Ovarian follicular expression of mRNA encoding the type I IGF receptor and IGF-binding protein-2 in sheep following five days of nutritional supplementation with glucose, glucosamine or lupins

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Abstract

The IGF system is associated with ovarian folliculogenesis. The effect of the IGFs mediated through the type I receptor (IGF-IR) and IGF-binding protein-2 (IGFBP-2), is to regulate the growth and atresia of follicles. To test if the mRNAs for IGF-IR and IGFBP-2 are differentially regulated in the follicle we used nutritional treatments that stimulate folliculogenesis and measured, by *in situ* hybridisation, their mRNAs expression. Groups of five anoestrous Merino ewes were fed wheat straw (control) or the control diet supplemented with lupins (500 g/day). Other ewes were fed the control diet and infused with glucose (50 mmol/h) or with glucosamine (3.5 mmol/h). Intravaginal progestagen sponges were inserted for 12 days, and nutritional treatments were started 5 days before progestagen removal. Follicular development was studied after an artificial follicular phase, simulated by progestagen for 12 days and a regime of GnRH pulses given for 36 h following progestagen withdrawal, when the animals were killed. The ovaries were collected and stored at -80°C until sectioning at $10\text{ }\mu\text{m}$. Every 25–28th and 29–32nd section was probed for IGF-IR and IGFBP-2 using ^{35}S -labelled oligonucleotide probes. None of the nutritional treatments affected the number or size of follicles positive for IGF-IR, but glucose ($P < 0.001$) and lupin ($P < 0.001$) treatments reduced the follicular concentration of mRNA. The nutritional treatments all increased the number of follicles positive for IGFBP-2 ($P < 0.05$) and reduced their mean diameter ($P < 0.05$) and with the exception of lupin feeding, the concentration of mRNA ($P < 0.05$). The results show that all treatments affected the intrafollicular IGF system and suggest that IGF-IR and IGFBP-2 are nutritionally regulated in the follicle. However, the effects of treatments were variable and suggest the existence of multiple regulatory mechanisms that allow for normal variation in composition and balance of the ruminant diet.

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Introduction

The relationship between nutrition and reproduction has been of interest to reproductive physiologists since the end of the 19th century (Heape 1899, Marshall 1904, 1905). Much of this research has focused on the effects of nutrition on ovulation rate and twinning (Clark 1934, Lindsay 1983, Lindsay *et al.* 1991, L'Anson *et al.* 1994, Robinson 1996). There is now a broad consensus that the effects of nutrition on ovulation rate and twinning are mediated by an effect of nutrition on folliculogenesis and that this effect can act at more than one level in the hypothalamo–pituitary–ovarian axis (Landau *et al.* 1995, Boukhliq *et al.* 1996, Robinson

1996, Rhind & McNeilly 1998, Armstrong *et al.* 2002, Gong 2002). Severe undernutrition suppresses the secretion of gonadotrophin-releasing hormone (GnRH) interfering with the normal ovarian cyclicity leading to the inhibition of folliculogenesis, the failure of ovulation and increasing the possibility of infertility (Boukhliq *et al.* 1996, Cameron 1996, Robinson 1996, Kafi & McGowan 1997, Nottle *et al.* 1997, Bossis *et al.* 1999). However, increased nutrition can stimulate folliculogenesis by a direct action on the follicle that is largely independent of either luteinising hormone (LH) or follicle-stimulating hormone (FSH) secretion (Ritar & Adams 1988). The flushing effect in

ewes, whereby increased feeding stimulates folliculogenesis, ovulation rate and twinning, is an example of this phenomenon (Leury *et al.* 1990, Scaramuzzi & Campbell 1990, Scaramuzzi *et al.* 1993, Hinch & Roelofs 1986).

