0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(10):3748–3758

doi: 10.1210/en.2002-220291

Does Cortisol Mediate Endotoxin-Induced Inhibition of Pulsatile Luteinizing Hormone and Gonadotropin-Releasing Hormone Secretion?

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Bacterial endotoxin (lipopolysaccharide), a commonly used model of immune/inflammatory stress, inhibits reproductive neuroendocrine activity and concurrently induces a profound stimulation of the hypothalamo-pituitary-adrenal axis. We employed two approaches to test the hypothesis that enhanced secretion of cortisol mediates endotoxin-induced suppression of pulsatile GnRH and LH secretion in the ovariectomized ewe. First, we mimicked the endotoxin-induced increase in circulating cortisol by delivering the glucocorticoid in the absence of the endotoxin challenge. Within 1–2 h, experimentally produced increments in circulating cortisol suppressed pulsatile LH secretion in a dose-dependent fashion. Second, we blocked the endotoxin-induced stimulation of cortisol secretion using the drug metyrapone, which inhibits the 11-β hydroxylase enzyme necessary for cortisol biosynthesis. In the absence of a marked stimulation of cortisol secretion, endotoxin still profoundly inhibited pulsatile GnRH and LH secretion. We conclude that, although enhanced cortisol secretion may contribute to endotoxin-induced suppression of reproductive neuroendocrine activity, the marked stimulation of the glucocorticoid is not necessary for this response. Our findings are consistent with the hypothesis that immune/inflammatory stress inhibits reproductive neuroendocrine activity via more than one inhibitory pathway, one involving enhanced secretion of cortisol and the other(s) being independent of this glucocorticoid. (*Endocrinology* 143: 3748–3758, 2002)

ACTIVATION OF THE immune system by inflammatory and infectious disease, injury, physical trauma, and a variety of noxious stimuli interrupts the ovarian cycle by suppressing reproductive neuroendocrine activity as well as ovarian responsiveness to gonadotropic hormones (1–6). Bacterial endotoxin, a lipopolysaccharide component of gram-negative bacteria commonly used as a model of immune/inflammatory stress (7), disrupts ovarian cyclicity in cattle, monkeys, and sheep (8–10). At a neuroendocrine level, endotoxin inhibits pulsatile GnRH secretion, suppresses pituitary responsiveness to GnRH, and concurrently stimulates hormones of the neuroendocrine stress axis: arginine vasopressin, CRH, ACTH, cortisol, and progesterone (11–15). Each of these stress axis hormones is a potential mediator of endotoxin-induced suppression of GnRH and LH secretion. In the monkey, for example, CRH and arginine vasopressin mediate LH inhibition in response to the cytokine IL-1, which acts as an intermediate in many responses to endotoxin (16, 17). Observations in sheep suggest both ACTH and cortisol can inhibit LH secretion and pituitary responsiveness to GnRH (18–22).

In the present study, we tested the hypothesis that cortisol mediates the inhibitory effects of endotoxin on pulsatile GnRH and LH secretion in sheep, a model species in which the secretory profiles and temporal associations of GnRH and LH can be monitored with high resolution. We employed two approaches. First, we delivered cortisol in the absence of an endotoxin challenge to determine if a cortisol rise, similar to that induced by endotoxin, is sufficient to suppress pulsatile LH secretion. Second, we suppressed cortisol synthesis during an endotoxin challenge to determine if increased secretion of cortisol is necessary for suppression of pulsatile LH and GnRH secretion.

Abbreviation: CV, Coefficient of variation.

Materials and Methods

Animals

This study was conducted between November 1999 and April 2001 on adult Suffolk ewes (70–110 kg). Before the experiments, the animals were maintained outdoors under standard husbandry conditions at the Sheep Research Facility near Ann Arbor, MI. For the experiments, the ewes were moved indoors under a simulated natural photoperiod. Each ewe was ovariectomized as this allows expression of robust pulses of GnRH and LH secretion, thereby facilitating detection of suppressed pulsatility following cortisol or endotoxin. Further, this model ensures that changes in GnRH and LH secretion are not caused by altered ovarian steroid secretion. Core body temperature was monitored with battery-operated, temperature-sensitive telemetry transmitters (DataCol5, Minimitters Inc., Sunriver, OR). The transmitters were implanted ip at the time of ovariectomy and tied to the uterine broad ligament. All procedures were approved by the Committee for the Use and Care of Animals at the University of Michigan.

General experimental design

This study consisted of five experiments. Because the rationale and designs of later experiments were based on results of the initial ones, experimental design details are presented in *Results*. Due to their sequential nature, the experiments spanned both the breeding and anestrous seasons. Each individual experiment contained both control

and experimental treatments. Thus, all experiments could be interpreted independently. The following procedures are generally applicable.

One day before each experiment, ewes were penned individually in the same room or in groups of three, and indwelling jugular catheters were inserted for sampling peripheral blood. In two of the five experiments, pituitary portal blood was collected by continuous withdrawal using a technique described in detail by Caraty *et al.* (23). For this purpose, a sampling apparatus was surgically implanted into the hypophyseal region. The sheep were then allowed to recover and pituitary portal blood was collected 2 wk later. This procedure allows pituitary portal and peripheral blood to be sampled at frequent intervals from animals that are not anesthetized, sedated, or physiologically compromised. Ewes were provided with hay and water during portal blood collection.

Treatments

Cortisol (hydrocortisone sodium phosphate, aqueous solution, 50 mg/ml; Merck and Co., West Point, PA) suspended in sesame oil vehicle or vehicle alone was injected sc. Cortisol doses were determined in pilot studies to increase the serum cortisol concentration within the range of values observed in ewes during endotoxin challenge (~60–150 ng/ml). Endotoxin (*Escherichia coli* lipopolysaccharide, Serotype 055:B5; Sigma, St. Louis, MO) was dissolved in nonpyrogenic saline (10 μg/ml) and injected iv at either 40 or 400 ng/kg. Both doses induce fever, inhibit reproductive neuroendocrine function, stimulate the neuroendocrine stress axis, and provoke transient sickness behaviors to varying degrees (*e.g.* lethargy, labored breathing, and diarrhea) (11, 12). Metyrapone in capsules (Ciba-Geigy, Basel, Switzerland) was used to inhibit cortisol synthesis. This drug, which blocks the 11-β hydroxylase enzyme necessary for cortisol synthesis, has been found to be effective in sheep (24). For use, metyrapone was removed from the capsules and administered as seven hourly injections at a dose of 11.3 mg/kg, im. This dose was based on that used in humans (25); pilot studies in our laboratory confirmed that it inhibits endotoxin-induced cortisol secretion in ewes without evoking overt adverse side effects. Vehicle for metyrapone consisted of an aqueous solution containing 10% polyethylene glycol/10% glycerol/1% gelatin. Progesterone was administered sc by implanting one 50 × 75-mm SILASTIC brand (Dow Corning, Midland, MI) packet that produces an early luteal phase level of serum progesterone (~1 ng/ml) in ovariectomized ewes (26).

Hormone assays

LH was measured in duplicate aliquots of plasma (10–200 μl) using a modification (27) of a previously described RIA (28, 29). Values are expressed in terms of NIH-LH-S12. Mean intra- and interassay coefficients of variation (CV) were 6.4% and 8.0%, respectively, and assay sensitivity for 200-μl aliquots averaged 0.8 ng/ml (37 assays). Cortisol was measured in duplicate 50-μl aliquots of plasma using the Coat-a-Count kit (Diagnostic Products Corp., Los Angeles, CA), previously validated for use in sheep (12). Mean intra- and interassay CV were 3.5% and 8.0%, respectively, and assay sensitivity averaged 0.8 ng/ml (15 assays). Progesterone was determined in duplicate 100 μl aliquots using the Coat-a-Count progesterone assay kit (Diagnostic Products Corp.), previously validated for use in sheep (30). Intra- and interassay CV were 6.1% and 9.0%, respectively, and assay sensitivity averaged 0.05 ng/ml (14 assays). GnRH was measured in duplicate in methanol extracts of approximately 200 μl aliquots of portal plasma using a previously described RIA (31, 32). Intra- and interassay variation averaged 10.2% and 14.6%, respectively, and assay sensitivity averaged 0.16 pg/ml (six assays).

