Effect of nutritional supplementation on quantities of glucose transporters 1 and 4 in sheep granulosa and theca cells

S. A. Williams^{1,2}, D. Blache², G. B. Martin², R. Foot¹,
M. A. Blackberry² and R. J. Scaramuzzi^{1*}

¹Department of Veterinary Basic Sciences, University of London, London NW1 0TU, UK;
and ²Faculty of Agriculture (Animal Science), University of Western Australia, Nedlands,
Western Australia, Australia

The stimulatory effect of nutritional supplementation on ovarian activity in sheep has been linked to an increase in glucose availability that, with insulin, directly decreases follicular steroidogenesis. Glucose uptake occurs by glucose transporters, but it is not known which glucose transporters are present in the sheep ovary or whether they are affected by nutritional stimulation. The aim of this study was to determine whether widely distributed glucose transporter 1 (GLUT1) or insulin-responsive GLUT4 are present in the granulosa or theca cells of sheep ovarian follicles, and whether their concentrations are affected by nutritional stimulation. Merino ewes ($n = 49\text{--}51$ per group) were stimulated nutritionally for 5 days before luteolysis with lupin grain or with one of two regimens of a glucogenic mixture, administered orally, which increases blood glucose concentrations towards the upper end of the

normal range. Water was used as a control. Ovaries ($n = 3$ per group) were dissected and the granulosa cells and thecal shell from individual follicles were examined for glucose transporters using western blotting. GLUT1 concentration was 7–18 times higher in the granulosa than in the theca cells. GLUT4 was detected at a similar concentration in both types of cell. Nutritional treatment had no effect on the concentration of GLUT1 or GLUT4 in either tissue, and did not increase ovulation rate, despite increased concentrations of glucose and insulin. Concentrations of glucose transporters were not correlated with follicular concentrations of oestradiol or androstenedione. The presence of GLUT1 and GLUT4 in the granulosa and theca of sheep follicles indicates that the transporters have a role within the ovary in the modulation of follicular function.

Introduction

Ovarian activity in sheep is influenced by nutrition and body reserves (for a review, see Downing and Scaramuzzi, 1991). Short-term nutritional supplementation (4–6 days) or, more specifically, an intravenous glucose infusion, can increase ovulation rate, indicating that the ovarian response to nutritional supplementation may be mediated by glucose (Stewart and Oldham, 1986; Nottle *et al.*, 1990; Downing *et al.*, 1995). Glucose can act at various sites and in various ways. Action at hypothalamo–pituitary sites does not appear to mediate the effect on ovulation rate because there are no changes in the blood concentrations of LH and FSH (Downing and Scaramuzzi, 1991). Similarly, glucose alone does not appear to affect ovarian function directly, but steroidogenesis is inhibited when glucose is infused with insulin into the ovarian vein (Downing *et al.*, 1999). This treatment decreases negative feedback and allows extra oestrogenic follicles to be selected to restore homeostasis, as proposed by Downing and Scaramuzzi (1997). The implication is that changes in insulin-mediated

glucose uptake within the ovary will modulate follicular function.

Glucose enters cells via a family of glucose transporters, of which glucose transporter 1 (GLUT1) and GLUT4 will be considered with respect to ovarian tissue because of their physiological characteristics. GLUT1 provides basal glucose uptake in most tissues and has been detected in ovarian tissue of rats and mice (Kol *et al.*, 1997; Kodaman and Behrman, 1999; Zhou *et al.*, 2000). In contrast, GLUT4 is tissue-specific and is the predominant insulin-responsive glucose transporter in muscle and adipose tissue (Bell *et al.*, 1990), but has not been detected in the ovary (Kol *et al.*, 1997; Kodaman and Behrman, 1999; Zhou *et al.*, 2000). However, the actions of glucose and insulin in the sheep ovary indicate that GLUT4 is likely to be expressed in this organ, providing insulin-stimulated glucose uptake.

When glucose is taken up, it may affect follicular function through one of at least three interlinked mechanisms: (i) changes in energy availability, independent of changes in GLUT, that alter the endocrine biochemistry and hence steroidogenic capacity of the follicular cells; (ii) alterations in the function or expression of glucose transporters in ovarian tissues that, in turn, alter glucose uptake, biochemistry and steroidogenic capacity; and (iii) a

*Correspondence
Email: rscara@rvc.ac.uk

combination of (i) and (ii), that is, changes in circulating glucose and insulin lead to an increase in glucose uptake that alters steroidogenic capacity which, in turn, affects the function or expression of the glucose transporters.

