The effect of sex steroids on the degradation of LRH by hypothalamic homogenates

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Abstract. Extract of hypothalami was prepared which contained peptidase capable of degrading LRH. The degradation of LRH by this extract either alone or under the influence of oestrogens, androgens and cholesterol, when added to the extract was measured. Oestrone, oestradiol and oestriol (1 pg to 100 pg) stimulated mean peptidase activity significantly \(P < 0.001\) in a dose-dependent manner. Testosterone (0.1 ng to 10 ng) also caused a dose-dependent increase in degradation of LRH, the two highest doses used significantly increasing the mean activity \(P < 0.001\). Only the highest dose of androstenedione (10 ng) or dehydroepiandrosterone (10 ng) caused a significant increase of the mean LRH degradation \(P < 0.05\). Neither cholesterol nor dihydrotestosterone increased peptidase activity when added to the extract.

It is suggested that it is possible that these peptidase enzymes could occupy a role in the negative feedback of steroids on the hypothalamus.

The ability of the hypothalamus to degrade physiologically active peptides in vitro has been documented for some time (Hooper 1962). It was subsequently found that the minimum activity of the peptidase was inversely related to the time of release of luteinizing hormone (Griffiths & Hooper 1972). Moreover these enzymes had more activity towards luteinizing hormone releasing hormone (LRH) than any other peptides such as oxytocin (Griffiths & Hooper 1974). The catabolism of LRH has now been demonstrated to occur during incubations with extracts of hypothalamic tissue derived from rabbits (Griffiths et al. 1974a), rats (Griffiths et al. 1974b) and sheep (Swift & Crighton 1979a).

Castration resulted in decreased degradation of LRH by these hypothalamic peptidase whereas treatment with sex steroids resulted in an increase of the peptidase activity (Griffiths & Hooper 1973a,b).

It has been demonstrated that the addition of oestradiol, progesterone or corticosterone directly to the peptidase preparation enhanced the degradation of LRH (Swift & Crighton 1979b).

This communication confirms some of the findings of the previous work and extends the results to include other androgenic and oestrogenic steroids.

Materials and Methods

1. Preparation of hypothalamic peptidase

Hypothalamic tissue was collected from the skulls of sheep which were judged to be ewes after slaughter at a local abattoir. The tissue was transported to the laboratory in chilled medium 199 (Difco Laboratories). The method of enzyme extraction was essentially that of Griffiths & Hooper (1974). Briefly, in the laboratory, the tissue was blotted, weighed and homogenised in 5 volume 0.25 M-sucrose with a glass/Teflon homogeniser. A nuclear cell debris fraction was collected by centrifugation at 500 g for 10 min at 4°C. The tissue supernatant fraction was then centrifuged at 25 000 g for 1 h at 4°C. The supernatant fraction was then dialysed against distilled water for 24 h at 4°C. The protein content of the diffusate was then determined by a biuret micromethod (Baily 1962) using bovine serum albumin as standard (Sigma chemicals, fraction V; 15% Na2). The protein concentration of the extract was adjusted to 100 μg non-diffusable protein/ml with a 3:1 mixture of 0.01
m-phosphate buffer (containing 0.9% sodium chloride, 0.01% sodium merthiolate and 0.25% egg albumin (pH 7.2)) and 0.05 m-phosphate buffer (pH 7.25).

2. Incubation of synthetic LRH with hypothalamic peptidase

Incubations (15 min) were performed at 37°C with the extract at 100 μg/ml protein in a total volume of 1 ml. The enzyme preparation was incubated with 500 ng synthetic LRH either alone or in the presence of steroid hormones. A pre-incubation period (10 min) of the extract and steroid hormones was allowed before the addition of 500 ng of synthetic LRH which initiated the incubation. The following steroids were added to the incubation to achieve the following incubations:

- Oestrone, oestradiol and oestriol (at 1 pg, 10 pg and 100 pg/ml);
- testosterone, 5 dihydrotestosterone (DHT), androstenedione (A), dehydroepiandrosterone (DHA) (at 0.1 ng, 1 ng and 10 ng/ml) and cholesterol (at 100 μg, 200 μg and 400 μg/ml).

Control incubation were arranged in the following four ways:

(a) Pre-boiled (15 min) extract and 500 ng LRH;
(b) Buffer and 500 ng synthetic LRH;
(c) Extract and buffer;
(d) Extract containing 100 pg oestradiol and 500 ng synthetic LRH.

