The effect of monosaccharide sugars and pyruvate on the differentiation and metabolism of sheep granulosa cells *in vitro*

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Abstract

The objective of this study was to investigate the effect of three monosaccharides or pyruvate on the ability of gonadotrophins to induce cellular proliferation and differentiation of cultured sheep granulosa cells. Lactate production and levels of mRNA expression for the glucose transporters SLC2A1, SLC2A4, SLC2A5 and SLC2A8 were also determined. No energy source in the culture media reduced cell number (50%) and oestradiol (E₂) production. Dose and type of monosaccharide had a highly significant ($P < 0.001$) effect on FSH-induced differentiation of the granulosa cells, and there was a highly significant interaction ($P < 0.001$). Glucose supported higher levels of E₂ production than fructose, which was in turn higher than galactose ($P < 0.001$). In contrast, pyruvate at low doses supported similar levels of E₂ production as glucose, but higher doses were markedly inhibitory to E₂ production ($P < 0.001$). Cells responded positively to insulin ($P < 0.001$) in the presence of all three monosaccharides. Glucose and the high doses of fructose resulted in the accumulation of lactate ($P < 0.001$), but pyruvate, galactose and the low dose of fructose resulted in low lactate production. SLC2A5 expression was not detected and SLC2A8 expression was not affected, but SLC2A1 and SLC2A4 expression was depressed ($P < 0.05$) by culture in the presence of fructose and glucose. These data show that glucose, metabolised under anoxic conditions to lactate, is the preferred energy substrate to support the gonadotrophin-induced differentiation of ovine granulosa cells *in vitro*, and that fructose and pyruvate, but not galactose, are alternative energy substrates despite marked differences in the way these substrates are metabolised.

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Introduction

In mammalian species, ovulation rate is determined by the number of follicles selected from the ovulatory wave of follicles, which in turn is controlled primarily by the levels and actions of key reproductive hormones (Webb & Campbell 2007). Although ovulation rate is influenced by genetically determined factors, it is also influenced by environmental factors such as nutrition; and ovulation rates can adapt to align with nutritional status. Extreme energy restriction can lead to suppressed development of ovulatory follicles and anoestrus through inhibition of GnRH pulsatility (Jolly *et al.* 1995, McShane *et al.* 1994, Katz & Vollenhoven 2000). However, increasing energy supply over a maintenance level leads to an increase in fecundity by an effect on follicle development and ovulation, which appears to act through a variety of mechanisms at both central and local levels (Scaramuzzi *et al.* 2006).

Glucose is a critical energy providing substrate for many cellular processes through its catabolism to

produce ATP. Much of the current research suggests an indirect effect of glucose on ovarian function via nutritionally induced metabolic hormones such as insulin, insulin-like growth factor 1 (IGF1) and leptin (Peluso *et al.* 1984, Davis *et al.* 1990, Munoz-Gutierrez *et al.* 2002, 2004, 2005). Conversely, glucose may also act directly on the ovary. Evidence of glucose transporter expression (SLC2A1 and SLC2A4) on granulosa and theca cells of ovine antral follicles (Williams *et al.* 2001) and ovarian arteriovenous difference studies both suggest that significant amounts of glucose can be taken up by the ovary (Rabiee *et al.* 1997). In addition, glucose infused in combination with insulin into the ovarian artery of ewes with ovarian autotransplants directly suppressed androstenedione and oestradiol (E₂) production from the dominant follicle without affecting peripheral gonadotrophin concentrations (Downing *et al.* 1999).

Many foods contain other monosaccharides in addition to glucose, which may also mediate nutritional

effects on follicular function. The supplementation of diets with lupins, which are high in galactose (van Barneveld 1999), stimulated folliculogenesis and increased fecundity in ewes (Knight *et al.* 1975). Enzymes involved in the metabolism of galactose are abundantly and actively expressed in the ovary, implicating it as a site of uptake, metabolism and utilisation (Heidenreich *et al.* 1993). Furthermore, significant plasma–follicular fluid concentration gradients in the human preovulatory follicle also suggest that galactose is both taken up and directly metabolised by ovarian follicles (Jozwik *et al.* 2007).

