Veno-arterial differences of immunoreactive and biologically active luteinizing hormone across the head in the cow

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SUMMARY

Immunoreactive (I) and biologically active (B) luteinizing hormone (LH) was measured in jugular vein and carotid arterial plasma of four cows 110–140 min after luteinizing hormone releasing hormone (LHRH) injection. Plasma was separated on Ultragel AcA54 and fractions corresponding to those containing authentic bovine luteinizing hormone (bLH) were assayed. Immunoreactivity in venous or arterial plasma occurred mostly in fractions corresponding in molecular weight to bLH. Low activity was measured in several other fractions. Biological activity was also high in the same fractions from venous plasma, though it was not so pronounced in arterial plasma.

A veno–arterial difference across the head for immunoreactive LH was found in all animals, and there was a significant difference in biologically active LH (mouse Leydig cell assay). The mean B:I value for immunoreactive LH was similar in venous and arterial plasma for three out of four animals, 0·72 and 0·66, respectively. In a fourth cow with a clinical history of infertility, the B:I value was low in both venous and arterial plasma, 0·19 and 0·08, respectively. The results indicate that infertility in this latter instance was associated with LHRH-induced secretion of a compound similar in molecular size and immunoreactivity to LH but deficient in biological potency.

INTRODUCTION

A recent study has shown that the ratio of biologically (B) to immunologically (I) active concentrations of luteinizing hormone (LH) in the bovine pituitary is ⁓onsiderably less than unity (B:I, 0·52±0·02), and .hat this ratio changes little during the reproductive cycle (Moss *et al.* 1988). This discrepancy between biological and immunological activity may be due to molecular heterogeneity of stored hormone since LH, in common with other glycoproteins, shows a considerable degree of microheterogeneity both in the polypeptide chain and in its attached carbohydrate (Hartree *et al.* 1985; Ward *et al.* 1986). Heterogeneity as reflected by assays based on a biological or immunological end-point (Reichert 1971; Prentice & Ryan 1975) may derive from differential processing of asparagine-linked oligosaccharides (Green & Baenziger 1988*a*,*b*) and from the feedback effect of gonadal steroids on the extent of glycosylation within pituicytes (Chappel 1985). Furthermore, Robertson *et al.* (1977) reported that differences exist in the nature of synthesized and secreted forms of LH molecules, and Veldhuis *et al.* (1987) showed that

there is a preferential release of biologically active LH in response to endogenous luteinizing hormone releasing hormone (LHRH) and to low-dose LHRH administration in man.

The purpose of the present study was to determine the biological and immunological reactivity of LH in jugular vein plasma of cows at the time of peak LH concentrations after injection of LHRH. Samples were taken simultaneously from arterial blood to determine the B:I ratio in recirculation since this would show whether the properties of hormone supplied to tissues (e.g. ovaries) were similar to those of LH in the jugular vein. Plasma was fractionated chromatographically to establish whether the majority of activity was associated with compounds that eluted at a position corresponding with authentic LH. Experiments were carried out in cyclic and pregnant animals and in a mature cow with clinical infertility to examine whether the stage of the reproductive cycle had any effect on the B:I ratio as reported at puberty in children (Reiter *et al.* 1982; Marrama *et al.* 1983) and rats (Buckingham & Wilson 1985), though apparently not in heifers (Dodson *et al.* 1988).

MATERIALS AND METHODS

Sampling

Sampling catheters were placed in a carotid artery previously exteriorized in a skin-covered loop and in a jugular vein. Venous and arterial blood (10 ml) was collected simultaneously in separate heparinized syringes at 15-min intervals for 45 min before and up to 180 min after an intravenous injection of LHRH (200 μg) and thyrotrophin-releasing hormone (TRH) (10 μg) in 2 ml 0·9 % (w/v) NaCl. (TRH was included for the purpose of studying prolactin release but only LH results are reported here.) Blood samples were kept on ice until centrifuged. Plasma obtained 110–140 min after LHRH stimulation was pooled and stored at 4 °C for c. 24 h.

