Is the canine corpus luteum an insulin-sensitive tissue?

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Abstract

This study aimed to determine in the canine corpus luteum throughout the dioestrus (1) the influence of insulin on glucose uptake; (2) the regulation of genes potentially involved; and (3) the influence of hypoxia on glucose transporter expression and steroidogenesis, after treatment with cobalt chloride (CoCl₂). Glucose uptake by luteal cells increased 2.7 folds ($P < 0.05$) in response to insulin; a phenomenon related to increased expression of glucose transporter (GLUT) 4 and phosphorylation of protein kinase B (AKT). The gene expression of insulin receptor and *SLC2A4* (codifier of GLUT4) genes after insulin stimulation increased on day 20 post ovulation (p.o.) and declined on day 40 p.o. ($P < 0.05$). Regarding potentially involved molecular mechanisms, the nuclear factor kappa B gene *RELA* was upregulated on days 30/40 p.o., when *SLC2A4* mRNA was low, and the interleukin 6 (*IL6*) gene was upregulated in the first half of dioestrus, when *SLC2A4* mRNA was high. CoCl₂ in luteal cell cultures increased the hypoxia-inducible factor *HIF1A*/HIF1A and the *SLC2A4*/GLUT4 expression, and decreased progesterone (P4) production and hydroxyl-delta-5-steroid dehydrogenase 3 beta (*HSD3B*) mRNA expression ($P < 0.05$). This study shows that the canine luteal cells are responsive to insulin, which stimulates glucose uptake in AKT/GLUT4-mediated pathway; that may be related to local activity of *RELA* and *IL6*. Besides, the study reveals that luteal cells under hypoxia activate *HIF1A*-modulating luteal function and insulin-stimulated glucose uptake. These data indicate that insulin regulates luteal cells' glucose disposal, participating in the maintenance and functionality of the corpus luteum.

Key Words

- ▶ corpus luteum
- ▶ glucose uptake
- ▶ insulin
- ▶ *RELA*/p65
- ▶ hypoxia

Journal of Endocrinology
(2016) **231**, 223–233

Introduction

The control of the function of canine corpus luteum (CL) is performed by complex mechanisms involving luteotrophic hormones, as well as autocrine and paracrine factors (Okkens *et al.* 1990, Onclin *et al.* 2000, Hoffmann *et al.* 2004, Kowalewski *et al.* 2011, Papa & Hoffmann 2011, Kowalewski 2014).

According to Sonnack (M Sonnack, unpublished communications) the canine CL lasts 60 days after ovulation (p.o., day 0 is the day of ovulation), formation

(day 0–15), maintenance or stage of secretory activity (day 15–45) and regression (day 45–60). During the CL development, a proper nutritional supply for luteal cells must be ensured, which is accomplished by glucose, the major energy substrate for ovarian activity, as demonstrated in granulosa cells of women with polycystic ovary syndrome (Kim *et al.* 2014) and endometrium (França *et al.* 2015), follicle and CL of cows, (Nishimoto *et al.* 2006). In CL of bovines, the

expression of solute carriers family 2 members (SLC2A) 1, 3 and 4, which codify the glucose transporters GLUT1, GLUT3 and GLUT4, respectively was reported, and the authors inferred that GLUT1 and GLUT3 would act as main carriers (Nishimoto *et al.* 2006). In this study, the authors proposed just a supportive role for GLUT4, in spite of (1) the ~3-fold increase observed in *SLC2A4* expression through the CL development, (2) the fact that GLUT4 display the highest capacity of transport glucose (Uldry & Thorens 2004) and (3) the fact that it can be rapidly regulated by insulin (Uldry & Thorens 2004). In CL of dogs, our group has demonstrated a differential expression of glucose transporter 1 (*SLC2A1*/GLUT1), which accompanied hypoxia during the cycle (Papa *et al.* 2014), pointing out that the CL responds to physiological variations of oxygen tension during dioestrus (Nishimura & Okuda 2015). However, nothing is known about the *SLC2A4*/GLUT4 expression in CL of dogs.

