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Apoptotic germ cells regulate Sertoli cell lipid storage and fatty acid oxidation

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Abstract

The presence of lipid droplets (LD) and the utilization of fatty acids (FA) as a source of energy are Sertoli cell (SC) putative characteristics. It is well known that SCs can phagocyte and degrade apoptotic germ cells (AGC) resulting in increasing lipid content and ATP levels. A relationship between the regulation of lipid storage and of lipid oxidation in SC might be envisaged. The aim of this study was to analyze whether AGC and FA are able to simultaneously regulate molecular mechanisms involved in lipid storage and in FA oxidation in SC. The experimental model utilized in this study consisted in SC cultures obtained from 20-day-old rats that were co-cultured with AGC or treated with palmitic acid (PA, 500 µM) for 24 and 48 h. AGC and PA increase LD, triacylglycerol (TAG) content and mRNA levels of *Plin1*, *Plin2*, *Plin3* (proteins involved in TAG storage). Simultaneously, AGC and PA rise the extent of FA oxidation and mRNA levels of *Cpt1* and *Lcad* (proteins involved in FA degradation). Results also show that peroxisome proliferator-activated receptor (PPAR) transcriptional activity, transcription factor which participate in lipid metabolism regulation, increases by AGC and PA treatment in SC. Additionally, the presence of a PPARg antagonist decreases the upregulation of LD content and *Plin1* expression. Similarly, the presence of a PPARb/d antagonist reduces the increase in FA oxidation and *Cpt1* mRNA levels. Altogether these results suggest that AGC and FA, which probably generate PPAR ligands, regulate lipid storage and fatty acid utilization, contributing to the energy homeostasis in the seminiferous tubules.

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Introduction

The process of spermatogenesis and consequently male fertility are dependent upon the somatic cells that are present in the testis. Leydig cells are essential because of androgen production, and Sertoli cells (SCs) are absolutely required in order to provide an adequate and protected environment within the seminiferous tubules. SC carbohydrate metabolism presents some interesting characteristics. Glucose is metabolized and converted into lactate since spermatocytes and spermatids rely on this hydroxyacid for energy provision (Jutte *et al.* 1982). Hence, SCs do not use glucose as an essential source of energy; in fact, it has been shown that this cell type can survive in culture for at least 48 h in the absence of this carbohydrate (Riera *et al.* 2009). Additionally, it has been demonstrated that SCs present numerous lipid droplets (LD) and that the oxidation of fatty acids (FA) can yield much of the energy required by these cells (Kerr *et al.* 1984, Jutte *et al.* 1985, Xiong *et al.* 2009). It may be assumed that the LD constitute the storage of FA for SC energy demands and that the mechanisms involved in SC lipid storage and utilization might be somehow related.

It is well recognized that in the seminiferous tubules under physiological conditions, more than 75% of developing germ cells undergo apoptosis before completing the spermatogenic process (Huckins 1978). Additionally, it is known that during the spermiation process, cytoplasmic portions of elongated spermatids are shed and form residual bodies (Kerr & de Kretser 1974). It had been suggested that the LD within SCs are the result of breakdown products of phagocyted residual bodies and AGC (Kerr *et al.* 1984). A few years later, it was shown that SCs phagocyte and degrade AGC and residual bodies (Pineau *et al.* 1991) and there exists a temporal relationship between phagocytosis of residual bodies and increased number of LD (Ueno & Mori 1990). Additional *in vivo* studies utilizing different experimental models support the idea that a relationship between apoptosis of germ cells and lipid droplet content in SCs exists. Liu *et al.* (2012) demonstrated that transient scrotal hyperthermia induces lipid droplet accumulation in murine testes. The authors hypothesized that SCs phagocytose, degrade apoptotic cells and store residual lipids within LD. Furland *et al.* (2011) showed similar results after heat exposure of the testis. In addition, it has been shown that other testicular injuries such as

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irradiation (Abreu & David-Ferreira 1982) and vitamin E deficiency (Bensoussan *et al.* 1998) also induce lipid droplet accumulation in SCs. More recently, Xiong *et al.* (2009) analyzed the existing correlation among apoptosis of spermatogenic cells, lipid droplet formation and ATP production in seminiferous tubules in busulfan-treated animals. They demonstrated that there is a temporal relationship between germ cell apoptosis and increased lipid droplet formation in SCs and ATP level in seminiferous tubules. In addition, these authors presented evidence suggesting that phagocytized AGC constitute the source of FA that are utilized to generate ATP in SCs. In all the above-mentioned studies, the molecular mechanisms that may be involved in the observed effects have not been analyzed.

Peroxisome proliferator-activated receptor (PPAR), a (NR1C1), b/d (NR1C2) and g (NR1C3), are ligand-activated transcription factors that are members of the nuclear-hormone receptor superfamily (Issemann & Green 1990, Michalik *et al.* 2006). All PPARs operate as sensors of FA and FA derivatives and thus control metabolic pathways involved in lipid and energy metabolism (Green & Wahli 1994, Krey *et al.* 1997). There is a general agreement in that PPARa and PPARb/d regulate lipid catabolism while PPARg regulates lipid anabolism (Chawla *et al.* 1994, Wang *et al.* 2003). Results of our laboratory have shown that in SCs, pharmacological activation of PPARa and PPARb/d regulates the expression of genes, which proteins are involved in FA transport and catabolism. More precisely, the regulation of fatty acid transporter *Fat/cd36*, carnitine palmitoyl transferase 1 (*Cpt1*) – responsible for the entrance of acyl-CoA into the mitochondria and the rate-limiting step of mitochondrial b-oxidation (Rasmussen & Wolfe 1999) –, long-chain and medium-chain acyl-CoA dehydrogenases (*Lcad* and *Mcad* respectively), was demonstrated (Regueira *et al.* 2014). We have also shown that in SCs, pharmacological activation of PPARg regulates the expression of genes which proteins are related to FA transport and to lipid synthesis and storage, such as *Fat/cd36*, glycerol-3-phosphate acyltransferase (*Gpat*), diacylglycerol acyltransferase (*Dgat*) and perilipins (*Plins*) – proteins of the surface of LD (Gorga *et al.* 2017). The physiological activators of PPARs that may be involved in the regulation of lipid metabolism in the seminiferous tubules have not been clarified yet. Considering the above-mentioned information, a plausible hypothesis is that FA can alternately act as energetic substrate and as endogenous ligands for PPARs, whose activation participates in the regulation of lipid metabolism.

The aim of the present study was to analyze whether apoptotic germ cells (AGC) and FA are able to regulate molecular mechanisms involved in lipid storage and in FA oxidation in SCs.

