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Phosphofructokinase and Malate Dehydrogenase Participate in the *In Vitro* Maturation of Porcine Oocytes

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Contents

Oocyte maturation depends on the metabolic activity of cumulus–oocyte complex (COC) that performs nutritive and regulatory functions during this process. In this work, the enzymes [phosphofructokinase (PFK) and malate dehydrogenase (MDH)] were tested to elucidate the metabolic profile of porcine COCs during the *in vitro* maturation (IVM). Enzymatic activity was expressed in U/COC and U/mg protein (specific activity) as mean ± SEM. *In vitro* maturation was performed with 2-oxoglutarate (5, 10 and 20 mM) or hydroxymalonate (30, 60 and 100 mM) inhibitors of PFK and MDH, respectively. The PFK and MDH activities (U) remained constant during maturation. For PFK, the U were (2.48 ± 0.23) 10^{-5} and (2.54 ± 0.32) 10^{-5}, and for MDH, the U were (4.72 ± 0.42) 10^{-5} and (4.38 ± 0.25) 10^{-5} for immature and *in vitro* matured COCs, respectively. The specific activities were significantly lower after IVM, for PFK (4.29 ± 0.48) 10^{-3} and (0.94 ± 0.12) 10^{-3}, and for MDH (9.08 ± 0.93) 10^{-3} and (1.89 ± 0.10) 10^{-3} for immature and *in vitro* matured COCs, respectively. *In vitro* maturation percentages and enzymatic activity diminished with 20 mM 2-oxoglutarate or 60 mM hydroxymalonate ($p < 0.05$). Viability was not affected by any concentration of the inhibitors evaluated. The U remained unchanged during IVM; however, the increase in the total protein content per COC provoked a decrease in the specific activity of both enzymes. Phosphofructokinase and MDH necessary for oocyte IVM would be already present in the immature oocyte. The presence of inhibitors of these enzymes impairs the meiotic maturation. Therefore, the participation of these enzymes in the energy metabolism of the porcine oocyte during IVM is confirmed in this study.

Introduction

During the past years, the research in porcine species has focused on improving the reproductive technologies. However, this extensive research has not accomplished the difficulties that already exist, as the suboptimal results of oocyte *in vitro* maturation (IVM), the high incidence of polyspermy in the *in vitro* fertilization or the low survival during the *in vitro* embryo culture (Funahashi 2003). If oocyte maturation, which is a critical step in the *in vitro* embryo production, is not carried out in proper conditions, it will affect the subsequent embryo development (Yuan and Krisher 2012). Nowadays, the system used for IVM has not been standardized and approximately 10–30% of pig oocytes fail to reach the MII stage (Zhang et al. 2012).

Mammalian oocyte maturation depends on the surrounding cumulus cells. Cumulus cells perform regulatory and nutritional functions, providing different metabolites to the oocyte (Sutovsky et al. 1993; Sturmey and Leese 2003). The cumulus cells metabolize glucose (Zuelke and Brackett 1992; Cetica et al. 1999; Sutton et al. 2003), producing oxidative substrates that can be used by the oocyte as energy source (Buccione et al. 1990; Brackett and Zuelke 1993; Cetica et al. 1999). Accordingly, glycolysis has been proposed as the main fate of the glucose consumed by porcine cumulus–oocyte complexes (COCs) during IVM (Krisher et al. 2007; Alvarez et al. 2012). In somatic cells, the regulation of the glycolysis is thought to take place mainly by the enzyme phosphofructokinase (PFK; Nelson and Cox 2005). Likewise, a high PFK activity has been detected in bovine COCs and their surrounding cells (Cetica et al. 2002).

