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Does Cortisol Acting Via the Type II Glucocorticoid Receptor Mediate Suppression of Pulsatile Luteinizing Hormone Secretion in Response to Psychosocial Stress?

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This study assessed the importance of cortisol in mediating inhibition of pulsatile LH secretion in sheep exposed to a psychosocial stress. First, we developed an acute psychosocial stress model that involves sequential layering of novel stressors over 3–4 h. This layered-stress paradigm robustly activated the hypothalamic-pituitary-adrenal axis and unambiguously inhibited pulsatile LH secretion. We next used this paradigm to test the hypothesis that cortisol, acting via the type II glucocorticoid receptor (GR), mediates stress-induced suppression of pulsatile LH secretion. Our approach was to determine whether an antagonist of the type II GR (RU486) reverses inhibition of LH pulsatility in response to the layered stress. We used two animal models to assess different aspects of LH pulse regulation. With the first model (ovariectomized ewe), LH pulse characteristics could vary as a function of both altered GnRH pulses and pituitary responsiveness to GnRH. In this case, antagonism of the type II GR did not prevent stress-induced inhibition of pulsatile LH secretion. With the second model (pituitary-clamped ovariectomized ewe), pulsatile GnRH input to the pituitary was fixed to enable assessment of stress effects specifically at the pituitary level. In this case, the layered stress inhibited pituitary responsiveness to GnRH and antagonism of the type II GR reversed the effect. Collectively, these findings indicate acute psychosocial stress inhibits pulsatile LH secretion, at least in part, by reducing pituitary responsiveness to GnRH. Cortisol, acting via the type II GR, is an obligatory mediator of this effect. However, under conditions in which GnRH input to the pituitary is not clamped, antagonism of the type II GR does not prevent stress-induced inhibition of LH pulsatility, implicating an additional pathway of suppression that is independent of cortisol acting via this receptor. (*Endocrinology* 148: 1882–1890, 2007)

ALTHOUGH STRESS CAN disrupt gonadotropin secretion and ovarian cyclicity, the mechanisms and mediators of this suppression remain unclear (1–4). Of interest, stress-induced impairment of reproductive function is typically associated with activation of the hypothalamic-pituitary adrenal (HPA) axis, resulting in a concurrent rise in circulating glucocorticoids. This has led to the hypothesis that enhanced glucocorticoid secretion is relevant to reproductive suppression during stress. However, there is no conclusive evidence that glucocorticoids are essential mediators of stress-induced suppression of reproductive activity. On the one hand, exogenous glucocorticoids are sufficient to inhibit gonadotropin secretion (5–10). On the other hand, merely showing that glucocorticoids are sufficient does not establish a mediatory role. It must also be determined that enhanced glucocorticoid secretion is necessary to inhibit gonadotropin secretion. This can be achieved by demonstrating that blocking glucocorticoid secretion or action alleviates reproductive neuroendocrine suppression during stress.

Only a handful of studies have used the latter approach to test the necessity of glucocorticoids for stress-induced inhibition of LH secretion, and these yielded conflicting results. For instance, antagonism of the type II glucocorticoid receptor (GR) alleviated LH suppression during restraint stress in male rats (11). Interpretation of that finding, however, is compromised because the antagonist was found to stimulate LH secretion in the absence of stress (12). In contrast to those findings, blockade of enhanced glucocorticoid secretion during an immune/inflammatory stress failed to reverse suppression of pulsatile GnRH and LH secretion in sheep and rats (13, 14). Clearly, the necessity of enhanced glucocorticoid secretion for stress-induced suppression of gonadotropin secretion has not been convincingly demonstrated in any species and remains an important, open question in the area of stress biology.

The present study addressed the requirement of cortisol in mediating reproductive neuroendocrine suppression in sheep exposed to an acute psychosocial stress. Isolation is a type of psychosocial challenge that has been used extensively to study neuroendocrine effects of stress in sheep (15–17). Our initial work with isolation (unpublished), however, yielded inconsistent results with respect to activation of the HPA axis and inhibition of pulsatile LH secretion. Responses were sometimes very brief (1–2 h) or nondetectable. Therefore, our first goal here was to develop a robust paradigm of psychosocial stress that reliably stimulates cortisol and suppresses LH pulses. This paradigm, hereafter referred to as the layered-stress, consists of sequential, hourly application of

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Abbreviations: CV, Coefficients of variation; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary adrenal; PR, progesterone receptor.

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individual stressors: social isolation, restraint, blindfolding to remove visual awareness, exposure to predatory cues. Once the layered-stress paradigm was established, we used it in conjunction with two distinct animal models to test the hypothesis that glucocorticoids, acting via the type II GR, are necessary for stress-induced suppression of pulsatile LH secretion. First, we determined whether the suppression of LH pulses in ovariectomized ewes is prevented by an antagonist of the type II GR. Second, we used a pituitary-clamp model in which pulsatile GnRH input to the pituitary is fixed to determine whether the psychosocial stress inhibits pituitary responsiveness to GnRH and whether antagonism of cortisol action via the type II GR reverses this effect.

Materials and Methods

Experiments were conducted on mature Suffolk ewes maintained under standard husbandry conditions at the Sheep Research Facility near Ann Arbor, MI. The ewes were fed hay and alfalfa pellets and had free access to water and mineral licks. In all experiments, the ewes had been ovariectomized aseptically and under general anesthesia at least 4 months before use. Five days before the experiment, ewes were moved indoors, in groups of three to six. One day before sampling, ewes received a jugular cathether for blood collection. All procedures were approved by the Committee for the Use and Care of Animals at the University of Michigan.

Model of psychosocial stress

A layered-stress paradigm was developed (Fig. 1) to generate reliable responses from both the HPA and reproductive neuroendocrine axes. This paradigm commenced with isolation in a novel room that prohibited visual exposure to other sheep (first hour). After an hour, restraint in a 0.5 × 1.0 m pen was added (second hour). Beginning in the third hour, ewes were blindfolded. After the initial experiments, the layered-stress paradigm was lengthened by an hour to include exposure to olfactory and auditory cues of a predator (dog urine and compact disc recording of a barking dog) during the fourth hour. Previous studies indicate predatory cues stimulate the HPA axis of sheep without the need for physical contact with a predator (18–20). Furthermore, we have found that each individual stress within the layered-stress paradigm enhances cortisol secretion when applied alone, but responses are inconsistent, variable in magnitude, and often last only 1–2 h (McCrum, C., E. Wagenmaker, and F. Karsch; unpublished).

