Stress or acute adrenocorticotrophin treatment suppresses LHRH-induced LH release in the ram*

R. L. Matteri, J. G. Watson and G. P. Moberg

Department of Animal Science, University of California at Davis, CA 95616, U.S.A.

Summary. This study examined the effects of varying treatment durations and doses of adrenocorticotrophin (ACTH) as well as restraint stress on the LH response to exogenous LHRH. Injection of 80 i.u. of a concentrated ACTH preparation at 11, 6, 3 or 1.5 h before LHRH administration was effective in suppressing the LH response. Injection of 40, but not 20 or 10, i.u. ACTH 3 h before an LHRH challenge inhibited the magnitude of the LH response, while cortisol values did not vary between ACTH doses. Injection of 200 µg of synthetic ACTH_{1–24} also resulted in a reduced LH response when given 3 h before LHRH. Restraint stress caused elevated corticosteroid levels and reduced LHRH responsiveness. The results of this study suggest that stress may cause an inhibition of pituitary gland ability to respond to LHRH by way of an hormonal component of the adrenocortical axis. A glucocorticoid-independent mechanism may be involved.

Introduction

An inverse relationship between stress and normal reproductive efficiency has frequently been recognized in human and veterinary medicine (Whitacre & Barrera, 1944; Hagino, 1968; Doney, Gunn & Griffiths, 1973; Fries, Nillius & Pettersson, 1974; Moberg, 1976; Stoebel & Moberg, 1982a). It has also been demonstrated that abnormal ovarian physiology often accompanies increased adrenal activity (Selye, 1939; Garm, 1949). Although these were correlative observations, they raised the question of whether stress-induced activation of the adrenocortical axis could cause an inhibitory effect on reproduction. Accordingly, there have been many studies dealing with a possible hormonal aetiology for these findings (Baldwin & Sawyer, 1974; Boccuzzi *et al.*, 1975; Liptrap & McNalley, 1976; Barb, Kraeling, Rampacek, Fonda & Kiser, 1982; Matteri & Moberg, 1982; Stoebel & Moberg, 1982b).

Fuquay & Moberg (1983) have shown that two adrenocorticotrophin (ACTH) injections at 1 and 9 h before an exogenous luteinizing hormone-releasing hormone (LHRH) challenge would decrease the luteinizing hormone (LH) response in rams. To assess the physiological significance of these findings, it was necessary to compare the duration and degree of exogenous adrenocortical activation with that seen during physiological stress and to determine whether decreased pituitary responsiveness to LHRH resulted during natural and experimentally-induced adrenal stimulation. The present study therefore examined the effects of various treatment durations and doses of ACTH, as well as restraint stress, with respect to adrenal response and LH release.

Materials and Methods

General procedures

Rams of mixed breed, 1–2 years of age, were obtained from the flock of the University of California, Davis. The animals were kept in groups of about 10 in 7 × 8 m pens, under natural

* Reprint requests to Dr G. P. Moberg.

lighting conditions. Water was available *ad libitum* and pressed feed pellets were provided once daily. The experiments were carried out in the housing pens, in sections cordoned off to 1 × 2 m.

Veterinary grade ACTH (Adrenomone; Burns-Biotec Laboratories, Inc., Omaha, NE, U.S.A.) or synthetic ACTH (Adrenocorticotrophic Hormone, 1–24; Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) was given intramuscularly at various times before intravenous injection of LHRH (Sigma Chemical Company, St Louis, MO, U.S.A.), 10 µg in saline (9 g NaCl/l). There were no differences in LHRH responsiveness between uninjected and vehicle-injected control animals (unpublished data) and so no control vehicle injections were given in this study.