Glucose metabolized by the cumulus cells produce metabolites, mainly lactate that is used by the oocyte during maturation (Cetica et al. 1999; Sutton et al. 2003; Alvarez et al. 2012). Lactate is oxidized to pyruvate within the cytosol with the production of NADH in the reaction catalysed by the enzyme lactate dehydrogenase (Nelson and Cox 2005). The balance of metabolic intermediates between the cytosol and the mitochondria must be maintained to enable lactate metabolism to continue (Lane and Gardner 2005). In this regard, NADH must be oxidized within the mitochondria in the respiratory chain. As intact, mitochondria are impermeable to NADH (Cooper et al. 1985), there are indirect shuttles involved in the movement of electrons from NADH across the mitochondrial membranes. The most common mitochondrial shuttle present in cells is the malate–aspartate shuttle (Nelson and Cox 2005). This shuttle involves cytoplasmic and mitochondrial enzymes. Malate dehydrogenase (MDH) is a main component of this shuttle and performs a key function in the passage of reduction equivalents through the internal mitochondrial membrane as a result of the presence of cytosolic and mitochondrial isoenzymes (Clarenburg 1992). The activity of this enzyme is relatively high in bovine COCs, suggesting that the activity of this enzyme may be related not only with the tricarboxylic acid (TCA) cycle but also with the malate–aspartate shuttle (Cetica et al. 2003). In mouse, it has been determined that the malate–aspartate shuttle is a key regulator of carbohydrate metabolism determining developmental progression in the pre-implantation embryo (Lane and Gardner 2005).

Therefore, the question arises whether porcine COC has a similar metabolic behaviour during IVM. To our knowledge, there are few studies that demonstrate biochemically the relationship between the enzymatic

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activity in COCs and the processes involved in the oocyte IVM in this species. The study of the maximum *in vitro* catalytic activity of the key enzymes provides information about the maximum capacity of a given metabolic pathway and may be a good indicator of the metabolic processes occurring within the cells under study (Crabtree et al. 1979; Newsholme and Leech 1983; Phair 1997). In this context, the aim of this work was to study the participation of the enzymes PFK and MDH in the porcine oocyte maturation process by determining the enzymatic activity of these enzymes in immature and *in vitro* matured porcine COCs, and the effect of their inhibition on oocyte IVM.

Material and Methods

Materials

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

Collection of cumulus–oocyte complexes

Ovaries from slaughtered gilts were transported in a warm environment (28–33°C) for 2–3 h of journey to the laboratory. Ovaries were washed in 0.9% (w⁄v) NaCl containing 100 000 IU/l penicillin and 100 mg/l streptomycin. COCs were aspirated from 3- to 8-mm antral follicles using a 10-ml syringe and an 18-gauge needle. Oocytes surrounded by a multilayer and dense cumulus were selected.

Oocyte *in vitro* maturation

Groups of 50 COCs were cultured in 500-μl 199 medium (Earle's salts, L-glutamine 100 mg/l, 2.2 mg/l sodium bicarbonate, GIBCO, Grand Island, NY, USA), supplemented with 10% (v/v) foetal bovine serum (GIBCO), 0.57 mM cysteine, 50 mg/l gentamicin sulphate, 0.5 mg/l porcine follicle-stimulating hormone (Folltropin-V, Bioniche, Belleville, ON, Canada) and 0.5 mg/l porcine luteinizing hormone (Lutropin-V, Bioniche) under mineral oil at 39°C in 5% CO_2 in humidified air for 48 h (Abeydeera et al. 2001).

Preparation of enzymatic extracts

Immature or *in vitro* matured COCs were washed with PBS supplemented with polyvinyl alcohol (PVA) (1 mg/ml) and suspended in distilled water. The suspension was frozen at −20°C until use (maximum 2 months). For enzymatic determination, the frozen COC suspensions were thawed, homogenized and sonicated at 100 W in 50% cycle at 4°C using a VibraCell sonicator model 600W (Sonics & Materials Inc., Newton, CT, USA) for 4 min. After centrifugation of the homogenate (10 000 ***g***, 20 min, 4°C), the supernatants were collected and maintained at 4°C until enzyme assay. For both enzymes, the extracts were prepared to obtain a final amount of enzyme that ensure linear behaviour during the time of activity measurement, thus enabling to calculate the rate of absorbance variation per minute.

