Animal Reproduction Science 127 (2011) 154–163

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

The infusion of glucose in ewes during the luteal phase increases the number of follicles but reduces oestradiol production and some correlates of metabolic function in the large follicles

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ARTICLE INFO

Article history:
Received 29 April 2010
Received in revised form 17 July 2011
Accepted 18 July 2011
Available online 16 August 2011

Keywords:
Sheep
Nutrition
Reproduction
Follicle
Glucose
Oestradiol

ABSTRACT

Short-term nutritional supplementation stimulates folliculogenesis in ewes probably by insulin-mediated actions of glucose in the follicle. The aim of this study was to determine the effect of glucose on follicle number and granulosa levels of Aromatase P450 and phosphorylated Akt and AMPK. Twelve Ile-de-France ewes were allocated to two groups; one ($n = 7$) infused with saline and the other ($n = 5$) with glucose (10 mM/h) for 72 h in the luteal phase. At the end of infusion, ovaries were collected and all follicles >1 mm in diameter were dissected to recover granulosa cells. Aromatase P450 and phosphorylated Akt and AMPK were analysed by Western blotting of granulosa cell lysates. Blood plasmas collected before and during the infusions were analysed for progesterone, oestradiol, LH, FSH, glucose, insulin and IGF-I. The infusion of glucose significantly increased follicle number but, significantly reduced Aromatase P450 and phosphorylated Akt and AMPK in granulosa cells. The circulating concentration of glucose rose significantly 3 h after the start of the glucose infusion and remained elevated until 27 h then fell; the circulating concentration of insulin rose significantly by 3 h and remained elevated. The circulating concentration of oestradiol fell significantly by 32 h and remained low; the circulating concentrations of LH and FSH were unaffected. These data show that short-term infusion of glucose stimulated follicular growth but decreased Aromatase P450 in granulosa cells. The reduced levels of phosphorylated Akt and AMPK suggest that the phosphatidylinositol 3-kinase pathway has been inhibited by high concentrations of glucose. These data also suggest that there may be functional cross-talk between FSH and insulin signalling in granulosa cells.

1. Introduction

In the ewe, short-term nutritional supplementation with energy yielding substrates during the late luteal phase of the oestrous cycle is associated with an increased number of follicles (Haresign, 1981; McNeilly et al., 1987; Viñoles et al., 2002, 2005; Letelier et al., 2008; Somchit et al., 2007) and increased ovulation rate (Nottle et al., 1985; Teleni et al., 1989; Downing et al., 1995a) an effect that can be mimicked by intravenous glucose (Downing et al., 1995b). It has been suggested that the mechanism of this effect probably involves direct follicular actions of nutrition mediated by the intrafollicular insulin-glucose

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0378-4320/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.anireprosci.2011.07.017

system (Scaramuzzi et al., 2006). However, little is known about glucose-induced, insulin signalling in the follicle or its effects on intra-follicular actions of FSH such as aromatase activity and the secretion of follicular oestradiol.

There is considerable evidence to show that the follicle has a functional insulin-glucose system. The insulin receptor, a tyrosine kinase that autophosphorylates on ligand binding has been identified in the follicle (el-Roeiy et al., 1993; Samoto et al., 1993; Poretsky et al., 1999). The intracellular actions of insulin are mediated by a complex array of intracellular pathways (Taniguchi et al., 2006) and there are numerous phosphatases and intermediary and terminal kinases as well as scaffold and docking proteins responsible for a wide variety of tissue specific responses to insulin (Taniguchi et al., 2006; Zaid et al., 2008; Leney and Tavaré, 2009). For example, the insulin receptor substrate proteins (IRSs) interact with the insulin receptor and when phosphorylated, initiate insulin's intracellular responses. The IRSs have been detected in granulosa cells of several species (Wu et al., 2000; Yen et al., 2004) and in ovine theca cells (Somchit, 2008). In one study, it was shown that selective deletion of the gene for IRS-2 was associated with impaired folliculogenesis in mice (Neganova et al., 2007). Other kinases activated by insulin, although not exclusively, have been identified in follicles including PI3K, Akt and AMPK (Evans and Martin, 2000; Carvalho et al., 2003; Zeleznik et al., 2003; Ryan et al., 2007, 2008; Tosca et al., 2008). The protein phosphatase, PTEN, which can attenuate the insulin signal through de-phosphorylation, has also been identified in the follicle (Froment et al., 2005; Goto et al., 2007; Fan et al., 2008) and the glucose transport proteins, GLUT1 and GLUT4, have been detected in granulosa and theca cells from sheep and cattle (Williams et al., 2001; Nishimoto et al., 2006).

In this experiment we set out to test the hypothesis that the glucose-induced secretion of insulin acting via the PI3K signalling pathway increased the number of follicles in the ovary. To test the hypothesis glucose was infused systemically for 5 days and its effects on the phosphorylation state of two downstream kinases, (Akt and AMPK) in the insulin PI3K signalling pathway, the level of Aromatase P450 in granulosa cells and the concentration of oestradiol in jugular venous plasma.