To assay 11-desoxycortisol, the ImmuChem double antibody 11-desoxycortisol assay kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA) was modified by adding 10 μl charcoal-stripped plasma to standard curve and control tubes. The assay was validated for use in sheep. Assay specificity was checked by confirming parallelism between serial dilutions of ovine samples and 11-desoxycortisol standards. Recovery averaged 103% when assay buffer was spiked with known amounts of standard (ICN Pharmaceuticals, Inc.). Samples from ovariectomized ewes not treated with endotoxin produced values at or below assay sensitivity (0.2 ng/ml). Concentrations of 11-desoxycortisol in samples were determined in duplicate 10-μl aliquots of plasma diluted 1:10 in charcoal-stripped plasma from ovariectomized ewes. All samples were measured in a single assay. Mean intraassay CV was 11.1%, and assay sensitivity averaged 0.2 ng/ml.

Data analysis

LH and GnRH pulses were identified using the Cluster pulse detection algorithm (33). Cluster sizes for peaks and nadirs were defined as 1 and 2 for LH, and 1 and 1 for GnRH. The t-statistic used to identify a significant increase and decrease was 2.6 and 2.6 for LH, and 3.8 and 3.8 for GnRH. LH and GnRH pulse amplitudes were defined as the difference between the peak of a pulse and its preceding nadir. Total pulsatile LH and GnRH outputs were calculated as the product of the number of pulses × mean pulse amplitude. GnRH in pituitary portal blood was calculated as a collection rate (pg/min) rather than concentration. This minimizes errors due to contamination of portal samples with peripheral blood or cerebrospinal fluid (both judged to be negligible in this study), or due to changes in the rate of portal blood flow.

Before statistical analysis, plasma hormone concentrations were log transformed and pulse frequencies were square root transformed to normalize variability across a broad range of values. All experiments consisted of a baseline period when no treatment was given and one or more periods when treatments were applied. To identify treatment effects, mean values for baseline and treatment periods were obtained in every ewe for each parameter (*e.g.* plasma cortisol, LH pulse measures, core body temperature, *etc.*). Next, a two-way, repeated-measures ANOVA (treatment × time) was used to identify significant interactions for all parameters between the control and experimental groups. ANOVAs for LH parameters excluded data during the first hour after treatment to allow time for treatments to take effect; values were also omitted for 1 h pretreatment to equalize the pre/posttreatment duration for analysis. When a significant treatment by time interaction was observed, *post hoc* analysis was conducted to identify specific treatment effects. This consisted of paired *t* tests comparing pre *vs.* posttreatment values in experiments 2–5, in which the same ewes received two treatments (control and experimental) in a cross-over design. *Post hoc* analysis in experiment 1, in which each ewe received three treatments, consisted of successively excluding data from one treatment and repeating the ANOVA on the remaining two. Significance level was set at $P \leq 0.05$.

Results

Experiment 1: does an increase in circulating cortisol, similar to that induced by endotoxin, suppress pulsatile LH secretion?

This experiment tested the hypothesis that the endotoxin-induced increase in circulating cortisol is sufficient to suppress pulsatile LH secretion. The experiment was performed on nine ovariectomized ewes during the anestrous season (April and May). Each ewe received three treatments in randomized sequence at 1-wk intervals according to a cross-over design: vehicle (sc injection every 30 min), low-dose cortisol (12.5 μg/kg sc, every 30 min for 4 h), and high-dose cortisol (50 μg/kg sc, every 30 min for 4 h). Blood was sampled every 12 min from 6 h before to 6 h after onset of treatment.

During the vehicle run of the experiment, serum cortisol remained at a basal concentration (7.2 ± 1.6 ng/ml; Fig. 1A). The low dose of cortisol elevated the mean serum cortisol concentration to approximately 60 ng/ml during hours 2–4 of treatment (Fig. 1B). This increment is similar to that observed in response to a low dose of endotoxin (40 ng/kg), which inhibits pulsatile LH secretion primarily by suppressing pituitary responsiveness to GnRH (Ref. 15; see also experiment 4 below). The high dose of cortisol produced a serum cortisol increment to approximately 160 ng/ml (Fig. 1C). This is similar to the increase in circulating cortisol observed in response to a high dose of endotoxin (400 ng/

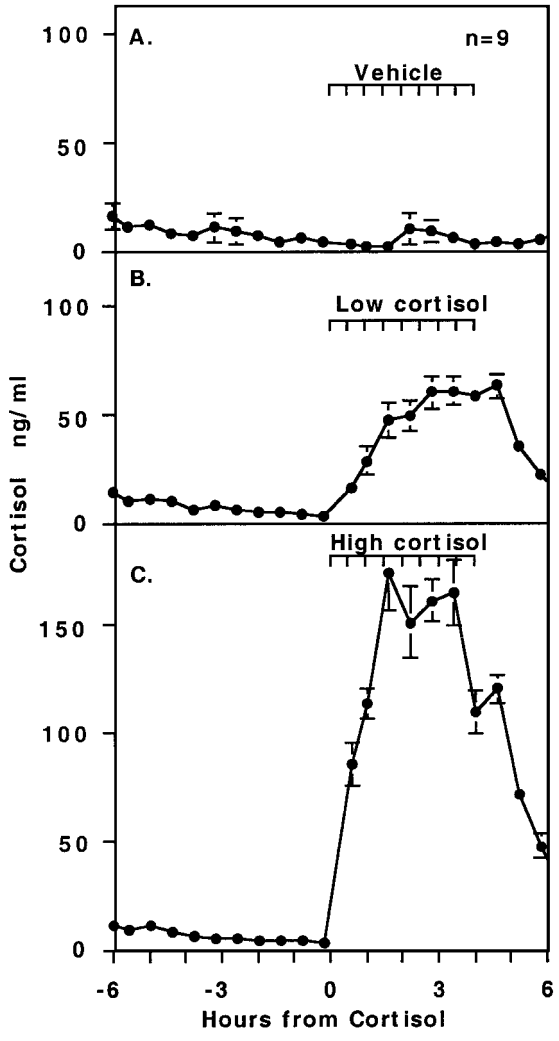

FIG. 1. Mean ± SEM plasma cortisol concentration in ewes treated with (A) vehicle sc every 30 min, (B) low-, and (C) high-dose cortisol (12.5 μg/kg or 50 μg/kg sc every 30 min, respectively) in experiment 1. *Horizontal line with tic marks* depicts period of 30-min injections. This was a cross-over experiment in which each of nine ewes received all three treatments. Where no SEM bar appears, the value is smaller than the data symbol.

kg), which inhibits pulsatile GnRH release as well as pituitary responsiveness to GnRH (Refs. 12 and 15; see also experiments 2 and 5 below). After the 4-h treatment period, cortisol declined toward pretreatment values.

LH responses during each run of the cross-over experiment are illustrated for two ewes in Fig. 2; mean values for all nine ewes are presented in Table 1. Vehicle did not significantly influence any aspect of pulsatile LH secretion. In contrast, the repeated measures ANOVA revealed that both doses of cortisol suppressed LH (Table 1). Specifically, the low dose reduced the mean serum LH concentration by 28% (pre *vs.* posttreatment means, $P \leq 0.01$), due to a lowering of total pulse output (39%, $P \leq 0.05$) and a tendency for decreased pulse amplitude (42%, $P \leq 0.1$) but not frequency. The high dose of cortisol produced a more pronounced suppression of mean LH (45%, $P \leq 0.001$) due to significant suppressions of both LH pulse frequency (12%, $P \leq 0.05$) and total pulse output (65%, $P \leq 0.05$), and again a tendency for reduced pulse amplitude (56%, $P \leq 0.1$).