The production and function of GLUT1 and GLUT4 are influenced by a range of factors, including insulin, glucose and ovarian steroids. Effects of glucose or insulin would be involved in mode of action (ii) proposed above, but how the nutrients affect the glucose transporters is uncertain because these factors can both increase and decrease expression of the genes, and the type of response differs among tissues (for a review, see Klip *et al.*, 1994). However, many of these contradictory observations are from studies *in vitro* or studies using rodent models of insulin-resistance, rather than from normal animals responding to physiological stimuli. In addition, specific studies need to be conducted in ruminants because their primary energy source is not glucose.

Ovarian steroids modulate GLUT1 production and function, an effect that would be involved in mode of action (iii) proposed above. Oestradiol increases the production of GLUT1 mRNA and protein, and subsequently increases glucose uptake across the blood–brain barrier and by the uterus of rats (Shi and Simpkins, 1997; Welch and Gorski, 1999). In contrast, high concentrations of androstenedione inhibit glucose transport in erythrocytes, presumably through an action on GLUT1 (Lacko *et al.*, 1975; Krupta and Deves, 1980; May and Danzo, 1988). These studies need to be extended to include GLUT4 and ovarian tissues.

Identification and localization of glucose transporters in the sheep follicle will improve our understanding of the way in which glucose affects ovarian activity in sheep. Therefore, the present study on the granulosa and theca tissue of large sheep follicles investigated the following: (i) whether GLUT1 and GLUT4 are expressed in the follicles; (ii) whether their concentrations respond to nutritional supplementation, in association with changes in the concentrations of glucose and insulin; and (iii) whether the concentrations of GLUT1 are correlated with concentrations of oestradiol and androstenedione in follicular fluid. These hypotheses were tested in ewes that were fed a nutritional supplement or were administered orally with a glucogenic mixture that increased plasma glucose concentrations within the normal range (Rodriguez Iglesias *et al.*, 1996).

Materials and Methods

Animals

The study was carried out in Western Australia (31°56'S) early in the breeding season. Merino ewes ($n = 201$) were divided into four groups with similar mean and variance (mean $\pm$ SEM) for live weight (55.9 ± 0.4 kg), age (4.3 ± 0.0 years) and body condition score using the method of Jefferies (1961) (3.3 ± 0.0 on a scale of 0.5 (very lean) to 5.0 (very fat)). The ewes had a high body condition score at the

time of allocation to treatment so poor quality grazing was provided for the duration of the study. Oestrous cycles were synchronized by the insertion of intravaginal pessaries containing progestagen (Chronogest; Intervet Australia Pty Ltd, Baulkham Hills, NSW) for 12 days. Nutritional treatments were administered during days 9–13 of the subsequent oestrous cycle (defined as days 1–5 of the treatment period). Luteolysis was induced at the end of day 5 of the treatment period with an i.m. injection of 125 μ g prostaglandin analogue (Estrumate; Schering-Plough Animal Health Ltd, North Ryde, NSW). The ewes were weighed on the day before treatment (day –1) and at the end of day 5 of the treatment period. The ovulation rate was determined by laparoscopy on day 16, that is, day 8 of the subsequent cycle (Oldham and Lindsay, 1980).

Nutritional treatments

Ewes were treated for 5 days starting at time 0 (08:00 h) on day 1 with one of the following: (i) 200 ml water every 8 h (control); (ii) 250 g lupin grain every 8 h (8-Lupin), which stimulates ovulation rate (Stewart and Oldham, 1986; Nottle *et al.*, 1990); (iii) 200 ml glucogenic mixture every 8 h (8-Gluc); or (iv) 200 ml glucogenic mixture every 12 h (12-Gluc). The dose of 200 ml and the intervals between doses were determined from a dose–response trial. The glucogenic mixture comprised 70% (v/v) glycerol (Sigma Chemicals, Balcatta, WA), 20% (v/v) 1,2-propanediol (Sigma Chemicals) and 10% (v/v) water, and was administered using an adjustable automatic drench gun (Philips, Cox Surgical, Coulsdon). The 8-Lupin ewes were fed in a group, whereas ewes in the other three groups were treated individually.

Blood and tissue collection

Blood samples were taken from a sub-group of ten ewes per treatment by jugular venepuncture. Samples were taken 30 min before the first treatment of the day for each of the 5 days of treatment, and at 2 h intervals between the first two treatments on each of days 1, 3 and 5 to determine the effects of the treatments on glucose and insulin. Glucose measurement was carried out immediately (see below), and the plasma was separated and stored at -20°C .

A sub-sample of four other ewes per treatment group was given one extra treatment of glucogenic mixture or lupin grain at the end of the 5 days and the ewes were killed 2 h later. The ovaries were removed immediately, snap frozen in liquid nitrogen and stored at -70°C .