2. Materials and methods

2.1. Animals and their management

Twelve Ile-de-France ewes between 2 and 4 years old and with body condition scores ranging between 2 and 3 were used in the study. During the study, they were fed a basal diet of good quality hay and with water freely available at all times. Before starting the infusions, oestrus was synchronised using progestagen sponges (Chronogest; Intervet/Schering-Plough Animal Health, Angers, France) for 12 days followed by the injection of 500 IU of pregnant mare serum gonadotrophin, (PMSG-Intervet; Intervet/Schering-Plough Animal Health, Angers, France) per ewe at the time the progestagen sponges were removed resulting in oestrus two days later. Eight days following oestrus, the ewes were fitted with bilateral jugular cannulae. One cannula was used exclusively for blood collection and the other exclusively for infusion (saline or glucose). The cannulae were flushed with sterile heparinised (100 IU/mL) saline, sealed and tucked under a protective covering. The next day, at 0900 h, one group of ewes was infused with saline for 72 h at a rate of 10 mL/h ($n = 7$) and the other with glucose ($n = 5$; 10 mM/h) also for 72 h at 10 mL/h. The ewes were fed daily with hay immediately after the blood sample at 0900 h. The experiments were carried out in accordance with French and European regulations on the care and welfare of animals in research.

2.2. Blood sampling and plasma preparation

Samples (5 mL) of jugular venous blood were collected in fluoride EDTA tubes twice daily at 0900 h and 1200 h and in heparin–lithium vacuum tubes thrice daily at 0900 h, 1200 h and 1700 h. On the second day after the start of infusion, blood samples (5 mL) were also collected in heparin–lithium vacuum tubes every 15 min for a six hour period from 1200 h. Within an hour of blood collection, the samples were centrifuged at 2500 × *g* for 10 min. Three aliquots of approximately 0.5 mL of plasma were stored at −20 °C.

2.3. Tissue collection

Following the end of infusion, the ewes were ovariectomised under general anaesthesia, by mid-ventral laparotomy and their ovaries recovered and placed in sterile saline until dissection. Dissection was performed within an hour of ovariectomy. Individual follicles >1 mm in diameter, were dissected and measured using a millimetre scale. All dissected follicles were slit open in 100 μL of 1× Eagles basic medium, (GIBCO Invitrogen, Cergy-Pontoise, France). Granulosa cells were removed by scraping the interior surface of the follicle wall gently, with a platinum loop. The granulosa cell suspension was homogenised by aspirating it several times. A sample of 5 μL was removed and used to determine the physiological state of the follicle as described below. The remaining granulosa cell suspension was centrifuged, the supernatant was then removed and the granulosa cell pellet stored at −80 °C for Western blot analysis.

2.4. Determination of follicle quality

To determine the physiological state of follicles a smear of granulosa cells was prepared for each follicle on a histological slide and stained with Haematoxylin–Carazzi (Monget et al., 1993). The state of the follicles was assessed by microscopic examination of the stained smear and follicles were classified as atretic, early atretic or non-atretic (normal) as described (Monniaux, 1987). Normal follicles were characterised by the presence of mitotic cells in the smear. Early atretic follicles were normal follicles that also contained some pycnotic cells as well as mitotic ells. Atretic follicles were characterised by many pycnotic bodies and no mitotic granulosa cells.

2.5. Hormone and metabolite analyses

The samples collected in fluoride EDTA tubes were analysed for glucose. Samples collected in heparin–lithium tubes were analysed as follows: the thrice daily samples were analysed for insulin, IGF-I, FSH and oestradiol, the samples collected every 15 min over a 6-h window on day 2 of the infusion were analysed for LH and the daily sample at 0900 h was analysed for progesterone. All assays were carried out in duplicate.

2.6. Assays for IGF-I, LH, FSH and progesterone

A commercial kit for human IGF-I (Cis Bio-International B6P 32F91192 Gif sur Yvette Cedex, France) was used to assay IGF-I. The method is a "sandwich" immunoradiometric assay. Two monoclonal antibodies were prepared against two different antigenic sites of IGF-I. One of these was used to coat assay tubes and the other was radiolabeled with iodine 125. Before assay, the samples were acid extracted and saturated with recombinant human IGF-II to ensure complete dissociation of IGF-I from IGF binding proteins. To validate the method for ovine IGF-I, a plasma sample from a fat ewe was diluted in buffer or in plasma from a thin ewe were assayed to establish parallelism; the regression coefficients were 0.99 and 0.92 respectively. The inter-plate coefficients of variation for plasma samples containing 33 and 378 ng/mL of human IGF-I were 4.3 and 8.4%, respectively. Plasma samples were analysed by ELISA to determine the concentrations of FSH, LH (Faure et al., 2005) and progesterone (Canépa et al., 2008). The limit of detection of LH was 0.01 ng/mL and the intra-assay coefficient of variation was 13.6%. The limit of detection of FSH was 0.1 ng/mL and the intra-assay coefficient of variation was 12.2%. The limit of detection of progesterone was 0.25 ng/mL and the intra-assay coefficient of variation was 14.3%.

