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RESEARCH ARTICLE

Sperm metabolism in pigs: a role for peroxisome proliferator-activated receptor gamma (PPARγ)

M. Santoro[1,2,*], C. Guido[1,3,*], F. De Amicis[1,3], D. Sisci[1,3], D. Vizza[1,3], S. Gervasi[4], A. Carpino[1,5,†] and S. Aquila[1,3,†,‡]

[1]Centro Sanitario, [2]Post-graduate School in Clinical Pathology and [3]Department of Pharmaco-Biology, University of Calabria, 87036 Arcavacata di Rende (CS), Italy, [4]University of Magna Græcia (CZ), Italy and [5]Department of Cellular Biology, University of Calabria, 87036 Arcavacata di Rende (CS), Italy

*These authors contributed equally to this work
†Joint senior authors
‡Author for correspondence (aquisav@libero.it)

SUMMARY

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear hormone receptor expressed predominantly in adipose tissue, also implicated in energy homeostasis. In this study, we used western blotting and immunofluorescence techniques to demonstrate for the first time that pig spermatozoa express PPARγ. To investigate the functional role of PPARγ in pig sperm, we evaluated its action on different events that characterize the biology of sperm cells, i.e. motility, capacitation, viability and acrosome reaction, using the PPARγ-agonist 15-deoxy-12,14-prostaglandin J2 (PGJ2). In responses to PGJ2 treatment, motility, cholesterol efflux and tyrosine phosphorylation were increased, which broadens the role of PPARγ from that previously described in the literature, as it also acts to improve sperm functionality. To further our understanding of the significance of PPARγ in pig sperm, we focused its effects on lipid and glucose metabolism. Evaluation of triglyceride content and lipase, acyl-CoA dehydrogenase and G6PDH activities suggests that PPARγ induces energy expenditure in pig spermatozoa. These data represent a meaningful advance in the field of sperm energy metabolism. Taken together, our results demonstrate for the first time that PPARγ is expressed by pig sperm, thus improving its functionalities in terms of motility, capacitation, acrosome reaction, survival and metabolism.

Key words: spermatozoa, prostaglandin, reproduction, nuclear receptor, PPARgamma.

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. PPARγ is a nuclear fatty acid receptor that has been implicated in energy homeostasis and in many pathological processes (Knouff and Auwerx, 2004). Particularly, it modulates lipid homeostasis in metabolically active sites, such as the liver, macrophage, adipocytes and muscle (Rangwala and Lazar, 2004; Kota et al., 2005). PPARγ is mainly expressed in adipose tissue (Gurnell, 2005), but this receptor is also present in several cell types (Nicol et al., 2005; Wang et al., 2006; Kostadinova et al., 2005). It also acts as a regulator of inflammation (Celinski et al., 2011) and is activated by endogenous arachidonic acid metabolites such as 15-deoxy-12,14-prostaglandin J2 (PGJ2) (Desvergne and Wahli, 1999; Kobayashi et al., 2005).

Intensive studies and compelling evidence have demonstrated a close link between energy status and reproductive function (Quandt, 1984; Moschos et al., 2002). In mice, the loss of the PPARγ gene in oocytes and granulosa cells resulted in impaired fertility (Cui et al., 2002). In our previous study, we demonstrated that human sperm express PPARγ and the functionality of this receptor was also investigated (Aquila et al., 2006). Many studies have shown that transcriptional factors as nuclear receptors, in addition to their classic genomic action, also regulate cellular processes through their nongenomic mechanisms (Cato et al., 2002). Different nuclear receptors, such as progesterone receptors (Shah et al., 2005; De Amicis et al., 2011), estrogen receptor α, estrogen receptor β and androgen receptors (Aquila et al., 2004; Aquila et al., 2007; Guido et al., 2011), were found to be present in ejaculated human spermatozoa, regulating some cellular processes. Ejaculated mammalian spermatozoa are highly differentiated attractive cells showing intriguing features; in particular, they go through two different physiological conditions: a quiescent metabolic state in the male genital tract, and an enhanced energy metabolism to accomplish their functional maturation, known as capacitation, in the female genital tract (Rathi et al., 2001; Yanagimachi, 1994; Suarez, 2008). In recent years, a new picture of the sperm cell is emerging: it expresses various receptor types (Travis and Kopf, 2002; Aquila et al., 2007), and it also produces their ligands, suggesting that through an autocrine short loop it may modulate its own functions independently by the systemic regulation (Aquila et al., 2005a; Aquila et al., 2005b). Nevertheless, sperm need to have a finely regulation of metabolism to affect the changes in signaling pathways encountered during their life. However, the mechanisms underlying the signaling events associated with the change in sperm energy metabolism are, to date, poorly understood.

