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Pituitary expression of LHβ- and FSHβ-subunit mRNA, cellular distribution of LHβ-subunit mRNA and LH and FSH synthesis during and after treatment with a gonadotrophin-releasing hormone agonist in heifers

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Abstract. The aim was to examine transcriptional and post-transcriptional regulation of LH and FSH biosynthesis. Female cattle were allocated to three groups: (*i*) Group 1, control ($n = 6$), synchronized to be at around Day 11 of the oestrous cycle on Day 31; (*ii*) Group 2 ($n = 6$), treated with gonadotrophin-releasing hormone (GnRH) agonist (deslorelin) for 31 days; and (*iii*) Group 3 ($n = 6$), treated with deslorelin for 28 days. All animals were slaughtered on Day 31. For animals in Group 2, pituitary content of LHβ-subunit mRNA was suppressed 60% ($P < 0.001$) and LH 95% ($P < 0.001$), whereas FSHβ-subunit mRNA was suppressed 25% ($P > 0.05$) and FSH 90% ($P < 0.001$). Three days after treatment with deslorelin (Group 3) LHβ-subunit mRNA and LH remained suppressed (50% and 95%, respectively; $P < 0.001$). At the same time, FSHβ-subunit mRNA did not differ from controls ($P > 0.05$) whereas FSH remained reduced by 80% ($P < 0.001$). The ratio of LHβ-subunit mRNA present in the nucleus versus cytoplasm of gonadotroph cells was reduced ($P < 0.05$) in heifers during treatment with deslorelin (0.59 ± 0.05) compared with the ratio in control heifers (1.31 ± 0.22) and heifers 3 days after discontinuation of treatment (1.01 ± 0.05). The findings indicated that treatment with GnRH agonist can suppress LHβ-subunit mRNA expression without any significant effect on FSHβ-subunit mRNA. As LH and FSH contents were suppressed to a greater degree than their β-subunit mRNAs, it would appear that treatment with a GnRH agonist might influence gonadotrophin biosynthesis by a post-transcriptional mechanism(s). For LHβ-subunit mRNA, this would appear not to be reduced export of message from the nucleus.

Introduction

The regulation of LH and FSH biosynthesis is generally thought to occur predominantly at the level of transcription (Brown and McNeilly 1999). There is evidence to suggest, however, that there exist multiple control points from the expression of LH and FSH genes to biosynthesis of these glycoproteins (D'Occhio *et al.* 2000*a*; Staton *et al.* 2000). Some control points are at the post-transcriptional level and may include alternate splicing, nuclear export, mRNA stability, and translation initiators and inhibitors (Staton *et al.* 2000). In cultured rat gonadotroph cells, progesterone and estradiol increased the stability of LHβ-subunit mRNA (Park *et al.* 1996) and testosterone increased the stability of FSHβ-subunit mRNA (Paul *et al.* 1990). In female rats, inhibin reduced the stability of FSHβ-subunit mRNA (Burger *et al.* 2001) whereas ovariectomy increased FSHβ-subunit mRNA levels, apparently by a non-transcriptional mechanism (Dalkin *et al.* 1993). Mechanisms for post-transcriptional regulation of LH and FSH may prove to be of particular importance in the integrated response of gonadotroph cells to stimulation from gonadotrophin releasing hormone (GnRH) and feedback regulation by the gonads.

Treatment with agonists of GnRH provides an experimental model to study mechanisms of transcriptional and post-transcriptional control of gonadotrophins. Exposure to GnRH agonists leads to gonadotroph cell dysfunction that includes a reduction in pituitary content of gonadotrophins (D'Occhio *et al.* 2000*b*). The latter was associated with reduced pituitary content of LHβ-subunit mRNA in male cattle (Melson *et al.* 1986; Aspden *et al.* 1996, 1997) and female sheep (McNeilly *et al.* 1991; Brooks and McNeilly 1994). After cessation of treatment with GnRH agonist, secretion of LH remained suppressed for some time in male and female cattle (Bergfeld *et al.* 1996*a*, 1996*b*), men (Hall *et al.* 1999) and women (Broekmans *et al.* 1996). It was not established in the

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aforementioned studies whether continued disruption of LH secretion after treatment with GnRH agonist was the result of transcriptional and/or post-transcriptional mechanisms.

In the present study, LHβ-subunit mRNA, FSHβ-subunit mRNA and LH and FSH glycoprotein contents of the anterior pituitary gland were characterized in female cattle during and after treatment with GnRH agonist. The distribution of LHβ-subunit mRNA between nuclear and cytoplasmic compartments was also determined. The findings would provide additional information towards understanding the mechanisms for transcriptional and post-transcriptional regulation of gonadotrophin synthesis, and whether these mechanisms can be differentially regulated for LH and FSH. The study formed part of a broader programme of research that is examining the utility of GnRH agonist treatment for controlled, reversible suppression of gonadotroph cell function (D'Occhio *et al.* 2000*b*).