The ewe's genetic potential for folliculogenesis is particularly sensitive to environmental modification. Thus the ewe is capable of a wide variation in both ovulation rate and litter size and nutrition is perhaps the most significant environmental factor affecting these (Martin *et al.* 1992). Thus the ewe is an excellent model to study nutritional influences on folliculogenesis. The mechanism that links nutrition to folliculogenesis is probably mediated by the changing concentrations of blood metabolites and/or nutrients acting directly on the follicle (Scaramuzzi & Campbell 1990, Scaramuzzi *et al.* 1993, Downing *et al.* 1995a–c, Gong 2002).

In recent years considerable effort has been expended in attempts to identify the metabolic and nutritional factors linking nutrition with folliculogenesis. The insulin-like growth factor (IGF) system is a likely candidate system that has not been extensively investigated in the sheep, although there are strong grounds for expecting that the system is involved in the nutritional regulation of ovine folliculogenesis (Monget & Martin 1997). First, hepatic production of IGF-I is itself nutritionally regulated (Renaville *et al.* 2002) and secondly IGF-I has a stimulatory effect on granulosa cells (Maggofin & Erickson 1994, Monniaux *et al.* 1994, Campbell *et al.* 1995, Khalid & Haresing 1996, Deaver & Bryan 1999). In sheep it stimulates oestradiol production (Scaramuzzi *et al.* 1999) and in sheep and cattle it interacts with insulin at physiological levels to influence both cellular proliferation and oestradiol production (Monniaux *et al.* 1994, Campbell *et al.* 1995, Schams *et al.* 1999, Vendola *et al.* 1999, Gong 2002). Physiological concentrations of IGF-I enhance FSH-stimulated steroidogenesis, LH receptor induction and the deposition of proteoglycans. Granulosa cells also synthesise IGF-I-binding proteins that participate in regulating local responses to IGFs and the availability of follicular IGFs depends on IGF-binding proteins (IGFBPs) and between them they modulate folliculogenesis (Monget *et al.* 1993, 1996, 2002, Monniaux *et al.* 1994, Spicer *et al.* 1995). This experiment set out to test the hypothesis that the intrafollicular IGF system is a mediator of the effect of nutrition on follicular development of the ewe. We did this by determining the effects of nutrition on the patterns of mRNA expression for components of the IGF system in ovine follicles. In this paper, as part of an ongoing study of the nutritional regulation of the IGF system in the follicle, we describe the pattern of mRNA expression for the type I IGF receptor (IGF-IR) and IGFBP-2 in ewes given nutritional treatments that stimulate folliculogenesis. The treatments we chose were the infusion of glucose and supplementation with lupin grain, both of which increase ovulation rate in sheep (Downing *et al.* 1995a,c, Muñoz-Gutiérrez *et al.* 2002). In addition we also tested a more speculative treatment (glucosamine infusion) because there is evidence

to suggest that glucosamine is an essential component of energy-sensing pathways (the hexosamine pathway) in muscle (Wang *et al.* 1998) and we reasoned that it might have a similar role in the follicle.

Material and Methods

Animals and treatments

Twenty-one anoestrous Merino ewes were treated for 12 days with medroxyprogesterone sponges (Repromap; Upjohn, Rydalmare, NSW, Australia). At the same time, the animals were allocated randomly to one of four treatment groups: (i) basal straw diet plus jugular infusion of saline (control group, $n = 5$); (ii) basal straw diet plus 50 mmol glucose/h by jugular infusion ($n = 5$); (iii) basal straw diet plus 500 g lupin grain/day ($n = 6$); and (iv) basal straw diet plus 3.5 mmol glucosamine/h by jugular infusion ($n = 5$). The nutritional treatments lasted for 120 h and ended at the time of sponge removal. The day before the experiment started, bilateral jugular venous cannulae were inserted under xylocaine-induced local anaesthesia. One catheter was used exclusively for infusions and the other for collecting blood samples. An artificial follicular phase to stimulate normal follicular development was induced using GnRH. Intravaginal progestagen sponges (Chronogest; Intervet Australia Pty Ltd, Lane Cove, NSW, Australia) were inserted into anoestrous ewes and left in place for 12 days. Starting at sponge removal, each ewe was treated with a regime of i.v. pulses of GnRH (Sigma, St Louis, MO, USA). The pulses were administered in three stages: 500 ng every 4 h from 0 to 12 h; 250 ng every 2 h from 14 to 24 h; and 200 ng every 1 h from 25 to 36 h. The animals were then killed with an overdose (5–6 g) of i.v. pentobarbital 156 h after starting nutritional treatments and 36 h after sponge removal. The ovaries were removed within 5 min, frozen in dry ice and stored at -80°C (Muñoz-Gutiérrez *et al.* 2002).

