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Reactive oxygen species (ROS) production triggered by prostaglandin D_2 (PGD_2) regulates lactate dehydrogenase (LDH) expression/activity in TM4 Sertoli cells

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ABSTRACT

Reactive oxygen species (ROS) regulate testicular function in health and disease. We previously described a prostaglandin D_2 (PGD_2) system in Sertoli cells. Now, we found that PGD_2 increases ROS and hydrogen peroxide (H_2O_2) generation in murine TM4 Sertoli cells, and also induces antioxidant enzymes expression suggesting that defense systems are triggered as an adaptive stress mechanism that guarantees cell survival. ROS and specially H_2O_2 may act as second messengers regulating signal transduction pathways and gene expression. We describe a stimulatory effect of PGD_2 on lactate dehydrogenase (LDH) expression via DP1/DP2 receptors, which is prevented by the antioxidant *N*-acetyl-L-cysteine and the PI3K/Akt pathway inhibitor LY 294002. PGD_2 also enhances Akt and CREB/ATF-1 phosphorylation.

Our results provide evidence for a role of PGD_2 in the regulation of the oxidant/antioxidant status in Sertoli cells and, more importantly, in the modulation of LDH expression which takes place through ROS generation and the Akt-CREB/ATF-1 pathway.

1. Introduction

In spite of the large amount of studies in semen, there has been little research so far on the contribution of prostaglandins (PGs) to testicular physiology/pathology.

Cyclooxygenase 2 (COX2), the inducible and rate-limiting isozyme of PG biosynthesis, is expressed in different testicular cell populations including Sertoli cells (Frungieri et al., 2015). In fact, several authors reported the production of PGs and the existence of prostanoid receptors in Sertoli cells (Ishikawa and Morris, 2006; Kristensen et al., 2011; Matzkin et al., 2012; Winnal et al., 2007). Sertoli cells transduce signals from FSH and testosterone into the synthesis of factors that are required to support spermatogenesis. In a recent study, our group demonstrated that FSH and testosterone exert a stimulatory effect on COX2 expression and the production of some PGs ($PGF_{2\alpha}$ and 15d-PGJ_2) in Sertoli cells from sexually inactive adult hamsters kept under a short-day photoperiod, through a mechanism that involves Erk1/2 phosphorylation (Matzkin et al., 2012). We also found that $PGF_{2\alpha}$ and

Abbreviations: AR, androgen receptor; AMH, antimüllerian hormone; Cat, catalase; CLU, clusterin; CRE, cyclic-AMP-responsive element; CREB, CRE binding protein; COX2, cyclooxygenase 2; GDNF, glial cell line derived neurotrophic factor; Gpx, glutathione peroxidase; H-PGDS, hematopoietic PGD synthase; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; L-PGDS, lipocalin PGD synthase; NAC, *N*-acetyl-L-cysteine; NR5A1, nuclear receptor subfamily 5, group A, member 1; PDHA1, pyruvate dehydrogenase E1 alpha subunit; PGD_2, prostaglandin D_2; PGs, prostaglandins; PI3K, phosphatidylinositol 3-kinase; Pxr1, peroxiredoxin 1; ROS, reactive oxygen species; SCO, Sertoli cell only; SHBG, sex hormone binding globulin; Sod1, superoxide dismutase 1; SOX9, SRY-box 9.

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15d-PGJ_2 via FP receptor and the nuclear PPARγ receptor, respectively, inhibit FSH- and testosterone-induced uptake of [2,6-^{3}H]-2-deoxy-D-glucose, a non-metabolizable glucose analogue in Sertoli cells from reproductively quiescent adult hamsters (Matzkin et al., 2012). The transport of glucose through the plasma membrane is the rate-limiting step in glucose metabolism and, consequently, in lactate production (Riera et al., 2001, 2009). The provision of adequate levels of lactate is one of the most important Sertoli cell functions to guarantee germ cell development. The conversion of pyruvate to lactate is catalyzed by the enzyme lactate dehydrogenase (LDH). LDH is encoded by the *Ldh a*, *Ldh b* and *Ldh c* genes (Li, 1989). Combinations of these gene products result in the tetrameric LDH isozymes LDH-1 (B4), LDH-2 (A1B3), LDH-3 (A2B2), LDH-4 (A3B1), LDH-5 (A4) and LDH C4 (Gupta, 1999, 2012). Whereas LDH C4 isozyme is only present in spermatozoa of the mature testis (Gupta, 1999; Markert et al., 1975), the other isozymes have been found in variable proportions in different somatic cell populations including Sertoli cells (Santiemma et al., 1987).

