Effect of steroids and nitric oxide on pituitary hormone release in ovariectomized, peripubertal rats

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Abstract

The purpose of this study was to determine the effects of the duration of steroid depletion on the steroid-induced luteinizing hormone and prolactin surges in ovariectomized, peripubertal female rats. Additionally, the role of nitric oxide (NO) in mediating the surge responses was determined. Peripubertal, 6-week-old, female Sprague-Dawley rats were ovariectomized. One or three weeks later, animals were injected with 17 β -estradiol (50 μ g, sc) followed 48 h later by progesterone (2.5 mg, sc). Effects of NO were examined by administering L-arginine (300 mg/kg, ip). The response of ovariectomized, adult females to steroid treatment was also determined.

One and three weeks after ovariectomy, steroid replacement produced an LH and prolactin surge in peripubertal animals. However, both the magnitude and duration of the LH surge was greater 3 weeks after ovariectomy. While L-arginine significantly enhanced the magnitude of the LH surge 1 week after ovariectomy, by 3 weeks L-arginine caused a decrease in the duration, but not the magnitude of the surge. In contrast, L-arginine did not affect either the magnitude or duration of the prolactin surge one week after ovariectomy, but diminished the magnitude after 3 weeks of steroid depletion. In adults, steroids induced significant increases in both LH and prolactin. These results demonstrate that sensitivity to NO stimulation of LH, but not prolactin secretion, is modulated by the duration of gonadal steroid hormone depletion. The differences in the responsiveness of LH and prolactin to steroid-induced stimulation in peripubertal animals demonstrate that these hormones are regulated by NO through different mechanisms.

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Introduction

Puberty is due to a number of neural changes that occur during embryonic and prepubertal development that lead to the activation of the gonadotropin releasing hormone (GnRH) neurons and the onset of regular, hormone cyclicity and ovulation (see Gore 2002 for review). Circulating levels of ovarian steroid hormones rise as GnRH neuronal pulse amplitude and frequency increase (Sisk *et al.* 2001). The gonadal steroids, especially estrogen, affect the activity of hypothalamic neurons and the sensitivity of the anterior pituitary gland (Gore 2001), but puberty occurs due to neuronal activation that is independent of gonadal steroids (Gore 2002 for review).

Once cyclicity is established after puberty, rats have a luteinizing hormone (LH) and prolactin (PRL) surge during the afternoon of proestrus (Gay *et al.* 1970, Kalra *et al.* 1972). These surges can be reliably reproduced in ovariectomized females using different methods of estrogen and progesterone replacement. Steroids may be administered by sequential injections (Heimke *et al.* 1987,

Bonavera *et al.* 1993) or by implanting steroid pellets (e.g. Le *et al.* 1997, Yen & Pan 1999), although implants produce constant high levels of steroid hormones that desensitize the pituitary gland to GnRH stimulation (for review see Gharib *et al.* 1990). Sequential injections of estrogen and progesterone most closely resemble the estrous cycle of the rat and produce an LH surge in mature rats from 1 day to 8 weeks after ovariectomy (Legan *et al.* 1973, Caligaris *et al.* 1974, Blake 1977, Adler *et al.* 1983, Clough & Rodriguez-Sierra 1983, Rubin *et al.* 1985, Pi 1986, Bonavera *et al.* 1993).

Both the LH and PRL surges are induced by estrogen and progesterone, but the hypothalamic neural mechanisms regulating these surges are different (Neill *et al.* 1971, Caligaris *et al.* 1974). Prolactin is primarily under tonic, inhibitory control from hypothalamic dopamine, but estrogen modulates prolactin secretion by acting on hypothalamic dopamine and directly on anterior pituitary lactotropes (see Freeman *et al.* 2000 and Ben-Jonathan & Hnasko 2001 for reviews). Increased estrogen, which stimulates the pre-ovulatory prolactin surge (Neill *et al.*

1971), is concentrated in dopaminergic neurons in the arcuate nucleus of the hypothalamus (Sar 1984). Furthermore, prolactin is regulated by a number of releasing factors that are also potential targets of estrogen modulation. LH release is controlled by the pulsatile secretion of hypothalamic GnRH (see Herbison & Pape 2001 for review). Increased estrogen produces increases in GnRH pulses and LH release (see Etgen *et al.* 1999 for review). Estrogen may act directly on GnRH neurons, probably through estrogen receptor β (Hrabovszky *et al.* 2000) or indirectly through transynaptic mechanisms or glial cell interaction (see Herbison & Pape 2001 for review).

