Effect of mouse epidermal growth factor on plasma concentrations of LH, FSH and testosterone in rams

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Summary. Two experiments were conducted to examine the effects of mouse epidermal growth factor (EGF) on the concentrations of testosterone, LH and FSH in jugular blood plasma and on the pituitary responsiveness to LHRH. In 20 rams treated with subcutaneous doses of EGF at rates of 85, 98 or 113 µg/kg fleece-free body weight, mean plasma LH and testosterone concentrations were significantly reduced ($P < 0.05$) at 6 h after treatment but not at 24 h. EGF treatment at 130 µg/kg fleece-free body weight suppressed the plasma content of these hormones for up to 48 h. Mean plasma FSH concentrations decreased significantly ($P < 0.05$) for up to 48 h after EGF treatment, the effect being most pronounced in rams with mean pretreatment FSH values ≥ 0.5 ng/ml. Intravenous injections of 1.0 µg LHRH given to each of 5 rams before and at 6 h, 24 h and 72 h after EGF treatment produced LH and testosterone release patterns which paralleled those obtained in 5 control rams similarly treated with LHRH.

These results suggest that, in rams, depilatory doses of mouse EGF temporarily impair gonadotrophin and androgen secretion by inhibiting LHRH release from the hypothalamus. Such treatment appears to have no effect on the responsiveness of the pituitary to LHRH.

Keywords: testosterone; gonadotrophins; sheep; EGF; LH; FSH

Introduction

In male mice, endogenous epidermal growth factor (EGF) is important in maintaining spermatogenesis but appears to have little or no effect on the plasma concentrations of FSH and testosterone (Tsutsumi *et al.*, 1986). However, several studies *in vitro* have shown different effects of mouse EGF with respect to the control of steroidogenesis by Leydig cells. In freshly prepared rat or mouse Leydig cells cultured in the presence or absence of LH, EGF stimulates androgen production (Verhoeven & Cailleau, 1986) but inhibits it if the cells are cultured for 10 days previously (Hsueh *et al.*, 1981; Welsh & Hsueh, 1982). Further, when a clonal strain of Leydig tumour cells which have receptors for both hCG and mouse EGF are exposed to mouse EGF, gonadotrophin-induced progesterone production is reduced (Ascoli, 1981).

In ewes, intravenous (i.v.) infusion of mouse EGF (100 µg/kg body weight) reduced LH concentrations by inhibiting the release of LHRH from the hypothalamus (Radford *et al.*, 1987a). It is not known whether treatment of rams with dose rates of mouse EGF required to induce partial or complete break of wool fibres for biological wool harvesting (85–130 µg/kg body weight) produces similar effects on the hypothalamus and gonadotrophin secretion or has any influence on steroidogenesis in the testis.

Accordingly, the present study was undertaken to examine the effects of mouse EGF treatment on the reproductive endocrine system in adult Merino rams.

Materials and Methods

Animals. Thirty Merino rams aged 4–6 years and weighing 53–72 kg were used during late summer to study the effects of mouse EGF on plasma concentrations of the reproductive hormones (Exp. 1) and on pituitary responsiveness to LHRH therapy (Exp. 2). For 3 months before the start of EGF treatment, the rams were kept in group pens in an animal house and fed a pelleted mixture (60:40 w/w) of ground lucerne hay and oat grain at a daily rate equivalent to 900 g per animal. Water was available *ad libitum*. The animals were weighed weekly to ensure that they maintained constant body weight. At 1 week before the start of EGF treatment, the rams were transferred to individual metabolism cages and the above diet was continued until the completion of the experiment.

Preparation of mouse EGF. In Exp. 1, EGF was prepared by genetic engineering methods (Allen *et al.*, 1987) and used at a concentration of 1.5 mg/ml. In Exp. 2, EGF was prepared from mouse submaxillary glands by the method of Savage & Cohen (1972) and purified (Radford *et al.*, 1987a). The protein was dissolved in sterile saline (9 g NaCl/l) at appropriate concentrations to obtain a total infused dose over 24 h of 105 µg/kg fleece-free body weight per treated ram.