Experiment 2: does metyrapone reverse endotoxin-induced inhibition of pulsatile LH secretion?

Experiment 1 revealed that an acute increase in circulating cortisol, similar to that induced by endotoxin, is sufficient to suppress pulsatile LH secretion. Here we tested the hypothesis that increased cortisol secretion is necessary for endotoxin-induced suppression of pulsatile LH secretion. This experiment was conducted during the breeding season using a cross-over design in which each of two treatments was given to 5 ewes in random sequence: endotoxin (400 ng/kg, iv) plus metyrapone; endotoxin plus vehicle for metyrapone. Jugular blood was sampled and core body temperature monitored at 10-min intervals for 12 h. During the first 3 h, no treatment was given (Period I). During the second 3 h (Period II), metyrapone or vehicle was injected hourly. At the start of the final 6 h (Period III), endotoxin was given and the hourly injections of metyrapone or its vehicle were continued for another 4 h. Thus, the first 3 h (Period I) provided a baseline; the second 3 h (Period II) tested the response to metyrapone; the final 6 h (Period III) tested the response to endotoxin in the presence or absence of metyrapone. For statistical analysis, data from Periods I and II were first compared for a metyrapone effect. Next, values before endotoxin (Periods I and II) were compared with values after endotoxin or vehicle (Period III). Note: Preendotoxin values for cortisol and 11-desoxycortisol included only Period I as metyrapone itself affected these hormones.

Time courses for all the parameters in a representative ewe during both the vehicle and metyrapone runs are illustrated in Fig. 3A; mean (± SEM) values for all five ewes are presented in Fig. 3B. Two-way repeated measures ANOVA indicated endotoxin stimulated plasma cortisol ($P \leq 0.001$), 11-desoxycortisol ($P \leq 0.001$), progesterone ($P \leq 0.01$), and core body temperature ($P \leq 0.001$). Metyrapone suppressed this stimulation of cortisol (values postendotoxin 84.2 ± 9.2 *vs.* 16.8 ± 3.1 ng/ml for vehicle and metyrapone groups, respectively, $P \leq 0.001$). Nevertheless, the mean plasma cortisol concentration postendotoxin in the metyrapone group exceeded the pretreatment baseline (6.5 ± 0.1, $P \leq 0.05$). Metyrapone enhanced endotoxin-induced stimulation of 11-desoxycortisol ($P \leq 0.001$) and progesterone ($P \leq 0.05$), but it did not affect the generation of fever.

Endotoxin alone suppressed pulsatile LH secretion, and this response was not reversed by metyrapone (Fig. 3). Statistical analysis indicated endotoxin suppressed the mean LH concentration (40%, $P \leq 0.05$), total pulsatile LH output (47%, $P \leq 0.05$), and LH pulse frequency (45%, $P \leq 0.01$; summary of LH results in Table 2). Metyrapone did not lessen any of these suppressive effects of endotoxin. Furthermore, metyrapone itself did not significantly affect LH secretion during the 3 h before endotoxin.

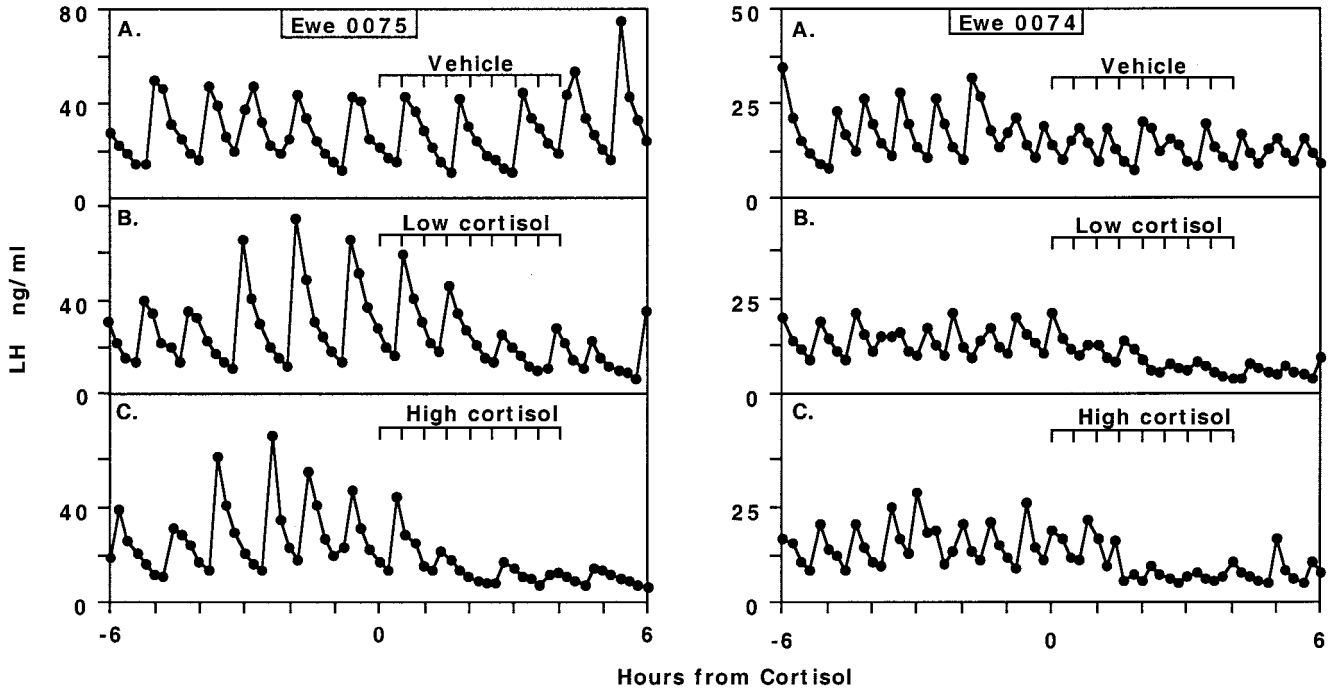

FIG. 2. LH pulse patterns in two representative ewes treated with (A) vehicle, (B) low-, and (C) high-dose cortisol during three runs of the cross-over experiment (experiment 1). *Horizontal line* with tic marks depicts period of 30-min injections. Plasma cortisol concentrations produced by these treatments are shown in Fig. 1.

TABLE 1. Effects of a low (12.5 μg/kg·0.5 h) or a high (50 μg/kg·0.5 h) dose of cortisol on LH secretion

	Mean LH (ng/ml)		LH total output (frequency × amplitude)		LH pulse frequency (pulses/5 h)		LH pulse amplitude (ng/ml)	
	Before[a]	After[b]	Before	After	Before	After	Before	After
Experiment 1								
Vehicle	21.5 ± 1.8	19.7 ± 1.9	89.7 ± 15.4	71.0 ± 13.4	4.8 ± 0.3	5.1 ± 0.2	18.4 ± 2.6	14.7 ± 3.6
Low cortisol	21.5 ± 1.9	15.5 ± 1.1[c]	91.0 ± 10.9	55.8 ± 8.5[c]	5.3 ± 0.3	5.3 ± 0.2	18.3 ± 3.3	10.6 ± 1.8
High cortisol	20.4 ± 1.7	11.3 ± 0.9[c]	97.5 ± 16.9	37.0 ± 3.4[c]	5.3 ± 0.2	4.7 ± 0.2[c]	18.7 ± 3.5	8.2 ± 1.0

[a] Before, mean (± SEM) values −6 to −1 h before cortisol treatment.
[b] After, mean (± SEM) values +1 to +6 h after start of cortisol treatment.
[c] Significant time effect (before *vs.* after cortisol, see text for *P* values).

Experiment 3: does the heightened progesterone rise induced by endotoxin plus metyrapone inhibit pulsatile LH secretion?