Materials and methods

Materials

[9,10-^{3}H(N)]-PA was purchased from New England Nuclear Co. (Boston, MA, USA). Culture medium and all other drugs and reagents were purchased from Sigma-Aldrich. Sprague–Dawley rats were housed and used according to the guidelines recommended by the National Institute of Health and approved by the Institutional Ethical Committee – Comité Insititucional de Cuidados y Uso de Animales de Laboratorio (CICUAL) – Hospital de Niños 'Dr. Ricardo Gutierrez' N°2018-001.

SC isolation and culture

SCs from 20-day-old Sprague–Dawley rats were isolated as previously described (Meroni *et al.* 2002). Briefly, decapsulated testes were digested with 0.1% collagenase and 0.006% soybean trypsin inhibitor in Hanks' balanced salt solution for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine–2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germinal cells. The SC suspension, collected by sedimentation, was resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 20 mM HEPES, 100 IU/mL penicillin, 2.5 µg/mL amphotericin B, 1.2 mg/mL sodium bicarbonate, 10 µg/mL transferrin, 5 µg/mL insulin, 5 µg/mL vitamin E and 4 ng/mL hydrocortisone. SCs were cultured in 25 cm^2 flasks, 6-, 24-multiwell plates or 8-well chamber Permanox slides (5 µg DNA/cm^2) at 34°C in a mixture of 5% CO_2:95% air.

No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to SC cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture, as examined by phase contrast microscopy.

Germ cells isolation and apoptotic germ cell preparation

Germ cells were isolated as previously described (Galardo *et al.* 2014). Testes from 30-day-old rats were decapsulated and digested with 0.1% collagenase (C0130 Sigma-Aldrich) and 0.006% soybean trypsin inhibitor (T9003 Sigma-Aldrich) in Hanks' balanced salt solution (HBSS) for 5 min at room temperature. The collagenase solution was diluted four-fold with HBBS and seminiferous tubules allowed to sediment for 2 min. The supernatant was discarded and the tubular pellet was washed twice with gentle shaking. Seminiferous tubules were cut into 2 mm segments and then digested with 0.05% collagenase, 0.003% soybean trypsin inhibitor and 0.003% DNA (DN25, Sigma-Aldrich) for 15 min at room temperature, while carefully transferring the suspension from one tube to another with a pipette. The suspension was diluted with one volume HBSS and material allowed to sediment for 5 min. The supernatant was transferred to a tube containing sufficient 2% bovine serum albumin (BSA) to make the final concentration

0.2% BSA. The suspension was allowed to settle for 10 min. Germ cells remaining in suspension were collected by centrifugation at 400 ***g*** for 3 min at 4°C. The resulting pellet was washed twice with HBSS containing 0.2% BSA and 0.003% DNA. The final cell pellet was resuspended in a 1:1 mixture of Dulbecco's Modified Eagle's Medium-Ham's F-12 Medium with the addition of 15 mM $NaHCO_3$, 100 IU/mL penicillin, 2.5 mg/mL amphotericin B, 20 mM Hepes, pH 7.4 (DMEM-F12) and seeded on a discontinuous four-layer (20, 25, 32, 37%) Percoll density gradient. The gradient was centrifuged at 800 ***g*** for 30 min at 4°C. The fractions at the 25–32% interface were collected. To remove Percoll, four volumes of DMEM-F12 were added and centrifugation at 400 ***g*** for 5 min at 4°C was performed. Germ cells were resuspended in DMEM-F12 supplemented with 10 mg/mL transferrin, 5 mg/mL insulin, 5 mg/mL vitamin E and 4 ng/mL hydrocortisone. Germ cell preparations were seeded at a density of 2×10^6 cell/mL in tissue culture flasks and cultured at 34°C in a mixture of 5% CO_2:95% air for 18 h. During this initial period, the few SCs contaminating the germ cell preparation were attached to the plastic surface. Purified germ cells were obtained by carefully removing the medium and centrifuging at 400 ***g*** for 5 min at 4°C, and resuspended in Minimum Essential Media supplemented with 15 mM $NaHCO_3$, 100 IU/mL penicillin, 2.5 mg/mL amphotericin B, 20 mM Na Hepes, pH 7.4, 10 mg/mL transferrin and 4 ng/mL hydrocortisone. In order to characterize the germ cell types present in the suspension, the preparation was evaluated by measuring the DNA content as previously described (Galardo *et al.* 2014). Briefly, cells were resuspended in DMEM:F12 supplemented with 50% fetal bovine serum and fixed in ice-cold 70% ethanol. Propidium iodide was added to fixed cells to a final concentration of 50 mg/mL. Flow cytometry was performed using a FACS Caliber (Becton Dickinson). The preparation contained 27% tetraploid cells (spermatocytes) and 63% haploid cells (spermatids) (Fig. 1A).

AGC were prepared as previously described (Xiong *et al.* 2009). Briefly, germ cells were seeded in six-multiwell plates in a density 5×10^5 cells/cm^2 and cultured at 34°C in a mixture of 5% CO_2:95% air for 48 h. After this incubation, germ cells were collected by centrifuging at 400 ***g*** for 5 min at 4°C and resuspended in an adequate volume of culture medium. In order to evaluate apoptosis, the acridine orange/ethidium bromide 4 µg/mL (1:1) staining assay was utilized (Liu *et al.* 2015). The presence of AGC was determined in freshly isolated cells and in cells cultured for 48 h. No apoptotic germ cells were observed in freshly isolated cells while all germ cells were apoptotic after 48 h in culture (AGC).

Culture conditions

SCs were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. SCs were cultured under basal (Basal) conditions or co-cultured with AGC (5×10^5 cells/cm^2) for 24 or 48 h. After the co-culture period, AGC were removed by gentle aspiration. Subsequently, and in order to eliminate the AGC that remained attached to SC monolayers, SC cultures were treated with hypotonic solution (20 mM Tris/HCl, pH 7·4) for 2 min (Riera *et al.* 2002). In parallel cultures, SCs were treated with 1% BSA (Control) or with PA (500 µM/1% BSA) for 24 or 48 h. SC monolayers either co-cultured with AGC or treated with PA were utilized to determine: number of LD, TAG content, FA oxidation, mRNA levels of *Fat/cd36*, *Gpat1*, *Dgat1*, *Plin1-4*, *Cpt1*, *Lcad*, *Mcad*, *Ppara*, *Pparb/d and Pparg*. Additionally, SC cultures were co-cultured with AGC or treated with PA in the absence or presence of 50 µM T0070907 (T007) – PPARg antagonist – or 20 µM GSK3787 (GSK) – PPARb/d antagonist – for 48 h. After these treatments, LD content, FA oxidation, *Plin1* and *Cpt1* mRNA levels were evaluated in SC monolayers.