In the current study we show for the first time that pig spermatozoa express PPARγ, broadening the role of this nuclear receptor, as it is also able to act on sperm physiology.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA) protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal PVP coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide, 15-deoxy-12,14-prostaglandin J2 (PGJ2), the irreversible PPARγ antagonist GW9662 (GW), phosphate buffered saline (PBS) and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Earle's balanced buffered solution (BBS) without calcium, without magnesium, without phenol red, without $NaHCO_3$ was obtained from Genaxxon Bioscience (Milan, Italy; product no. C4228.0500, unsupplemented Earle's medium). Acrylamide bisacrylamide was purchased from Labtek Eurobio (Milan, Italy). Triton X-100 and Eosin Y were purchased from Farmitalia Carlo Erba (Milan, Italy). A gel band purification kit, the ECL Plus western blotting detection system, Hybond^{TM} ECL^{TM} and Hepes sodium salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Cholesterol-oxidase–peroxidase (CHOD–POD) enzymatic colorimetric, triglyceride, acyl-CoA dehydrogenase, lipase activity and glucose-6-phosphate dehydrogenase (G6PDH) assays were purchased from Inter-Medical (Biogemina Italia, Catania, Italy). Goat polyclonal actin antibody (Ab) (1-19), polyclonal rabbit anti-PPARγ Ab, rabbit anti-p-Akt1/Akt2/Akt3 S473 (AKTS) Ab, rabbit anti-p-Akt1/Akt2/Akt3 T408 (AKTT) Ab, total anti-Akt1/Akt2 Ab, rabbit anti-p-MAPK 42/44 Ab, total anti-MAPK 42/44 Ab, rabbit anti-p-Bcl2 Ab and total anti-Bcl2 Ab, were purchased from Cell Signaling (Milan, Italy); polyclonal rabbit anti-phosphotyrosine (PY99) Ab, peroxidase-coupled anti-rabbit and anti-goat Ab, anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugated Ab and Protein A/G-agarose plus were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Semen samples and spermatozoa preparations

The investigation was conducted on semen from eight fertile male pigs (*Sus scrofa domestica* Erxleben 1777; large white) kept at the Swine Artificial Insemination Centre (Rende, Cosenza, Italy). The animals were 20 to 30 months old and they weighed from 260 to 330 kg. Individual fresh ejaculates were collected using the gloved hand method and filtered immediately using Universal Semen bags (Minitub, Tiefenbech, Germany). Individual sperm-rich ejaculates were collected. Semen analysis showed normal sperm parameters in all the ejaculates [200–300 ml volume, total spermatozoa per ejaculate: 30×10^9–60×10^9, progressive sperm motility 70–90%, sperm abnormalities (clumping, abnormal tails, abnormal acrosome, etc.): 20–30%]. Semen was transported within half an hour to the laboratory. It was diluted 1:10 with Tris-buffered saline (TBS) buffer and centrifuged on a discontinuous Percoll density gradient (72%/90%) to remove eventual bacteria and debris. Samples were then centrifuged at 700 ***g*** for 30 min and the upper phase was used to determinate the cholesterol levels, while the pellet containing sperm was lysed to perform western blots and triglyceride and acyl-CoA dehydrogenase assays, and determine G6PDH and lipase activity. Prior to centrifugation, several aliquots were used to perform sperm motility and viability analyses. Spermatozoa preparations were as previously described (Aquila et al., 2002).

Processing of ejaculated sperm

Percoll-purified sperm were washed once with unsupplemented Earle's medium for 10 min at 800×***g***, and then incubated in the same medium for 30 min at 39°C and 5% CO_2, without (control) or with treatment (experimental). Some samples were incubated in Earle's BBS medium supplemented with 25 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} $CaCl_2$, 0.6% BSA and 1 mmol l^{-1} pyruvate (pH 7.4) (capacitating medium), used only for the capacitated sample in the immunofluorescence assay. When the cells were treated with an inhibitor, a pre-treatment of 15 min was performed and subsequently the sperm were incubated with the substances reported in the manuscript for 30 min.

To evaluate pig sperm viability, experiments were performed under three conditions: (1) in freshly ejaculated sperm, before any incubation (without or with increasing PGJ2) at time 0 min; (2) after being purified by Percoll, treated without or with PGJ2 at time 0 min; and (3) Percoll-purified sperm incubated without PGJ2 or with increasing PGJ2 concentrations at 30 min as described above.

Evaluation of sperm motility and viability

Sperm motility was assessed by means of light microscopy examining an aliquot of each sperm sample in the absence (NC) or presence of increasing PGJ2 concentrations (1 to 20 μmol l^{-1}) and/or 10 μmol l^{-1} GW alone or combined with 10 μmol l^{-1} PGJ2. Sperm motility was expressed as percentage of total motile sperm. An independent observer scored at least 200 cells.

Viability of pig spermatozoa was assessed using the DNA-specific fluorochrome propidium iodide (PI). Sperm suspension (1×10^9 ml) was exposed to PI (12 μmol l^{-1}) for 5 min at room temperature. Then spermatozoa were fixed by adding 1 ml of 12.5% (w/v) paraformaldehyde in 0.5 mol l^{-1} Tris (pH 7.4) and the slides were immediately examined under an epifluorescence microscope (Olympus BX41, Olympus Italia, Segrate, Milan, Italy), observing a minimum of 200 spermatozoa per slide (×100 objective).