To verify efficacy of the layered-stress paradigm, nine ovariectomized ewes (six in the breeding season and three in the nonbreeding season) were penned together during a 4-h control period when no stress was applied. Over the next 3 h, ewes were exposed to the layered stress (fourth hour with predator cues not used). Jugular blood was sampled at 12-min intervals in the nonbreeding season; the sampling interval was reduced to 6-min in the breeding season to account for the seasonal increase in LH pulse frequency (21).

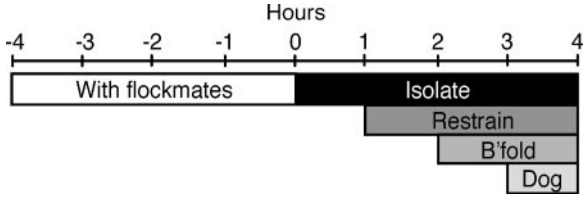

FIG. 1. Layered-stress paradigm. Time is depicted as hours relative to the onset of stress. Before stress, ewes are housed with flockmates. The stress period begins with isolation in a novel room. One hour later, the stress of restraint is added and, beginning in the third hour, ewes are blindfolded (B'fold). In later experiments (experiment 1, part 2; and experiment 2), a fourth hour of stress was added when ewes were exposed to predator cues (dog odor and sound of a barking dog).

Experiment 1: does a type II GR antagonist reverse stress-induced suppression of pulsatile LH secretion?

Our approach was to administer RU486 before exposure to the layered-stress paradigm. RU486 is a commonly used, nonspecific antagonist of both the type II GR and the progesterone receptor (PR) (22–24). In all experiments, RU486 was obtained from Sigma (St. Louis, MO), suspended in vehicle (equal parts sesame oil and 75% ethanol), and delivered by im injection at a dose of 10 mg/kg body weight. RU486 or its vehicle was administered at two time points, 12 and 24 h before initiation of stress. We previously determined that this dose of RU486 blocks cortisol-induced suppression of pulsatile LH secretion and that this reversal is attributed to the antagonism of the type II GR, not PR (25). This experiment was conducted in two parts during the nonbreeding season.

Part 1. Twelve ovariectomized ewes were allocated to two groups: stress plus RU486 or stress plus vehicle (n = 6/treatment). Jugular blood was collected for 7 h at 12-min intervals for measurement of LH and cortisol. Sampling commenced 8 h after the final injection of RU486 or vehicle. During the first 4 h of blood collection, animals were not stressed. For the next 3 h, ewes were exposed to the layered-stress paradigm as described in Fig. 1 (note, fourth hour with predatory cues not used in this experiment).

Part 2. Part 1 suggested that RU486 was unable to reverse the suppressive effects of stress on pulsatile LH secretion. Therefore, we repeated the experiment using a more powerful design to minimize the effect of variability among animals. In part 2, each ewe was sampled during both a no-stress run and a stress run, thus allowing each animal to serve as its own control. Also, beginning in this experiment, the layered-stress paradigm was lengthened to include exposure to predatory cues during the fourth hour (see section above, model of psychosocial stress). This 4-h paradigm was used in all remaining experiments.

Twenty-four ovariectomized ewes were allocated to a control group and an experimental group (n = 12/group). Ewes in both groups were monitored on two occasions, a no-stress run and a stress run conducted 2 d apart. The control group received no further treatment, whereas the experimental group received vehicle during the no-stress run and RU486 during the stress run. The no-stress run (±vehicle) was always conducted first and the stress run (±RU486) second, to avoid carryover effects of the treatments. Details of RU486 and vehicle administration are provided in part 1. For both runs, jugular blood was collected at 10-min intervals for 9 h. During the stress run, all ewes were exposed to the layered-stress paradigm for the latter 4 h of blood collection.

Experiment 2: does a type II GR antagonist reverse stress-induced inhibition of pituitary responsiveness to GnRH?

A recent study suggests that isolation/restraint stress inhibits pituitary responsiveness to GnRH pulses in gonadectomized sheep (26). Cortisol also inhibits pituitary responsiveness to GnRH pulses (10) and this action is mediated by the type II GR (25). Therefore, experiment 2 determined whether cortisol, acting via the type II GR, is necessary for stress-induced suppression of pituitary responsiveness to GnRH.

Pituitary-clamp model. This experiment used a pituitary-clamp model described previously (10, 27). Briefly, endogenous GnRH pulses were chronically blocked in ovariectomized ewes by constant delivery of a luteal phase level of estradiol during the nonbreeding season (28). Physiological GnRH boluses (5 ng/kg, iv, over 6 min; Sigma) were then delivered hourly via a timer-controlled peristaltic pump for 6 d to reactivate the gonadotrope and stabilize pituitary responsiveness to GnRH. This GnRH treatment produces GnRH pulses in pituitary portal blood having amplitudes approximately 2-fold greater than GnRH observed in portal blood of ovariectomized ewes during the nonbreeding season (27). The amplitude of LH pulses induced by the unvarying GnRH boluses in this model provides an index of pituitary responsiveness to GnRH.

Part 1. Eighteen ovariectomized ewes were randomly allocated to one of three groups (n = 6/group): stress plus RU486, stress plus vehicle, or no stress plus vehicle (control). The ewes were maintained in individual pens in a common room and received hourly GnRH boluses for 6 d to

stabilize gonadotrope responsiveness. RU486 or vehicle was then administered as in experiment 1. On the sixth day, jugular blood was collected at 12-min intervals for 9 h to assess LH pulse amplitude. For the first 5 h, no further treatment was applied. During the next 4 h, ewes in the stress plus vehicle and stress plus RU486 groups were relocated to novel isolation rooms and exposed to the 4-h layered-stress paradigm. Ewes in the no-stress control group remained in their home pens. Beginning 3 h before blood collection and continuing through the sampling period, ewes were disconnected from the peristaltic pump and the hourly boluses of GnRH were delivered manually (over 20 sec). This allowed pulsatile GnRH to be continued during the first hour of stress when ewes were isolated but not confined to pens.