Phosphofructokinase assay

The PFK activity was determined in extracts of immature or *in vitro* matured COCs (n = 300) as described by Cetica et al. (2002) with minimum modifications. The enzymatic activity was measured in a Shimadzu spectrophotometer model UV-160 (Shimadzu Corporation, Tokyo, Japan) at 340 nm for 10 min at 37°C in 100 mM TRIS-HCl buffer pH 8.2 supplemented with (assay concentrations) 10 mM $MgCl_2$, 10 mM NH_4Cl, 4 mM fructose 6-phosphate, 0.4 mM NADH, 2 mM ATP and 1 mM AMP in the presence of the auxiliary enzymes: 1.4 U aldolase, 40 U phosphotriose isomerase and 5 U glycerol-3-phosphate dehydrogenase. An enzymatic unit of PFK was defined as the quantity of enzyme that catalysed the production of 1 μM fructose 1,6-bisphosphate per minutes that was equal to the oxidation of 2-μmol NADH per minutes.

Malate dehydrogenase assay

The MDH activity was determined in extracts of immature or *in vitro* matured COCs (n = 300) as described by Cetica et al. (2003) with minimum modifications. The activity was measured at 340 nm for 3 min in 90 mM glycine buffer pH 10 supplemented with (assay concentrations) 40 mM malate and 1.2 mM NAD. An enzymatic unit of MDH was defined as the quantity of enzyme that catalysed the reduction of 1 μmol NAD per minutes.

Determination of total proteins

Total protein concentration was determined in enzymatic extract supernatants using the method described by Lowry et al. (1951). Briefly, 10 μl of enzymatic extract was added into Biuret reagent and further incubated for 10 min at room temperature. Thereafter, 100 μl Folin–Ciocalteu reagent was added into the reaction mixture and further incubated at room temperature in darkness for other 30 min. The calibration curve was made using different dilutions of a solution of BSA (1 mg/ml). All determinations were done at 660 nm.

Evaluation of oocyte meiotic maturation

After culture, oocytes were denuded, fixed in glutaraldehyde (2% (v/v) in PBS) for 15 min, stained for 15 min (1% Hoechst 33342 in PBS) and finally washed in PBS containing 1 mg/l PVA and mounted on glass slides. Oocytes were examined under an epifluorescence microscope at ×100 and ×400 magnification (using 330–380 nm and 420 nm excitation and emission filters, respectively) (Coy et al. 2005). Oocytes that showed metaphase II chromosome configuration were considered meiotically mature.

Evaluation of oocyte viability

Cumulus cells and oocyte viabilities were assessed by incubation for 10 min at 37°C in PBS added with 2.5 μg/l fluorescein diacetate fluorochrome. COCs were

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washed in PBS before being observed in an epifluorescence microscope (Jenamed 2, Carl Zeiss, Jena, Germany) using 450–490 nm and 520 nm excitation and emission filters, respectively, at ×100 magnification. Live cells were distinguished from dead ones based on their green fluorescence (Figure S1; Alvarez et al. 2013).

Effect of enzymatic inhibitors on the *in vitro* maturation

Cumulus–oocyte complexes were matured under the conditions described above with the addition of increasing concentrations of inhibitors of the enzymes under study: 2-oxoglutarate or hydroxymalonate, inhibitors of PFK and MDH, respectively (Chang et al. 2009). In all treatments, the final pH of maturation media was carefully adjusted to 7.4. Osmolarity of media was adjusted to be among 270 and 330 mOsm. Meiotic maturation and viability rates were evaluated in each case.

Effect of inhibitors on enzymatic activity

The enzymatic activity of PFK and MDH was determined in the presence of the minimum concentration of 2-oxoglutarate or hydroxymalonate that inhibits IVM. The inhibitors were added to the assay buffer during the enzyme assay.

