IGF-I stimulation of luteinizing hormone secretion, IGF-binding proteins (IGFBPs) and expression of mRNAs for IGFs, IGF receptors and IGFBPs in the ovine pituitary gland

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Abstract

Circulating concentrations of insulin-like growth factor-I (IGF-I) are reduced in juvenile sheep during nutritional growth restriction and the associated delay in puberty. Since exogenous IGF-I has been shown to stimulate luteinizing hormone (LH) secretion, it is postulated that endogenous IGF-I may act as a stimulatory metabolic signal to the pubertal ovine hypothalamo-pituitary axis, yet its site of action is unknown. Using coronal hypothalamic and pituitary sections from pubertal ewe lambs, *in vitro* autoradiography was used to localise ¹²⁵I-labelled IGF-I binding, and gene expression for components of the IGF system was localised by *in situ* hybridisation using oligonucleotide probes. High concentrations of ¹²⁵I-IGF-I binding were seen in the pars tuberalis (PT) and pars distalis (PD) of the pituitary, and relatively little in the hypothalamus; binding in the PT but not the PD was displaced by excess unlabelled IGF-I. Large amounts of mRNA were detected for the type-1 receptor (IGF-1R) and for IGF-binding protein (IGFBP)-5, localised to the PT and PD, and less intense specific hybridisation signals were obtained with mRNAs for IGF-II, type-2 receptor

(IGF-2R) and IGFBP-3. There was some evidence for specific hybridisation to IGFBP-4 mRNA in the PT. IGF-I, IGFBP-1 and IGFBP-2 mRNAs were not detected in PT and PD. None of the genes were expressed in hypothalamic tissue. Western-ligand binding on PD extracts from male castrates revealed by their molecular weights the likely presence of IGFBPs-2, -3, and -5. Finally, cultured PD cells from abattoir-killed sheep were challenged with IGF-I (0.1, 1, 10 or 30 nM) or luteinizing hormone-releasing hormone (LHRH, 10 nM) alone, or both together. Basal LH output was stimulated by 10 nM IGF-I ($120 \pm 11.2\%$, $P > 0.05$), 30 nM IGF-I ($148 \pm 12.8\%$, $P < 0.01$), and LHRH alone ($200 \pm 16.1\%$, $P < 0.001$); there was no additive or subtractive effect of LHRH and IGF-I given together. Thus, an intrapituitary IGF system exists in sheep and the present results are consistent with an endocrine role for IGF-I in nutritional modulation of LH secretion at the level of the pituitary gland.

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Introduction

It is postulated that insulin-like growth factor-I (IGF-I) is partly responsible for nutritional modulation of reproductive function in mammals. Circulating concentrations of IGF-I are elevated at puberty in rodents (Handelsman *et al.* 1987), primates (Mauras *et al.* 1996) and ruminants (Adam *et al.* 1995). Exogenous IGF-I administered to female rhesus monkeys by subcutaneous infusion accelerated puberty, probably by stimulating luteinizing hormone-releasing hormone (LHRH) neuronal activity (Wilson 1998); moreover, centrally administered IGF-I both stimulated LHRH output and significantly advanced puberty in rats, suggesting that IGF-I is a metabolic trigger for puberty, acting at the hypothalamic level (Hiney *et al.* 1996). However, an earlier study indicated that there may

be fundamental species differences in the central distribution of receptors for IGF-I, since ¹²⁵I-IGF-I binding was localised in the hypothalamic median eminence (ME) of rats, but in the pars tuberalis of the pituitary (PT) and not in the ME of sheep (Williams *et al.* 1995). It may therefore be unwise to extrapolate putative IGF-I feedback mechanisms between species. Furthermore, given their fundamentally different nutritional physiology, differences in central nutritional feedback between monogastric and ruminant species may perhaps be anticipated. IGF-I stimulation of luteinizing hormone (LH) release from cultured anterior pituitary cells has been demonstrated for rats (Soldani *et al.* 1994) and pigs (Whitley *et al.* 1995), but not for a ruminant species.

