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# Comparison of leucine enkephalin and adrenocorticotrophin effects on adrenal function in fetal and adult sheep*

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**Summary.** At Day 120–125 of gestation equimolar amounts of ACTH and leu-enkephalin injected *in vivo* provoked similar rises in plasma cortisol concentrations in chronically catheterized fetuses. There was no concomitant change in plasma DHEA concentrations, or in maternal cortisol concentrations. At term (Days 135–140) 2 out of 5 animals responded similarly to both leu-enkephalin and ACTH injections with a rise in plasma cortisol concentrations, but the other 3 animals, in which basal cortisol concentrations had already risen, showed no response to either agonist. In adult sheep, ACTH provoked a significant increase in the plasma cortisol concentrations, but equimolar amounts of leu-enkephalin were without effect. There was a significant output of cortisol in response to ACTH administration by collagenase-dispersed adrenal cells from term sheep fetuses *in vitro*. Leu-enkephalin had no effect on cortisol output from dispersed adrenal cells when added by itself, or with ACTH. We conclude that leu-enkephalin is able to function as a stimulator of pituitary–adrenal function during fetal life. The lack of effect of leu-enkephalin on adrenal cells implies that its action is exerted not directly at the adrenal gland, but indirectly at the level of the hypothalamus or pituitary through stimulation of the release of other corticotrophic substances.

## Introduction

During pregnancy in some species, the placenta as well as the fetal pituitary synthesizes a variety of peptides that are structurally related to pro-opiomelanocortin (POMC; Liotta & Krieger, 1980) or are presumably derived from pre-proenkephalin (Tan & Yu, 1981). However, the functions of these peptides in mother and fetus are not entirely understood. Carson & Challis (1982) have shown that leucine-enkephalin, injected into newborn lambs, provoked a rise in the concentration of cortisol in plasma in a dose-dependent fashion. Studies of adult rats have indicated that this effect might be mediated through the central release of adrenocorticotrophin (ACTH; De Souza & Van Loon, 1982), although a direct effect on the adrenal itself cannot be excluded (Lymangrover, Dokas, Kong, Martin & Saffran, 1981).

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In the present study, we have extended our earlier observations (Carson & Challis, 1982) to determine whether leucine-enkephalin influenced plasma cortisol concentrations during fetal life and in adult sheep. Since several studies have indicated that the responsiveness of the sheep fetal adrenal gland to ACTH stimulation rises in late pregnancy (Madill & Bassett, 1973; Wintour *et al.*, 1975), we compared the effects of equimolar bolus injections of leu-enkephalin with those of ACTH at two different times before parturition. We also measured changes in the plasma concentrations of dehydroepiandrosterone (DHEA), a potential oestrogen precursor which is secreted as its sulphoconjugate from the adrenal gland of the human fetus (Jaffe *et al.*, 1981). In the present experiments, we measured free DHEA rather than its sulphoconjugate because the sheep fetal adrenal is reported to have relatively low sulpho-transferase activity (Anderson, Pierrepoint, Turnbull & Griffiths, 1973). Finally, we initiated experiments to determine the site of action of leu-enkephalin by examining its effects on cortisol output from fetal adrenal cells isolated from sheep fetuses near term.

## Materials and Methods

### *In-vivo studies*

Experiments were performed with 6 chronically catheterized fetal sheep. Under general anaesthesia (Challis & Patrick, 1981) and using sterile techniques, catheters were implanted into the fetal carotid artery and jugular vein at Day 110–115 of pregnancy. Details of the catheterization procedures used have been published elsewhere (Challis & Patrick, 1981).

All fetal experiments were begun 4–8 days after surgery. Studies were performed on the same fetuses at Days 120–125 and 135–140 of gestation. On successive days injections of 1 ml sterile saline (0·154 M-NaCl); adrenocorticotrophin 1–24 (Cortrosyn: Organon Ltd, Toronto, Canada; 10 μg); or leucine-enkephalin (Des-Tyr'-leucine enkephalin: Sigma, St Louis, MO, U.S.A; equimolar with the ACTH injection) were given through the jugular venous catheter. On each occasion, the saline test was conducted first, the order of ACTH and leu-enkephalin injection was randomized, and in some experiments a second control test with saline was given on Day 4. On each sampling day, samples of fetal blood (1 ml) and maternal blood (4 ml) were withdrawn at −30, 0, +10, +30 and +60 min relative to the bolus injection of test substance, which was administered in 1 ml saline. All blood samples were collected into heparinized tubes that were standing in ice. The samples were centrifuged at 1500 *g* for 10 min at 4°C. The plasma was stored at −20°C until subsequent analysis.

