Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes

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Intracellular communication between the cumulus cell complex and the oocyte is essential for numerous processes during oocyte maturation. The aim of this study was to determine the interaction between oocyte-secreted factors and the metabolic activity of bovine cumulus cell complexes during *in vitro* maturation (IVM). Cumulus–oocyte complexes (COCs) were aspirated from ovaries derived from an abattoir and divided into four treatment groups: (i) intact COCs, (ii) oocyctomized complexes (OOX), in which the ooplasm was microsurgically removed, (iii) OOX co-cultured with denuded oocytes (OOX+DO) and (iv) DO. The complexes were cultured individually in IVM media. After 0–4, 10–14 and 20–24 h of culture, the utilization of oxygen, glucose, pyruvate and L-lactate by the complexes was measured. The metabolic activity of the DO was undetectable. There were no significant differences in metabolic measurement among any of the treatment

groups, indicating that the metabolism of the cumulus complex is not affected by the presence of the oocyte. When metabolic activity for the complexes was analysed relative to time in culture, there was an approximate twofold increase in the consumption of oxygen, glucose and pyruvate over the 24 h period ($P < 0.05$), although production of L-lactate remained constant. The relationship between total glucose uptake and L-lactate production indicated that the majority of glucose consumed at the start of culture was being utilized via glycolysis, but by the cessation of the maturation period, there was significant utilization of glucose elsewhere, possibly for the formation of cumulus extracellular matrix. These results indicate that metabolism of COC does not reflect biochemical activity of the oocyte. Nevertheless, the metabolic requirements of the COC increase throughout maturation.

Introduction

The environment in which oocytes are cultured during *in vitro* maturation (IVM) plays an important role in subsequent embryo development. The types and concentrations of energy substrates added to IVM medium alter the metabolic profiles and maturation of oocytes. Glucose, in particular, is an important energy substrate and its addition to medium in appropriate concentrations leads to improved maturation and blastocyst development (Rose-Hellekant *et al.*, 1998; Lim *et al.*, 1999; Khurana and Niemann, 2000; Zheng *et al.*, 2001). Glucose has several fates during IVM: glycolysis leads to the production of ATP and substrates such as pyruvate for further energy production. Alternatively, the metabolism of glucose via the pentose phosphate pathway not only supplies substrates that contribute to ooplasmic integrity,

but is also linked to the regulation of meiotic maturation within the oocyte (Downs *et al.*, 1998; Downs and Utecht, 1999). Studies focusing on the metabolism of oocytes have shown that during maturation factors that are associated with improved embryo development, such as oocytes collected from adult donors or oocytes matured *in vivo*, are accompanied by increased glucose utilization through glycolysis and the pentose phosphate pathway, when compared with oocytes from prepubertal donors or oocytes matured *in vitro* (Gandolfi *et al.*, 1998; Steeves and Gardner, 1999a; Durkin *et al.*, 2001). Furthermore, conditions that promote higher glycolytic activity during IVM also improve developmental capacity (Krisher and Bavister, 1999). Despite cumulus cells playing an important role in the utilization of energy substrates by the oocyte, there is minimal information regarding the metabolic profiles of the cumulus–oocyte complex (COC), and most studies focus on the profiles of denuded oocytes. In the absence of somatic cells, little glucose is utilized by the oocyte (Rieger and

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Loskutoff, 1994; Saito *et al.*, 1994). Hence, the oocyte is reliant on cumulus cells to metabolize glucose to intermediates such as pyruvate, the preferred substrate of the oocyte (Biggers *et al.*, 1967). Therefore, an increased understanding of the metabolic activity of the intact COC may allow the development of improved *in vitro* maturation conditions and hence improved developmental outcomes.