Preparation of ovaries

Ovaries from each ewe were thawed and dissected and all visible antral follicles were collected in 25 mmol Hepes l^{-1} , 1 mmol EDTA l^{-1} , 255 mmol sucrose buffer l^{-1} containing 0.2 mmol di-isopropylfluorophosphate l^{-1} and 1 μ g pepstatin A ml^{-1} , pH 7.4 (HES; all reagents from Sigma, Poole). The six largest follicles (2–8 mm diameter) from

three ewes per group were dissected individually in a known volume of HES and separated into granulosa cells, thecal shell and diluted follicular fluid, and stored at -80°C until further analyses for glucose transporters (granulosa and theca) or measurement of steroid concentrations (follicular fluid were performed). The volume of antral fluid collected from each follicle was calculated from the follicle diameter using the following formula: $\text{volume} = 0.52(\text{diameter}^{2.70})$ (Carson *et al.*, 1981).

The granulosa and theca fractions were homogenized in HES buffer using 1.5 ml micropestles (Eppendorf, Hamburg). As a positive control, a sample of sheep muscle was collected from a local abattoir, frozen in liquid nitrogen and stored at -80°C , and then prepared in the same way as the follicular samples. The homogenate was centrifuged at 500 *g* for 5 min at 4°C . The supernatant was decanted and centrifuged at 80 000 *g* for 60 min at 4°C . The resultant pellet was re-suspended in HES. Protein concentrations of the unknown samples were measured in duplicate against an ovalbumin standard (0.12–2.00 mg ml^{-1} ; grade V, Sigma) within one 96-well plate using the method of Bradford (1976). Protein from follicular samples was precipitated using 5% (v/v) of 72% (w/v) trichloroacetic acid (Sigma ultra; Sigma) and the pellet was re-suspended in Laemmli SDS sample buffer that was neutralized using 2 mol Tris l^{-1} so that all samples were of the same concentration. A minimum of 20 μg protein was required for analyses of glucose transporters and samples that did not contain this amount were not analysed.

Protein separation of ovarian samples and immunoblotting

The techniques for protein separation and transfer were as described by Brant *et al.* (1992) and will be described only briefly with modifications. Follicular samples and sheep muscle (positive control) were separated on a 10% (w/v) SDS-PAGE resolving gel and transferred electrophoretically to a nitrocellulose membrane (Protran; Schleicher Schuell UK Ltd, London). The same membrane was used for detecting both GLUT1 and GLUT4 because many of the follicles yielded small amounts of protein using rabbit polyclonal antibodies specific to the carboxy terminus of GLUT1 at a 1:500 dilution (Davies *et al.*, 1987) or GLUT4 at a 1:400 dilution (Brant *et al.*, 1993). The second antibody was donkey anti-rabbit IgG conjugated to peroxidase (Amersham Pharmacia Biotech UK Ltd, Little Chalfont) at 1:1000 dilution in Tris-buffered saline containing Tween 20 (20 mmol Tris l^{-1} , 150 mmol NaCl l^{-1} , 0.02% (v/v) Tween 20; TBST) with 1% (w/v) dried milk for enzymatic chemiluminescence (ECLplusTM) detection. The immunoblots were developed using ECLplusTM according to the manufacturer's instructions (Amersham Pharmacia Biotech UK Ltd) and the membrane was exposed to X-ray film (Kodak, Technical Photosystems). Multiple exposures were obtained at different time intervals to optimize the visibility of bands. The antibodies were removed using

stripping buffer and the membrane was used for GLUT1 detection using ECLTM. The stripping procedure was tested by repeating the immunoprobings procedure without the primary antibody and was found to be 100% effective because after a 30 min exposure no bands were detectable.

Western blot analysis

The blots were analysed using Molecular AnalystTM/PC Software version 1.4 (Bio-Rad Labs Ltd, Hemel Hempstead). Average absorbance using Volume Analyst (volume absorbance $\times \text{mm}^2$) was measured in a box drawn around the GLUT4 band, and a box of equal size was drawn beside the GLUT4 band for measurement of the background. GLUT1 was not analysed in the same way because blots had occasional small dark dots that would have given false high background measurements. Instead, a perpendicular line was drawn through the GLUT1 band and the density profile analysed. The GLUT1 peak value was identified and assessed using Profile Analyst (absorbance $\times \text{mm}$). Background absorbance was determined by drawing another line between the bands. The concentrations of protein in follicular samples are expressed as a percentage of the positive control (muscle). The inter-blot coefficient of variation was 11.7% as calculated from four positive controls. Samples were analysed in three sets and treatments were spread evenly throughout the sets.