Along with glucose and galactose, fructose is a physiologically significant monosaccharide, particularly in human nutrition (Elliott *et al.* 2002), and is also the predominant blood sugar in foetal ruminants (Daniels *et al.* 1974). Fructose is absorbed via a fructose-specific member of the solute carrier family, SLC2A5 (formerly GLUT5), which is expressed mainly in the small intestines and testes (Burant *et al.* 1992). Fructose is an important energy substrate within the testis, being taken up from the seminal fluid and utilised by the mature spermatid as its main source of metabolic energy (Burant *et al.* 1992). Conversely, there is currently little evidence for a direct effect of fructose on the ovary. In one study, SLC2A5 mRNA expression was not detected in the rat ovary (Kol *et al.* 1997), and in the ruminant, fructose is thought to be rapidly metabolised to glucose in the liver (Luick *et al.* 1957) which may then trigger insulin release. In the primate and the human, fructose has little effect on insulin and leptin release or on the concentrations of blood glucose (Curry 1989, Havel 1997, Teff *et al.* 2004), but in sheep, we have observed increased insulin secretion in response to a challenge with fructose that differs in terms of duration and magnitude to challenges with identical doses of glucose (Onions *et al.* 2009). Furthermore, both direct ovarian and systemic infusions of fructose were observed to have an effect on ovarian steroidogenesis (Onions *et al.* 2009).

In summary, in addition to glucose, there is evidence to suggest that other monosaccharides such as galactose and fructose may also have effects on the ovary, which could mediate important changes in follicular development. Therefore, the objective of this study was to investigate the effect of dose and type of monosaccharide on the ability of gonadotrophins to induce cellular proliferation and differentiation of cultured granulosa cells in sheep.

Results

Effect of dose and type of monosaccharide on E_2 production and cell number

The first set of experiments examined the effect of dose and type of monosaccharide on cell number and E_2 production by cells cultured in the presence of a

Figure 1 The effect of culturing ovine granulosa cells from small immature follicles (between 1 and 3 mm in diameter) under conditions known to induce cellular differentiation with increasing doses of three different monosaccharides on (a) cell number, (b) oestradiol production and (c) oestradiol production corrected for cell number after 192 h of culture. For clarity, only results from the physiological (2.1 mM) and superphysiological (16.7 mM) doses of monosaccharide have been included in this figure. Values are mean $\pm$ s.e.m. Different letters indicate statistically significant differences ($P < 0.05$).

physiological dose of insulin (10 ng/ml). Absence of monosaccharide in the culture media resulted in around a 50% suppression in the number of viable cells after 192 h of culture ($P < 0.001$; Fig. 1a) and correspondingly low levels of E_2 production (Fig. 1b, c). Regardless of dose or type of monosaccharide, hormone production by granulosa cells from small follicles exposed to low doses of FSH exhibited a standard steroidogenic induction

profile (Campbell *et al.* 1996), characterised by a highly significant increase in E_2 ($P < 0.001$) production with time in culture peaking at 192 h (see for example Fig. 2). Dose and type of monosaccharide, however, had a highly significant effect on the characteristics of this response ($P < 0.001$), and there was a highly significant interaction between these two parameters ($P < 0.001$). Over all doses, glucose (5.5 ± 0.07 log pg/ml) resulted in significantly higher levels of E_2 production than fructose (5.0 ± 0.07 log pg/ml; $P < 0.001$), which in turn was higher than galactose (4.8 ± 0.08 log pg/ml; $P < 0.01$). Surprisingly, there was no significant difference in E_2 production at 192 h between low (2.1 mM) and high (16.7 mM) doses of each monosaccharide (Fig. 1b), although the high dose of galactose resulted in a significant depression ($P < 0.01$) relative to both fructose and glucose (Fig. 1b). Examination of the effect of dose and type of monosaccharide on cell number after 192 h of culture revealed similar trends to those observed for E_2 production with highly significant effects of dose and type of monosaccharide and a highly significant interaction ($P < 0.001$). Thus, glucose resulted in the highest cell numbers with levels around 80% of the initial plating density (60 ± 2.6 kcells/well) with again no effect of dose of glucose over a large range of concentrations (Fig. 1a). Cell survival in the presence of fructose (54 ± 2.5 kcells/well; $P < 0.05$) was slightly lower than that observed for glucose but was much lower in the presence of galactose (38 ± 2.2 kcells/well; $P < 0.01$). There were no differences in cell number within monosaccharides between the low or high doses

tested (Fig. 1a; $P > 0.05$). When E_2 production was corrected for cell number (Fig. 1c), there were still highly significant effects of dose and type of monosaccharide and a highly significant interaction ($P < 0.001$). The main trends from this analysis were that E_2 production from cells cultured across all doses of glucose (1.62 ± 0.09 log pg/kcell) was significantly higher ($P < 0.001$) than those cultured in the presence of galactose (1.34 ± 0.10 log pg/kcell) or fructose (1.30 ± 0.08 log pg/kcell), which did not differ. In contrast, while there was no difference between high and low doses of glucose and fructose, the high dose of galactose (16.7 mM) induced a significant inhibitory effect on E_2 production per cell relative to the low dose of this sugar (Fig. 1c; $P < 0.05$).