The longer exposure to hypoxia was reported to increase transcription of the gene *Slc2a1* in several cell types (Zhang *et al.* 1999). Later on, it was described that hypoxia also increases *Slc2a3* and *Slc2a4* expression in brain (Royer *et al.* 2000). Finally, it was described that *Slc2a4* increases in response to muscle contraction, which reduces the intracellular oxygen tension, and that involves increased binding of the hypoxia-inducible factor 1A (*Hif1a*) into the *Slc2a4* promoter region (Lima *et al.* 2009). Additionally, some studies further suggest that the *Hif1a* can also regulate the plasma membrane translocation of GLUT4 (Sakagami *et al.* 2014), known as the insulin-sensitive glucose transporter, primarily described in insulin-sensitive tissues, as adipose tissue and skeletal and cardiac muscles (James *et al.* 1988, Slot *et al.* 1991, Marette *et al.* 1992, Klip *et al.* 1996), reinforcing the effect of hypoxia upon regulation of GLUT4.

Although the CL requires high glucose uptake, works so far have explored neither the effect of insulin as inducer of glucose uptake in this organ nor whether physiological hypoxic conditions interfere with that effect when CL is forming, secreting or regressing. The transcriptional regulation of the *Slc2a4* gene involves the control of several transcriptional factors including the enhancer *Hif1a* and the repressor RELA, former transcription factor p65, codified by the gene *Rela* (Furuya *et al.* 2013). Besides, interleukin 6 (*IL6*) has also been suggested as a repressor of *Slc2a4* gene (Franckhauser *et al.* 2008, Poletto *et al.* 2015).

Thus, this study was designed to investigate the involvement of insulin on glucose uptake by luteal cells,

as well as potentially involved positive and negative regulators of this effect. Given the importance of CL hypoxia-induced adaptation and its inductive role of glucose uptake, the effects of CoCl_2 , a mimicker of hypoxic environment and inducer of HIF1A (Metzen *et al.* 2003, Grasselli *et al.* 2005) on the expression of GLUT1/*SLC2A1* and GLUT4/*SLC2A4*, as well as on luteal cells progesterone (P4) production were also investigated.

Materials and methods

Ethics statement

Written consent was obtained from all owners of the dogs and the procedures were performed according to ethical principles approved by the Ethics Committee for the Use of Animals at the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (Protocol numbers: 1432/2008 and 2080/2010).

Dogs and sample collection

Corpora lutea (CLs) were collected from 34 healthy cross-bred female dogs aged between 2 and 8 years via ovariohysterectomy (OSH) on specific days of dioestrus. After the onset of pro-oestrous bleeding, blood samples were collected on alternate days to determine P4 concentrations. When plasma P4 levels reached 5 ng/mL, it was considered the day of ovulation (Concannon *et al.* 1989). For cell culture experiments, CLs were collected as follows: on days 20 (highest P4 plasma concentrations) and 40 (highest oestradiol (E2) plasma concentrations) post ovulation (p.o.) for (insulin treatment ($n=4$ per day); on day 30 p.o. (full secretory activity) for glucose uptake assay ($n=3$) and over dioestrus ($n=21$) for the cobalt chloride treatment.

Anaesthetic and surgical procedures were performed as described previously (Papa *et al.* 2014). Before anaesthesia, blood samples (5 mL) were collected for metabolic and hormonal analyses. For immunohistochemistry, one CL per dog was fixed in 4% buffered formalin for 24 h and embedded in paraffin. For real-time PCR and Western blotting, one CL per dog per technique was immediately frozen in liquid nitrogen and stored at -80°C , and for cell culture, all CLs from each dog ($n=4$ dogs per group) were kept in ice-cold phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin-amphotericin B solution (A5955; Sigma-Aldrich).

Table 1 List of primers used for real time (TaqMan) PCR.