One potential mediator of estrogen action on the hypothalamus is nitric oxide (NO). NO is an endogenously produced gaseous neurotransmitter that is formed from the substrate L-arginine via the action of the enzyme, nitric oxide synthase (NOS) (Bredt 1999). Once formed, NO exerts its effects on target neurons by influencing the function of second messengers, such as cyclic GMP (see Dawson & Snyder 1994, Krumenacker *et al.* 2004 for reviews). GnRH neurons in the hypothalamus are surrounded by NOS-positive neurons, suggesting that NO regulates these neurons (Grossman *et al.* 1994, Bhat *et al.* 1996, Herbison *et al.* 1996, Bredt 1999). NO stimulates GnRH release (Bonavera *et al.* 1993) and synthesis (Wang *et al.* 1998) and may play a role in the induction of the steroid-induced LH surge (Bonavera *et al.* 1993, 1994, Aguan 1996). While the role of NO in mediating the steroid-induced prolactin surge is not clear, NO stimulates prolactin release and inhibits dopaminergic neuronal activity in the median eminence (Yen & Pan 1999). Furthermore, excitatory amino acids (Abbud & Smith 1993), particularly glutamate (Van Den Pol & Trombley 1993) stimulate prolactin release (see Freeman *et al.* 2000 for review), and excitatory amino acids induce NO release (Dhandapani & Brann 2000).

The goal of this study was to examine the effects of NO supplementation and the duration of steroid depletion, induced by ovariectomy, on the steroid-induced LH and prolactin surges in peripubertal female rats. The results indicate that there is an age-related sensitivity to steroid hormone replacement and to NO stimulation that is modulated by the duration of gonadal steroid hormone depletion.

Materials and Methods

Animals and treatments

Female rats (*Ratus rattus*) of the Sprague Dawley strain were purchased from Harlan Laboratories (Indianapolis, IN, USA). All rats were ovariectomized at either 6 weeks of age, prior to vaginal opening, or as mature adults (~16 weeks of age). Animals were housed in the animal facility at Miami University on a 12 h light:12 h darkness cycle (lights on at 0300 h). Food and water were available *ad libitum*. All procedures were performed in accordance

with the National Institutes of Health (NIH) guidelines and were approved by the Miami University Institutional Animal Care and Use Committee (IACUC).

Surgical preparation and gonadal steroid treatment

Peripubertal rats (6 weeks old) were examined, and only those that did not have vaginal openings were used in these studies, i.e. those that have not yet started estrous cyclicity (Docke & Dorner 1974, Urbanski & Ojeda 1987). Typically, vaginal openings occur in our animal population between 39–45 days of age. Bilateral ovariectomies were performed on all animals under isoflurane/oxygen gas anesthesia. Peripubertal animals were allowed to recover for 7 or 21 days. Adult animals were given a 21-day recovery period. We previously examined the steroid-induced LH and prolactin surges in adult animals after 7 days of steroid depletion (Brown *et al.* 2004). Following recovery, rats were injected with 17 β -estradiol (E; 50 μ g/kg, sc) or sesame oil at 0700 h (day 1). The day after the estradiol injection and one day prior to their use in an experiment, indwelling jugular catheters were surgically implanted in each rat under isoflurane/oxygen gas anesthesia as previously described (Jaworski-Parman *et al.* 1997, Callahan *et al.* 2000). On the day of the experiment (day 3), animals received progesterone (P; 2.5 mg/kg, sc) or sesame oil at 0700 h. This regimen of steroid replacement is known to induce an LH surge in mature, ovariectomized rats (Heimke *et al.* 1987).

