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Abstract: The purpose of this study was to evaluate the existence of a possible simultaneous regulation of fatty acid (FA) metabolism and lactate production by PPAR α and PPAR β/δ activation in Sertoli cells (SC). SC cultures obtained from 20-day-old rats were incubated with WY14643 or GW0742 — pharmacological activators of PPAR α and PPAR β/δ respectively. The fatty acid transporter CD36, carnitine palmitoyltransferase 1, long- and medium-chain 3-hydroxyacyl-CoA dehydrogenases mRNA levels were analyzed. An increase in the above-mentioned genes in response to activation of both nuclear receptors was observed. Additionally, PPAR β/δ activation increased lactate production as a consequence of increased pyruvate availability by inhibiting the Pyruvate Dehydrogenase Complex. Altogether, these results suggest that in SC, PPAR α activation participates in the regulation of FA metabolism. On the other hand, PPAR β/δ activation regulates FA metabolism and lactate production ensuring simultaneously the energetic metabolism for SC and germ cells.

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Professor W E Rainey
Editor-in-chief
Molecular and Cellular Endocrinology

July 20th, 2013

Dear Professor Rainey:

We are enclosing a new version of our manuscript entitled "Activation of PPARα and PPARβ/δ regulates Sertoli cell metabolism" by Regueira M, Riera MF, Galardo MN, Pellizzari EH, Cigorraga SB and Meroni SB to be considered for publication in the Molecular and Cellular Endocrinology.

We have dealt with all the reviewers' comments. Particularly, we have done most of the experiments suggested by the reviewers and additional results have been added to the manuscript. Detailed answers to the criticisms that have arisen from the reviewers are listed in the next pages.

This work has not been, and will not be, submitted for publication elsewhere until the journal has reached a decision on whether to publish the paper.

Thank you for considering our paper for publication in the Molecular and Cellular Endocrinology. We look forward to hearing from you.

Very sincerely yours

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Reviewer #1: In this paper regulatory mechanisms of metabolism are uncovered in rat Sertoli cells. Activation of both PPAR<alpha> and PPAR<beta>/<delta> increases expression of genes involved in fatty acid oxidation. In addition, activation of PPAR<beta>/<delta> but not of PPAR<alpha> stimulates lactate production that serves as an energy source for germinal cells. The latter is mediated by increased phosphorylation of pyruvate dehydrogenase that is expected to result in increased availability of pyruvate for lactate synthesis. The data give new insights into the metabolic regulation in testis. The data are well described and documented. Altogether, although the information provided in this paper is novel and relevant, the amount of data is rather limited.

Major comments

1) Although there are many figures, figure 1-4 prove the same i.e. the increased expression of PPAR<alpha> and PPAR<beta>/<delta> target genes after administration of the respective ligands. This could be shortened in the text and more compacted in the figures. Also figure 7 and 8 could be combined.

The figures 1-4 and 6-8 were combined as requested by the reviewer.

2) Besides the demonstration that PPAR<alpha> and PPAR<beta>/<delta> ligands cause an increased expression of genes involved in mitochondrial <beta>-oxidation, it would be stronger evidence if it was shown that this effectively results in more fatty acid oxidation in Sertoli cells.

We certainly agree with the reviewer that measuring fatty acid oxidation would be the gold standard for our work. We have the intention to measure fatty acid oxidation as described by Mulvihill et al., 2011. We have had problems at the time of purchasing ^{3}H palmitate due to restrictions in our institution for purchasing doses higher than 250 uCi of this radioisotope. We are in the process of obtaining authorization from local nuclear regulatory authorities. Bureaucracy takes some time in our country. Unfortunately, we are not able right now to perform this determination.

A major point of regulation of fatty acid oxidation is at the level of fatty acyl-CoA transport from the cytosol into the mitochondria where it is oxidized. The rate limiting enzyme in this process is CPT-1. Acetyl-CoA carboxylase (ACC) plays an important role in regulating CPT1 activity. ACC carboxylates Acetyl-CoA forming malonyl-CoA, which allosterically inhibits CPT-1 activity. Therefore, a decrease in ACC activity by phosphorylation, decreases malonyl Co-A levels, increases CPT1 activity and thereby increases the uptake of fatty acyl-CoA into the mitochondria for subsequent oxidation. Instead of measuring fatty acid oxidation, we determined the levels of phosphorylation of ACC (P-ACC). The following figure shows that WY as well as GW treatments increase P-ACC levels, which probably reflects an increase in fatty acid oxidation with PPAR α and PPAR β/δ activation in Sertoli cells. This experiment was repeated twice. We have not included these data in the manuscript but will be willing to do so, in case the reviewer requires it.

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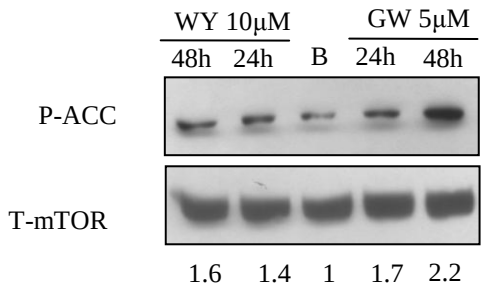

Figure legend: Sertoli cells were incubated for 24 and 48h with 10 μM WY or 5 μM GW. Cell extracts were utilized for Western blot analysis using antibodies specific for P-ACC or T-mTOR. Numbers below the autoradiography show pooled data of the two independent experiments indicating the fold variation in the level of ACC phosphorylation (ratio of P-ACC to T-mTOR in each sample) relative to BASAL (B).

3) The effect of activated PPAR<beta>/<delta> on phosphorylation of PDCs is likely mediated by increased expression of PDKs, as also mentioned by the authors. It would be much stronger evidence if this could indeed be demonstrated. In particular, PDKs are also target genes for PPAR<alpha> (this was not mentioned in the manuscript) whereas WY does not cause increased PDC phosphorylation in Sertoli cells. Therefore, analysis of PDK expression could clarify the differential effects of PPAR<alpha> and PPAR<beta>/<delta> agonists on lactate production.

In order to analyze the relevance of PDKs on the regulation of lactate production, we have investigated the regulation by WY and GW of PDKs and PDPs.
We have observed that treatment with GW increases PDK1 and PDK4 expression while treatment for 48 hs with WY increases PDK2, PDK3 and PDK4 expression. However, only WY treatment increases PDP1 expression. These results suggest that PDP1 may be responsible, at least in part, for the basal levels of P-PDC that are observed with WY treatment. These results have been included in the manuscript and discussed accordingly.

4) In the legends to figure 1,3 and 4 the number of independent experiments are not mentioned.

The number of independent experiments was included in the figure legends.

5) In figure 5 only a representative experiment is shown although 3 repeats were performed. It would be better to show the pooled data of all the experiments.

Figure 5 was changed including the pooled data of all experiments

6) The sentence "The role of PPARs in the regulation of gene expression has been studied primarily in liver and adipose tissue" with a reference to a paper of 2001, is not reflecting the literature of the last decade. Plenty of papers were published showing important roles of PPARs in many different tissues.

The sentence was changed and several papers showing the role of PPARs in different tissues were included.

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7) In the discussion, the physiological relevance of the findings should be better highlighted e.g. what is the testicular phenotype of PPAR knockout mice? Which endogenous ligands of PPARs could be present at certain time points

PPAR α deficient mice were viable, fertile, and healthy and lacked any observable gross defects, thus implying that PPARα is not essential in embryonic development or in male fertility (Lee et al., 1995). In regard to PPAR β/δ KO, Barak et al., (2001) have demonstrated that PPAR β/δ has an essential role in placentation, such that its deficiency results in frequent embryonic lethality. Surviving PPAR β/δ null mice, while rare, are generally healthy and fertile.
Although these studies are important to understand the physiological role of PPARs, in our opinion these findings do not strengthen our work discussion. Consequently, we have not included these findings in the manuscript but will be willing to do so, in case the reviewer requires it.