2.7. Oestradiol

Oestradiol was determined by radioimmunoassay (Ben Said et al., 2007) of solvent extracted plasma. Samples (500 μL) of plasma were extracted in 3 mL of solvent (cyclohexane and ethyl acetate, 1:1 by volume) for 2 h and then allowed to stand so that a plasma pellet formed at the bottom of the tube. The pellet was quickly frozen in liquid nitrogen, and the organic phase transferred to a glass tube. After evaporation of the solvent under compressed air, the residue was dissolved in 200 μL 0.1 M gelatine-phosphate buffer (0.72 M $Na_2HPO_4{\cdot}2H_20$, 0.28 M $Na_2H_2PO_4{\cdot}2H_20$, 0.155 M NaCl and NaN_3 [1 g/L]) at pH 7. Tubes were left overnight at 4 °C. The radioimmunoassay was performed using an oestradiol radioimmunoassay kit (Estradiol-2 kit P2210; Diasorin, SA, Antony, France). The limit of detection of oestradiol was 0.39 pg/mL and the intra-assay coefficient of variation was 20.5%.

2.8. Glucose

The concentration of glucose in plasma was determined by colorimetry using the glucose oxidase method. The reagents were supplied as a kit (Glucose Assay Kit; Sigma Aldrich Inc., Saint-Quentin Fallavier, France) and the assay method followed the instructions provided by the manufacturer of the kit. Plasma samples were diluted to obtain concentrations that fell within the range of the standard curve (20–80 μg of glucose per mL). The sensitivity of the assay was 2 mg/dL.

2.9. Insulin

Concentrations of insulin in plasma were determined by ELISA using a commercial kit for ovine Insulin (Mercodia AB, Sweden). The assay method followed the instructions provided by the manufacturer of the kit. The range of the assay was from 0.05 to 3.0 ng/mL and the sensitivity of the assay was 0.025 ng/mL. The antibody did not cross react with either the C-peptide or pro-insulin.

2.10. Antibodies for Western blotting

All antibodies were obtained from commercial sources. The analysis of Akt used a rabbit polyclonal antibody to Akt (Cell Signalling Technology, Beverly, MA, USA) and a rabbit polyclonal antibody to phospho-Akt1/2/3 (Ser 473; Santa Cruz Biotechnology Inc., Heidelberg, Germany). The analysis of AMPK used a rabbit polyclonal antibody to AMPK (Thr 172; Cell Signalling Technology, Beverly, MA, USA) and a rabbit polyclonal antibody to phospho-AMPK (Cell Signalling Technology, Beverly, MA, USA). A mouse monoclonal antibody was used to analyse Aromatase P450 (ABD Serotec, Düsseldorf, Germany) and a mouse monoclonal antibody to vinculin was used as an internal standard (Sigma Aldrich Inc., Saint-Quentin Fallavier, France). All antibodies were used at a dilution of 1/1000. The secondary antibodies that were used were either a goat anti-mouse IgG (Laboratoires Eurobio, Courtaboeuf, France) or a goat anti-rabbit IgG (Laboratoires Eurobio, Courtaboeuf, France).

2.11. Western blotting

Western blotting was used to determine the levels and the ratio of phosphorylated Akt to total Akt and phosphorylated AMPK to total AMPK in non-atretic follicles greater than 2 mm in diameter. Lysates of granulosa cells were prepared on ice, by adding 100 μL of a lysis buffer (150 mM NaCl, 10 mM Tris Base, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 100 mM NaF, 9 mM $Na_2HPO_4{\cdot}2H_2O$, 1% Triton X100, 0.5% NP 40, H_2O_D, pH 7.4) to the granulosa cell pellet followed by centrifugation at 13,200 rpm for 30 min at 4 °C. The concentration of protein in the supernatants was determined by colorimetry using the BCA protein assay reagent (Interchim, Montluçon, France). Aliquots of lysate containing 30 μg of protein were re-suspended in Laemmli buffer (glycerol 50%, SDS 10%, Hepes 1 M – pH7.6, beta-mercaptoethanol 25%, bromophenol blue) and then analysed by Western blotting.

Granulosa cell lysates were subjected to electrophoresis on 10% (vol/vol) SDS-polyacrylamide gels for 2.5 h at 80 V, in the running buffer (H_2O_D, 50 mM Tris Base, 400 mM Glycine, 2% EDTA 0.1 M, 1% SDS 10%). The proteins were

then transferred onto nitrocellulose membranes for 1.5 h at 80 V, in transfer buffer (H_2O_D, 20 mM Tris Base, 200 mM Glycine, 20% Methanol, 0.1% SDS 10%). After washing in TBS Tween (TBST; H_2O_D, 2 mM Tris Base, 15 mM NaCl, 0.1% Tween 20, pH 7.4), the membranes were incubated for 1 h at room temperature with TBST containing 5% dry milk powder to saturate non-specific sites. Subsequently, membranes were incubated overnight at 4 °C with primary antibodies in TBST containing 5% dry milk powder.