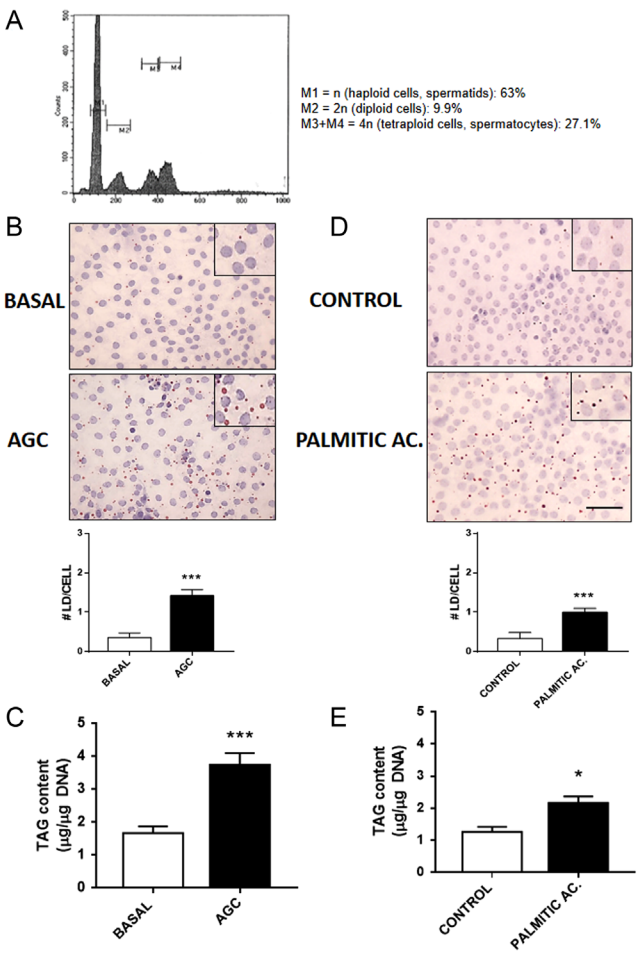

Figure 1 Effects of AGC and palmitic acid on LD formation and TAG content in Sertoli cell. (A) Purity of germ cell isolation. Isolated germ cells were analyzed to determine DNA content by flow cytometry using a FACS Caliber (Becton Dickinson). Results are expressed as % of total isolated cells. (B, C, D and E) Sertoli cells were co-cultured for 48 h with AGC (5×10^5 cell/cm^2) or incubated with palmitic acid (500 µM/1% BSA). (B and D) Upper panels: microphotographs of Sertoli cell monolayers stained with ORO. Inset: magnification of the microphotographs. Bar = 50 µm. Lower panels: LD content in Sertoli cell monolayers. Values represent mean ± S.D. of number of LD per Sertoli cell in one representative experiment out of three. ***$P < 0.001$ vs Basal or Control (1% BSA). (C and E) TAG levels were determined in cell lysates and expressed as µgTAG/µg DNA (means ± S.D.) $n = 3$. ***$P < 0.001$ vs Basal; *$P < 0.05$ vs Control (1% BSA).

Cell viability tests performed after a 48-h incubation period showed that none of the experimental conditions tested modify cell viability (data not shown).

Real-time quantitative PCR (RT-qPCR)

Purified SCs were utilized to isolate total RNA using TRI Reagent (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of RNA was estimated by spectrophotometry at 260 nm. Reverse transcription (RT) was performed on 2 µg RNA at 42°C for 50 min with a mixture containing 200U MMLV reverse transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix (Invitrogen).

RT-qPCR was performed by a Step One Real Time PCR System (Applied Biosystems). The specific primers for RT-qPCR are listed in Table 1. Amplification was carried out as recommended by the manufacturer: 25 µL reaction mixture containing 12.5 µL of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µL of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to *Hprt1*. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.

ORO staining

The LD were visualized by Oil Red O staining (ORO) (Wang *et al.* 2006). Briefly, SCs cultured in eight-well chamber Permanox slides were fixed with 10% formalin for 1 h and stained with ORO solution (Sigma, ORO saturated solution in isopropanol:water, 3:2) for 30 min. First, the background was removed by washing the cells with water for 1 min, and then hematoxylin staining was performed. The LD present in SCs were analyzed under an Eclipse 50i microscope with a DS Fi1 digital camera and NIS Elements version 3.0 BR, imaging software (Nikon Instruments Inc.).The ratio LD to cell nucleus was calculated utilizing ImageJ-NIH software. Two hundred SCs were analyzed in each experimental condition performed in quadruplicate. Results were expressed as number of lipid droplet per cell (mean ± S.D.).

Triacylglyceride measurement

Cells cultured in 25 cm^2 flasks were washed twice with phosphate-buffered saline (PBS) at room temperature. SC monolayers were then treated with trypsin 0.05% w/v and EDTA 0.02% w/v in PBS, pH 7.4 for 2 min at 34°C until cells were detached. Trypsin action was stopped by the addition of 0.3% soybean trypsin inhibitor in PBS pH 7.4. Cells collected by centrifugation at 800 ***g*** for 5 min were resuspended in PBS pH 7.4 and homogenized by ultrasonic irradiation. Adequate aliquots were destined for triacylglyceride (TAG) and DNA determinations. TAG was measured by a routinely used method based on the colorimetric determination of the glycerol released upon the action of lipoproteinlipase on the triacylglycerides (TG Color, GPO/PAP AA; Wiener Lab, Rosario, Argentina). Results were expressed as µg TAG/µg DNA.

Fatty acid oxidation assay

Fatty acid oxidation was performed measuring the release of ^3H_2O to the incubation medium from [^{3}H]-palmitate. Briefly, after the experimental conditions, SCs cultured in 24-multiwell plates were incubated for 4 h at 34°C with culture medium (500 µL/well) containing 1% fatty acid free BSA, 2 µCi/mL of [9,10(n)-^{3}H]-PA, 10 µM PA and 0.25 mM L-carnitine. Triplicate incubations were performed. After the incubation period, medium was collected and treated on ice for 30 min with an equal volume of 10% trichloroacetic acid. Thereafter a centrifugation at 13,000 ***g*** for 5 min was performed and the supernatant recovered and treated with five volumes of methanol:chloroform (2:1) and two volumes of KCl:HCl 2 M. Phases were separated by centrifugation at 3000 ***g*** for 5 min. An adequate aliquot of the aqueous phase was taken and counted in a liquid scintillation spectrophotometer. Results are expressed as pmol of palmitic acid/h/µg DNA.