Effect of dose and type of monosaccharide in relation to insulin on E_2 production

Insulin controls the utilisation and metabolism of monosaccharides and therefore, the next series of experiments examined the effect of the dose of insulin on granulosa cell differentiation at three doses of each of the three monosaccharides. The results of this analysis showed highly significant effects of insulin, the type and dose of monosaccharide, and the interactions among these three parameters (all $P < 0.001$) on FSH-mediated induction of E_2 production (Fig. 3). In the absence of monosaccharide, the dose of insulin had no significant effect on E_2 production, but in the presence of each monosaccharide, insulin induced a highly significant ($P < 0.001$) increase in E_2 production. In line with our previous experimental series, the individual monosaccharides differed significantly in their ability to support the induction of E_2 production ($P < 0.001$) with glucose (5.12 ± 0.10 log pg/ml) being more potent than fructose (4.62 ± 0.09 log pg/ml), which in turn was more potent than galactose (3.94 ± 0.16 log pg/ml). Similarly, E_2 production was not significantly different between low (2.1 mM) and high (16.7 mM) doses of glucose (Fig. 3c) and fructose (Fig. 3a), whereas the high dose of galactose was inhibitory compared to the low dose of galactose (both $P < 0.05$) at the zero and 100 ng/ml doses of insulin (Fig. 3b).

In comparison, in this experiment, type of monosaccharide had no significant effect on cell number, and while highly significant ($P < 0.001$) effects of dose of monosaccharide and dose of insulin were observed on overall cell number, these differences were due primarily to low cell numbers at low doses of sugar and insulin, so that cell number was not affected by insulin for doses > 1 ng/ml (data not shown). Accordingly, analysis of the E_2 production data corrected for cell number resulted in the same main effects with very highly significant ($P < 0.001$) differences for type of monosaccharide, dose of monosaccharide and dose of insulin (data not shown).

Figure 2 The effect of increasing doses of pyruvate on the induction of oestradiol production by granulosa cells from small immature follicles (between 1 and 3 mm in diameter) cultured for 48, 96, 144 and 192 h. Cells were also cultured in the presence of 1 ng/ml FSH, 10 ng/ml insulin and 10 ng/ml LR3-IGF1. Values are mean $\pm$ S.E.M. Asterisks indicate statistically significant differences ($P < 0.05$).

Figure 3 The effect of increasing doses of insulin on oestradiol production by granulosa cells from small immature follicles (between 1 and 3 mm in diameter) cultured for 192 h in the absence of, or in the presence of, physiological (2.1 mM) or super-physiological (16.7 mM) doses of (a) fructose, (b) galactose or (c) glucose. Cells were also cultured in the presence of 1 ng/ml FSH and 1 ng/ml LR3-IGF1. Values are mean $\pm$ S.E.M. Different letters indicate statistically significant differences ($P < 0.05$).

Effect of pyruvate on granulosa cell differentiation

Pyruvate is one of the major products of glycolysis, and thus represents the most likely intermediate energy source following monosaccharide metabolism. This series of experiments, therefore, examined the effect of increasing doses of pyruvate on the induction profiles of E_2 production. The results of these studies are presented in Fig. 2. Dose of pyruvate had a highly significant effect on E_2 production, and there was a highly significant interaction with time of culture ($P < 0.001$). Thus, lower doses of pyruvate resulted in classic induction profiles

with a low initial concentration in the first 48 h of culture followed by a rapid increase at 96 h, which remained elevated and stable after 144 and 192 h of culture (Fig. 2). In contrast, with doses of pyruvate above 8.3 mM, there were significant depressions in the production of E_2 with the maximum level of production being reached after 96 h and falling progressively thereafter (Fig. 2). The maximum levels of E_2 production induced in granulosa cells cultured in the presence of low doses of pyruvate were similar to those observed under the same conditions in the presence of glucose (Figs 2 and 3).

Lactate production

Lactate production, by cells cultured in the presence of physiological (2.1 mM) and supra-physiological (16.7 mM) doses of monosaccharide and pyruvate, was determined. There was no significant effect of time in culture on lactate production, so data were pooled across all times (Fig. 4). Culture of granulosa cells in the presence of glucose resulted in levels of lactate production which were two orders of magnitude higher than in the total absence of monosaccharide ($P < 0.001$). There was no significant stimulation of lactate production in the presence of either dose of galactose or pyruvate, whereas the high dose of fructose, but not the low dose, resulted in significant levels of lactate production ($P < 0.001$).