Blood samples (4 ml) were taken by jugular venepuncture with evacuated collection tubes. Samples were taken at –10, 0, 10, 20, 30, 60, 90, 120, 150 and 180 min in Exps 1, 2 and 3 (time 0 = time of LHRH injection). During the stress experiment (Exp. 4), the –10 min sample was replaced with a –30 min sample. Blood samples were immediately placed on ice until they could be centrifuged. Aliquot samples were placed in a –20°C freezer within 30 min of collection. Serum LH concentrations were subsequently determined. Corticosteroid levels were also assayed in selected samples.

Preliminary experiments showed that the first of a series of LHRH injections given to a ram produces a greater LH release than do subsequent injections (unpublished data). This has also been reported for young bulls (Chantaraprateep & Thibier, 1978) and heifers (Li & Wagner, 1983) given serial LHRH injections. We found that consistency of responsiveness could be improved by administering 10 µg LHRH 3–7 days before the first injection in a series of experimental LHRH challenges. The experimental trials were performed 3–7 days apart unless otherwise specified.

Experiment 1. In the treatment trials of this experiment, 80 i.u. ACTH (Adrenomone) were given i.m. at 11, 6, 3 or 1.5 h before administration of 10 µg LHRH (N = 9, 7, 10 and 10, respectively). Each animal underwent control and treatment trials to obtain data which could be analysed by paired statistical methods. During the control periods, the only treatment received was an LHRH injection. The order of treatment and control trials was randomly varied for each animal.

Experiment 2. To examine the effect that various doses of ACTH might have on pituitary responsiveness to LHRH, experimental animals underwent treatment trials consisting of i.m. administration of 40, 20 or 10 i.u. ACTH (Adrenomone) 3 h before the injection of 10 µg LHRH (N = 4, 12 and 11, respectively). As in Exp. 1, control treatments entailed LHRH administration only and each animal served as its own control. To assess the repeatability of LH responsiveness within animals, 6 rams were subjected to two serial control trials. The sequence of treatment and control trials was randomly assigned to each animal.

Experiment 3. Since the ACTH preparation used in the preceding two experiments was a domestic animal pituitary extract, synthetic ACTH was used to confirm the specific effect of this peptide. On the first day of the experiment, 4 rams received the control treatment of LHRH injection only and 3 rams were injected i.m. with 200 µg synthetic ACTH_{1–24} (in saline with 16% gelatine) 3 h before the administration of LHRH. The gelatine was added to mimic the Adrenomone vehicle used in the previous experiments. After 1 week, the animals that initially received the ACTH were given the control treatment and the previous control animals were treated with synthetic ACTH.

Experiment 4. To determine whether psychological stress would have an effect similar to exogenous ACTH on the pituitary gland's ability to respond to LHRH, a restraint protocol was developed. This procedure entailed restraining the animals on their sides, with the front and hind legs secured separately. Just enough longitudinal tension was applied to the ropes to prevent the animals from physically harming themselves during any struggling that might occur. The rams were restrained in this fashion for 40 min and released for 20 min. This was repeated for 6 consecutive hours. After 3 h of restraint, 10 µg LHRH were administered as in the previous experiments. Half of the 16 rams were exposed to stress, while the remaining animals underwent

control trials in the first test period. The tests were reversed for each animal 1–3 weeks later. Control trials consisted of an LHRH injection while the animals were moving freely in the presence of familiar flock-mates.

Hormone assay procedures. Serum LH concentrations were determined by a modification of the double-antibody radioimmunoassay developed by Niswender, Reichert, Midgley & Nalbandov (1969). The changes in procedure were as follows. Iodination of LH, using the iodogen method previously described (Moberg, Watson, Stoebe & Cook, 1981), was carried out with ^{125}I , rather than ^{131}I . Iodination grade LH was donated by Dr H. Papkoff. The tracer concentration was set to 40 000 c.p.m./100 μl buffer. Gelatine (Knox Gelatine, Inc., Englewood Cliffs, NJ, U.S.A.), rather than freeze-dried egg white was used to make the 0.1% protein-phosphate buffer solution. Goat anti-rabbit gamma globulin (Antibodies, Inc., Davis, CA, U.S.A.) at a working dilution of 1:180 was substituted for the sheep anti-rabbit antibody solution. The primary antibody (rabbit anti-ovine LH serum; preparation 2-OLH-R, from the laboratory of the late Dr I. I. Geschwind) was used at a concentration of 1:100 000. Finally, the incubation time between addition of the radiolabelled hormone and the secondary antibody was extended to 48 h.