Experiments were performed in 6 non-pregnant sheep during anoestrus. Under general anaesthesia vinyl catheters were introduced into the femoral artery and femoral vein as described previously. The experimental design was as described for the fetuses but using 10-fold greater amounts of $ACTH_{1-24}$ and leu-enkephalin. Injections were made through the femoral vein catheter. Blood samples (5 ml) were withdrawn from the femoral arterial catheter at the same time intervals as in the fetal experiments.

### *In-vitro studies*

In the pregnant sheep, intrauterine pressure was monitored continuously from an intra-amniotic catheter (Challis & Patrick, 1981). Signals from the pressure transducer were amplified to record continuously on a Grass Model 78 polygraph with a scale set from 0 to 100 mmHg. The appearance of Type B labour-like contractions (Lye, Sprague, Mitchell & Challis, 1983) of amplitude greater than 10 mmHg were indicative of early labour. At this time, general anaesthesia was induced by intravenous pentobarbitone sodium (Abbott, Montreal, Quebec). The fetuses were removed rapidly and weighed. The fetal adrenal glands were dissected out, cleaned, weighed and dispersed into single cell suspensions using 0·05% collagenase as described previously

(Glickman & Challis, 1980). Fetal adrenal cells were counted and were diluted to a concentration of 100 000 cells/ml in Krebs–Ringer bicarbonate buffer (Dawson, Elliott, Elliott & Jones, 1969). The cells were added to plastic tubes containing various amounts of $ACTH_{1-24}$, leucine-enkephalin or combinations of ACTH and leucine-enkephalin, to give a final concentration of 50 000 cells/ml. The cells were incubated for 4 h at 37°C under 95% $O_2$–5% $CO_2$ and the incubations were stopped by freezing. Cell viability at the beginning, and in some experiments at the end, of the incubation period was assessed by the exclusion of trypan blue stain, and was consistently greater than 95%.

*Radioimmunoassay*

Cortisol was measured by radioimmunoassay using an antibody raised in our laboratory, and employing procedures that have been evaluated with respect to fetal and maternal sheep plasma, and incubation media from isolated adrenal cells (Glickman & Challis, 1980; Challis *et al.*, 1981). The intra- and inter-assay coefficients of variation were 7·8 and 8·0% respectively. Dehydroepiandrosterone (DHEA) was measured using an antibody and radioimmunoassay procedure as reported previously (Challis, Socol, Murata, Manning & Martin, 1980). The recovery of known amounts of DHEA added to fetal plasma was $y = 0{\cdot}91\ x + 7{\cdot}2$ where $y$ = amount added and $x$ = amount recovered. All DHEA samples were measured in one assay. The intra-assay coefficient of variation was 8·9%.

*Statistical analysis*

Throughout the text all results are presented as means ± s.e.m. for the number of observations indicated. For the purposes of analysis the mean basal concentration of steroid in plasma was calculated from the value at −30 min and in the 0 min sample. We used two-way unbalanced analysis of variance and Duncan's Multiple Range Test to determine the effects of treatment and of time on cortisol concentrations in plasma or incubation media.