In view of the observation that peripherally administered IGF-I can acutely stimulate LH secretion in sheep

(Adam *et al.* 1997a), the objectives of the present studies were to determine *in vitro* whether its likely site of endocrine action on gonadotrophin secretion in this species is at the level of the hypothalamus or the pituitary gland. IGF-I mediates its effects through binding to a type-1 IGF receptor (IGF-1R) on target cells (LeRoith *et al.* 1995), where its actions are modulated by IGF-binding proteins (IGFBPs) (Jones & Clemmons 1995). We therefore investigated ^{125}I -IGF-I binding and gene expression for IGFs, their receptors and binding proteins along the ovine hypothalamo-pituitary tract. In addition, western-ligand binding was used to determine IGFBP activity in anterior pituitary homogenates. Lastly, since high concentrations of gene expression for components of the IGF system were found in the pituitary rather than the hypothalamus of this species, we sought to establish whether IGF-I induced LH release from ovine anterior pituitary cells in culture.

Materials and Methods

Tissue collection for in vitro autoradiography and in situ hybridisation

Brains and pituitary glands were obtained from four pubertal Suffolk $\times$ Greyface ewe lambs that had been reared on unrestricted food for another study (Adam *et al.* 1998). They were euthanised by lethal injection with sodium pentobarbitone (Euthesate, Willows Francis Veterinary, Crawley, UK) at 8 months of age. Post-mortem examination of their ovaries revealed the presence of at least one corpus luteum, indicating that they had each initiated oestrous cyclicity and were in the luteal phase at death. Following decapitation, brains and attached pituitaries were rapidly removed, frozen in isopentane chilled over dry-ice, and stored at -80°C . Coronal cryostat sections ($20\ \mu\text{m}$) of hypothalamic and pituitary tissue were cut, thaw-mounted onto slides double-coated with gelatin and poly-L-lysine, and stored at -80°C .

In vitro autoradiography

Recombinant human IGF-I (Bachem UK Ltd, Essex, UK) was iodinated and autoradiographic localisation of ^{125}I -IGF-I binding sites was performed as described in detail previously (Williams *et al.* 1995). Briefly, paired sections were washed at 4°C for 6 min in high-salt, low-pH buffer ($0.2\ \text{M}$ NaAc, $0.5\ \text{M}$ NaCl with 0.1% BSA, pH 3.5), in order to dissociate bound IGF-I, then washed for 20 min in $50\ \text{mM}$ Tris-HCl (pH 7.4 and containing $5\ \text{mM}$ MgCl_2). They were then incubated with $50\ \text{pM}$ ^{125}I -IGF-I (specific activity approximately $250\ \text{d.p.m./fmol}$) either with or without $50\ \text{nM}$ IGF-I (Bachem UK Ltd) in buffer containing 0.2% BSA and $1\ \text{kiU}$ aprotinin/ml at 4°C for 24 h. Sections were then washed in $50\ \text{mM}$ Tris-HCl (pH 7.4 and containing

$5\ \text{mM}$ MgCl_2) for 15 min at 4°C , rinsed in distilled water and air-dried; X-AR OMAT film (Kodak, Rochester, NY, USA) was then exposed to the sections for 2 weeks. Approximately 30 pairs of hypothalamic and pituitary sections were used from each sheep.