Part 2. Part 1 demonstrated that RU486 prevented stress-induced suppression of pituitary responsiveness to GnRH. Part 2 was conducted to confirm these findings using a more powerful design in which each ewe serves as its own control (similar to experiment 1, part 2).

Twelve ovariectomized ewes were set up in the pituitary-clamp model and received GnRH boluses for 6 d to stabilize gonadotrope responsiveness. The ewes were then allocated to a control group or an experimental group (n = 6/treatment) and monitored during a no-stress run and a stress run. The experimental group received vehicle during the no-stress run (first run) and RU486 during the stress run (second run). The control group received neither RU486 nor vehicle during the no-stress and stress runs. Details of the RU486 and GnRH treatments, blood sampling, and stress procedure were the same as described for part 1 of this experiment. After the first run of the experiment, ewes were reconnected to the peristaltic pump for GnRH delivery until 3 h before the second run, conducted 2 d later.

Hormone assays

LH concentrations were determined in duplicate aliquots (25–100 μl) of plasma using a modification (29) of a previously described RIA (30, 31). Values are expressed in terms of NIH-LH-S12. The mean intra- and interassay coefficients of variation (CV) were 4.2% and 5.2%, respectively, and assay sensitivity averaged 0.55 ng/ml (44 assays).

Total plasma cortisol concentrations were determined in duplicate 50-μl aliquots of unextracted plasma using the Coat-A-Count cortisol assay kit (Diagnostic Products Corp., Los Angeles, CA), validated for use in sheep (32). Mean intra- and interassay CV were 7.8% and 8.0%, respectively (36 assays). Assay sensitivity averaged 0.57 ng/ml.

Progesterone was assayed in duplicate 100-μl aliquots of unextracted plasma using the Coat-A-Count progesterone assay kit (Diagnostic Products Corp.), validated for use in sheep (33). Mean intra- and interassay CV were 5.1% and 2.8%, respectively (two assays). Assay sensitivity averaged 0.16 ng/ml.

Data analysis

LH pulses were identified (model development and experiment 1) using the Cluster pulse-detection algorithm (34). Cluster sizes for peaks and nadirs were set at either 1 or 2 and the t-statistic used to identify a significant increase and decrease was 2.6. LH pulse amplitude was defined as the difference between the peak value and its preceding nadir. Total pulsatile output was calculated as the product of pulse frequency (pulses/h) × mean pulse amplitude. For every ewe, average values for each LH parameter (mean LH concentration, average amplitude, frequency, total pulsatile output) were calculated across the prestress and stress periods or corresponding times when no stress was imposed. In experiment 2, amplitudes of the LH responses to the exogenous GnRH boluses were averaged across the prestress and stress period in each ewe as an index of pituitary responsiveness. Before statistical analysis, hormone concentrations were log transformed because SDs were directly proportional to the concentration, a relationship typical for the measurement of circulating hormones. To adjust for heterogeneity of variance, pulse frequencies were square root transformed.

Analyses were then conducted to address two objectives. First, differences in LH pulse parameters between the prestress and stress periods were analyzed by ANOVA to determine effects of the layered-stress paradigm. Second, ratios of the stress to prestress values were calculated in each ewe for all LH pulse parameters and analyzed to determine whether stress effects differed in the presence and absence of RU486 (stress × RU486 interaction). In part 1 of experiments 1 and 2, which involved sampling during only one run, ratios were analyzed by ANOVA. In part 2 of experiments 1 and 2, which involved sampling during two runs (stress run and no-stress run), ratios were analyzed across runs by repeated measures ANOVA.

Mean cortisol concentrations were calculated for the prestress and stress period. The peak concentration was identified as the greatest value during the stress period. Mean values during the prestress and stress periods, and peak values were log transformed and analyzed by *t* test to identify differences in the presence and absence of RU486. Significance level was set at $P < 0.05$.

Results

Model of psychosocial stress

Figure 2 illustrates profiles of cortisol and LH in two representative ewes exposed to the layered-stress paradigm (ewe 1054 during the nonbreeding season and 1049 during the breeding season). In all nine ewes, this stress elicited robust activation of the HPA axis and unambiguous suppression of pulsatile LH secretion. Mean (±SEM) plasma cortisol concentrations generally remained low and stable before stress and were elevated to maximal values >10-fold above baseline within 2 h after stress onset (prestress *vs.* stress mean ± SEM, 5.9 ± 2.2 *vs.* 78.8 ± 6.9 ng/ml). Coinciding with enhanced secretion of cortisol was suppression of pulsatile LH secretion. Statistical analysis revealed a reduction of mean LH concentration, LH pulse amplitude and total pulsatile output (prestress *vs.* stress mean ± SEM: mean LH, 19.1 ± 3.0 *vs.* 13.8 ± 2.0 ng/ml, $P < 0.001$; LH pulse amplitude, 16.4 ± 3.6 *vs.* 7.1 ± 1.6 ng/ml, $P < 0.05$; total pulsatile output, 16.0 ± 3.0 *vs.* 5.6 ± 1.2 ng/ml, $P < 0.005$). In addition, there was a trend for an inhibition of LH pulse frequency (prestress *vs.* stress, 1.14 ± 0.13 *vs.* 0.89 ± 0.17 pulses/h; $P = 0.07$).

