Effects of estrogen and estrous cycle on glucocorticoid and catecholamine responses to stress in sheep

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Komesaroff, Paul A., Murray Esler, Iain J. Clarke, Meryl J. Fullerton, and John W. Funder. Effects of estrogen and estrous cycle on glucocorticoid and catecholamine responses to stress in sheep. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E671–E678, 1998.—There have been relatively few studies of the effects of estrogen on hormonal responses to stress. We therefore studied changes in ACTH, cortisol, norepinephrine (NE), and epinephrine (Epi) after stress induced by a barking dog (audiovisual stressor) and insulin-induced hypoglycemia (metabolic stressor) in ovariectomized sheep treated with estradiol or placebo and in intact sheep in the follicular and luteal phases of the estrous cycle. Both stressors produced acute increases in ACTH, cortisol, Epi, and NE. A high physiological dose of estradiol significantly reduced the ACTH and cortisol responses to both stressors but did not affect Epi and NE responses. Plasma ACTH and cortisol responses to both stressors and Epi and NE responses to insulin were lower in the follicular than in the luteal phase, but catecholamine responses to the audiovisual stressor did not change during the estrous cycle. We conclude that in sheep, estrogen attenuates glucocorticoid responses to stress and that hormonal changes during the estrous cycle affect glucocorticoid responses to both metabolic and audiovisual stressors and catecholamine responses to a metabolic stressor.

catecholamines; adrenal gland; hypothalamic-pituitary-adrenal axis

The mechanisms involved in the actions of sex steroids on the hormonal responses to stress and, in particular, the role of estrogens have received limited attention. Using a well-established model (14, 21), we have therefore examined the effects of estradiol administration and placebo on responses to two stressors in ovariectomized sheep. Levels of ACTH, cortisol, norepinephrine (NE), and epinephrine (Epi) were determined after an audiovisual stressor and also after insulin-induced hypoglycemia (a metabolic stressor).

A question of physiological interest is whether stress responses are altered during the estrous cycle, in which endogenous levels of progesterone as well as of estradiol vary in a cyclical pattern, levels of progesterone being higher in the luteal phase of the cycle and those of estradiol in the follicular phase (26). Accordingly, in a separate experiment, we sought to examine whether there is variation in the responses of these hormones during the estrous cycle in ewes by determining the responses of ACTH, cortisol, NE, and Epi after both audiovisual and hypoglycemic stress in the follicular and luteal phases of the estrous cycle.

MATERIALS AND METHODS

Animals. For the experiment examining the effects of estrogen administration, two groups of eight ewes were studied in two separate studies involving two doses of estrogen. The sheep were ovariectomized at least 4 wk before they were studied. Each received a subcutaneous implant containing either estradiol or placebo 24 h before study and was subjected sequentially to audiovisual (barking dog) and insulin-induced hypoglycemic stressors.

For the experiment involving cycling sheep, eight mature Corriedale ewes were studied during the middle of the breeding season in late autumn. The estrous cycles were synchronized by administration of the prostaglandin analog cloprostenol, as described in Synchronization of reproductive cycles, and the sheep were studied during the luteal and follicular phases of the cycle. On each occasion, the sheep were subjected sequentially to audiovisual and insulin-induced hypoglycemic stress.

Jugular vein cannulas were inserted on the day before study. During the course of the experiments, the sheep were allowed access to both food and water. Approval for all experiments was obtained from the Baker Institute Animal Ethics Committee.

Estrogen treatment. Of the first group of eight sheep, four received a subcutaneous implant containing sufficient estradiol known on the basis of previous experiments (15) to produce plasma estradiol levels in the low normal physiological range ("low dose"), and four received implants containing placebo. Of the second group, four received implants containing an estrogen dosage known (15) to produce plasma estradiol levels in the high physiological range ("high dose"), and...
four received implants containing placebo. The estrogen used was in the form 17β-estradiol (Sigma) and was contained in Silastic tubes (Dow Corning) 3 cm long with an internal diameter of 3.35 mm, as previously described (15, 19). For the low-dose experiment, one such tube was inserted; for the high-dose experiment, four tubes were used.