Western blot analysis of sperm proteins

Western blot analysis was used to identify PPARγ in pig spermatozoa. Sperm samples, washed twice with unsupplemented Earle's medium for 10 min at 800×***g***, were incubated without or with the indicated treatments, and then centrifuged for 5 min at 5000×***g***. The pellet was resuspended in lysis buffer as previously described (Aquila et al., 2002). Equal amounts of protein (80 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with an appropriate dilution of the indicated Ab. The bound of the secondary Ab was revealed with the ECL Plus western blotting detection system according to the manufacturer's instructions. The negative control consists of a sperm lysate that was immunodepleted of PPARγ (Aquila et al., 2004) (i.e. the lysate was preincubated with anti-PPARγ Ab for 1 h at room temperature, and immunoprecipitated with Protein A/G-agarose). To further verify the specificity of our Ab anti-PPARγ, the blot was probed with the normal rabbit serum instead of the primary Ab. As an internal control, all membranes were subsequently stripped (glycine 0.2 mol l^{-1}, pH 2.6 for 30 min at room temperature) of the first Ab and reprobed with anti-β-actin. The protein bands were quantified by scanning densitometry (Imaging Densitometer GS-700, Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was also performed to evaluate protein tyrosine phosphorylations.

Immunofluorescence assay

Following Percoll separation, sperm cells were rinsed three times with 0.5 mmol l^{-1} Tris-HCl buffer, pH 7.5, and fixed with absolute methanol for 7 min at –20°C. After methanol removal, sperm cells were washed in TBS containing 0.1% Triton X-100 and were treated for immunofluorescence. PPARγ staining was carried out after blocking with normal horse serum (10%) using a rabbit polyclonal anti-human PPARγ as the primary Ab and an anti-rabbit IgG FITC

conjugated (1:100) Ab as the secondary Ab. Sperm cells incubated with normal rabbit serum instead of the primary Ab were utilized as the negative control. The slides were examined under a fluorescence microscope (Olympus BX41), and a minimum of 200 spermatozoa for each slide was scored.

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured in duplicate by a CHOD–POD enzymatic colorimetric method according to manufacturer's instructions in the incubation medium from human spermatozoa. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) or in capacitating medium for 30 min at 39°C and 5% CO_2. Some samples were incubated in the presence of increasing PGJ2 concentrations (1 to 20 μmol l^{-1}). Other samples were incubated in the presence of 10 μmol l^{-1} GW, either alone or combined with 10 μmol l^{-1} PGJ2. At the end of the sperm incubation the culture media were recovered by centrifugation, lyophilized and subsequently dissolved in 1 ml of buffer reaction. The samples were incubated for 10 min at room temperature, and then the cholesterol content was measured with the spectrophotometer at 505 nm. The cholesterol standard used was 200 mg dl^{-1}. The limit of sensitivity for the assay was 0.03 mg dl^{-1}. Inter- and intra-assay variations were 0.03 and 0.02%, respectively. Results are presented as cholesterol amount (mg dl^{-1}) in culture medium from 10×10^6 sperm and are given as means ± s.e.m.

Triglyceride assay

Triglycerides were measured in duplicate using a glycerol phosphate oxidase–peroxidase enzymatic colorimetric method according to the manufacturer's instructions (Biogemina Italia, Catania, Italy) in sperm lysates and as previously described (Aquila et al., 2006; Aquila et al., 2009). Data are presented as mg 10^{-9} sperm.

Lipase activity assay

Lipase activity was evaluated by the method of Panteghini et al. (Panteghini et al., 2001) based on the use of DGGR [1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester] as substrate, as previously described (Aquila et al., 2006; Aquila et al., 2009).

Acyl-CoA dehydrogenase activity assay

Acyl-CoA dehydrogenases are a class of enzymes that function to catalyze the initial step in each cycle of fatty acid β-oxidation. An assay of acyl-CoA dehydrogenase was performed on sperm, using a modification of the method described by Lehman et al. (Lehman et al., 1990) as previously described (Aquila et al., 2006).

G6PDH activity assay

An assay of G6PDH activity was performed on sperm lysates in the same experimental conditions as those described above. The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm, as previously described (Aquila et al., 2005b; Aquila et al., 2006; Aquila et al., 2009).

Acrosome reaction

The evaluation of the acrosome reaction was performed using FITC-peanut agglutinin (PNA). At the end of incubation, sperm cells were washed three times with 0.5 mmol l^{-1} Tris-HCl buffer (pH 7.5) and were allowed to settle onto slides. Smears, dried in air, were dipped in absolute methanol for 15 min and left at room temperature. The samples were then incubated with a solution of FITC-PNA in a humid chamber at room temperature. After 30 min, the slides were washed with PBS to remove the excess label. Scoring of the staining was immediately assessed, using an epifluorescence microscope (Olympus BX41) according to a published scoring system (Cheng et al., 1996). A minimum of 200 live sperm was examined for each treatment, as previously reported (Aquila et al., 2011). Values are expressed as percentage of acrosome-reacted cells.

Statistical analysis

The experiments for western blot and immunofluorescence analysis were performed in at least three independent experiments. The data from motility and viability experiments, and the CHOD–POD enzymatic colorimetric method, triglyceride, acyl-CoA dehydrogenase, G6PDH and lipase activity assays were obtained from at least six replicate experiments using duplicate determinations and are presented as means ± s.e.m. The differences in mean values were calculated using ANOVA with a significance level of $P\leq0.05$.