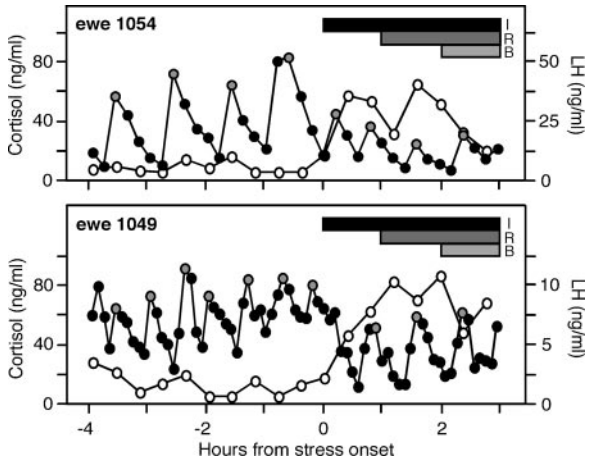

FIG. 2. Profiles of circulating LH (*black circles*) and cortisol (*open circles*) in two representative ovariectomized ewes exposed to the layered-stress paradigm during the nonbreeding season (ewe 1054) or breeding season (ewe 1049). The layered-stress paradigm is depicted at the *top* of each panel and consisted of isolation (I, 0 h), followed by restraint (R, 1 h) and blindfold (B, 2 h). Predator cues were not employed in this experiment. Peaks of LH pulses are identified by *gray circles*.

TABLE 1. Effects of stress and RU486 on plasma cortisol concentrations[a]

Cortisol	Vehicle or No RU486	RU486
A. Prestress cortisol (ng/ml)		
Experiment 1		
Part 1 (3-h stress paradigm)	6.2 ± 1.1	10.8 ± 4.3
Part 2 (stress run only)	9.6 ± 1.5	12.8 ± 2.9
Experiment 2		
Part 1	6.1 ± 1.2	8.7 ± 5.8
Part 2 (stress run only)	8.9 ± 1.3	9.4 ± 1.3
B. Stress cortisol (ng/ml)		
Experiment 1		
Part 1 (3-h stress paradigm)	43.5 ± 6.7	48.0 ± 6.1
Part 2 (stress run only)	37.3 ± 4.9[c]	49.7 ± 6.7[c]
Experiment 2		
Part 1	31.9 ± 7.5[b]	50.0 ± 5.3[b]
Part 2 (stress run only)	37.8 ± 8.9[b]	69.6 ± 8.9[b]

[a] All values are mean ± SEM of the average value for each ewe during the prestress and stress periods.
[b] $P < 0.05$; significant increase in cortisol values in the presence of RU486.
[c] $P < 0.07$; trend for a significant increase in cortisol values in the presence of RU486.

Experiment 1: does a type II GR antagonist reverse stress-induced inhibition of pulsatile LH secretion?

Part 1. Again, the layered-stress paradigm increased plasma concentrations of cortisol in all ewes, reaching maximal levels 1–2 h after stress onset (53.8 ± 5.6 ng/ml; data not shown). Neither prestress values (Table 1A) nor the mean plasma cortisol concentrations during stress (Table 1B) were significantly affected by RU486.

In ewes treated with vehicle, the layered stress suppressed mean LH concentration by 12% ($P < 0.05$), LH pulse amplitude by 42% ($P < 0.05$) and total pulsatile output by 34% ($P < 0.05$; Table 2). Frequency was not affected. RU486 did not lessen any of these suppressive effects of stress ($P > 0.1$). Furthermore, treatment with RU486 did not affect LH secretion during the 4-h prestress sampling period ($P > 0.1$; Table 2), indicating lack of an antagonist effect in the absence of stress.

Part 2. Profiles of cortisol and LH in a representative ewe from the control and experimental (RU486) groups are shown in Fig. 3. Plasma cortisol remained low during the no-stress run and before stress during the stress run and values did not significantly differ between groups (Table 1A and Fig. 3). Plasma cortisol concentrations reached maximal values 1–2 h after stress onset and tended to be increased by RU486 (44.7 ± 6.2 *vs.* 65.6 ± 10.5 ng/ml, control *vs.* RU486, $P < 0.07$). Mean cortisol values during the 4-h stress period also tended to be enhanced by RU486 ($P < 0.07$; and Table 1B).

The layered-stress paradigm markedly suppressed pulsatile LH secretion in control ewes (no RU486) compared with values during the no-stress run (run × time interaction, $P < 0.05$; Fig. 3A and Table 3, control group). Statistical analysis revealed a reduction in mean LH concentration by 18% ($P < 0.005$), LH pulse amplitude by 37% ($P < 0.001$), and total pulsatile output by 31% ($P < 0.01$). Frequency of LH pulses was not affected by stress ($P > 0.1$). As in part 1, RU486 did not reverse any of the suppressive effects of stress (group × run interaction, $P > 0.1$; Fig. 3B and Table 3, experimental group). Although the interaction was not significant statistically, it is noteworthy that the absolute reduction of mean LH values and LH pulse amplitude during the stress run in ewes treated with RU486 was less than half of that observed in control ewes not treated with the antagonist (5% *vs.* 18% reduction in mean LH; 18% *vs.* 37% reduction in amplitude; Table 3). LH pulse amplitude began to increase in some ewes near the end of the stress period, both in the presence and absence of prior treatment with RU486 (*e.g.* Fig. 3B).

Experiment 2: does a type II GR antagonist reverse stress-induced inhibition of pituitary responsiveness to GnRH?

Part 1. Figure 4 depicts LH and cortisol profiles in two ewes representative of each of the three treatment groups tested in the pituitary-clamp model: no stress plus vehicle control, stress plus vehicle, or stress plus RU486. Plasma cortisol levels generally remained low and stable during the prestress period and through the end of sampling in no-stress controls (overall mean: 6.8 ± 2.0 ng/ml; Fig. 4). As in experiment 1, RU486 did not significantly affect the basal plasma cortisol concentration (Table 1A). However, RU486 significantly enhanced both the maximal plasma cortisol concentration during stress (53.9 ± 7.2 ng/ml *vs.* 75.4 ± 5.6 ng/ml, vehicle *vs.* RU486, respectively; $P < 0.05$) and the overall mean value during the 4-h stress period ($P < 0.05$; Table 1B).