It is well known that granulosa cells play a critical role in the growth and development of the oocyte. Inter-cellular communication between cumulus cells and the oocyte occurs via paracrine factors and through gap junctions (Albertini *et al.*, 2001). Cumulus cells facilitate the transfer of nutrients and factors essential for oocyte development, such as metabolites, amino acids, signal transduction molecules and other factors (Colonna and Mangia, 1983; Larsen and Wert, 1988; Buccione *et al.*, 1990a; Eppig, 1991). Cumulus cells also play an important role in the regulation of cytoplasmic and nuclear maturation of the oocyte. For example, the transmission of regulatory molecules such as cAMP and purines via gap junctions allows for the maintenance of meiotic arrest within the oocyte (Eppig and Downs, 1984). Indeed, there is a positive relationship between the developmental potential of the oocyte and cumulus cell morphology, such as cumulus cell compaction (Shioya *et al.*, 1988; Madison *et al.*, 1992; Lonergan *et al.*, 1994; Goud *et al.*, 1998; Khurana and Niemann, 2000), number of cells (Hashimoto *et al.*, 1998) and the degree of expansion.

More recently, the importance of the two-way communication axis between oocytes and granulosa cells has been revealed. Oocytes secrete paracrine growth factors that regulate a broad range of granulosa cell functions by modulating fundamental control elements. For example, oocytes regulate expression of LH receptors, kit ligand, inhibin subunits and expression of extracellular matrix molecules (Canipari *et al.*, 1995; Eppig *et al.*, 1997; Lanuza *et al.*, 1998; Joyce *et al.*, 1999). As such, oocytes not only promote growth of granulosa cells (Vanderhyden *et al.*, 1992; Gilchrist *et al.*, 2001) and of the follicle, but also regulate differentiation processes such as steroidogenesis and physical remodelling of the follicle (Vanderhyden, 1993; Eppig *et al.*, 2002). The primary recipients of oocyte paracrine signalling are cumulus cells. Cumulus cells have a phenotype that is distinct from that of mural granulosa cells (which line the wall of the follicle), and indeed, maintenance of the cumulus cell phenotype is dependent on oocyte secretions (Eppig *et al.*, 1997; Li *et al.*, 2000). Therefore, paracrine signalling by the oocyte is essential for normal cumulus cell function, as well as for maintaining a functional morphogenic gradient across the ovarian follicle.

As the oocyte regulates such a broad range of cumulus cell functions, it was hypothesized that oocyte paracrine secreted factors would modulate cumulus cell

metabolism. As the capacity of the oocyte to utilize glucose is positively correlated with developmental potential, it may be possible to use the metabolic parameters of the cumulus complex as a predictive marker of oocyte quality. The aim of this study was to determine whether oocyte-secreted factors influence the metabolism of the COC.

Materials and Methods

Materials

Unless specified, all chemicals and reagents were purchased from Sigma (St Louis, MO).

Collection and culture of oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory in warm (30–35°C) saline. Follicles, 3–8 mm in diameter and of non-atretic appearance (Yang and Rajamahendran, 2000), were aspirated using an 18-gauge needle attached to a 10 ml syringe and collected in aspiration media (Hepes-buffered TCM-199 (ICN Biochemicals, Costa Mesa, CA), supplemented with 0.23 mmol sodium pyruvate l^{-1} and 50.0 μ g heparin ml^{-1}). Complexes from all aspirated oocytes with smooth, ungranulated ooplasm and intact, compact cumulus vestments with more than five cell layers were selected and washed twice in Hepes-buffered TCM-199. COCs were divided randomly into four treatment groups ($n=36$ per treatment): (i) intact COCs, (ii) oocyctomized complexes (OOX), (iii) OOX co-cultured with denuded oocytes (OOX + DO) and (iv) DO. Six replicate experiments were performed. OOX were produced by microsurgical removal of the oocyte, leaving the cumulus cell complex intact (Buccione *et al.*, 1990b), and DO were generated by removing cumulus cells from COCs by vortexing. COC, OOX, OOX + five DO and five DO were then washed in maturation media and cultured individually in pre-equilibrated 10 μ l drops of maturation media (bicarbonated-buffered TCM-199 supplemented with 0.23 mmol sodium pyruvate l^{-1} , 4.0 mg BSA ml^{-1} (ICN Biochemicals), 10.0 μ g hCG ml^{-1} (Pregnyl, Organon, Oss) and 20.0 iu FSH l^{-1} (Puregon, Organon) overlaid with mineral oil and incubated at 39°C with 5% CO₂ in humidified air. Culturing OOX together with five DO in a 10 μ l drop gives a concentration of 0.5 oocyte per μ l, which is within the typical range used to examine oocyte effects on follicular cells (Gilchrist *et al.*, 2001).