L-Arginine administration

In a second study, separate groups of steroid-treated, ovariectomized, peripubertal females received L-arginine (150 mg/kg, ip) or saline at 1000 or 1200 h on the day of the experiment following the protocol of Yen and Pan (1999). Steroids, sesame oil, and L-arginine were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Blood sampling and tissue collection

On the day of the experiment, blood samples (0.8 ml) were withdrawn through the previously implanted jugular cannula at 0900, 1100, 1200, 1300, 1400, 1600 and 1800 h. Blood volume was immediately replaced with an equal volume of sterile, heparinized (50 U/ml) saline to prevent alterations in hormone levels due to any changes in volume (Brown *et al.* 2004). All blood samples were kept at 4°C until the end of the experiment. Following the last blood sample, rats were killed and the uterus (including the cervix) was removed from each rat and the fat was removed. Uteri were blotted on absorbent paper, and wet uterine weights were recorded. Wet uterine weights are reported as a percentage of body weight. The blood was centrifuged (1000 $\times$ g) and the plasma was collected and stored at -20°C until subjected to radioimmunoassay.

Radioimmunoassay (RIA)

Plasma samples were assayed in duplicate for LH and PRL using reagents provided by the National Hormone and Peptide Program (NHPP), the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), and Dr A F Parlow (Harbor-UCLA Research and Education Institute Torrance, CA.). Hormone levels are expressed in terms of rat PRL Reference Prep-3 or rat LH Reference Prep-3. Goat anti-rabbit gamma globulin was purchased from Antibodies Inc. (Davis, CA, USA). ¹²⁵I-Labeled PRL and ¹²⁵I-labeled LH were purchased from Covance Laboratories (Vienna, VA, USA). Plasma samples from a given experiment were assayed in a single RIA. The lower limit of detection for PRL and LH was 0.8 ng/ml. Intra-assay and interassay coefficients of variation were less than 8% and 12% respectively.

Statistical analysis

All data were analyzed using a repeated measures ANOVA design using Miami University Statistical Analysis System, *t*-tests were employed to determine whether various sample group means differed sufficiently to indicate differences in the corresponding population means. These tests were performed using the Bonferroni multiple comparison approach in order to control the overall experimental error. The overall error rate was controlled at $\alpha = 0.1$ for hormone data and $\alpha = 0.05$ for uterine weight. Because ANOVA designs assume that all groups have normally distributed errors with constant variance, LH values for the 6-week-old animals given a 3-week recovery period were log transformed prior to analysis to deal with outliers and the fact that variability in this group was much larger than in the other groups.

Results

Ovariectomy plus steroid hormones (estrogen + progesterone): basal and peak hormone levels

In peripubertal animals, there was a significant increase in basal levels of LH, but not PRL, by three weeks post-ovariectomy (Table 1). Regardless of age at the time of

ovariectomy or the duration of steroid depletion, steroid replacement induced significant LH and PRL surges in adult and peripubertal females (Table 1). However, the LH surge was significantly greater in the peripubertal animals after three weeks of steroid depletion. In contrast, the magnitude of the PRL surge was greatest in adult, ovariectomized animals (Table 1).

Hormone levels in peripubertal females following one week of steroid depletion

When administered one week after ovariectomy, steroid hormones induced a significant increase in LH in peripubertal females and L-arginine administration significantly increased the magnitude of the LH surge (Fig. 1). Although a PRL surge was also induced by this steroid treatment, L-arginine did not have any effect on the prolactin response (Fig. 2).

Hormone levels in peripubertal females following three weeks of steroid depletion

When administered three weeks after ovariectomy, steroid hormones induced a significant increase in the magnitude and duration of the LH surge in peripubertal females (Fig. 3) compared with one week of steroid depletion (Fig. 1) and with adults (Fig. 4, Table 1). Administration of L-arginine did not increase the magnitude of the LH surge. In fact, the duration of the surge decreased with L-arginine supplementation (Fig. 3).