Primer	Primer sequence	Accession number	Amplicon length
HIF1A – for	5'-GCTGCTGGAGACACAATCATATCTT-3'	XM860420	92
HIF1A – rev	5' ACATCATTATACAACGGAACCTCCTCAAG-3'		
HIF1A – probe	5'-CAATGACACAGAAACTG-3'		
HSD3B – for	5'-GGGTACTCAGCTCCTGTTGGAA-3'	NM_001010954.1	102
HSD3B – rev	5'-GCCACCTCTATGGTGTGGTAT-3'		
HSD3B – probe	5'-TGCCCAGGCTAGTGTGCCGATCTT-3'		
IL6 – for	5'-AAAGAGCAAGGTAAAGAATCAGGATG-3'	XM_843327-1	148
IL6 – rev	5'-GCAGGATGAGGTGAATTGAAGTGATT-3'		
IL6 – probe	5'-GTGATGTCTGCTCGGTAAG-3'		
INSR – for	5'-GCAGGARGAGGTGAATTGTTGTG-3'	AIBJWTL_R	108
INSR – rev	5'-CCGAGACCTCAGTTTCCCA-3'		
INSR – probe	5'-AAAACGAGGCCCGAGGATTT-3'		
RELA – for	5'-GCAGAAAGAGGACATTGAAGTGATT-3'	NW_876266-1	118
RELA – rev	5'-TCGGTGTACATCAGCTTGAGAAA-3'		
RELA – probe	5'-CCAGGCTGGGAGGCCCGA-3'		
SLC2A1 – for	5'-CAGCCAGAGTCCCCTGTATCTA-3'	XM_539554	99
SLC2A1 – rev	5'-GTCCCCACCTTCAGGTACTG-3'		
SLC2A1 – probe	5'-CACCCCAGACTTCACC-3'		
SLC2A4 – for	5'-GCCTGCCAGAAAGAGTCTGAAG-3'	NM_001159327	91
SLC2A4 – rev	5'-GCTTCGCTTCTCCTCCTT-3'		
SLC2A4 – probe	5'-CAGTGCCCCAGATACAT-3'		
PPIA	Pre-designed assay from Life Technologies Prod. No. Cf03986523-gH		

Luteal cell culture

CL samples were immediately washed with fresh PBS containing 1% antibiotic-anti-mycotic solution (A5955, Sigma-Aldrich), and cut into small pieces. These pieces were transferred to 1 mL Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), 20 mM HEPES (Sigma-Aldrich), 1% antibiotic-anti-mycotic solution and 1 mg/mL collagenase type 1 (C0130; Sigma-Aldrich). The incubation lasted for 1 h under stirring (60 rotations/min) at 37°C. The suspension was centrifuged at 200g for 10 min, re-suspended in DMEM and filtered through a cell strainer (70 µm; BD Falcon; BD Biosciences, Durham, NC, USA). The filtrate was centrifuged at 200g for 10 min, re-suspended in an erythrocyte lysis buffer (0.16 M NH₄Cl, 0.01 M Tris-HCl pH 7.2–7.4) diluted in DMEM (v/v) for 10 min, centrifuged at 200g for 10 min,

and re-suspended in DMEM. For glucose uptake study, luteal cells were seeded in 6-well plates, whereas for insulin and cobalt chloride (CoCl₂) treatments, cells were seeded in 24-well plates. Furthermore, for GLUT1 and GLUT4 immunocytochemistry, luteal cells were seeded in 24-well plates containing coverslips and fixed with 95% methanol. Subsequently, cells were kept in an incubator at 37°C and 5% CO₂. After ten days of culture (90% confluence), the cells were subjected to serum-free medium for 24 h (except for glucose uptake) and treated following different protocols as described below.

Determination of 2-deoxyglucose uptake

Glucose uptake analysis was performed as described previously (Campello *et al.* 2012), using 2-deoxy-[1,2-³H]-D-glucose 0.2 mCi·mL⁻¹; Perkin Elmer) for 5 min, after

Table 2 List of antibodies used for immunohistochemistry and Western blotting.

Antibodies	Isotype	Epitope	Dilution IHC	Dilution WB	Supplier (order n°)
GLUT1	Goat polyclonal IgG	C-terminus human	–	0.4	Santa Cruz (C-20; sc-1605)
GLUT4	Rabbit polyclonal IgG	C-terminus human	0.5	1.4	Millipore (07-1404)
HIF1A	Rabbit polyclonal IgG	Human HIF1A amino acids (432–528)	–	0.4	Novus Biologicals (NB100-134)
IL6	Mouse monoclonal IgG2a	Recombinant full length human protein	0.3	0.4	Abcam (ab 9324)
INSR	Rabbit polyclonal IgG	N-terminus human	0.1	0.5	Abcam (ab 78424)
P65	Mouse monoclonal IgG3	Human p65	0.3	0.4	Millipore (MAB 3026)
ACTB	Mouse monoclonal	N-terminal	–	0.4	Sigma-Aldrich (Clone AC-15; A1978)

a 20-min stimulus with 100nM insulin. Incorporated radioactivity was measured, results were calculated and expressed as both cpm/ μ g protein and cpm/ 10^6 cells.