Materials and methods

The experimental protocol used was endorsed by the Animal Ethics Committee of Central Queensland University according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 1997, prepared by the NH&MRC, CSIRO and the Australian Agricultural Council.

Gonadotrophin-releasing hormone agonist implant formulation and implantation

The GnRH agonist deslorelin (D-Trp^6-Pro^9-des-Gly^{10}-GnRH ethylamide; Karten and Rivier 1986) was formulated into slow-release implants that contained 5 mg of deslorelin (D'Occhio and Aspden 1996) (Peptech Animal Health, Dee Why, NSW, Australia). When incubated *in vitro*, each implant released approximately 25 μg of deslorelin every 24 h (determined by high-performance liquid chromatography and absorbance at 278 nm) (J. Walsh, unpublished results). Implants were placed subcutaneously in the dorsal surface of the ear under aseptic conditions using a standard surgical trocar.

Experimental design

Eighteen, 2-year-old Zebu (Brahman) cross-bred female cattle (heifers, 441 ± 13 kg) were block randomized on live weight to three groups: (*i*) Group 1 (control, $n = 6$), synchronized to be at Day 11 of the ovarian cycle on Day 31 of the study; (*ii*) Group 2 ($n = 6$), received two deslorelin implants for 31 days; and (*iii*) Group 3 ($n = 6$), received two deslorelin implants for 28 days. All heifers were slaughtered on Day 31. Pituitary glands were recovered and first hemisected midsagittally then bisected again to produce quarters, with recording of dorsal and ventral quarters. Pituitary quarters were plunged into liquid nitrogen and stored at −80°C until processed.

An additional group of control heifers (Group 4, $n = 3$) synchronized as for Group 1, and a further group of heifers treated with two deslorelin implants (Group 5, $n = 3$), were used to test for pituitary desensitization to GnRH on Day 28. Desensitization was determined by measuring changes in plasma concentrations of LH after injection of natural-sequence GnRH (50 μg, i.m.). Blood samples were taken immediately before injection of GnRH (Time 0), then 30, 60 and 120 min after injection. Increases in plasma LH (ΔLH) after injection of GnRH were calculated by subtracting the concentrations at Time 0 from respective maximal concentrations after injection of GnRH.

Oestrous cycles of control heifers (Groups 1 and 4) were synchronized by injection of oestradiol benzoate (1 mg i.m.; CIDIROL®, Genetics Australia, Baccus Marsh, Victoria, Australia) and intravaginal placement for 10 days of a progesterone-releasing device (CIDR®, Genetics Australia). Heifers were given a luteolytic dose of prostaglandin (500 μg i.m. cloprostenol; estroPLAN®) at the time of CIDR® removal. For control heifers to be in the mid-luteal phase of the oestrous cycle at the time of slaughter, oestrus synchronization was started 3 weeks before slaughter.

Pituitary RNA extraction

Total cellular RNA was extracted using Sigma Tri Reagent™ (Sigma Chemical Co., St Louis, MO, USA) according to Sigma Technical Bulletin MB-205. Briefly, a ventral quarter anterior pituitary from each animal was weighed and then homogenized in 5 mL of Tri Reagent. Samples were left for 5 min at ambient temperature followed by the addition of 1.0 mL of chloroform and were then vortexed for 15 s. The resulting mixture was centrifuged at 12 000*g* for 15 min at 4°C. The upper aqueous layer containing the RNA was transferred to a fresh tube and the RNA precipitated by the addition of 2.5 mL of isopropanol followed by centrifugation at 12 000*g* for 10 min at 4°C. The resulting RNA pellets were washed in 75% ethanol, air dried, and then dissolved in 200 μL of diethylpyrocarbonate (DEPC)-treated, autoclaved, Milli-Q purified water. Concentrations of total cellular RNA were estimated by spectrophotometry at 260 nm with the RNA purity validated by the absorbance ratio A260 nm : A280 nm (1.70 ± 0.03).

Northern blot analysis

Northern blot analysis was undertaken as described elsewhere (Aspden *et al.* 1996). Aliquots of total cellular RNA (25 μg) were separated by electrophoresis on a glyoxal denaturing agarose (1.2%) gel and blotted overnight by capillary action using 20 × SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0) solution onto a Hybond-N nylon membrane (Amersham, Buckinghamshire, UK). The blot was baked for 2 h at 80°C and exposed to ultraviolet radiation on a transilluminator for 1 min to fix the RNA to the membrane.