In each ewe, all GnRH boluses induced increases in circulating LH and no extraneous LH pulses were observed, indicating endogenous GnRH pulses were effectively abolished in the pituitary-clamp model. LH responses to the GnRH boluses were regular and did not vary systematically across the sampling period in no-stress control ewes (Fig. 4A and Table 4; Fig. 5A illustrates mean LH responses over time). Stress alone reduced LH pulse amplitude by approximately 35% on average ($P < 0.05$; Fig. 4B and Table 4), with maximal suppression of approximately 55% evident 1.5 h

TABLE 2. Effects of stress and RU486 on LH pulse parameters in experiment 1, part 1[a]

	Mean LH (ng/ml)		LH pulse amplitude (ng/ml)		LH pulse frequency (pulses/h)		Total pulsatile output (frequency × amplitude)	
Group[c]	Prestress	Stress	Prestress	Stress	Prestress	Stress	Prestress	Stress
Stress + Vehicle[d]	24.4 ± 2.3	21.5 ± 2.9[b]	14.7 ± 1.8	8.5 ± 2.5[b]	1.08 ± 0.11	1.06 ± 0.13	15.2 ± 1.2	10.1 ± 1.7[b]
Stress + RU486[d]	25.3 ± 3.1	22.4 ± 2.5[b]	11.9 ± 1.6	9.3 ± 1.7[b]	1.25 ± 0.16	0.78 ± 0.17[b]	16.7 ± 2.2	8.7 ± 2.3[b]

[a] All values are mean ± SEM (n = 6).
[b] Significant time effect (prestress *vs.* stress; $P < 0.05$).
[c] No significant group effects were detected, indicating no difference in the effect of stress in the presence and absence of RU486.
[d] Vehicle or RU486 was injected 12 and 24 h before the initiation of stress (*i.e.* before sampling in the prestress and stress periods).

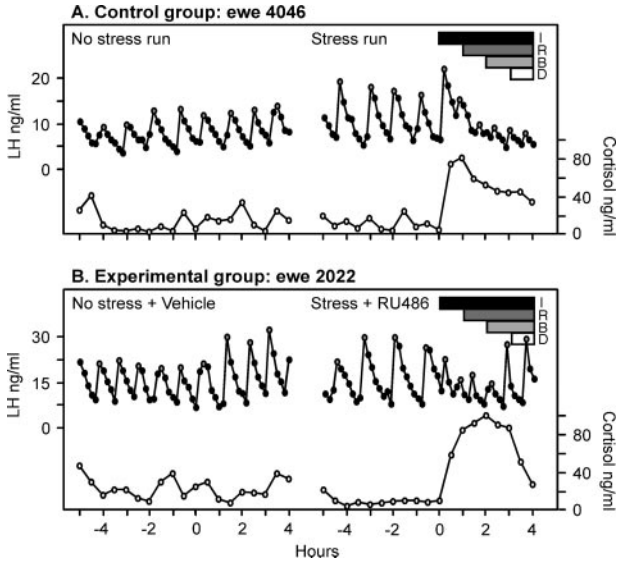

FIG. 3. Patterns of pulsatile LH (*black circles*) and cortisol (*open circles*) secretion during both the no-stress run and stress run in one representative ewe from the control (A) and experimental group (B, RU486) in experiment 1, part 2. The layered-stress paradigm is depicted at the *top* of the panel during the stress run and consisted of isolation (I, 0 h), followed by restraint (R, 1 h), blindfold (B, 2 h) and dog cues (D, 3 h). Peaks of LH pulses are identified by *gray circles*.

after the onset of stress (Fig. 5A). This demonstrates the layered-stress paradigm suppresses pituitary responsiveness to GnRH. In marked contrast, stress did not elicit a significant decrease in LH pulse amplitude in ewes treated with RU486 ($P > 0.1$; Fig. 4C and Table 4). The change in values during stress did not differ from that in no-stress controls ($P > 0.1$; Fig. 5A and Table 4), indicating the antagonist alleviated the suppressive effect of stress. Furthermore, RU486 did not affect LH pulse amplitude in the absence of stress, as indicated by values during the prestress period (Table 4).

Part 2. Profiles of cortisol and LH in a representative ewe from the control (stress alone) and experimental (stress plus RU486) groups are shown in Fig. 6. Plasma cortisol remained generally low and stable during the no-stress run and before stress, and prestress values were not significantly affected by RU486 (Fig. 6 and Table 1A). Cortisol reached maximal concentrations 2–3 h after the onset of stress, and again, mean values during the 4-h stress period were significantly enhanced by RU486 ($P < 0.05$; Table 1B).

In control ewes, the layered-stress paradigm reduced LH pulse amplitude by approximately 45% compared with values during the no-stress run, again indicating suppression of pituitary responsiveness to GnRH during stress (run × time interaction, $P < 0.01$; Fig. 6A and Table 5). RU486 reversed this effect. LH pulse amplitude was not significantly affected by stress in experimental ewes treated with RU486 before stress (run × time interaction, $P > 0.1$; Fig. 6B and Table 5). Furthermore, the statistical analysis revealed the effect of stress on LH pulse amplitude was significantly different in the presence *vs.* the absence of RU486 (group × run interaction, $P < 0.05$; Table 5). The average response to the hourly GnRH boluses illustrated that suppression of pituitary responsiveness was maintained throughout the 4-h stress period in control ewes and alleviated by antagonism of the type II GR in experimental ewes (Fig. 5B).

Plasma progesterone. Concentrations of plasma progesterone were determined in three samples obtained within 1 h before stress and in six samples obtained during the 3 h after stress onset in ewes that received vehicle or RU486 in experiment 2 (parts 1 and 2). Before stress, the plasma progesterone concentration was uniformly undetectable (<0.16 ng/ml). Mean plasma progesterone concentration during stress (all ewes) was 0.19 ± 0.01 ng/ml, with most values remaining undetectable.