Statistical analysis

The enzymatic activities are expressed as enzymatic units (U) and specific activities (SA). The U are expressed in U per COC; meanwhile, SA are expressed in U per mg protein. Data are expressed as mean ± SEM. Enzymatic activities in immature and *in vitro* matured COCs were compared using a Student′s *t*-test. Statistical analyses of meiotic maturation and viability percentages were made by chi-squared analysis for nonparametric data. The inhibitory effect on the enzymatic activity of each enzyme was determined by comparing the U in the presence or absence of the inhibitor using the Student′s *t*-test for paired samples. The p value used to determine significance in all tests was 0.05.

Results

Enzymatic activities

Phosphofructokinase and MDH activities expressed in enzymatic units per COC remained without changes during IVM. However, a significant decrease in the specific activity was observed for both enzymes when immature and *in vitro* matured COCs were compared. The activity (U/COC and U/mg protein) of MDH was approximately the double of the PFK activity for both immature and *in vitro* matured COCs (Table 1). An increase in total protein content per COC was observed during IVM (2.66 ± 0.42 and 6.79 ± 0.12 μg protein/COC for immature and *in vitro* matured COCs, respectively); this increase was statistically significant ($p < 0.05$).

Effect of enzymatic inhibitors on the *in vitro* maturation

To study the participation of these enzymes during the oocyte IVM process, increasing concentrations of enzymatic inhibitors (2-oxoglutarate or hydroxymalonate for PFK and MDH, respectively) were added to the IVM medium. The meiotic maturation rate diminished ($p < 0.05$) with 20 mM 2-oxoglutarate compared with control (Fig. 1); meanwhile, the addition of hydroxymalonate from 60 mM also diminished the meiotic maturation rate ($p < 0.05$; Fig. 2). None of the concentrations of the inhibitors evaluated affected COCs viability (Table 2).

Effect of inhibitors on enzymatic activity

The enzymatic activity of PFK and MDH in the presence of the minimum inhibitory concentration of the inhibitor of each enzyme was determined. The U with the inhibitors of PFK and MDH (20 mM 2-oxoglutarate or 60 mM hydroxymalonate, respectively) were significantly lower respect to their respective controls (Table 3).

Discussion

Oocyte maturation is the final step of oocyte development and allows the oocyte to be fertilized and to develop into an embryo. The COC requires an enormous quantity of ATP to accomplish the dynamic changes that occur during maturation. During IVM, high activities of PFK, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase and MDH have been detected (Tsutsumi et al. 1992; Downs and Utecht 1999; Cetica et al. 2002, 2003). Specific activity (U/mg protein), which is a measure of enzyme purity (Nelson and Cox 2005), was significantly lower in *in vitro* matured COCs. The increase in COC protein

Table 1. Phosphofructokinase (PFK) and malate dehydrogenase (MDH) activities in immature and *in vitro* matured cumulus–oocyte complex (COCs)

	Units per COC		Units per mg protein	
	Immature	Mature	Immature	Mature
PFK	$(2.48 \pm 0.23)\ 10^{-5\ a}$	$(2.54 \pm 0.32)\ 10^{-5\ a}$	$(4.29 \pm 0.48)\ 10^{-3\ a}$	$(0.94 \pm 0.12)\ 10^{-3\ b}$
MDH	$(4.72 \pm 0.42)\ 10^{-5\ a}$	$(4.38 \pm 0.25)\ 10^{-5\ a}$	$(9.08 \pm 0.93)\ 10^{-3\ a}$	$(1.89 \pm 0.10)\ 10^{-3\ b}$

Values are means ± SEM of 5–6 replicates. For each enzyme, the superscripts a and b indicate significant differences ($p < 0.05$) between immature and *in vitro* matured COCs.