Gonadotrophin subunit cDNA probes were used to assess relative pituitary mRNA contents. Bovine LHβ-subunit (Maurer 1985) and FSHβ-subunit (Maurer and Beck 1986) cDNA probes were kindly supplied by Dr D. L. Hamernik (Endocrinology & Reproductive Sciences IRG Center for Scientific Review, NIH, Bethesda, MD, USA), and the rat α-subunit cDNA probe (Godine *et al.* 1982) was kindly supplied by Dr I. J. Clarke (Prince Henry's Institute of Medical Research, Melbourne, Victoria, Australia). A human β-actin cDNA (cat. no. G800-1; Clontech, Palo Alto, CA, USA) probe was used to determine and correct differences as a result of loading.

Prehybridization was for 2 h in 15 mL of hybridization buffer, which consisted of 50% formamide, 50 mM phosphate buffer (pH 8.0), 5 × SSC, 0.2% (w/v) bovine serum albumin (BSA), 0.2% (w/v) polyvinylpyrrolidone-40, 0.2% (w/v) ficoll, *Escherichia coli* tRNA (40 μg mL^{-1}) and herring sperm DNA (20 μg mL^{-1}). Aliquots (25 ng) of cDNA probes were prime labelled ('Rediprime II'; Amersham) randomly with [α-^{32}P]dCTP ('Readyview'; Amersham). In each hybridization 1 × 10^6 c.p.m. mL^{-1} of incorporated probe was used. The hybridizations were for 16 h at 42°C in a Hybaid hybridization oven. Following hybridization, the membrane was washed twice at room temperature for 20 min in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) and exposed to Kodak Biomax MS Scientific Imaging Film. Higher stringency washes were undertaken when required to reduce background further. Autoradiography results were quantified using densitometry (Kodak DC40 camera, Kodak Digital Science 1D Image Analysis Software). The blot was stripped for re-probing by placing in 95°C distilled water containing 0.1% (w/v) SDS for 5 min. Loading adjustments for

LHβ-, FSHβ- and α-subunit mRNA were made relative to the amount of β-actin in each lane.

cRNA probe synthesis

The pSP65 plasmid containing a 547 bp bovine LHβ-subunit cDNA probe (Maurer 1985) was linearized using the restriction enzyme Xbal, proteinase K treated at 37°C for 1 h and extracted twice using phenol–chloroform. After centrifugation the pellet was dissolved in DEPC-treated water to a concentration of 0.4 μg μL^{-1}.

The linearized cDNA template was used for the transcription of antisense [^{35}S]UTP-labelled cRNA probes using SP6 polymerase in accordance with the transcription kit manufacturer's recommendations (RNA Transcription Kit; Stratagene, La Jolla, CA, USA), and used for hybridizations the same day. Unincorporated nucleotides were removed using a Sephadex G50 spin column.

It was not possible to transcribe a sense probe from this plasmid as only the SP6 site was available for transcribing the antisense probe. For negative controls a sense probe against the steroidogenic enzyme P450 scc was transcribed as described by Xu *et al.* (1995). Sections of heart and kidney tissues were also hybridized with the antisense probe as further negative controls.

For hybridization the labelled probes were diluted in hybridization buffer (50% (v/v) formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 1 × Denhardt's solution (0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) BSA), 10 mM dithiothreitol, 500 μg mL^{-1} yeast transfer RNA, 10% dextran sulfate) to 2×10^7 c.p.m. mL^{-1}.

In situ *hybridization*

In situ hybridization was based on the method of Xu *et al.* (1995). Sections (7 μm) of anterior pituitary tissue were cut at −20°C using a cryostat and placed onto chilled microscope slides (Superfrost Plus; Erie Scientific Co., Portsmouth, NH, USA). Two slides per animal were prepared for antisense probing and one slide per animal for probing to detect non-specific binding. Sections were then fixed using 4% (v/v) formaldehyde in 0.01 M phosphate-buffered saline (PBS) for 5 min. Slides were then washed in PBS followed by dehydration in an ethanol series, then stored at −80°C in a dessicated air-tight box until hybridization, approximately 1 week later.

Sections were prepared immediately prior to hybridization by rehydrating in 2 × SSC (0.6 M sodium chloride, 0.06 M sodium citrate, pH 7.0), acetylated in 0.25% acetic anhydride in 0.1 M triethanolamineHCl, pH 8.0, for 10 min and then washed in 2 × SSC. Sections were then dehydrated in an ethanol series and air-dried. Hybridization was undertaken using 50 μL of diluted probe on each section in a humidified container at 55°C for 20 h. Slides were then washed twice in 2 × SSC. An aliquot (200 μL, 50 μg mL^{-1}) of ribonuclease-A (Sigma Chemical Co.) in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0, was dispensed on each section and slides incubated at 37°C for 1 h.