The expression of SLC2A (GLUT) mRNAs

For SLC2A5 mRNA, a standard curve was successfully constructed using cDNAs from samples of sheep intestine, but no positive signal was detected in cultured granulosa cells. Two further alternate primers sets

Figure 4 Lactate accumulation in media from granulosa cells from small immature follicles (between 1 and 3 mm in diameter) cultured in the absence of, or in the presence of, low or high doses of three different monosaccharides and pyruvate. Data have been combined from across different time points. Values are mean $\pm$ S.E.M. Asterisks indicate $P < 0.001$ for comparison with zero dose.

Figure 5 mRNA expression of *SLC2A* in granulosa cells from small immature follicles (between 1 and 3 mm in diameter) after culture in the presence of low or high doses of three different monosaccharides. Data have been combined from across different time points. Dashed horizontal line represents the level of mRNA expression in freshly isolated cell at time zero. Values are mean $\pm$ S.E.M. of the ratio of *SLC2A* to 18S expression, converted to a percentage and subjected to log transformation. Different letters indicate statistically significant differences ($P < 0.05$).

designed from the ovine or bovine sequence were tested, but again no positive signal for *SLC2A5* mRNA was detected in granulosa cells.

Conversely, mRNA expression of *SLC2A1*, *SLC2A4* and *SLC2A8* was detected in granulosa cells at time zero and

in granulosa cells cultured for up to 192 h in the presence of low and high doses of monosaccharide (Fig. 5). There were no significant effects of time in culture on *SLC2A* mRNA expression within treatment groups, and these data were, therefore, pooled for further analysis. Both *SLC2A1* (Fig. 5a) and *SLC2A4* (Fig. 5b) expression exhibited generally similar responses, although there were some differences. Thus, galactose had no effect on *SLC2A1* or *SLC2A4* expression, whereas higher doses of both fructose and glucose resulted in a depression in both *SLC2A1* and *SLC2A4* expression, although the effect of high dose fructose on *SLC2A4* expression did not reach statistical significance. In addition, *SLC2A4* expression was also significantly ($P < 0.05$) suppressed by the low dose of glucose (Fig. 5b). In contrast, dose and type of monosaccharide had no effect on *SLC2A8* expression in cultured granulosa cells (Fig. 5c).

Discussion

The results of this study have shown that while glucose is the preferred energy substrate to support the gonadotrophin-induced differentiation of ovine granulosa cells *in vitro*, fructose and pyruvate, but not galactose, represent alternative energy sources despite marked differences between these substrates in the way they are metabolised. Furthermore, we have shown that while cultured granulosa cells respond to insulin in a dose-dependent manner and express a number of glucose transporters (*SLC2A1*, *SLC2A4* and *SLC2A8*), expression of both *SLC2A1* and *SLC2A4* is regulated at a local level by the concentration of glucose and/or fructose.

To our knowledge, this is the first study showing a direct requirement for glucose in the gonadotrophin-induced differentiation of ovarian granulosa cells. Glucose is a critical metabolic fuel in mammals, and while most mammals derive their glucose from the diet, ruminants do not. The ruminant is dependent on the biosynthesis of glucose from dietary precursors such as propionic acid, a product of anaerobic fermentation of carbohydrate in the rumen, and from gluconeogenic amino acids absorbed from the small intestine (Scaramuzzi *et al.* 2006). The ability of granulosa cells to survive under culture conditions in the complete absence of pyruvate or monosaccharide may be explained by the ability of these cells to utilise amino acids such as glutamine in the culture media or cellular glycogen for gluconeogenesis, but further work is needed to test this hypothesis. The requirement for glucose to induce cellular differentiation of granulosa cells shown in this study is consistent with *in vivo* studies showing that treatment with non-metabolisable 2-deoxy-D-glucose resulted in cessation of oestrous cycles in cattle (McClure *et al.* 1978) and prevention of the LH surge in sheep (Funston *et al.* 1995). Furthermore, it has been shown that there is a significant uptake of glucose by the ovary in sheep (Scaramuzzi *et al.* 2010)

and cattle (Rabiee *et al.* 1997). It has been previously shown that significant quantities of lactate are produced by the ovary *in vivo* (Rabiee *et al.* 1997), and the results of the present study indicate that the likely source of this lactate is the anaerobic metabolism of glucose by granulosa cells. This idea is consistent with the fact that the membrana granulosa cell layer in ovarian follicles is avascular and that intra-follicular oxygen tensions are very low (Murray *et al.* 2009).