Table 1 Primers for RT-qPCR.

	Forward	Reverse
Fat/cd36	5′-ACCAGGCCACATAGAAAGCA-3′	5′-CACCAATAACGGCTCCAGTAA-3′
Gpat1	5′-TGCCCAATTCATCAGAATAC-3′	5′-TGGGATACTGGGGTTGAAAA-3′
Dgat1	5′-TTCATCTTTGCTCCTACTTTGTG-3′	5′-GTGAAAAAGAGCATCTCAAG-3′
Plin1	5′-GATCTGGGATTCTGCTTTGC-3′	5′-CTGGAGCACATTCTCCTGTTC-3′
Plin2	5′-TCTGAACCAGCCAACATCTG-3′	5′-CACCACCCCTGAGACTGTG-3′
Plin3	5′-TAGCCTGATGGAATCTGTGAAAC-3′	5′-GCTTAGATGGGTCCTTTTC-3′
Plin4	5′-TACATCCTTTGTGGAGCACTTAG-3′	5′-GGGGTCTACTGCTGTTTGTA-3′
Cpt1	5′-GGAACTCAAACCCATTCGTC-3′	5′-GTTGGATGGTGTCTGTCTCT-3′
Lcad	5′-AAAGGTCTGGGAGTGATTGG-3′	5′-CCATTCTCCACCAAAAAGAGG-3′
Mcad	5′-CGAGCACAACACACAAAACC-3′	5′-TTCCTCTCTGGCAAACTTCC-3′
Ppara	5′-TGTCGAATATGTGGGGACAA-3′	5′-TCTTCGAGCTTCGATCACAC-3′
Pparb/d	5′-GCACATCTACAATGCCTACCTG-3′	5′-TGATGAAGGGTGCGTTATGG-3′
Pparg	5′-TCCTCCTGTTGACCCAGAGC-3′	5′-CATCACAGAGAGGTCCACAG-3′
Hprt1	5′-AGTTCTTTGCTGACCTGCTG-3′	5′-TTTATGTCCCCCGTTGACTG-3′

Specific primers utilized for RT-qPCR of the following genes: *Fat/cd36*, *Gpat1*, *Dgat1*, *Plin1-4*, *Cpt1*, *Lcad*, *Mcad*, *Ppara*, *Pparb/d*, *Pparg*, *Hprt1*.

Transfection and luciferase reporter assay

SCs were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Afterward transfection was performed with the plasmids pGL3PPREx3TK-LUC or pGL3-Basic (Promega Corporation) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The plasmid pMIR-REPORT (Promega Corporation) expressing the *β-galactosidase* gene was used for normalization. The medium was replaced 24 h after transfection, and cells were co-cultured with AGC or treated with PA for 24 h. Then, cells were washed with PBS and total lysates prepared using passive lysis buffer. Samples were processed with the Dual-Luciferase Reporter system (Promega Corporation) and luciferase activity measured using Junior LB9509 luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activity was normalized to β-galactosidase activity and expressed as arbitrary units.

Other assays

DNA was determined by the method of Labarca and Paigen (1980). A cell viability test was performed in cells cultured on 96-multiwell using a commercial kit (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega Corporation).

Statistical analysis

All experiments were run in triplicates and repeated 3–4 times. One-way ANOVA and *post hoc* analysis using Tukey–Krämer's multiple comparisons test were performed using GraphPad InSat version 6.00 (GraphPad Software). *P* values <0.05 were considered statistically significant.

Results

AGC and PA regulate the number of LD and TAG content in SCs

As previously shown by Xiong *et al.* (2009), we observed that co-culture with AGC increases the amount of LD in SCs (Fig. 1B). As a consequence of this observation, we decided to evaluate if the increase in the number of LD was accompanied by an increase in lipid content. TAG content after SC co-culture for 48 h with AGC (5×10^5 cell/cm^2) was determined. Figure 1C shows that AGC increased TAG content in SCs.

In order to evaluate the participation of FA in the regulation of lipid metabolism, PA was selected. The rationale to select the latter FA is based on the fact that it is the most abundant FA in SCs, in phospholipids and TAG of germ cells and of residual bodies (Hurtado de Catalfo & Gómez Dumm 2005, Oresti *et al.* 2013). SCs were incubated for 48 h with 500 µM PA and LD number and TAG content were evaluated. Figure 1D and E show that, similar to what has been observed in the co-culture with AGC, incubations with PA increased LD number and TAG content in SCs.

AGC and PA regulate the expression of genes involved in FA transport and TAG storage in SCs

In order to evaluate possible molecular mechanisms that may be responsible for the observed effects of the co-culture with AGC and the treatment with PA on the number of LD and in TAG content, we decided to determine the mRNA levels of genes which proteins participate in FA transport, TAG synthesis and storage. Namely, *Fat/cd36*, *Gpat1*, *Dgat1* and *Plin1-4* were evaluated. Figure 2A shows that co-culture of SCs with AGC increased *Fat/cd36* mRNA levels in a 48-h co-culture period, whereas Fig. 2B and C show that it did not modify *Gpat1* and *Dgat1* expression at any time tested. Figure 2D shows that PA inhibited *Fat/cd36* expression while it did not modify *Gpat1* and *Dgat1* mRNA levels at any time evaluated (Fig. 2E and F). In addition, Fig. 3 shows that after 48 h of co-culture with AGC, SCs increased the expression of *Plin1-3* (Fig. 3A, B and C). Figure 3 also shows that incubation with PA increased *Plin1* mRNA levels after 24 and 48 h (Fig. 3E)