In contrast to the follicular samples, GLUT1 was not detectable in the positive control so GLUT1 content could not be compared quantitatively. Hence, analysis of the GLUT1 protein in the follicular fractions was restricted to comparison of the ratio of granulosa GLUT1:thecal GLUT1 across the treatment groups.

Hormone, metabolite and glucose analysis

Glucose was measured in whole blood using a Medisense[®]2 glucose meter (Medisense Inc., Bedford, MA). The normal reading range was 1.1–33.3 mmol l^{-1} . Coefficients of variation for fresh human blood for low (2.2 ± 0.16 mmol l^{-1}), medium (4.5 ± 0.18 mmol l^{-1}), medium high (11.1 ± 0.35 mmol l^{-1}) and high (14.2 ± 0.67 mmol l^{-1}) quality controls were 7.7, 4.1, 3.1 and 4.7%, respectively.

Insulin was measured by a double-antibody radioimmunoassay (Tindal *et al.*, 1978) using ovine insulin standards (23.2 iu mg^{-1} ; Sigma-Aldrich Pty Ltd, Castle Hill, NSW). The samples were assayed as duplicate 100 μl aliquots and the limit of detection was 0.03 ng ml^{-1} . The intra-assay coefficients of variation for the low (0.32 ng ml^{-1}), medium (0.99 ng ml^{-1}) and high (1.60 ng ml^{-1}) quality controls were 10.7, 8.2 and 4.9%, respectively.

Oestradiol and androstenedione concentrations in follicular fluid were determined by radioimmunoassay (Campbell, 1998) and in both cases all the samples were measured in a single assay. The intra-assay coefficients of variation for the assays were 10.2 and 11.7% for the oestradiol and androstenedione assays, respectively.

Table 1. Body condition score and live weight at allocation to treatment, weight before and after 5 days of treatment, and weight lost during treatment in ewes given nutritional supplements for 5 days

	Control (<i>n</i> = 51)	8-Lupin (<i>n</i> = 49)	8-Gluc (<i>n</i> = 50)	12-Gluc (<i>n</i> = 51)
Body condition score at allocation	3.3 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.1
Weight at allocation (kg)	56.6 ± 1.0	55.7 ± 0.9	55.6 ± 0.9	55.7 ± 0.9
Weight on day 0 (kg)	57.8 ± 0.9	56.6 ± 0.9	56.8 ± 0.9	56.7 ± 0.9
Weight on day 5 (kg)	55.6 ± 0.8	55.5 ± 1.0	54.4 ± 0.9	54.5 ± 0.9
Weight change during the 5 days (kg)	-2.2 ± 0.3	-1.1 ± 0.3 ^a	-2.4 ± 0.3 ^a	-2.1 ± 0.3

Values are mean ± SEM.

Control: 200 ml water every 8 h; 8-Lupin: 250 g lupin grain every 8 h; 8-Gluc: 200 ml glucogenic mixture every 8 h; 12-Gluc: 200 ml glucogenic mixture every 12 h.

Groups with the same superscripts are significantly different (^a*P* < 0.05).

Statistical analysis

Two ewes in the 8-Gluc group from which blood samples were taken had no visible corpora lutea and so their hormone data are omitted from the study. Live weight and live weight change were assessed using one-way ANOVA with *post hoc* comparisons using Scheffe's test. Chi-squared tables (2 × 3) were used to analyse ovulation rates and size classes of follicles: < 2 mm, 2–4 mm and > 4 mm in diameter. The concentrations of GLUT4 in the granulosa and theca samples were analysed using a non-parametric ANOVA for randomized blocks, with nutritional status (treatments) and western blot batch (blocks) as the sources of variation. Simple regression analyses were used to test whether there were relationships among glucose transporter concentrations, steroid concentrations and follicle size, and between live weight and mean GLUT4 concentrations in theca or granulosa for each ewe. The granulosa:theca ratios of GLUT1 were analysed using a one-way ANOVA. Glucose and insulin data were analysed using time points common to all groups. The effects of treatment and time on concentrations of glucose and insulin were assessed using a repeated measures ANOVA. At the time of treatment within each treatment group, a peak glucose concentration was recorded if the concentration increased by more than twice the standard deviation of the previous time point.

Results

Ovulation rate and live weight

The control (*n* = 45), 8-Gluc (*n* = 44) and 12-Gluc (*n* = 44) groups had the same ovulation rate (1.4), which did not differ significantly from that of the 8-Lupin group (1.6; *n* = 43). Three or four ovulations were observed in two ewes from each group that received nutritional supplements, but not in ewes in the control group. Three ewes in the 8-Gluc group did not ovulate.