Standards were made from NIAMMD O-LH-23. Minimum assay sensitivity was determined as 0.6 ng/ml. Inter- and intra-assay variabilities were 10.1 and 6.2%, respectively. The standard and serum pool dilutions produced parallel curves.

Corticosteroid levels were determined by a competitive protein-binding assay as previously described (Moberg *et al.*, 1981). Tritiated cortisol ([1,2,6,7- ^3H]cortisol) was obtained from Amersham, Arlington Heights, IL, U.S.A. Standards were prepared from cortisol purchased from Sigma Chemical Co. Minimum assay sensitivity was 3.9 ng/ml. Inter- and intra-assay variabilities were determined as 9.1 and 6.7%, respectively.

Statistical analysis. All LH data were converted to area under the LHRH response curve ((ng $\times$ min)/ml). These converted values were analysed with the aid of biomedical statistical software (BMDP Statistical Software, Department of Biomathematics, University of California, Los Angeles, CA, U.S.A.). Two-way analysis of variance was employed for all experiments, blocking by treatment and animal. A paired *t* test was also used to compare the LH response areas in Exps 1, 2 and 3 (Sokal & Rohlf, 1969). The serum corticosteroid values from Exp. 4 were analysed by this test at each sample time. The remaining cortisol values were analysed by Student's *t* test for differences between 2 means.

Results

Experiment 1

The results of varying the ACTH treatment time before LHRH are shown in Text-fig. 1(a) and Table 1. Analysis by paired *t* test and 2-way analysis of variance (ANOVA) show that administration of ACTH (Adrenomone) at each pretreatment time resulted in a reduction of stimulated LH release. Variances in the levels of LH suppression (Text-fig. 1a) were sufficiently large to prevent detection of differences between treatment trials. This, coupled with the demonstration of significant between-animal differences by ANOVA (Table 1), reflects the large amount of variability in absolute LH responsiveness that existed between the animals used in this study.

The degree of treatment effectiveness also varied considerably between animals and treatment-resistant animals were observed throughout this study. In the treatments for which a significant effect was detected, about 20% of the animals did not show LH response curve areas <15% of control (lower 95% confidence limit for repeated control trials, as determined from values obtained in 6 animals). Available corticosteroid data for unaffected animals ranged from 250 to 1000% of control release rates during experimentation.

Text-fig. 1. The amount of LH suppression due to injection of (a) 80 i.u. ACTH at various times before an LHRH challenge and (b) various doses of ACTH. The LH area values indicate the mean $\pm$ s.e.m. change (treatment – control) in LH response curve areas. The numbers in parentheses indicate the sample size. * $P < 0.05$; ** $P < 0.01$ (paired t test).

Table 1. Results for the 2-way analysis of variance (treatment $\times$ animal) for effect of treatment on LH response of rams

Exp.	Treatment	Dose	Time before LHRH (h)	Treatment effect	Between-animal variation	No. of rams
1	ACTH*	80 i.u.	1.5	$P < 0.005$	$P < 0.007$	10
	ACTH	80 i.u.	3.0	$P < 0.004$	$P < 0.022$	10
	ACTH	80 i.u.	6.0	$P < 0.031$	$P < 0.018$	7
	ACTH	80 i.u.	11.0	$P < 0.006$	$P < 0.0002$	9
2	ACTH*	40 i.u.	3.0	$P < 0.001$	$P < 0.001$	4
	ACTH	20 i.u.	3.0	$P < 0.347$	$P < 0.005$	12
	ACTH	10 i.u.	3.0	$P < 0.309$	$P < 0.013$	11
3	ACTH ₁₋₂₄	200 μ g	3.0	$P < 0.013$	$P < 0.0003$	7
4	Restraint	6 h total	3.0	$P < 0.048$	$P < 0.516$	16

* Adrenomone.