Slides were then washed at 55°C in 2 × SSC, 0.1% β-mecaptoethanol (BME) for 15 min; 1 × SSC, 0.1% BME for 15 min; 1 × SSC, 50% formamide, 0.1% BME for 30 min; 0.1 × SSC, 0.1 BME for 15 min (twice), dehydrated in an ethanol series and allowed to air dry.

Sections were dipped in Hypercoat LM-1 emulsion (Amersham), allowed to partially dry for 2 h, and stored in a light-proof desiccated box for 4 days at 4°C. Slides were then developed with Kodak Dektol developer, and fixed with Kodak fixer. The sections were then lightly stained with hematoxylin and eosin.

Image analysis

Photographs of sections were taken at ×1000 magnification using a Nikon CoolPix 995 digital camera. Nuclear and cytoplasmic hybridization intensity was quantified by image analysis using the public domain National Institutes of Health 'ImageJ' software (Garverick *et al.* 2002). Ten cells showing hybridization (five from each antisense probed section) were analysed for silver grain density over the nucleus and cytoplasm for each animal and the results for each animal averaged prior to analysis. The cytoplasmic region was defined as the area around the nucleus bounded by a distance of 5 mm from the nuclear envelope on the ×1000 photographs. Analysis of nuclear and cytoplasmic regions from cells in the sense-probed sections was used for background subtraction.

Hormone assays

Ventral quarter pituitaries were weighed and then homogenized in sucrose lysis buffer (10 mM Tris, 0.1% BSA (w/v), 0.1% NaN_3 (w/v), sucrose 25 mM; pH 7.4; 4°C) (Brown and Reeves 1983). Homogenates were centrifuged at 10 000*g* for 30 min at 4°C and dilutions of supernatants used for assay. Anterior pituitary homogenate supernatant concentrations of LH and FSH were determined using double-antibody radioimmunoassays (D'Occhio *et al.* 1986). The same LH assay was used to determine plasma concentrations of LH in Groups 4 and 5 in response to injection of GnRH. Intra- and inter-assay coefficients of variations for LH and FSH assays were <10% based on duplicate samples. Sensitivities of the assays were 0.2 ng mL^{-1} of USDA bovine (b)LH-B-5, and 0.12 ng mL^{-1} of bovine FSH (AFP-5332B, National Hormone and Pituitary Program, Harbour-UCLA Medical Centre, Torrance, CA, USA).

Statistical analyses

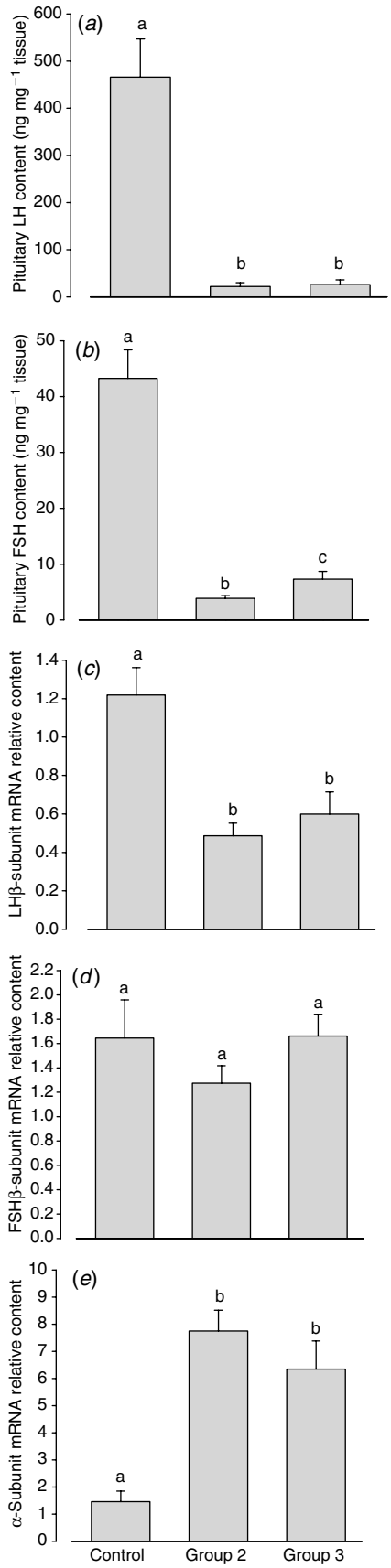
Hormonal and mRNA data were analysed by analysis of variance procedures using SPSS ANOVA, with least significant difference comparisons of group means. The paired sample *t*-test was used to compare density of hybridization between the nucleus and cytoplasm within groups. When necessary, data were $\log_{10}$ transformed to improve homogeneity of variance. Results for plasma LH (ng mL^{-1}), pituitary LH and FSH content (ng mg^{-1} tissue), pituitary LHβ-, FSHβ- and α-subunit mRNAs (relative arbitrary units) and *in situ* hybridization for LHβ-subunit mRNA (relative arbitrary units) are means ± SEM.