Synchronization of reproductive cycles. The reproductive cycles of eight sheep were synchronized by the injection (im) of 125 μg of the prostaglandin F-2α analog cloprostenol (Estrumate, Pitman-Moore, North Ryde, NSW, Australia), which causes regression of the corpus luteum and precipitates a normal follicular phase (1, 8). The onset of estrus occurs 40–70 h after injection (8, 34). The sheep were studied 11 days later, during the luteal phase of the estrous cycle. All sheep then received another injection of cloprostenol on the evening after the study and a further injection 11 days later. The second study was conducted 2 days after the third injection, during the late follicular phase of the cycle.

Collection of blood samples. Blood was taken, as previously described (21), at 10-min intervals for 1 h to establish an undisturbed baseline, after which the sheep were exposed to 5 min of audiovisual stress. Blood samples were taken at 2.5, 5, 10, 20, 30, 40, 50, and 60 min after commencement of this stress. After a 10-min break, baseline samples were once again taken each 10 min for 1 h. Sheep then received insulin sufficient to produce profound hypoglycemia, and blood samples were taken at 20-min intervals for 160 min, after which glucose was administered. Blood volume was replaced by normal saline as blood was collected. Selected samples were subsequently analyzed for ACTH, cortisol, NE, Epi, glucose, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and progesterone.

 Induction of stress. To induce an audiovisual “emotional” stress, a barking dog was introduced into the sheep shed for 5 min. The same dog was used on each occasion and was encouraged to bark equally at each of the four sheep and to jump over the dividers that separated them in a common pen. The method used (and the dog) is identical to that previously employed (14, 21).

To produce hypoglycemia, the sheep were injected with 100 units (1.5 units/kg) of recombinant human Actrapid insulin (CSL-Novo, Parkville, Australia) as an intravenous bolus. Hormone extraction and assays. ACTH was adsorbed from plasma onto porous Vycor glass and measured by specific RIA as previously described (14). The interassay coefficient of variation was 7% (n = 4), and the sensitivity was 15 pg/ml. Cortisol was measured in unextracted plasma by specific RIA (14). The intra-assay coefficient of variation was 8% (n = 32), and sensitivity was 10 nmol/l. Epi and NE were extracted from plasma onto activated alumina and assayed by HPLC with electrochemical detection by a modification of a previously described method (13). In view of difficulties experienced with the use of 3,4-dihydroxybenzylamine, N-methyl dopamine was employed as the internal standard. The intra-assay coefficient of variation was 8% (n = 32), the interassay coefficient of variation was 10% (n = 10), and the sensitivity of the assay was 12 pg/ml. Plasma glucose was assayed by the glucose oxidase colorimetric method (21). FSH and LH were measured by RIA as described previously (5, 22). The sensitivity of the assay was 0.2 ng/ml for FSH and 0.23 ng/ml for LH. Estradiol was also measured by RIA as previously described (21). All measurements fell below the sensitivity of this assay, which was 30 pmol/l.

Statistics. Comparisons were made between baseline and stressed values of ACTH, cortisol, Epi, and NE for individual sheep and between estrogen-treated and control sheep and between luteal phase and follicular phase for these hormones for group data. Specifically, response curves were compared by ANOVA with repeated measures, and comparisons were made of the areas under the curve for each of the stress experiments using a paired Student’s t-test; in addition, the time to peak levels was compared between the two groups using paired Student’s t-test. For analysis of plasma glucose data, areas under the curve and average gradients were analyzed by a Student’s t-test after logarithmic transformation. Results were taken to be statistically significant at the P < 0.05 level.

RESULTS

Plasma estrogen, progesterone, FSH, and LH levels. In the experiment involving estrogen administration, estrogen, FSH, and LH were measured for each sheep on one occasion at the commencement of the experiment. As expected, on no occasion did estradiol levels in plasma exceed the sensitivity of the assay. In the sheep that received placebo, plasma LH and FSH concentrations were 1.9–6.1 U/l and 3.1–6.5 U/l, respectively. In all sheep treated with estrogen, LH and FSH were undetectable (data not shown).