Regarding the endogenous ligands of PPARs, a paragraph in the discussion was included.

Minor comments
1) The 'Highlights' are not well formulated because there is no referral to the cell type that is being investigated

The highlights were changed including the cell type.

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Reviewer #2: Your manuscript entitled "Activation of PPAR<alpha> and PPAR<beta>/<delta> regulates Sertoli cell metabolism" has been recommended for publication in Molecular and Cellular Endocrinology. The results of this study were interesting although they did not address the functional specificity of all the PPARs expressed in Sertoli cells in vivo. The endogenous PPAR activators are still currently unknown, however, induction of these activators are likely to be regulated by Sertoli/germ cell interactions that vary during spermatogenesis. This suggests that significant cross-talk is likely to occurs in vivo between PPAR<beta>/<delta> catabolic regulation of FA metabolism and PPAR<gamma> anabolic regulation of FA in the Sertoli cells. Future studies, therefore, should also include the functional role that PPAR<delta> plays in Sertoli cell metabolism since it is the most abundant PPAR isoform that is expressed in vivo in Sertoli cells.

Several evidences point out that PPAR α and PPAR β/δ regulate fatty acid catabolism while PPAR γ regulates fatty acid anabolism. In Sertoli cells, fatty acid catabolism has been proposed as an important energy source, while glucose metabolism has been proposed to be mainly destined to the production of lactate in order to supply energy to germ cells. Supporting this last proposal, we have observed that Sertoli cells are viable in the absence of glucose for quite a long period of time in culture (Riera, et al., 2010). In this context, we were interested in understanding the mechanisms related to fatty acid catabolism, particularly those resulting from PPARα and PPAR β/δ activation. As for the pattern of expression of PPARs in Sertoli cells, some controversy exists. Braissant et al. (1996) have shown that PPAR γ expression is weak while that of PPAR β/δ is strong in adult Sertoli cells. More recently Thomas et al., (2011) have studied the developmental pattern of expression of PPARs during spermatogenesis and have demonstrated that PPAR γ is the predominant isoform expressed in 20-day-old Sertoli cells. In the latter report, it has also been shown that this transcription factor is involved in the regulation of expression of several genes participating in lipid metabolism. These discrepancies on the levels of expression of PPARs may result from different methodological approaches and from the age of the animals utilized in these studies. We agree with the reviewer that it is important to analyze the functional role of PPAR γ in the regulation of fatty acid metabolism in Sertoli cells. However, it should be kept in mind that activation of PPAR γ would result in energy expenditure rather than in energy provision.

We have evaluated the role of PPAR γ activation by rosiglitazone on the parameters analyzed in the present study. Sertoli cells were stimulated for 24 and 48 h with rosiglitazone (RSG: 1 and 10 μM) and FAT/CD36, CPT1, LCAD, MCAD expression was evaluated. The following table shows that LCAD expression augmented, while FAT/CD36, CPT1 and MCAD expression did not change under the described experimental conditions.

Effect of Rosiglitazone (RSG) on FAT/CD36, CPT1, LCAD and MCAD expression. Experiments were repeated three times.

	FAT/CD36	CPT1	LCAD	MCAD
BASAL	1	1	1	1
RSG 10μM 24h	1.2±0.1	1.2±0.1	1.8±0.2*	1.0±0.2
RSG 10μM 48h	1.2±0.1	1.3±0.1	2.3±0.4*	1.0±0.3

A paragraph in the discussion was added. It discusses the possible endogenous PPARs activators.

Activation of PPAR α and PPAR β/δ regulates Sertoli cell metabolism

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ABSTRACT

The purpose of this study was to evaluate the existence of a possible simultaneous regulation of fatty acid (FA) metabolism and lactate production by PPAR α and PPAR β/δ activation in Sertoli cells (SC). SC cultures obtained from 20-day-old rats were incubated with WY14643 or GW0742 —pharmacological activators of PPAR α and PPAR β/δ respectively. The fatty acid transporter CD36, carnitine palmitoyltransferase 1, long- and medium-chain 3-hydroxyacyl-CoA dehydrogenases mRNA levels were analyzed. An increase in the above-mentioned genes in response to activation of both nuclear receptors was observed. Additionally, PPAR β/δ activation increased lactate production as a consequence of increased pyruvate availability by inhibiting the Pyruvate Dehydrogenase Complex. Altogether, these results suggest that in SC, PPAR α activation participates in the regulation of FA metabolism. On the other hand, PPAR β/δ activation regulates FA metabolism and lactate production ensuring simultaneously the energetic metabolism for SC and germ cells.

KEY WORDS: Fatty Acid oxidation; Pyruvate Dehydrogenase Complex; Lactate; Testis.

1. INTRODUCTION

The process of spermatogenesis and consequently male fertility is dependent upon the somatic cells that are present in the testis. Leydig cells are essential because of androgen production, and Sertoli cells –somatic cells of the germinal epithelium- are absolutely necessary in order to provide an adequate and protected environment within the seminiferous tubules. Studies on Sertoli cell glucose metabolism have shown that these cells actively metabolize this sugar, but the vast majority is converted to lactate and is not oxidized via the Krebs cycle (Robinson and Fritz, 1981). The very high rate of lactate production by Sertoli cells, which has been well documented, has a physiological importance as it provides the energetic substrate to germinal cells in the adluminal compartment (Grootegoed et al., 1984; Jutte et al., 1983). In this metabolic context, it has been demonstrated that palmitate can be oxidized to CO_2 and it has been suggested that the oxidation of fatty acids (FA) can yield much of the energy required by Sertoli cells (Jutte et al., 1985). Nevertheless, the mechanisms involved in FA utilization were not analyzed at the time of the above-mentioned study and are poorly known so far in Sertoli cells.

Needless to say, FA must enter into the cell in order to be metabolized. Biochemical and biophysical approaches have provided evidence for the existence of two distinct processes in cellular FA uptake: passive diffusion through the lipid bilayer and protein-facilitated transport (Luiken et al., 2002; Schwenk et al., 2010). One of the proteins involved in FA uptake is FAT/CD36, an integral membrane glycoprotein which has been found in a wide variety of cells (Abumrad et al., 1993; Coburn et al., 2000; Ibrahimi and Abumrad, 2002). Once FA are incorporated into the cells, they are activated by covalently linking to a coenzyme A forming an acyl-CoA derivative and then the carnitine palmitoyl transferase 1 (CPT1) is responsible for the entrance of acyl-CoA into the mitochondria (Rasmussen and Wolfe, 1999). The process known as β-oxidation is responsible for the oxidation of acyl-CoA and consequently the production of energy. The first step in β-oxidation is the α-β-dehydrogenation of the fatty acyl-CoA ester by a family of specific chain length acyl-CoA dehydrogenases (Ghisla and Thorpe, 2004). This family includes long chain (LCAD) and medium chain (MCAD) dehydrogenases enzymes. β-oxidation involves the stepwise removal of acetyl-CoA molecules from the shrinking FA chain. The acetyl-CoA molecules feed the Krebs cycle for further oxidation to water and carbon dioxide to obtain energy (Drynan et al., 1996).

The regulation of these molecular events necessary for oxidation of FA is strictly controlled and varies within different tissues (Lopaschuk et al., 1994; McGarry and Foster, 1980; Rasmussen and Wolfe, 1999).