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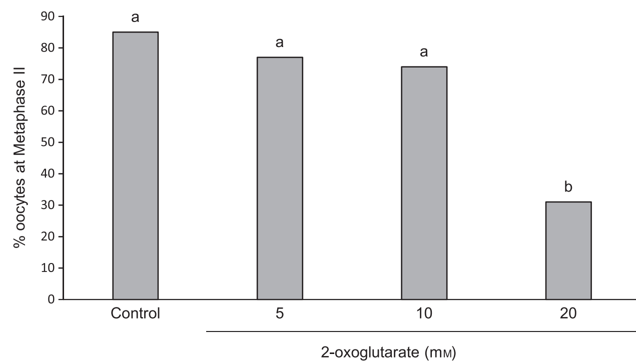

Fig. 1. Percentages of meiotic maturation in cumulus–oocyte complexes (COCs) cultured with different concentrations of 2-oxoglutarate, n = 91–106 for each treatment. [a,b] Bars with different superscripts are significantly different ($p < 0.05$)

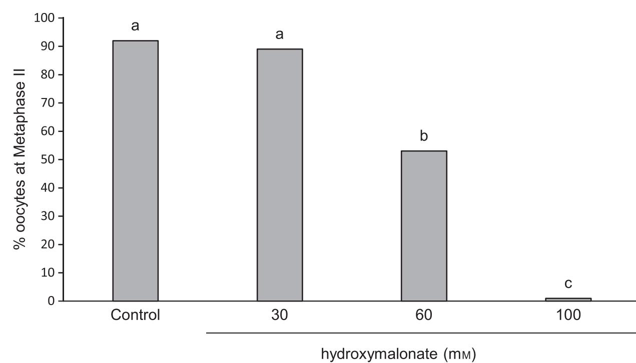

Fig. 2. Percentages of meiotic maturation in cumulus–oocyte complexes (COCs) cultured with different concentrations of hydroxymalonate, n = 91–106 for each treatment. [a–c] Bars with different superscripts are significantly different ($p < 0.05$)

Table 2. Percentages of live oocytes and live cumulus in cumulus–oocyte complex (COCs) matured in the presence of different inhibitors

	2-oxoglutarate			
	0 mM	5 mM	10 mM	20 mM
% live oocytes	100	89	100	95
% live cumulus	89	100	100	100
	Hydroxymalonate			
	0 mM	30 mM	60 mM	100 mM
% live oocytes	100	95	95	90
% live cumulus	95	100	100	100

n = 28–30 COCs for each treatment, no significant differences between treatments.

content would be the cause of the decrease in specific activity. This increase in protein content during IVM was previously reported in porcine (Alvarez et al. 2012) and bovine COCs (Gutnisky et al. 2007). Similar results in the evaluation of specific activity of PFK and MDH have been found in bovine COCs during IVM (Cetica et al. 2002, 2003).

Table 3. Inhibition of phosphofructokinase (PFK) and malate dehydrogenase (MDH) activities in immature cumulus–oocyte complex (COCs)

	Without inhibitor	With inhibitor	% inhibition
PFK	$(2.60 \pm 0.14)\ 10^{-5\ a}$	$(1.08 \pm 0.20)\ 10^{-5\ b}$	59 ± 7*
MDH	$(4.95 \pm 0.56)\ 10^{-5\ a}$	$(1.10 \pm 0.27)\ 10^{-5\ b}$	77 ± 5**

Enzyme activities are expressed in units per COC (U). Values are means ± SEM of 5–6 replicates. For each enzyme, the superscripts a and b indicate significant differences ($p < 0.05$) in the presence or absence of specific inhibitors. Inhibition obtained by the presence of 20 mM 2-oxoglutarate (*) or 60 mM hydroxymalonate (**).

In porcine species, glycolysis appears to be the main fate of glucose during IVM (Krisher et al. 2007; Alvarez et al. 2012, 2013). This is consistent with the high activity of PFK detected in porcine COCs remaining to be established the relative participation of this enzyme in oocytes and cumulus cells separately, as mammalian oocytes have low capacity to utilize this substrate (Steeves and Gardner 1999; Purcell and Moley 2009; Sutton-McDowall et al. 2010), possibly due to the limited amount of PFK (Cetica et al. 2002).