Follicle counts

Forty-two frozen ovaries were sectioned serially at $10\ \mu\text{m}$ using a cryostat at -20°C and the frozen sections were used to count and measure the population of antral follicles and to measure the pattern of mRNA expression for aromatase. These data have been reported in a previous publication (Muñoz-Gutiérrez *et al.* 2002). The remaining sections were available for additional analyses and every 25th to 32nd were selected so that all antral follicles could be examined in at least two series of sections, by *in situ* hybridisation (ISH) to determine the expression of the mRNAs for IGF-IR and IGFBP-2.

ISH

The follicular expression of mRNAs for IGF-IR and IGFBP-2 were determined on dehydrated and fixed sections. The sections were probed with $[\alpha\text{-}^{35}\text{S}]\text{dATP}$ - (SJ 1334;

Amersham Pharmacia Biotech; Bucks, UK) labelled probes for IGF-IR and IGFBP-2 (sense; 45mers synthetic single-stranded oligonucleotide). The oligonucleotides (5 ng) were end-labelled using deoxynucleotidyl transferase (Promega, UK, Delta House, Chilworth Research Centre, Southampton, UK). The probe sequences were based on published cDNA sequences for IGF-IR and IGFBP-2 (Perks *et al.* 1995, Perks & Wathes 1996). The IGF-IR oligonucleotide 45mer was synthesised using the specific sequence 5'-ctc acg gtc atc cgc ggc tgg aaa ctc ttc tac aac tac gcc ctg-3' (Ullrich *et al.* 1986, Genbank accession number NM_000875). The IGFBP-2 oligonucleotide 45mer was synthesised using the specific sequence 5'-gcg cca gcc ccg agc agg ttg cag aca atg gcg agg agc act ctg-3' (Delhanty & Han 1992, Genbank accession number S44612).

Established ISH procedures (Perks 1994, Wathes *et al.* 1996, Leung 1997, Muñoz-Gutiérrez *et al.* 2002) were used with minor modifications. Briefly, the sections were impregnated with the reaction mixture containing labelled probe in hybridisation buffer (100 000 c.p.m. per 100 µl hybridisation buffer per slide) covered with a Parafilm coverslip, and incubated overnight at 49 °C. After incubation, the sections were washed at room temperature in citrate buffer (15 mmol/l sodium chloride, 15 mmol/l sodium citrate, pH 7.0, containing 0.2% (w/v) sodium thiosulphate pentahydrate) in a shaking bath for 30 min followed by 1 h at 60 °C. The slides were dehydrated in a gradient of ethanol, air dried and exposed to Kodak Biomax MR-1 film for 5 days. The sense probes were used as the negative control. Sections of ovine placenta were used as positive control because there is strong expression of both IGF-IR and IGFBP-2 in this tissue (Reynolds *et al.* 1997).

Photographic development

The manufacturer's (LM-1; Amersham Pharmacia Biotech) instructions were followed. Briefly, dried slides were dipped into the emulsion for 5 s at 43 °C and allowed to dry at room temperature and then on a metal tray pre-cooled with dry ice for 10 min. The slides were then placed in a light-tight box with anhydrous silica gel. After 45 days of exposure, the slides were dipped into a developer (Phenisol; Ilford Limited, Ilford, Essex, UK) for 5 min and then into a stop bath of 0.5% acetic acid (v/v) for

1 min. The developed slides were immersed in a fixative (47% (w/v), sodium thiosulphate pentahydrate) and washed before counterstaining with Harris' haematoxylin and eosin (Muñoz-Gutiérrez *et al.* 2002).