RESULTS

Pig sperm contains PPARγ

Using an Ab raised against the carboxyl terminus of the human PPARγ protein, we explored the presence of PPARγ in ejaculated pig sperm by western blot analysis. A band corresponding to the molecular mass values of 70 kDa (Fig. 1A) was evident in pig sperm extracts, as in the human sperm used as positive control. The band was not detected in the negative control (lane 2), performed as previously described (Aquila et al., 2007), or when the non-immune rabbit serum (Fig. 1B) was used instead of the primary Ab, further confirming the specificity of the Ab for PPARγ.

PPARγ is located prevalently in the midpiece

In the majority of the sperm population, as detected by the immunofluorescence assay with the same Ab as that used for western blot, PPARγ was found in the apical region of the head, in the subacrosomial region and prevalently in the midpiece, while the signaling was almost absent in the tail (Fig. 2A). Capacitated sperm

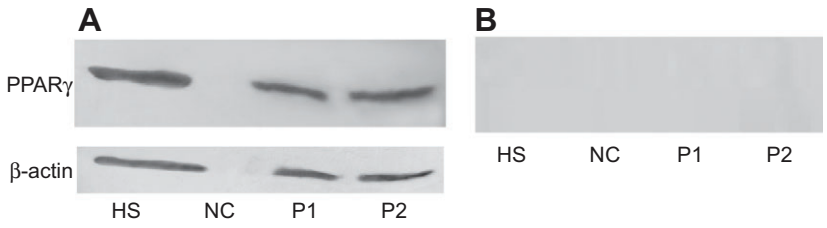

Fig. 1. Ejaculated pig spermatozoa contains PPARγ. (A) Western blot of PPARγ protein in pig spermatozoa; expression in two samples of ejaculated spermatozoa from normal pigs (P1, P2). Human sperm extract was used as control (HS). The negative control (see Materials and methods) is represented in lane 2 (NC), β-actin was used as loading control. (B) To further verify the specificity of our anti-PPARγ antibody (Ab), the blot was probed with the normal rabbit serum instead of the primary Ab. The autoradiographs show the results of one representative experiment.

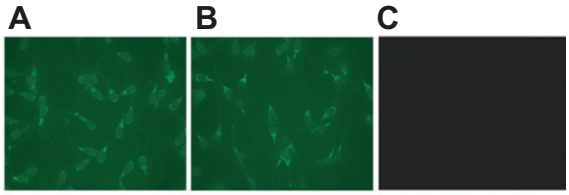

Fig. 2. PPARγ compartmentalization in ejaculated pig spermatozoa. (A) PPARγ immunolocalization in uncapacitated sperm. (B) PPARγ immunolocalization in capacitated sperm. (C) Sperm cells incubated replacing the anti-PPARγ Ab with normal rabbit serum were utilized as negative control. The images shown are representative examples of experiments that were performed at least three times with reproducible results.

are shown in Fig. 2B, and it appears that the location of the receptor mirrors that observed in uncapacitated sperm. No fluorescent signal was obtained when the normal rabbit IgG was used instead of the primary Ab (Fig. 2C).

PGJ2 influences motility through PPARγ in pig sperm

Sperm motility indicates the ability of sperm to move properly towards an egg. In all experiments, the natural PPARγ ligand PGJ2 (at concentrations of 1, 10 and 20 μmol l^{-1}) induced an increase in sperm motility that was significant at the 10 μmol l^{-1} concentration. Interestingly, the effect was reduced by the specific GW antagonist, suggesting a PPARγ-mediated action (Fig. 3).

PGJ2 induces capacitation in pig sperm

Successively, we investigated whether the PPARγ agonist was able to influence the functional maturation of sperm by evaluating its action on capacitation. This unique process has been correlated with functional and biochemical changes in sperm, including cholesterol efflux (Travis and Kopf, 2002) and tyrosine phosphorylation of sperm proteins (Visconti et al., 1995). Washed sperm were treated with increasing concentrations of PGJ2 (1, 10 and 20 μmol l^{-1}). At the end of incubation the samples were centrifuged, and the upper phase was employed to determinate cholesterol levels, while the pellet (sperm) was lysed to study protein tyrosine phosphorylation. The outcomes indicate that cholesterol efflux increased from the 1 μmol l^{-1} to the 10 μmol l^{-1} PGJ2 treatment, while the 20 μmol l^{-1} did not induce any further effect (Fig. 4A). Similar results were obtained for tyrosine phosphorylations of the sperm proteins (Fig. 4B). Both processes were attenuated using the specific PPARγ-antagonist GW, suggesting an involvement of this receptor.

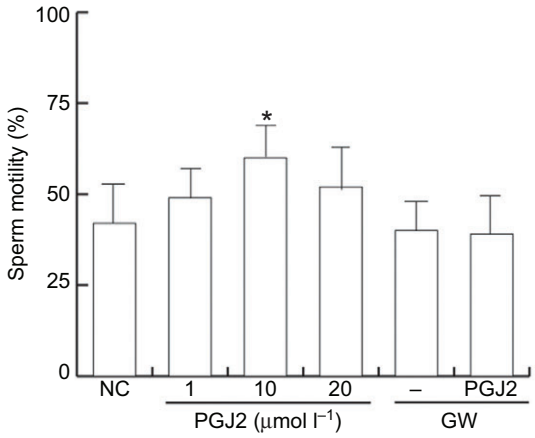

Fig. 3. Effects of 15-deoxy-12,14-prostaglandin J2 (PGJ2) on sperm motility are PPARγ-mediated. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 39°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentrations of PGJ2 (1, 10, 20 μmol l^{-1}), or with 10 μmol l^{-1} irreversible PPARγ antagonist GW9662 (GW) alone or combined with 10 μmol l^{-1} PGJ2. Sperm motility is expressed as a percentage of total motile sperm. Data are means ± s.e.m. of six independent experiments performed in duplicate. *P<0.05 *versus* control.