## Results

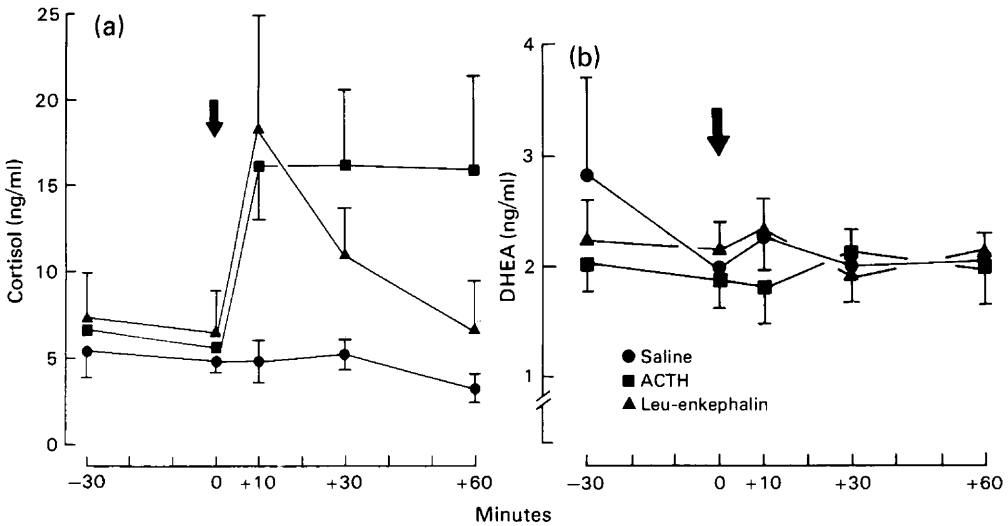
*In-vivo studies*

*Fetuses at Day 120–125.* The mean basal cortisol concentration in fetuses at 120–125 days of gestation was 5·37 ± 1·55 ng/ml (N = 6). There was no effect of saline injection on plasma cortisol concentrations. There was a significant increase in plasma cortisol concentrations at +10 min after injection of ACTH or leu-enkephalin. The differences in mean cortisol concentrations at +30 and +60 min were not statistically significant ($P > 0{\cdot}05$). The increment (Δ) in plasma cortisol after each of these two agonists was similar (Text-fig. 1a). The mean basal cortisol concentration had risen to 10·25 ± 3·33 ng/ml (N = 6) at the start of the second saline injection. Again, the saline had no significant effect on plasma cortisol concentrations, values being 12·45 ± 3·10 ng/ml at +10 min, 7·65 ± 2·79 ng/ml at +30 min and 4·78 ± 1·58 ng/ml at +60 min.

In contrast to the effects on plasma cortisol, there were no significant effects of any treatment on plasma DHEA concentrations at Days 120–125. In the pre-injection samples the concentration of DHEA was 2–3 ng/ml, and the values fluctuated around 2 ng/ml for the duration of the study (Text-fig. 1b).

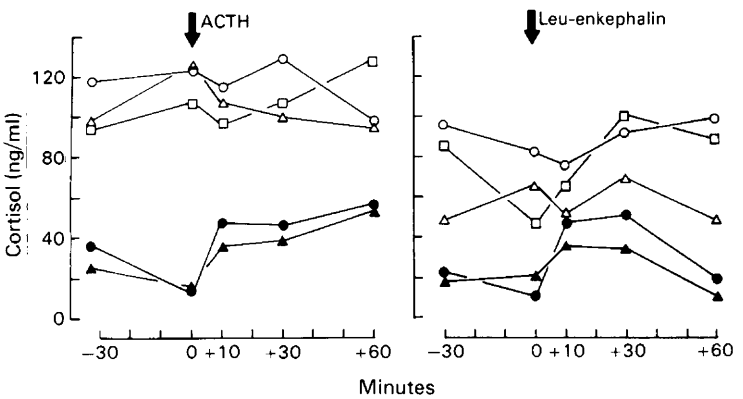
Maternal cortisol concentrations varied between 5 and 10 ng/ml. There was no significant effect on the maternal plasma cortisol concentration after administration of saline, ACTH or leu-enkephalin to the fetus (data not shown).

*Fetuses at Day 135–140.* The response (increment) in plasma cortisol after administration of ACTH or leu-enkephalin to fetuses later in gestation appeared to vary according to the basal cortisol concentration. There were no significant effects of leu-enkephalin or ACTH when the




**Text-fig. 1.** The concentrations of (a) cortisol and (b) dehydroepiandrosterone in the plasma of chronically catheterized fetal sheep after bolus injection at time 0 of $ACTH_{1-24}$, leucine-enkephalin or saline. Each value is the mean ± s.e.m. for observations on 6 fetuses at Day 120–125 of pregnancy.

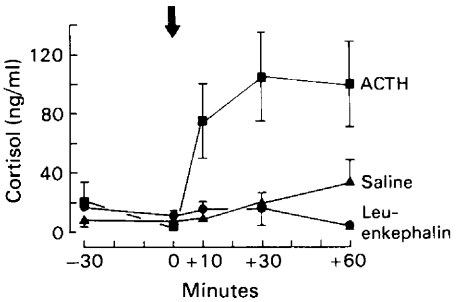
cortisol concentrations for all the fetuses in each group were combined. However, it was noticeable that, in 2 fetuses in which the basal cortisol concentration ranged between 20 and 40 ng/ml, there were increments in plasma cortisol of 20–30 ng/ml after ACTH or leu-enkephalin administration. In 3 different fetuses estimated to be <24 h before delivery, in which the basal cortisol concentrations fluctuated between 50 and 120 ng/ml, there was no obvious change in the plasma cortisol concentration after the injection of either agonist (Text-fig. 2).