Effect of insulin on luteal *INSR* and *SLC2A4* expression

Luteal cells from CLs obtained on days 20 and 40 p.o. were maintained in DMEM without serum for 24h. These cells were then treated with 100nM insulin (I6634; Sigma-Aldrich). After 24h, the culture medium was removed, TRIzol was added to the cells and they were collected and processed for real-time PCR analysis.

Gene expression assessed by real-time PCR

Total RNA extraction was performed using the TRIzol reagent, following manufacturer's instructions (Life Technologies). RNA concentration was measured using the NanoDrop 2000 (Thermo Fisher Scientific), whereas the purity and integrity of the samples were determined using 2% agarose gel.

Real-time PCR (qPCR) was carried out as described previously (Kowalewski *et al.* 2006). Briefly, the total RNA was subjected to reverse transcription using the SuperScript III kit, according to manufacturer's instructions (Life Technologies). PCR analyses were performed with an automated fluorometer (ABIPrism 7500, Life Technologies), using 96-well optical plates. Each sample (25ng total RNA) was analyzed at least in duplicates. Primers (Table 1) were ordered from Applied Biosystems (Life Technologies). Relative quantification was performed by normalizing the target genes signals with cyclophilin A signal (as reference gene), as determined by the NormFinder software (Andersen *et al.* 2004). The amplification efficiency was analyzed by the LinRegPCR programme (Ramakers *et al.* 2003), followed by Pfaffl's method (Pfaffl 2001).

Immunohistochemistry for *INSR*, *GLUT4*, *IL6* and *p65* and immunocytochemistry for *GLUT4*

Immunohistochemistry and immunocytochemistry were performed based on procedures described previously (Mariani *et al.* 2006). Sections (2 μ m thick) from two CL samples per dog, or cells grown over coverslips, analyzed. Primary antibodies are presented in Table 2. Negative controls were established by using the corresponding isotype control (normal rabbit IgG; Santa Cruz Biotechnology) or by replacing the primary antibody with

buffer in subsequent reactions. Mouse skeletal muscle was used as a positive control for *INSR* and *GLUT4*, and human carcinoma tissue was used as a positive control for *p65* and *IL6*.

Protein expression assessed by Western blotting

For total protein extraction, isolated cells and tissue samples were homogenized in NET-2 lysis buffer (50mM Tris-HCl (pH 7.4), 300mM NaCl and 0.05%

Figure 1

Glucose uptake, AKT, GLUT4 and GLUT1 analyses in canine luteal cells from 30-day p.o. corpora lutea, stimulated or not (basal) with insulin for 20 min. (A) Glucose uptake. (B) Protein expression of phosphorylated AKT (phospho-AKT) and total AKT. Immunocytochemistry for GLUT4 (C and D) and GLUT1 (E and F), before (C and E) and after (D and F) the addition of insulin. Positive signals were observed, as indicated by the brown colour in the luteal cells. Data are mean $\pm$ S.E.M. of 4 wells for glucose uptake and 3 wells for AKT per group. In each well, the cells are from CLs from different animals. Different letters represent significant difference ($P < 0.001$). Bars = 50 μ m. Inserts = negative controls.

NP-40) containing 1 µL/mL protease inhibitor cocktail (Sigma-Aldrich), and centrifuged at 10,000g for 10 min at 4°C. The supernatant was used as a total cellular extract. Nuclear protein extraction was prepared based on procedures described previously (Andrews & Faller 1991). The total protein concentration in the samples was determined by the Bradford method (Bradford 1976).

Whole cellular and nuclear extracts (25 or 30 µg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (HIF1A) and 12% (GLUT1, GLUT4, INSR, IL6 and p65) gels and electrophoretically transferred onto a nitrocellulose membrane (Fátima *et al.* 2013). Immunodetection was performed using the primary antibodies listed in Table 1, and peroxidase-labelled anti-rabbit, anti-mouse and anti-goat antibodies at 1:7500 dilution as secondary antibodies (Amersham Biosciences, GE Healthcare Life Science). The blots were visualized using an Enhanced Chemiluminescence (ECL) Kit (Amersham Biosciences, GE Healthcare Life Science) and images were captured by ChemiDoc MP Image system (Bio-Rad Laboratories). Beta-actin (ACTB) was used as loading control.