Results

Pituitary LH and FSH contents

Results for pituitary LH and FSH contents are shown in Fig. 1. Pituitary LH content was reduced ($P < 0.001$) for heifers treated for 31 days with deslorelin (23 ± 3 ng mg^{-1} tissue) compared with control heifers (466 ± 81 ng mg^{-1} tissue). Pituitary LH content continued to be lower ($P < 0.001$) than control values 3 days after the discontinuation of treatment with deslorelin (26 ± 4 ng mg^{-1} tissue). Pituitary content of LH in heifers 3 days after discontinuation of deslorelin treatment did not differ ($P > 0.05$) from that of heifers slaughtered with implants intact.

Pituitary FSH content was reduced ($P < 0.001$) for heifers treated for 31 days with deslorelin (3.87 ± 0.49 ng mg^{-1} tissue) compared with control heifers (44.13 ± 4.28 ng mg^{-1} tissue). Pituitary FSH content continued to be lower ($P < 0.001$) than control values 3 days after the discontinuation of treatment with deslorelin (7.32 ± 1.39 ng mg^{-1} tissue). In contrast, pituitary content of FSH in heifers 3 days after discontinuation of deslorelin treatment was higher ($P < 0.05$) than in heifers slaughtered with implants intact.



Pituitary LHβ-, FSHβ-, and common α-subunit mRNA contents

Autoradiography results for the Northern blots are shown in Fig. 2. Results for anterior pituitary LHβ-, FSHβ- and α-subunit mRNA relative contents are shown in Fig. 1. Pituitary LHβ-subunit mRNA relative content was reduced ($P < 0.001$) for heifers treated for 31 days with deslorelin (0.49 ± 0.06) compared with control heifers (1.22 ± 0.14). Pituitary LHβ-subunit mRNA relative content continued to be lower ($P < 0.001$) than control values 3 days after the discontinuation of treatment with deslorelin (0.60 ± 0.11). Pituitary relative content of LHβ-subunit mRNA in heifers 3 days after discontinuation of deslorelin treatment did not differ ($P > 0.05$) from that of heifers slaughtered with implants intact. Pituitary FSHβ-subunit mRNA relative content did not differ ($P > 0.05$) among controls (1.65 ± 0.31), Group 2 (1.27 ± 0.14) and Group 3 (1.66 ± 0.18).

Pituitary α-subunit mRNA relative content was increased ($P < 0.001$) for heifers treated for 31 days with deslorelin (7.75 ± 0.76) compared with control heifers (1.46 ± 0.39). Pituitary α-subunit mRNA relative content continued to be higher ($P < 0.001$) than control values 3 sdays after the discontinuation of treatment with deslorelin (6.34 ± 1.04). Pituitary relative content of α-subunit mRNA in heifers 3 days after discontinuation of deslorelin treatment did not differ ($P > 0.05$) from that of heifers slaughtered with implants intact.

In situ *hybridization for LHβ-subunit mRNA*

The results of image analysis for density of silver grains representing LHβ-subunit mRNA hybridization in the nucleus and cytoplasmic regions of gonadotroph cells are shown in Table 1. Representative photographs at ×1000 magnification of the *in situ* hybridization results for LHβ-subunit mRNA in individual anterior pituitary gonadotroph cells are shown in Fig. 3. Nuclear, cytoplasmic and nuclear plus cytoplasmic hybridization were greater in gonadotroph cells from control heifers than in cells of heifers in Groups 2 and 3. The ratio of nuclear to cytoplasmic hybridization was reduced ($P < 0.05$) in heifers during treatment with deslorelin (Group 2) compared with the same ratio in Groups 1 and 3 (Table 1).

Pituitary response to gonadotrophin-releasing hormone test

Control heifers showed a greater increase ($P < 0.05$) in plasma LH after injection of GnRH (ΔLH, $2.64 \pm$

Fig. 1. Anterior pituitary (*a*) LH; (*b*) FSH; (*c*) LHβ-; (*d*) FSHβ-; and (*e*) common α-subunit mRNA contents in mid-luteal control heifers, heifers treated with deslorelin for 31 days and slaughtered with implants intact (Group 2), and heifers 3 days after discontinuation of 28 days of deslorelin treatment (Group 3). Results are means ± SEM; $n = 6$ per group. [a,b,c]Means without a common superscript differ ($P < 0.05$).

$0.80\,ng\,mL^{-1}$) compared with heifers receiving deslorelin (ΔLH, $0.02 \pm 0.04\,ng\,mL^{-1}$).