Chromatographic separation

Plasma (5·0 ml) was fractionated at 4 °C on a column of Ultragel AcA54 (gel dimension 83 × 2·6 cm; LKB, Bromma, Sweden) equilibrated with 0·1 mM phosphate buffer saline (PBS), pH 7·2. The same buffer was used for all subsequent elutions. The column (C26/100; Pharmacia, Uppsala, Sweden) was calibrated with molecular weight markers before application of the sample. Fractions (11·5 ml) collected at a rate of 23 ml/h using an LKB fraction collector were stored at 4 °C and assayed for biological activity and immunological reactivity within 48 h. Void volume of the column was 115 ml.

In vitro bioassay

Biologically active LH in PBS fractions was measured using an in vitro bioassay, based upon LH-stimulated testosterone production by isolated mouse Leydig cells, adapted from the method described by Van Damme et al. (1974). Testes removed at autopsy from five 7–9-week-old F_1 (CBA♂ × Balb/cJ♀) mice previously housed in a light-(14 h light : 10 h dark) and temperature-(22 °C) controlled room were decapsulated and suspended in 10 ml sterile 10 mM Eagles minimum essential medium (pH = 7·4). (Gibco, Europe Ltd) containing 10 mM Hepes (Sigma Chemical Co. Ltd), 0·1 % bovine serum albumin (Sigma) and 0·2 % kanamycin solution (Gibco). Testes were aspirated six to eight times through a polytetrafluoroethylene tube (~ 100 × 3·0 mm) fitted to a 10 ml plastic syringe and the resulting tissue suspension was then filtered through a 60 μ nylon gauze to remove tubules and connective tissue. The filtrate was centrifuged (10 min, room temperature, 130 g) and the pellet resuspended in fresh Eagles minimum essential medium. The suspension was centrifuged once more under similar conditions and the pellet resuspended in 2 ml Earle's balanced salt solution (10 × concentration) without sodium bicarbonate

(Gibco) containing 0·1 % kanamycin, 0·1 % bovine serum albumin and 250 mM Hepes.

Leydig cells were isolated from this crude testicular cell suspension using a Percoll gradient as described by Schumacher et al. (1978). Cell viability was determined by the Trypan blue exclusion dye test and varied from 78 to 86 %.

Cell suspension aliquots (100 μl) containing 0·2 × 10⁵ Leydig cells were incubated with 100 μl standard (0–8 ng/ml NIH-LH-B4; NIH, Bethesda, Maryland) or 100 μl sample in triplicate for 2 h in a shaking water bath at 34 °C in an atmosphere of 95 % O_2 : 5 % CO_2. Standards and samples were then heated to 56 °C for 30 min to inactivate the cells and to denature binding proteins. Samples were cooled, stored at 4 °C for c. 24 h and assayed for testosterone concentrations by radioimmunoassay after extraction with 4·0 ml diethyl ether.

Antiserum was raised in a ewe against testosterone-3-BSA conjugate. Cross-reactions of other steroids were as follows: dihydrotestosterone, 52·0 %; 5-androstane-3,17β-diol, 34·4 %; androstenedione, 0·8 %; androsterone, < 0·5 %; dehydroepiandrosterone, < 0·5 %; and oestradiol-17β, 0·1 %. Mean sensitivity was 0·22 ± 0·026 ng/ml (4 assays) and extraction efficiency 88·4 ± 9 % (4 assays). Intra- and interassay coefficients of variation were 6·1 and 14·6 % respectively.

Radioimmunoassay of LH

Immunoreactive LH in 0·4 ml eluted fractions was measured using the technique described by Jenkin, Heap & Symons (1977) except that a second antibody was used to separate free and antibody-bound radioligand. The bovine standard NIH-LH-B4 was used for the construction of calibration lines. Assay sensitivity, estimated as twice the standard deviation of binding of radioligand in the absence of the unlabelled ligand, was 0·53 ng/ml. Intra- and interassay coefficients of variation were 12·9 and 14·7 % respectively.