Oxygen consumption assay

The oxygen consumption of COC, OOX, OOX + DO and DO was assayed at 0–4, 10–14 or 20–24 h of culture. The protocol for the oxygen assay has been described by Houghton *et al.* (1996) and is based on the fluorescent

properties of pyrene, an oil soluble compound that is excited at 340 nm, the fluorescence of which dissipates in a linear manner in the presence of increasing oxygen concentrations. Oxygen assay chambers were constructed using 5 μ l PCR micropipettes (Drummond Scientific Company, Broomall, PA) and a stainless steel plunger. One μ l of 1 mmol pyrene l^{-1} dissolved in mineral oil was drawn into the micropipettes, followed by 2 μ l Hepes-buffered maturation media containing a single COC, OOX, OOX+one DO, or one DO. An airtight seal was made at the open end of the chamber and the plunger was fixed using sealing wax. Negative control (0% O_2) chambers were constructed in a similar manner, but with 1 mmol oxyrase l^{-1} (Oxyrase Inc., Mansfield, OH) in 60 mmol glucose l^{-1} (equilibrated overnight to remove O_2) replacing the media and positive controls (20% O_2) were constructed with media alone. The fluorescence emission of pyrene at the pyrene-media and the pyrene-plunger interfaces were measured using a fluorophotometric-inverted microscope (Leica, Wetzlar). Measurements were taken at intervals of 30 min over a 4 h period. In between measurements, the chambers were maintained at 39°C. Oxygen consumption by each complex was determined using a computer program that describes the movement of oxygen from the pyrene-oil into the media as oxygen is consumed by the complex (Houghton *et al.*, 1996). At the completion of the assay, the chambers were dismantled and the complexes and spent media were stored in separate 96-well plates (Falcon) overlaid with mineral oil at -80°C .

DNA quantification

The DNA content of individual complexes or oocytes collected at the completion of the oxygen assay was quantified using PicoGreen dye (Molecular Probes, Eugene, OR), a fluorescent nucleic acid dye that has a high affinity for double-stranded DNA compared with single-stranded DNA and other nucleic acids. Extraction and preparation of the samples and quantification of the DNA were performed entirely in 96-well plates. Total DNA was extracted from complexes by adding 50 μ l of lysis buffer (50 mmol Tris-HCl l^{-1} , 1 mmol EDTA l^{-1} (pH 7.6), supplemented with 500 μ g proteinase K ml^{-1}) and incubating for 3.5 h at 50°C and then for 10 min at 80°C. Lysates were subsequently treated with 50 μ l of 10 μ g DNase-free RNase ml^{-1} (Roche Diagnostics, Basel) and incubated for 30 min at 40°C. Excess liquid was evaporated from the wells and 100 μ l Tris-EDTA buffer was added to each well. PicoGreen was prepared according to the manufacturer's instructions and added to each sample. Fluorescence was measured using a FLUOstar Galaxy microplate reader and software (BMG Labtechnologies Pty Ltd, Offenbury) with excitation set at 485 nm and emission at 520 nm. Standard curves (lambda DNA) were used to determine the DNA content of the samples. The interassay coefficient of variation

was <3%. RNase treatment does not affect DNA concentration or contribute to background fluorescence.

Metabolism assays

Glucose, L-lactate and pyruvate consumption and production during the oxygen assay were determined by measuring the concentrations of each of the substrates in the spent media using microfluorometric assays (Leese and Barton, 1984; Gardner and Leese, 1986). All of the metabolic measurements were expressed per ng of DNA to account for the variable number of cumulus cells within each complex.