Sertoli cells also produce prostaglandin D_2 (PGD_2) and express DP receptors (Matzkin et al., 2012; Moniot et al., 2009; Samy et al., 2000). Although the involvement of PGD_2 in the activation of Sertoli-expressed genes that are required for germ cell development and maintenance during organogenesis (i.e. Sox9, Notch1 and Cyp26B1) has already been addressed (Moniot et al., 2009; Rossitto et al., 2015), the role of this eicosanoid in the response of Sertoli cells to oxidative stress has not been explored so far. We have recently reported a marked increase in the production of PGD_2 in testes of short-lived mice undergoing oxidative processes (Matzkin et al., 2016). In this regard, we have also found that levels of reactive oxygen species (ROS) are higher in peritubular cells of patients with impaired spermatogenesis and fibrosis than in peritubular cells from men with normal spermatogenesis (Kampfer et al., 2012). In the same infertility patients, we had previously described the existence of a testicular PGD_2 system (Schell et al., 2007) and the participation of the PGD_2-metabolite 15d-PGJ_2 in the testicular generation of ROS (Schell et al., 2010). *Catalase* (*Cat*) levels are also significantly elevated in peritubular cells of infertile patients suggesting that these cells present an improved ability to handle oxidative stress (Kampfer et al., 2012). In addition, we have seen that the testicular concentration of the antioxidant melatonin positively correlated with the expression of the antioxidant enzymes *superoxide dismutase 1* (*Sod1*), *peroxiredoxin 1* (*Pxr1*) and *Cat* in infertile patients (Rossi et al., 2014).

Considering these preliminary data, we decided to investigate whether the PGD_2 system participates in the modulation of the oxidant-antioxidant status in Sertoli cells. Despite the low oxygen tensions that characterize the testicular micro-environment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids and the presence of systems that generate ROS including the mitochondria and a variety of enzymes such as the xanthine- and NADPH- oxidases and the cytochrome P450s (Aitken and Roman, 2008). It has been well established that an excessive production of ROS is harmful to the cell and consequently, counter-defense systems are activated (Aitken and Roman, 2008). However, low and regulated ROS production may be relevant to Sertoli cell activity under physiological conditions (Galardo et al., 2014; Valko et al., 2007). In this context, ROS might act as secondary messengers responsible for signal transduction pathways which control gene expression (Hossain et al., 2015). Thus, bearing in mind the critical contribution of Sertoli cells to the supply of lactate into the spermatogenic environment, the involvement of PGD_2-dependent ROS generation in the physiological regulation of *Ldh a* gene expression in these somatic cells of the tubular compartment was investigated.

2. Materials and methods

2.1. Cell culture

The TM4 Sertoli cell line derived from immature mouse Sertoli cells [American Tissue Culture Collection (ATCC® CRL1715™), Riversville, MD, USA] was cultured in RPMI1640 medium (Gibco, NY, USA) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO, USA). For all experiments, cells had been subcultured no more than 10 times. TM4 Sertoli cells were incubated in FCS-free medium for 24 h to synchronize the cell cycle. To drive G0 cells into cell cycle, we treated serum-starved TM4 Sertoli cells with medium containing FCS. After approximately 16–20 h, when cells entered into S phase, they were incubated at 37 °C under a humid atmosphere with 5% CO_2, in the presence or absence of the following chemicals: PGD_2 (0.1 nM, 10 nM, 0.1 μM and 1 μM, Sigma-Aldrich), *N*-acetyl-L-cysteine (NAC; 100 μM, 1 mM and 5 mM, Sigma-Aldrich) and hydrogen peroxide (H_2O_2; 1 mM, Merck Millipore, KGaA, Darmstadt, Germany). In some experiments, TM4 Sertoli cells were pre-incubated for 1 h in the presence of phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 (10 μM, Sigma-Aldrich), the DP1 receptor antagonist BW A686C (1 μM and 10 μM, Cayman Chemical, Ann Arbor, MI, USA) or the DP2 receptor antagonist CAY 10471 (1 μM and 10 μM, Cayman Chemical).

PGD_2, LY 294002, BW A686C and CAY 10471 were dissolved in absolute ethanol (Merck Millipore) and then further diluted in RPMI1640. An appropriate volume of ethanol (5–10 μl/ml medium RPMI1640) was added to control experiments to account for possible effects of ethanol. NAC and H_2O_2 were diluted in RPMI1640, which was then used as vehicle for control incubations.