One interpretation of experiment 2 is that the large increase in cortisol secretion is not necessary for endotoxin-induced suppression of pulsatile LH secretion. However, metyrapone enhanced endotoxin-induced secretion of progesterone, a steroid that itself can suppress pulsatile LH secretion (34). This progesterone was presumably of adrenal origin as the ewes were ovariectomized. To test the possibility that the heightened progesterone response suppressed LH secretion, we monitored LH pulses in response to such a progesterone increment in the absence of endotoxin. Five ovariectomized ewes were either sham implanted or treated with a progesterone implant according to a cross-over design in which each ewe served as its own control. Jugular blood was sampled every 10 min from 3 h before to 6 h after progesterone or sham implantation. The experiment was conducted in the early anestrous season (March). The progesterone implant elevated the mean plasma progesterone level to 1.3 ± 0.2 ng/ml. This was slightly higher than the mean value in ewes treated with endotoxin plus metyrapone in experiment 2, but similar to the peak plasma progesterone concentration in those ewes. This progesterone elevation did not inhibit any aspect of pulsatile LH secretion (Table 3). Thus, the heightened progesterone response to endotoxin cannot account for suppression of LH in the metyrapone-treated ewes of experiment 2.

Experiment 4: does metyrapone reverse the suppressive effect of endotoxin on pituitary responsiveness to GnRH?

Experiments 2 and 3 suggest enhanced secretion of cortisol is not necessary for endotoxin to suppress pulsatile LH secretion. There is, however, another explanation. Previous findings in ewes indicate that the 400-ng/kg dose of endotoxin used in experiment 2 can inhibit LH pulsatility via two mechanisms, suppressing hypothalamic GnRH secretion and blunting pituitary responsiveness to GnRH (12, 15). Perhaps cortisol mediates inhibition at only one of these levels, in which case, reversal of suppression at that level would be

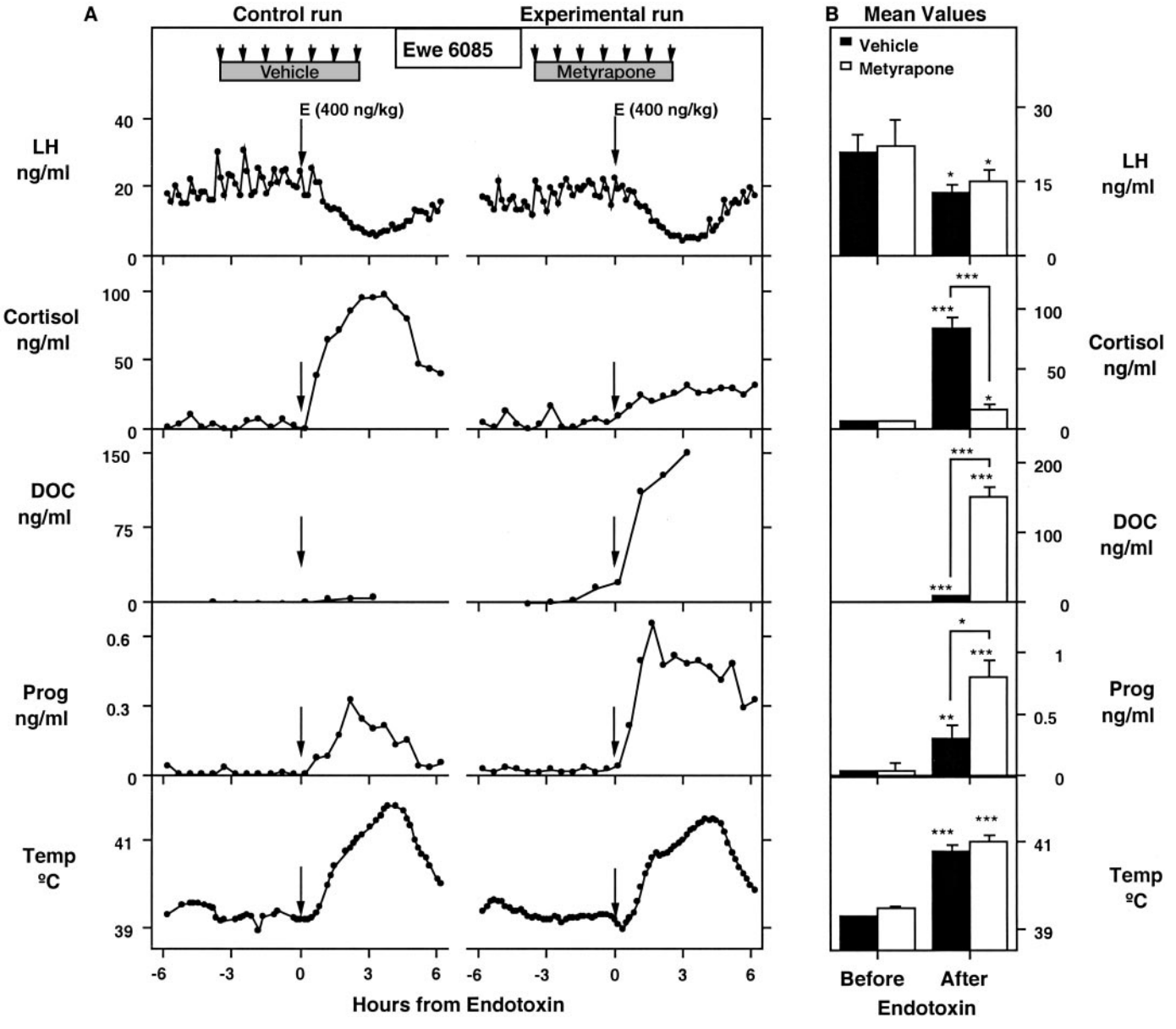

FIG. 3. A, Plasma LH, cortisol, 11-desoxycortisol (DOC), progesterone (Prog), and core body temperature (Temp) in one representative ewe following vehicle or metyrapone treatment in conjunction with a high dose of endotoxin (E, 400 ng/kg) in experiment 2. Vehicle and metyrapone were administered according to a cross-over design. *Shaded horizontal bars* with *arrowheads* at top depict half-hourly injections of metyrapone or vehicle. B, Mean (± SEM; n = 5) plasma LH, cortisol, 11-desoxycortisol, progesterone, and core body temperature before endotoxin (−6 to −1 h for LH, progesterone and temperature, or −6 to −3 h for cortisol and 11-desoxycortisol) and after endotoxin (1–6 h) in ewes pretreated with vehicle (*closed bars*) or metyrapone (*open bars*). Significant differences before and after endotoxin, or between vehicle and metyrapone-treated ewes, are denoted by the *asterisks* (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

masked because inhibition still occurs at the other level. Specifically, elevated cortisol might be needed to inhibit pituitary responsiveness to GnRH, but reversal of this effect by metyrapone would not be evident because suppression of GnRH secretion is not dependent upon elevated cortisol. Our next two experiments addressed this possibility. Here, we examined whether elevated cortisol is necessary for the pituitary effect of endotoxin; the next experiment tested if cortisol mediates the hypothalamic effect.

To test whether cortisol mediates the pituitary effect of endotoxin, we took advantage of our prior finding that a low dose of endotoxin suppresses LH pulses in ovariectomized ewes primarily by inhibiting pituitary responsiveness to GnRH (15). This experiment was conducted in two parts. Part 1 tested whether metyrapone reverses the inhibitory effect of low-dose endotoxin on LH pulses; part 2 was a terminal experiment on these same ewes to confirm that a low dose of endotoxin suppresses LH pulses independent of a reduction in GnRH secretion.