Discussion

Treatment of heifers with the GnRH agonist deslorelin was associated with a 60% reduction in anterior pituitary LHβ-subunit mRNA and 95% reduction in LH glycoprotein content. For FSH, there was a 25% reduction in FSHβ-subunit mRNA and 90% reduction in FSH glycoprotein content. Therefore, for both LH and FSH, the relative reduction in glycoprotein content was substantially greater than the relative reduction in mRNA. It could be concluded from these findings that both gene transcription and mRNA translation are influenced by treatment with GnRH agonist. Under the conditions of the present study, post-transcriptional regulation may have been greater for FSH as the reduction in FSHβ-subunit mRNA (25%) was not significant whereas the reduction in FSH glycoprotein (90%) was highly significant. In rats, ovariectomy and immunization against inhibins both increased pituitary FSHβ-subunit mRNA by a non-transcriptional mechanism (Dalkin *et al.* 1993). In a more recent study in rats, inhibin reduced the stability of FSHβ-subunit mRNA (Burger *et al.* 2001). A similar effect of inhibin on FSHβ-subunit mRNA stability was obtained with sheep pituitary cells in culture (Clarke *et al.* 1993). In the current study, levels of FSHβ-subunit mRNA in heifers treated with deslorelin did not differ significantly from control values. Therefore, the reduced pituitary content of FSH in heifers treated with deslorelin would seem to be related to post-transcriptional regulation by means other than FSH mRNA stability.

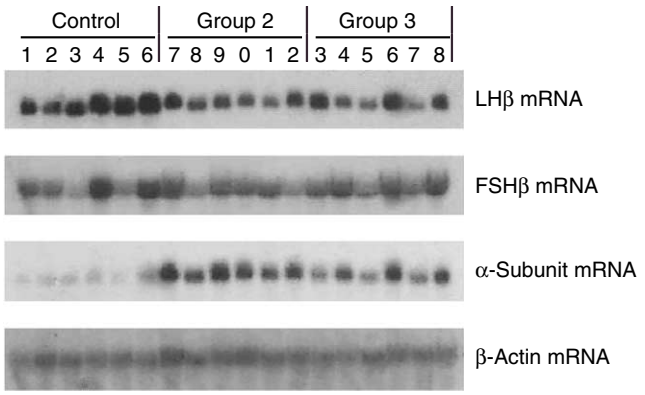


Fig. 2. Northern blot autoradiography results of probing for pituitary LHβ-, FSHβ-, and α-subunit mRNA and β-actin mRNA in heifers: Group 1 (control, synchronized mid-luteal, lanes 1–6); Group 2 (deslorelin implants for 31 days and slaughtered with implants intact, lanes 7–12); and Group 3 (3 days after discontinuation of 28 days of deslorelin treatment, lanes 13–18).

Three days after the cessation of treatment with deslorelin neither LHβ-subunit mRNA or LH glycoprotein had shown any major changes, whereas FSHβ-subunit mRNA was similar to controls and FSH glycoprotein content had shown a small but significant rise of 10% (from 90% to 80% reduction). Collectively, these findings could be interpreted to suggest that LH and FSH can be differentially regulated at both the transcriptional and post-transcriptional levels. In support of differential regulation of LH and FSH, pituitary content of FSH in heifers was similar to control levels 14 days after discontinuation of chronic delsorelin treatment, whereas pituitary content of LH was reduced at 14 days but recovered by 28 days (M. J. D'Occhio, G. A. Challen, W. J. Aspden, unpublished results).

The reduced anterior pituitary content of LHβ-subunit mRNA in heifers treated with deslorelin was consistent with findings in entire (Aspden *et al.* 1997) and castrated (Aspden *et al.* 1996) bulls. Similarly, FSHβ-subunit mRNA was reduced in castrated bulls treated with deslorelin (Aspden *et al.* 1996). In the latter study, there were no differences in pituitary FSH content between control bulls and bulls treated with GnRH agonist. It is considered unlikely that reduced pituitary content of FSH in heifers treated with deslorelin in the present study was the result of increased FSH release, as plasma concentrations of FSH were not elevated in heifers (McLeod *et al.* 1985; Gong *et al.* 1996), cows (D'Occhio *et al.* 1989) and bulls (Melson *et al.* 1986) treated chronically with GnRH agonist.