After washing in TBST, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (final dilution 1:7000; Laboratoires Eurobio, Courtaboeuf, France) or horseradish peroxidase-conjugated anti-mouse IgG (final dilution 1:10,000; Laboratoires Eurobio, Courtaboeuf, France) for 2 h at room temperature in TBS Tween containing 5% dry milk powder. After washing in TBS Tween, the signal was detected by enhanced chemiluminescence (PerkinElmer, Life and Analytical Sciences, Courtaboeuf, France). The membranes were exposed on GE Healthcare film (PerkinElmer, Life and Analytical Sciences, Courtaboeuf, France), and then developed (Kodak AL4) and fixed (Kodak LX24) and dried. The films were analysed and the blots were quantified using ScionImage (4.0.3.2 version; Scion Corporation, Frederick, MA, USA).

2.12. Statistical analyses

All experimental data are presented as means ± SEM. The data were analysed using the statistical software program SPSS for Windows version 17.0. Follicle diameter and the data from Western blotting were analysed using Student's *t*-test. The blood concentrations of glucose, insulin, IGF-I, oestradiol and FSH were analysed using a repeated measures ANOVA run under the general linear model. Pulses of LH were identified (Cohen-Tannoudji, 1988; Martin et al., 1980) and the parameters of LH secretion were analysed either by Student's *t*-test (mean LH and basal LH) or the Mann–Whitney *U* test (pulse frequency and pulse amplitude).

3. Results

3.1. General observations

Following the removal of progestagen sponges and the injection of PMSG, one ewe failed to ovulate (progesterone concentration <1 ng/mL), the data from this ewe has been excluded from the analyses. No abnormal reactions were noted in any of the ewes during the infusions. The mean body weight of ewes infused with saline (50.1 ± 1.0 kg; mean ± SEM) was not significantly different ($P = 0.536$) from that of ewes infused with glucose (51.8 ± 1.0 kg).

3.2. Insulin and glucose

The concentration of glucose rose significantly within 4 h of the start of infusion and remained significantly elevated at 24 h and 28 h, but it had returned to pre-infusion concentrations by 48 h (Fig. 1). The concentration of insulin rose significantly within 4 h of the start of infusion and

Table 1
The effect of glucose infused at 10 mM/h for 72 h during the late luteal phase of the oestrous cycle on the mean ± SEM concentration of IGF-I (ng/mL).

Treatment group	Time relative to the start of infusion (h)											
	−24	−20	−14	0	4	10	24	28	34	48	58	72
Saline ($n = 6$)	148 ± 10.0	174 ± 24.5	160 ± 19.8	139 ± 12.2	148 ± 14.1	145 ± 11.4	139 ± 11.4	141 ± 13.3	136 ± 11.9	139 ± 11.6	139 ± 10.6	134 ± 9.28
Glucose ($n = 5$)	151 ± 14.8	163 ± 12.8	156 ± 12.4	165 ± 16.7	159 ± 13.2	166 ± 15.5	186 ± 19.8	187 ± 17.9	181 ± 17.9	202 ± 19.4*	198 ± 21.3*	191 ± 21.2*

* $P < 0.05$.

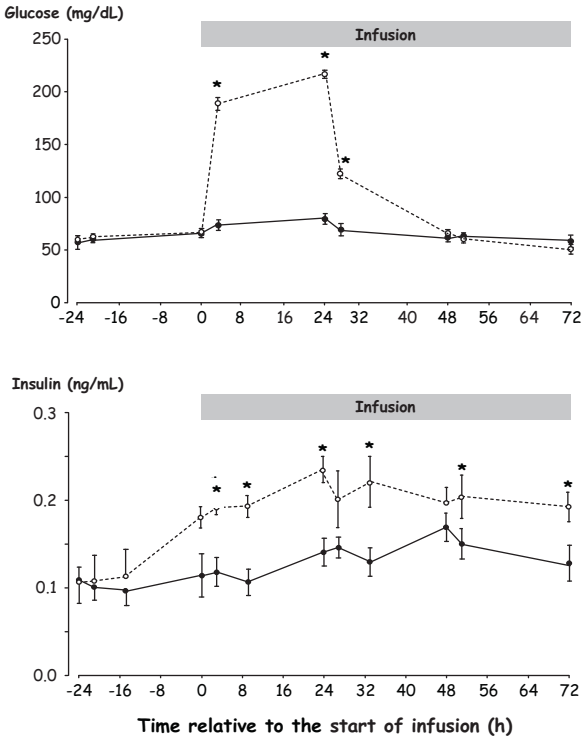

Fig. 1. The effect of glucose infused at 10 mM/h for 72 h during the late luteal phase on the plasma concentrations of glucose and insulin. Black circles – saline infused (control, $n = 6$); white circles – glucose infused ($n = 5$). An asterisk indicates a significant difference between treatments within times ($P < 0.05$).