After incubations, cells were used for RNA extraction followed by RT-qPCR, protein extraction followed by immunoblotting, assessment of LDH activity or quantification of intracellular PGD_2 concentrations. Media were frozen at −70 °C until the levels of PGD_2 secreted to the incubation media were determined by immunoassay.

2.2. RT-PCR and RT-qPCR analyses

Total RNA was isolated from confluent TM4 Sertoli cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and from microdissected human Sertoli cells using the Paradise Plus Reagent system (Applied Biosystems, Bedford, MA, USA) according to the manufacturers' instructions. RT reactions were performed using dN6 random primers as previously described (Rossi et al., 2012).

PCR conditions were 95 °C for 5 min, followed by 30–40 cycles of 94 °C for 1 min, 55–60 °C (annealing temperature) for 1 min, 72 °C for 1 min, and a final incubation at 72 °C for 5 min. When required, a second PCR amplification using nested primers was also carried out. The following oligonucleotide primers were used for amplification of *18S* rRNA (5′- ACACGGACAGGATTGACAGATT and 5′-CGTTCGTTATCGGAATTAACCA), mouse *DP1* receptor (5′-TGCAACCTGGGTGCCATG and 5′-GGCTTGGAGGTCTTCCGA), human *DP1* receptor (first set 5′-TGCAACCTCGGCGCCATG and 5′-GCTCGGAGGTCTTCTGCT; hemi-nested set 5′-CAACCTCTATGCGATGCA and 5′-GCTCGGAGGTCTTCTGCT), mouse *DP2* receptor (5′-CATGTGCTACTACAACTTGC and 5′-GCAGACTGAAGATGTGGTAGG), human *DP2* receptor (first set 5′-TATGTGCTACTACAATGTGC and 5′-GCAGGCTGAACACGTGGTAGG; hemi-nested set 5′-TATGTGCTACTACAATGTGC and 5′-GTGGCTCGAGGCGATGATCG), *COX2* (5′-GCTTCAAACAGTTTCTCTACAA and 5′-CATTTCTTCCCCCAGCAAC), *lipocalin PGD synthase* (*L-PGDS*; 5′-CTCCACCACTGACACGGAG and 5′-TCCTCAGGAAAAACCAGTGT), *hematopoietic PGD synthase* (*H-PGDs*; 5′-GAATAGAACAAGCTGACTGGC and 5′- AGCCAAATCTGTGTTTTTGG), *nuclear receptor subfamily 5, group A*,

member 1 (*NR5A1*; 5′-CCCTGTTGGATTACACCTTG and 5′-GTTGCCAAATGCTTGTGGTA), *glial cell line derived neurotrophic factor* (*GDNF*; 5′-TTGCAGCGGTTCCTGTGAAT and 5′-TCTTAGAATATGGTAAACCAGGTTGTCA), *clusterin* (*CLU*; 5′ CCACGCCATGAAGATTCTCCTGC and 5′-CTCCCTGGACGGCGTTCTGA) and *sex hormone binding globulin* (*SHBG*; 5′-GACATTCCCCAGCCTCATGCA and 5′-TGCCTCGGAAGACAGAACCACG).

For mouse *DP1* receptor, *COX2*, *L-PGDS*, *CLU* and *SHBG* oligonucleotide primers were designed as homologous to regions of different exons. PCR products were separated on 2% agarose gels and visualized with ethidium bromide. The identity of the cDNA products was confirmed by sequencing, using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer.

qPCR assays were performed using oligonucleotide primers for *Sod1* (5′-AAAGCGGTGCGTGCTGAA and 5′-CAGGTCTCCAACATGCCTCT), *glutathione peroxidase* (*Gpx*; 5′-CCTCAAGTACGTCCGACCTG and 5′-CAATGTCGTTGCGGCACACC), *Pxr1* (5′-CACCCAAGAAACAAGGAGGA and 5′-GAGATACCTTCATCAGCCTT), *Cat* (5′-CCGACCAGGGCATCAAAA and 5′- CATTGGCGATGGCATTGA), *Ldh a* (5′-CATTGTCAAGTACAGTCCACACT and 5′-TTCCAATTACTCGGTTTTTGGGA) and *Gapdh* (5′-GACGGCCGCATCTTCTTGT and 5′- AACGACCTTCACCATTTTGTCT) which was selected as the housekeeping gene. Reactions were conducted using SYBR Green PCR Select Master Mix and the ABI PRISM 7500 sequence detector System (Applied Biosystems). Following the mathematical model of Pfaffl (2001), the relative levels of mRNA expression were determined for each sample as previously described (Rossi et al., 2012).