Incubations were terminated by boiling (10 min) in a waterbath. The tubes were sealed and stored at −20°C until required for assay for residual LRH. Each incubation was performed as five replicates and on at least three occasions with different hypothalamic preparations.

3. Assay of LRH

The details of the assay method are described elsewhere (Swift & Crichton 1979a). The incubation media were diluted 1:50 before radioimmunoassay for LRH. An antiserum (Lot R42) donated by Dr. T. M. Nett, Colorado State University (final dilution 1:100 000) was used.

The sensitivity of the assay, defined as the smallest amount of unlabelled LRH which caused a significant displacement (P < 0.05) of tracer ligand from antiserum was 12 pg. The inter- and intra-assay variations were 5.4% (n = 5) and 2.6% (n = 5) respectively. Neither the effect of boiled extract nor oestradiol (100 pg) gave a significant displacement of binding of the antiserum to LRH standards over the range of the standard curve.

4. Chromatography of the oestrogens

In some incubations it was desirable to find if any effect mediated by one oestrogen could be due to interconversion to another oestrogen. As such the three oestrogens (5 mg) were incubated separately with the hypothalamic peptidase preparation (5 ml) for 30 min. At the end of the incubation the medium was rendered alkaline (pH 8.5) and washed with diethyl ether (15 ml). The ether extract was evaporated under a stream of nitrogen and the residue suspended in a minimum volume of chloroform:methanol (1:1) and chromatographed on TLC plates in toluene:ethanol (9:1) with oestrone, oestradiol and oestriol as authentic markers. The oestrogens were detected by spraying the plates with 2% sulphuric acid and heating at 100°C for 20 min.

5. Metabolism of tritiated testosterone during incubation with the hypothalamic peptidase

Tritiated testosterone (50 000 cpm [1,2,6,7,-H]testosterone, 1 ng, Radiochemical Centre, Amersham) was incubated with the peptidase preparation (1 ml). After 30 min the medium was shaken with diethyl ether (5 ml). The ether was evaporated in a stream of nitrogen. The residue was dissolved on a small volume of chloroform: methanol mixture (1:1) and chromatographed on paper in the solvent system toluene:petroleum ether:methanol:water: 5:5:7:3 by volume. The radioactive material was located by an actiograph scanner and its position compared with that of known authentic standards (oestrone, oestradiol, oestriol, testosterone, DHA and androstenedione).

6. Statistical analyses

The results were analysed by either Student's 't'-test or Duncan's Multiple Range test, as appropriate.

![Fig. 1.](image)

Effects of oestradiol and cholesterol on the degradation of LRH (500 ng) when incubated with hypothalamic extract. Each column is the mean of five incubations. Vertical bars represent ± SEM. Column (a) buffer with 500 ng LRH added; (b) pre-boiled extract with 500 ng LRH; (c) endogenous LRH present in the extract; (d) residual LRH when 500 ng LRH was incubated with the extract; (e) residual LRH when 500 ng LRH was incubated with the extract containing 100 pg oestradiol; (f-h) residual LRH when 500 ng LRH was incubated with the extract containing 100, 200 or 400 μg of cholesterol, respectively.
Results

1. Degradation of LRH in vitro and the effects of steroids

Pre-boiled hypothalamic extract did not degrade LRH significantly and the residual LRH in the extract after dialysis and dilution was not significantly different from zero. The incubation of 500 ng LRH with the hypothalamic extract for 15 min resulted in a 26% loss of radioimmunoassayable LRH (see Fig. 1 columns b, c and d).

To ensure that the results were not due to varying steroid sensitivity of different hypothalamic preparations, hypothalamic extract to which oestradiol had been added was incubated with LRH (500 ng) in each batch. The inter-assay variation obtained within these incubates was 12.9% (n = 7) (Fig. 1 column e).

Cholesterol at the three concentrations used, did not cause a significant increase in hypothalamic peptidase activity (Fig. 1 column f, g and h). Oestrone, oestradiol and oestriol all caused dose-dependent increase of the LRH degrading ability of the hypothalamic extract. All dose levels significantly increased the enzymic activity as compared with the extract without steroid added (P < 0.001, Fig. 2 columns b–k). The same dose of each oestrogen caused a similar increase in hypothalamic peptidase activity and larger doses of each oestrogen stimulated higher rates of degradation of exogenous LRH. The 10 pg and 100 pg dose effects being significantly greater than the 1 pg dose for oestrone (P < 0.01) and oestradiol (P < 0.001) but not significantly different for oestriol. Testosterone caused a dose-dependent increase of hypothalamic degradation of LRH (Fig. 3 columns d, e and f), the 1 ng and 10 ng doses causing a significant increase of the peptidase activity (P < 0.001). Androstenedione and DHA did not cause a dose-dependent increase in the degradation of LRH for only at the highest dose used (10 ng) were the results significantly different from the control (P < 0.05) (Fig. 3 columns g–i). DHT at any of the doses used did not stimulate the peptidase action (Fig. 3 columns m, n and o).