The live weights of the ewes did not differ significantly among treatments, but ewes in the 8-Lupin group lost

significantly less weight during the 5 days of nutritional treatment than did ewes in the 8-Gluc group (Table 1). In the 12-Gluc and 8-Gluc groups, the ewes with an ovulation rate of 2 had a significantly higher mean live weight than the ewes with an ovulation rate of 1, but this difference was not observed in the control or 8-Lupin groups (Fig. 1).

Glucose

At the start of the nutritional treatment (*t* = 0 on day 1), blood glucose concentrations increased in the three groups given oral treatments (Fig. 2). The peak glucose concentration was lower in the control group than it was in the 8-Gluc (*P* < 0.05) and 12-Gluc groups (*P* < 0.001). During the remainder of the sampling period, there were no further peak glucose concentrations in the control group but there were peak concentrations of approximately half the amplitude on day 6 in the 8-Gluc group and days 3 and 5 in the 12-Gluc group. In the 8-Lupin group, glucose concentrations did not increase at any of the feeding times. Blood glucose concentrations were significantly higher in the 8-Lupin group than in the control group for the duration of the sampling period (*P* < 0.0001). Glucose concentrations in the 8-Gluc and 12-Gluc groups during the sampling period were significantly higher than those in the control or 8-Lupin groups (*P* < 0.0001), but were not different from one another (Fig. 2).

Insulin

Plasma insulin concentrations increased at the time of the first treatment in the control, 8-Gluc and 12-Gluc groups (Fig. 3). The concentrations in the control group remained relatively constant after the first 8 h of the sampling period. The insulin concentrations of the 8-Lupin, 8-Gluc and 12-Gluc groups were significantly higher than those in the control group for the duration of the study (*P* < 0.0001). Although the insulin concentrations in the 8-Lupin group increased after the first 24 h of the sampling period, the concentrations in the 8-Gluc and 12-Gluc groups were significantly higher for the duration of the

Fig. 1. Relationship of live weight to ovulation rate after nutritional supplementation for 5 days preceding a prostaglandin-induced luteolysis of the previous oestrous cycle in sheep treated with 200 ml water at 8 h intervals (Control), 250 g lupin grain at 8 h intervals (8-Lupin) or 200 ml of a glucogenic substance at 8 (8-Gluc) or 12 h intervals (12-Gluc). Horizontal bars indicate the mean weight. Columns with the same letter have significantly different mean weights ($^aP < 0.005$; $^bP < 0.01$).

sampling period ($P < 0.0001$). In the 8-Gluc group, the insulin concentrations were at least twice the starting concentration for the first 72 h, declining to control concentrations on day 5. The profile in the 8-Gluc group was significantly different from that in the 12-Gluc group, in which the increase in plasma insulin concentrations was not as pronounced ($P < 0.0001$; Fig. 3).

Number of follicles

The numbers of follicles in each of the three size classes per ewe in the control group differed from those in both the 8-Gluc and the 12-Gluc groups, but not from those in the 8-Lupin group ($P < 0.05$; Table 2). The sizes of follicles in the 8-Lupin group also differed from those in both the 8-Gluc and the 12-Gluc groups ($P < 0.05$; Table 2).

Glucose transporters

GLUT1 was detected in all granulosa and theca samples and there were higher concentrations in the granulosa than in the theca cells (Fig. 4). The granulosa:theca ratios of GLUT1 concentrations were 8.5 ± 2.3 , 6.7 ± 4.0 , 7.9 ± 1.1 and 17.6 ± 12.9 for the control, 8-Gluc, 12-Gluc and 8-Lupin groups, respectively. These values were not significantly different and were not related to follicle size, or to the concentrations of oestradiol or androstenedione in the follicular fluid.

GLUT4 was detected in the theca and granulosa for all follicles (Fig. 4, Table 3) but the GLUT4 concentrations in the largest follicles and the mean content per ewe in either type of cell did not differ among groups. GLUT4 detected in granulosa or theca cells is shown according to follicle size, oestradiol concentration and androstenedione concentration (Tables 4 and 5). There was no relationship among

Fig. 2. Blood glucose concentrations (mean $\pm$ SEM) during 5 days of nutritional treatment preceding a prostaglandin-induced luteolysis in sheep treated with (a) 200 ml water at 8 h intervals (Control), (b) 250 g lupin grain at 8 h intervals (8-Lupin), (c) 200 ml of a glucogenic mixture at 8 h intervals (8-Gluc) or (d) 200 ml of a glucogenic mixture at 12 h intervals (12-Gluc). The arrows indicate the treatment times: every 8 h for (a), (b) and (c) and every 12 h for (d). The asterisks indicate peak values that are higher than the value in the previous point by more than 2 standard deviations of the assay.

any of these variables and GLUT4 concentrations, or between live weight and mean GLUT4 concentrations within ewes.