2.3. Immunoblotting

TM4 Sertoli cell protein homogenates (5–50 μg), prepared as previously described (Rossi et al., 2012) were loaded onto 15% tricine-SDS-polyacrylamide gels, electrophoretically separated, and blotted onto nitrocellulose membrane. Protein concentrations were measured by the method of Lowry et al. (1951). We performed immunoblots as described (Rossi et al., 2012) using a mouse monoclonal anti-actin antibody (1:5000, Calbiochem, La Jolla, CA, USA), a rabbit monoclonal anti-Cat antibody (1:1500, Epitomics Inc., Burlingame, CA, USA), a rabbit monoclonal anti-Pxr1 antibody (1:3000, Abcam, Milton, Cambridge, UK), a rabbit polyclonal anti-phospho Akt kinase serum (p-Akt; 1:500, Cell Signaling Technology Inc., Beverly MA, USA), a rabbit polyclonal anti-Akt kinase serum (1:500, Cell Signaling Technology Inc.), a rabbit polyclonal anti-phospho extracellular signal-regulated kinase (p-Erk) 44/42 (1/2) MAP kinase serum (1:500, Cell Signaling Technology Inc.), a rabbit polyclonal anti-Erk1/2 kinase serum (1:500, Cell Signaling Technology Inc.), a mouse monoclonal anti-phospho P38 MAP kinase antibody (p-P38; 1:500, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), a rabbit monoclonal anti-P38 kinase antibody (1:500, Cell Signaling Technology Inc.), a rabbit polyclonal anti-phospho CRE binding protein (p-CREB) and anti-phospho ATF-1 (p-ATF-1) serum (1:500, Cell Signaling Technology Inc.) or a rabbit polyclonal anti-phospho pyruvate dehydrogenase E1 alpha subunit (p-PDHA1) serum (1:1000, Novus Biological, Littleton, CO, USA).

2.4. Immunocytochemical analyses

Formaldehyde-fixed TM4 Sertoli cells were used for immunodetection of SRY-box 9 (SOX9) and the antimüllerian hormone (AMH) as previously described (Matzkin et al., 2012). Cells were incubated overnight at 4 °C with a rabbit polyclonal anti-SOX9 serum (1:25, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or a rabbit polyclonal anti-AMH serum (1:100, for further information about the antiserum see Dutertre et al., 1997). For control purposes, the first antiserum was omitted or incubations were carried out with normal non-immune sera.

2.5. ROS measurement

Generation of ROS was determined with the cell-permeant fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Invitrogen, Darmstadt, Germany) as described (Rossi et al., 2014). H_2DCFDA diffuses into the cell, where it is enzymatically converted by intracellular esterases and oxidized into the high fluorescence compound DCF, which allows the determination of H_2O_2, peroxynitrite anions and peroxyl radicals. Approximately 4×10^3 TM4 Sertoli cells per well were plated on white bottom 96-well plates in extracellular fluid (140 nM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 25 mM glucose; pH 7.4). After 2 h incubation, cells were loaded with 10 μM H_2DCFDA for 30 min at 37 °C and 5% CO_2. Then, cells were treated with or without PGD_2 (1 μM) in the presence or absence of NAC (1 mM) for 2 h at 37 °C. DCF generation was measured over time using a fluorometer (Fluostar BMG Labtech, Offenburg, Germany) at 492 nm excitation and 520 nm emission.

2.6. H_2O_2 measurement

The production of H_2O_2 was determined with the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes). In the presence of horseradish peroxidase (HPR) the Amplex® Red reagent reacts in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Consequently, resorufin generation allows the detection of the H_2O_2 released from biological samples.

Approximately 2×10^3 TM4 Sertoli cells were plated on black bottom 96-well plates in 120 μl of working solution containing 40 μM Amplex® Red reagent and 0.1U/ml HPR, and incubated in the presence or absence of PGD_2 (0.1 μM and 1 μM) for 2 h at 37 °C. Resorufin generation was measured over time using a fluorometer (Fluostar BMG Labtech) at 544 nm excitation and 590 nm emission.