2. Chromatography of the oestrogens

When any of the oestrogens were chromatographed on the TLC system only one spot was visible which corresponded to the original oestrogen added to the medium.

3. Metabolism of tritiated testosterone

Only one radioactive peak was found in the extracted medium which corresponded to that of testosterone.
fig. 3

Effects of testosterone, A, DHA and DHT on the degradation of LRH (500 ng) when incubated with hypothalamic extract. Each column is the mean of five replicates. Vertical bars represent ± SEM. Column (a) residual LRH after incubation of LRH (500 ng) with pre-boiled extract; (b) residual LRH after incubation of LRH (500 ng) with the extract; (c) residual LRH after incubation of LRH (500 ng) with the extract containing 100 pg of oestradiol; (d–f) residual LRH after incubation of LRH (500 ng) with the extract containing 0.1, 1, or 10 ng of testosterone, respectively; (g–i) residual LRH after incubation of LRH (500 ng) with the extract containing 0.1, 1, or 10 ng of A, respectively; (j–l) residual LRH after incubation of LRH (500 ng) with the extract containing 0.1, 1, or 10 ng of DHA, respectively; (m–o) residual LRH after incubation of LRH (500 ng) with the extract containing 0.1, 1, or 10 ng of DHT, respectively.

Discussion

The results presented in this study confirms some of the work reported earlier (Swift & Crighton 1979b) and extends those findings to include two further oestrogens and a series of androgens. The previous work had found that the three steroids tested (oestradiol, progesterone and corticosterone) all increased hypothalamic peptidase activity although there are great differences in structure and function of these steroid molecules and it was considered possible that the effect may not be specific to any particular group of steroids but due to their cyclopentanophenanthrene ring structure. However, neither cholesterol nor DHT altered the rate of degradation of LRH indicating that the hypothalamic peptidase shows some specificity.

The elevation of peptidase activity by the three oestrogens was greater than any of the other steroids investigated, although the concentration of oestrogen used was 100-fold less than that of any other steroid but within the limits of oestrogen concentration in ewe plasma. The stimulatory activity of each oestrogen bears no correlation with the relative biological activity (as assessed by uterine growth) associated with the oestrogen. The effect on the peptidase cannot be due to the rapid interconversion of one oestrogen to another as significant metabolism could not be detected after incubation. It may well be that the stimulation of the peptidase could be associated with the interaction with the phenolic A ring common to all three oestrogens.

The increase of peptidase activity after incubation with testosterone and the high dose of A and DHA, but not DHT, may have indicated that the androgens (when used at concentrations higher than that normally found in ewe plasma) were aromatised during incubation as has been demonstrated to occur in the hypothalami of many species (Naftolin et al. 1975). However, the effect of testosterone did not appear to be mediated by aromatisation to oestradiol. It should be remembered that less than 0.1% conversion of testosterone to oestra¬

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show sex specific reactions to testosterone whereby the activity of 'male-derived' peptidases are stimulated by testosterone pre-treatment but 'female-derived' peptidases are not. Although care was taken during the collection of hypothalami we cannot be absolutely certain that all the tissue taken was from ewes. Thus increased peptidase activity due to androgen addition might be argued to have been derived from male hypothalami. However, as three different preparations of the peptidase was used with the same result it is thought unlikely that the effect of testosterone was due to male derived peptidase.

The possible physiological role that these enzymes play is complex and any extrapolation between these in vitro results and that which may occur naturally in vivo must be considered with caution. It is well established that after gonadectomy gonadotrophin levels rise. It has also been found that there was an associated suppression of the peptidase activity of the hypothalamus after gonadectomy but that this may be reversed by oestradiol (Griffiths & Hooper 1973a) or testosterone (Griffiths & Hooper 1973b). Thus it may be possible that these enzymes, if allowed to come into contact with LRH in vivo, may perhaps play a central role in the steroidal negative feedback system of gonadotrophin release by decreasing the amount of biologically active LRH released from the hypothalamus.

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References


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