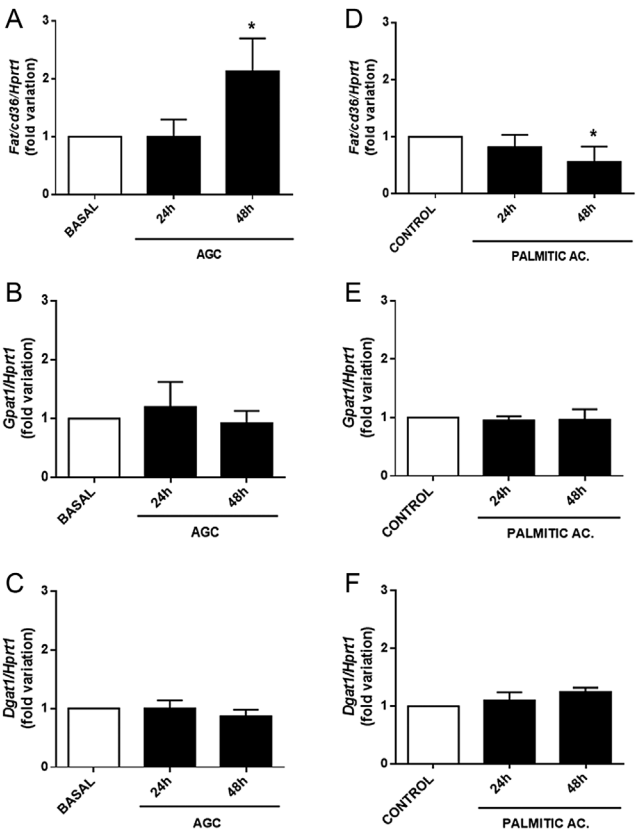

Figure 2 Effects of AGC and palmitic acid on *Fat/cd36*, *Gpat1* and *Dgat1* mRNA levels in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) (A, B and C) or treated with palmitic acid (500 µM/1% BSA) (D, E and F) for variable periods of time (24 and 48 h). Total cellular RNA was then extracted and RT-qPCR was performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. $n = 3$, *$P < 0.05$ vs Basal or Control (1% BSA).

and *Plin2*, *Plin3* mRNA levels after 48 h of incubation (Fig. 3F and G). Neither the co-culture with AGC nor the incubation with PA resulted in the modification of *Plin4* mRNA levels (Fig. 3D and H).

AGC and PA regulate FA oxidation in SCs

As mentioned in the introduction, it has been shown that phagocytosis of AGC increases the amount of LD, and it has been suggested that breakdown products of LD provide FA that are utilized to produce ATP in SCs. Based on these previous data, we decided to evaluate a possible regulation of FA oxidation by either co-culture with AGC or by incubation with PA. Figure 4 shows that FA oxidation increased after 48 h of co-culture with AGC and after 24 and 48 h cultures with 500 µM PA (Fig. 4A and B).

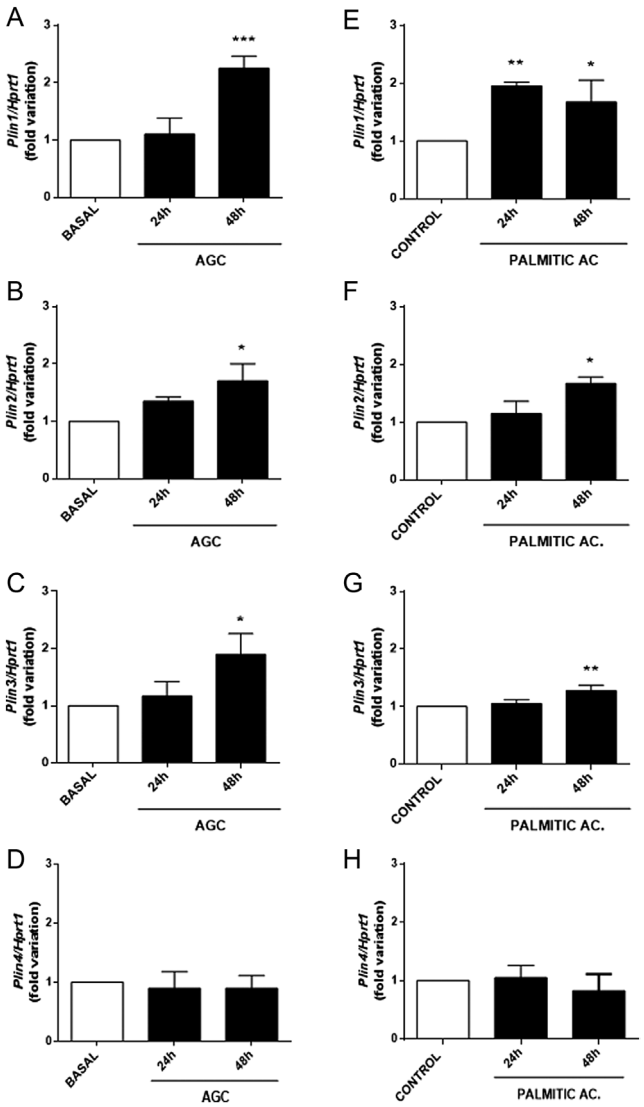

Figure 3 Effects of AGC and palmitic acid on Plins mRNA levels in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) (A, B, C and D) or treated with palmitic acid (500 µM/1% BSA) (E, F, G and H) for variable periods of time (24 and 48 h). Total cellular RNA was then extracted and RT-qPCR was performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. $n = 3$, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs Basal or Control (1% BSA).

AGC and PA regulate the expression of genes involved in FA oxidation in SCs

With the aim to determine if the observed increase in FA oxidation could be accounted for by the regulation of expression of genes which proteins are related to FA catabolism, a possible modification in *Cpt1*, *Lcad* and *Mcad* mRNA levels in the presence of AGC and of PA was evaluated.

Figure 5 shows that co-culture with AGC increased *Cpt1* and *Lcad* expression after 48 h of co-culture (Fig. 5A and B), while it did not modify *Mcad* mRNA levels at any time tested (Fig. 5C). Figure 5 also shows results obtained under PA treatment. PA increased *Cpt1* gene expression in a 48-h incubation period (Fig. 5D), *Lcad* expression in a 24-h incubation period (Fig. 5E) and did not modify *Mcad* expression at any time tested (Fig. 5F).

AGC and PA regulate PPAR transcriptional activity in SCs

Considering that we have recently observed that PPARa, PPARb/d and PPARg pharmacological activation has a relevant role in the regulation of lipid metabolism in the seminiferous tubule (Regueira *et al.* 2014, 2015, Gorga *et al.* 2017), we designed a set of experiments in order to determine a possible regulation of *Ppara*, *Pparb/d* and *Pparg* gene expression and of PPARs transcriptional activity as a result of co-culture with AGC and of PA treatment.

Figure 6A, B, C and E, F, G show that neither the co-culture with AGC nor the treatment with PA altered mRNA levels of *Ppara*, *Pparb/d* and *Pparg*.

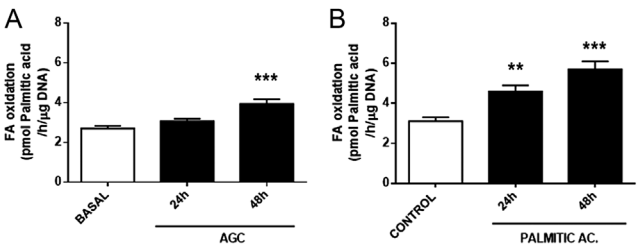

Figure 4 Effects of AGC and palmitic acid on fatty acid oxidation levels in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) or treated with palmitic acid (500 µM/1% BSA) (A or B respectively) for variable periods of time (24 and 48 h). Fatty acid oxidation was assessed by measuring ^3H_2O produced in the incubation medium. Results are expressed as pmol of palmitic acid/h/µg DNA (means ± S.D. $n = 3$. $^{**}P < 0.01$; $^{***}P < 0.001$ vs Basal or Control (1% BSA)).