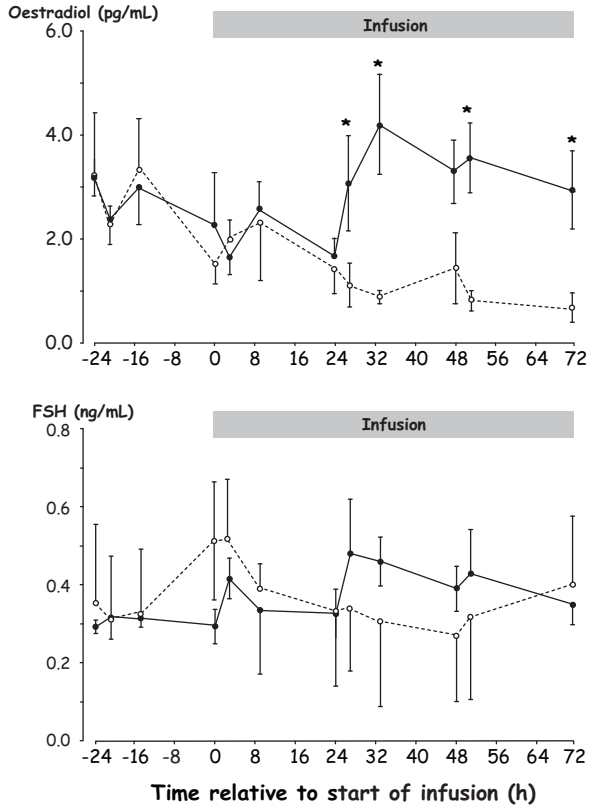

Fig. 2. The effect of glucose infused art 10 mM/h for 72 h during the late luteal phase on the plasma concentrations of 17 β oestradiol and FSH. Black circles – saline infused (control, $n = 6$); white circles – glucose infused ($n = 5$). An asterisk indicates a significant difference between treatments within times ($P < 0.05$).

was elevated at all subsequent sampling times (Fig. 1), at 28 h and 48 h although above control concentrations the increase was not significant but at all other times the increase was significant (8, 24, 34, 52 and 58 h; all $P < 0.05$).

3.3. Insulin-like growth factor I (IGF-I)

Although the concentration of IGF-I rose within 4 h ($P = 0.213$) of the start of infusion (Table 1) the increase was not significant until 48 h ($P = 0.015$) and it remained significantly elevated at 58 h ($P = 0.023$) and 72 h ($P = 0.052$).

3.4. The number of follicles

The infusion of glucose significantly ($P = 0.017$) increased the total number of follicles greater than 1 mm in diameter (Table 2) and significantly ($P = 0.031$) reduced the average diameter of all follicles over 2 mm in diameter; 3.36 ± 0.15 mm for controls compared to 2.96 ± 0.14 mm for glucose. There was a significant ($P = 0.018$) increase in the number of small (1.0–2.0 mm) follicles and a trend ($P = 0.060$) towards an increase in the number of medium (2.0–3.5 mm) follicles (Table 2). There was no effect on the number of large follicles ($P = 0.213$). The total number of non-atretic follicles was not affected by treatment but the number of atretic follicles was significantly ($P = 0.017$) greater in ewes infused with glucose compared to control ewes (Table 2). However, if the numbers of atretic follicles are expressed as a percentage of the total number of follicles the difference is no longer significant ($P = 0.273$).

3.5. Oestradiol and FSH

The concentration of FSH was not significantly affected by treatment (Fig. 2) however the concentration of oestradiol was significantly decreased by 28 h after the start of the infusion and remained lower than controls for the duration of the experiment. The reductions were significant at 34, 52 and 58 h ($P < 0.05$) but not at 48 h (Fig. 2).

3.6. Progesterone and LH

The concentrations of progesterone confirmed that 11 of the 12 ewes were in the luteal phase of the oestrous cycle during the period of infusion. There were no differences in the concentration of LH between the two groups nor were there any significant differences in the basal concentration of LH, the frequency of LH pulses or the amplitude of LH pulses (Table 3).

3.7. Aromatase P450, Akt and AMPK in granulosa cells

Western immunoblotting was able to detect Aromatase P450, Akt, phospho-Akt, AMPK, phospho-AMPK in

Table 2
The effect of glucose infused at 10 mM/h for 72 h during the late luteal phase of the oestrous cycle on the mean (±SEM) total number of follicles greater than 1 mm in diameter, the number of small, medium and large follicles and the number and percentage of non-atretic follicles.

Treatment	The number of follicles				
	Total (>1 mm)	Small (1 to <2 mm)	Medium (2 to <3.5 mm)	Large (>3.5 mm)	Non-atretic (>2.0 mm)
Saline (control) ($n=6$)	16.7 ± 2.60	10.0 ± 2.24	4.71 ± 1.02	2.20 ± 0.49	3.70 ± 0.92
Glucose ($n=5$)	36.4 ± 6.48*	28.2 ± 6.40*	6.80 ± 0.66**	1.40 ± 0.51	4.40 ± 0.51

* $P<0.05$.
** $P=0.06$.