In the experiment involving cycling sheep, estrogen, progesterone, and LH were measured in each sheep on one occasion at the commencement of the experiment. Once again, as expected, on no occasion did estradiol levels exceed the sensitivity of the assay. Mean progesterone levels were 13.6 ± 1.7 nmol/l (mean ± SE) during the luteal phase and 1.3 ± 0.5 nmol/l during the follicular phase. Mean LH levels were 2.9 ± 0.2 ng/ml during the luteal phase and 13.3 ± 2.0 ng/ml during the follicular phase.

Glucose. The effects of insulin on plasma glucose levels before and after low- and high-dose estrogen are shown in Fig. 1, A and B. The low dose of estrogen did not affect glucose levels. However, in sheep treated with the high dose of estrogen, baseline glucose levels and minimum glucose levels attained were significantly higher, and the time taken to reach an arbitrary point of severe hypoglycemia (1.5 mmol/l) was shorter in placebo-treated animals than in estrogen-treated animals. However, all sheep achieved severe hypoglycemia, and the absolute fall in glucose levels, the rate of fall of plasma glucose, and the time taken to reach nadir were similar between the two groups.

Baseline glucose levels were greater (P < 0.05) in the follicular phase than those measured at corresponding time points in the luteal phase of the cycle, as shown in Fig. 1C. However, subsequent values were not different at the two different stages of the estrous cycle. All sheep achieved profound hypoglycemia, with the minimum glucose levels and the time taken to reach them being identical. Following the achievement of hypoglycemia, the plasma glucose levels remained low until the conclusion of the experiment. Neither the areas under the two curves nor the rates of fall in plasma glucose concentrations differed significantly between the two phases of the estrous cycle.

Plasma ACTH and cortisol responses. Mean ACTH responses to audiovisual stress and insulin-induced hypoglycemia after estrogen treatment are shown in Fig. 2, A and B, and cortisol responses are presented in...
As expected, exposure to both the audiovisual stressor and insulin-induced hypoglycemia produced abrupt elevation in ACTH and cortisol concentrations, with the effects of hypoglycemia exceeding those of audiovisual stress. After insulin administration, the increases in hormone levels resembled the falls in glucose. Plasma levels of both ACTH and cortisol had returned to baseline at the time of glucose infusion. In the experiment involving the low dose of estrogen, cortisol levels were indistinguishable between estrogen- and placebo-treated sheep after both audiovisual and hypoglycemic stress. Plasma ACTH levels were lower after dog stress with estrogen treatment compared with placebo; however, the two curves were not statistically different by any of the measures used. Similarly, there was no statistical difference in the ACTH responses to insulin administration between estrogen- and placebo-treated animals, although in this case in the estrogen-treated sheep, the average levels were lower for the first 100 min, after which they increased markedly, largely because of a very high level achieved by one sheep. In the high-dose experiment, the baseline cortisol levels in the placebo- and estrogen-treated sheep were similar, as were the times taken for cortisol concentrations to reach a peak after each stress. However, the absolute increases after both dog stress...
tation, the rise in hormone levels paralleled the fall in glucose. Plasma levels of both ACTH and cortisol had returned to baseline at the time of glucose infusion. Baseline plasma ACTH levels were similar between the two phases. Peak plasma ACTH levels were significantly higher in the luteal phase in comparison with the follicular phase after both dog (P < 0.01) and hypoglycemic (P < 0.025) stress. In addition, for both stressors, the total plasma ACTH response, as measured by the area under the concentration vs. time curve, was greater (P < 0.01 and P < 0.05, respectively) in the luteal phase. The findings with respect to plasma cortisol levels were similar. Baseline values were equivalent between the two phases, but peak levels were significantly higher in the luteal phase after both dog (P = 0.02) and hypoglycemic (P = 0.025) stress in comparison with the follicular phase. In addition, for both stresses, the areas under the concentration vs. time curve were greater in the luteal phase (P < 0.02 and P < 0.04).