Peroxisome proliferator-activated receptor –PPAR–, α (NR1C1), β/δ (NR1C2), and γ (NR1C3), are ligand-activated transcription factors that are members of the nuclear-hormone receptor superfamily (Issemann and Green, 1990; Michalik et al., 2006). All PPARs function as sensors of FA and FA derivatives and thus control metabolic pathways involved in lipid and energy metabolism (Green and Wahli, 1994; Krey et al., 1997). In the liver, PPAR α is activated by both saturated and polyunsaturated FA and their derivatives and among other biological actions its activation promotes FA catabolism (Jump et al., 2005). PPAR β/δ has also been shown to enhance FA catabolism in skeletal muscle and adipose tissue and it has been recently shown that it may participate in the hepatic response to starvation (Sanderson et al., 2009; Wang et al., 2003). As for PPAR γ, its activation has been involved in adipocyte proliferation and differentiation and in improving glucose homeostasis and insulin sensitivity by promoting FA storage (Rangwala and Lazar, 2004; Siersbaek et al., 2010). In essence, PPAR α and PPAR β/δ function as catabolic regulators while PPAR γ mostly regulates anabolic lipid metabolism (Chawla et al., 1994; Jump et al., 2005; Tontonoz et al., 1995; Wang et al., 2003). Specific pharmacological activators of PPAR α and PPAR β/δ, such as WY14643 and GW0742 respectively, are essential tools for the study of the association between PPAR activation and different biological functions (Desvergne and Wahli, 1999; Dressel et al., 2003).

As mentioned before, Sertoli cell carbohydrate metabolism presents some interesting characteristics. Glucose is not an important source of energy for Sertoli cells, in fact it has been shown that this cell type can survive in culture for at least 48 hours in the absence of glucose (Riera et al., 2009). Glucose is metabolized to lactate since germ cells situated beyond the blood testis barrier rely on Sertoli cell production of this hydroxyacid to obtain energy. Among the molecular mechanisms that have been shown to participate in lactate production, glucose transport mediated by GLUTs, LDH isoenzymes -reversibly catalyzing the interconversion of pyruvate in lactate- and lactate transport across the plasma membrane, mediated by monocarboxylate transporters (MCTs), may be mentioned (Nehar et al., 1997, 1998; Riera et al., 2001, 2002).

In the present study, we postulated a role for PPAR α and PPAR β/δ activation in the expression of genes involved in FA catabolism in Sertoli cells. In addition, we

hypothesized that PPAR α and PPAR β/δ activation can simultaneously regulate FA and glucose metabolism in order to ensure the provision of energy to Sertoli and germ cells respectively.

To evaluate these hypotheses we investigated: (1) the regulation of the expression of genes involved in FA metabolism by activation of PPAR α and PPAR β/δ and (2) a role of PPAR α and PPAR β/δ activation in lactate production in Sertoli cells.

2. MATERIALS AND METHODS

2.1 Materials

[2,6-^{3}H]-2-deoxy-D-glucose (2-DOG) was purchased from NEN (Boston, MA, USA). Culture media were purchased from Invitrogen (Carlsbad, CA, USA). WY14643 and GW0742 and all other drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2 Sertoli cell isolation and culture

Sertoli cells 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 Sertoli cell 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. Sertoli cells were cultured in 25 cm^2 flask, 6-, 24- or 96-multiwell plates (5 μg DNA/cm^2) at 34ºC 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 Sertoli cell 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.

2.3 Culture conditions

Sertoli cells were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Stimulation with WY14643 (WY) or GW0742 (GW) was performed with variable doses and for variable periods of time. The conditioned media and the cells treated for 48 h with variable doses of WY or GW were harvested and used to evaluate lactate production and glucose uptake respectively. Cells incubated for 24- or 48-h with WY or GW were used to evaluate FAT/CD36, CPT1, LCAD, MCAD, GLUT1, LDHA, MCT4, PDK1-4 and PDP1-2 mRNA levels and phospho-Pyruvate Dehidrogenase Complex (P-PDC) protein levels.

2.4 Real-time PCR (RQ-PCR)

Total RNA was isolated from Sertoli cells cultured in 6-multiwell plates with TRI Reagent (Sigma-Aldrich). 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 SuperScript II reverse transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix (Invitrogen).

Real-time PCR was performed by a Step One Real Time PCR System (Applied Biosystems, Warrington, UK). The specific primers for real-time PCR were: 5´-ACCAGGCCACATAGAAAGCA-3´ and 5´-CACCAATAACGGCTCCAGTAA-3´ for FAT/CD36; 5´-AAAGGTCTGGGAGTGATTGG-3´ and 5´-CCATTCTCCACCAAAAAGAGG-3´ for LCAD; 5´-CGAGCACAACACACAAAACC-3´ and 5´-TTCCTCTCTGGCAAACTTCC-3´ for MCAD; 5´-GGAGTGTCGGTTTAGGTTGC-3´ and 5´-GCTGTGAAACGGAGAATGGA-3´ for GLUT1; 5’-TGTGGTGAACCGCTTTGG-3’ and 5’-CAGACCCAAGCCAGTGATG-3’ for MCT4; 5’-TCCAGGGAGACCTAAAGCTG-3’ and 5’-CGTGGTTGGTTCTGTAATGC-3’ for PDK1; 5’-GACCCAGTCTCCAACCAGAAC-3’ and 5’-GGGATCAATGCTGCCAATGTG-3’ for PDK2; 5’-GTCGCCGCTCTCTATCAAAC-3’ and 5’-AGCCAGTCGCACAGGAAG-3’ for PDK3; 5’-CGAAGATGCCTTTGAGTGTG-3’ and 5’-TGGTGAAGGTGTGAAGGAAC-3’ for PDK4; 5’-ACAGGAGAATGTGTGTGTGTCC-3’ and 5’-

TGGCATCAGAGAACAGTGGTAG-3' for PDP1 and 5'-AGAGGATTCGCCCAGTGTC-3' and 5'-AAGTGGAGGTGGAGTGTTTTTC-3' for PDP2; 5´-TGGCACCACACTTTCTACAAT-3´ and 5´-GGTACGACCAGAGGCATACA-3´ for β actin. 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 β actin. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 seg, 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.

2.5 Northern blot analysis

Total RNA was isolated from Sertoli cells cultured in 25 cm^2 tissue culture flasks using the TRI Reagent (Sigma-Aldrich). The amount of RNA was estimated by spectrophotometry at 260 nm. For Northern blot analysis 10 µg total RNA was electrophoresed on a 1% agarose-10% formaldehyde gel. After migration, RNAs were transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buenos Aires, Argentina) by capillary transfer with 10 x SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.4) and fixed with U.V. Stratalinker (Stratagene Cloning Systems, La Jolla, CA, USA). cDNA probes were labeled with [α-^{32}P]deoxy-CTP (Amersham Pharmacia Biotech) using a random-primed labeling kit (Prime-a-Gene Labeling System, Promega Corporation, Madison, USA). The cDNA probes used were the following: CPT1a, a 785b probe previously obtained using a RT-PCR technique with specific primes (5'-CACGAAGCCCTCAAACAGAT-3' and 5'-AATGTGCGACGATACAGCAG-3'), LDH A, a rat 3'UTR 0.4 kb insert, Pst I-Bgl II (kindly gifted by Dr. Jungmann, Chicago USA) and a 18S oligonucleotide. Blots were prehybridized for 5 h at 42ºC in 50% formamide, 0.75 M NaCl, 20 mM sodium phosphate (pH 7.5) and 1 mM EDTA, 5 x Denhart's solution, 10% dextran sulfate, 0.5% SDS and 100 µg/ml herring sperm DNA. Hybridization was then performed overnight at 42ºC in the same hybridization buffer containing 1-4 x 10^6 cpm/ml ^{32}P-labeled probe. Membranes were washed utilizing

different astringency conditions depending on the probe utilized. Membranes were exposed to Kodak X-Omat S films (Eastman Kodak, Rochester, NY, USA) at -70 ºC, for variable periods of time according to the probe utilized. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image software (Scion Corporation, Fredereck, MD, USA). The 18S signal was used to standardize mRNA contents.