Statistical analyses

Comparisons of the means of metabolic parameters were performed using two-way ANOVA for the three treatments (COC, OOX and OOX + DO) and the three time-points during maturation. As there were no interactions between time of maturation and treatment group, main effects of time of maturation and treatment are presented. The relationship between glucose uptake and L-lactate production was tested by linear regression analyses. All of the statistical analyses were performed using SigmaStat version 2.0 computer software (SPSS Inc., Chicago, IL).

Results

The utilization of oxygen, pyruvate, glucose and L-lactate by intact COCs, OOX and OOX + DO was compared at 0–4, 10–14 or 20–24 h of culture to investigate the relationship between oocyte secreted factors and the metabolic activity of bovine cumulus cell complexes throughout IVM. The metabolism of DO was also measured, but was undetectable for all of the metabolites measured (data not shown). There were no differences in the consumption of oxygen, pyruvate or glucose or production of L-lactate between intact COCs, OOX or OOX + DO (Fig. 1). This finding demonstrates that oocyte secreted factors do not alter metabolic activity of cumulus cells.

At 20–24 h, the consumption of oxygen (Fig. 2a; 94.0 ± 14.9 pl ng^{-1} DNA h^{-1}), pyruvate (Fig. 2b; 4.69 ± 0.85 pmol ng^{-1} DNA h^{-1}) and glucose (Fig. 2c; 42.4 ± 6.38 pmol ng^{-1} DNA h^{-1}) was significantly greater than the consumption at 0–4 h (oxygen: 50.2 ± 5.64 pl ng^{-1} DNA h^{-1} , $P = 0.026$; pyruvate: 2.15 ± 0.22 pmol ng^{-1} DNA h^{-1} , $P < 0.05$; glucose: 23.5 ± 3.60 pmol ng^{-1} DNA h^{-1} , $P < 0.05$) for oxygen, pyruvate and glucose, respectively). When present at the concentrations used during the present study (5.56 mmol glucose l^{-1} and 0.23 mmol pyruvate l^{-1}) glucose was the preferred substrate throughout the entire culture period, and several-folds more glucose was

Fig. 1. The influence of oocyte-secreted factors on the metabolism of bovine cumulus cell complexes was determined by measuring the consumption of (a) oxygen, (b) pyruvate and (c) glucose, and (d) the production of L-lactate by intact cumulus–oocyte complexes (COC), oocyctomized complexes (OOX) and OOX co-cultured with denuded oocytes (OOX + DO). Data are expressed per ng of DNA and each bar represents the pooled means $\pm$ SEM. There were no significant differences between any of the treatments for all of the metabolites measured, regardless of culture time.

Fig. 2. The utilization of metabolites by bovine cumulus cell complexes was measured at three time points during *in vitro* maturation. The consumption of (a) oxygen, (b) pyruvate and (c) glucose, and (d) the production of L-lactate were measured. Data are expressed per ng of DNA and each bar represents means $\pm$ SEM. ^{ab}Values with different superscripts are significantly different ($P < 0.05$).

taken up compared with pyruvate. L-lactate was the only metabolite measured in which a net production into the medium was observed and production remained constant over the entire 24 h culture period (Fig. 2d).

For every molecule of glucose consumed, two molecules of L-lactate are produced via the glycolytic

pathway. The relationship between total glucose uptake and L-lactate production was examined at each time-point to estimate the proportion of glucose being utilized for lactate production. At the beginning of the IVM period, the relationship between glucose and L-lactate was highly significant (Fig. 3a; $P < 0.001$,

Fig. 3. The relationship between total glucose consumption and L-lactate production by bovine cumulus cell complexes via the glycolytic pathway was examined using linear regression analyses after (a) 0–4 h, (b) 10–14 h or (c) 20–24 h of culture. Points represent individual complexes.