**Text-fig. 2.** The effect of equimolar $ACTH_{1-24}$ or leucine-enkephalin on the concentration of cortisol in the plasma of 5 individual sheep fetuses in late pregnancy. Two fetuses with basal cortisol concentrations of 20–40 ng/ml (●, ▲) responded to both agonists, whereas 3 fetuses with basal cortisol concentrations of 50–120 ng/ml (□, ○, △) did not respond.

The mean concentration of DHEA in fetal plasma on Days 135–140 was 2·01 ± 0·19 ng/ml (N = 6). There was no significant effect of any treatment on fetal DHEA concentrations at this time (data not shown).

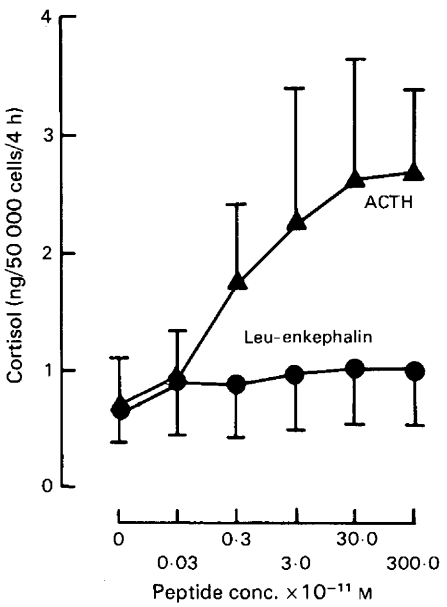
*Adult sheep.* The mean pre-injection concentration of cortisol in the adult sheep was 11·05 ± 2·32 ng/ml (N = 6). There was no significant change in the plasma cortisol concentration after saline or leu-enkephalin injection. However, equimolar injection of ACTH produced a significant increase in plasma cortisol concentration, with an increment of 80–90 ng/ml (Text-fig. 3).




**Text-fig. 3.** The effect of $ACTH_{1-24}$, leucine-enkephalin or saline on plasma cortisol concentrations in adult anoestrous sheep. Each value is the mean ± s.e.m. for observations on 6 animals.

*In-vitro adrenal responses*

Collagenase-dispersed adrenal cells prepared from term fetal sheep produced a significant increase in cortisol output in response to ACTH treatment *in vitro* (Text-fig. 4). On average, the increment was 1·89 ng/50 000 cells for 4 h. There was no significant response of the cells in terms of cortisol output to leucine-enkephalin. In two experiments, each performed in duplicate, ACTH dose–response curves were performed in the presence of fixed amounts of leu-enkephalin ($0·3 \times 10^{-10}$ and $30 \times 10^{-10}$ M). Addition of leu-enkephalin did not influence the ability of the cells to secrete cortisol in response to ACTH stimulation.




**Text-fig. 4.** The output of cortisol by dispersed adrenal cells, from term fetal sheep, incubated with $ACTH_{1-24}$ (▲) or leucine-enkephalin (●). Each value is the mean ± s.e.m. for observations on 4 fetuses.

## Discussion

Bolus injections of equimolar amounts of $ACTH_{1-24}$ or leucine-enkephalin both provoked a significant increase in the plasma cortisol concentration in fetal sheep at Day 120–125 of gestation. In fetuses nearer term (Days 135–140), when basal cortisol concentrations had risen, two response patterns emerged. In 2 out of 5 animals, >48 h before labour, both agonists stimulated a similar rise in plasma cortisol, indicating no selective change in fetal adrenal response to their effects during late pregnancy. However, in 3 out of 5 fetuses, <48 h before parturition, there was no response to either ACTH or leu-enkephalin. Basal cortisol concentrations in these fetuses were higher, and it might be suggested that the adrenal was already secreting cortisol maximally in response to endogenous ACTH, which is known to be rising at this time (Rees, Jack, Thomas & Nathanielsz, 1975; Jones, 1983). This finding is consistent with the loss in minute-to-minute variability in the plasma cortisol concentration of fetuses during the last 4–5 days before parturition (Challis *et al.*, 1981). A similar poor response to exogenous ACTH is seen in lambs at 4 h of age, in which basal cortisol concentrations are also high (Carson & Challis, 1981).

We found no change in the maternal cortisol concentration associated with fetal leu-enkephalin or ACTH injections. This finding is consistent with the lack of transplacental transfer of these peptides (Jones, Luther, Ritchie & Worthington, 1975), and suggests a lack of effect of leu-enkephalin administration to the fetus on the secretion of biologically active corticotrophic substances into the maternal compartment.