2.7. Live cell imaging studies

TM4 Sertoli cells were grown overnight in a glass bottomed cultured dish (μ-Dish, Ø 35 mm; Ibidi, Martinsried, Germany) under standard conditions. Then, cells were incubated in the presence or absence of PGD_2 (1 μM) in RPMI1640 serum-free medium under a humid atmosphere with 5% CO_2 at 37 °C for 24 h. Optimal incubation conditions (5% CO_2, 37 °C, 95% relative humidity) were created by using an Ibidi heating system and an Ibidi gas incubation system. Time-lapse series were generated by taking a picture every 20 min for 24 h with a ProgRes MF camera (Jenoptik, Jena, Germany), the Micro-Manager 1.3 Microscopy Software (Ron Vale's laboratory at UCSF, San Francisco, CA, USA) and a transmitted light microscope (Axiovert 135; Zeiss, Oberkochen, Germany).

2.8. Proliferation assay

Approximately 9×10^3 TM4 Sertoli cells were plated on 96-well plates and incubated for 24 h in the presence or absence of PGD_2 (0.1 nM, 10 nM and 1 μM). Cellular metabolic activity was determined using the CellTiter 96 AQueous One Solution cell-proliferation assay (Promega Corporation, Madison, WI, USA) as previously described (Rossi et al., 2014).

2.9. LDH activity assay

Total LDH activity was determined using the LDH-P unitest kit (Wiener Laboratories, Rosario, Argentina) which includes pyruvate

and NADH as substrate and co-factor, respectively, of the reaction catalyzed by the LDH enzyme.

Approximately 1×10^6 TM4 Sertoli cells were resuspended in 100 μl 0.9% NaCl, disrupted by ultrasonic irradiation and centrifuged at 15,800*g* for 10 min at 4 °C. Supernatants were used to determine total LDH activity spectrophotometrically at 340 nm following the manufacturer's protocol. From each sample, an aliquot was preserved for protein determinations. Results are expressed as μmol/min (IU) per mg protein.

2.10. Patients and human testicular samples

The study was designed in agreement with the Helsinki Declaration and its last modification in Tokyo 2004 on human experimentation and was approved by the Ethics Committee of the Institute of Biology and Experimental Medicine (Buenos Aires, Argentina) and the Ethics Committee of the Durand Hospital (Buenos Aires, Argentina). Informed consent was obtained from all the patients included in the study.

Twenty men (27–42 years old) with idiopathic infertility and non-obstructive azoospermia without an infection process who presented a normal karyotype were registered in this study. Patients with known etiology of infertility, such as genitourinary infections, mumps orchitis, varicocele, hypogonadotropic hypogonadism, chromosome anomalies, obstruction or agenesia of seminal ducts were excluded.

Patients were assessed and diagnosed by open testicular biopsy. A small piece of testicular tissue was immersed in Bouin's solution and submitted to histopathological diagnosis. Biopsies were classified either as hypospermatogenesis or Sertoli cell only (SCO) appearance according to McLachlan et al. (2007). The hypospermatogenesis group included men in which the biopsy specimens contained less than 17 mature spermatids per tubule but all stages of spermatogenesis are present. SCO syndrome term is used when there are no tubules containing germ cells (Silber and Rodriguez-Rigau, 1981; Silber et al., 1997).

A second piece of testicular tissue was preserved for molecular biology studies. Ethical and legal considerations excluded the possibility of studying testicular samples from healthy men.

2.11. PGD_2 immunoassay

PGD_2 concentrations in TM4 Sertoli cells and human testicular biopsies were determined using a commercial kit (Cayman Chemical) as previously described (Matzkin et al., 2012). In order to prevent further PG chemical degradation samples were treated with methoxylamine hydrochloride (MOX HCl). The levels of PGD_2 secreted from TM4 Sertoli cells to the incubation media were also quantified. After MOX HCl treatment and acidification using 1 M