Image analysis

After exposure, the autoradiographic images of the ovarian sections were quantified for specific labelling using an image analysis system (Seescan, Cambridge, UK) to measure the absorbance of specific areas identified in the adjacent slides. The emulsion-coated slides were also used to confirm the cellular localisation of the IGF-IR and IGFBP-2 signals. The results from the autoradiographs are expressed as arbitrary units (units of absorbance with a linear range of 0.01–2.10). The non-specific counts (estimated from the sense probe) were subtracted from the total counts (estimated from the anti-sense probe) to produce a mean value for specific hybridisation in each follicle (Muñoz-Gutiérrez *et al.* 2002).

Statistical analysis

The total number of follicles, the number of positive follicles, the diameter of positive follicles and the relative density of expression were all analysed for treatment effects using a split-plot ANOVA. When significant effects were detected, further post-hoc tests using Tukey's test (SAS 1995) were carried out to examine specific treatment effects. The proportions of probe positive follicles between treatments were tested by the Chi² test.

Results

One animal in the lupin-fed group had an infected uterus and persistent corpus luteum and was excluded from the results and the analysis.

Ovarian IGF-IR mRNA

All antral follicles, consisting of 1404 follicles in 40 ovaries from 20 sheep were tested for both IGF-IR and IGFBP-2 mRNA expression. The number of follicles positive for IGF-IR was not affected by the treatments (Table 1). Although the glucose-infused and lupin-fed groups tended to have more follicles positive for the IGF-IR than control and glucosamine-infused ewes, the differences were not

Table 1 The number and average diameter of IGF-IR-positive follicles and IGF-IR expression (arbitrary units of absorbance) of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days. Values are means $\pm$ S.E.M. $n = 5$ in each treatment.

Treatment	Number of IGF-IR-positive follicles per ewe	Diameter of IGF-IR-positive follicles (mm)	IGF-IR mRNA concentration (units of absorbance)
Control	10.6 $\pm$ 4.0	1.8 $\pm$ 0.13	0.12 $\pm$ 0.007 ^a
Glucose infusion	20.4 $\pm$ 9.8	1.7 $\pm$ 0.11	0.05 $\pm$ 0.003 ^b
Lupin-fed	13.2 $\pm$ 6.4	1.57 $\pm$ 0.13	0.07 $\pm$ 0.007 ^{b,c}
Glucosamine infusion	10.8 $\pm$ 9.4	1.79 $\pm$ 0.18	0.10 $\pm$ 0.016 ^{a,c}

Values with different superscripts within columns differ significantly ($P < 0.001$).

statistically significant. There was an effect of treatment on the proportion of follicles positive for IGF-IR ($P = 0.027$). Compared with controls (23.8%) glucose infusion had no effect (20.0%), but lupin feeding (17.7%) and glucosamine infusion (16.8%) resulted in lower proportions of follicles positive for IGF-IR. The mean diameter of follicles positive for IGF-IR was not affected (Table 1).

The expression of mRNA for IGF-IR was localised in the granulosa and theca cell layers (Fig. 1) and the concentration of mRNA varied with treatment ($P = 0.001$; Table 1). Control and glucosamine-infused ewes had high expression of IGF-IR and the group means were not significantly different from each other (Table 1) but they were significantly different from the groups' means

Figure 1 Antisense (AS; panels a, d and f) and sense (S; panels b, e and g) autoradiographic localisation of mRNA for IGF-IR in ovaries from GnRH-stimulated anoestrous Merino ewes. Note the intense expression of IGF-IR (panels d and f) in both the granulosa ('g') and theca ('t') cells compared with the follicular antrum ('fa') or the ovarian stroma. Scale bars represent (a and b) 2.5 mm and (d–g) 10 μ m.

for the lupin-fed and glucosamine-infused ewes. The follicles from lupin-fed and glucose-infused ewes had low levels of mRNA expression for IGF-IR and again the group means were not significantly different from each other (Table 1).