One surprising aspect of our data was the ability of glucose to support gonadotrophin-induced differentiation of granulosa cells at such low doses. Doses in the physiological range (1.1–2.1 mM; Somchit *et al.* 2007, Nandi *et al.* 2008, Scaramuzzi *et al.* 2010) were as effective as the tenfold higher dose (16.7 mM) commonly found in most commercial culture media. This suggests that in commercial, widely used media, glucose is present in vast excess to that required to provide energy via glycolysis, and therefore may also be exerting an effect on cellular differentiation and function via alternative mechanisms. One of the most widely studied of these alternate pathways is the hexosamine signalling pathway which, via the input of glucose and production of glucosamine-6-phosphate, is able to sense and respond to nutritional fluctuations by altering specific gene expression (Marshall *et al.* 1991, Obici & Rossetti 2003, Curi *et al.* 2005). While the hexosamine pathway has mainly been studied in muscle and fat, it has been shown to be an important pathway in energy metabolism of cumulus–oocyte complexes (Thompson 2006), and genes shown to be responsive to increased glucose via the hexosamine pathway, such as leptin (Wang *et al.* 1998, McClain *et al.* 2000), basic fibroblast growth factor (McClain *et al.* 1992) and transforming growth factor- α (McClain *et al.* 1992), have been shown to modulate ovarian steroidogenesis (Campbell *et al.* 1994, Scaramuzzi & Downing 1995, Spicer & Francisco 1997, Kendall *et al.* 2004). In addition, systemic infusion of glucosamine has been shown to stimulate follicular development in a similar manner to that seen following glucose infusion (Munoz-Gutierrez *et al.* 2002), demonstrating that the hexosamine signalling pathway can induce changes in the ovine ovary and implying glucose may elicit its effects via this pathway. Further work is, therefore, required to confirm whether the hexosamine signalling pathway does have a role in the gonadotrophin-induced differentiation of sheep granulosa cells *in vitro*.

The current study has also confirmed and extended previous findings (Williams *et al.* 2001, Nishimoto *et al.* 2006), showing that ruminant granulosa cells express both the non-insulin-dependent and insulin-dependent glucose transporters, SLC2A1 and SLC2A4 respectively. The levels of both SLC2A1 and SLC2A4 expression did not vary with the differentiative status of granulosa cells, and high levels of *SLC2A1* and *SLC2A4* mRNA were detected in freshly isolated cells at time zero and for up

to 192 h in cultured cells not exposed to glucose. The down-regulatory effect of glucose on *SLC2A1* and *SLC2A4* mRNA expression in cultured granulosa cells is consistent with similar inhibitory effects of high glucose reported in the literature (review: Klip *et al.* (1994)). However, in cultured cells, down-regulation of SLC2A1, but not SLC2A4, expression has most commonly been observed in response to elevated glucose, whereas depression in SLC2A4 protein and mRNA expression has been commonly observed in response to *in vivo* exposure to glucose in many species (review: Klip *et al.* (1994)). It, therefore, appears likely that both *SLC2A1* and *SLC2A4* mRNA expression in sheep granulosa cells is regulated by local mechanisms that respond to differences in the availability of substrate or products of metabolism. The expression of *SLC2A4* mRNA by sheep granulosa cells is consistent with the ability of granulosa cells cultured in the presence of glucose to respond to insulin in a dose-dependent manner (Fig. 3). However, the inclusion of insulin in the culture is essential for granulosa cell survival in this serum-free system (Campbell *et al.* 1996), and at higher doses, insulin interacts with the type 1 IGF receptor and therefore mimics many of the stimulatory effects of IGF1 on granulosa cell differentiation (Campbell *et al.* 1996). It is, therefore, possible that the stimulatory effect of high levels of insulin in amplifying the differentiative effects of FSH in granulosa cells (Campbell *et al.* 1996) operates via mechanisms other than through modulation of energy supply (Lopaczynski 1999). This view is supported by the fact that the granulosa cells cultured in the presence of fructose and galactose also responded to the higher doses of insulin.