Cobalt chloride (CoCl₂) treatment

Luteal cells collected on days 10, 30 and 60 p.o. were treated for 24 h with 500 µM cobalt chloride (CoCl₂; Sigma-Aldrich). The concentration was chosen based on a preliminary dose-response curve. After the treatment, the medium was collected and stored at -20°C until progesterone determination by validated chemiluminescence immunoassay (Papa *et al.* 2014). Finally, the cells were processed for RNA or protein extraction as described above.

Figure 2

Gene expression of the insulin receptor (*INSR*) and the solute carrier 2A4 (*SLC2A4*) in canine luteal cells from 20-day (A) and 40-day (B) p.o. and treated with 100 nM insulin for 24 h. Relative expression (RGE) of target genes was normalized by cyclophilin A. Data are mean ± s.e.m. of cells from 4 CLs from different animals in each day. Asterisks indicate significant differences ($P < 0.05$).

Statistical analysis

All the experiments were performed at least 3 times with a minimum of three replicates. Data were tested according to their homogeneity and normality of variances, transformed to square to normalize the distributions and presented as mean ± s.e.m. Statistical differences between control and treated groups were calculated using unpaired *t*-test with Welch's correction. In each analysis, critical significance level was $P < 0.05$. All calculations were performed using the GraphPad Prism 5 programme (GraphPad Software).

Results

Glucose uptake by luteal cells under insulin stimulation

To investigate the role of insulin in the canine CL, we tested the capacity of luteal cells from 30-day p.o. CLs to uptake glucose in response to insulin. Glucose uptake was 2.7-fold increased after insulin stimulus (Fig. 1A), expressed by both micrograms of protein ($P < 0.01$) and number of cells ($P < 0.05$). Additionally, we verified that despite unchanged total AKT content, insulin increased ($P < 0.001$) the AKT phosphorylation (Fig. 1A and B). GLUT4 and GLUT1 staining revealed that both were present in luteal cells; however, only GLUT4 showed a qualitative signal increase in the presence of insulin (Fig. 1C and D).

Figure 3

INSR (A), *SLC2A4* (B), *IL6* (C) and *RELA* (D) mRNA expression in canine corpora lutea (CLs) from 10 to 60 days after the ovulation, determined by real-time (TaqMan) PCR. Relative expression (RGE) of target genes was normalized by cyclophilin A. Data are mean ± s.e.m. of 4 CLs from different animals. Bars with different letters represent significant differences ($P < 0.05$).

Effect of insulin on *INSR* and *SLC2A4* gene expression in luteal cells

After determining the ability of luteal cells to alter the insulin-stimulated glucose uptake, we investigated the effect of 24-h insulin treatment upon the insulin receptor (*INSR*) and the *SLC2A4* gene expression in luteal cells from 20-day and 40-day p.o. corpora lutea. The results show that in luteal cells from day 20 p.o. insulin increased the expression of *INSR* and *SLC2A4* (Fig. 2A), whereas in cells from day 40 p.o., the insulin effect was completely opposed (Fig. 2B).

INSR, *SLC2A4*, *IL6* and *RELA* mRNA expression in the CL throughout dioestrus

The mRNA expression of *INSR* (Fig. 3A) and *SLC2A4* (Fig. 3B) in luteal tissue was the lowest on day 40 p.o. ($P < 0.05$), a moment in which the *RELA* (Fig. 3D) expression was the highest. Besides, the highest expression of *INSR* and *SLC2A4* was observed on day 20 p.o., when

RELA expression was low. In general, the results show that *INSR* and *SLC2A4* expression were parallelly regulated throughout the dioestrus, and in opposition to that observed in the *RELA* expression. Finally, the highest expression of *IL6* (Fig. 3C) was concomitant with the highest levels of *INSR* and *SLC2A4* (20 p.o.); however, *IL6* expression in other moments of the dioestrus seems to not correlate with the *INSR* or *SLC2A4* expression.