Plasma Epi and NE levels. Data for Epi before and after low- and high-dose estrogen are presented in Fig. 4, A and B; data for NE are presented in Fig. 5, A and B. Plasma levels of both Epi and NE increased markedly in response to both stressors. Substantial variations were seen both within and between individual sheep; nonetheless, some clear patterns emerged. As with ACTH and cortisol, the changes in plasma levels of Epi after hypoglycemia were significantly greater than after dog stress. However, the responses of NE were similar after dog and hypoglycemic stress. Overall, estrogen treatment did not affect the changes in plasma levels of either Epi or NE after either stress. Baseline levels of Epi, the absolute increases in plasma levels, and the times taken to reach peak values were all similar between estrogen- and placebo-treated sheep for both dog and hypoglycemic stress. The same was found with NE, although here, in comparison with Epi, there was much more variation. The times between the injection of insulin and the responses of either ACTH or catecholamines did not differ significantly in the two experiments. Despite the fact that with high-dose estrogen severe hypoglycemia was delayed in comparison with placebo treatment, the onset of the catecholamine responses did not change, suggesting that the variations observed were not related to different levels of blood glucose attained or to the time course of hypoglycemia.

Mean Epi and NE responses in follicular and luteal phases are presented in Figs. 4C and 5C. Both Epi and NE increased markedly in response to both stressors. As in previous experiments, substantial variations were seen both within and between individual sheep. There was no difference between follicular and luteal phases in the baseline levels of either hormone before the application of stress. For both Epi and NE, the responses to insulin were markedly greater in the luteal phase than in the follicular phase (P < 0.03 and P = 0.01, respectively). However, the response to dog stress did not change significantly for either hormone between the two phases of the estrous cycle.
These studies suggest that, in ovariectomized sheep, estrogen attenuates the glucocorticoid responses to both audiovisual and insulin-induced hypoglycemic stress but does not have an effect on catecholamine responses to either stressor. In addition, in mature sheep across the estrous cycle, the ACTH and cortisol responses to both audiovisual and insulin-induced hypoglycemic stress and Epi and NE responses to hypoglycemic stress are greater in the luteal phase than in the follicular phase ($P < 0.03$), but differences do not reach significance in case of audiovisual stress ($P = 0.18$).

**DISCUSSION**

Several previous studies have also shown an effect of estrogen on glucocorticoid responses to stress, and some have suggested an effect on catecholamine responses. For example, it has been shown (23) that estrogen administration to postmenopausal women attenuates the ACTH, cortisol, androstenedione, and NE responses to mental stress, although, in this case, somewhat surprisingly, the responses of premenopausal and postmenopausal women were indistinguishable. It has also been shown that estrogen administration to young men blunts the Epi and NE responses to mental stress (12).

Our results are also consistent with results from studies on women during the menstrual cycle. For example, in one study in normal women, the luteal phase was associated with greater stroke volume responses and lower vascular tone than the follicular phase (17); in another, women in the luteal phase reacted significantly more to the cold pressor test but...
were all identical, suggesting that the stress in the unlikely, because in this experiment too, the rate of fall therefore confounds the results. This would also seem argued that this altered the hypoglycemic stress and higher than in the luteal phase, and it might thus be plasma glucose levels were similar for estrogen- and progesterone may have an independent effect on stress responses, since a progesterone analog, tetrahydroprogesterone has been shown to have anxiolytic effects.

In our study, estrogen clearly had an effect on plasma glucose levels, and this may have contributed to differences in the responses to hypoglycemia. In this case, the stimulus is mainly hypoglycemia itself (9), with the initiation of the hormonal response occurring abruptly once a threshold is crossed (3, 20), although the rates of fall and the absolute levels of plasma glucose that are reached are also of some importance. In the present case, although (as expected) the decreased insulin sensitivity after estrogen administration resulted in higher baseline and trough levels, the dose of insulin administered in all cases was sufficient to induce profound hypoglycemia, and the times of onset of hypoglycemia and the decrements and rates of fall of plasma glucose levels were similar for estrogen- and placebo-treated animals. Similarly, in the intact sheep, the baseline glucose levels in the follicular phase were higher than in the luteal phase, and it might thus be argued that this altered the hypoglycemic stress and therefore confounds the results. This would also seem unlikely, because in this experiment too, the rate of fall and the time to nadir and the absolute value of nadir were all identical, suggesting that the stress in the follicular phase was not diminished with respect to the luteal phase (3, 20); indeed, the absolute fall in the follicular phase was greater than that in the luteal phase, which would suggest a greater rather than a smaller stress. Furthermore, in both cases, the results were consistent between the audiovisual and the hypothalamic components of the experiment, suggesting that the effects noted were real ones. Accordingly, we believe that, as in our previous study (21), the comparisons of the catecholamine responses after insulin-induced hypoglycemia are valid.

