Some effects of epidermal growth factor on reproductive function in Merino sheep

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Summary. During the i.v. infusion of a depilatory dose (100 µg/kg bodyweight) of mouse epidermal growth factor (EGF) into ovariectomized Merino ewes the frequency of pulsatile LH release was significantly reduced. However, the amplitude of pulses of LH secretion, either those naturally occurring or those induced by LHRH injection, was unchanged or only slightly reduced.

Similar infusions of mouse EGF were made in progestagen-treated anoestrous Merino ewes in which LH secretion was maintained by injections of LHRH. These ewes did not experience oestrus or ovulate in response to PMSG injected 1 day after mouse EGF treatment (2 days before progestagen withdrawal); both responses occurred in controls. The EGF-treated ewes experienced oestrus and ovulated following progestagen–PMSG treatment 6 weeks later.

These results suggest that (1) mouse EGF inhibits the hypothalamic pulse generator responsible for LH release in the ewe but has little if any effect on pituitary sensitivity to LHRH; and (2) mouse EGF apparently has a direct effect on the ovaries, temporarily impairing their ability to ovulate in response to exogenous gonadotrophin.

Introduction

The treatment of sheep with mouse epidermal growth factor (EGF) can cause shedding of the fleece (Moore *et al.*, 1981). This depilatory effect is being investigated as a method of harvesting wool and, at the doses being used for the purpose, mouse EGF has been reported to have significant hormonal effects in sheep, some of which could affect the reproductive performance of ewes. For example, after a depilatory dose of mouse EGF given to ewes on Days 9 or 10 of the oestrous cycle there was no apparent effect on progesterone secretion but the next oestrus was delayed for several days (Shaw *et al.*, 1985). In the same study, when the EGF was given 24 h after cloprostenol-induced luteolysis, there was decreased frequency of pulsatile luteinizing hormone (LH) release and increased pulse amplitude by the end of the 24-h infusion of EGF. Over the next several days, oestradiol-17β and LH secretion decreased and secretion of follicle-stimulating hormone (FSH) increased. The ewes failed to experience oestrus, but subsequent progesterone secretion was apparently unchanged.

The present study was undertaken to extend these findings and in particular to determine the site(s) of action of mouse EGF on the reproductive endocrine system in Merino ewes.

Materials and Methods

The EFG was prepared from the submaxillary glands of adult male mice and by the method of Savage & Cohen (1972). The gland extract was fractionated on a Biogel P10 column and the fraction containing EGF was separated on DEAE-cellulose. The fraction corresponding to the major peak was concentrated, dialysed and lyophilized. For infusion the EGF was dissolved in sterile 0.15 M-NaCl at a concentration of 15 µg/ml.

The sheep were housed indoors in individual pens and fed 800 g of a pelleted mixture (60:40 w/w) of lucerne chaff and oats once daily. Water was available *ad libitum*.

Experiment 1: effect of depilatory doses of mouse EGF on LH secretion in ovariectomized ewes. Eight Merino ewes, weighing 40–47 kg, which had been ovariectomized 2 months previously, were used. Each had a vinyl cannula (1.0 mm i.d., 1.5 mm o.d.; Dural Plastics, Sydney, NSW, Australia) inserted into each jugular vein, one for infusion and one for blood sampling and injection. One day later 4 ewes were infused with 4.5 mg EGF ($\approx 100 \mu\text{g/kg}$) dissolved in 300 ml 0.15 M-NaCl at the rate of 12.5 ml/h for 24 h; the 4 control ewes received infusion of 300 ml 0.15 M-NaCl alone over the same period. Two ewes in each treatment group were injected through the other cannula with 250 ng luteinizing hormone-releasing hormone (LHRH, I.C.I., Melbourne, Victoria, Australia) in 1 ml 0.15 M-NaCl at 90-min intervals before, during and after the infusion (Fig. 1). Heparinized blood samples were collected from this cannula every 15 min during the same periods. The blood samples were stored in ice water and centrifuged within 2 h of collection. The resultant plasma samples were stored at -10°C until assayed for mouse EGF and LH.