2.6 Western blot analysis

Cells cultured in 6-multiwell plates were washed once with PBS at room temperature. Then, 200 μl of PBS containing 2 μl of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50nM okadaic acid and 2 mM PMSF was added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. For Western blot analysis, 200 μl of 2X Laemmli buffer (4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 0.004% w/v bromophenol blue, and 0.125 M Tris-HCl, pH 6.8) was added and thoroughly mixed. Samples were immersed in boiling water bath for 5 min and then immediately settled on ice. Proteins (40 μg in each lane) were resolved in 10% SDS-PAGE (10% acrylamide/bisacrylamide for the resolving gel and 4.3% acrylamide/bisacrylamide for the stacking gel) in a mini protean 3 cell (Bio-Rad). After SDS-PAGE, gels were electrotrasferred at 100 V for 60 min onto PVDF membranes (Hybond-P, Amershem Pharmacia Biotech) using a mini trans-blot cell (Bio-Rad, Hercules, CA, USA). Membranes were probed with specific antibodies that recognized the phosphorylated form of Pyruvate Dehydrogenase complex (P-PDC) (Piruvate dehydrogenase E1-α subunit (P-Ser293) antibody, Novus Biologicals, Littleton, CO, USA) or total Akt (T-Akt) (Akt antibody, Cell Signalig Technology, Inc., Danvers, MA, USA). A 1:1000 dilution of primary antibodies, as indicated by the manufacturer, was used. For chemiluminescent detection of the blots, a commercial kit from Cell Signaling Technology was used. The intensities of the autoradiographic bands were estimates by densitometry scanning using NIH Image Software (Scion Corporation). Levels of T-Akt were used as loading control.

2.7 Measurement of 2-deoxyglucose (2-DOG) uptake

Glucose transport was studied using the uptake of the labeled non-metabolizable glucose analogue 2-DOG. Cells cultured on 24-multiwell plates were washed two times with glucose-free PBS at room temperature. Sertoli cells were then incubated at 34ºC in

0.3 ml glucose-free PBS containing [2,6-^{3}H]-2-DOG (0.5μCi/ml) for 30 min. Unspecific uptake was determined in incubations performed in the presence of a 10000-fold higher concentration of unlabeled 2-DOG. At the end of the incubation period, dishes were placed on ice and expensively washed with ice-cold PBS until no radioactivity was present in the washings. Cells were then dissolved in 0.4% sodium deoxycholate/0.5 M sodium hydroxide and counted in a liquid scintillation spectrophotometer. Parallel cultures receiving identical treatments to those performed before the 2-DOG uptake assay and were destined to DNA determinations. 2-DOG incorporation into the cells was normalized, considering the amount of DNA in the cultures.

2.8 Lactate determination

Conditioned media obtain from cells cultured in 24-multiwell plates were used to determine lactate production. Lactate was measured by a standard method involving conversion of NAD^{+} to NADH. The amount of NADH was determined as the rate of increase of absorbance at 340 nm. A commercial kit from Sigma-Aldrich was used.

2.9 LDH activity measurement

Cells cultured in 24-multiwell plates were disrupted by ultrasonic irradiation in 0.9% NaCl and aliquots were preserved for DNA determinations. The remaining material was centrifuged at 15800g for 10 min. The supernatant was used to measure total LDH activity, which was determined by a routinely used spectrophotometric method (Wiener lab., Rosario, Argentina). Results were expressed as mIU/μg DNA.

2.10 Other assays

A cell viability test was performed in cells cultured on 96-multiwell using a commercial kit (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega Corporation). DNA was determined by the method of Labarca and Paigen (1980). Protein content was determined by Lowry's assay.

2.11 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 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). P values < 0.05 were considered statistically significant.

3. RESULTS

3.1 PPAR α and PPAR β/δ activation regulates the expression of genes involved in fatty acid metabolism in Sertoli cells

In order to evaluate the effects of PPAR α and PPAR β/δ on the expression of genes involved in FA metabolism, Sertoli cells were incubated for different periods of time —24 and 48 hours— and with variable doses of WY14643 (WY, 1 and 10 μM) or GW0742 (GW, 1 and 5 μM), pharmacological activators of PPAR α and PPAR β/δ respectively. The levels of expression of the following genes, which participate in FA metabolism, were evaluated: FAT/CD36, CPT1, LCAD and MCAD. Cell viability tests performed after a 48-h incubation period showed that neither WY nor GW modify cell viability at the doses tested (data not shown).

3.1.1 Regulation of FAT/CD36 expression

The left panel in Figure 1A —24 and 48 h incubations with 10μM WY— and the right panel in the same figure —48 h incubations with 1 and 10 μM WY— show that only in 48-h incubations with 10μM WY an increase in FAT/CD36 mRNA levels was observed. Right and left panels in Figure 1B, depicting similar experiments performed with the PPAR β/δ activator, show that only in 48-h incubations with 5μM GW an increase in FAT/CD36 mRNA levels was observed.

3.1.2 Regulation of CPT1 expression

Figure 1C (left panel) shows that activation of PPAR α with 10μM WY increased CPT1 mRNA levels in 24-h incubations. Figure 1C (right panel) shows that a statistically significant increase in CPT1 levels with 10μM WY in 24-h incubations was observed. As for the activation of PPAR β/δ, left panel in figure 1D shows that GW 5μM produced a time-dependent increase in CPT1 mRNA levels. The right panel in figure 1D shows that both 1 and 5μM GW doses significantly increased CPT1 mRNA levels in 48-h incubations.

3.1.3 Regulation of LCAD and MCAD expression

The left panel in Figure 1E shows that activation of PPAR α with 10μM WY increased LCAD mRNA levels at both incubation periods analyzed, and right panel in the same figure shows that in 24-h incubations, both doses of WY utilized in this study promoted an increment in LCAD mRNA levels. Fairly the same results were obtained activating PPAR β/δ (Figures 1E, left and right panels).

In a similar way, the left panel in Figure 1F shows that in 24- and 48-h incubations, activation of PPAR α with 10μM WY increased MCAD mRNA levels. The right panel in the same Figure shows that in 24-h incubations just the 10μM dose of WY increased MCAD mRNA levels. Furthermore, the left panel in Figure 1G shows that in 24- and 48-h incubations, activation of PPAR β/δ with 5μM GW increased MCAD mRNA levels and, the right panel in the same Figure shows that in 24-h incubations, the 5μM GW dose merely promoted an increase in MCAD mRNA levels.

3.2 PPAR α and PPAR β/δ activation promotes differential effects on lactate production in Sertoli cells

The next set of experiments was performed in order to analyze a possible effect of PPAR α and PPAR β/δ activation on Sertoli cell lactate production. Sertoli cells were incubated with WY14643 (WY) or GW0742 (GW) and lactate levels in 48 hour conditioned media were determined. Additionally, glucose incorporation into the cell and mRNA levels of GLUT1, LDH A and MCT4 were evaluated.

Figure 2A shows that the two doses of WY utilized in this study, 1μM and 10μM, did not modify lactate production in 48-h incubations. Interestingly, GW 5μM increased lactate production as shown in Figure 2B. These results prompted us to investigate a possible differential role of PPAR α and PPAR β/δ activation on the regulation of molecular mechanisms that participate in the production of lactate. Figures 3A and 3B show that treatments for 48-h with 1μM and 10μM WY (Figure 3A) or with 1μM and 5μM GW (Figure 3B) did not modify 2-DOG uptake. Similarly, Figures 3C and 3D show that treatments with WY and GW did not modify GLUT1 mRNA levels at any time studied. As for LDH, figures 3E and 3F show that neither WY nor GW modified LDH A mRNA levels in Sertoli cells. Concomitantly, Table 1 shows that LDH activity was not modified either. In addition, figures 3G and 3H show that the above-mentioned treatments did not modify MCT4 mRNA levels.

3.3 PPAR β/δ activation regulates the phosphorylation levels of the Pyruvate Dehydrogenase Complex (PDC) in Sertoli cells

Despite the differential effect of PPARs activators on lactate production, we were unable to demonstrate any differential effects on the regulation of several mechanisms that participate in the production of this hydroxiacid. Searching for an explanation to the observed differential effect, we decided to explore a possible regulation of pyruvate availability which is essential for LDH activity.