RESULTS

The elution profile from Ultragel AcA54 showed that most plasma proteins eluted between fractions 12 and 19, authentic bovine LH between fractions 20 and 23 (apparent M_r c. 35000–40000) and free iodine and degraded products between fractions 40 and 45. Negligible biological or immunological activity (< 0·1 %) eluted in the void volume.

Immunoreactivity in fractionated jugular vein plasma was high in fractions 20 to 23 in all animals (Fig. 1). The sum of these fractions represented 29 ± 3 % of the total immunological reactivity in all fractions. Fractionation profiles showed low immunological reactivity in several other fractions
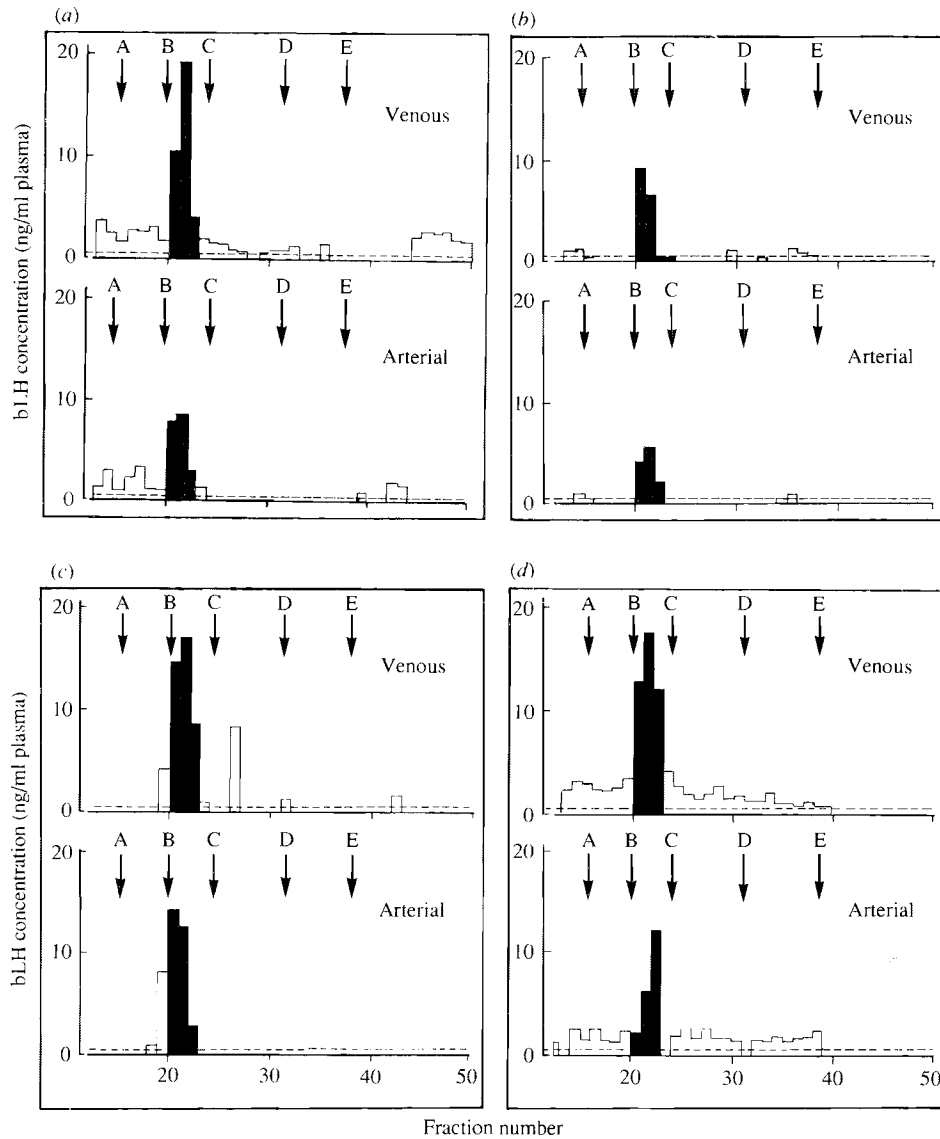
Fig. 1. Distribution of immunoreactive bovine luteinizing hormone (bLH) in jugular venous and carotid arterial plasma after gel filtration (Ultragel AcA 54). Results are shown for cows (a) C42, (b) A104, (c) E30 and (d) D46 treated with LH releasing hormone (200 μg, i.v.) and sampled 110–140 min later. (Total concentration of LH and reproductive status of animals are given in Table 1.) (   ), limits of assay sensitivity; (■), fractions in which authentic bLH is eluted. Elution of various molecular size markers is indicated by vertical arrows: A, bovine serum albumin (M_r 67000); B, ovalbumin (M_r 45000); C, carbonic anhydrase (M_r 30000); D, cytochrome c (M_r 12300); E, potassium dichromate (M_r 5000).