In situ hybridisation

The *in situ* hybridisation procedure was performed as described previously, using single stranded 45-base oligo-nucleotide probes for IGF-I, IGF-II, IGF-1R, IGF-2R, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5 (Stevenson *et al.* 1994, Reynolds *et al.* 1997, Wathes *et al.* 1998, Osgerby *et al.* 1999). These probes have all been validated for use in tissues from the ewe. Briefly, sections were fixed in 4% paraformaldehyde in $0.01\ \text{M}$ PBS, washed in $0.01\ \text{M}$ PBS and dehydrated sequentially in 70 and 95% ethanol. Antisense and sense oligonucleotide probes were ^{35}S -labelled at the 3'-ends using terminal deoxynucleotidyl transferase (Pharmacia Biotechnology, St Albans, UK). Paired sections were hybridised with $110\ 000\ \text{c.p.m.}$ antisense or sense probe and incubated overnight at 42°C . After incubation, they were washed in saline sodium citrate ($1 \times \text{SSC}$)/ 0.2% sodium thiosulphate pentahydrate for 30 min at room temperature, then in $1 \times \text{SSC}$ / 0.2% sodium thiosulphate pentahydrate for 60 min at 55°C . Finally, slides were rinsed for 1 min in each of the following: $1 \times \text{SSC}$, $0.1 \times \text{SSC}$, 70% ethanol and 95% ethanol. Once dry, all slides were exposed to Hyperfilm- β -max (Amersham International plc, Amersham, Buckinghamshire, UK). Selected individual slides were subsequently dipped in photographic emulsion (LM1; Amersham International plc), stored at 4°C , then developed and counterstained with haematoxylin and eosin. For each probe, approximately 12 pairs of hypothalamic and pituitary sections were used from each sheep. Sections of ovine uterus and/or placenta, which had previously been shown to express the various components of the IGF system, were included within each batch of slides processed to provide positive controls.

Western-ligand binding

Anterior pituitary glands were collected from eight Suffolk $\times$ Greyface male castrate sheep that had received unrestricted food as part of another study (Adam *et al.* 1997b). The glands were dissected free of connective tissue and frozen glands were homogenised at a concentration of $100\ \text{mg}$ tissue per ml ice-cold SDS/cholate buffer, sonicated for 15 s, and centrifuged at $13\ 600\ \text{g}$ for 10 min at 4°C (after Funston *et al.* 1995a). The supernatant was removed, stored at -80°C , then subjected to SDS-PAGE followed by Western-ligand blot analysis to determine the presence of IGFBPs (using the methods of Funston *et al.* 1995a, with minor adjustments). Bradford protein determinations were first carried out on the

anterior pituitary homogenates; aliquots were then diluted to 50 µg protein/15 µl homogenisation buffer before being mixed with 15 µl gel loading buffer (62.5 mM Tris, 2% SDS, 0.02% bromophenol blue, 10% glycerol, pH 6.8). Samples were boiled for 4 min, placed on ice for 5–10 min, loaded into wells and electrophoresed through a 3% stacking gel and a 12.5% separating gel in non-reducing conditions (Hossenlop *et al.* 1986). Protein molecular weight markers were electrophoresed at the same time in adjacent lanes. Separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine in 15% methanol), and IGFBP activity was detected by incubating membranes with ^{125}I -IGF-I (after Lee *et al.* 1991). After electro-transfer, membranes were air-dried, incubated overnight at 4 °C in TBS (10 mM Tris (pH 7.4), 150 mM NaCl and 0.05% sodium azide) containing 1% polyvinylpyrrolidone (PVP40), washed in TBS for 2×5 min, then incubated in pairs at 4 °C with ^{125}I -IGF-I (5000 000 c.p.m.) in TBS containing 0.1% Tween-20 and 1% BSA. After incubation, membranes were washed for 2×10 min in TBS containing 0.1% Tween-20, then for 3×10 min in TBS, before being air-dried; X-AR OMAT film (Kodak) was then exposed to the membranes at 4 °C for 2 weeks.

Pituitary cell culture

Pituitary glands were aseptically removed from abattoir-killed sheep (male castrates) during the breeding season and used for five culture experiment replicates ($n=3$ glands per culture). The anterior lobe was dissected out and the cells enzymatically dispersed and cultured as previously described (Ross *et al.* 1996). Cells were suspended at 5×10^5 /ml in culture medium (Dulbecco's modified Eagle's medium (DMEM) containing 12.5% lamb serum), plated at 0.5 ml cell suspension per well in a 24-well plate and cultured at 37 °C. On Day 4 of culture, medium was discarded and plates were rinsed with serum-free medium. Cells were then cultured in 0.5 ml fresh serum-free medium and challenged (six wells per treatment) with IGF-I (recombinant human, Bachem UK Ltd; 0.1, 1, 10 or 30 nM) or LHRH (10 nM) alone or in combination (10 nM LHRH and 0.1 or 30 nM IGF-I). Four hours after treatment, medium was collected, stored at -20 °C and assayed for LH.