Pyruvate is not only a substrate for LDH but also, through its conversion to acetyl-CoA, it feeds the Krebs cycle. The conversion of pyruvate to acetyl-CoA is catalyzed by PDC, an enzymatic complex which is negatively regulated by phosphorylation. The phosphorylation status of PDC is regulated by a family of four pyruvate dehydrogenase kinases (PDK 1-4) and two pyruvate dehydrogenase phosphatases (PDP 1-2). In this way, those processes which increase PDC phosphorylation will result in a decreased utilization of the ketoacid in the Krebs cycle and will increase the pool of available pyruvate to be converted into lactate by LDH.

Figure 4A shows that PPAR α activation, which did not modify lactate production, did not modify P-PDC levels either. On the other hand, Figure 4B shows that PPAR β/δ activation, which increased lactate production also increased P-PDC levels.

We next explored the participation of the different isoforms of PDKs and/or PDPs in the regulation of P-PDC levels. For this purpose, the expression of PDKs and PDPs in response to PPAR α and PPAR β/δ activation was analyzed. Figure 5 shows that 24- and 48-h treatments with 10 μM WY increased mRNA levels of PDK2 (Figure 5 C) and PDK4 (Figure 5 G). Increased levels of PDK3 mRNA were only observed in 24-h incubations with 10 μM WY (Figure 5 E). No changes in PDK1 mRNA levels in any of the WY experimental conditions tested were observed (Figure 5 A). Figure 5 also shows that 24- and 48-h treatments with 5 μM GW increased PDK1 mRNA levels (Figure 5 B). Increased PDK4 mRNA levels were only observed in 48-h incubations with 5 μM GW (Figure 5 H). The same experimental conditions did not modify neither PDK2 (Figure 5 D) nor PDK3 mRNA levels (Figure 5 F). As for PDPs, only a 48-h treatment with 10 μM WY promoted an increase in PDP1 mRNA levels (Figure 5 I). No other experimental conditions tested modified the expression of PDPs (Figure 5 J, K, L).

4. DISCUSSION

Spermatogenesis in mammals is a precisely controlled process, comprising extensive genomic and cellular remodeling from spermatogonia to haploid cells and the final release of spermatozoa. This process requires a continuous cross talk between germ cells and Sertoli cells which exert multiple tasks critical for germ cell differentiation (Russell et al., 1993). In this context, the regulation of Sertoli cell metabolism is essential to provide nutrients to germ cells and to fulfill its own energy requirements. Energetic metabolism in the seminiferous tubule has been considered to have features of its own. The most important energetic metabolite used by the spermatogenic cells is lactate, which is produced from glucose in Sertoli cells (Boussouar and Benahmed, 2004). Consequently, Sertoli cells cannot rely on glucose for its own energy requirements and it has been postulated that this cell type utilizes FA as energy source (Jutte et al., 1985). Curiously, FA metabolism in Sertoli cells has received little attention since then. The presence of a mitochondrial FA oxidation system (Fukasawa et al., 2010), the demonstration of the existence of proteins which transport fatty acids — FAT/CD36 (Gillot et al., 2005) and CPT1 (Adams et al., 1998) —, and the evidence of the presence of PPAR α and β/δ (Braissant et al., 1996) in Sertoli cells indicate that FA oxidation may indeed be an important energy source in this cell type. Noteworthy, no previous studies have analyzed a possible relationship between PPAR α and PPAR β/δ activation and Sertoli cell metabolism.

The present study shows that activation of PPAR α and PPAR β/δ regulates the expression of genes involved in FA catabolism in Sertoli cells. The role of PPARs in the regulation of gene expression has been studied in different tissues (Burkart etl al., 2007; Chakravarthy et al., 2007; Sanderson et al., 2009; Wan et al., 2010; Gan et al., 2011; Cour Poulsen et al., 2012). Noticeably, the expression of PPARs varies in different tissues (Braissant et al., 1996; Burkart et al., 2007; Kliewer et al., 2001). In the testis, PPAR β/δ is the PPAR most abundantly transcribed while PPAR α is weakly expressed. PPAR α and PPAR β/δ seem to be expressed mainly in Sertoli cells (Braissant et al., 1996). Noteworthy, the existence of a developmental pattern of PPAR gene expression in Sertoli cells during spermatogenesis has been recently demonstrated (Thomas et al., 2011).

As mentioned in the introduction, the facilitated transport of FA is mediated by an integral transmembrane protein, namely FAT/CD36 (Bonen et al., 2004; Holloway et al., 2008). FAT/CD36 knockout mice revealed that this protein has an important role in FA and lipoprotein metabolism (Febbraio et al., 1999, 2002). In Sertoli cells, FAT/CD36 has been found in the vicinity of residual bodies engulfed by the Sertoli cells during the spermiation process suggesting a role of this protein in the phagocytosis of apoptotic germ cells and residual bodies (Gillot et al., 2005). Our results show that PPAR α and PPAR β/δ activation in Sertoli cells increases FAT/CD36 mRNA levels. FAT/CD36 may favor FA uptake and also phagocytosis of apoptotic germ cells and residual bodies, which may represent an additional source of FA available for oxidation in Sertoli cells (Xiong et al., 2009).

As for the genes involved in FA oxidation, previous reports have demostrated a relationship between CPT1, LCAD and MCAD expression and PPARs activation in several cell types as: cardiac myocytes (Brandt et al., 1998; Gilde et al., 2003), pancreatic beta-cells (Wang et al., 2003), human skeletal muscle cells (Krämer et al., 2007) and the hepatic cell line HepG2 (Gulick et al., 1994). In Sertoli cells, we observed that PPAR α and PPAR β/δ agonists stimulate in a similar way the expression of the three genes involved in FA oxidation —CPT1, LCAD and MCAD. These results provide evidence for a physiological role of PPARs in Sertoli cell lipid metabolism.

Even though several lines of evidence showed overlapping of functions of PPAR α and PPAR β/δ as transcriptional regulators of FA oxidation (Muoio et al., 2002), recent observations suggest that this may not be the case for the regulation of the expression of other genes, which are also involved in energetic metabolism. For instance, it has been shown that PPAR α and PPAR β/δ direct distinct metabolic regulatory programs in the mouse heart. Particularly, an increased utilization of glucose in response to PPAR β/δ activation has been observed in cardiomyocytes (Burkart et al., 2007). Opposite effects, i.e. decreased glucose utilization, have been observed in human adipocytes (Ribet et al., 2010).

As mentioned in the introduction, Sertoli cell carbohydrate metabolism presents some interesting characteristics and the importance of analyzing to what extent activation of the two PPARs was involved in glucose metabolism became apparent. Glucose, which does not seem to be an important source of energy for Sertoli cells (Riera et al., 2009), is metabolized to lactate in this cell type. In this respect, we have observed different

roles of PPAR α and PPAR β/δ activation in the regulation of lactate production in Sertoli cells. Activation of PPAR β/δ, but not of PPAR α, increased lactate production. This increase was not accompanied by the regulation of several mechanisms that have been previously shown to be targets of hormonal regulation of lactate production (Nehar et al., 1997, 1998; Riera et al., 2001, 2002). Specifically, no regulation of glucose uptake, GLUT1 mRNA levels, LDH activity or LDH A and MCT4 mRNA levels occurred in response to PPAR α and PPAR β/δ activation. We then hypothesized that an increased availability of pyruvate could account for the increased lactate production. Such increase in pyruvate levels could be the consequence of a lower conversion to acetyl-CoA by phosphorylation of PDC with the subsequent inactivation of this complex. We have observed an increase in P-PDC levels in response to PPAR β/δ activation. This result underlines the importance of this nuclear receptor in the control of pyruvate metabolism through the Krebs cycle in Sertoli cells. Phosphorylation status of PDC is regulated by members of a family of pyruvate dehydrogenase kinases (PDKs) and pyruvate dehydrogenase phosphatases (PDPs) (Holness and Sugden, 2003; Patel and Korotchkina, 2001). It has been shown that some PDKs are PPARs target genes in human kidney cells (Degenhardt et al., 2007) and in myotubes (Muoio et al., 2002). Therefore, we decided to investigate a possible regulation of PDKs and PDPs by PPAR α and PPAR β/δ activation. WY and GW treatments increased the expression of different PDKs, supposedly contributing to increase the phosphorylated state of PDC. Noticeably, only WY treatment increased PDP1 mRNA levels and this result suggests that under PPAR α activation PDP1 might be counterbalancing the effects of PDKs on the levels of P-PDC.