PPARγ influences viability by inducing AKT, BCL2 and MAPK 42/44 phosphorylation in pig spermatozoa

Sperm survival is an important aspect of ejaculate quality that determines fertilization success. From our results it emerges that PPARγ increases sperm viability from 1 to 10 μmol l^{-1} PGJ2, while 20 μmol l^{-1} did not induce further effect (Fig. 5A). These effects were obtained in the samples after incubation, while in freshly ejaculated sperm or after being purified by Percoll, both before any incubation, did not result in any substantial variation.

To investigate this sperm activity from a molecular point of view, we evaluated the effect of PGJ2, exploring the most important signaling involved in cell survival and previously investigated in human and pig sperm, the PI3K/Akt pathway (Fisher et al., 1998; Aquila et al., 2004) and MAPK 42/44 phosphorylation (Almog and Naor, 2008). As shown in Fig. 5, increasing doses of PGJ2 (1, 10 and 20 μmol l^{-1}) resulted in a significant increase in the AKTS (Fig. 5B) and AKTT (Fig. 5C) phosphorylations. AKT, the major downstream PI3K signal transducer, is fully activated when both S473 and T308 phosphorylations were enhanced (Aquila et al., 2007). In the same vein, the PPARγ agonist induced MAPK 42/44 phosphorylation (Fig. 5D). BCL2, a key protein in survival signaling (Ito et al., 1997) phosphorylated at Serine 70, the physiologically relevant phosphorylation site necessary for its full survival function, is enhanced upon exposure to PGJ2 (Fig. 5E). All these events were reduced by GW, confirming the involvement of PPARγ.

PPARγ modulates triglycerides, lipase activity and β-oxidation of the fatty acids in pig sperm

The main role of PPARγ is in lipid metabolism; therefore, we evaluated the intracellular level of triglycerides, lipase activity and the β-oxidation of the fatty acids under PPARγ agonist treatments. Upon the abovementioned treatments, sperm triglyceride levels were decreased (Fig. 6A) and, concomitantly, lipase activity and β-oxidation of the fatty acids were augmented (Fig. 6B,C). GW was able to attenuate the PPARγ-agonist effects.

PPARγ affects G6PDH activity and acrosome reaction in pig spermatozoa

In somatic cells, the role of PPARγ in glucose metabolism has been explored (Lenhard et al., 1997). The effect of glucose on the fertilizing ability of sperm appears to be mediated by the pentose phosphate pathway (PPP) (Miraglia et al., 2010), and G6PDH is the key rate-limiting enzyme in this metabolic pathway, and has been shown to be functional in human spermatozoa (Aquila et al., 2009). As shown in Fig. 7A, PGJ2 activates G6PDH activity at concentrations of 10 and 20 μmol l^{-1}. Thus, our results indicate a regulatory role of PPARγ in sperm glucose metabolism. The effect of PGJ2 on acrosome reaction (Fig. 7B) showed a pattern of response similar to that observed in our experiments and that previously reported (Joyce et al., 1987).

DISCUSSION

PPARγ, a nuclear hormone receptor, has an important role in the control of energy, lipid and glucose homeostasis (Rangwala and