INSR, GLUT4, IL6 and p65 protein expression in the CL throughout dioestrus

The *INSR*, GLUT4, IL6 and p65 proteins were detected by immunohistochemistry in luteal cells at all studied stages (Fig. 4). To verify whether the mRNA regulations observed in the canine CLs were accompanied by changes in the respective proteins, Western blotting analysis of the proteins was performed. *INSR* expression (Fig. 5A) was higher on day 10 p.o., which was also observed by

Figure 4

Immunohistochemical detection of *INSR*, GLUT4, IL6 and p65 in canine corpora lutea (CLs) from 10 to 60 days after ovulation. Positive *INSR*, GLUT4, IL6 and p65 signals were observed at all stages of CLs. Inserts = negative controls. Bars = 50 μ m.

Figure 5

Expression of INSR (A), GLUT4 (B), IL6 (C), total p65 (D) and nuclear p65 (E) proteins in canine corpora lutea (CLs) from 10 to 60 days after ovulation. In each panel, representative Western blotting images are shown. Results were to ACTB expression. Data are expressed as the mean $\pm$ S.E.M. of 4 CLs from different animals. Different letters indicate a significant difference ($P < 0.05$).

immunohistochemistry. GLUT4 expression (Fig. 5B) showed a peak on day 30 p.o., which was also observed by immunohistochemistry. However, these regulations were not in parallel with the mRNA regulation, indicating the occurrence of post-transcriptional modulations.

We also studied the proteins of the above-mentioned genes that are known to regulate GLUT4 expression either

Figure 6

Expression of *HIF1A*, *SLC2A1* and *SLC2A4* mRNAs (A) and *HIF1A*, *GLUT1* and *GLUT4* proteins (B) in canine luteal cells 24-h treated with 500 μ M CoCl_2 . Gene expression was analyzed by qPCR and proteins by Western blotting in a whole cellular homogenate. Relative expression (RGE) of target genes was normalized by cyclophilin A. Representative immunoblots are shown. B-actin (ACTB) was used as loading control. Data are mean $\pm$ S.E.M. of 12 plates from 4 different animals from days 10, 30 and 60 p.o. Different letters represent significant difference ($P < 0.001$).

Figure 7

Progesterone secretion (A) to the medium ($\text{ng} \cdot \mu\text{g}^{-1}$ de protein) and (B) *HSD3B* mRNA expression (B) by canine luteal cells 24-h treated with 500 μ M CoCl_2 . Data are mean $\pm$ S.E.M. of 12 plates from 4 different animals from days 10, 30 and 60 p.o. Asterisk represents significant difference ($P < 0.05$).

positively (IL6) or negatively (p65). Quantification of IL6 showed the highest expression at day 30 p.o., decreasing thereafter (Fig. 5C; $P < 0.05$); similar to that observed in the immunohistochemistry. We quantified p65 in a total cellular extract and nuclear samples of luteal cells (Fig. 5D and E), and observed that in both the p65 expression was highest on day 30 p.o. This high level of expression was followed by a decline on day 50 p.o. After day 50 p.o., nuclear p65 was undetectable by Western blotting.

Cobalt chloride stimulates *HIF1A/HIF1A*, *SLC2A1/GLUT1* and *SLC2A4/GLUT4* expression and inhibits progesterone production in canine luteal cells

It is well determined that CL physiologically undergoes hypoxia; thus, we investigated the effect of CoCl_2 on luteal cells obtained on days 10, 30 and 60 p.o. Since no effect of the day (10, 30, 60 p.o.) was observed after statistical analysis over P4 production and hydroxyl-delta-5-steroid dehydrogenase 3 beta (*HSD3B*) mRNA expression, we considered just the effect of treatment, that is, control cells and CoCl_2 -treated cells. As expected, CoCl_2 increased the expression of *HIF1A* mRNA and *HIF1A* protein, and that was accompanied by similar increase in the mRNA and protein expression of *SLC2A1/GLUT1* and *SLC2A4/GLUT4* (Fig. 6).

Additionally, 24-h CoCl_2 treatment of the luteal cells (Fig. 7) reduced both the P4 secreted to the medium, and the *HSD3B* mRNA expression, which codifies the key enzyme for P4 synthesis.

Discussion

To the best of authors' knowledge, this is the first study to present data that luteal cells are able to uptake glucose

under insulin stimulus in an AKT-mediated way, and followed by GLUT4 protein increase. In addition, expression of genes related to insulin biological effectiveness as *INSR*, *SLC2A4*, *IL6* and *RELA* has been characterized. Besides, the influence of mimicking low O₂ concentrations on the expression of *HIF1A*/*HIF1A*, *SLC2A1*/*GLUT1* and *SLC2A4*/*GLUT4* mRNAs and proteins, as well as on the *HSD3B* mRNA expression and P4 production throughout dioestrus was also analyzed.