Discussion

Despite extensive evidence that stress-like increments in glucocorticoids are sufficient to inhibit gonadotropin secretion (5–10), the necessity of glucocorticoids for suppression of reproductive neuroendocrine activity during stress had not been convincingly demonstrated in any species. Here we developed a paradigm of layered psychosocial stressors that robustly stimulates cortisol and unambiguously inhibits pulsatile LH secretion in the ovariectomized ewe. Using that paradigm, we determined this stress-induced suppression of LH secretion reflects, at least in part, a reduction of pituitary responsiveness to GnRH, a finding that reinforces the recent observation of Stackpole *et al.* (26). In addition, we demon-

TABLE 3. Effects of stress and RU486 on LH pulse parameters in experiment 1, part 2[a]

Group[c]	Mean LH (ng/ml)		LH pulse amplitude (ng/ml)		LH pulse frequency (pulses/h)		Total pulsatile output (frequency × amplitude)	
	Prestress	Stress	Prestress	Stress	Prestress	Stress	Prestress	Stress
Control group								
No-stress run	18.2 ± 2.6	17.5 ± 1.9[b]	12.5 ± 1.9	11.9 ± 1.6[b]	1.12 ± 0.06	1.10 ± 0.04	13.5 ± 1.9	12.8 ± 1.5[b]
Stress run	20.2 ± 2.6	16.5 ± 2.5[b]	15.6 ± 1.9	9.8 ± 2.0[b]	1.02 ± 0.06	1.08 ± 0.06	15.4 ± 1.6	10.7 ± 2.4[b]
Experimental group (RU486)[d]								
No-stress run	14.7 ± 1.3	16.1 ± 1.3[b]	12.1 ± 2.0	12.3 ± 1.7[b]	1.00 ± 0.06	1.10 ± 0.07	11.8 ± 1.5	13.0 ± 1.7[b]
Stress run	19.5 ± 1.4	18.5 ± 1.5[b]	17.6 ± 2.0	14.4 ± 2.9[b]	0.93 ± 0.08	1.04 ± 0.07	15.7 ± 1.6	13.1 ± 1.6[b]

[a] All values are mean ± SEM (n = 12) across the prestress and stress period or corresponding times during the no-stress run.
[b] Significant run × time interactions (prestress *vs.* stress; $P < 0.05$–0.005, see text for details) were detected within each group, indicating suppressive effects of stress in both the presence and absence of RU486.
[c] No significant group × run interactions were detected, demonstrating no significant difference in effect of stress in the presence and absence of RU486.
[d] RU486 was injected 12 and 24 h before the initiation of stress (*i.e.* before sampling in the prestress and stress periods).

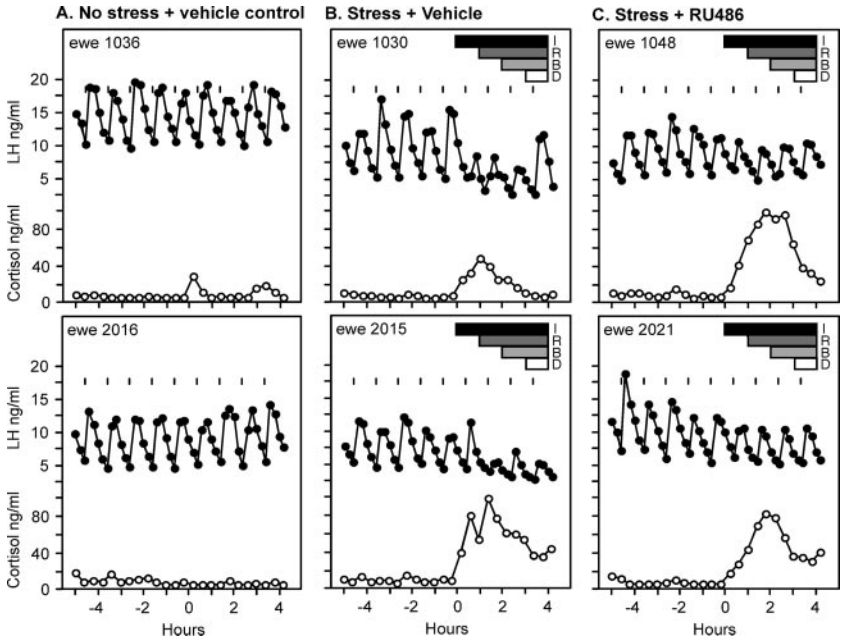

FIG. 4. LH and cortisol responses in two representative ewes from each of the three groups in experiment 2, part 1: no-stress vehicle control (A, *top and bottom panels*), stress plus vehicle (B, *top and bottom panels*), stress plus RU486 (C, *top and bottom panels*). The layered-stress paradigm is depicted at the *top* of the panel in ewes that were stressed, as described in Fig. 3. *Tick marks* indicate time of administration of hourly GnRH (5 ng/kg, iv) boluses to induce each LH response.

strated that antagonism of the type II GR prevented this stress-induced suppression of pituitary responsiveness in an animal model in which pulsatile GnRH input to the pituitary was held constant. As we observed previously (25), the antagonist alone did not affect LH secretion in the absence of stress. Collectively, these findings lead to the important conclusion that psychosocial stress inhibits pulsatile LH secretion, in part, by suppressing pituitary gland responsiveness to GnRH; cortisol, acting via the type II GR, is a necessary mediator of this effect.

Equally important, however, is our finding that antagonism of the type II GR did not unambiguously prevent stress-induced suppression of pulsatile LH secretion in ovariectomized ewes of experiment 1, when GnRH input to the pituitary was not clamped at a fixed rate. Two notable differences in the animal models of experiments 1 and 2 may account for these differing outcomes. One is the presence (experiment 2) *vs.* absence (experiment 1) of estradiol. Considering the growing literature that suggests gonadal steroids modify the disruptive effects of stress on reproductive neuroendocrine activity (4, 19, 35), perhaps the presence of estradiol in experiment 2 enhanced the efficacy of cortisol in mediating LH inhibition and enabled RU486 to reverse this suppression. The second major difference in animal models pertains to pulsatile GnRH input to the pituitary, which was held constant in experiment 2 but allowed to vary during stress in experiment 1. Substantial evidence implicates the hypothalamus as a target whereby stress inhibits LH secretion (1, 2, 4, 35), and it is possible that the layered-stress paradigm induces factors that inhibit pulsatile GnRH release as well as mediators (*e.g.* cortisol) that suppress pituitary responsiveness to GnRH. Based on this thinking, we are presently testing the hypothesis that the layered stress inhibits pulsatile GnRH secretion into pituitary portal blood and that this effect is independent of cortisol acting via the type II GR. Such a mechanism would accommodate the present findings as well as our recent observation that cortisol inhibits pulsatile LH secretion in ovariectomized ewes without reducing pulsatile GnRH secretion (10). Collectively, these considerations lead to our present conceptual model: psychosocial stress suppresses pulsatile LH secretion by influencing multiple neuroendocrine sites, with cortisol me-