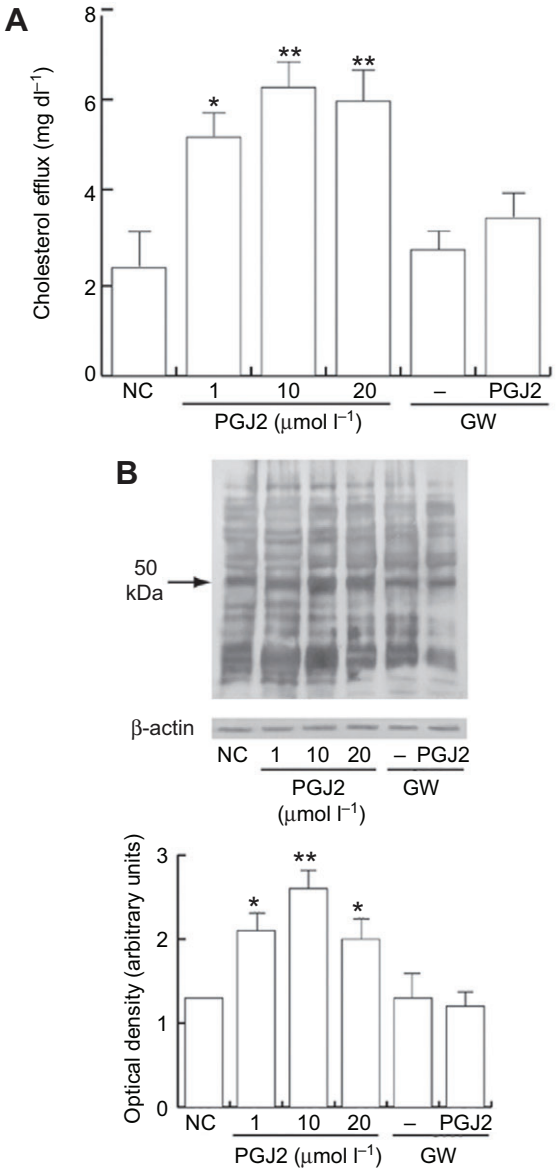

Fig. 4. Cholesterol efflux and tyrosine phosphorylation of sperm proteins increase upon PGJ2 exposure. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 39°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentrations of PGJ2 (1, 10, 20 μmol l^{-1}), or with 10 μmol l^{-1} GW alone or combined with 10 μmol l^{-1} PGJ2. (A) Cholesterol in culture medium from ejaculated pig spermatozoa was measured by enzymatic colorimetric assay. Data are means ± s.e.m. of six independent experiments performed in duplicate. Results are presented as cholesterol amount (mg dl^{-1}) in culture medium from 10×10^6 sperm. (B) Eighty micrograms of sperm lysates were used for western blot analysis of protein tyrosine phosphorylations. Upper: quantitative representation after densitometry on the 50 kDa band. Data are means ± s.e.m. of six independent experiments. The autoradiograph shows a representative experiment. Lower: Densitometric evaluation of the proteins. *P<0.02 *versus* control; **P<0.01 *versus* control.

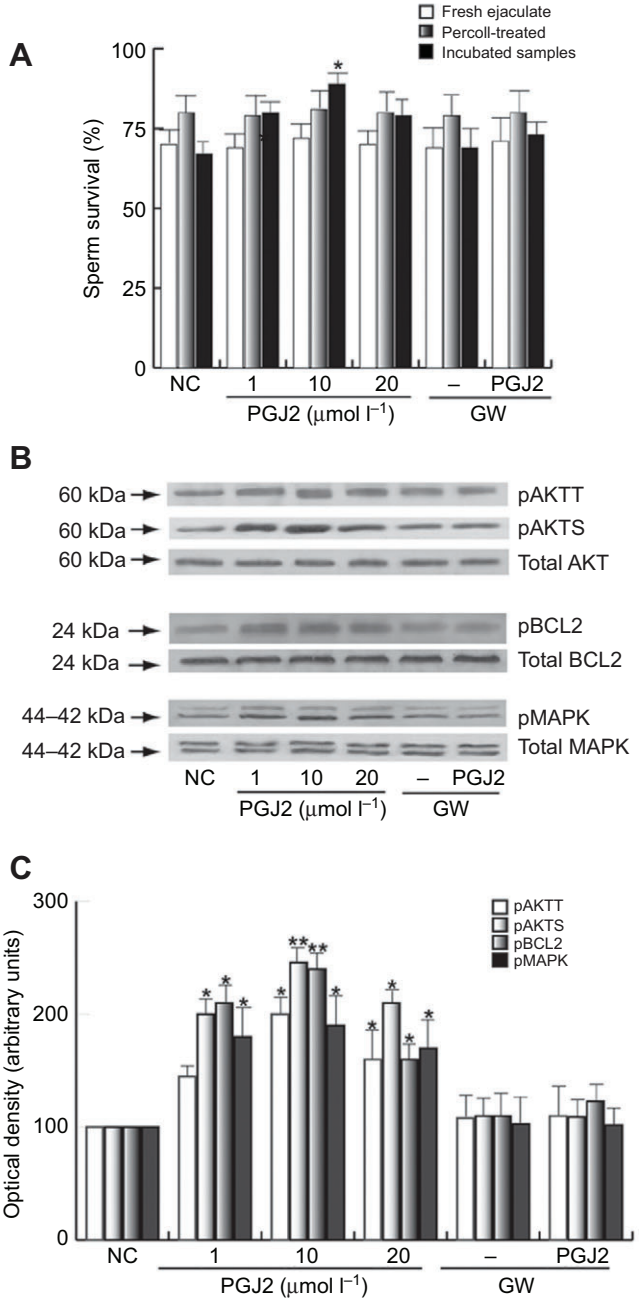

Fig. 5. PGJ2 increases survival as well as AKT, MAPK 42/44 and Bcl2 phosphorylation in pig sperm. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 39°C and 5% CO_2, and treated as indicated. (A) Viability upon increasing PGJ2 concentrations in freshly ejaculated sperm and after being purified by Percoll, before any incubation (with or without PGJ2) and in incubated sperm. (B) Exactly 80 μg of sperm lysates were used for western blot analysis of AktS, AktT, MAPK 42/44 and BCL2 phosphorylation. Loading controls: total AKT, total MAPK 42/44 and total BCL2. Molecular weights are shown for each protein. (C) Densitometric evaluation of the proteins. The autoradiographs are representative examples of experiments that were performed at least six times with repetitive results. *P<0.05 *versus* control; **P<0.01 *versus* control.

Lazar, 2004; Kota et al., 2005). Sperm energy management is currently a nebulous topic that needs to be examined in depth given the peculiarities of this cell type. In the present study, we showed for the first time that PPARγ is expressed in pig spermatozoa. Intriguingly, PPARγ was shown to have an effect on motility, survival, capacitation and sperm metabolism.