Estrogen doses in this experiment were chosen on the basis of previous studies that employed an identical methodology (15, 26). These have shown mean estradiol levels to be in the range of 3–5 pmol/l in ovariectomized sheep, 7–10 pmol/l after the low-dose estradiol treatment, and 14–18 pmol/l after high-dose estradiol. These values are within the physiological range for cycling sheep. The lower limit of sensitivity of all available commercial estradiol assays is at least 25 pmol/l, as a result of which all the levels in our study were undetectable. The attenuation of the glucocorticoid responses by estrogen was statistically significant with the high-dose but not with the low-dose regimen, suggesting that the effects we have observed are likely to be of relevance in the physiological context. In the intact sheep, as expected, progesterone levels were lower and LH levels were markedly higher in the follicular than in the luteal phase.

In these experiments, on each occasion, dog stress preceded hypoglycemia, raising the question of whether there is either a facilitatory or an inhibitory effect of the former stress on the latter one. This possibility, however, was explicitly tested and excluded in our earlier study (9). Despite this, it should be noted that these results in sheep may not apply to other species, in which prior stress has on occasion been shown to produce a facilitatory (29) or an inhibitory effect or no effect at all (11). In our experiments on intact sheep, testing was in each case conducted in the luteal phase before the follicular phase rather than in random order, raising the possibility of an effect of one phase on the other. We do not feel that this is likely, however, because our previous study showed no changes in responses when testing was conducted 2 wk apart; and, in any case, the fact that in vivo repeated cycling leads to mutual interactions between phases suggests that such an effect would not alter the conclusions of this experiment.

Although the mechanisms of the effects of estradiol on glucocorticoid levels are uncertain, it appears probable that it acts via ACTH and thus the pituitary or hypothalamus rather than directly on the adrenal gland. This is also likely to be the main pathway underlying the changes observed in the stress responses during the estrous cycle, although a contribution from changing progesterone levels is also likely, as discussed above, and a contribution from LH and perhaps FSH cannot be excluded. This is consistent with evidence obtained from women with hypothalamic
amenorrhea, in whom a blunted response to corticotropin-releasing hormone administration and increased cortisol levels were observed (2). Similarly, in ovariectomized rhesus monkeys, a reduced responsiveness to hypoglycemic stress is found that can be largely reversed with estradiol through an effect on gonadotropin-releasing hormone pulse generator activity (7). It is likely that these effects of estradiol on the hypothalamic-pituitary-adrenal axis are modulated, at least in part, by changes in glucocorticoid receptor (GR) numbers and/or function. In rats, it has been shown that estradiol abolishes the autologous downregulation of GR seen in hippocampus and hypothalamus (16). Furthermore, there is evidence of gender-specific differences in the gene expression of hippocampal and hypothalamic GR and of an effect of exogenous estradiol on GR mRNA levels (27).

In conclusion, this study shows that, in ovariectomized ewes, administration of estradiol at physiological levels attenuates the glucocorticoid responses to audiovisual and hypoglycemic stress and that, in mature cycling ewes, the cortisol and ACTH responses to audiovisual and hypoglycemic stress and the Epinephrine (Epi) and NE responses to hypoglycemic stress are greater in the luteal phase than in the follicular phase of the estrous cycle. These results raise questions as to whether similar effects are found in cycling women. Despite indirect evidence that this is the case, in view of the well-established species differences in the effects of estrogen on hormonal responses to stress, caution should be exercised in applying these physiological responses in humans. Further studies are needed to address this question directly.

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