Table 3
The effect of glucose infused at 10 mM/h for 72 h during the late luteal phase of the oestrous cycle on the mean (±SEM) concentration of LH, the basal concentration of LH, the frequency of LH pulses and the amplitude of LH pulses. There were no significant differences between treatments.

Treatment	Mean LH (ng/mL)	Basal LH (ng/mL)	Pulse frequency (pulses per 6 h)	Pulse amplitude (ng/mL)
Saline (control) ($n=6$)	0.152 ± 0.079	0.041 ± 0.007	0.57 ± 0.43	0.59 ± 0.46
Glucose ($n=5$)	0.060 ± 0.020	0.031 ± 0.006	0 40 ± 0.24	0.38 ± 0.15

granulosa cell lysates from individual follicles greater than 2.5 mm in diameter (Fig. 3).

The infusion of glucose reduced significantly ($P=0.034$) the expression of Aromatase P450 in granulosa cell lysates from follicles greater than 2 mm in diameter (Table 3). When the follicles were analysed within their physiological state (normal and early atretic) the effect of glucose was not significant ($P=0.210$) in follicles that were early atretic and tended towards significance ($P=0.062$) in normal follicles (Table 3). The level of Aromatase P450 was significantly ($P=0.022$) reduced in early atretic follicles compared to normal follicles (Table 4).

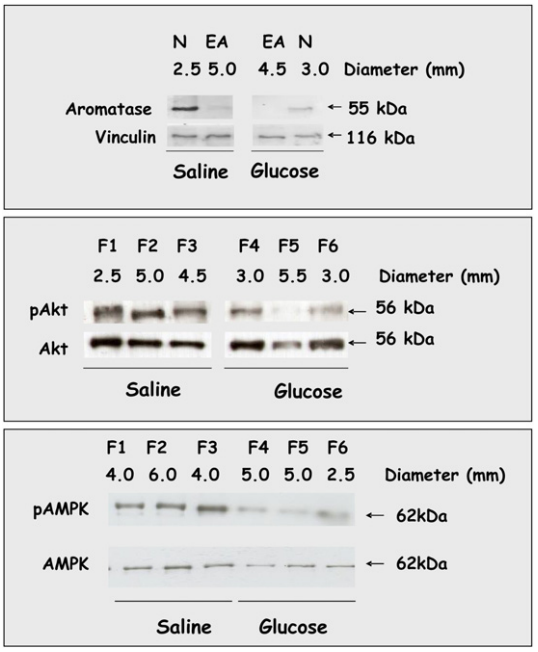

Fig. 3. Western immunoblot showing bands for Aromatase P450 at 55 kDa and for vinculin (an internal standard) at 116 kDa [top panel] in granulosa cells lysates from four individual, non-atretic follicles (N) or early atretic (EA) follicles and of pAKT at 56 kDa and Akt at 55 kDa [middle panel] and pAMPK at 62 kDa and AMPK at 62 kDa [bottom panel] in granulosa cells lysates from six individual follicles F1 to F6 from ewes infused with glucose at 10 mM/h for 72 h or from saline-infused control ewes.

The level of total Akt was not significantly ($P=0.415$) different between treatments but the level of phosphorylated Akt ($P=0.0009$) and the ratio of phosphorylated to total Akt ($P=0.0008$) were both significantly lower in granulosa cell lysates from ewes infused with glucose (Table 5). The levels of total Akt ($P=0.339$) and phosphorylated Akt ($P=0.351$) and the ratio of phosphorylated to total Akt ($P=0.234$) were not significantly different in granulosa cell lysates from normal and early atretic follicles.

The level of total AMPK was similar between treatments but the level of phosphorylated AMPK and the ratio of phosphorylated to total AMPK were both significantly lower in granulosa cell lysates from ewes infused with glucose (Table 5).

4. Discussion

These data show that a short-term infusion of glucose increased the total number of follicles suggesting that follicle growth has been stimulated by the infusion of 10 mM/h of glucose. The effect of glucose was strongest in small (1.0–2.0 mm) follicles. In medium (2.0–3.5 mm) follicles this effect was weaker with only a trend ($P<0.10$) towards significance and the effect was not seen in large (<3.5 mm) follicles. The number of non-atretic follicles was also increased by approximately 16% in ewes infused with glucose but again this difference was not significant. Because the number of small follicles was increased by the infusion of glucose a consequence was a reduction in their average diameter. In addition there may have also been an effect on the large follicles themselves because the average diameter of large follicle in ewes infused with glucose was reduced by around 35% but, because of the small number of follicles available for analysis this reduction was not significant and thus this suggestion is in need of experimental confirmation. It is quite clear that at least in this experimental model, glucose increased the number of small and medium sized follicles between 1 and 3.5 mm in diameter but not the number of large follicles with diameters

Table 4
The effect of glucose infused at 10 mM/h for 72 h during the late luteal phase of the oestrous cycle on the mean ± SEM diameter of non-atretic and early atretic follicles and their levels of Aromatase P450 in granulosa cell lysates expressed as a ratio with an internal standard, vinculin.

