66.1 ± 1.6
g, respectively; $P < 0.005$). Although this difference
was initially minimized immediately after culling, by
d 5 of life and continuing throughout the study, litters
of control dams were again heavier than litters of food-
restricted dams (Fig. 1).

**Plasma hormone concentrations.** Plasma LH con-
centration was depressed significantly by food restric-
tion ($P < 0.0001$), and the administration of naloxone
did not alter LH levels in either dietary treatment group
(Fig. 2).

There was a significant effect ($P < 0.0005$) of dietary
Treatment on plasma FSH concentration, such that food
restriction resulted in depressed FSH (Fig. 3). There was
also a marginal effect of drug treatment, such that the
administration of naloxone resulted in higher overall cir-
culating concentrations of FSH, compared with saline-
treated rats (13.8 ± 0.6 vs. 12.0 ± 0.6 µg/L; $P = 0.09$).

Plasma prolactin concentrations were transformed
to their natural logarithms before analysis by ANOVA.
Data presented here represent back transformations of
these data (Fig. 4). The interaction among diet group,
drug group and time after drug administration was sig-
nificant ($P < 0.05$) in explaining the variability among
these data. Food restriction resulted in significantly
lower plasma concentrations of prolactin, compared
with those of control rats. This effect was greater for
saline-treated dams and became less important over
time (Table 1).

**DISCUSSION**

These data clearly support our hypothesis, which is
based on previous work from our laboratory, that food
restriction during lactation results in depressed plasma
gonadotropin concentrations in the rat (McGuire 1994,
McGuire et al. 1995). However, we expected that this
depression of circulating hormones would be relieved

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\begin{align*}
\frac{\text{Control}}{\text{Food-restricted}} \quad \frac{\text{Food-restricted}}{\text{Control}}
\end{align*}
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FIGURE 2 The effects of food intake and naloxone administration on plasma luteinizing hormone (LH) concentration during lactation among control and food-restricted rats. Data are expressed as means ± SEM. Food-restricted dams \( n = 36 \) had significantly lower plasma LH concentrations than controls \( n = 32, P < 0.0001 \). There was no effect of naloxone administration on plasma LH concentrations, regardless of dietary treatment group.

by treatment with naloxone, an opioid receptor antagonist. Results presented here suggest that, in both well-fed and food-restricted lactating rats, LH concentration was not changed by injection of naloxone. Naloxone administration did, however, have a marginal effect on increasing FSH concentrations in rats of both diet groups. Because group size was relatively limited in this investigation \( n = 4-6 \) per group killed, we suggest that further studies investigating these effects employ more generous sample sizes.

Consistent with data published previously by our group, plasma prolactin concentrations were depressed by 50% food restriction [Kliwer and Rasmussen 1987, Schulze and Rasmussen 1993], but this effect was lessened and even reversed with naloxone administration. The inhibitory effect of naloxone on plasma prolactin concentrations in the well-nourished rat has been documented previously [Blank et al. 1980, Sirinathsinghji and Martini 1984]. The marginal rise in plasma prolactin at 30 min post-injection in the food-restricted group remains puzzling to us and deserves further study. Nonetheless, these data clearly demonstrate a dissociation of the previously hypothesized inverse link between prolactin and gonadotropin concentrations. This is consistent with data previously published by us and others [McGuire et al. 1995, Sirinathsinghji and Martini 1984].

To interpret these data fully, one must first consider whether the naloxone dose and window of time studied were adequate to ensure the detection of a response, if it occurred. In a study of ovariectomized, nonlactating female rats, Dyer et al. [1985] demonstrated a signifi-

FIGURE 3 The effects of food intake and naloxone administration on plasma follicle-stimulating hormone (FSH) concentration during lactation among control and food-restricted rats. Data are expressed as means ± SEM. Food-restricted dams \( n = 36 \) had significantly lower plasma FSH concentrations than controls \( n = 32, P < 0.0005 \). Naloxone administration tended to increase plasma FSH concentrations \( P = 0.09 \) compared with the saline-treated rats.
FIGURE 4 The effects of food intake and naloxone administration on plasma prolactin (Prl) concentration during lactation among control and food-restricted rats. Data are expressed as means ± SEM, n = 36 (food-restricted) or 32 (controls). There was a significant interaction among diet group, drug group and time of killing (P < 0.05), such that food restriction lowered plasma prolactin concentrations, but this effect was lessened with naloxone administration and diminished over time.