Experiment 2: effect of depilatory doses of mouse EGF on pituitary sensitivity to LHRH and on ovarian response to exogenous gonadotrophin in anoestrous ewes. Intravaginal sponges containing 60 mg medroxyprogesterone acetate (Repromap; Upjohn, Sydney, NSW, Australia) were inserted into 6 anoestrous ewes, weighing 40–44 kg. After 8 days each ewe was fitted with a cannula in each jugular vein. One day later 3 ewes were infused over 24 h with 4.5–4.8 mg ($\approx 100 \mu\text{g/kg}$) EGF in 0.15 M-NaCl, and 3 with 300 ml 0.15 M-NaCl alone. LHRH (250 ng in 1 ml 0.15 M-NaCl) was injected through the other cannula every 3 h, from 4 h before to 12 h after the start of infusion, and from 4 h before to 9 h after the end of infusion. Heparinized blood samples were collected from this cannula immediately before, then at 5, 10, 15, 30, 45, 60, 90 and 120 min after each injection. These samples were treated and stored as in Exp. 1 and assayed for mouse EGF and LH. All 6 ewes were injected i.v. with 750 i.u. PMSG (Folligon; Intervet, Sydney, NSW, Australia) 24 h after the end of infusion. The vaginal sponges were removed 48 h later, and the ewes were run with vasectomized rams wearing Sire-sine harnesses and crayons (Hortico, Melbourne, Victoria, Australia). The ewes were observed daily for crayon marks indicative of mating, and 5 days after sponge removal their ovaries were examined by laparoscopy under local anaesthesia. The intravaginal sponge–PMSG treatment was repeated twice so that the PMSG injections were given 2, 23 and 44 days after the start of infusion (Fig. 1).

Hormone assays. Mouse EGF was measured by radioimmunoassay as described by Panaretto *et al.* (1982). The same mouse EGF was used for antiserum production, iodination and standard. The antiserum, raised in a rabbit, was used at a concentration of $1:2 \times 10^4$ at which it bound 35–40% of the trace-iodinated EGF. There was no cross-reaction with human EGF (Chiron Corp., Emeryville, California, U.S.A.) and no immunoreactivity could be detected in the plasma of sheep before mouse EGF infusion or in the plasma of rats. The sensitivity of the assay, defined as the mass of hormone equivalent to 2 s.d. below that bound by the antiserum with tracer alone, was 0.95 ng/ml. The intra- and inter-assay coefficients of variation (CV), based on values obtained for two pools of plasma within each assay, were 4% and 7% respectively.

LH was measured by a double-antibody radioimmunoassay using an antiserum raised in a rabbit against a locally purified ovine LH preparation (ALW 59.80.3). The same ovine LH was iodinated and NIH-LH-S15 was used as standard. The antiserum, at a concentration of $1:10^6$, bound 30–35% of the iodinated hormone. There was <3% cross-reaction with ovine FSH, ovine prolactin and ACTH, <10% cross-reaction with ovine GH and a 20% cross-reaction with ovine TSH. The sensitivity of the assay was 0.13 ng/ml. The intra- and inter-assay CV, based on values obtained for three pools of plasma assayed within each assay, were 8 and 14% respectively.

Statistical analysis. A peak concentration of LH obtained one or two samples after a preceding nadir, and exceeding the latter by at least twice the coefficient of variation of the assay, was taken as representing an endogenous pulse of LH secretion. The amplitude was the difference between the value of the nadir and that of the peak. The amplitude of an LH peak induced by the injection of LHRH was determined by subtracting the concentration of the sample obtained immediately before injection from that of the first or second sample after injection. Differences in pulse amplitudes and frequencies were assessed using Student's *t* test.

Results

Experiment 1

Plasma mouse EGF concentrations and attachment of fleeces. In the ewes infused with 98–102 μg EGF/kg the concentrations of EGF in the plasma rose slowly to reach values of 18–27 ng/ml (Fig. 2). Shedding of wool began 5–7 days later. It was then removed by hand leaving the animals completely nude on the woolgrowing regions of the body and the ewes were protected by coats (R. J. Davis, Wahrenonga, NSW, Australia) for the next 4 weeks. The attachment of wool was unaffected in the control ewes.

Changes in secretion of LH. In the saline-infused ewes not given LHRH, LH was released episodically at intervals of 30–45 min, mean $\pm$ s.e.m. pulse amplitude being 11.7 ± 2.7 ng LH/ml (Fig. 1). There was no change in frequency or amplitude during infusion. In the ewes injected

Fig. 1. Outline of protocol of Exp. 2.

Fig. 2. Changes in plasma concentrations of LH (—●—) and mouse EGF (···■···) in ovariectomized Merino ewes infused over 24 h with saline, or mouse EGF (100 µg/kg body wt) in saline (Exp. 1). Within each panel the bottom 2 ewes were injected periodically with 250 ng LHRH (↓).

periodically with LHRH, LH values peaked within 30 min of each injection, usually 15 min, and there was no significant change in the amplitude of the induced pulses (20.2 ± 1.1 ng LH/ml) throughout the experiment.