As for the possible physiological ligands for PPARs little is known. It has been proposed that fatty acids and a wide range of lipid derived molecules may act as physiological ligands of PPARs (Bensinger and Tontonoz 2008). However, these molecules are not clearly defined. In the testis, apoptotic spermatogenic cells and residual bodies are phagocytosed and degraded by Sertoli cells during spermatogenesis. A temporal relationship between phagocytosis of residual bodies and increased lipid droplets in Sertoli cells has been observed (Kerr et al., 1984; Ueno & Mori 1990; Sasso-Cerri et al., 2001). On the other hand, it has been demonstrated that spermatogenesis can be compromised by inactivation of hormone-sensitive lipase, which is involved in fatty acid supply, suggesting that accurate fatty acid metabolism is critical for male

reproduction (Chung et al., 2001). In this context, Xiong et al. (2009) demonstrated that lipids, present in lipid droplets, are a source of energy for Sertoli cells. It is tempting to speculate that these lipid droplets may also be the source of molecules which may act as endogenous ligands for PPARs, as recently proposed by Mottillo et al. (2012) in brown adipocytes.

Altogether our findings, which are summarized in Figure 6, indicate that PPAR α and PPAR β/δ activation in Sertoli cells stimulate the expression of genes involved in FA transport and oxidation. In addition, results presented herein demonstrate that PPAR α and PPAR β/δ exert a differential regulation on lactate production. Considering that PPAR β/δ is the most abundant PPAR isoform and that its activation simultaneously promotes FA oxidation and increased pyruvate availability for the conversion to lactate, it is tempting to speculate that this nuclear receptor may have a relevant physiological role in the seminiferous tubules. Future studies will be necessary to determine whether this is a general paradigm applicable to other tissues or if it is particularly important in Sertoli cell energetic function.

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FIGURE LEGENDS

Figure 1: Effect of WY and GW on FAT/CD36, CPT1, LCAD and MCAD mRNA levels in Sertoli cells. Sertoli cells were incubated for variable periods of time (24 and 48 h) with 10 μM WY (**A, C, E, G left panel**) or 5 μM GW (**B, D, F, H left panel**). Additionally, Sertoli cells were incubated with 1 and 10 μM WY (**A, C, E, G right panel**) or 1 and 5 μM GW (**B, D, F, H right panel**) during the incubation periods indicated in the figure. Total cellular RNA was then extracted. A, B, E, F, G and H: RQ-PCR was performed. The comparative ΔΔCt method was used to calculate relative gene expression. C and D: Northern blot analysis was performed. Membranes were hybridized with labeled cDNA probes for CPT1. The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments performed indicating the fold variation in mRNA levels (ratio of CPT1 mRNA to 18S in each sample) relative to basal. Results are expressed as means±S.D. of three independent experiments performed, **P<0.01; *P<0.05 versus BASAL.

Figure 2: Effect of WY and GW on lactate production in Sertoli cells. Sertoli cells were maintained under BASAL conditions or stimulated with 1 and 10 μM WY for 48 h **(A)**. Additionally, Sertoli cells were maintained under BASAL conditions or stimulated with 1 and 5 μM GW for 48 h **(B)**. Lactate was determined in the conditioned media. Pooled data of three independent experiments performed are shown. Values are expressed as means±S.D.. ***P<0.001 versus BASAL.

Figure 3: Effect of WY and GW on glucose uptake and on GLUT1, LDH A and MCT4 mRNA levels in Sertoli cells. Sertoli cells were incubated for 48 h in the absence or presence of 1 and 10 μM WY **(A)** or in the absence or presence of 1 and 5 μM GW **(B).** Glucose uptake assay (2-DOG uptake) was performed after the corresponding incubation period. Additionally, Sertoli cells were incubated for variable

periods of time (24 and 48 h) with 10 μM WY (**C, E, G**) or 5 μM GW (**D, F, H**). C, D, G and H: total cellular RNA was then extracted and RQ-PCR was performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means±S.D of three independent experiments performed. E and F: Northern blot analysis was performed. Membranes were hybridized with labeled cDNA probes for LDH A. The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments performed indicating the fold variation in mRNA levels (ratio of LDH A mRNA to 18S in each sample) relative to basal. No statistically significant differences were found.

Figure 4: Effect of WY and GW on P-PDC levels in Sertoli cells. Sertoli cells were incubated for variable periods of time (24 and 48 h) with 10 μM WY (**A**) or 5 μM GW (**B**). Cell extracts were utilized for Western blot analysis using antibodies specific for P-PDC or T-Akt. The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments indicating the fold variation in phosphorylation (ratio of P-PDC to T-Akt in each sample) relative to BASAL. Results are expressed as mean±S.D., *P<0.05 versus BASAL.

Figure 5: Effect of WY and GW on PDKs and PDPs mRNA levels in Sertoli cells. Sertoli cells were incubated for variable periods of time (24 and 48 h) with 10 μM WY (**A, C, E, G, I, K**) or 5 μM GW (**B, D, F, H, J, L**). Total cellular RNA was then extracted and RQ-PCR was performed. The comparative ΔΔCt method was used to calculate relative gene expression. Results are expressed as means±S.D of three independent experiments performed, **P<0.01; *P<0.05 versus BASAL.

Figure 6: A schematic model of the mechanisms involved in the FA and lactate metabolism in Sertoli cells. The activation of PPAR α and PPAR β/δ induces the transcription of FAT/CD36, CPT1, LCAD and MCAD, leading to an increase in FA transport and oxidation (pink pathway). On the other hand, only the activation of PPAR β/δ promotes lactate increment and increases P-PDC levels, by the induction of the PDKs transcription (blue pathway).

HIGHLIGHTS

1. In Sertoli cells PPARα and PPARβ/δ regulate expression of genes involved in fatty acid metabolism
2. PPARβ/δ activation increases Sertoli cell lactate production
3. PPARβ/δ activation increases Phosphorylated Pyruvate Dehydrogenase Complex levels in Sertoli cells

Table 1: Effects of WY14643 (WY) and GW0742 (GW) on the LDH activity

	mIU/µg DNA
BASAL	22.9±1.8
WY 10 µM 48h	25.2±0.1
GW 5 µM 48h	22.2±0.1

Sertoli cells were incubated under BASAL conditions or stimulated for 48 h with 10 mM WY or 5 mM GW. LDH activity was determined on cells harvested at the end of the treatment. Results are expressed as mean± S.D., of triplicate incubation in one representative experiment out of three. No statistically significant differences were found.