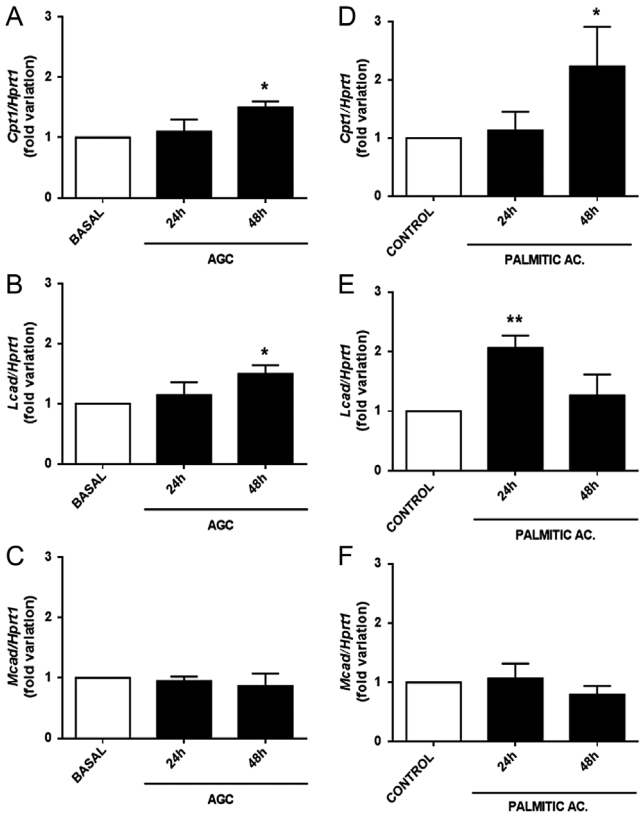

Figure 5 Effects of AGC and palmitic acid on *Cpt1*, *Lcad* and *Mcad* mRNA levels in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) (A, B and C) or treated with palmitic acid (500 µM/1% BSA) (D, E and F) for variable periods of time (24 and 48 h). Total cellular RNA was then extracted and RT-qPCR was performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. $n = 3$, $^*P < 0.05$; $^{**}P < 0.01$ vs Basal or Control (1% BSA).

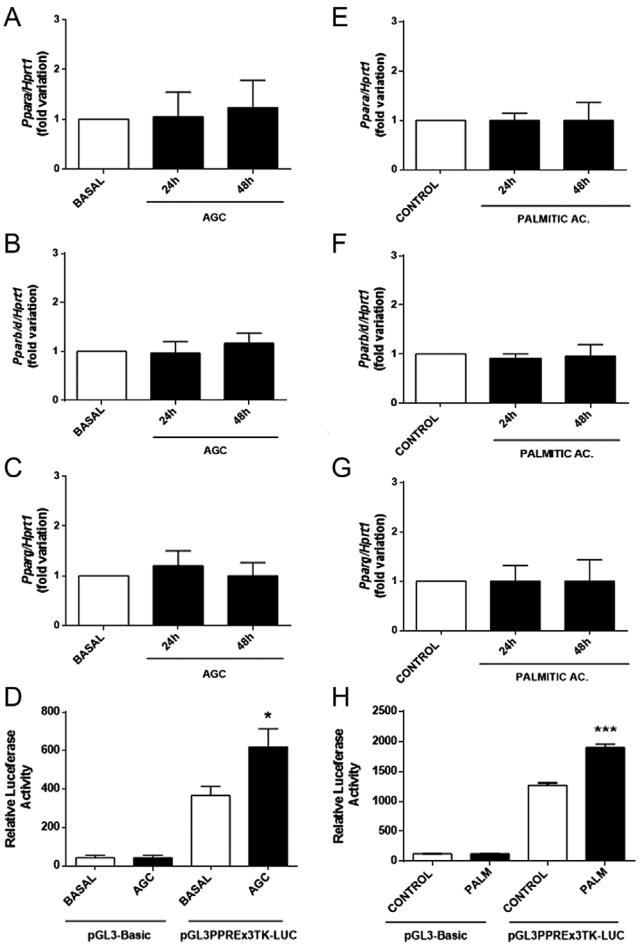

Figure 6 Effects of AGC and palmitic acid on *Ppars* mRNA levels and transcriptional activity in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) or treated with palmitic acid (500 µM/1% BSA) for variable periods of time (24 and 48 h). (A, B, C and E, F, G) Total cellular RNA was extracted. RT-qPCR was performed to analyze *Ppars* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. $n = 3$. (D and H) Sertoli cells transiently transfected with pGL3PPREx3TKpLUC/pMIR-REPORT or pGL3-Basic/pMIR-REPORT were co-cultured with AGC (5×10^5 cell/cm^2) or treated with palmitic acid (500 µM/1% BSA) for 24 h. Results are expressed as Relative Light Units for Firefly luciferase activity normalized to b-galactosidase activity. Data represent the means ± S.D. of triplicate incubations in one representative experiment out of three. $^*P < 0.05$; $^{***}P < 0.001$ vs Basal or Control (1% BSA).

In order to analyze the possible regulation of PPARs transcriptional activity, a plasmid containing three PPAR response elements (PPRE) was utilized. Figure 6D shows that SCs co-cultured with ACG raised luciferase activity, and Fig. 6H shows that SCs treated with PA also increased luciferase activity, which means an increase in the transcriptional activity of PPARs.

PPARg involvement in the regulation by AGC and PA of TAG storage

Next, we explored the participation of PPARg in the upregulation of the number of LD and the expression of *Plin1* – relevant gene in TAG storage – exerted by AGC and by PA. To achieve this goal, SCs were co-cultured with AGC or treated with PA in the absence or presence of 50 µM T007 – PPARg pharmacological antagonist. Figure 7A and C show that the upregulation of LD content in response to the co-culture with AGC or to PA treatment was diminished in the presence of T007. Similarly, Fig. 7B and D show that the upregulation of *Plin1* mRNA levels in response to the co-culture with AGC or to PA treatment was reduced in the presence of T007.