Steroid treatment also induced a PRL surge in peripubertal females (Fig. 5) that was similar to the surge induced by steroids after one week of steroid depletion (Fig. 2), but significantly less than the PRL surge in adults (Fig. 6). The magnitude and duration of the PRL surge was significantly decreased by L-arginine administration.

Uterine weight

Regardless of the duration of steroid depletion, there was a significant increase in uterine weight following estrogen and progesterone treatment (Fig. 7). However, by three weeks post-ovariectomy, the increase in uterine weight was significantly less than the increase after one week.

Table 1 Basal and peak LH and PRL levels in female rats following ovariectomy. Results, expressed as $\mu\text{g/ml}$, are means $\pm$ S.E.M. Adult females were 16 weeks old and peripubertal animals were 6 weeks old at the time of the ovariectomy. The post-ovariectomy recovery period and the number of animals in each group are in parentheses.

Animal group	Basal LH	Peak LH	Basal PRL	Peak PRL
Peripubertal (1 wk; <i>n</i> = 8)	5.8 $\pm$ 1.1	28.1 $\pm$ 7.0*	24.6 $\pm$ 8.0	284.2 $\pm$ 16.6*†
Peripubertal (3 wks; <i>n</i> = 11)	18.8 $\pm$ 2.8‡	179.9 $\pm$ 32.8*†‡	44.9 $\pm$ 11.1	231.4 $\pm$ 59.5*†
Adult (1 wk; <i>n</i> = 7)	12.6 $\pm$ 1.9	34.7 $\pm$ 12.6*	23.5 $\pm$ 15.7	716.4 $\pm$ 278.1*
Adult (3 wks; <i>n</i> = 15)	17.4 $\pm$ 2.1	63.1 $\pm$ 14.4*	63.2 $\pm$ 21.3	1745.7 $\pm$ 182.6*

Peak levels of LH and PRL were significantly greater than basal levels, i.e. levels at 0900 h, within the same group. The time of the steroid-induced LH peak was 1300 or 1600 h in peripubertal animals after one or three weeks of steroid depletion respectively. In adult animals, LH levels peaked at 1400 h. PRL levels reached peak values at 1100 h in peripubertal animals, regardless of the recovery time, whereas in adult animals, PRL levels reached peak values at 1200 h.

All *P* values $\leq$ 0.006. *Significantly different from basal levels in same treatment group; †significantly different from peak levels in adults; ‡significantly different from peripubertal group, 1 week after ovariectomy.

Figure 1 The effect of steroid hormone (EP, ◆) replacement on LH levels in peripubertal females, 1 week post-ovariectomy. Peak LH levels were significantly increased with supplemental L-arginine (Larg, ■). * $P < 0.0053$, significantly different from baseline (0900 h); † $P < 0.0053$, significantly different between treatment groups.

Steroid replacement also increased uterine weight in the adult females (Fig. 8) and the increase was similar to that observed in peripubertal females after 3 weeks of steroid depletion.

Discussion

The results of this study indicate that increases in NO, due to L-arginine administration, increased the magnitude of the steroid-induced LH surge in peripubertal, ovariectomized female rats, but the stimulatory effects of NO are related to the duration of steroid depletion. Furthermore, basal levels of LH were elevated in peripubertal females 3 weeks after ovariectomy and these animals were more responsive to steroid-induced LH secretion. One possible explanation for these results is that as the interval between ovariectomy and steroid replacement increased, circulating levels of gonadotropins increased due to loss of negative feedback (Sagrillo *et al.* 1996,

Figure 2 The effect of steroid hormone (EP, ◆) replacement on PRL levels in peripubertal females, 1 week post-ovariectomy. Peak PRL levels were not different between the control and L-arginine (Larg, ■) supplemented groups. * $P < 0.0053$, significantly different from baseline (basal levels, i.e. levels at 0900 h).