Experiment 1: effect of mouse EGF on plasma testosterone, LH and FSH concentration. Rams were allocated at random to 4 groups (N = 5 per group) and treated with subcutaneous injections of EGF at dose rates of 85, 98, 113 or 130 µg/kg fleece-free body weight (Groups 1–4, respectively). Fleece-free body weight was defined as the liveweight minus the estimated weights of the wool and horns.

From each ram, 4 blood samples (5 ml) were obtained 20-min apart, commencing at 24 h (09:30 h) before and at 6, 24, 48 and 72 h after EGF treatment. All blood samples were obtained by jugular venepuncture, held on ice until centrifugation within 2 h of collection and the plasma stored at –10°C until assayed for LH and testosterone. For determination of FSH, a single blood sample (5 ml) was obtained from each ram before and at 3, 6, 9, 11, 15.5, 22, 24 and 30 h after treatment and on each of the 9 subsequent days. The same samples were used to determine plasma EGF concentrations prevailing up to 30 h from start of the EGF treatment.

Experiment 2: effect of mouse EGF on pituitary responsiveness to LHRH. Mouse EGF (105 µg/kg fleece-free body weight) was infused over 24 h into each of 5 rams via an indwelling jugular catheter at a rate of 12.5 ml/h while 5 control rams received sterile saline delivered at the same rate. At 24 h before (09:30 h) and at 6, 24 and 72 h after the start of the infusions, each ram was injected (i.v.) with 1.0 µg LHRH (I.C.I., Melbourne, Victoria, Australia). In association with each LHRH treatment, blood samples (4 ml) were obtained from each ram via jugular catheters before the LHRH injection and, afterwards, at 10-min intervals for 1 h and then at 20-min intervals for a further 2 h.

Hormone assays. All hormones were measured using radioimmunoassay. In the mouse EGF assay (Panaretto *et al.*, 1982), the sensitivity, defined as twice the s.d. of the zero point of the standard curve, was 0.21 ng/ml. The coefficients of variation (CV) for plasma samples with mean concentrations of 5.5, 10.8 and 24.4 ng/ml were all <10%. The sensitivity of the LH assay (Radford *et al.*, 1987a) ranged from 0.09 to 0.23 ng/ml and for pooled ovine plasma samples with mean concentrations of 0.9, 2.9 and 5.0 ng/ml the intra- and inter-assay CV were <10% and <15%, respectively. In the FSH assay (Radford *et al.*, 1987b) the sensitivity ranged from 0.06 to 0.13 ng/ml and for 2 pooled sheep plasma samples with mean concentrations of 0.9 and 3.9 ng oFSH-RP-1/ml the intra- and inter-assay coefficients of variation were all <10%.

Testosterone concentrations were measured in duplicate. The samples and standards (20 µl) were extracted for 5 min with 2 ml toluene:hexane (2:1, v/v), the solvent phase was evaporated to dryness under N₂ and the single-antibody technique of Garnier *et al.* (1978) was then used. An antiserum raised in a sheep against testosterone-3-carboxymethyloxime-BSA (M. S. F. Wong & R. I. Cox, C.S.I.R.O., Division of Animal Production, P.O. Box 239, Blacktown, NSW, Australia) was used at a dilution of 1:60 000. Cross-reactivity with dihydrotestosterone, 4-androstene-3β,17β-diol and androstenedione was 31%, 30% and 1.3% respectively. All oestrogen and progesterone compounds had <1% cross-reactivity. Mean non-specific binding was 2.8%, the sensitivity of the assay was 0.1 ng/ml and for pooled sheep plasma samples with mean concentrations of 0.6, 3.5 and 7.3 ng/ml the inter- and intra-assay CV were <8% and <10% respectively.