GLUT4 expression, the limiting step of insulin-stimulated glucose uptake, was highest in the middle of the CL maintenance period (day 30 p.o.); thus, that moment was chosen to confirm the hypothesis that canine luteal cells are sensitive to insulin. In fact, insulin induced a 2.7-fold increase in the glucose uptake within 20 minutes, similar to that described in classical insulin-sensitive tissues such as adipose tissue and skeletal muscle (James *et al.* 1988, Klip & Pâquet 1990, Slot *et al.* 1991, Marette *et al.* 1992). Besides, in response to insulin, phospho-AKT was 4-fold increased, indicating a classic activation of insulin signalling, as described previously for insulin-induced glucose uptake by oocytes and cumulus cells (Purcell *et al.* 2012).

As P4 and E2 plasma levels variate throughout dioestrus, and E2 has been reported to be able to modulate *SLC2A4*/*GLUT4* expression, we tested the effect of insulin on *INSR* and *SLC2A4* genes' expression in luteal cells obtained from CLs harvested at the limits of the maintenance/secretory phase; that is at days 20 and 40 p.o., when the highest levels of P4 and E2, respectively, were observed. Interestingly, insulin increased the *SLC2A4* and *INSR* mRNAs on day 20, but decreased them on day 40 p.o., suggesting that the previous hormonal ambient of the CLs modulated the insulin effect.

SLC2A4 mRNA and GLUT4 protein have been shown to be regulated by E2, and that is differently modulated by the oestrogen receptors 1 (ESR1) and 2 (ESR2), as demonstrated in adipose and muscular tissues (Barros *et al.* 2009). Using knockout mice for ESR1 and ESR2, Barros and collaborators reported that ESR1 increases, whereas ESR2 represses the expression of GLUT4 (Barros *et al.* 2009). Using selective ESR1 and ESR2 agonists in 3T3L1 adipocytes, the enhancer effect of ESR1 and the repressor effect of ESR2 on *SLC2A4*/*GLUT4* expression were confirmed (Campello *et al.* 2012). Therefore, the effect of E2 upon *SLC2A4*/*GLUT4* expression may depend on the balance between ESR1 and ESR2. The expression of *ESR1* and *ESR2* in canine CL over dioestrus has been already described (Hoffmann *et al.* 2004, Papa & Hoffmann 2011). During the first half of dioestrus

(formation and maintenance/secretory phase, *ESR1* is more abundant decreasing in the second half (end of maintenance and regression phases). On the other hand, *ESR2* remains unchanged over dioestrus. Associating these findings to this study, we can infer that insulin is able to increase expression of *SLC2A4* on day 20 p.o. probably due to the predominant action of *ESR1*, despite low E2 levels (Papa *et al.* 2014). Conversely, on day 40 p.o. (end of the maintenance/secretory phase), high E2 levels and predominant action of *ESR2* contribute to the repressive effect of insulin on *SLC2A4* expression. Therefore, these data we reported for luteal cells regarding insulin effect on *SLC2A4* gene seem to be related to E2/*ESR1*/*ESR2* balance, similarly to those described in the adipose and muscle tissues.

Regarding the *INSR* gene regulation and gonadal hormones, E2 has already been proposed as enhancer of *INSR* mRNA expression in hypothalamus (Clegg *et al.* 2006), however, nothing is known about either its hormonal regulation in the female reproductive organs or the *ESR1/2* participation.

Comparing the effects of insulin in cultured luteal cells, similar regulations were observed directly in the CLs harvested throughout the dioestrus. *SLC2A4* and *INSR* mRNAs were highest at day 20, and lowest at day 40, suggesting that endogenous insulin is playing the same role that was observed *in vitro* in luteal cells, and in accordance with the *ESR1*/*ESR2* balance.

Regulation of *SLC2A4* expression is dependent on the binding activity of nuclear factor kappa B (NFkB), mainly the isoforms NFkB1 and *RELA*, to the promoter region of *SLC2A4* in muscle and adipose tissues. Besides, NFkB-binding activity also participates on the *ESR1*- and *ESR2*-mediated effects of E2 in adipocytes (Campello *et al.* 2012). Not surprisingly, when evaluating *RELA* expression in canine CL, we found an upregulation of this gene and respective protein on day 40 p.o., thus suggesting the same regulation of *SLC2A4* mRNA that has been described for NFkB in adipose and skeletal muscle tissues or cells (Silva *et al.* 2005, Campello *et al.* 2012, Furuya *et al.* 2013).