Using an Ab raised against the carboxyl terminus of the human PPARγ protein, western blot analysis showed a positive signal for PPARγ at the same molecular weight as was obtained in human spermatozoa used as positive control (Aquila et al., 2006). The

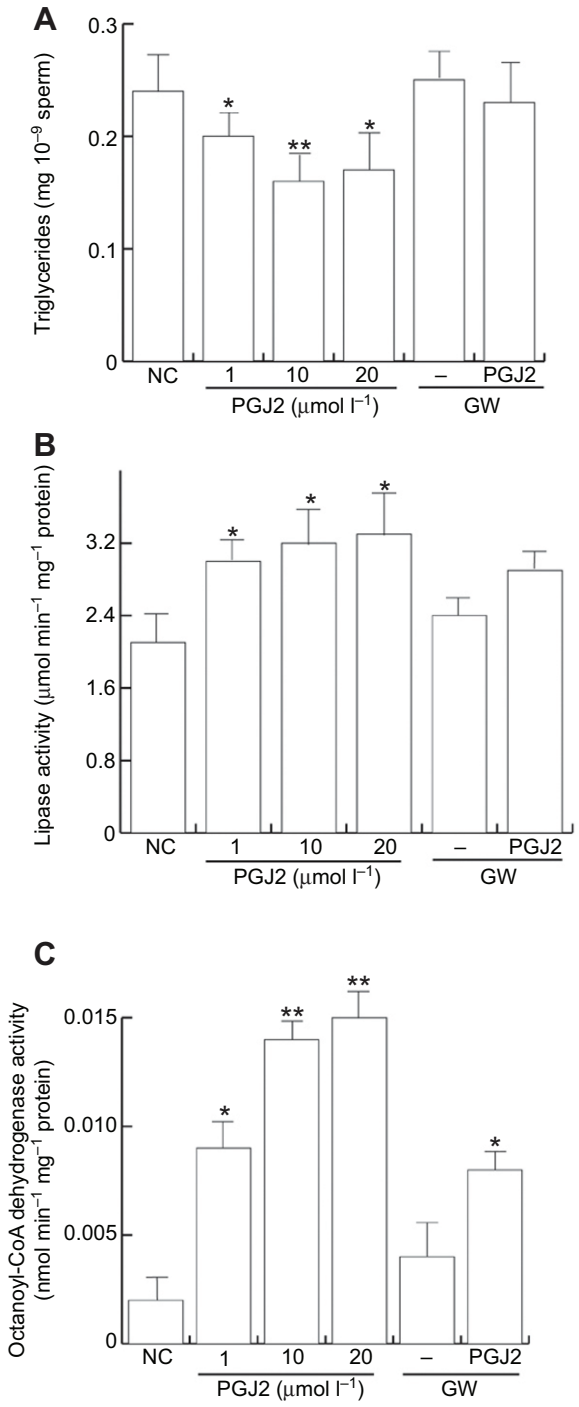

Fig. 6. PGJ2 modulates lipid metabolism in pig sperm. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (NC) for 30 min at 39°C and 5% CO_2 and treated as indicated. Assays of triglyceride (A), lipase activity (B) and acyl-CoA dehydrogenase activity (C) were performed as described in the Materials and methods. Data are means ± s.e.m. of six independent experiments performed in duplicate. **P*<0.05 *versus* control; ***P*<0.01 *versus* control.

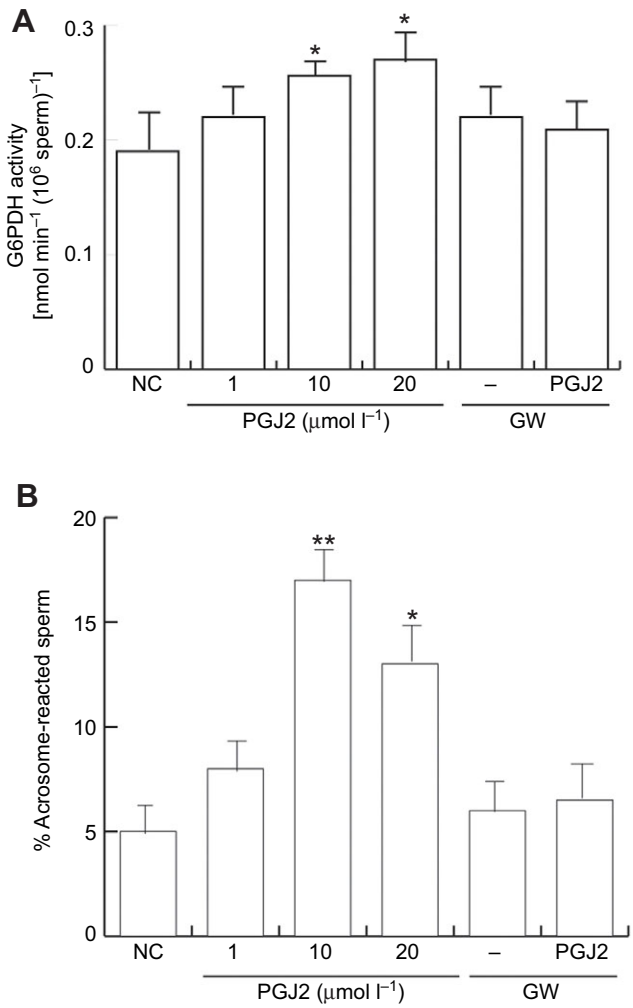

Fig. 7. G6PDH activity and acrosome reaction increased upon PGJ2 exposure. Sperm were washed with unsupplemented Earle's medium and were treated as indicated. (A) G6PDH activity was performed as described in the Materials and methods. Data are means ± s.e.m. of six independent experiments performed in duplicate. Data are expressed as nanomoles per minute per 10^9 sperm. **P*<0.05 *versus* control. (B) Acrosome reaction was determined as described in the Materials and methods and the values are expressed as percentage of acrosome-reacted cells. Data represent means ± s.e.m. of four independent experiments each done in duplicate. **P*<0.05 *versus* control; ***P*=0.02 *versus* control.