The response pattern of adult sheep to equimolar injections of ACTH or leu-enkephalin was different from that of the fetuses. In adult animals, ACTH, but not leu-enkephalin, provoked a rise in the concentration of cortisol in plasma, suggesting that the leu-enkephalin response is peculiar to fetuses and neonatal sheep (Carson & Challis, 1982), but is lost some time thereafter. These results raised the question of the site of action of leu-enkephalin on the adrenal response.

To examine this issue, we incubated adrenal cells from fetal sheep with ACTH or leu-enkephalin. Although these cells were responsive to ACTH, they did not respond significantly to leu-enkephalin over the concentration range studied, nor in two experiments did leu-enkephalin influence the response pattern to ACTH. There are several possible explanations for these results. It is conceivable that the fetal adrenal response to leu-enkephalin changes with gestational age, and term fetal adrenals may have been inappropriate for this study. However, the in-vivo results in the present experiments, and our previous results for newborn lambs (Carson & Challis, 1982), indicated that term fetal adrenals should have responded to leu-enkephalin if the effect was a direct one. We cannot exclude the possibility that our digestion procedures destroyed adrenal leu-enkephalin receptors. However the mild nature of the digestion procedure and the significant response of the fetal adrenal cells to ACTH would seem to negate this suggestion. In superfused rat adrenocortical tissue, methionine enkephalin produced a greater effect in potentiating the stimulation of corticosteroid output by a subsequent challenge with ACTH than it did as a direct corticotrophic agonist (Lymangrover *et al.*, 1981). However, in the present study, leu-enkephalin was equally devoid of activity when given by itself or concomitantly with ACTH.

These findings contrast with several reports showing direct effects of opioids on adrenal function. In contrast to the results of Lymangrover *et al.* (1981), others have shown that morphine decreases the ACTH responsiveness of rat adrenals. High concentrations of β-endorphin stimulated corticosterone output from isolated rat adrenal cells, although with a potency less than 2% that of ACTH (Shanker & Sharma, 1979), whereas lower concentrations of β-endorphin inhibited ACTH-stimulated or basal corticosterone output (Szalay & Stark, 1981). Racz *et al.* (1980) reported that methionine-enkephalin and leucine-enkephalin suppressed corticosterone output by rat adrenal tissue *in vitro*.

A further possible explanation for the discrepancy between our in-vivo and in-vitro results, and perhaps for the change in response in adult sheep, is that in fetuses and newborns (Carson & Challis, 1982) leu-enkephalin acts at the hypothalamus or pituitary to provoke the release of ACTH

or other corticotrophic agents. De Souza & Van Loon (1982) reported a significant rise in plasma ACTH concentrations in rats, 5–10 min after administration of the potent enkephalin analogue D-ala$^2$-met-enkephalinamide. Hypophysectomy or pre-treatment with dexamethasone blocked the increase in corticosterone after analogue administration, supporting strongly the suggestion of an intermediary role of ACTH release in the corticosteroid response. These results are consistent with studies showing an increase in plasma corticosterone concentrations in mice after intracerebroventricular administration of methionine-enkephalin (Gibson, Ginsburg, Hall & Hart, 1979), and support a hypothalamic site of action for the opiate. The further possibility that growth hormone is released after leu-enkephalin injection (Dupont, Cusan, Labrie, Coy & Li, 1977) and stimulates the fetal adrenal *in vivo* (Devaskar, Devaskar, Voina, Velayo & Sperling, 1981) cannot be excluded at present.

If the major site of leu-enkephalin action is at the hypothalamus, then the peptide has to cross the blood–brain barrier to exert its effect. Partridge & Mietus (1981) found that enkephalins have low permeability across the blood–brain barrier of the cow, and that the capillaries in this species had high enkephalinase activity. It is possible that the loss of leu-enkephalin effect compared to ACTH in adult sheep over that found in fetuses is associated with decreased permeability and with increased enkephalinase activity at the blood–brain barrier in the adult.

We have been unable to demonstrate changes in the concentration of DHEA in fetal plasma after administration of leu-enkephalin at 120–125 or 135–140 days of pregnancy. These results are consistent with observations suggesting that the fetal adrenal is not a major source of $C_{19}$ steroids during late pregnancy (Flint, Anderson, Steele & Turnbull, 1975).

At present, we can only speculate on the physiological significance of our results. The identity of the trophic hormone(s) to the ovine fetal adrenal gland remains unclear. In other species, enkephalins are synthesized in placental (Tan & Yu, 1981) and adrenal medulla (Wilson, Chang & Viveros, 1980) tissue. The present study raises the possibility that, if isolated in the sheep fetus, enkephalins may function as neurotransmitters and be implicated in the regulation of pituitary–adrenal function during fetal life.

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