Once absorbed, fructose can be metabolised by conversion to fructose-6-phosphate by hexokinases and hence enter directly the glycolytic pathway, or it can be converted into fructose-1-phosphate by fructokinase from which the trioses dihydroxyacetone and glyceraldehyde are synthesised before complete metabolism to pyruvate (Zubay 1998). In farm animals, in which fructose is the principle sugar in semen, high levels of SLC2A5 expression are observed in spermatozoa, and fructose is metabolised by hexokinases directly via the glycolytic pathway (Jones & Connor 2000) with the resultant production of large amounts of lactate (Jones & Connor 2004). In the current study, we were unable to detect SLC2A5 expression in ovarian granulosa cells, but fructose was capable of supporting relatively high levels of cell survival, the FSH-induced induction of E₂ production and the accumulation of significant amounts of lactate when included at high doses. The failure to find SLC2A5 expression in sheep ovarian somatic cells is in agreement with the study of Kol *et al.* (1997) in the rat ovary, and it is, therefore, likely that granulosa cells may contain other SLC2A capable of transporting fructose. These include SLC2A2, SLC2A7, SLC2A8 and SLC2A11 (Zhao & Keating 2007). In the present study, we have

confirmed the finding of [Pisani *et al.* \(2008\)](#) that SLC2A8 (formerly GLUT8) is expressed by sheep granulosa cells. Expression of SLC2A8 was not affected by exposure to either fructose or galactose, both of which are thought to be preferentially transported by this factor, and this finding is consistent with the current view that SLC2A8 primarily catalyses the transport of sugar through intracellular membranes rather than across the plasma membrane ([Schmidt *et al.* 2009](#)). Although we found that granulosa cells cultured in the presence of fructose were able to respond to insulin in a dose-dependent manner, the fact that *SLC2A4* mRNA expression responded differentially to identical doses of glucose and fructose suggests that SLC2A4 was not acting as a major fructose transporter in sheep granulosa cells. In the ruminant, dietary fructose is thought to be rapidly metabolised to glucose in the liver ([Luick *et al.* 1957](#), [Boda 1964](#)), but in sheep, we have observed insulin responses to a fructose challenge which differ in terms of duration and magnitude to identical doses of glucose ([Onions *et al.* 2009](#)), and both direct ovarian and systemic infusions of fructose had stimulatory effects on ovarian androstenedione and E₂ secretion ([Onions *et al.* 2009](#)). The current study supports these *in vivo* findings and shows that fructose may have a direct effect on ovarian function, but more work is required to clarify the physiological basis and mechanism of these effects.

The main products of galactose metabolism are glucose (in the form of glucose-1-phosphate which then enters the glycolytic pathway) and UDP-galactose, which is required for glycoconjugation of proteins and lipids ([Forges *et al.* 2006](#)). In contrast to fructose, a number of SLC2As that transport galactose (GLUT1, GLUT2, GLUT3 and GLUT8) have been reported in the ruminant ovary (([Nishimoto *et al.* 2006](#); GLUT1 and GLUT3); ([Pisani *et al.* 2008](#); GLUT1, GLUT3 and GLUT8); ([Williams *et al.* 2001](#); GLUT1)). It is, therefore, surprising that galactose was the least well utilised of the sugars tested by the granulosa cells, and it is significant that very little lactate was synthesised in cells cultured in the presence of galactose. This suggests that galactose does not enter glycolysis in granulosa cells, despite the theoretical availability of this pathway. The ovary in rodents and humans has an abundance of the enzymes required to metabolise galactose utilising the Leloir pathway ([Liu *et al.* 2000](#)), but little is known of the patterns of expression of these enzymes in ruminants. The accumulation of galactose or its metabolites is associated with impaired ovarian function in the human, which arises typically because of a deficiency or a mutation of galactose-1-phosphate uridylyltransferase, one of the key enzymes required for the metabolism of galactose. The pathogenesis of this ovarian dysfunction is unclear, but in addition to direct toxicity, it includes deficient galactosylation of glycoproteins and glycolipids, induction of apoptosis and modulation of the expression of intra-ovarian growth factors such as growth

differentiation factor 9 ([Forges *et al.* 2006](#)). These toxic effects of galactose on ovarian function are consistent with our observed inhibitory effects of high doses of galactose on FSH-induced differentiation of granulosa cells and the fact that direct ovarian infusion of galactose in sheep led to a depression in ovarian E₂ and androstenedione secretion ([Onions *et al.* 2009](#)). The role of galactose in the ruminant, particularly in relation to possible effects of feeding rations rich in galactose, such as lupins ([van Barneveld 1999](#)), on ovarian function is, therefore, a research priority.