Figure 3 The effect of steroid hormone (EP, ◆) replacement on LH levels in peripubertal females, 3 weeks post-ovariectomy. Administration of gonadal steroids induced a significant increase in LH levels, but L-arginine (Larg, ■) treatment inhibited the duration of the surge. * $P < 0.0053$, significantly different from baseline (0900 h); † $P < 0.0053$, significantly different between treatment groups.

Gore & Roberts 1997, Herbison 1998). Loss of negative feedback causes the pituitary to be more responsive to GnRH stimulation (Legan *et al.* 1973) and results in increased LH release following steroid hormone treatment. Another possibility is that, as the animal ages, it becomes more responsive to steroid hormone stimulation. It is clear that as maturation occurs, a number of developmental changes occur that influence the activity of neural factors required for the induction of the LH surge, including maturation of the gamma-aminobutyric acid (GABA) (see Ojeda *et al.* 2003) and glutamatergic pathways, as well as n-methyl-D-aspartate (NMDA) receptors (Urbanski & Ojeda 1987, Brann *et al.* 1997, Gore 2001). However, previous results from our laboratory indicate that age alone cannot explain this increased responsiveness to steroids (Brown *et al.* 2004). The same regimen of steroid hormone replacement used in this study was administered to

Figure 4 The effect of estrogen plus progesterone (E + P) administration on LH levels in adult females, 3 weeks post-ovariectomy. LH levels surged at 1400 h. * $P < 0.0053$, significantly different from baseline (0900 h).

Figure 5 The effect of steroid hormone (EP, $\blacklozenge$) replacement on PRL levels in peripubertal females, 3 weeks post-ovariectomy. PRL levels surged at 1100 h and remained significantly elevated at 1200 h. Administration of L-arginine (Larg, $\blacksquare$) decreased the magnitude and duration of the PRL surge. * $P < 0.0053$, significantly different from baseline (0900 h); † $P < 0.0053$, significantly different between treatment groups.

9-week-old females one week after ovariectomy, and the steroid-induced LH response was not greater than the levels reported in this study for 6-week-old, peripubertal animals (Brown *et al.* 2004). Therefore, it is more likely that it is the duration of the steroid depletion that alters the sensitivity of the pituitary and/or hypothalamus to steroid stimulation (Legan *et al.* 1973, King & Letourneau 1994), resulting in an increased LH surge.

The magnitude of the steroid-induced LH surge in peripubertal animals (28.1 ± 7.0 ng/ml at 1400 h) after one week of steroid depletion was similar to levels previously reported for adults (34.74 ± 12.5 ng/ml at 1400 h; Brown *et al.* 2004). L-Arginine supplementation significantly increased the magnitude of the LH surge in these peripubertal, ovariectomized females. The involvement of NO in mediating the steroid-induced LH surge has already been shown in adult, ovariectomized females (Bonavera *et al.* 1993, 1996, Brann *et al.* 1997), but this is the first report showing that NO enhances the LH surge in peripubertal

Figure 6 The effect of estrogen plus progesterone (E + P) administration on PRL levels in adult females, 3 weeks post-ovariectomy. PRL levels surged at 1100 h and remained significantly elevated at 1200 h. * $P < 0.0053$, significantly different from baseline (0900 h).

Figure 7 The effect of vehicle (oil) or steroid hormone replacement (EP), administered at 1 or 3 weeks after ovariectomy, on uterine weight as a percentage of body weight in peripubertal females. * $P < 0.0056$, significantly different from controls; † $P < 0.0056$, significantly different from steroid-treated females after one week of steroid depletion.

animals. However, the facilitatory effects of NO are dependent on steroid exposure because after 3 weeks of steroid depletion, NO actually decreased the magnitude and duration of the LH surge. One possible explanation for the decrease in the LH response to L-arginine is that the longer duration of estrogen depletion caused a decreased sensitivity of NOS. It has been reported that estrogen increased hypothalamic NOS activity (McCann *et al.* 1998, Gouveia & Franci 2004) and gene expression (Sahu 1998), as well as NOS activity and protein levels in the pituitary (Garrel *et al.* 1998). Therefore, following the longer period of steroid depletion, NOS activity and/or levels may have been reduced. A second possibility for the decreased LH response to L-arginine supplementation is that NOS levels decreased as the animals matured. An age-related decrease in hypothalamic NOS gene expression has been reported, but this occurred in middle-aged rats (Sahu 1998). Therefore, it seems unlikely that the decreased LH response to L-arginine is due to an