Corticosteroid concentrations were significantly elevated ($P < 0.001$) after ACTH injections compared to control values (Text-fig. 2). These ACTH-stimulated values were not different from one another, except at 14 h, when the mean concentration was less than at 6 h ($P < 0.05$).

Experiment 2

When the dose of ACTH that was administered was varied 40 and 80 i.u. ACTH inhibited pituitary gland responsiveness to LHRH (Text-fig. 1b). Some suppression was obtained with 10 and 20 i.u. ACTH but it was not significant (see also Table 1). As in Exp. 1, significant between-animal differences were observed (Table 1). Serum corticosteroid values were greater than in controls ($P < 0.001$) and did not diminish with decreasing ACTH dosage. The cortisol concentrations (mean $\pm$ s.e.m.) 3 h after 0, 10, 20, 40 or 80 i.u. ACTH were 6.8 ± 0.8 ($N = 49$), 44.5 ± 5.3 ($N = 8$), 49.8 ± 5.7 ($N = 8$), 56.8 ± 6.1 ($N = 5$) and 45.5 ± 4.2 ($N = 8$) ng/ml, respectively.

Text-fig. 2. The mean $\pm$ s.e.m. corticosteroid response to an i.m. injection of 80 i.u. ACTH. * $P < 0.01$ (t test) compared with corresponding control value. The no. of animals is given in Text-fig. 1(a) and data from some pilot animals are also included.

Text-fig. 3. The LH response areas of rams in Exp. 3 treated with synthetic ACTH₁₋₂₄. Individual animals are denoted by a-g.

Experiment 3

The administration of ACTH₁₋₂₄ significantly decreased pituitary gland sensitivity to LHRH (Text-fig. 3; Table 1). A large degree of between-animal variation was again present and not all animals were affected by the ACTH₁₋₂₄ treatment.

Text-fig. 4. The LH response areas of rams in Exp. 4 during stress. Individual animals are denoted by a-p.

At 3 h after ACTH₁₋₂₄ injection, the mean $\pm$ s.e.m. serum corticosteroid concentration was 11.3 ± 2.0 ng/ml compared to 8.6 ± 1.8 ng/ml in the control trial. These values did not differ from each other, although the mean concentration in the ACTH-treated rams was greater ($P < 0.05$) than baseline values in Exps 1 and 2. The control values of this experiment were not significantly different from those seen in the other experiments.

Experiment 4

The LH data obtained during restraint stress are shown in Text-fig. 4. An analysis of the LH response areas (Table 1) demonstrated an effective suppression of LH release due to stress. This was the only experiment in this study in which between-animal variation was not significant. Serum corticosteroids were elevated throughout the sample collection period (Text-fig. 5). Stress-stimulated cortisol values 3 h after the beginning of the restraint procedure were not different from the corticosteroid concentrations seen 3 h after the injections of ACTH (Exp. 1). Although not

Text-fig. 5. The mean $\pm$ s.e.m. corticosteroid concentrations in rams in Exp. 4. * $P < 0.05$; ** $P < 0.01$ compared with corresponding control value (paired t test).

every animal was affected with respect to LH suppression, corticosteroid secretion was increased by stress in each ram (ranging from 250 to 650% of control values).

Discussion

Administration of ACTH has been shown to cause a reduction of basal LH secretion in the bull (Johnson, Welsh & Juniewicz, 1982), inhibition of the preovulatory LH surge in the dairy cow (Stoebel & Moberg, 1982b) and gilt (Barb *et al.*, 1982), and suppression of the LH response to LHRH in the dairy heifer (Matteri & Moberg, 1982) and ram (Fuquay & Moberg, 1983). In addition to supporting these previous findings, this report defines ACTH treatment time and dose relationships with respect to suppression of the LHRH-induced LH response. It is also demonstrated that behavioural stress causes a similar reduction in pituitary gland sensitivity to LHRH.