immuno-histochemical assays demonstrated that PPARγ protein was detectable in pig sperm, with specific signals being located in the apical region of the head, in the subacrosomial region and in the midpiece, while a weak label was obtained in the tail. These results are consistent with those from in human sperm (Aquila et al., 2006).

Furthermore, we evaluated the signaling in the capacitated sperm and it was not substantially different from that obtained in uncapacitated sperm.

Regarding motility, our results are in agreement with recent studies on human spermatozoa, where prostaglandins are reported to enhance this process (Aitken et al., 1986; Aitken and Kelly, 1985), and with our previous study, where we showed that the effects of these compounds are mediated by PPARγ (Aquila et al., 2006).

In this study, upon PGJ2 exposure we observed a significant increase in cholesterol efflux and tyrosine phosphorylation of sperm proteins. All these effects were PPARγ-mediated because they were reversed by the irreversible PPARγ antagonist GW. Data presented herein also demonstrated that PGJ2, by inducing the phosphorylation of classical key survival proteins such as MAPK 42/44, PI3K, AKT and BCL2, is involved in the survival of pig sperm. During their

life cycle, sperm pass through two different physiological conditions: uncapacitated, during which sperm remain in a quiescent metabolic state, when in the male genital tract or upon ejaculation, accumulating and/or economizing energy substrates; and capacitated, when sperm travel through the female genital tract, increasing the metabolic rate to acquire the competence to fertilize the oocyte.

A key role for PPARγ in energy metabolism in somatic cells has been established, and during the switch from the quiescent to the capacitated state the sperm show an increased metabolic rate, which is probably necessary for the changes in sperm signaling related to the capacitation process. The connection between the events of capacitation and the variations in sperm energy metabolism is not well understood. Our data on the analyses of lipid metabolism suggest a lipolytic effect of PPARγ, similar to the results shown for human sperm. Therefore, we hypothesize that during capacitation, when energy expenditure increases, PPARγ works to mobilize lipid reserves, providing additional metabolic fuel to sustain the capacitation process. The effect of glucose on the fertilizing ability of spermatozoa appears to be mediated by its metabolism through the PPP (De Amicis et al., 2011). Interestingly, our data showed that PPARγ was able to modulate in a dose-dependent way the activity of G6PDH, the key rate-limiting enzyme in the PPP. It is important to point out that in all our experiments, we obtained different responses using low or high PGJ2 concentrations; in fact, 1 and 10 $\mu mol\,l^{-1}$ were stimulatory, whereas the higher PGJ2 concentration (20 $\mu mol\,l^{-1}$) did not induce any further effect than that obtained with 10 $\mu mol\,l^{-1}$. Differences in ligand levels may result in a diverse response, as it was demonstrated in somatic cells (Castoria et al., 2003) as well as in human sperm (Aquila et al., 2005b).

Elevated levels of prostaglandins have been reported in seminal plasma (Templeton et al., 1978; Rodríguez-Martínez et al., 2009) and in cervical mucus (Charbonnel et al., 1982); in addition, sperm synthesize prostaglandins (Rodríguez-Martínez et al., 2009; Roy and Ratnam, 1992). Different studies have shown that prostaglandins enhance the fertilizing ability of sperm (Aitken et al., 1986; Aitken and Kelly 1985; Joyce et al., 1987), and our results on the acrosome reaction confirmed this role of prostaglandins. The autonomous capability of sperm to release prostaglandins suggests that they may act on PPARγ through an autocrine short loop to regulate energy management during the capacitation process (Aquila et al., 2006).

In conclusion, PPARγ influences pig sperm biology and physiology by regulating motility, capacitation, acrosome reaction, survival and metabolism.

LIST OF ABBREVIATIONS

Ab	antibody
AKT	RAC-PK-alpha
BBS	balanced buffered solution
BCL2	B-cell lymphoma 2
BSA	bovine serum albumin
CHOD–POD	cholesterol-oxidase–peroxidase
DGGR	1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin)ester
ECL	enhanced chemiluminescence
FITC	fluorescein isothiocyanate
G6PDH	glucose-6-phosphate dehydrogenase
GW	irreversible PPARγ antagonist GW9662
MAPK	mitogen-activated protein kinase
NC	uncapacitated sample without treatment
PBS	phosphate buffered saline
PGJ2	15-deoxy-12,14-prostaglandin J2
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PNA	peanut agglutinin
PPAR	peroxisome proliferator-activated receptor
PPP	pentose phosphate pathway
TBS	Tris-buffered saline

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