As pyruvate is the primary output of the glycolysis of glucose, it was expected that the inclusion of this carboxylate in the culture media would mimic the responses observed with glucose. However, while pyruvate at low equimolar doses supported FSH-induced differentiation of granulosa cells to similar levels as those observed for glucose, in several other respects, the response of the cells differed markedly between these two energy substrates. Thus, higher doses of pyruvate were strongly inhibitory to E₂ production, whereas high doses of glucose were not. Furthermore, cells cultured in the presence of pyruvate produced negligible quantities of lactate, whereas provision of glucose as a substrate resulted in the accumulation of large amounts of lactate. This latter observation suggests that the granulosa cells were able to metabolise exogenous pyruvate aerobically via the Krebs cycle but that pyruvate derived endogenously by the metabolism of glucose was preferentially shunted to produce lactate. A possible explanation for this unexpected finding may lie in the mechanisms regulating pyruvate transport. Carboxylates such as pyruvate and lactate are transferred across cell membranes by monocarboxylate transporters (MCTs, also known as SLC16As), which currently make up a family of 14 proteins related by sequence homology ([Merezhinskaya & Fishbein 2009](#)). The MCTs classically transport metabolites across plasma membranes, but they may also function in sub-cellular membranes and have different substrate specificities ([Merezhinskaya & Fishbein 2009](#)). Little is known of the actions of these MCTs in the ovary, but their presence would be predicted given that a primary role of MCT1–4 is lactate efflux in cells generating large amounts of lactic acid from anaerobic glycolysis ([Merezhinskaya & Fishbein 2009](#)). Given that pyruvate requires translocation to the mitochondria for aerobic respiration, the presence and differential expression of MCTs in ovarian somatic cells may, therefore, explain the unexpected finding that exogenous and endogenous pyruvate are differentially metabolised by granulosa cells. However, more work is required to confirm this suggestion. Similarly, the depressive effects of high doses of pyruvate on granulosa cell differentiation may be related to changes in the expression of MCTs, but because pyruvate can also be metabolised by numerous alternative pathways, a number of other mechanisms may explain this observation.

In conclusion, this study has provided clear evidence that glucose, metabolised under anoxic conditions to lactate, is the preferred energy substrate to support the gonadotrophin-induced differentiation of ovine granulosa cells *in vitro* and that while cultured granulosa cells respond to insulin in a dose-dependent manner, paradoxically, expression of both SLC2A1 and SLC2A4 is inhibited *in vitro* by high doses of glucose. Furthermore, these data have shown that fructose and pyruvate, but not galactose, are alternative energy substrates for granulosa cell differentiation, but that there are marked differences between these substrates in the way they are metabolised. More work is, therefore, required to elucidate the underlying mechanisms resulting in these differences and to relate the findings of these cell culture studies to the established effects of nutrition on reproductive function in this and other species.

Materials and Methods

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich. Ovine ovaries were obtained from a commercial abattoir. Small immature follicles between 1 and 3 mm in diameter were dissected from a number of ovaries to negate any individual animal effects, and granulosa cells were isolated using the procedure described previously for ovine tissues (Campbell *et al.* 1996). Granulosa cells at a density of 75 000 viable cells/well in 96-well flat bottom plates (Nunc, Nunc) with 250 µl of media were used to examine the influence of different monosaccharides on cell proliferation and E₂ production. Granulosa cells, at an equivalent density of 450 000 cells/well in 24-well flat bottom plates (Nunc, Nunc, Roskilde, Denmark) with 1 ml of media, were used to examine the effect of different monosaccharides on glucose transporter expression. Granulosa cell culture media were supplemented with 1 ng/ml of FSH (NIDDK-oFSH-20), 1–10 ng/ml LR3-IGF and varying doses of insulin. These combination of factors have been shown previously to be optimal for induction of E₂ production by sheep granulosa cells cultured under serum-free conditions (Campbell *et al.* 1996). The granulosa cell culture medium was McCoy's 5a, which was

custom prepared with no glucose or pyruvate (JRH Biosciences Inc., Lenexa, KS, USA and BioConcept, Allschwil, Switzerland) and subsequently supplemented with 0, 1.1, 2.1, 4.2, 8.4 and 16.7 mM of glucose, fructose or galactose. These doses were designed to span the normal physiological range (1.1–2.1 mM; Somchit *et al.* 2007, Nandi *et al.* 2008, Scaramuzzi *et al.* 2010) to a maximum of 16.7 mM that represented the normal rate of glucose supplementation in commercial preparations of these media. Similarly, media were supplemented with pyruvate across a similar equimolar dose range to a maximum of 33 mM. The McCoy's 5a was also supplemented with sodium bicarbonate (2.2 mg/ml), HEPES (15 mM), BSA (1 mg/ml), Penstrep (100 U/ml penicillin and 0.1 mg/ml streptomycin), L-glutamine (0.365 mg/ml), transferrin (5 µg/ml) and selenium (0.1 µg/ml).