Statistical analysis. The data of Exp. 1 were analysed using the analysis of variance appropriate for repeated measurements (in time) on the same animal.

Large variability between rams was noted with both pre- and post-treatment measurements and a post-analysis grouping into 'high' and 'low' rams was done to check on the similarity in response for this grouping. Additionally, single degree of freedom contrasts were calculated for each ram to highlight specific treatment patterns such as the eventual recovery of hormone concentrations to pre-treatment values. Such contrasts were statistically tested using the *t* test after due allowance was made for the multiple comparison effect of selecting a subset of possible comparisons from those available. Thus, an individual contrast was tested at the α/k level where α is the experiment-wise error rate (commonly 5% or lower) and k refers to the degrees of freedom of the factor from which the contrast was selected.

No statistical tests were done for Exp. 2. As the magnitude of the response varied from ram to ram, no convenient summary of the response would provide the data for a simple statistical hypothesis. Since the pattern rather than the

Fig. 1. Mean ($\pm$ s.e.m.) plasma concentrations for LH and testosterone in rams before and at various times after treatment with (a) 85, (b) 98, (c) 113 or (d) 130 μ g mouse EGF/kg fleece-free body weight (5 rams per group). *Mean value differed significantly ($P < 0.05$) from corresponding pre-treatment value.

magnitude of the response was being examined, the required inference was made from a graphical presentation of the results.

Results

Plasma EGF concentration and fleece removal

For each treatment group in Exp. 1, plasma concentrations of EGF (ng/ml) increased progressively to reach a maximum level by 11.0–15.5 h after dosing (mean $\pm$ s.e.m. 12.8 ± 2.5 ; 13.1 ± 1.1 ; 16.9 ± 3.5 ; 19.9 ± 1.1 ; Groups 1–4, respectively) and declined thereafter to reach low levels (1–2 ng/ml) by 30 h. In Exp. 2, the plasma concentrations of EGF increased continuously over the infusion period, reaching a mean peak value 23.2 ± 2.8 ng/ml by the end of the infusion, after which time the concentrations fell to 1–2 ng/ml within 2.5 h.

In Exp. 1, the fleeces were easily removed by hand at 8–10 days after EGF treatment in all rams given doses of 113 μ g/kg body weight or above, in 60% of the animals given 98 μ g/kg and in none of the animals given 85 μ g/kg. In Exp. 2 with an infusion of 105 μ g/kg body weight, 40% of the animals had fleeces that could be easily removed. Wool attachment was normal in the control rams.

Fig. 2. Pooled mean ($\pm$ s.e.m.) plasma FSH concentrations (■—■) for 20 rams before and at various times after treatment with EGF (see 'Results'). ●—● and ▲—▲ represent mean values for rams with pretreatment FSH values ≥ 0.5 ng/ml ($N=8$) and <0.5 ng/ml ($N=12$).

Experiment 1

As shown in Figs 1 and 2, for each of the treatment groups, the mean LH and testosterone concentrations decreased significantly ($P < 0.05$) by 6 h after injection of EGF although there was no significant difference between the treatment groups. In Group 4, the mean LH and testosterone plasma concentrations were still significantly ($P < 0.05$) lower than pre-treatment values at 24 h after treatment but the treatment group $\times$ time interaction was not statistically significant. In all treatment groups, the mean concentrations of the 2 hormones increased subsequently to higher than pretreatment levels before returning to near normal levels.

There was no statistical evidence of a dose level, or a dose level $\times$ time interaction on plasma FSH concentrations obtained over the 10 days after EGF treatment. Pooling the values from all animals showed that the mean plasma FSH concentrations were significantly lower ($P < 0.05$) than the pre-treatment values for up to 48 h after EGF treatment, then rose to higher than pretreatment values over the next 1–2 days before returning to normal. Although there was considerable variation between rams in the magnitude of these changes in FSH concentration, the percentage decrease in both the 'high' and the 'low' groups was approximately 20%. The decrease was most evident in the 8 rams in which the prevailing pretreatment FSH concentrations were ≥ 0.5 ng/ml (see Fig. 2), although a similar pattern of response occurred in the rams with low initial FSH concentrations.