Discussion

It is now clear that the theca and granulosa cells of the sheep ovarian follicle contain both GLUT1 and GLUT4, but that the distribution of GLUT1 and the amounts of GLUT4 were not affected by nutritional treatment, even when there

Table 2. Number and size of all follicles dissected from ovaries of ewes given nutritional supplements for 5 days

Follicle size	Control ^{ab}	8-Lupin ^{cd}	8-Gluc ^{ad}	12-Gluc ^{bc}
Large (> 4 mm)	2.0 ± 0.4	3.0 ± 0.4	2.8 ± 0.6	3.3 ± 0.3
Medium (2–4 mm)	7.8 ± 1.7	10.0 ± 2.7	5.0 ± 1.4	4.8 ± 1.4
Small (< 2 mm)	32.0 ± 8.3	47.0 ± 13.9	47.3 ± 8.6	45.8 ± 12.1

Values are mean ± SEM.
n = 4 per treatment group.
Control: 200 ml water every 8 h; 8-Lupin: 250 g lupin grain every 8 h; 8-Gluc: 200 ml glucogenic mixture every 8 h; 12-Gluc: 200 ml glucogenic mixture every 12 h.
Groups with the same superscripts are significantly different (^{abc}*P* < 0.05; ^d*P* = 0.054).

Fig. 3. Plasma insulin concentrations (mean ± SEM) during 5 days of nutritional treatment preceding a prostaglandin-induced luteolysis in sheep treated with (a) 200 ml water at 8 h intervals (Control), (b) 250 g lupin grain at 8 h intervals (8-Lupin), (c) 200 ml of a glucogenic mixture at 8 h intervals (8-Gluc) or (d) 200 ml of a glucogenic mixture at 12 h intervals (12-Gluc). The arrows indicate the treatment times: every 8 h for (a), (b) and (c) and every 12 h for (d).

Fig. 4. Western blotting showing the glucose transporters 1 and 4 (GLUT1 and GLUT4) detected in the granulosa (g) and theca (t) of three follicles (A, B and C) from one ewe in the group treated with 200 ml of a glucogenic mixture at 12 h intervals (12-Gluc) for 5 days. Arrows indicate the calculated molecular mass of the proteins. In contrast to the follicular samples, the positive control had undetectable concentrations of GLUT1, so comparison of relative concentrations of GLUT1 was not possible. Therefore, analysis of the GLUT1 protein in the follicular fractions was restricted to comparisons among treatment groups of the granulosa:theca ratio of GLUT1. The relative concentration of GLUT1 and GLUT4 in the two types of cell is markedly different: the GLUT1 concentration is higher in the granulosa cells than in the theca cells, whereas for GLUT4 there is no visible difference in the concentration between the two types of cell. GLUT1 is represented by a diffuse band with a molecular mass between 47.5 kDa and 55 kDa. In contrast, the GLUT4 protein band is narrow and has a molecular mass of about 48 kDa in the positive control samples compared with about 52 kDa in the ovarian samples. The difference was attributed to differences in tissue-specific glycosylation (Lis and Sharan, 1993).

were changes in the concentrations of glucose and insulin. Moreover, the distribution of GLUT1 and the amounts of GLUT4 were not related to follicle size, or concentrations of oestradiol or androstenedione. Therefore, nutritional supplements that increase glucose and insulin concentrations do not appear to affect ovarian glucose transporter concentrations in ewes.

The presence of GLUT1 in the sheep ovary is in agreement with findings in rodents (Kol *et al.*, 1997; Kodaman and Behrman, 1999; Zhou *et al.*, 2000) but, in contrast to findings in rodents, GLUT4 is also present in the

Table 3. Glucose transporter 4 (GLUT4) detected as a percentage of the positive control (muscle) in the theca and granulosa of all follicles from ewes given nutritional supplements for 5 days

	Control	8-Lupin	8-Gluc	12-Gluc	Total
Total thecal GLUT4 in all follicles (percentage of positive control)	80 ± 25 (n = 9)	78 ± 32 (n = 7)	121 ± 23 (n = 12)	44 ± 9 (n = 10)	83 ± 12 (n = 38)
Total granulosa GLUT4 in all follicles (percentage of positive control)	75 ± 31 (n = 6)	84 ± 33 (n = 6)	55 ± 40 (n = 6)	63 ± 14 (n = 8)	69 ± 14 (n = 26)

Values are mean ± SEM.