Ovarian IGFBP-2 expression

The number, the percentage and the diameter of follicles positive for IGFBP-2 and the concentration of mRNA for IGFBP-2 in granulosa cells were all affected by treatments (Table 2; Fig. 2). The treated groups all had greater numbers of follicles positive for IGFBP-2 than the control group ($P=0.038$; Table 2). There also were significant differences among the three treatment groups (Table 2) with the greatest in the glucose-infused group and lowest in the glucosamine-infused group. The lupin-fed group was intermediate between the glucose-infused and glucosamine-infused groups (Table 2). The glucose-infused ewes had a higher percentage ($P=0.003$) of their antral follicle population positive for IGFBP-2 (26.70%) than the glucosamine-infused (16.70%), lupin-fed (15.54%) and control (7.18%) groups. The presence of mRNA for IGFBP-2 was only detected in the granulosa cell layer and was not seen in the thecal cell layer of any follicle (Fig. 2). Ewes infused with either glucose or glucosamine had significantly ($P=0.040$) greater concentrations of mRNA IGFBP-2 in the granulosa cells than control or lupin-fed ewes (Table 2).

Ovarian P_{450} aromatase mRNA, IGF-IR mRNA and IGFBP-2 mRNA

This set of ovaries contained 31 aromatase-positive follicles spread across all groups and these data have been previously reported (Muñoz-Gutiérrez *et al.* 2002). In brief, of the 31 aromatase-positive follicles, 15 (48.3%) were also positive for IGF-IR (Fig. 3) and their distribution was not affected by treatment ($P=0.09$). The percentages by treatment were: control (50.0%), glucose-infused (40.0%), lupin-fed (37.5%) and glucosamine-infused (44.4%).

In contrast, the number of aromatase-positive follicles also showing IGFBP-2 expression was affected by treatment ($P=0.022$). From 31 aromatase-positive follicles, 23 (74.2%) were positive for IGFBP-2 (Fig. 3). These follicles

were found in all treatments. The percentages by treatment were: control (100%), glucose-infused (100%), lupin-fed (62%) and glucosamine-infused (44%).

None of the treatments affected ($P=0.99$) the number of aromatase-positive follicles that were positive for both IGF-IR and IGFBP-2 (Fig. 3). Of the 23 follicles aromatase-positive and IGFBP-2-positive only 15 were also positive for IGF-IR. The percentage of aromatase positive follicles positive for both IGF-IR and IGFBP-2 was 62.2%. The percentages of follicles by treatment that were positive for all three probes were 50, 40, 40 and 50% for control, glucose-infused, lupin-fed and glucosamine-infused respectively.

Discussion

The aim of this work was to find out if components of the ovarian IGF system are regulated by nutritional treatments known to stimulate folliculogenesis, and our findings show that both the IGF-I receptor and IGFBP-2 have differential responses to these treatments. In summary, our data show that all three treatments affected the IGF system, suggesting that both IGF-IR and IGFBP-2 nutritional regulation is probably at the level of the follicle itself. The effects of the three treatments were not identical, suggesting different mechanisms that presumably take account of natural variation in the composition (carbohydrate, free fatty acids and protein) of the ruminant diet, are operating to regulate nutritional responses in the ovine follicle.