Injections of ACTH at 11, 6, 3 or 1.5 h before LHRH administration were all effective in reducing the subsequent LH response. This suggests that stress may be capable of inducing a relatively long refractory period of gonadotrophin secretion. Since this ACTH-induced LH suppression also occurs in the ewe (Barb *et al.*, 1982; Matteri & Moberg, 1982; Stoebel & Moberg, 1982b), significant reductions in fertility might result if stress is experienced during such a period before the preovulatory LH surge. In fact, stress has been linked to lower ovulation rate in the rat (McKay, Wood & Reinke, 1975) and ewe (Doney *et al.*, 1973). Stress has also been shown to cause an inhibition of preovulatory LH release in the dairy heifer (Stoebel & Moberg, 1982a). Stress-induced adrenocortical activation may therefore be linked to low fertility.

Although the Adrenomone preparation used in the majority of these experiments was highly concentrated in ACTH activity, it was possible that the presence of some substance other than ACTH was producing the observed LH suppression. Injection of 200 μ g ACTH₁₋₂₄, a synthetic ACTH, was effective in reducing pituitary LHRH responsiveness, even though the corticosteroid response was short-lived compared to that produced by Adrenomone treatment. It would therefore

appear that prolonged glucocorticoid elevation is not a prerequisite for inducing decreased LHRH sensitivity.

Injections of 40 and 80 i.u. ACTH, but not 10 or 20 i.u. resulted in statistically significant reductions in LH release when administered 3 h before the LHRH challenge. Nevertheless, the corticosteroid concentrations did not diminish as the ACTH dosage was reduced, and animals that did not show LH suppression had corticosteroid concentrations higher than in controls. This suggests that a glucocorticoid-independent mechanism may be involved.

It has been reported for the sow (Liptrap, 1970) and the cow (Liptrap & McNalley, 1976) that administration of ACTH, but not cortisol, can produce polycystic ovarian syndrome. ACTH is a potent inhibitor of LHRH-stimulated LH secretion in the dairy heifer, but cortisol infusions producing serum corticosteroid concentrations similar to those seen during ACTH treatment had only a weak inhibitory effect (Matteri & Moberg, 1982). A similar suppression of LHRH-induced LH release was produced by ACTH, but not cortisol, in intact and adrenalectomized rams (Fuquay & Moberg, 1983). Similarly, ACTH treatment can inhibit the post-ovariectomy LH rise in adrenalectomized adult female rats (Ogle, 1977). The results of these studies are in apparent discord with reports demonstrating that glucocorticoid treatment can inhibit the preovulatory LH surge in the dairy cow (Stoebel & Moberg, 1982b) and gilt (Barb *et al.*, 1982). One possible reason for the discrepancy is that cortisol treatment may affect some central nervous system site(s), causing interference with the release of LHRH from the hypothalamus, while ACTH administration directly inhibits pituitary gland LH secretion. Although glucocorticoids may be exerting some effect on pituitary LHRH sensitivity (Baldwin & Sawyer, 1974; Chantarapruteep & Thibier, 1978; Matteri & Moberg, 1982), a possible involvement of an extra-adrenal product cannot be discounted. ACTH may reduce LH secretion by a direct effect or by the induction of an unknown secondary mechanism.

The results produced by restraint stress demonstrate coincident adrenocortical activation and a depression of the LH response to LHRH similar to that induced by ACTH treatment. This in itself is not proof of a causal relationship between adrenocortical stimulation and LH inhibition. However, when combined with the observations from the remainder of this study, the data suggest that some hormonal component(s) of the pituitary-adrenal axis is involved in stress-induced reduction of pituitary LHRH sensitivity.

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