Control: 200 ml water every 8 h; 8-Lupin: 250 g lupin grain every 8 h; 8-Gluc: 200 ml glucogenic mixture every 8 h; 12-Gluc: 200 ml glucogenic mixture every 12 h.

Table 4. Glucose transporter 4 (GLUT4) detected as a percentage of the positive control in the theca of sheep follicles categorized according to follicle size, and follicular fluid oestradiol and androstenedione concentrations in ewes given nutritional supplements for 5 days

	Control	8-Lupin	8-Gluc	12-Gluc	Total
Follicle size					
< 4 mm	73 ± 31 (n = 4)	8 ± 0 (n = 2)	81 ± 23 (n = 4)	– (n = 0)	63 ± 9 (n = 10)
≥ 4 mm	85 ± 41 (n = 5)	106 ± 38 (n = 5)	141 ± 31 (n = 8)	44 ± 9 (n = 10)	90 ± 13 (n = 28)
Oestradiol					
< 10 ng ml ⁻¹	84 ± 41 (n = 4)	62 ± 49 (n = 4)	143 ± 66 (n = 3)	59 ± 11 (n = 6)	80 ± 19 (n = 17)
≥ 10 ng ml ⁻¹	76 ± 36 (n = 5)	99 ± 44 (n = 3)	113 ± 24 (n = 9)	21 ± 6 (n = 4)	85 ± 72 (n = 21)
Androstenedione					
Undetectable	80 ± 31 (n = 7)	78 ± 32 (n = 7)	124 ± 25 (n = 11)	36 ± 13 (n = 5)	88 ± 15 (n = 30)
Detectable	78 ± 55 (n = 2)	– (n = 0)	90 (n = 1)	51 ± 13 (n = 5)	62 ± 14 (n = 8)

Values are mean ± SEM.

Control: 200 ml water every 8 h; 8-Lupin: 250 g lupin grain every 8 h; 8-Gluc: 200 ml glucogenic mixture every 8 h; 12-Gluc: 200 ml glucogenic mixture every 12 h.

sheep ovary, within the thecal shell and granulosa cells. The apparent difference between species may be a result of differences in digestive physiology: rats are monogastric whereas sheep are ruminants. A second explanation is that the rodent studies were limited to immature animals and GLUT4 might be expressed only in mature or large follicles. Finally, it is possible that GLUT4 has a specific role in the sheep ovary, namely mediation of the short-term nutritional stimulation of ovulation rate, which has not been documented in rodents.

The expression of GLUT1 indicates an important role for this transporter in providing basal glucose uptake in granulosa and theca cells, as seen in other tissues. However, the observation that there is more GLUT1 in granulosa cells than in theca cells is more significant. As oestradiol stimulates GLUT1 (Shi and Simpkins, 1997; Welch and Gorski, 1999), it is possible that the granulosa cells produce more GLUT1 in response to high local concentrations of oestradiol, either in the granulosa cytoplasm or the

oestradiol-rich follicular fluid. However, there was no relationship between ovarian steroid concentrations and the concentrations of GLUT4 or the granulosa:theca ratios of GLUT1. An alternative explanation for higher concentrations of GLUT1 in the granulosa than in the theca cells is that the granulosa layer is avascular, whereas the theca is not (Bassett, 1943). The lack of a direct blood supply may hinder nutrient uptake and a higher concentration of GLUT1 may overcome this.

Insulin-regulated GLUT4 in the granulosa and theca cells may have two roles. The first is that insulin-stimulated glucose uptake in follicular cells provides precursors for the glycogen that is stored within the granulosa of sheep antral follicles (Hay and Moor, 1975; Cran *et al.*, 1979). It is highly unlikely that a small organ like the ovary acts as a storage site for whole body glucose homeostasis, but it is likely that glycogen is stored for local use, as it is in muscle cells. GLUT4 would enable insulin-stimulated uptake of excess glucose for storage as glycogen in granulosa cells, to

Table 5. Glucose transporter 4 (GLUT4) detected as a percentage of the positive control in the granulosa of sheep follicles categorized according to follicle size, and follicular fluid oestradiol and androstenedione concentrations in ewes given nutritional supplements for 5 days