suggesting the presence of other cross-reacting compounds. The amount of the dominant compound (fractions 20 to 23) was greater in venous than in arterial plasma (Fig. 1; Table 1).

Biological activity in jugular venous plasma was also high in fractions 20 to 23 (Fig. 2). The sum of these fractions represented $34 \pm 7\%$ of the total immunological reactivity in all fractions. Biological activity in fractionated arterial plasma did not show a consistent peak and, as in venous samples, activity was recorded in several fractions with a wide range of apparent molecular sizes.

The immunoreactivity associated with the dominant compound (fractions 20 to 23) was always higher

Table 1. *Immunological reactivity (I) and biological activity (B) of luteinizing hormone (LH) in jugular venous (V) and carotid arterial (A) plasma collected 110–140 min after LHRH injection in four cows at different times of the reproductive cycle. Samples were fractionated by gel chromatography on Ultragel AcA54 and fractions 20 to 23 containing bovine LH were analysed*

Animal	Reproductive status	LH (ng/ml)				V–A difference		B:I ratio	
		I		B					
		V	A	V	A	I	B	V	A
C42	Pregnant (259 days)	35·7	19·7	24·8	16·6	16·0	8·2	0·69	0·84
A104	Pregnant (269 days)	18·0	12·3	13·0	4·4	5·7	8·6	0·72	0·36
E30	Not pregnant (cyclic)	44·0	37·6	33·3	29·3	6·4	4·0	0·76	0·78
D46	Not pregnant (infertile)	45·4	22·6	8·4	1·7	22·8	6·7	0·19	0·08
Mean ± s.e.m. ($\log_e$)		3·51 ±0·21	3·06 ±0·23	2·85 ±0·31	2·05 ±0·64	2·37 ±0·34	1·89 ±0·18	−0·65 ±0·33	−0·99 ±0·55
Mean (back-transformed)		33·4	21·3	17·3	7·8	10·7	6·6	0·52	0·37

in venous than in arterial plasma, the mean veno–arterial difference across the head being 10·7 ng/ml. Biological activity in the same fractions was also higher in venous than in arterial plasma ($P < 0.01$, paired t test) and the mean veno–arterial difference was 6·6 ng/ml. The veno–arterial differences for immunoreactivity and biological activity were not significantly different.

The B:I ratio, a measure of biological potency, for fractions 20 to 23 was similar for jugular vein plasma in three out of four animals (0·72±0·02), but in D46, a cow with a history of infertile cycles, the biological potency was low (Table 1; Fig. 2). The comparable B:I values for arterial plasma in three out of four animals were 0·66±0·15, whereas in D46 it was also low (0·08). In all four experiments, biological potency was less than unity.