Part 1. This experiment was conducted on 6 ovariectomized ewes, five of which had been treated with the high dose of endotoxin in experiment 2. Each ewe was treated with a low dose of endotoxin (40 ng/kg, iv bolus) plus metyrapone or

TABLE 2. Effects of metyrapone or vehicle on LH secretion following the 400-ng/kg dose of endotoxin

	Mean LH (ng/ml)		LH total output (frequency × amplitude)		LH pulse frequency (pulses/5 h)		LH pulse amplitude (ng/ml)	
	Before[a]	After[b]	Before	After	Before	After	Before	After
Experiment 2								
Vehicle + endotoxin	20.8 ± 3.8	12.6 ± 1.8[c]	57.0 ± 11.1	26.8 ± 8.3[c]	6.8 ± 0.6	3.8 ± 1.1[c]	8.3 ± 1.5	6.9 ± 0.8
Metyrapone + endotoxin	22.4 ± 5.1	15.1 ± 2.2[c]	64.0 ± 23.9	31.3 ± 7.9[c]	7.4 ± 0.5	2.6 ± 0.4[c]	8.7 ± 2.9	4.0 ± 1.1

[a] Before, mean (± SEM) values −6 to −1 h from endotoxin.
[b] After, mean (± SEM) values +1 to +6 h from endotoxin.
[c] Significant time effect (before *vs.* after endotoxin, see text for *P* values).

TABLE 3. Effects of progesterone or sham implants on LH secretion

	Mean LH (ng/ml)		LH total output (frequency × amplitude)		LH pulse frequency (pulses/3 h)		LH pulse amplitude (ng/ml)	
	Before[a]	After[b]	Before	After	Before	After	Before	After
Experiment 3								
Sham implant	23.7 ± 3.8	24.7 ± 3.5	59.3 ± 5.2	64.0 ± 8.2	4.2 ± 0.4	4.2 ± 0.2	14.9 ± 2.3	15.1 ± 1.5
Progesterone implant	23.4 ± 5.6	21.3 ± 4.9	55.7 ± 9.0	49.0 ± 12.9	4.0 ± 0.3	4.4 ± 0.4	14.0 ± 2.1	10.9 ± 2.9

[a] Before, mean (± SEM) values −3 to 0 h from implant.
[b] After, mean (± SEM) values +3 to +6 h from implant.

endotoxin plus vehicle using a cross-over design (same protocol as in experiment 2). Jugular blood was sampled at 6-min intervals, more frequently than experiment 2 because this study was done in the late breeding season (December) when LH pulse frequency in ovariectomized ewes is near its annual maximum (35). Core body temperature was monitored at 10-min intervals.

Time courses for all parameters during both the vehicle and metyrapone runs of the experiment are illustrated in Fig. 4A for a representative ewe. Mean values (± SEM) for all animals are presented in Fig. 4B. As in experiment 2, endotoxin stimulated ($P \leq 0.001$) plasma cortisol, although to a lesser extent than did the higher dose of endotoxin, and this response was suppressed by metyrapone ($P \leq 0.001$). Furthermore, the low dose of endotoxin induced a significant rise in progesterone ($P \leq 0.001$), which was enhanced by metyrapone ($P \leq 0.001$), and induced fever.

The low dose of endotoxin suppressed pulsatile LH secretion; this response was not affected by metyrapone (Fig. 4; Table 4, part 1). Specifically, this endotoxin dose suppressed the mean plasma LH concentration (28%, $P \leq 0.01$), total pulsatile LH output (29%, $P \leq 0.01$), and LH pulse amplitude (28%, $P \leq 0.01$) but not frequency. Metyrapone did not alter any of these suppressive effects of endotoxin.

Part 2. We next sought to confirm the previous finding (15) that the low dose of endotoxin suppresses principally the pituitary responsiveness to GnRH (*i.e.* reduces pulsatile LH secretion without a corresponding inhibition of GnRH). The same six sheep used for part 1 were prepared surgically for pituitary portal blood collection. Two weeks later, both peripheral and pituitary portal blood were sampled for 6 h before and 6 h after the low dose of endotoxin (40 ng/kg). Jugular blood was sampled over 5-min intervals due to the high frequency LH pulses expected at the time of the experiment (February). Pituitary portal blood was obtained over 10-min intervals because limitation of sample volume following endotoxin prevented more frequent sampling. Core body temperature was assessed every 10 min. Technical difficulties prevented successful portal blood collection in two ewes; results are thus presented for only four animals.

The low dose of endotoxin did not significantly alter any aspect of GnRH pulsatility: mean value, pulse frequency, pulse amplitude or total pulse output (Fig. 5, same representative ewe as in part 1; Table 4, *bottom*, composite results). In contrast, this treatment significantly inhibited the mean plasma LH concentration ($P \leq 0.05$) and total pulsatile LH output ($P \leq 0.01$). Of particular interest relative to a pituitary effect, nearly half of the GnRH pulses after low-dose endotoxin were not accompanied by an identified LH pulse (compare GnRH with LH pulse frequency pre- *vs.* postendotoxin in Table 4, part 2; see also Fig. 5A). This reinforces the prior conclusion (15) that the low dose of endotoxin suppresses LH primarily at the level of the pituitary gland.

Experiment 5: does metyrapone reverse endotoxin-induced suppression of pulsatile GnRH secretion?

This final experiment tested the hypothesis that elevated cortisol secretion is necessary for the suppressive effects of endotoxin on pulsatile GnRH secretion. Twelve ewes were ovariectomized and surgically prepared for pituitary portal blood collection during the anestrous season (April–June). Two weeks later, the ewes were treated with the high dose of endotoxin (400 ng/kg) plus metyrapone or endotoxin plus vehicle according the same protocol used in experiment 2. This dose of endotoxin is known to inhibit pulsatile GnRH secretion as well as pituitary responsiveness to GnRH (12, 15, 36). To avoid repeated portal blood sampling of the same ewes, separate animals were used for vehicle (n = 6) and metyrapone (n = 6) treatments (*i.e.* not a cross-over design). Jugular and portal blood were sampled over 10-min intervals and core body temperature was recorded every 10 min.

The time courses of GnRH, LH and cortisol in a representative vehicle and metyrapone-treated ewe are shown in Fig. 6A; mean values for all ewes are presented in Fig. 6B. As in experiment 2, endotoxin stimulated plasma cortisol ($P \leq 0.001$) and metyrapone suppressed this response ($P \leq 0.001$).