Table 1. Relative density (arbitrary units) of hybridization in the nucleus and cytoplasm for LHβ-subunit mRNA in pituitary gonadotroph cells of mid-luteal control heifers, heifers treated with deslorelin for 31 days and slaughtered with implants intact (Group 2), and heifers 3 days after discontinuation of 28 days of deslorelin treatment (Group 3)

Results are means ± SEM

Group	Relative density (arbitrary units)			
	Nucleus	Cytoplasm	Nucleus + cytoplasm	Nucleus : cytoplasm
Control	$9.58 \pm 1.10^{a,x}$	$7.78 \pm 0.96^{a,x}$	17.37 ± 1.72^{a}	1.31 ± 0.22^{a}
Group 2	$2.12 \pm 0.18^{b,x}$	$3.57 \pm 0.10^{b,y}$	5.69 ± 0.20^{b}	0.59 ± 0.05^{b}
Group 3	$3.00 \pm 0.41^{b,x}$	$2.95 \pm 0.31^{b,x}$	5.95 ± 0.70^{b}	1.01 ± 0.05^{a}

[a,b]Columns without a common superscript differ ($P < 0.05$).
[x,y]Rows without a common superscript differ ($P < 0.05$).

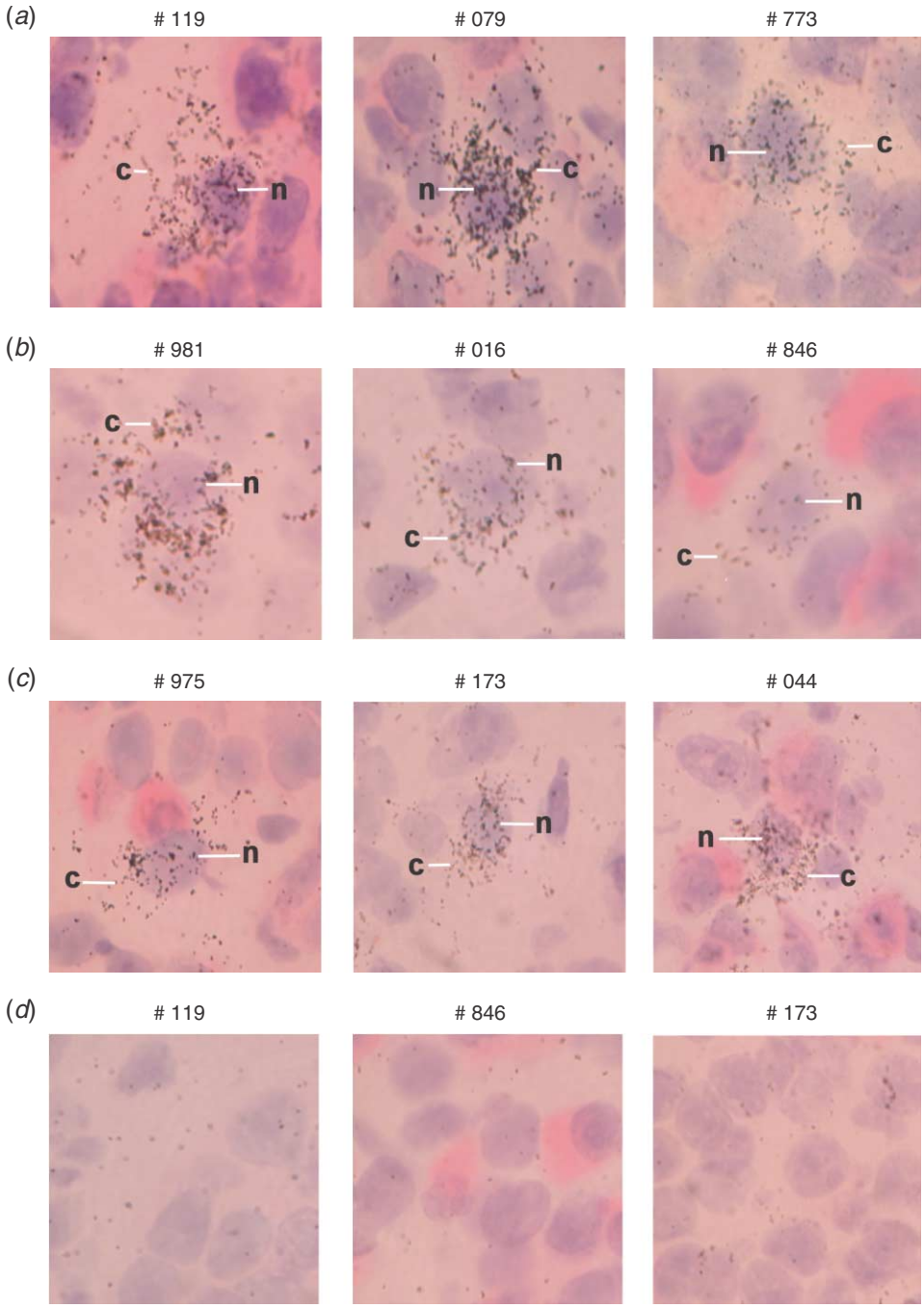


Fig. 3. Representative photographs (×1000) of *in situ* hybridization for LHβ-subunit mRNA in anterior pituitaries of three control heifers (Row A, Heifers 119, 079, 773), three Group 2 heifers (deslorelin implants for 31 days and slaughtered with implants intact; Row B, heifers 981, 016, 846), and three Group 3 heifers (3 days after discontinuation of 28 days of deslorelin treatment; Row C, heifers 975, 173, 044). Photographs show hybridization to LHβ-subunit mRNA in individual anterior pituitary gonadotroph cells, indicated by silver grains that appear as small dark spots. Nuclear hybridization (n) and cytoplasmic hybridization (c) are indicated by the white indicator lines. Only gonadotroph cells show hybridization to the LHβ-subunit probe and contain resultant silver grains and, hence, other cell types in the photographs show only a random background level of silver grains. Representative sense negative control probings are shown in Row D.

The continued presence of LHβ-subunit mRNA and FSHβ-subunit mRNA in gonadotroph cells that were presumably down-regulated by agonist treatment suggested that there can occur constitutive expression of LH and FSH genes. Downregulation was demonstrated by the absence of a LH response to injection of natural-sequence GnRH. Apparent constitutive expression of LH and FSH genes could represent a mechanism that ensures the maintenance of basic functionality of gonadotroph cells in the absence of GnRH stimulation. Interestingly, whereas expression of LHβ- and FSHβ-subunit mRNA was maintained at a relatively high level, translation of the respective mRNAs was quite low. There is evidence that a number of stimulatory factors can induce gonadotrophin synthesis and release independent of GnRH, although this remains a controversial area (Evans 1999). The possible involvement of these factors in the present study cannot be excluded.