$r^2 = 0.31$), indicating that a large proportion of the glucose consumed was converted to L-lactate at linear rates. At 10–14 h, this relationship was still evident

(Fig. 3b; $P = 0.045$, $r^2 = 0.12$), albeit not as pronounced as the relationship at the start of IVM. At 20–24 h, there was no longer a relationship between the rate of glucose consumption and L-lactate production (Fig. 3c; $P = 0.178$, $r^2 = 0.06$). This finding, coupled with the constant L-lactate production over time, indicates that although a substantial proportion of total glucose utilization occurred via glycolysis, increasingly less glucose could be accounted for by L-lactate production, indicating an alternative fate.

In concurrent studies, the IVM system used in the present study resulted in blastocyst production rates of > 40% (data not shown), demonstrating that oocytes derived from this system behave typically of bovine oocytes matured *in vitro*.

Discussion

To date, apart from morphological features, there are no non-invasive methods that can be applied before IVF and *in vitro* embryo culture to predict the developmental potential of an oocyte. The aim of the present study was to determine whether oocyte-secreted factors (the activity of which may indicate oocyte 'health') influence the metabolism of oxygen, pyruvate, glucose and L-lactate by bovine cumulus cell complexes, as well as to create a metabolic profile of COC metabolism throughout IVM. The present study has demonstrated that the measured metabolic parameters are not affected by the presence of the oocyte. Cumulus complexes that were in physical contact with the oocyte, as well as complexes co-cultured with oocytes, had metabolic activities indistinguishable from those of complexes in which the oocyte had been removed (OOX). Furthermore, this was the case at each of the time points measured throughout the IVM period. Oocyte-secreted factors operate in a strictly concentration-dependent manner (Buccione *et al.*, 1990b; Lanuza *et al.*, 1998; Gilchrist *et al.*, 2001), and it is most unlikely that the lack of cumulus cell response to co-culture with oocytes was due to insufficient oocyte factors, as OOX complexes co-cultured with DO at a density of 0.5 oocytes per μ l is sufficient to elicit a response in cumulus cells (Li *et al.*, 2000; Gilchrist *et al.*, 2001). Furthermore, the lack of difference among treatment groups is unlikely to be due to the insensitivity of the metabolic assays, as these techniques have been used to demonstrate significant differences in the metabolic activity of embryos (Leese and Barton, 1984; Houghton *et al.*, 1996; Thompson *et al.*, 1996).

It is perhaps surprising that oocytes do not seem to regulate cumulus cell metabolism, given that, in general, the oocyte plays a role in the regulation of most cumulus cell functions that have been examined to date (Eppig, 2001). However, there are precedents for cumulus cell activities that are independent of oocyte regulation. Most

notably, FSH-stimulated expansion of pig and bovine COC occurs independently of the oocyte (Prochazka *et al.*, 1991; Singh *et al.*, 1993; Ralph *et al.*, 1995; Nagyova *et al.*, 1999). However, this is a species-specific phenomenon as rodent COC mucification is absolutely dependent on the presence of the oocyte (Buccione *et al.*, 1990b; Salustri *et al.*, 1990). In addition, it has been demonstrated that pig oocytes secrete factors that enable the expansion of both rat and mice cumulus cells (Vanderhyden, 1993; Nagyova *et al.*, 2000).

The observation that the metabolic activity of the cumulus cell is independent of the oocyte is in contrast to previous studies that demonstrated that LH increased glycolytic activity in intact bovine COC, but not in OOX (Zuelke and Brackett, 1992). However, there are numerous differences between the two studies. For example, the source of LH was different and the IVM media used in the present study contained hCG, whereas Zuelke and Brackett (1992) used bovine LH purified from pituitary extracts. In addition, total glucose uptake was measured throughout IVM, compared with the activities of specific pathways at the end of IVM (Zuelke and Brackett, 1992). The present study demonstrated that by 24 h of culture, a large proportion of the glucose consumed was not utilized via glycolysis. It is possible that only the specific glycolytic pathway that was examined in the previous study is affected by oocyte-secreted factors.