Treatment	State	Number of follicles	Diameter (mm)	Aromatase (ratio aromatase:vinculin)
Saline (control) ($n = 6$)	Non-atretic	22	3.66 ± 0.214	1.39 ± 0.284
	Early atretic	8	2.88 ± 0.125	0.73 ± 0.094
	Combined	30	3.45 ± 0.172	1.21 ± 0.216
Glucose ($n = 5$)	Non-atretic	22	3.23 ± 0.246**	0.901 ± 0.127***
	Early atretic	12	2.63 ± 0.109*	0.598 ± 0.110**
	Combined	34	3.01 ± 0.170*	0.794 ± 0.093*

* $P < 0.05$.
** $P = 0.089$.
*** $P = 0.061$.

greater than 3.5 mm and this observation is in broad agreement with numerous other investigations where ewes have been infused with glucose (Muñoz-Gutiérrez et al., 2002, 2004) or fed diets that cause short-term increases in the blood concentrations of glucose (Viñoles et al., 2002, 2005; Letelier et al., 2008; Somchit et al., 2007). These published data suggest that the increase in the ovulation rate that was induced by these treatments is due to an increase in the number of medium sized follicles (2.0–3.5 mm in diameter).

The patterns of insulin and glucose in blood suggest that the ewes were able to cope with the imposed glucose load. Initially the concentration of glucose rose to very high levels that stimulated the secretion of insulin. The increased blood concentrations of insulin were able to clear the increased load of blood glucose and by 28 h the concentration of glucose had fallen back into its physiological range and by 48 h they were back at control concentrations. The concentrations of insulin remained elevated reflecting restored glucose homeostasis in the presence of a high glucose load. The removal of glucose is most likely to have been by GLUT4-mediated uptake, primarily by adipose tissue, but because granulosa cells also contain GLUT4 (Williams et al., 2001; Nishimoto et al., 2006), granulosa cells will also have taken up increased amounts of glucose.

The infusion of glucose also stimulated the secretion of IGF-I and IGF-I is a potent stimulator of both follicle growth and follicular steroid and inhibin secretion (Campbell, 1988). The effect of glucose was strongest in follicles less than 2.0 mm in diameter and because small follicles can grow independently of gonadotrophin support it is also possible that an effect of glucose was to stimulate the growth of gonadotrophin-independent follicles. Thus it is possible that the increased number of small and medium sized follicles was caused by the elevated concentrations of IGF-I. Interestingly, the infusion of glucose reduced the blood concentration of oestradiol despite the presence of elevated blood concentrations of IGF-I suggesting that the intra-follicular bio-availability of IGF-I is suppressed in large follicles compared to small and medium sized follicles (Monget et al., 1993).

The increased rate of follicle growth as indicated by the increase in the number of follicles, has taken place in the absence of any detectable changes in the circulating concentrations of either LH or FSH suggesting that the effect of the infusion of glucose has been to modify follicular responsiveness to gonadotrophins. While these data suggest a local effect of glucose in the follicle they do not demonstrate a direct action of glucose because a consequence of its infusion was to also increase the circulating concentrations of insulin and IGF-I and these two hormones also have local effects of granulosa cell function and these could be independent of glucose.

Although the number of large follicles was not affected by the infusion of glucose their endocrinological function was, and its effects were inhibitory. The level of Aromatase P450 in granulosa cells and the circulating concentrations of oestradiol were both reduced in the ewes that were infused with glucose. These data suggest a specific inhibitory effect of glucose on the synthesis of oestradiol by granulosa cells. In the rat, *in vivo* treatment with insulin reduced FSH-stimulated oestradiol production (Peluso et al., 1991) and in the ewe nutritional treatments that increased insulin and glucose concentrations suppressed circulating concentrations of oestradiol (Viñoles et al., 2002, 2005; Letelier et al., 2008; Somchit, 2008). A reduced concentration of oestradiol would, according to the theory of negative feedback, lead to a compensatory increase in the secretion of FSH. This was not seen in this study and nor has it been seen in other similar investigations. This inconsistency is often attributed to inadequacies in the sampling regimes and/or to problems with the available assays for FSH and while these explanations are plausible there is another possibility. This is inhibin, because it is equally possible that the increased number of small and medium follicles has led to increased inhibin secretion that counteracts the effects of reduced oestradiol in the FSH negative feedback system. Thus, this study if nothing else, serves to focus future research on the effects of nutrition on the intrafollicular inhibin system which was not studied in this experiment.

In the present study, we have shown that a short-term infusion of glucose at 10 mM/h affected some components of insulin signalling pathways in granulosa cells. The kinase Akt, is a downstream kinase in the insulin and IGF-I signalling pathways, it is thought to mediate the proliferative effects of both insulin and IGF-I. The kinase, Akt is present in granulosa cells and it has been localised to the granulosa cells in primordial follicles and to the basal granulosa in preantral and antral follicles (Meng et al., 2007; Goto et al., 2007); it was not detected in atretic follicles (Meng et al., 2007). We observed a decrease in phosphorylated Akt in granulosa cells following treatment with systemic glucose, indicating a low level of Akt activity and decreased

Table 5
The effect of glucose infused at 10 mM/h for 72 h during the late luteal phase of the oestrous cycle on the mean (±SEM) levels of total Akt (T-Akt) and phosphorylated Akt (p-Akt) and of total AMPK (t-AMPK) and phosphorylated AMPK (p-AMPK) and their ratios in lysates of granulosa cell from non-atretic follicles.