TABLE 4. Effects of stress and RU486 on LH pulse amplitude in experiment 2, part 1[a]

Group[b]	LH pulse amplitude (ng/ml)	
	Prestress	Stress
No stress + vehicle control[d]	7.4 ± 1.7	8.4 ± 2.4
Stress + vehicle[d]	10.1 ± 1.8	6.7 ± 1.2[c]
Stress + RU486[d]	8.8 ± 1.3	8.0 ± 1.5

[a] All values are mean ± SEM (n = 6) across the prestress and stress period or corresponding times in the no-stress group.
[b] Significant group effect ($P < 0.05$) was detected.
[c] Significant time effect (prestress *vs.* stress; $P < 0.05$).
[d] Vehicle or RU486 was injected 12 and 24 h before the initiation of stress (*i.e.* before sampling in the prestress and stress periods).

TABLE 5. Effects of stress and RU486 on LH pulse amplitude in experiment 2, part 2[a]

Group[c]	LH pulse amplitude (ng/ml)	
	Prestress	Stress
Control group		
No-stress run	5.2 ± 1.2	5.3 ± 1.1[b]
Stress run	7.0 ± 2.0	3.9 ± 1.1[b]
Experimental group (RU486)[d]		
No-stress run	6.0 ± 0.8	5.4 ± 0.9
Stress run	11.6 ± 1.5	9.1 ± 1.4

[a] All values are mean ± SEM (n = 6) across the prestress and stress period or corresponding times during the no-stress run.
[b] Significant run × time interaction (prestress *vs.* stress; $P < 0.01$) was only detected in the control group.
[c] Significant group × run interaction ($P < 0.05$) was detected, indicating the effect of stress differed in the presence and absence of RU486.
[d] RU486 was injected 12 and 24 h before the initiation of stress (*i.e.* before sampling in the prestress and stress periods).

diating inhibition only at the pituitary level in ovariectomized ewes.

It is important to consider an alternative interpretation to explain the different outcomes of experiments 1 and 2. Specifically, the estradiol treatment used to block endogenous GnRH pulses in experiment 2 might have enhanced expression of PR, enabling stress-induced progesterone secretion to inhibit LH pulse amplitude. In this case, the efficacy of RU486 in reversing inhibition of pituitary responsiveness to GnRH might reflect antagonism of progesterone action via PR (or possibly cortisol acting via PR) rather than cortisol actions via the type II GR. We consider this alternative interpretation improbable for three reasons. First, secretion of progesterone was not substantially enhanced by the layered-stress paradigm; values remained undetectable or barely detectable during stress. Even if progesterone had been elevated to a level sufficient to inhibit LH pulsatility, the action of progesterone on LH pulses is very different from the effect we observed in response to stress. Progesterone acts via the hypothalamus to reduce the frequency of episodic GnRH secretion, not via the pituitary to lower its response to GnRH (36). Thus, the suppressive effect of stress on LH pulse amplitude in experiment 2 cannot be attributed to progesterone; it is more likely due to cortisol, which was elevated to a level sufficient to inhibit pituitary responsiveness to GnRH (10, 25). Second, there is strong evidence that the effect of cortisol to reduce pituitary responsiveness is mediated by the type II GR, not PR. In contrast to RU486, a selective PR antagonist (ORG 31,710) did not prevent cortisol-induced suppression of pituitary responsiveness to GnRH (25). Moreover, a selective agonist of the type II GR (dexamethasone) mimics the action of cortisol in reducing the pituitary response to GnRH (37). Third, estradiol is not needed for cortisol to inhibit pituitary responsiveness to GnRH, or for RU486 to reverse this effect (10), indicating that induction of PR by estradiol is not a likely explanation for the responses we observed in experiment 2. Collectively, these considerations favor our interpretation that the suppressive effect of stress on pituitary responsiveness to GnRH is due to cortisol actions via the type II GR, and does not involve enhanced PR or progesterone.

When placing our conclusions into a broader context, we emphasize an important qualification: it is unlikely that cortisol is the sole mediator of stress-induced suppression of pituitary responsiveness to GnRH. Clearly, cortisol is a key factor and the finding that antagonism of the type II GR reverses the suppressive effect of the layered stress on pituitary responsiveness could be taken as evidence that other mediators are not important. However, we caution against this interpretation for several reasons. The recent observation that psychosocial stress reduces the LH response to GnRH in hypothalamo-pituitary disconnected ewes, which do not express a cortisol rise in response to stress, suggests that stress-induced factors other than cortisol can inhibit pituitary responsiveness (26). In addition, increased secretion of cortisol is not necessary for the reduction in pituitary responsiveness to GnRH in ovariectomized ewes challenged with an immune/inflammatory stress (13). Not only does this implicate involvement of other intermediates, it also suggests the rel-

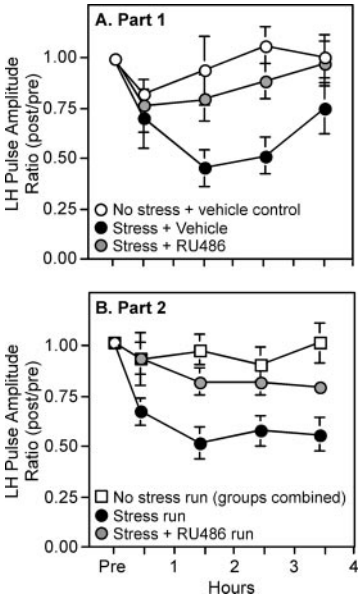

FIG. 5. LH pulse amplitude for each hour over the 4-h stress period in experiment 2. Amplitude is expressed as a ratio of each hourly poststress LH response to the mean prestress LH response (±SEM; no SEM indicates value is smaller than data point). LH responses were induced by hourly GnRH (5 ng/kg, iv) boluses. A, In part 1, ewes were allocated to one of three groups: no-stress vehicle control (*open circles*), stress plus vehicle (*black circles*), or stress plus RU486 (*gray circles*). B, In part 2, ewes were sampled during a stress run in the absence (*black circles*) and presence (*gray circles*) of RU486. *Open squares* depict the combined results of the two groups (RU486 and no antagonist) during the no-stress run.