In the EGF-infused ewes not given LHRH the inter-pulse interval was initially about 45 min. This increased to at least 60 min by 3 h from the start of infusion by which time basal levels of LH had begun to fall. During the next sampling period (Fig. 2) basal levels were between 0 and 2 ng LH/ml, the inter-pulse interval was at least 75 min, and in 1 of the 2 ewes pulse amplitude was significantly lower than before infusion (13.2 ± 2.0 compared with 5.7 ± 0.8 ng LH/ml, $t = 6.6$, d.f. = 5, $P < 0.01$). There was no significant change in pulse amplitude in the other. Pulse frequency and basal concentrations of LH remained low in both ewes until at least 3 h after the end of infusion. By the next day pulse frequency, amplitude, and basal levels had returned to preinfusion values. In the ewes periodically injected with LHRH, basal concentrations of LH were very low by mid-way through the infusion but then began to rise with the continued injection of LHRH. There was no significant change in the amplitude of the LHRH-induced pulses in one ewe (16.3 ± 0.9 ng LH/ml) throughout the experiment, and a small though significant decrease in amplitude (17.7 ± 1.5 compared to 12.7 ± 1.0 ng LH/ml, $t = 3.01$, d.f. = 9, $P < 0.02$) in the other.

Experiment 2

Plasma concentrations of mouse EGF and attachment of fleeces. The concentrations of EGF reached 21–42 ng/ml in the plasma of the ewes given 103–111 μ g EGF/kg. Shedding of wool started 5–7 days later and the ewes were treated as in Exp. 1. Wool growth was unaffected in the control ewes.

LH secretion in response to LHRH injection. Each injection of LHRH stimulated LH secretion. There were no significant differences in response associated with the infusion of mouse EGF in any ewe and accordingly no differences in response between EGF and saline-infused ewes (Fig. 3).

Fig. 3. Changes in plasma concentrations of LH in anoestrous Merino ewes injected periodically with 250 ng LHRH (↓): ●—●, ewes infused with mouse EGF (100 μ g/kg) over 24 h (N = 3), ○---○, saline-infused controls (N = 3). ■···■, Plasma concentrations of mouse EGF. Vertical bars indicate s.e.m.

Response to PMSG. All control ewes experienced oestrus 2–4 days after sponge removal, and all but one shed multiple ova (Table 1). The EGF-treated ewes neither experienced oestrus nor ovulated after the first PMSG injection, but one came into oestrus and all shed multiple ova after the second PMSG injection and all experienced oestrus and shed multiple ova after the third PMSG injection (Table 1).

Table 1. Oestrus and ovulation in ewes after PMSG–progestagen treatments at intervals after infusion of mouse EGF in Exp. 2

Days from EGF infusion to injection of PMSG*	Treatment (3 ewes per group)	No. of ewes in oestrus 2–4 days after sponge removal	No. of CL present 5 days after sponge removal
2	EGF	0	0
	Control	3	4, 4, 8
23	EGF	1	3, 5, 7
	Control	3	1, 4, 13
44	EGF	3	2, 2, 7
	Control	3	1, 3, 4

*750 i.u. PMSG injected i.v. 48 h before removal of intravaginal progestagen sponge.

Discussion

Despite considerable differences between ewes in the concentrations of mouse EGF in the plasma at the end of infusion, all treated ewes shed their wool. In Exp. 1, mouse EGF caused a significant reduction in the frequency of pulsatile LH release in ovariectomized Merino ewes, indicating a direct inhibitory effect of EGF on the putative hypothalamic pulse generator responsible for endogenous LHRH release (Lincoln *et al.*, 1985). No such unequivocal statement can be made about the effect of EGF on the amount of LHRH released per pulse. There was no change in the amplitude of the endogenous LH pulses in one EGF-treated ewe, suggesting that neither the amount of LHRH released nor the sensitivity of the pituitary to LHRH had been affected. Similarly, no change in pituitary sensitivity to injected LHRH was detected in any of the intact ewes in Exp. 2. On the other hand, the reduced pituitary response to injected LHRH in one EGF-treated ovariectomized ewe (Fig. 2f), and smaller endogenous LH pulses in another (Fig. 2b) could represent either reduced pituitary sensitivity or reduced amounts of LHRH secreted. The reasons for these apparently different responses are unknown. There was no evidence that LH pulse amplitude increased towards the end of the EGF infusion, as reported for mid-cycle ewes by Shaw *et al.* (1985), but the hormonal status of the ewes involved in the two studies was very different.

In Exp. 2, there was no evidence of endogenous LH release in any ewe. This suggests that the LH pulses, induced to ensure that the ovaries received at least a normal level of gonadotrophin support, were provided more frequently than necessary. None the less, there is no reason to believe that this influenced the results. These showed that the ovaries of the EGF-treated ewes were incapable of ovulating in response to exogenous gonadotrophin given 1 day after treatment, and that the ability to ovulate was recovered within several weeks. This temporary inability to ovulate suggests that mouse EGF affected the ovaries directly rather than initially by way of reduced gonadotrophin secretion. This suggestion is based on the fact that pretreatment patterns of LH secretion were rapidly re-established after the EGF treatment of the ovariectomized ewes in Exp. 1, and there is no reason to believe that the hypothalamo–pituitary system of intact ewes would

recover any less quickly. The direct ovarian effect, and its reproductive consequences, are currently under investigation.

The results provide evidence that mouse EGF induces temporary inhibition of reproductive function in the ewe, with both the hypothalamus and ovaries being involved.

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