DISCUSSION

Bovine LH released from the pituitary in response to LHRH injected intravenously was fractionated by molecular sieving using Ultragel AcA54 gel chromatography. Separation in this instance depended on differences in molecular size rather than charge heterogeneity achieved by isoelectric focusing (Keel & Grotjan 1984; Keel *et al.* 1987). Immunological reactivity in fractions corresponding to the elution position of authentic LH was consistently greater in venous than in arterial plasma taken 110 to 140 min after agonist challenge. This was also true for biological activity in which values were significantly greater in venous than arterial samples. The veno–arterial difference across the head was similar for measurements of immunoreactivity and biological activity which is consistent with the action of LHRH

in producing a substantial release of pituitary LH in the cow, as in other species.

The immunoassay and biological assays utilized the same standard preparation of LH so that direct comparisons between the two assays are appropriate. The ratio of B:I was less than unity in each experiment indicating the presence in jugular vein and carotid artery plasma of immunoreactive compounds with diminished biological potency after LHRH stimulation. Most B:I values were similar to those obtained with bovine anterior pituitary (Moss *et al.* 1988), but lower than those in man (Veldhuis *et al.* 1987). In the present study, low biological potency was observed in cow D46, an animal with a clinical history of infertility in which the B:I ratio was only 0·19 and 0·08 in jugular vein and carotid artery plasma, respectively. These results indicate that infertility was associated with the secretion of an immunologically reactive form(s) of LH possibly deficient in receptor binding and therefore biological activity, as reported for certain isoforms of follicle-stimulating hormone (FSH) (Ulloa-Aguirre & Chappel 1982; Foulds & Robertson 1983). The finding that the B:I ratio was similar for compounds in the jugular vein and carotid artery implies that the potency of LH was not affected selectively by metabolism during recirculation.