The expression of mRNA for the IGF-IR was detected in granulosa and theca cells of antral follicles and particularly small antral follicles, confirming earlier observations (Perks *et al.* 1995). There was no effect of the nutritional treatments on the number or the average diameter of follicles positive for IGF-IR but there were differences in the concentration of message in the follicle wall. The infusion of glucose and the feeding of lupin grain both reduced the concentration of message compared with controls. This finding suggests that one effect of these treatments is to modify IGF-I-stimulated steroidogenesis in the follicle. The concentration of mRNA in the follicle wall was determined using quantitative densitometry on photographic images of whole ovaries on photographic film. This technique detects those follicles expressing mRNA in the follicle wall but it is

Table 2 The number and average diameter of IGFBP-2-positive follicles and IGFBP-2 expression (arbitrary units of absorbance) of GnRH-treated anoestrous Merino ewes infused with glucose or glucosamine, or fed a supplement of lupin grain for 5 days. Values are means $\pm$ S.E.M. $n=5$ in each treatment.

Treatment	Number of IGFBP-2-positive follicles per ewe	Diameter of IGFBP-2-positive follicles (mm)	IGFBP-2 mRNA concentration (units of absorbance)
Control	4.6 $\pm$ 0.6 ^a	2.53 $\pm$ 0.38 ^a	0.18 $\pm$ 0.019 ^a
Glucose infusion	24.6 $\pm$ 1.9 ^b	2.13 $\pm$ 0.13 ^a	0.26 $\pm$ 0.033 ^b
Lupin-fed	12.8 $\pm$ 1.2 ^c	1.85 $\pm$ 0.23 ^b	0.19 $\pm$ 0.014 ^a
Glucosamine infusion	8.8 $\pm$ 1.4 ^d	1.76 $\pm$ 0.17 ^b	0.23 $\pm$ 0.016 ^b

Values with different superscripts within columns differ significantly ($P < 0.05$).

Figure 2 Antisense (AS; panels a, c, e and g) and sense (S; panels b, d, f and h) autoradiographic localisation of mRNA for IGFBP-2 in ovaries from GnRH-stimulated anoestrous Merino ewes. Note the intense expression of IGFBP-2 (panels e and g) is confined to the granulosa cell layer ('g') and expression is not seen in the theca ('t'), the follicular antrum ('fa') or the ovarian stroma. Scale bars represent (a-d) 2.5 mm and (e-h) 10 µm.

unable to differentially quantify expression from the granulosa and theca cell layers. Consequently, it is not possible to tell if the nutritional treatments affected the concentrations of mRNA in the granulosa or theca layers of the follicle.

Our interpretation of these results is that the lower concentration of IGF-IR message would lead to a reduced number of IGF-IR receptors in the plasma membrane

(Estívariz & Ziegler 1997) and therefore reduced IGF-I-stimulated steroidogenesis in the follicle. The effect of this change would be to reduce the secretion of oestradiol and negative feedback on FSH secretion leading to a transient increase in FSH secretion (Campbell 1988) and the selection of additional dominant follicles to restore the endocrine homeostasis between oestradiol and FSH. The physiological and biological consequences of this

Figure 3 Antisense (AS; panels a, c and e) and sense (S; panels b, d and f) autoradiographic localisation of mRNA for aromatase ('ar') (a and b), IGF-IR (c and d) and IGFBP-2 (e and f) in ovaries from GnRH-stimulated anoestrous Merino ewes. Note that specific hybridisation is confined to the follicle wall of some but not all, antral follicles. The sections are sequential sections 40 μ m apart. The scale bars represent 2.5 mm.

effect would be an increase in ovulation rate and twinning rate (Scaramuzzi & Campbell 1990, Scaramuzzi *et al.* 1993).

The cellular expression of mRNA for IGFBP-2 was determined by examining microscope slides coated with exposed photographic emulsion, under a microscope, and confirmed the expression of mRNA for IGFBP-2 in follicular granulosa cells (Besnard *et al.* 1996, Perks & Wathes 1996). There were effects of all three nutritional treatments on both the pattern of follicular expression and the concentration of message within follicles (Table 2). All treatments increased the total number but not the percentage of follicles expressing the IGFBP-2 gene. These results suggest that the nutritional treatments increased the recruitment of antral follicles and that development of IGFBP-2 expression by follicles is part of the recruitment process in small follicles (1.5–2.5 mm in diameter). The average diameter of follicles positive for IGFBP-2 was significantly decreased by all the nutritional treatments, again suggesting that one effect of nutritional treatments is directed towards follicles undergoing recruitment. There were also nutritionally

induced differences in the concentration of mRNA for IGFBP-2 in the follicle wall. The infusion of glucose and glucosamine both increased the concentration of message compared with controls. This finding suggests that one effect of these treatments is to increase the local intrafollicular concentrations of IGFBP-2.