Interestingly, MDH activity was approximately the double of the PFK activity as it has been observed in bovine (Cetica et al. 2002, 2003). Although MDH is not considered a key enzyme that controls the flow of the TCA cycle, it plays a main role in the control of the TCA cycle because it catalyses the reaction that regenerates oxaloacetate to initiate the cycle (Nelson and Cox 2005). Furthermore, high MDH activity might be related not only with the TCA cycle but also with the malate–aspartate shuttle system for transport of reduction equivalents through the internal mitochondrial membrane, as it has been proposed in bovine COCs (Cetica et al. 2003). These findings are also in accordance with those observed by Lane and Gardner (2005) in mouse and highlight the role of this shuttle as a key regulator of embryo metabolism.

Despite of the importance of glucose, it has been demonstrated that endogenous lipids are important for oocyte maturation in pig as porcine oocytes contain a substantial high amount of triglyceride specially compared to those of human and mice (Sturmey and Leese 2003). The free fatty acids derived from the triglycerides are oxidized by beta-oxidation pathway and metabolized in the TCA cycle. Glucose may be used to provide oxaloacetate to prime the TCA cycle, as oxaloacetate can be derived from pyruvate via the enzyme pyruvate carboxylase (Nelson and Cox 2005). In a preliminary work, we detected isocitrate dehydrogenase (IDH)–NAD activity in the porcine COC for the first time (Breininger et al. 2013). Isocitrate dehydrogenase, which is considered the main key enzyme of the TCA cycle, has three isoenzymes, two mitochondrial and one cytosolic. However, only the mitochondrial isoenzymes use NAD as electron acceptors (Nelson and Cox 2005). This finding, contrary to the results found in cow where no NAD–IDH activity was recorded in immature and *in vitro* matured COCs (Cetica et al. 2003), supports the idea of the importance of the TCA cycle in the metabolism of porcine oocytes.

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This study also highlights the importance of the enzymes PFK and MDH during IVM of porcine oocytes. The results indicate that culturing pig COCs in the presence of inhibitors of PFK and MDH compromises the oocyte maturation. Moreover, the addition of the inhibitors at the same concentration that impairs IVM also inhibits enzymatic activity in both studied enzymes. This process that includes nuclear and cytoplasmic maturation requires enormous energy from various substrates, including glucose, amino acids and lipids (Sutton et al. 2003). The inhibition of glycolytic pathway and TCA cycle may be diminishing the energetic state of the oocyte and therefore affecting nuclear maturation and probably cytoplasmic maturation.

In conclusion, porcine immature COC, which are the morphological and functional units that participate in the maturation process, has high enzymatic activity of PFK and MDH. The enzymatic units remained unchanged during IVM; however, the increase in the total protein content of the COC provoked the decrease in the specific activity of both enzymes. The PFK and MDH necessary for oocyte IVM would be already present in the immature COC. Their synthesis during IVM would be minimal or null. Further studies are required to evaluate the gene expression of these enzymes during IVM. We confirm the inhibition of PFK and MDH of porcine COCs with 2-oxoglutarate and hydroxymalonate, respectively. The inhibition of both enzymes impairs the meiotic maturation of the porcine oocytes. Therefore, the participation of PFK and MDH in the energy metabolism of porcine oocyte during IVM is confirmed in the present study.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Elizabeth Breininger performed the experiments to evaluate enzymatic activity, the analysis and interpretation of data and drafted the manuscript. Bruno Vecchi Galenda performed the experiments about *in vitro* maturation. Gabriel Alvarez and Cynthia Gutnisky contributed to perform the experiments to evaluate enzymatic activity and to the analysis and interpretation of data. Pablo Cetica designed the experiments, contributed to the analysis and interpretation of data and drafted the article.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Fluorescein diacetate fluorochrome to study cumulus cells and oocytes viability. Live cells display green fluorescence.

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