Although some studies have demonstrated a negative effect of IL6 on *SLC2A4*/*GLUT4* expression in adipocytes (Lagathu *et al.* 2003, Poletto *et al.* 2015), IL6-induced enhancer effect on *SLC2A4*/*GLUT4* expression was reported in skeletal muscle cells (Al-Khalili *et al.* 2006), indicating that IL6 regulations may vary accordingly to the cell type. Our data support the hypothesis that in luteal cells, IL6 plays an enhancer effect on *SLC2A4*/*GLUT4* during formation/maintenance phases of dioestrus, as the upregulation of IL6 mRNA and protein

were highest in the days 20 and 30 p.o., respectively. Besides, we must consider that the variations in IL6 production observed in CLs may be participating on the regulation of ESR1/ESR2 expression, as it has already been reported in a cell line A2780 from human ovarian carcinoma (Wang *et al.* 2014).

Hypoxia can be considered a physiological state for luteal cells (Nishimura & Okuda 2015) and highly influences the cellular homeostasis. Cells increase HIF1A (hypoxia-inducible factor 1A) expression as the oxygen tension decreases (Tarhonskaya *et al.* 2015), and the canine CL response to hypoxia and HIF1A expression has been correlated to increased *SLC2A1*/GLUT1 expression (Papa *et al.* 2014). The influence of HIF1A in *SLC2A4*/GLUT4 expression has also been described in skeletal muscle cells (Silva *et al.* 2005, Lima *et al.* 2009), in which reduced oxygen tension activates HIF1A and enhances *SLC2A4* transcription. This work showed that *SLC2A4* expression parallels that of *SLC2A1* in canine CL (Papa *et al.* 2014) over dioestrus, and cells subjected to CoCl₂ increased expression of *HIF1A*, *SLC2A1* and *SLC2A4* mRNAs and respective proteins. In fact, HIF1A is able to bind to an E-box domain present in the promoter region of *SLC2A4* gene, and once activated by reduction in intracellular oxygen tension, as in response to contraction of skeletal muscle, its binding activity increases enhancing *SLC2A4* transcription (Lima *et al.* 2009). Thus, strong regulations observed in GLUT1 and GLUT4 expression, as well as in glucose uptake in CL, seem to be highly related to cellular oxygen disposal throughout the dioestrus.

Altogether, our results point towards the ability of luteal cells to uptake glucose in response to insulin stimulus, which includes insulin in the list of luteotrophic factors. Additionally, the data revealed that INSR and GLUT4 expression in luteal cells varies according to the phase of CL lifespan and that can be related to IL6 and RELA/p65 expression. Besides, the study demonstrates that CoCl₂ enhances *SLC2A4* and *HIF1A* mRNA expression in luteal cells, indicating that hypoxia plays an important role in the regulation of *SLC2A4*/GLUT4 expression during the development of CL.

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.

Funding

This work was supported by FAPESP (grant numbers 2008/54835-8, 2010/07373-9 and 2011/17768-3) and CAPES (025/2011).

Author contribution statement

All authors participated in the experimental design, data analysis and interpretation, and revised the manuscript for intellectual content. L M M C S performed canine luteal cell culture, CoCl₂ experiments and wrote part of the manuscript. R S S performed canine luteal cell culture, insulin stimulation experiments and wrote part of the manuscript. R M L and T S D V performed ovariectomies and C L sample collection. A B A-V performed glucose uptake analysis in cultured canine luteal cells. U F M critically read the manuscript and participated in very fruitful discussions. P C P conceived the studies, performed IL6 and RELA/p65 analyses and wrote the manuscript.

Acknowledgements

The authors thank Dr Ivana Carvalho from CEPRA NGO and the Zoonosis Control Center of Guarulhos for providing access to the dogs and keeping them in nice atmosphere. We thank all dogs' guardians, who agreed to dispose ovarian tissue for this study.

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Received in final form 3 September 2016

Accepted 27 September 2016

Accepted Preprint published online 27 September 2016