Figure 1

Figure 1

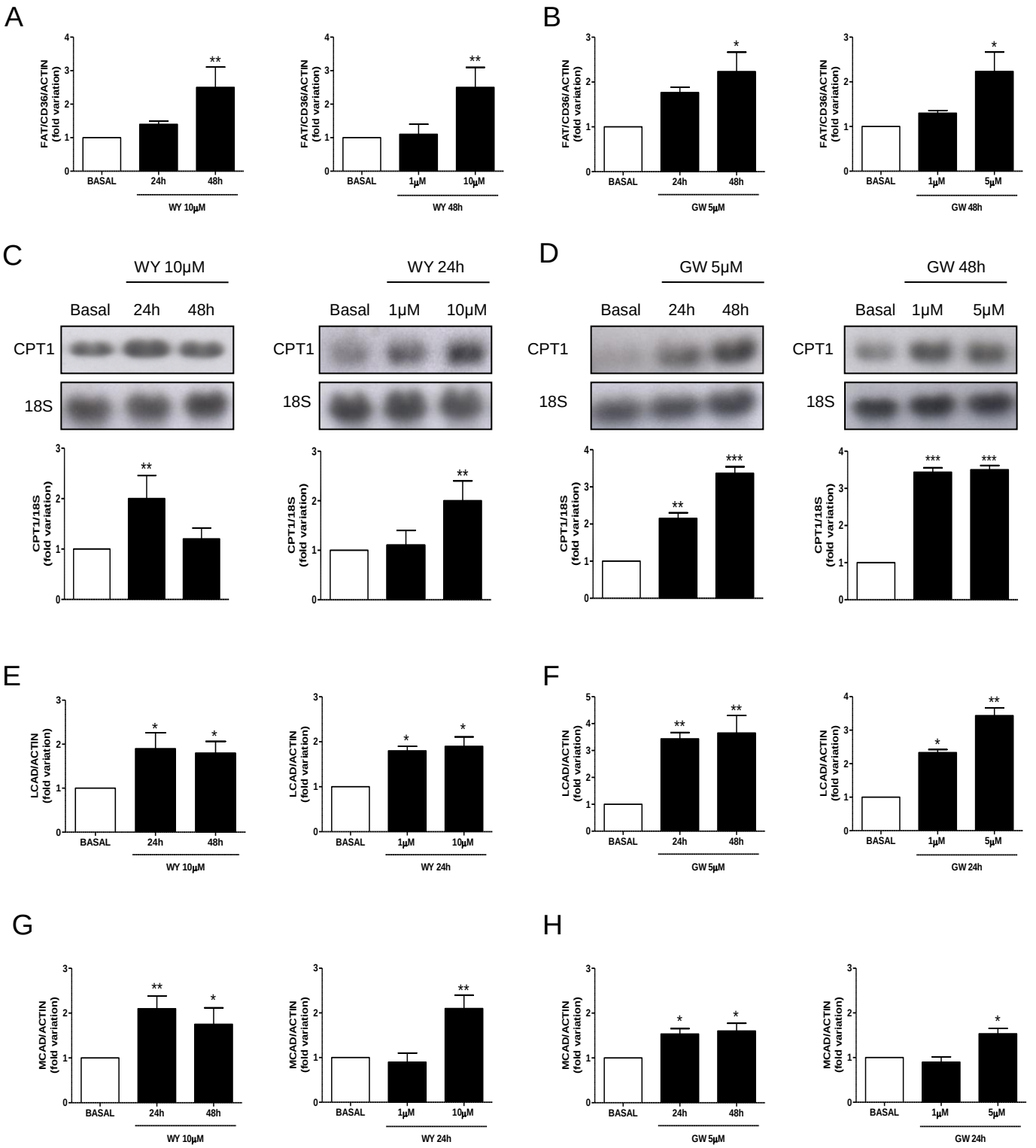

Figure 2

Figure 2

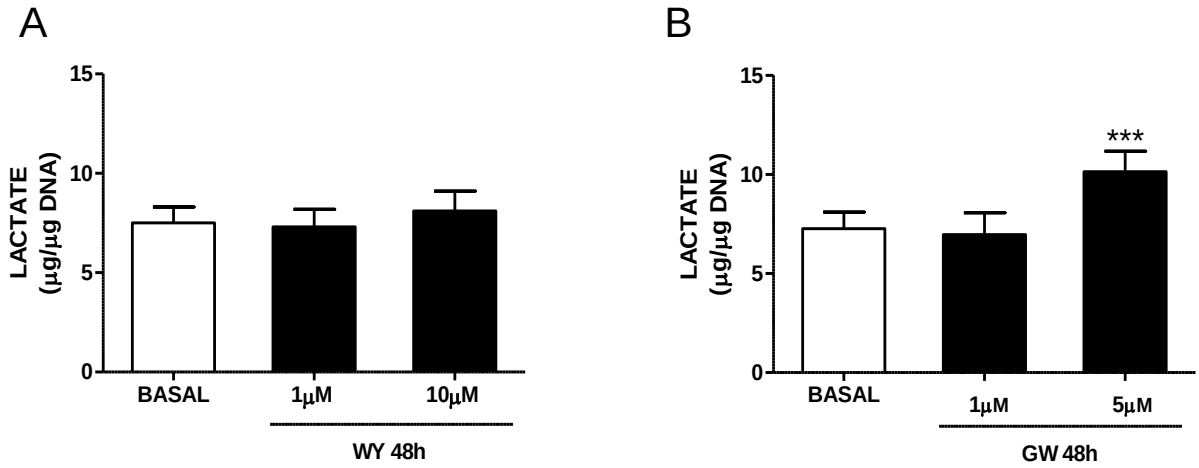

Figure 3

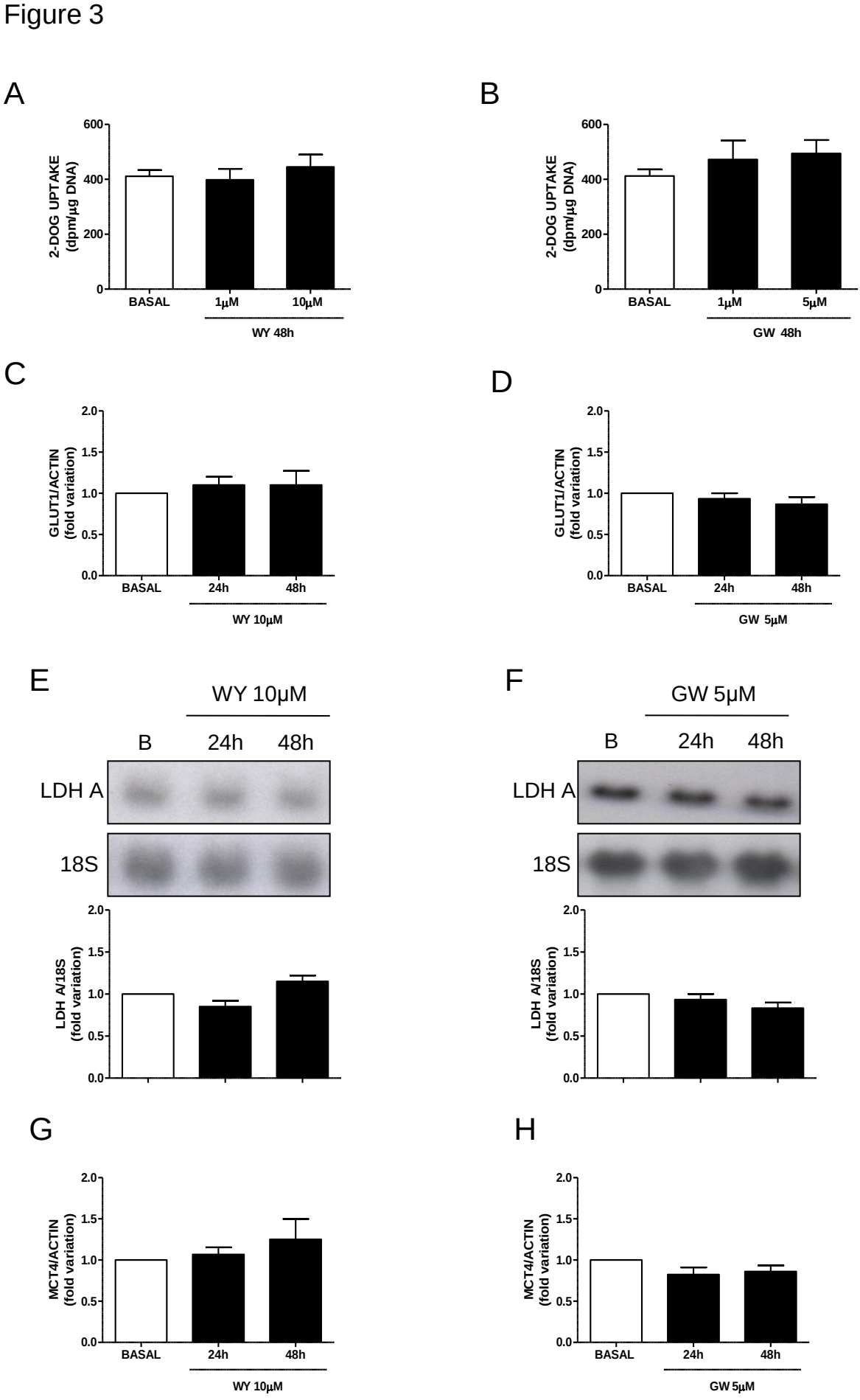
Figure 3
A
2-DOG UPTAKE (dpm/µg DNA)
600
400
200
0
BASAL
1µM
10µM
WY 48h
B
2-DOG UPTAKE (dpm/µg DNA)
600
400
200
0
BASAL
1µM
5µM
GW 48h
C
GLUT1/ACTIN (fold variation)
2.0
1.5
1.0
0.5
0.0
BASAL
24h
48h
WY 10µM
D
GLUT1/ACTIN (fold variation)
2.0
1.5
1.0
0.5
0.0
BASAL
24h
48h
GW 5µM
E
WY 10µM
B
24h
48h
LDH A
18S
LDH A/18S (fold variation)
2.0
1.5
1.0
0.5
0.0
F
GW 5µM
B
24h
48h
LDH A
18S
LDH A/18S (fold variation)
2.0
1.5
1.0
0.5
0.0
G
MCT4/ACTIN (fold variation)
2.0
1.5
1.0
0.5
0.0
BASAL
24h
48h
WY 10µM
H
MCT4/ACTIN (fold variation)
2.0
1.5
1.0
0.5
0.0
BASAL
24h
48h
GW 5µM