For 96-well plate cultures, each dose of monosaccharide or pyruvate was replicated in quadruplicate, and cultures were maintained for 192 h with 175 µl (70%) of the media exchanged every 48 h with equivalent fresh media. Spent media were retained, stored at –20 °C, for subsequent analysis for E₂ concentrations. The number of viable cells was estimated using neutral red staining at the end of the culture (Campbell *et al.* 1996). Steroid production per cell was calculated for the final time point. Each experiment was repeated at least three times.

For 24-well plate cultures, physiological (2.1 mM mg/ml) and superphysiological (16.7 mM) doses of each monosaccharide were replicated in duplicate per treatment per time point, and cultures were maintained for up to 192 h with 700 µl (70%) of the media being exchanged every 48 h with equivalent fresh media. Cell lysates for molecular analysis were collected at each time point and stored frozen in RTL buffer with 1% β-mercaptoethanol prior to analysis. Spent media were retained, stored at –20 °C, for subsequent analysis of E₂ concentrations. Each experiment was repeated at least three times.

Concentrations of E₂ in unextracted culture media were determined using a previously described RIA (Campbell *et al.* 1996). The sensitivity of the assay was 1.4 pg/ml. The intra- and inter-assay coefficients of variation (CV) were <10%.

Concentrations of lactate in the culture media were determined using a commercial enzymatic assay kit for L-lactate (Randox, Belfast, Co., Antrim, UK) and a clinical

Table 1 Details of quantitative PCR primers and PCR conditions for semi-quantification of SLC2A (GLUT) expression levels in ovine granulosa cells cultured in the presence of different monosaccharides.

SLC2A	Primers	PCR conditions	References
SLC2A1	Sense: 5'-TTAACCGCAACGAGGAGAAC-3' Antisense: 5'-AACAGCTCCAGGATGGTGAC-3'	95 °C for 10 min, 95 °C for 20 s, 59 °C for 30 s and 72 °C for 1 min. 40 cycles	Accession no. U89029
SLC2A4	Sense: 5'-GGAGCTGGTGTGGTCACACA-3' Antisense 5'-GGAGCAGAGCCACAGTCATCA-3'	94 °C for 15 min, 94 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s. 40 cycles	Benomar <i>et al.</i> (2006)
SLC2A5	Sense: 5'-AGTCATCTCCATCATCGTCCT-3' Antisense: 5'-GTACCCGCCACCATGTAGGCAG-3'	94 °C for 10 min, 94 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. 40 cycles	Rizos <i>et al.</i> (2004)
SLC2A8	Sense: 5'-CTGGCATCTACAAGCCCTTC-3' Antisense: 5'-TGAACCTGGCCTCTCAAAG-3'	95 °C for 10 min, 95 °C for 20 s, 59 °C for 30 s and 72 °C for 1 min. 40 cycles	Accession no. AF 495799

chemistry autoanalyser (RX Imola, Randox, Belfast, Co.), run with sample volumes increased from 2 to 20 µl to increase sensitivity. The sensitivity of the assay was 0.043 mM. The intra- and inter-assay CV were <1%.

Solute transporter (GLUT) mRNA expression

First-strand cDNA libraries were synthesised using the IT 1st strand synthesis kit (ABgene, Epsom, Surrey, UK), and utilising total RNA extracted from ovine granulosa cells using the RNeasy mini kit (Qiagen). These were semi-quantified by real-time PCR using the 7500 fast real-time PCR system (Applied Biosystems, Warrington, UK) with arbitrary SLC2A expression levels being normalised against starting amount differences using a 18S endogenous control. Samples were amplified in triplicate for determination of levels of expression of SLC2A1, SLC2A4, SLC2A5 and SLC2A8 utilising the primers and PCR conditions detailed in Table 1.

Data analysis

Each experiment was repeated at least three times, and the significance of treatment effects was determined by ANOVA utilising either SPSS (SPSS UK Ltd, Surrey, UK) or Systat (SSI, San Jose, CA, USA) statistical packages. The primary end points determined were hormone production per 48 h time point (48, 96, 144 and 192 h) expressed as concentration after correction for the residual amount of media left in the wells when the media were changed, the number of viable cells at the end of culture and mass of hormone produced per 1000 cells in 48 h. For clarity, only responses to the physiological (2.1 mM) and superphysiological (16.7 mM) doses of monosaccharide have been presented for illustrative purposes in Fig. 1, whereas the results of the main effect analysis presented in the text utilised the complete dose–response. Data presented are least-squares means and S.E.M. The normality and homogeneity of variances of the data were determined using appropriate tests and data transformed as required.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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