LH assay

LH concentrations in culture medium were quantified by radioimmunoassay kit (National Institute of Digestive and Kidney Disorders (NIDDK), Rockville, MD, USA) in one assay run and expressed in terms of the reference standard NIDDK-oLH-1-2 (Adam *et al.* 1997a). The intra-assay coefficient of variation was 0.13 and the assay sensitivity was 0.05 ng/ml.

Figure 1 ^{125}I -labelled IGF-I binding to coronal sections of ovine hypothalamus and pituitary in the absence (a, c) and presence (b, d) of 50 nM unlabelled IGF-I. PT, pars tuberalis; PD, pars distalis; 3 V, third ventricle.

All chemicals were purchased from Sigma UK (Poole, Dorset, UK) unless otherwise stated.

Statistical analysis

LH concentrations in the five replicate pars distalis (PD) cell cultures following control, IGF-I and LHRH challenges were analysed by ANOVA (two-factor with replication) to determine significant treatment effects. Data (means and s.e.m. values) were then transformed to express treatment effects as percentage stimulation of LH above the controls.

Results

In vitro autoradiography

Autoradiographic localisation revealed high concentrations of ^{125}I -IGF-I binding in the pars tuberalis (PT) and PD of the pituitary, but less in the hypothalamus (Fig. 1). Binding in the PT but not the PD was displaced by excess unlabelled IGF-I.

In situ hybridisation

Large amounts of mRNA were detected for IGF-1R and IGFBP-5, localised to the PT and PD of the pituitary, and

Figure 3 Photographic emulsions ($\times 40$ magnification) of ovine pituitary (pars distalis) sections hybridised with antisense oligonucleotide probes to (a) IGF-1R mRNA and (c) IGFBP-5 mRNA; (b) and (d) show adjacent sections hybridised with corresponding sense probes.

less intense specific hybridisation signals were obtained with mRNAs for IGF-II, IGF-2R and IGFBP-3 localised to the same regions (Figs 2 and 3). IGFBP-4 mRNA gave a weak hybridisation signal in the PT but not in the PD. The specificity of hybridisation to the IGFBP-2 antisense probe was unproved since this was of similar intensity to that of the sense probe in PT and PD tissue. There was little evidence for specific hybridisation of any of the antisense probes to hypothalamic tissue. In all sections, the pars intermedia (PI) of the pituitary hybridised intensely and non-specifically with both sense and antisense probes. IGF-I and IGFBP-1 mRNAs were not detected in the hypothalamus or in the pituitary, although positive signals were found in the positive control sections of ovine uterus (data not shown).

Western-ligand binding

Proteins with ^{125}I -IGF-I binding activity were detected in anterior pituitaries (Fig. 4) and identification was hypothesised according to their molecular weights (Hossenlop

et al. 1986, Funston *et al.* 1995a,b, Jones & Clemmons 1995). Binding was observed at approximately 36–40, 32 and 29 kDa (identified as IGFBP-3, IGFBP-2 and IGFBP-5 respectively).

Pituitary cell culture

Relative to control treatments, 10 ($P>0.05$) and 30 nM IGF-I ($P<0.01$) increased LH secretion in anterior pituitary cell cultures, but the lower concentrations of IGF-I had no effect (Fig. 5). LHRH stimulated LH release ($P<0.001$), with no additional effects from co-administered IGF-I.