Since elevated intrafollicular concentrations of IGFBP-2 are associated with atresia and reduced concentrations with follicle selection (Monget & Bondy 2000, Mazebourg *et al.* 2003), it is plausible to suggest that nutritionally regulated IGFBP-2 within the follicular antrum may be one mechanism by which nutrition stimulates ovulation rate in sheep. Nutritionally induced increases in the intrafollicular concentration of IGFBP-2 would be expected in follicles with increased mRNA expression and would complement nutritional effects on the IGF-IR by modifying tissue availability of IGFs in the follicle (Breier 1999, Renaville *et al.* 2002). This is because IGFBP-2 locally sequesters IGFs and reduces their bioavailability to the granulosa and theca cells of the follicle (Monget *et al.* 1993, 1996, Monget & Monniaux 1995, Monniaux *et al.*

1997, Monget & Bondy 2000, Renaville *et al.* 2002, Mazebourg *et al.* 2003) by changing the external concentration of free IGFs in the cellular environment, by modifying cell membrane permeability, and local IGF metabolism and/or compartmentalisation in the cell (Estívariz & Ziegler 1997), resulting in a reduction of IGF-stimulated steroidogenesis in the follicle (Gong 2002).

This experiment demonstrates that all three nutritional treatments were able to modify aspects of the intrafollicular IGF system. Specifically, they show that the concentrations of mRNA for the IGF-IR and IGFBP-2 in the ovarian follicle are both differentially regulated. However, the patterns of gene expression were not identical between nutritional treatments suggesting the nutritional regulation of folliculogenesis is mediated by more than one mechanism. This is not surprising considering the varied nature of natural diets that grazing ruminant animals consume and the evolutionary significance of ovulation. The treatments we utilised were two that looked exclusively at glucogenic mechanisms (glucose and glucosamine infusion) and a third (lupin grain) that increased amino acid uptake as well as being gluconeogenic, and all of these treatments are predicted to lead to reduced IGF-stimulated steroidogenesis by the follicle. This prediction remains to be tested along with the effects of the nutritional supplements on other components of the IGF system.

The composition of the diet also affects the IGF system (Breier 1999). Nutritional protein seems to have a more significant effect on IGF-I production than nutritional energy (Renaville *et al.* 2002) and energy derived from carbohydrates has a more potent effect on IGF-I blood levels than energy derived from energy supplements (Estívariz & Ziegler 1997). Some of the differential effects of diet observed in this study may therefore be associated with the nutritional treatments. Lupins are a high-quality source of both energy and protein while glucose and glucosamine are sources of energy alone. Glucose-infused ewes will have derived their energy from glucose while the energy in the glucosamine-infused ewes is derived from hepatic gluconeogenesis and fatty acid oxidation.

The fact that not all follicles positive for aromatase were also positive for IGF-IR or IGFBP-2 suggests that the intrafollicular IGF system is not essential for folliculogenesis. However, this does not preclude a regulatory role for the intrafollicular IGF system.

We conclude that at all three nutritional treatments affected the intrafollicular IGF system and that both the IGF-IR and IGFBP-2 components of the IGF system are differentially regulated by nutrition, most probably at the level of the follicle itself. Furthermore, nutritional regulation of the intra-ovarian IGF system may be responsible for nutritional effects on ovulation in sheep. The effects of the three treatments were highly variable suggesting that sheep have complex regulatory mechanisms that presumably take account of the wide variety in

the composition and balance of the nutrient intake in grazing sheep.

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