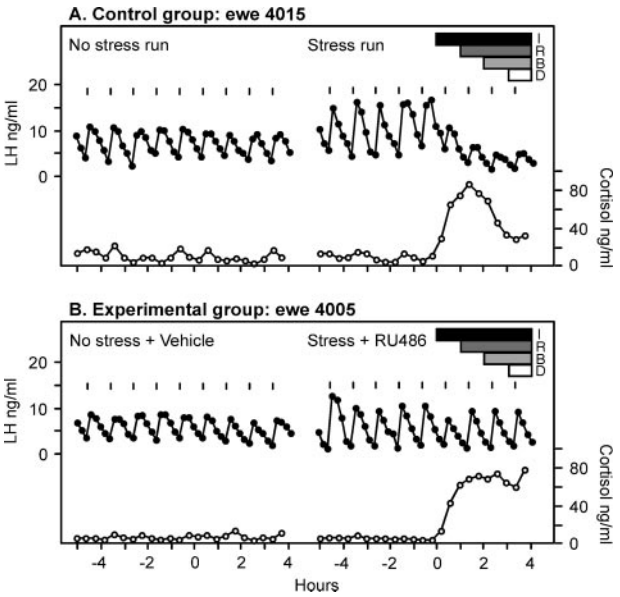

FIG. 6. LH (*black circles*) and cortisol (*open circles*) responses during both the no-stress run and stress run in one representative ewe from the control (A) and experimental group (B, RU486) in experiment 2, part 2. The layered-stress paradigm, depicted at the *top* of the panel during the stress run, is described in Fig. 3.

ative importance of each depends upon the nature of the stressor. For example, inhibition during immune/inflammatory stress is mediated by proinflammatory molecules such as cytokines and prostaglandins (1, 38, 39) whereas suppression of pituitary responsiveness by psychosocial stress is mediated by cortisol.

Finally, two questions need to be addressed. First, given that cortisol mediates stress-induced suppression of pituitary responsiveness to GnRH (experiment 2), why was RU486 ineffective (or less effective) in reversing the effect of stress on LH pulse amplitude in experiment 1, in which GnRH was not fixed? Although not statistically different, our observation in part 2 of experiment 1 that the suppressive effect of stress on the mean plasma LH concentration and LH pulse amplitude in the presence of RU486 was less than half of that seen in the absence of RU486 reveals two plausible explanations. One is that RU486 was less effective (or even ineffective) in antagonizing cortisol action via the type II GR in experiment 1. However, the enhancement of stress-induced cortisol secretion documents the efficacy of RU486 treatment in antagonizing negative feedback effects of endogenous cortisol via the type II GR. In each experiment that used the 4-h stress paradigm, cortisol values induced by stress either tended to increase (experiment 1, part 2; $P = 0.07$) or were increased (experiment 2, parts 1 and 2; $P < 0.05$) in the presence of RU486. The lack of an effect of RU486 on the stress-induced increase in plasma cortisol in experiment 1, part 1 may reflect the shorter layered-stress paradigm (3 h *vs.* 4 h in the other experiments). Nevertheless, we cannot discount the possibility that the estradiol used in the pituitary-clamp model improved the efficacy of RU486 in experiment 2. An alternative explanation for the finding that RU486 did not reverse stress-induced suppression of pulsatile LH secretion in experiment 1 is that GnRH input to the pituitary gland was not clamped and the layered-stress paradigm may inhibit LH pulse amplitude at multiple neuroendocrine levels. As postulated above, the layered stress might reduce GnRH pulse amplitude by a mechanism not requiring cortisol action via type II GR, and this could mask the effect of RU486 in reversing stress-induced suppression of pituitary responsiveness to GnRH.

The second question relates to the relevance of our study to suppression of reproductive fitness in response to stress encountered in real-life situations. In this regard, three of the stressors used in the layered-stress paradigm (isolation, restraint, threat of a predator) are likely to be encountered by sheep in the wild or in agricultural environments (40). The layered stress, however, was relatively acute, lasting only 3–4 h, and it would be highly interesting to examine responses during chronic stress. Pertinent to this, chronic psychosocial stress in women is considered a major contributor to certain menstrual cycle disorders including functional hypothalamic anovulatory syndrome (41, 42). Of interest to our investigation, this syndrome is associated with elevated plasma glucocorticoids and reduced LH secretion (43, 44). Thus, the psychosocial stress model used in the present study may prove to be relevant for understanding mechanisms of reproductive dysfunction associated with various clinical disorders and psychological stressors facing women in today's society.

In closing, the importance of using powerful experimental models is emphasized, not only models that reliably elicit clear-cut neuroendocrine responses to stress but also ones that can discriminate between hypothalamic and pituitary effects. Had we used the LH pulse pattern in ovariectomized ewes as the sole marker of reproductive neuroendocrine suppression, this study might not have disclosed an important mediatory role for cortisol. Our collective findings allow two conclusions. First, in an animal model in which pulsatile GnRH stimulation of the pituitary is fixed, psychosocial stress inhibits pulsatile LH secretion, in part, by reducing pituitary gland responsiveness to GnRH. This inhibitory influence is mediated by cortisol acting via the type II GR. Second, in a model in which GnRH input to the pituitary is not fixed, psychosocial stress may inhibit pulsatile LH secretion by additional mechanisms that are independent of cortisol acting via the type II GR. Thus, whereas cortisol action via this receptor is an important contributor to reproductive neuroendocrine suppression in response to psychosocial stressors, our findings emphasize the complexity of the stress response and reinforce the concept that multiple pathways mediate stress-induced reproductive dysfunction.

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