Discussion

Intense ^{125}I -IGF-I binding was localised in the pituitary tissue of the present sheep, with much less binding seen over the hypothalamic tissue, in agreement with the findings of Williams *et al.* (1995). High concentrations of

Figure 2 Autoradiographic localisation of mRNAs for IGF-1R, IGF-2R, IGFBP-2, -3, -4 and -5, and IGF-II following hybridisation of ovine hypothalamus and pituitary sections with antisense (A/S, left-hand side) or sense (S, right-hand side) oligonucleotide probes. PT, pars tuberalis; PD, pars distalis; PI, pars intermedia.

Figure 4 Ligand blot detection of IGFBPs in the anterior pituitary of two representative sheep. Identities of bands at 36–40, 32 and 29 kDa were estimated from Funston *et al.* (1995a,b) as IGFBP-3, IGFBP-2 and IGFBP-5 respectively.

specific ^{125}I -IGF-I binding in the PT were indicative of the presence of functional IGF-I receptors in this region, but the specificity of the intense binding seen in the PD was not confirmed by this method since it was not

Figure 5 Effect of IGF-I and LHRH on LH release by cultured ovine anterior pituitary cells. Results from five replicate experiments, each with six wells per treatment (2.5×10^5 cells per well), are expressed as a percentage of basal LH release (mean value $\pm$ S.E.M., 69.9 ± 5.01 ng/ml) in control cultures (C). *** $P < 0.001$, ** $P < 0.01$ compared with C.

displaced by an excess of unlabelled IGF-I. This lack of displacement is a recurrent feature of ovine PD tissue (LM Williams, personal communication) and could have been attributable to the abundance of IGFBPs, the gene and/or peptide expression of which was localised in the same tissue. However, large amounts of IGF-1R mRNA, localised to the same pituitary PT and PD tissues, verified the probable abundant presence of the type-1 receptor. In the earlier study (Williams *et al.* 1995), the ^{125}I -IGF-I binding over the PT appeared to represent a mixed population of functional IGF-I receptors and binding proteins; the present results now extend this observation to include the PD. ^{125}I -IGF-I binding was not seen in the PI; the strong hybridisation signals, seen in this region, to the IGF receptor and IGFBP oligonucleotide probes were detected at similar intensities with both sense and antisense probes and were therefore non-specific. This was possibly attributable to the high density of melanotrophs in the PI, since similar non-specific binding has been reported in uterine melanocytes (Stevenson *et al.* 1994).

The low level of specific ^{125}I -IGF-I binding seen throughout the hypothalamus was not accompanied by any IGF receptor or IGFBP mRNA in this region. This provided unconvincing evidence for endocrine actions of IGF-I within the hypothalamus compared with the overwhelming evidence for ^{125}I -IGF-I binding, IGF receptor and IGFBP gene expression in pituitary tissue. The ^{125}I -IGF-I was possibly binding in the hypothalamus to insulin receptors, which are widespread in the brain and have some affinity for IGF-I (Schwartz *et al.* 1992).

Although mRNA for the type-2 receptor was also detected in PT and PD tissues, this receptor does not bind IGF-I (Kornfeld 1992), and its presence could be to act as a clearance receptor for IGF-II (Baker *et al.* 1993) since IGF-II mRNA was localised in the same tissues. The function of this putative intrapituitary paracrine/autocrine role for IGF-II is unknown.

The present data demonstrate the presence in ovine PT and PD pituitary tissue of gene expression for IGFBP-3 and IGFBP-5 but not IGFBP-2. In addition, IGFBP-2, -3 and -5 peptides were detected by Western-ligand blotting in ovine anterior pituitaries, as previously reported for both ovine and bovine anterior pituitaries (Funston *et al.* 1995a,b; Snyder *et al.* 1999). This would suggest that the IGFBP-2 peptide in pituitary tissue is synthesised elsewhere; indeed, IGFBP-2 is found in circulating blood (Funston *et al.* 1995a,b). It is acknowledged that the relative amounts of IGFBP gene and peptide expression (examined here in luteal-phase ewes and male castrates respectively) may vary with reproductive status, since anterior pituitary IGFBP-2 is apparently increased by oestradiol in ewes (Clapper *et al.* 1998), and the relative amounts of the anterior pituitary IGFBPs vary with stage of the oestrous cycle in cows (Funston *et al.* 1995a). The precise functions of IGF binding proteins within the pituitary have yet to be elucidated but are likely to involve