	Control	8-Lupin	8-Gluc	12-Gluc	Total
Follicle size					
< 4 mm	31 (n = 1)	7 (n = 1)	6 (n = 1)	– (n = 0)	15 ± 8 (n = 3)
≥ 4 mm	84 ± 37 (n = 5)	19 ± 36 (n = 5)	65 ± 47 (n = 5)	63 ± 14 (n = 8)	76 ± 15 (n = 23)
Oestradiol					
< 10 ng ml ⁻¹	88 ± 62 (n = 2)	62 ± 3 (n = 3)	63 ± 47 (n = 5)	86 ± 21 (n = 4)	72 ± 17 (n = 14)
≥ 10 ng ml ⁻¹	69 ± 42 (n = 4)	106 ± 44 (n = 3)	13 (n = 1)	41 ± 10 (n = 4)	67 ± 22 (n = 12)
Androstenedione					
Undetectable	85 ± 37 (n = 5)	84 ± 33 (n = 6)	63 ± 47 (n = 5)	56 ± 22 (n = 4)	85 ± 17 (n = 20)
Detectable	26 (n = 1)	– (n = 0)	13 (n = 1)	71 ± 19 (n = 4)	21 ± 3 (n = 6)

Values are mean ± SEM.

Control: 200 ml water every 8 h; 8-Lupin: 250 g lupin grain every 8 h; 8-Gluc: 200 ml glucogenic mixture every 8 h; 12-Gluc: 200 ml glucogenic mixture every 12 h.

provide energy substrates for periods of rapid growth or high metabolic demand. This mechanism could also lead to non-specific enhancement of cell viability, which would enable follicles to ovulate that would not have done so under a lower nutritional regimen. The idea of non-specific nutritional enhancement is supported by the fact that there was no difference in the GLUT4 concentrations among follicle sizes, although follicles < 2 mm in diameter were not assessed in this study. Therefore, it is apparent that circulating concentrations of glucose and insulin do not affect the concentrations of follicular GLUT4 in sheep because differences in GLUT4 were not seen among treatments. The second possible role for GLUT4 is to allow an increase in ovarian glucose uptake if glucose and insulin are available, thereby enabling the ewe to respond to short-term changes in nutrition.

Ovulation rate was not increased by any of the treatments despite the fact that glucose and insulin concentrations were increased. Glucose and insulin concentrations also increased briefly in the control group and this increase was attributed to a stress response to the introduction of an unfamiliar procedure. Ovulation rate also depends on live weight and body condition, but in the control ewes there was no difference in live weight between ewes with ovulation rates of 1 or 2. In contrast, in the animals given the glucogenic mixture, ewes with an ovulation rate of 2 were heavier than ewes with an ovulation rate of 1. This finding indicates that heavier ewes are better able

to increase their ovulation rate in response to the increase in glucose availability, despite evidence to indicate that they are unable to respond to nutritional supplementation because they have already reached the limit imposed by their genetic potential (for a review, see Downing and Scaramuzzi, 1991). However, irrespective of treatment, GLUT4 concentrations were not related to live weight, and nutritional stimulation did not affect the GLUT4 concentrations or granulosa:theca ratios of GLUT1. There are three possible reasons: (i) owing to restrictions on the amount of tissue, this study could not differentiate between intracellular glucose transporters and glucose transporters that transport at the plasma membrane, and so the amount of GLUT4 actively transporting glucose may have been increased by nutritional treatment even though the total cellular amount was not affected; (ii) the glucose and insulin concentrations had returned to control values by the end of the study, when the ovaries were collected (although glucose uptake was not determined); and (iii) the observation that nutrition did not affect glucose transporter concentrations highlights the differences between glucose homeostasis in ruminants and non-ruminants. In addition, it is likely that there are differences in glucose homeostasis between physiologically normal ruminants and physiologically abnormal animals, such as the insulin-resistant rodent model, and there may be differences between studies *in vivo* and *in vitro* (for a review, see Klip *et al.*, 1994).

Ovulation rate may not have been increased by nutritional supplementation because of the high body condition score of the ewes, which may have reduced the magnitude of the response (for a review, see Downing and Scaramuzzi, 1991). This effect is evident from the ovulation rate of the control ewes (1.4 ± 0.1), which was high for Merinos and similar to that seen by Brien *et al.* (1976) in ewes fed with lupin grain. The ewes in the present study appeared to be close to the limit imposed by their genetic potential, which would explain why the ewes fed with lupin showed no increase in ovulation rate.

Further work into the distribution of another insulin-responsive glucose transporter that was discovered recently, GLUTX1/GLUT8, may increase understanding of how glucose stimulates ovulation rate (Carayannopoulos *et al.*, 2000; Doege *et al.*, 2000; Ibberson *et al.*, 2000).

In conclusion, GLUT1 and GLUT4 are present within the granulosa and theca cells of sheep antral follicles and there is more GLUT1 in the granulosa than in the theca cells. Therefore, there are two pathways for glucose uptake by the two types of cell that govern follicular development in the sheep ovary.

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