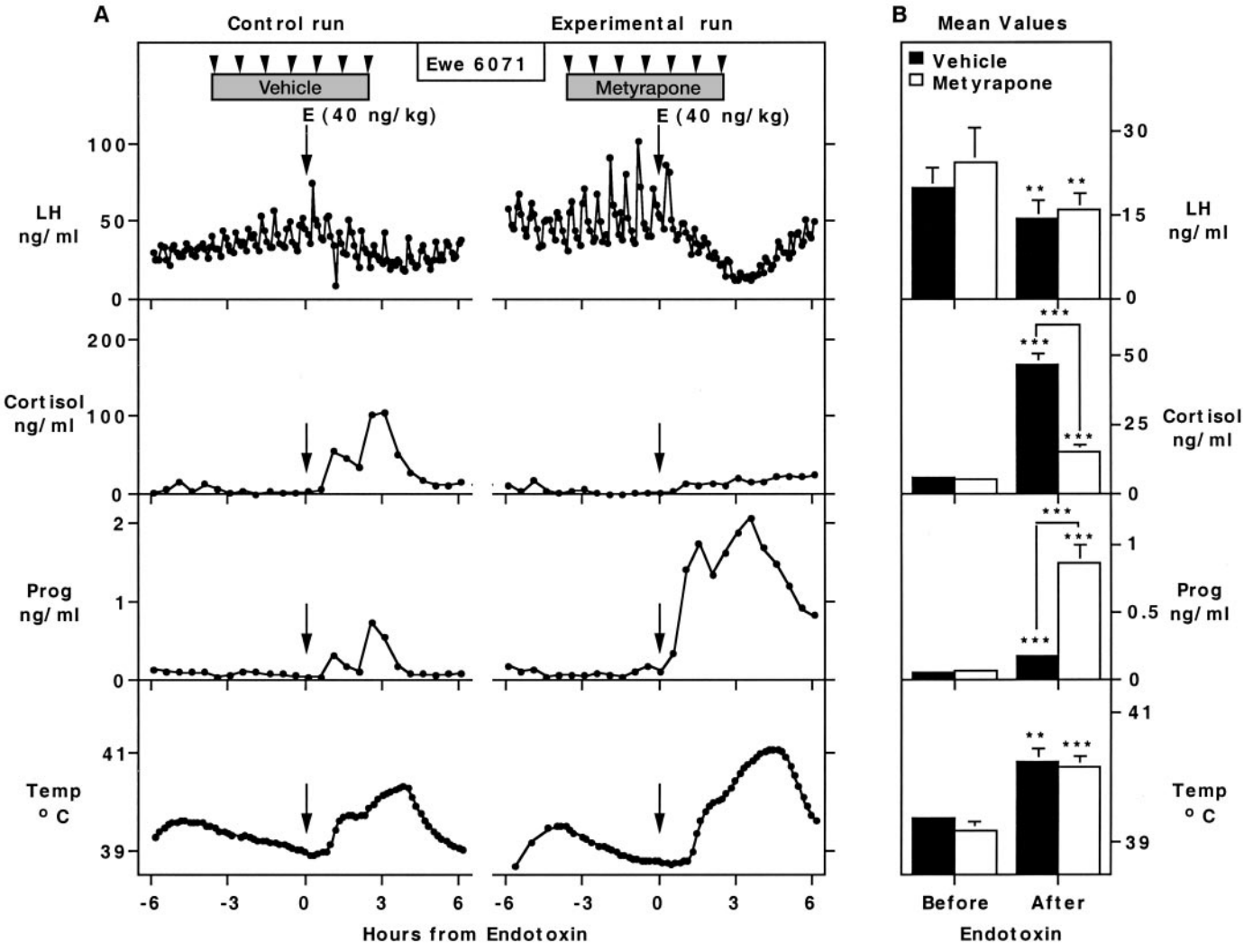

FIG. 4. A, Plasma LH, cortisol, progesterone (Prog), and core body temperature (Temp) in a representative ewe following vehicle or metyrapone treatment in conjunction with a low dose of endotoxin (E, 40 ng/kg) in experiment 4, part 1. Vehicle and metyrapone were administered according to a cross-over design. *Shaded horizontal bars with arrowheads at top* depict half-hourly injections of metyrapone or vehicle. B, Mean (± SEM; n = 6) plasma LH, cortisol, progesterone, and core body temperature before endotoxin (−6 to −1 h for LH, progesterone and temperature, or −6 to −3 h for cortisol) and after endotoxin (1–6 h) in ewes pretreated with vehicle (*closed bars*) or metyrapone (*open bars*) in conjunction with a low-dose endotoxin. Significant differences before and after endotoxin, or between vehicle and metyrapone-treated ewes, are denoted by the *asterisks* (**, $P < 0.01$; ***, $P < 0.001$).

TABLE 4. Effects of metyrapone or vehicle on LH secretion following the 40-ng/kg dose of endotoxin

	Mean (LH: ng/ml; GnRH: pg/min)		Total output (frequency × amplitude)		Pulse frequency (pulses/5 h)		Pulse amplitude (LH: ng/ml; GnRH: pg/min)	
	Before[a]	After[b]	Before	After	Before	After	Before	After
Experiment 4, part 1								
LH: Vehicle + endotoxin	20.0 ± 3.5	14.4 ± 3.3[c]	84.4 ± 15.3	60.5 ± 25.5[c]	8.7 ± 0.7	7.5 ± 1.0	9.7 ± 1.6	7.0 ± 2.3[c]
LH: Metyrapone + endotoxin	24.7 ± 5.9	15.9 ± 2.9[c]	123.3 ± 41.7	51.4 ± 13.4[c]	7.8 ± 0.6	6.5 ± 1.0	14.8 ± 4.7	7.6 ± 1.5[c]
Experiment 4, part 2								
GnRH: Endotoxin alone	1.3 ± 0.4	1.1 ± 0.4	32.7 ± 10.6	26.5 ± 10.8	8.5 ± 0.6	11.0 ± 0.7	4.4 ± 1.7	2.3 ± 0.8
LH: Endotoxin alone	27.4 ± 7.9	19.3 ± 5.7[c]	97.4 ± 35.5	37.7 ± 12.2[c]	8.3 ± 0.5	6.0 ± 0.7	12.1 ± 4.5	6.4 ± 1.8

[a] Before, mean (± SEM) values −6 to −1 h from endotoxin.
[b] After, mean (± SEM) values +1 to +6 h from endotoxin.
[c] Significant time effect (before *vs.* after endotoxin, see text for *P* values).

Further, metyrapone enhanced endotoxin-induced progesterone secretion ($P \leq 0.001$) but did not affect induction of fever (data not shown).

The high dose of endotoxin alone inhibited pulsatile GnRH and LH secretion (Fig. 6). Statistical analysis indicated that endotoxin reduced mean GnRH/LH values ($P \leq 0.05$),

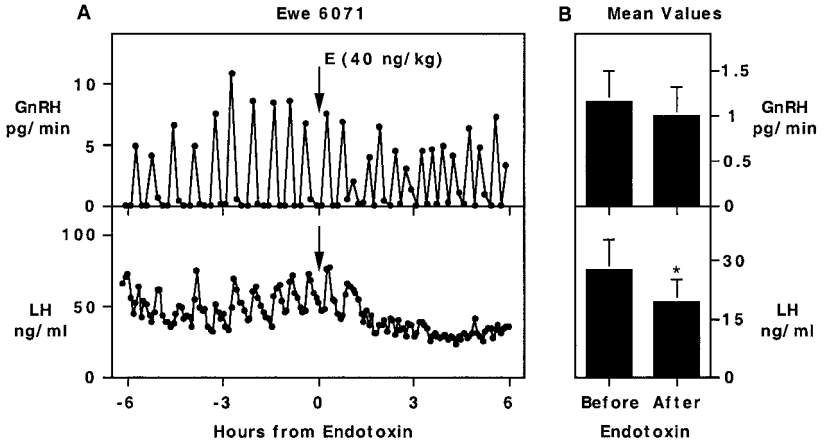

FIG. 5. A, Pituitary portal GnRH and peripheral plasma LH profiles in the same ewe as in Fig. 4 following a low dose of endotoxin (E, 40 ng/kg) in experiment 4, part 2. B, Mean (± SEM; n = 4) pituitary portal GnRH collection rate and circulating LH concentrations before (−6 to −1 h) and after (1–6 h) a low dose of endotoxin. Significant difference before *vs.* after endotoxin is denoted by the *asterisk* (*, $P < 0.05$).