Treatment	Akt				AMPK			
	Number of follicles	Total (t-Akt)	Phosphorylated (p-Akt)	Ratio (p-Akt to t-Akt)	Number of follicle	Total (t-AMPK)	Phosphorylated (p-AMPK)	Ratio (p-AMPK to t AMPK)
Saline (control) ($n = 6$)	14	96.0 ± 8.18	71.7 ± 7.79	0.78 ± 0.09	9	42.9 ± 2.51	68.3 ± 3.17	1.60 ± 0.056
Glucose ($n = 5$)	12	97.9 ± 4.66	44.9 ± 4.97*	0.45 ± 0.03*	3	38.0 ± 2.99	45.1 ± 1.45*	1.20 ± 0.056*

* $P < 0.05$.

insulin signalling along this pathway in granulosa cells after a 72 h infusion of glucose. A role for Akt in folliculogenesis although apparent, is not fully understood at this time (Ryan et al., 2007, 2008). These authors reported that the level of phosphorylated Akt was higher in dominant compared to subordinate follicles during the follicular wave in sheep and cattle, and they suggested a role for Akt in follicle growth and development (Ryan et al., 2007, 2008).

We have also observed decreased levels of phosphorylated AMPK following 72 h of glucose. As previously reported for ovarian tissue from several species (Tosca et al., 2005, 2006), AMPK is present in granulosa cells and seems to be involved in the regulation of the secretion of oestradiol via the MAPK-ERK1/2 signalling pathways. These authors concluded that AMPK regulates steroidogenesis in granulosa cells by acting on steroidogenic enzymes. In our study, we were unable to study the pattern of phosphorylation of the ERKs because of the lack of specific antibodies to ERK and phospho-ERK that could be used with ovine tissue. Clearly further studies are required to establish the role of the MAPK signalling pathway and ERK1/2 in our experimental model.

The levels of both phospho-Akt and phospho-AMPK were reduced in our experiment and this may be due to inactivation of the insulin receptor and the induction of insulin resistance. The duration of the infusion was 72 h and this interval was selected because we know that three to five days of nutritional supplementation of ewes with a diet high in energy-yielding substrates was sufficient to stimulate follicle growth and ovulation rate. The possibility exists that this treatment may be sufficient to induce insulin resistance and that the mechanism of nutritional stimulation of folliculogenesis sheep involves the induction of insulin resistance. Polycystic ovarian syndrome (PCOS) in women is an ovarian pathology that is strongly associated with insulin resistance even in the absence of excess bodyweight (Bernard et al., 2003). The disease is characterised by disturbed ovarian function and a polycystic and anovulatory morphology. Interestingly, when induced to ovulate women with PCOS tend to be hypersensitive to gonadotrophin stimulation and to have higher rates of twin births (Blankstein and Quigley, 1988). It has been suggested that PCOS is the consequence of an impaired PI3K insulin signalling pathway. The disease can be successfully treated by reducing insulin resistance by treatment with biguanides such as metformin (Bernard et al., 2002).

Our study demonstrates the potential importance of glucose and glucose-stimulated signalling pathways in the mediation of nutritional effects of follicle growth. The reduced levels of Aromatase P450 and oestradiol secretion were associated with reduced levels of both phospho-Akt and phospho-AMPK. It has been suggested (Silva et al., 2006) that the activity of Aromatase P450 is regulated by two alternative regulatory pathways, an inhibitory tonic ERK/MAPK pathway and a post-transcriptional PI3K pathway. Increased numbers of small follicles were also observed but because of the very low yields of protein from these follicles were unable to determine the levels of phospho-Akt and phospho-AMPK in individual follicles. Real time PCR may offer an alternative future approach for these investigations.

The secretion of oestradiol is primarily regulated by FSH acting through the cyclic AMP, protein kinase A pathway. In our study, the inhibition of the insulin-PI3K signalling pathway induced by glucose was associated with a reduction in the level of Aromatase P450 in granulosa cells. This suggests that the functional activity of the FSH signalling pathway was affected by the insulin-stimulated PI3K pathway. The level of this interaction remains to be established but our data suggests that it is beyond Akt.

Acknowledgements

The research was supported with grants from the BBSRC (Grant BB/0018420/1) and the European Union Framework 6 funding program (MEXC-CT-2006-042499). RJS was the recipient of an EU Marie Curie Chair of Excellence (MEXC-CT-2006-042499). We wish to thank Jean-Paul Petit for carrying out the ovariectomies, Jean-Baptiste Menassol for help with follicle dissection, Lionel Lardic for the oestradiol radioimmunoassay, Anne-Lyse Laine and the Laboratory of Hormonal Assays at the UMR PRC for the progesterone, LH and FSH ELISAs.

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