Figure 4

Figure 4

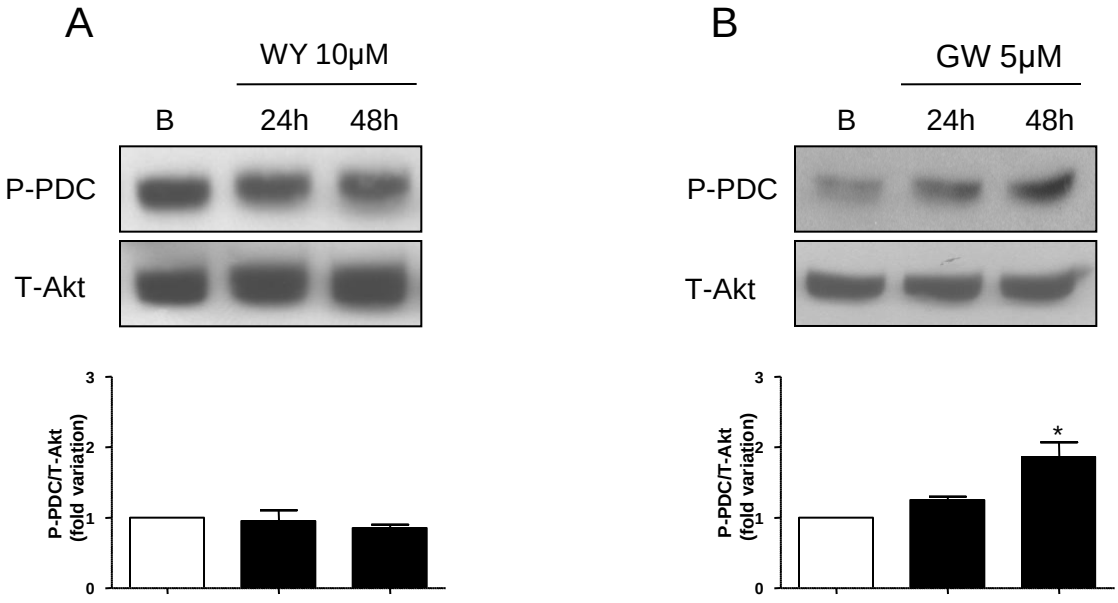

Figure 5

Figure 5

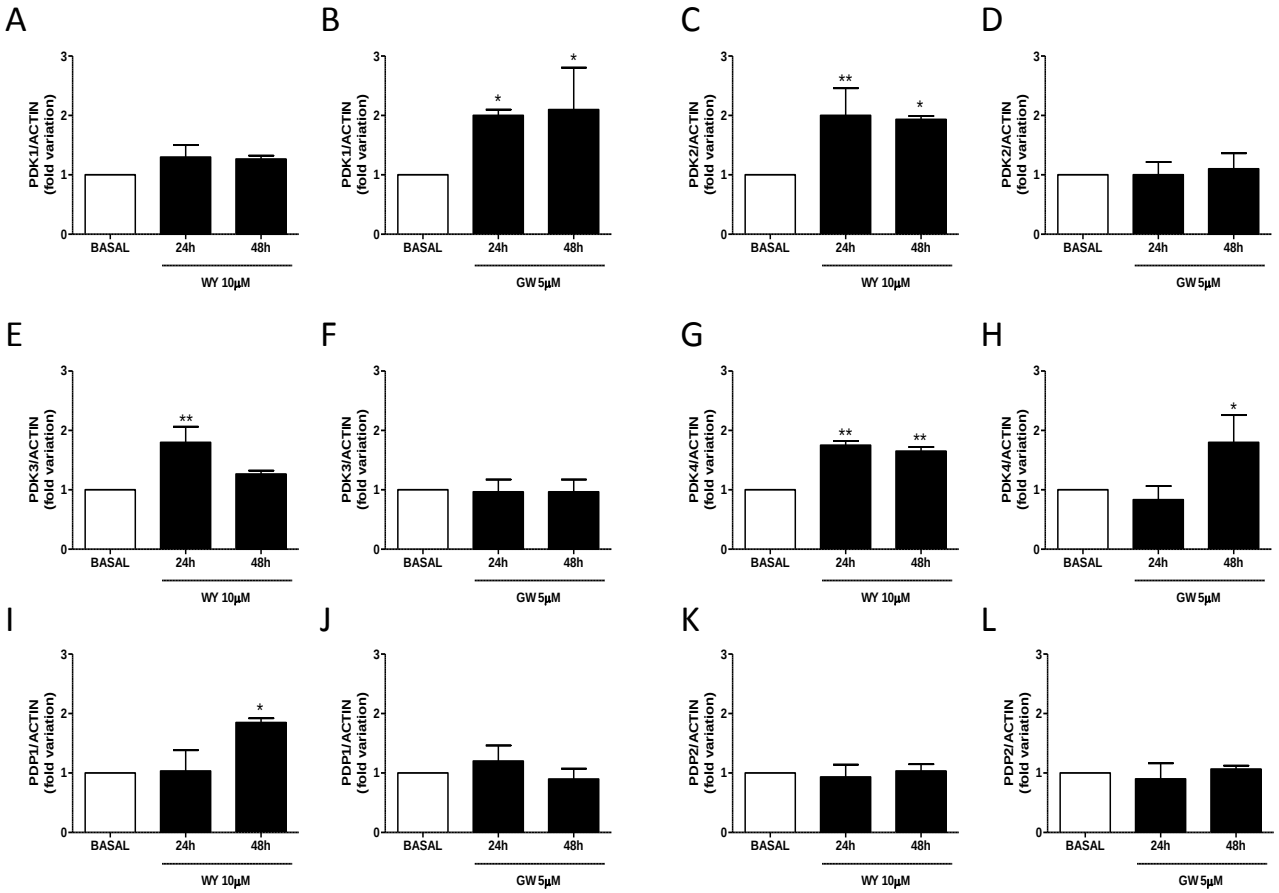

Figure 6

Figure 6