Anterior pituitary content of the common α-subunit mRNA was increased by treatment with deslorelin. Male rats (Lalloz *et al.* 1988) and female sheep (McNeilly *et al.* 1991) treated with GnRH agonist likewise showed an increase in common α-subunit mRNA. It was suggested that GnRH or GnRH agonists directly stimulate the expression of the α-subunit (Saunders *et al.* 1998). But in the present study it was shown that α-subunit mRNA content remained elevated 3 days after discontinuation of treatment with deslorelin, when it was assumed that deslorelin was no longer present systemically. Whatever the mechanism for increased expression of α-subunit during treatment with GnRH agonist, there is a delay in the return to normal expression similar to the delay of that of normal expression of LHβ- and FSHβ-subunits. The current study is the first to report that pituitary content of LH, and LHβ-subunit mRNA remain at treatment levels 3 days after discontinuation of treatment with a GnRH agonist, whereas common α-subunit mRNA content remains elevated following withdrawal of treatment.

The greater relative content of LHβ-subunit mRNA compared with LH glycoprotein content in the anterior pituitary of heifers treated with deslorelin suggested post-transcriptional regulation that may not involve significant changes in mRNA stability. A mechanism for post-transcriptional regulation of mRNA involves altered nuclear export. In the yeast *Saccharomyces cerevisiae*, nuclear export of mRNA was reduced by various stressors including heat-shock, high salt and ethanol (Saavedra *et al.* 1996). In the present study nuclear hybridization for LHβ-subunit mRNA was reduced relative to cytoplasmic hybridization during treatment with deslorelin. There was no difference between nuclear and cytoplasmic hybridization 3 days after withdrawal of treatment with deslorelin. These preliminary findings were interpreted to suggest that post-transcriptional regulation of LH synthesis during deslorelin treatment is related to factors downstream of nuclear export, such as direct translational inhibition (Day and Tuite 1998).

In summary, treatment with a GnRH agonist was associated with major decreases in anterior pituitary contents of LHβ-subunit mRNA, LH and FSH, and only a relatively small decrease in FSHβ-subunit mRNA. This indicated a differential effect on LH and FSH gene expression, and both transcriptional and post-transcriptional regulation of LH and FSH biosynthesis. The results in the present study reflected the function of gonadotroph cells that were under the influence of a GnRH agonist. It could be argued that the apparent differential regulation of transcriptional and post-transcriptional mechanisms associated with LH and FSH synthesis implied by the current findings may not occur under normal circumstances. Although there was no apparent regulation in expression of LH glycoprotein owing to reduction in LHβ-subunit mRNA nuclear export, evidence suggests that post-transcriptional regulation may be of particular importance in the function of gonadotroph cells (D'Occhio *et al.* 2000*a*; Staton *et al.* 2000). Accordingly, further studies are required to elucidate the significance of post-transcriptional regulation of LH and FSH mRNAs in the integrated response of gonadotroph cells to stimulation from GnRH, and feedback imposed by gonadal factors.

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