Figure 8 The effect of oil (control) or steroid hormone replacement (E/P) on uterine weight, expressed as a percentage of body weight, in adult females, 3 weeks after ovariectomy (OVX). * $P < 0.0056$, significantly different from controls.

age-related decline in NOS expression because the rats used in this study were young.

Steroid hormone replacement also caused a significant increase in prolactin secretion in peripubertal animals, and although the timing of the surge was similar to that seen in adult ovariectomized females, the magnitude of the surge was much lower. Neither the duration of steroid depletion, nor the administration of L-arginine increased the magnitude of the prolactin secretory response to steroid hormone administration. In fact, after three weeks of steroid depletion, L-arginine significantly decreased the magnitude and duration of the prolactin response to steroid treatment. Although NO appears to play a role in mediating the steroid-induced prolactin surge (Bonavera *et al.* 1994, Yen & Pan 1999), the effects of increased NO levels vary depending on the site of NO action and the methods employed to produce increases in NO. For example, increased levels of NO produced by administration of an NO donor, produced a decrease in prolactin secretion from hemipituitary glands collected from male rats (Duvilanski *et al.* 1996), suggesting NO inhibits prolactin secretion. However, NO was essential in mediating the interleukin-1 β -induced stimulated release of prolactin in cultured pituitary cells from male rats (Brunetti *et al.* 1995). On the other hand, at the hypothalamic level, NO stimulated prolactin secretion in the male by inhibiting hypothalamic tyrosine hydroxylase activity (Gonzalez *et al.* 1999). Similarly, inhibitors of NOS synthesis prevented the estrogen-induced inhibition of dopaminergic activity in the median eminence (Yen & Pan 1999), as well as the prolactin surge (Bonavera *et al.* 1994) in female rats. However, L-arginine administration, which also increases NO levels, did not have any effect on either dopaminergic activity or prolactin levels in ovariectomized, steroid-treated rats (Yen & Pan 1999). In our study, L-arginine was administered systemically following the protocol of Yen and Pan (1999). Our results, in agreement with Yen and Pan (1999), demonstrate that L-arginine had no effect on circulating levels of prolactin following one week of steroid depletion. Our results also confirm those of Velardez *et al.* (2003) who reported that increases in NO did not affect prolactin release from anterior pituitary cells collected from rats 2 weeks after ovariectomy and treated with 17 β -estradiol. After 3 weeks of steroid depletion, however, L-arginine significantly decreased the prolactin response to steroid hormone stimulation, suggesting that the inhibitory effects of NO, probably at the level of the anterior pituitary gland, were increased (Duvilanski *et al.* 1996).

Uterine weight was used as a marker of steroid hormone action. As expected, steroid hormone replacement produced an increase in uterine weight in all treatment groups. However, while steroid replacement increased uterine weight in ovariectomized, peripubertal females, the increase was greater after only one week of steroid depletion. In addition, the magnitude of the increase in uterine weight was similar in the peripubertal and adult,

ovariectomized females after 3 weeks of steroid depletion. These results indicate that, regardless of the age of the animal, the uterus loses sensitivity to steroid effects over time. We have previously reported that there is greater uterine growth when steroid replacement occurs in a cyclic, repeated pattern (Brown *et al.* 2004).

In conclusion, these results demonstrate that there is a sensitivity to NO stimulation of LH, but not prolactin, that is modulated by the duration of gonadal steroid hormone depletion. Furthermore, three weeks of steroid depletion increased basal levels of LH, but not prolactin indicating a loss of negative feedback in the regulation of LH. The differences in the responsiveness of LH and prolactin to steroid-induced stimulation in peripubertal animals demonstrate that these hormones are regulated by NO through different mechanisms.

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