PPARb/d involvement in the regulation by AGC and PA of FA oxidation

Finally, the participation of PPARb/d in the upregulation of FA oxidation and in the expression of *Cpt1* – relevant gene for FA oxidation – was explored. SCs were co-cultured with AGC or treated with PA in the absence

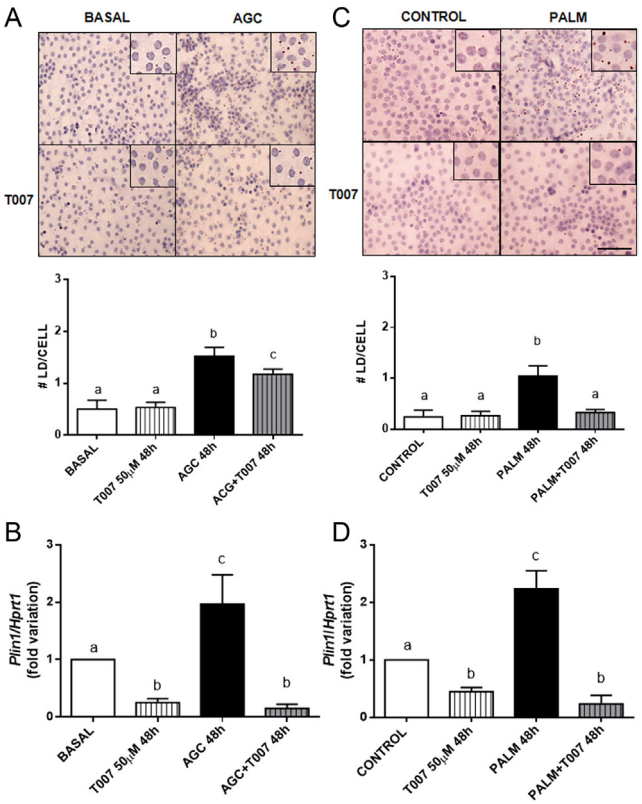

Figure 7 Effects of PPARg antagonist on the up-regulation of lipid droplet content and *Plin1* mRNA levels by AGC and palmitic acid in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) or treated with palmitic acid (500 µM/1% BSA) in the absence or presence of 50 µM T0070907 (T007) – PPARg antagonist – for 48 h. (A and C) Upper panels: microphotographs of Sertoli cells monolayers stained with ORO. Inset: magnification of the microphotographs. Bar = 50 µm. Lower panels: LD quantification in Sertoli cell monolayers. Values represent mean ± S.D. of number of LD per Sertoli cell in one representative experiment out of three. Different letters indicate statistically significant differences $P < 0.05$ vs Basal or Control (1% BSA). (B and D) Total cellular RNA was extracted. RT-qPCR was performed to analyze *Plin1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. $n = 3$, different letters indicate statistically significant differences $P < 0.05$ vs Basal or Control (1% BSA).

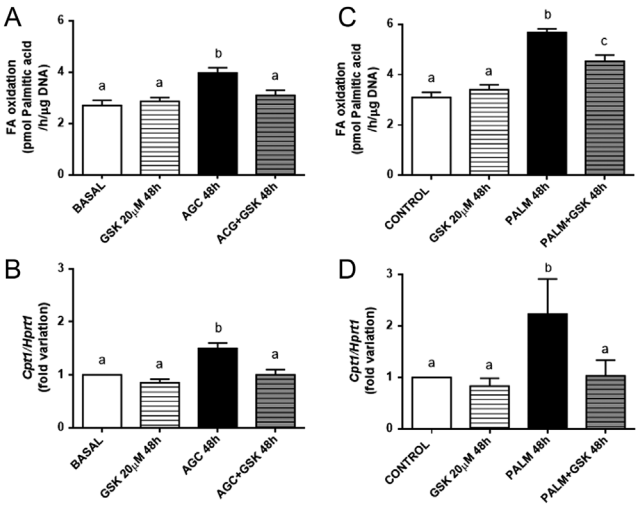

Figure 8 Effects of PPARb/d antagonist on the up-regulation of FA oxidation and *Cpt1* mRNA levels by AGC and palmitic acid in Sertoli cell. Sertoli cells were co-cultured with AGC (5×10^5 cell/cm^2) or treated with palmitic acid (500 µM/1% BSA) in the absence or presence of 20 µM GSK3787 (GSK) – PPARb/d antagonist – for 48 h. (A and C) Fatty acid oxidation was assessed by measuring ^3H_2O produced in the incubation medium. Results are expressed as pmol of palmitic acid/h/µg DNA (means ± S.D.) $n = 3$. Different letters indicate statistically significant differences $P < 0.05$ vs Basal or Control (1% BSA). (B and D) Total cellular RNA was extracted. RT-qPCR was performed to analyze *Cpt1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means ± S.D. $n = 3$, different letters indicate statistically significant differences $P < 0.05$ vs Basal or Control (1% BSA).

or presence of 20 µM GSK – PPARb/d pharmacological antagonists. Figure 8A and C show that the upregulation in the FA oxidation in response to the co-culture with AGC or to PA treatment was diminished in the presence of GSK. Likewise, the upregulation of *Cpt1* expression in response to the co-culture with AGC or to PA treatment was inhibited in the presence of GSK (Fig. 8B and D).

Discussion

SCs have an active phagocytic activity, which is essential to remove AGC and to maintain an adequate function of seminiferous tubule. Not only AGC but also residual bodies are engulfed by SCs and as a result, the amount of LD increases within this cell type (Kerr *et al.* 1984, Wang *et al.* 2006). As mentioned in the introduction, Xiong *et al.* (2009) have shown a correlation between the raise in the number of LD and the increase in ATP levels within SCs. These latter observations suggested the idea that FA are stored as neutral lipids in LD and that their hydrolysis provides free FA, which constitute an essential source of energy for SC. In this context, it has been shown that knockout mice for adipocyte triacylglycerol lipase (ATGL) – enzyme that catalyzes the rate-determining step of the degradation of TAG – show not only an increase in TAG content but a decrease in fatty acid oxidation and in the expression of genes related to its degradation in different tissues (Haemmerle *et al.* 2011, Tang *et al.* 2013). Altogether, these previous information led us to hypothesize that phagocytosis of AGC by SCs is related to the regulation of the expression of genes associated to synthesis and storage of TAG in LD, and it is also to the generation of FA or FA derivatives that regulate the expression of genes involved in their own oxidation. Considering that breakdown products of LD provide FA that are utilized to produce ATP in SCs and that it is known that PA is the most abundant fatty acid in SCs, germ cells and residual bodies (Hurtado de Catalfo & Gómez Dumm 2005, Oresti *et al.* 2013),

we decided to compare the effects of co-culture with AGC with those produced by PA treatment.