Experiment 2

For all control and EGF-treated rams, injection of LHRH at each test period resulted in an immediate and substantial rise in plasma LH concentration. This was followed within 20 to 30 min by a marked increase in plasma testosterone concentrations with a peak occurring at 60–90 min, after which the values declined (see Fig. 3).

Fig. 3. Mean testosterone (○—○) and LH (●—●) concentrations in plasma of (a) 5 EGF-treated and (b) 5 control rams, following intravenous injections of LHRH at 0, 6, 24 and 72 h after the start of EGF treatment.

Discussion

Treatment of rams with mouse EGF at dose rates of 85–130 $\mu\text{g/kg}$ fleece-free body weight caused a decline in both plasma LH and testosterone concentrations within 6 h of dosing. These depressions persisted for less than 24 h when the rams received $\leq 113 \mu\text{g/kg}$ but were more prolonged at the higher dose rate (< 48 h). Although a true diurnal rhythm of release of either LH or testosterone has not been demonstrated in rams, Ortavant *et al.* (1982) found that the frequency of plasma LH and testosterone peaks in groups of Préalpes-du-Sud and Ile-de-France rams during summer was minimal immediately after dawn and maximal 3 h later. In the present study, no blood samples were obtained over such periods, and so it is unlikely that temporal effects contributed to the depressions in plasma concentrations of the two hormones. Plasma concentrations of FSH also declined by 6 h but neither the magnitude nor the duration of FSH suppression following EGF treatment was related to dose of EGF. Accordingly, it appears that mouse EGF has differential effects on the secretion of LH and FSH.

In rat Leydig cells cultured for different periods, EGF can either enhance (Verhoeven & Cailleau, 1986) or suppress (Hsueh *et al.*, 1981; Welsh & Hsueh, 1982) gonadotrophin-stimulated testosterone production. In the present study, however, the fall in plasma testosterone concentration in the EGF-treated rams could be attributed to a suppression of LH secretion rather than to a direct inhibition of steroidogenesis by the Leydig cells. At all times whilst plasma concentrations of LH and testosterone were depressed, LHRH therapy with consequent LH release induced large increases in plasma concentrations of both hormones in EGF-treated rams, the response being similar to that obtained in the same rams before EGF treatment and to that produced concurrently in the control animals. Since plasma FSH concentrations were also temporarily depressed in EGF-treated rams and LHRH is necessary for the release of both FSH and LH (Schally *et al.*, 1971; Fraser, 1976), it appears that, in rams, mouse EGF (at the dose rates used) acts on the hypothalamus, temporarily impairing the secretion of LHRH and therefore LH secretion. This is in accord with conclusions from an earlier study on the effects of EGF treatment (100 µg/kg body weight) in ewes (Radford *et al.*, 1987a, b).

Morris *et al.* (1988) showed that EGF caused an increase in both basal and FSH-stimulated inhibin production by immature rat Sertoli cells in culture, presumably acting via the specific EGF-binding sites on these cells. Furthermore, the stimulatory effect of EGF on inhibin production was additive to that of FSH (Morris *et al.*, 1988). Although comparable EGF-binding sites have yet to be demonstrated in ram Sertoli cells, it is possible that, in the present study, EGF may have acted upon the Sertoli cells, causing a temporary increase in inhibin production which in turn contributed to the suppression of FSH release by the pituitary. Contrary to findings in ewes (Radford *et al.*, 1987b), EGF treatment in rams did not induce a prolonged elevation in plasma FSH concentration subsequent to an initial depression. This difference arises because the Sertoli cells survive the effects of EGF whereas, in ewes, ovarian follicles of ≥ 0.6 mm in diameter, which are the source of inhibin, are destroyed (Radford *et al.*, 1987b).

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