modulation of IGF actions (Jones & Clemmons 1995). They are apparently nutritionally sensitive, in that changes in the amount of IGFBPs are seen in the serum and hypothalamo-pituitary tracts of thin ewes with low circulating IGF-I relative to fatter ewes (Snyder *et al.* 1999). In summary, the presence of IGFBPs with the same localisation as IGF-1R is consistent with the intrapituitary nutritional feedback actions of IGF-I.

IGF-I actions within the ovine PT have previously been demonstrated in short-term cultures by its ability to regulate mitogen-activated protein kinase (Hazlerigg *et al.* 1996). Its precise physiological role in the PT is still open to speculation but the presence of gene expression for components of an IGF system, in addition to the specific ^{125}I -IGF-I binding, lends support to its significance.

Since gene expression for IGF-I itself was not detected in the present ovine pituitaries, the intrapituitary actions of IGF-I are likely to be endocrine rather than paracrine/autocrine. From photographic emulsion-coated sections, it could be seen that the most abundant mRNAs (IGF-1R and IGFBP-5) were homogeneously and abundantly distributed throughout the anterior pituitary, which is compatible with their expression by endocrine cells (Fig. 3). Clearly, the endocrine role of IGF-I in the ovine anterior pituitary is likely to include actions on other cell types, such as somatotrophs (Fletcher *et al.* 1995), but putative actions on gonadotrophin secretion were the focus of the present study.

Given that pituitary cells apparently had the machinery to respond to circulating IGF-I, the final evidence supporting our hypothesis was the demonstration that physiological concentrations of IGF-I stimulated LH release from cultured ovine pituitary cells. This effect was dose-dependent, as in eel pituitary cell cultures (Huang *et al.* 1998). LHRH alone provided greater LH stimulation and was unaffected by IGF-I co-administration. This suggested that the present actions of IGF-I on LH were direct rather than via enhanced LHRH-induced LH release, in contrast to the evidence for both mechanisms from rat pituitary cell culture data (Soldani *et al.* 1994, 1995). The stimulatory dose of IGF-I alone *in vitro* (30 nM) approximates to plasma concentrations in pubertal sheep (CL Adam & PA Findlay 1997, unpublished observations) and is therefore in the physiological range. However, the LHRH dose used in this experiment was probably excessive, producing maximal LH release, and it is acknowledged that different relative concentrations of IGF-I and LHRH may have produced different responses.

The available evidence points to IGF-I acting as a circulating messenger that can mediate nutritional effects on gonadotrophin secretion in sheep. The present findings are consistent with this action being at the level of the pituitary gland rather than the hypothalamus, unlike the rat model (Hiney *et al.* 1996), but in agreement with its site of feedback actions on ovine growth hormone

secretion (Fletcher *et al.* 1995). Accordingly, IGF-I concentrations in the cerebrospinal fluid of sheep during nutritional stimulation of gonadotrophin secretion were not elevated despite concomitantly raised concentrations in plasma (Miller *et al.* 1998), and IGF-I administered intracerebroventricularly to sheep had no effect on pulsatile LH secretion (L Chagas, D Blache & GB Martin, personal communication). Although the major focus of research into nutritional modulation of the gonadotrophic axis in the past has addressed actions at the hypothalamic level (e.g. Miller *et al.* 1998), it is now clear that actions at the pituitary provide further control. Since the precise regulation of gonadotrophin secretion is vital to reproductive function, it is perhaps not surprising that this should be achieved by the interaction of components at both the pituitary and hypothalamic level (Evans 1999).

In summary, the present data demonstrate the existence of an intrapituitary IGF system in the sheep and provide evidence consistent with an endocrine role for IGF-I in nutritional modulation of LH secretion at the level of the pituitary gland.

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