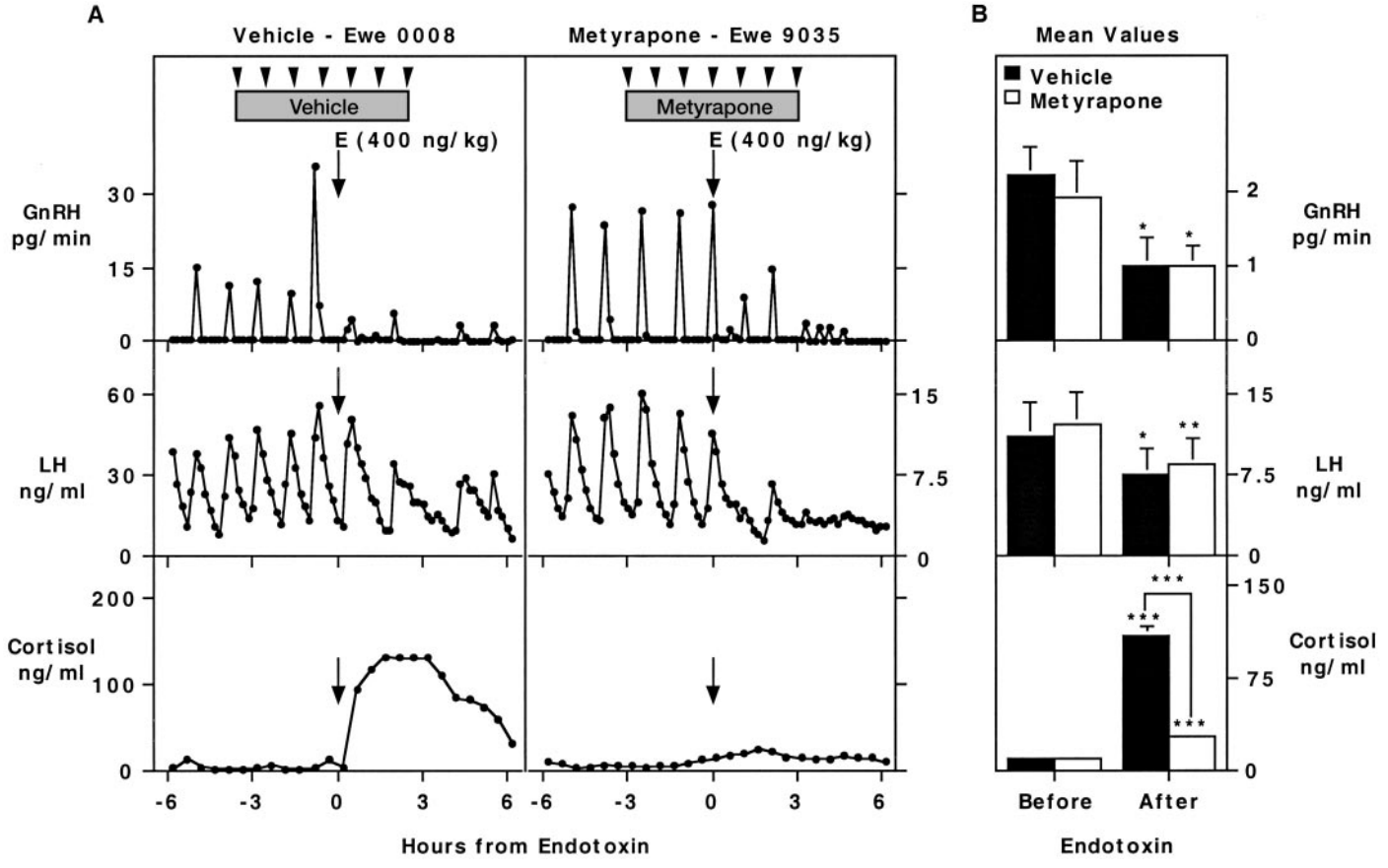

FIG. 6. A, Pituitary portal GnRH and peripheral plasma LH and cortisol profiles in one representative ewe following vehicle or metyrapone in conjunction with a high dose endotoxin (E, 400 ng/kg) in experiment 5 (not a cross-over design). *Shaded horizontal bars with arrowheads at top* depict half-hourly injections of metyrapone or vehicle. B, Mean (± SEM; n = 6) GnRH collection rate in pituitary portal blood and LH and cortisol concentrations in peripheral plasma before endotoxin (−6 to −1 h for LH and GnRH, or −6 to −3 h for cortisol) and after endotoxin (1 to 6 h) in ewes pretreated with vehicle (*closed bars*) or metyrapone (*open bars*) in conjunction with a high dose endotoxin. Significant differences before and after endotoxin, or between vehicle and metyrapone-treated ewes, are denoted by the *asterisks* (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

total GnRH/LH pulsatile output ($P \leq 0.01$), and GnRH/LH pulse amplitude ($P \leq 0.001$) Table 5). None of these suppressive effects was altered by metyrapone. Further, in both groups, endotoxin disrupted the highly regular rhythm of GnRH pulses typical of ovariectomized ewes. Following endotoxin, GnRH pulses sometimes occurred as brief volleys of very high frequency and low amplitude followed by periods devoid of pulses (*e.g.* no. 9035; Fig. 6). Overall, this resulted in stimulation of GnRH pulse frequency in both groups ($P \leq 0.01$, Table 5). Metyrapone by itself did not alter GnRH or LH pulses before endotoxin.

Finally, it should be noted that the nature of the sup-

TABLE 5. Effects of metyrapone or vehicle on GnRH and LH pulse parameters following the 400-ng/kg dose of endotoxin

	Mean (LH: ng/ml; GnRH: pg/min)		Total output (frequency × amplitude)		Pulse frequency (pulses/5 h)		Pulse amplitude (LH: ng/ml; GnRH: pg/min)	
	Before[a]	After[b]	Before	After	Before	After	Before	After
Experiment 5 GnRH								
Vehicle + endotoxin	2.2 ± 0.4	1.0 ± 0.4[c]	45.3 ± 13.6	18.9 ± 9.4[c]	4.7 ± 0.6	6.5 ± 1.0[c]	11.3 ± 2.1	3.3 ± 1[c]
Metyrapone + endotoxin	1.9 ± 0.5	1.0 ± 0.3[c]	44.7 ± 13.3	23.6 ± 8.1[c]	5.2 ± 0.6	7.2 ± 0.5[c]	10.5 ± 3.9	3.2 ± 1.2[c]
Experiment 5 LH								
Vehicle + endotoxin	11.0 ± 3.2	7.5 ± 2.3[c]	84.1 ± 29.5	23.1 ± 8.2[c]	4.5 ± 0.5	4.7 ± 0.7	17.1 ± 4.4	5.9 ± 2.9[c]
Metyrapone + endotoxin	12.2 ± 3.0	8.4 ± 2.4[c]	70.1 ± 19.4	12.9 ± 6.3[c]	5.0 ± 0.6	3.8 ± 0.5	13.2 ± 2.2	3.8 ± 1[c]

[a] Before, mean (± SEM) values −6 to −1 h from endotoxin.
[b] After, mean (± SEM) values +1 to +6 h from endotoxin.
[c] Significant time effect (before *vs.* after endotoxin, see text for *P* values).

pressive effect of endotoxin on LH pulses in this experiment (decreased amplitude), which was performed during the anestrous season, differed from that in experiment 2 (decreased frequency), which was performed in the breeding season. Nevertheless, the primary result in terms of the objective of this study was the same in both seasons: metyrapone did not reverse the inhibitory effect of endotoxin on pulsatile LH secretion.

Discussion

Despite extensive investigation, there continues to be a debate as to whether glucocorticoids play a role in suppressing reproductive neuroendocrine function in response to stress. Numerous studies suggest an increase in circulating glucocorticoids can inhibit gonadotropin secretion. In the orchidectomized rhesus monkey, for example, exogenous cortisol inhibits LH and FSH secretion, but the response requires 3–4 wk to become evident (37). In the male rat, glucocorticoids suppress the postcastration rise in circulating LH, at least in part by reducing pituitary responsiveness to GnRH (38). In gonadectomized sheep, iv infusion of cortisol suppresses LH secretion, inhibits responsiveness to GnRH and reduces GnRH-receptor gene expression in the pituitary gland (20–22, 39). Other studies, however, suggest glucocorticoids are not the predominant mediators of stress-induced suppression of gonadotropin secretion (see recent review by Tilbrook *et al.,* Ref. 40). Nevertheless, as emphasized in that review, the divergent findings may reflect different experimental conditions: chronic *vs.* acute exposure to elevated glucocorticoids, natural *vs.* synthetic glucocorticoids, presence *vs.* absence of gonadal steroids, and the sex and species of the experimental subject. Further, as pointed out in that review, different types of stressful situations most likely recruit different pathways that inhibit gonadotropin secretion and only some of these may involve cortisol, a point we return to later in this *Discussion*.

In the present study, we used endotoxin as a model of immune/inflammatory stress to test the hypothesis that enhanced secretion of cortisol mediates stress-induced suppression of pulsatile GnRH and LH secretion in the ovariectomized ewe. Prior work in ewes indicates a high dose of endotoxin (*e.g.* 400 ng/kg) inhibits reproductive neuroendocrine activity at both the hypothalamus and pituitary gland, whereas endotoxin at a low doses (*e.g.* 40 ng/kg) inhibits LH pulses without altering GnRH release, suggesting some of its effects are exerted at the pituitary (12, 15). The present study confirmed these observations. Here, we used both high and low doses of endotoxin and two complementary experimental approaches to test for a role of cortisol. One approach was to mimic the cortisol response to each of the two doses of endotoxin, but in the absence of endotoxin. The other approach was to block the cortisol rise in the face of endotoxin challenge (both doses) and determine if suppression of GnRH/LH pulses could be reversed.