The fact that the metabolic activities of cumulus cells were independent of oocyte-secreted factors indicates that the non-invasive measurements of substrate consumption or production by COCs used in the present study cannot be used to determine the biochemical activity (that is health) of an oocyte. This finding indicates that acquisition of developmental competence of bovine oocytes may also not be determined by such factors. This is also the case in feline oocytes, in which increased glucose metabolism is positively correlated with developmental potential; whereas the metabolism of cumulus cells removed before fertilization was not indicative of the developmental potential of the oocyte from which they originated (Spindler *et al.*, 2000). It is plausible that features of the *in vivo* environment such as follicle size, classification (that is subordinate or dominant), health or stage of the oestrous cycle of the donor at collection have a greater influence on developmental potential, as these factors have been associated with improved embryo development (Blondin and Sirard, 1995; Hagemann, 1999; Hagemann *et al.*, 1999).

The metabolic activity of the COC as a whole has not been widely characterized to date, as most studies have focused on the utilization of substrates by the oocyte. As the presence of the cumulus cell vestment is essential to oocyte maturation, the metabolism of the intact COC throughout IVM was investigated as opposed to that of the denuded oocyte alone. Although the oocyte does not have an effect on the metabolism

of the complex, the increased requirement for glucose during the maturation period is in agreement with the reported metabolic profile of the denuded oocyte (Rieger and Loskutoff, 1994; Steeves and Gardner, 1999b). Pyruvate consumption by COCs is also positively related to nuclear maturation (Downs *et al.*, 2002); hence increased culture time leads to higher requirements for pyruvate by the oocyte. Glycolysis was the predominant pathway for glucose utilization, as L-lactate production accounted for most of the glucose consumed. The oxygen uptake (which is indicative of oxidative phosphorylation) can be accounted for by the uptake of pyruvate (and subsequent utilization through the tricarboxylic acid cycle).

The constant rate of L-lactate production throughout IVM indicates that the proportion of consumed glucose that was utilized for ATP production via glycolysis for the COC did not change over the 24 h of culture. This finding is in agreement with the observation that phosphofructokinase activity (the rate limiting enzyme of glycolysis) remains constant throughout oocyte maturation (Cetica *et al.*, 2002). There are numerous pathways through which glucose can be used, and it is possible that during different stages of oocyte maturation, there are different requirements for the substrates that were measured. Previous studies have demonstrated that the pentose phosphate pathway is active throughout maturation and there is a positive relationship between increased glucose flux through this pathway and the induction of meiosis (Downs *et al.*, 1998). It is possible that towards the end of IVM the flux of glucose through the pentose phosphate pathway increases to allow for increased production of substrates involved in nuclear maturation. An additional route of glucose utilization is the conversion of glucose to extracellular matrix components. A large component of cumulus expansion is the increased synthesis of extracellular matrix, which is not only an energy expensive process but also requires the synthesis of matrix components. Glucose metabolized by cumulus cells can be utilized via the glycolytic pathway for energy production, but may also be used for the synthesis of glucosamine, a substrate for hyaluronic acid (Chen *et al.*, 1993), a major component of cumulus matrix. These pathways are most likely linked, as the addition of FSH has been associated with increased glucose consumption (Downs and Utecht, 1999) and mucification (Salustri *et al.*, 1989). Mucification occurs in the latter part of maturation and is important *in vivo* for the events after ovulation (Chen *et al.*, 1993). Therefore, it is more likely that the increased glucose uptake towards the end of IVM observed in this study is attributed to an increased extracellular matrix formation, particularly as pentose phosphate pathway activity has been shown to be low compared with glycolytic activity (Urner and Sakkas, 1999). Investigations are currently in progress to analyse further the role of glucose in cumulus matrix formation in the COC.

In conclusion, the present study has demonstrated that the metabolism of oxygen, pyruvate, glucose and L-lactate by bovine cumulus cell complexes is not affected by oocyte-secreted factors, hence non-invasive measurements of the utilization of these substrates by COCs is unlikely to represent biochemical activities of the oocyte associated with developmental competence. In addition, the present study has demonstrated that the metabolic requirements of the COC increased through IVM. It is possible that further investigation of the metabolism of the COC as a unit may aid in the optimization of culture conditions during IVM.

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