The results presented herein show that co-culture with AGC or PA treatment increase the number of LD and TAG content in SCs. In addition, the co-culture with AGC elicits an augmentation of *Fat/cd36* mRNA levels. To this respect, it is worth mentioning that FAT/CD36 is not only involved in the transportation of FA into the cells but also in the phagocytosis of residual bodies and AGC by SCs (Gillot *et al.* 2005). It may be presumed that the increase in mRNA levels of *Fat/cd36* can be translated in an increase in the amount of protein at the plasma membrane which ultimately will facilitate the entrance of FA and/or the phagocytosis of AGC.

The fact that PA treatment decreases *Fat/cd36* mRNA expression in SCs is not surprising. Noteworthy, it has been shown that an excess of FA inside the cells, considering its hydrophobic characteristics, can produce a noxious effect. In a concordant way, it has been demonstrated that increasing concentrations of PA diminish *Fat/cd36* expression in intestinal cells (Xie *et al.* 2012). Additionally, fatty acid overload has been implicated in the development of pathologies such as diabetes mellitus type 2 and cardiac failure (Glatz 2015). With all this background, it may be suggested that diminished *Fat/cd36* mRNA levels promoted by PA is the reflection of a negative feedback loop which prevents the harmful effect of excessive fatty acid levels within SCs. Further analysis is necessary in order to evaluate this assumption.

Although an augmentation in TAG levels under both experimental conditions utilized in the present study has been observed, we were unable to demonstrate regulation of the expression of enzymes involved in TAG synthesis, such as *GpatT1* or *Dgat1*. These observations suggest that the increase in TAG content is probably related to an increase in the enzyme activities of existing GPAT1 and DGAT1 by the rise in the availability of their substrates, namely acyl-CoAs.

It is known that LD are organelles that have a TAG-rich core enclosed by a single layer of phospholipids surrounded by specific proteins such as PLINs, which not only drive TAG storage but also regulate TAG hydrolysis. PLINs are the most abundant distinctive label of the LD machinery. The levels of PLINs have been correlated with the intracellular content of TAG and of LD (Bickel *et al.* 2009). Furthermore, it has been observed that the levels of PLINs increase under conditions of augmented fatty acid levels within the cells (Brasaemle 2007, Fan *et al.* 2009). We observed that co-culture with AGCs and the treatment with PA increase *Plin1*, *Plin2* and *Plin3* mRNA levels. It is tempting to speculate that the observed augmentation in LD number, TAG content and *Plins* expression in SCs might be related to the increase in fatty acid inward caused by the above-mentioned experimental conditions.

As mentioned in the introduction, Xiong *et al.* (2009) have shown that co-culture with AGC elicits an increase in ATP levels and in *Lcad* mRNA levels in SCs. Our results showing an increase in fatty acid oxidation in the presence of AGC are probably related to the increased ATP levels observed in the previous work. In addition, this investigation demonstrates that not only *Lcad* but also *Cpt1* mRNA levels increased as a consequence of apoptotic germ cell phagocytosis. In this respect, the increase in the expression of *Cpt1* and *Lcad* genes, which proteins promote an augmentation in the entrance and in the degradation of FA within the mitochondria, may be therefore partially responsible for the observed increase in fatty acid oxidation.

Although the physiological mechanisms that participate in the activation of PPARs in different tissues are not clear, it is well established that FA and their derivatives act as PPARs ligands (Bensinger & Tontonoz 2008). We have previously demonstrated that pharmacological activation of PPARa and PPARb/d regulates the expression of genes involved in fatty acid oxidation (Regueira *et al.* 2014) and additionally that activation of PPARg increases LD number, TAG levels and the expression of genes involved in TAG synthesis (Gorga *et al.* 2017). On this background, we postulated a participation of PPARs on the observed effects on lipid metabolism promoted by AGC and PA. There is evidence showing that activation of PPARs correlates with an increase in the expression of their own genes, which increases the amount of these transcription factors as a way to raise the sensibility of the system (Thach *et al.* 2016). These previous observations led us to evaluate if such a mechanism existed in SCs. Results showing unchanged *Ppara*, *Pparb/d* and *Pparg* mRNA levels under the experimental conditions tested suggest that it is not the case in SCs.

As it may be expected, a mechanism that results in the activation of PPARs involves the generation of their ligands under physiological conditions. To this respect, Mottillo *et al.* (2012) have demonstrated that the hydrolysis of brown fat gives endogenous ligands for PPARa and PPARb/d, which is accompanied by an increase in the expression of *Lcad* and *Mcad*. Furthermore, the fact that ATGL-knockout mice present a decrease in the expression of *Cpt1*, *Lcad*, *Mcad* and that the expression of these proteins is restored by treatment with a PPARs agonist, further support the relevance of the generation process of endogenous PPAR ligands (Haemmerle *et al.* 2011, Tang *et al.* 2013). Results obtained in this study, which show an increase in PPAR transcriptional activity by AGC and by FA, strongly suggest that PPARs ligands are being generated as a result of germ cell phagocytosis and of fatty acid entry into SCs. There exists a general agreement in that activation of PPARg regulates anabolic pathways while activation of PPARb/d regulates catabolic pathways. To this respect, it has been established that there is an association between PPARg

activation and the regulation of *Plin1* gene expression in different tissues (Arimura *et al.* 2004) and that the expression of *Plin1* by PPARg activation directs the final stages of differentiation of preadipocytes into adipocytes (Haj-Yasein *et al.* 2017). As for FA utilization, previous reports have shown a relationship between FA oxidation, *Cpt1* expression and PPARb/d activation in different cell types (Wang *et al.* 2003, Regueira *et al.* 2014, 2015). Our last set of experiments show, on the one hand, that the upregulation of LD content and *Plin1* expression in SCs cultured with AGC or treated with FA are inhibited by the presence of a specific PPARg antagonist. On the other hand, that the increase in FA oxidation and *Cpt1* mRNA levels in SCs under both experimental conditions employed are diminished in the presence of a specific PPARb/d antagonist. Altogether, our results reinforce the idea that PPARs are activated by ligands generated intracellularly under the experimental conditions utilized in order to regulate lipid metabolism in SC.

Conclusion

The results presented herein demonstrate that AGC and PA regulate lipid metabolism by, at least in part, regulation of the expression of genes involved in transport, lipid storage and fatty acid oxidation in SCs. Furthermore, our results show that activation of PPARs is involved in the regulation of TAG storage, FA oxidation and gene expression, which are promoted by co-culture with AGC and by PA treatment. It is postulated that phagocytosis by SCs of AGC, a physiologically occurring process, results in the production of FA or fatty acid derivatives which activate PPARs and regulate lipid storage and fatty acid utilization to regulate energy homeostasis in the seminiferous tubules.

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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