In the present study most of the immunoreactivity in jugular vein plasma was associated with compounds having an apparent M_r of c. 35000. This value corresponds with the apparent size of authentic bLH in this system but was slightly greater than that of published values probably on account of the use of gel filtration rather than polyacrylamide gel chromatography. Bovine LH is known to have two nonidentical subunits (α and β), noncovalently linked, with M_r of 14000 and 18000, respectively. Although the subunits
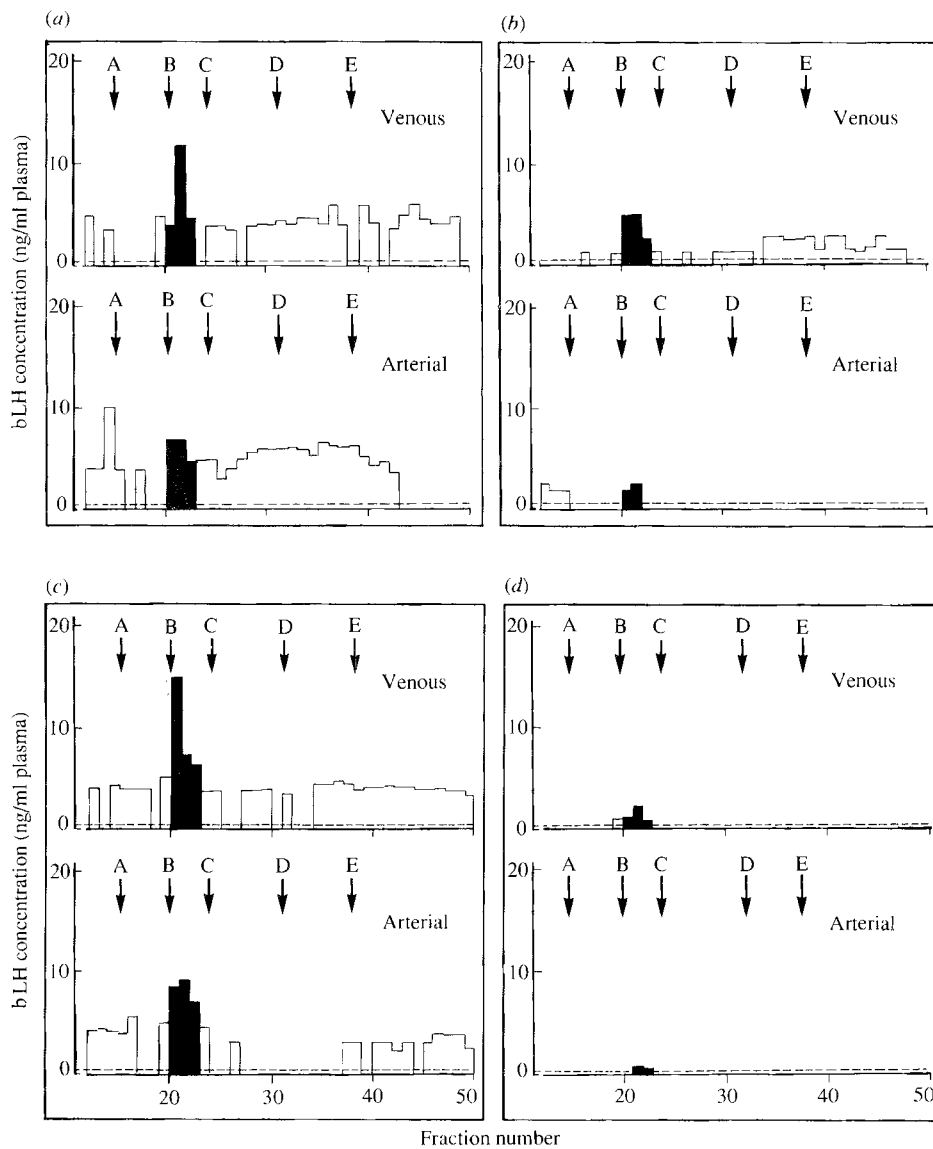
Fig. 2. Distribution of biologically active bovine luteinizing hormone (bLH) in jugular venous and carotid arterial plasma after gel filtration (Ultragel AcA54). Results are shown for cows (*a*) C42, (*b*) A104, (*c*) E30 and (*d*) D46 treated with LH releasing hormone (200 μg, i.v.) and sampled 110–140 min later. (Total concentration of LH and reproductive status of animals are given in Table 1.) (——), limits of assay sensitivity and (■), fractions in which purified bLH is expected to elute. Elution of various molecular size markers is indicated by vertical arrows: A, bovine serum albumin (M_r 67000); B, ovalbumin (M_r 45000); C, carbonic anhydrase (M_r 30000); D, cytochrome c (M_r 12300); E, potassium dichromate (M_r 5000).

have immunological reactivity they have little or no biological activity when dissociated (Jackson & Liu 1982). It is thus unlikely that any compounds detected by bioassay with an apparent M_r of < *c*. 30000 would represent subunits of LH.

Previous work using gel filtration of human pituitary extracts revealed the presence of a 'large'

LH molecule (Jackson & Liu 1982). Large molecular weight compounds were also identified in the present study between the void volume and peak activity. These compounds were detected by both assays in venous as well as in arterial plasma, though not in all animals studied. Compounds with an apparent smaller molecular size were also detected, possibly repre-

senting deglycosylated forms of LH or its free subunit. Free β-subunits in plasma have been reported to increase markedly after LHRH stimulation (Graesslin *et al.* 1976; Sawyer-Steffan *et al.* 1982; Meldrum *et al.* 1984).

The near lack of biologically active LH in samples from a cow with a clinical history of infertility was in agreement with observations in humans with delayed pubertal development (Reiter *et al.* 1982). Similarly peripubertal rats weighing < 60 g possess a biologically inactive pleiomorphic form of LH which may explain their failure to ovulate (Buckingham & Wilson, 1985). In both these studies the presence of immunologically reactive LH was recorded, as in the present study. Thus, LH concentrations measured by radioimmunoassay alone as an indicator of pituitary function in the cow may be inadequate to predict target organ response in the absence of a parallel biological test.

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