A significant increase in plasma LH concentration in fasted, nonlactating rats after injecting 1.5 mg naloxone hydrochloride per kg body weight. Blank et al. [1980] found that, by 15 min after injection, 2.5 mg naloxone hydrochloride/mg body weight was sufficient to depress plasma prolactin levels and increase LH concentration in rats. Further, studying ovariectomized, lactating rats given free access to food, Sirinathsinghji and Martini [1984] also found a significant effect of naloxone (dose rate ~2 mg/kg body weight) on LH, but not FSH, by 15 min after injection; this effect was maintained for 60 min after injection. Thus, we believe that our dose (3 mg naloxone hydrochloride/kg body weight) and window of investigation (0–60 min) was indeed adequate to detect an effect, if it occurred.

In contrast to previously published data (Sirinathsinghji and Martini 1984), naloxone administration in the present study did not result in increased LH concentrations in dams given free access to food. Further more, in the present study, FSH concentration was increased marginally after naloxone injection. We believe that our findings differ from those previously reported, because our animal model differed importantly from that used by others. Whereas other experimenters have studied the influence of endogenous opioid release on gonadotropin concentration in ovariectomized dams (food-restricted, nonlactating dams, Dyer et al. 1985; freely fed, lactating dams, Sirinathsinghji and Martini 1984), dams used in the present study were intact. We chose to use this experimental design, because we have previously shown a significant effect of food restriction on duration of postpartum anestrus of intact, lactating rats [McGuire et al. 1992], and we sought to understand better the strategies used by this same animal model. Furthermore, opiate binding site density in the hypothalamus of the rat appears to be inversely related to the presence of both estrogen and progesterone (Weiland and Wise 1990). Because the corpora lutea of lactating rats are highly steroidogenic and our intent was to understand the normal reproductive physiology of this animal model, we chose not to ovariectomize our rats. Thus, we suspect that our use of intact animals explains the differences seen between this and previously published studies.

Furthermore, previously published work considering the influence of opioids on gonadotropin release in lactating animals has focused on the response of animals deprived of suckling stimulus for an extended period of time before naloxone or β-endorphin was administered [Sirinathsinghji and Martini 1984, Taya and Sasamoto 1989]. Although this might be an appropriate experimental design to study the possible interactions among suckling, opioids and gonadotropins, we believe that this approach is not appropriate for studying the added effect of food restriction in the lactating animal. We wanted to study the normal physiology of lactating rats of differing nutritional backgrounds allowed to freely

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TABLE 1

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Drug group</th>
<th>Saline</th>
<th>Naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.6 ± 2.81 [16]</td>
<td>3.1 ± 2.42 [16]</td>
<td></td>
</tr>
<tr>
<td>Food-restricted</td>
<td>0.8 ± 1.9 [17]</td>
<td>1.3 ± 2.33 [18]</td>
<td></td>
</tr>
</tbody>
</table>

1 Figures represent means ± SEM; numbers in parentheses are numbers of animals.
2 Significantly different from saline-treated group (P < 0.05).
3 Marginally different from saline-treated group (P < 0.10).
nurse their litters until the time of drug administration. Thus, dams and pups were allowed to remain together until either saline or naloxone was administered.

We believe that data presented here accurately describe the influence of maternal food intake (ad libitum vs. food-restricted) on plasma gonadotropin concentrations as well as the possibility that endogenous opioids are mediating this effect. Interestingly, we found a possible effect of naloxone administration on FSH, but not LH concentrations. From previous work (McGuire et al. 1995), we have found that the effect of food restriction on plasma LH, but not FSH, might be influenced indirectly by the effect of food restriction on suckling characteristics in lactating rats. This idea fits well with conclusions drawn previously by Sirinathsinghji and Martini (1984) using the lactating dam deprived of suckling for 8 h before sampling. In that study, naloxone administration resulted in an elevation of LH, but not FSH concentration, suggesting that suckling itself countered immediately before samples were obtained, naloxone administration resulted in a marginal increase in plasma FSH, but not LH in both dietary treatments and postpartum infertility in lactating Gambian women. Am. J. Clin. Nutr. 39: 227–235.


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