In experiment 1, we delivered two doses of cortisol in the absence of endotoxin, producing plasma cortisol increments that approximated those induced by endotoxin. Both increments in circulating cortisol unambiguously suppressed pulsatile LH secretion. The smaller increment, which approximated the cortisol response to a low dose of endotoxin (40 ng/kg), suppressed the mean plasma LH concentration by 28%, which was equivalent to the 28% reduction produced by the low dose of endotoxin. The larger cortisol increment approximated the cortisol response to a higher dose of endotoxin (400 ng/kg) and caused greater suppression in the mean serum LH concentration (45%), which was comparable to that produced by the higher dose of endotoxin (40%). It should be noted that this experiment was conducted during the anestrous season when responsiveness to ovarian steroid negative feedback is greater than during the breeding season (35). Although a systematic test for a seasonal difference in responsiveness to cortisol has not yet been performed, we have found that both increments in plasma cortisol tested in experiment 1 inhibit LH pulses during the breeding season (Breen, K. M., and F. J. Karsch, unpublished observations).

The above findings clearly indicate that an acute increase in circulating cortisol itself is sufficient to inhibit pulsatile LH secretion, and that this may contribute to endotoxin-induced suppression of reproductive neuroendocrine activity in the ovariectomized ewe. To our knowledge, this is the first report that a stress-like level of cortisol can inhibit pulsatile LH secretion acutely, with suppression becoming evident within 1–2 h (see Fig. 1). Further, from the effects on LH pulse frequency and amplitude, it is tempting to speculate that cortisol, like endotoxin, might exert a dose-dependent inhibition at both the hypothalamic and pituitary levels. Although further experiments are needed, using preparations in which GnRH secretion and pituitary responsiveness are assessed directly, this possibility is consistent with interpre-

tations of prior studies in sheep and other species, including rodents and primates (20, 21, 37, 38).

Our second approach for testing a mediatory role of cortisol was to inhibit the cortisol response in the presence of an endotoxin challenge. For this purpose, we used metyrapone to block the 11-β hydroxylase enzyme that catalyzes conversion of 11-desoxycortisol to cortisol. Suppression of the increase in cortisol failed to prevent or even attenuate the inhibitory effects of endotoxin on any aspect of LH pulsatility (experiment 2). Follow-up experiments suggested enhanced secretion of cortisol is not needed for endotoxin to suppress either hypothalamic GnRH secretion or pituitary responsiveness to GnRH (experiments 4 and 5). It should be pointed out, however, that endotoxin-induced cortisol synthesis was not totally eliminated by metyrapone. For example, in experiment 2, the mean serum cortisol concentration increased from a basal value of 6–16 ng/ml following endotoxin in ewes treated with metyrapone. Although far less than the endotoxin-induced increment in circulating cortisol in the absence of metyrapone, which often exceeds 100 ng/ml, it is possible that this small rise in cortisol was sufficient to suppress GnRH and LH pulses. In a recent dose-response study, however, we observed that a rise in plasma cortisol to 16 ng/ml is below the threshold needed to suppress LH pulsatility in the ovariectomized ewe (Breen, K. M., and F. J. Karsch, unpublished observations). Our findings, therefore, provide strong evidence that increased cortisol secretion is not essential for endotoxin to suppress reproductive neuroendocrine activity.

Of interest, metyrapone enhanced the endotoxin-induced secretion of biosynthetic precursors for cortisol, progesterone, and 11-desoxycortisol. This probably reflected a spilling over of these intermediates into the circulation as they began to accumulate in the adrenal cortex consequent to the blockade of 11-β hydroxylase. It is unlikely, however, that these intermediates could account for suppressed GnRH or LH pulsatility in the absence of a typical cortisol response. The precursor 11-desoxycortisol is not considered to be biologically active (41) and the rise in circulating progesterone, in itself, was not of sufficient magnitude or duration to suppress LH pulses (experiment 3). Although it remains possible that heightened secretion of other precursors suppressed gonadotropin secretion in metyrapone-treated ewes, the more likely explanation is that adrenal steroids are not obligatory mediators of endotoxin-induced suppression of GnRH and LH pulses.

Collectively, our findings lead to the conclusion that enhanced secretion of cortisol is sufficient to suppress pulsatile LH secretion but this is not necessary for suppressive effects of endotoxin on reproductive neuroendocrine function in the ovariectomized ewe. It is important to integrate these seemingly paradoxical findings into a unified model to explain how endotoxin suppresses GnRH and LH secretion and, in particular, the role played by cortisol. One attractive model is that multiple inhibitory pathways are called into play by this immune/inflammatory stress and, in the absence of any one pathway, others effectively suppress gonadotropin secretion. In this regard, endotoxin elicits widespread pathophysiological responses and homeostatic adaptations (7). Other responses of neuroendocrine significance are enhanced production of proinflammatory cytokines, endogenous opioids, prostaglandins, and other intermediates, all of which have been implicated in the endotoxin-induced suppression of reproductive neuroendocrine function (for reviews see Refs. 2, 6, and 42). It seems likely, therefore, that these mediators are sufficient to inhibit gonadotropin secretion during immune/inflammatory stress. A cortisol-mediated pathway may ensure reproductive suppression during immune stress and, in addition, participate in gonadotropin suppression during any type of stressful encounter that activates the hypothalamo-pituitary-adrenal axis. These interpretations fit nicely with the concept that different types of stress activate different pathways to suppress reproductive neuroendocrine function (see review by Tilbrook *et al.*, Ref. 40).

In considering this interpretation, however, it is important to keep in mind that our studies were conducted in ovariectomized ewes. Others have shown that the inhibitory effects of cortisol on LH secretion are intensified by estradiol (21, 22, 39). Thus, in the presence of the ovaries, cortisol may play a more dominant role in endotoxin-induced suppression of reproductive neuroendocrine function. Another possibility is that cortisol-induced inhibition of LH may be blocked during an immune challenge. In this regard, endotoxin, acting via cytokines, may reduce glucocorticoid binding or the number of available binding sites in cells that mediate cortisol inhibition of LH secretion (43, 44).

Finally, beyond the issue of whether cortisol contributes to endotoxin-induced suppression of gonadotropin secretion, it is important to place our findings into a broader physiological context by considering their relevance to the disruptive effects of stress on the estrous cycle. Of considerable interest is the recent finding that infusion of cortisol, which produced a serum cortisol increment similar to that achieved by our low dose of cortisol, interrupted the follicular phase rise in estradiol, blocked the preovulatory LH surge and disrupted the estrous cycle of the ewe (45). In sheep, as in other species, high frequency LH pulses are needed to stimulate the follicular phase increase in estradiol secretion (46). Thus, it will be of keen interest to determine if the suppressive effects of cortisol on pulsatile LH secretion, as observed in the present study, contributes to stress-induced disruption of ovarian cyclicity.

Acknowledgments

The authors are indebted to Messrs. Douglas D. Doop and Gary McCalla for their care of the animals and assistance with animal experimentation. We also thank Dr. Vasantha Padmanabhan for her assistance with this study, Dr. Morton Brown for advice with the statistical analyses, Drs. Gordon D. Niswender and Leo E. Reichert, Jr. for supplying RIA reagents, and Dr. Johannes D. Veldhuis for providing the Cluster pulse detection algorithm.

Received March 11, 2002. Accepted June 4, 2002.

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Preliminary reports have appeared in *Biology of Reproduction* (Vol. 62, Supplement 1, 2000 Abstract 550) and *Stress* (vol. 5:101–112, 2002).

This work was supported by NIH-HD-30773 and the Office of the Vice President for Research at the University of Michigan. N.B. was sup-

ported by la Fondation pour la Recherche Médicale (SE000738-01) (France).

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