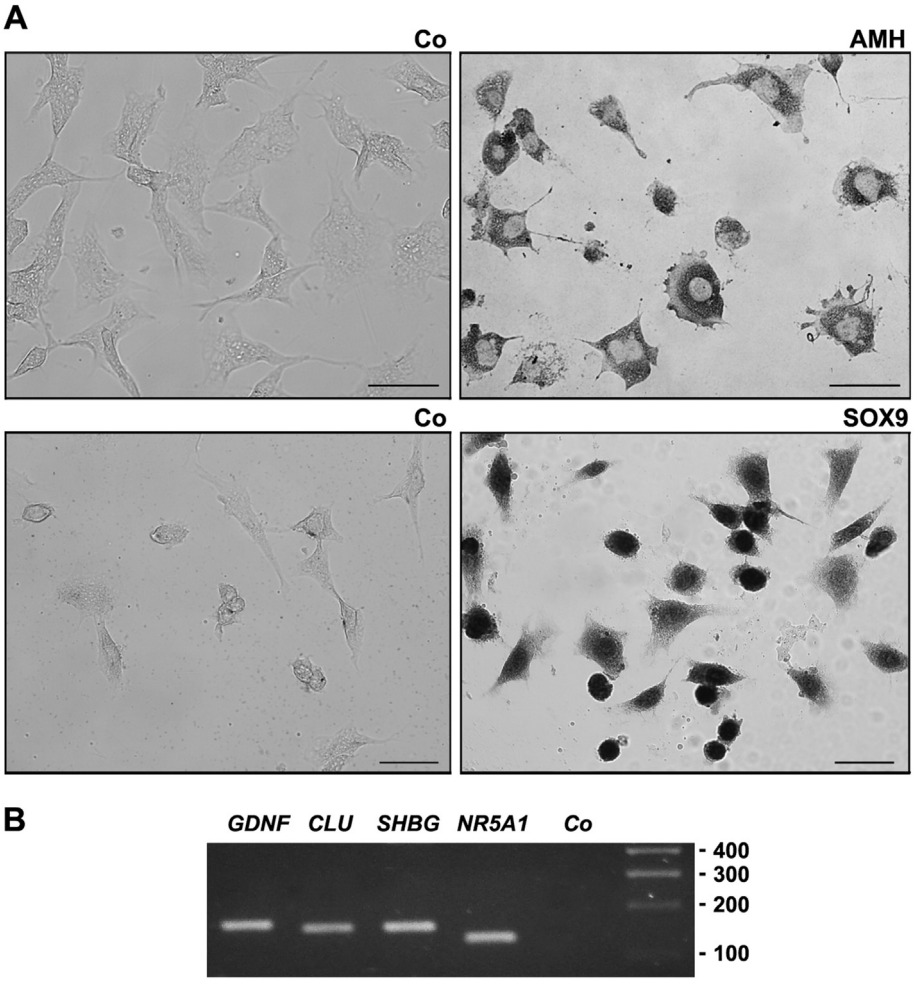

Fig. 1. *Identification of Sertoli cell markers expressed in murine TM4 cells.* (A) Positive immunostaining for AMH and SOX9 was found in TM4 cells. No reaction was observed when TM4 cells were incubated only with normal non-immune serum and the conjugated antibody (Co, control). Bar, 50 μm. (B) mRNA expression of *GDNF* (171 bp), *CLU* (160 bp), *SHBG* (161 bp) and *NR5A1* (134 bp) was detected by RT-PCR in TM4 cells. Co: non-template control.

citrate buffer (pH 4.7), media were injected into C18 columns and then eluted with ethyl acetate. Eluted fractions were evaporated to dryness under nitrogen stream and reconstituted in assay buffer.

The minimum detectable immunoassay concentration was 0.28 femtomole (fmol)/tube. Intra-assay and inter-assay coefficients of variation were less than 10% and less than 15%, respectively. PGD_2 levels were expressed as pmol per 10^6 cells and pmol per g tissue.

2.12. Immunohistochemical analysis followed by laser capture microdissection and RT-PCR

A rabbit polyclonal anti-androgen receptor (AR) serum (1:100, Santa Cruz Biotechnology Inc.) was used to detect the immunological expression of ARs. Afterwards, laser capture microdissection was performed as previously described (Rossi et al., 2014). Microdissected samples containing AR immunopositive Sertoli cells were used for RT-PCR as described above.

2.13. Statistical analyses

Data analyses were performed using GraphPad Prism® 5.03 (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using one-way ANOVA followed by Student's *t*-test for two comparisons or Student–Newman–Keuls test for multiple comparisons. Data are expressed as mean ± S.E.M.

For immunoblotting studies, bands were quantified by densitometry and normalized to the housekeeping gene using SCION IMAGE (SCION Corporation, Frederick, MD, USA).

3. Results

3.1. Expression of Sertoli cell markers in TM4 cells

Immunocytochemical assays showed cytoplasmic AMH and nuclear SOX9 expression in TM4 Sertoli cells (Fig. 1A). Furthermore, the mRNA expression of *NR5A1*, *GDNF*, *CLU* and *SHBG* was detected in TM4 Sertoli cells by RT-PCR studies (Fig. 1B).

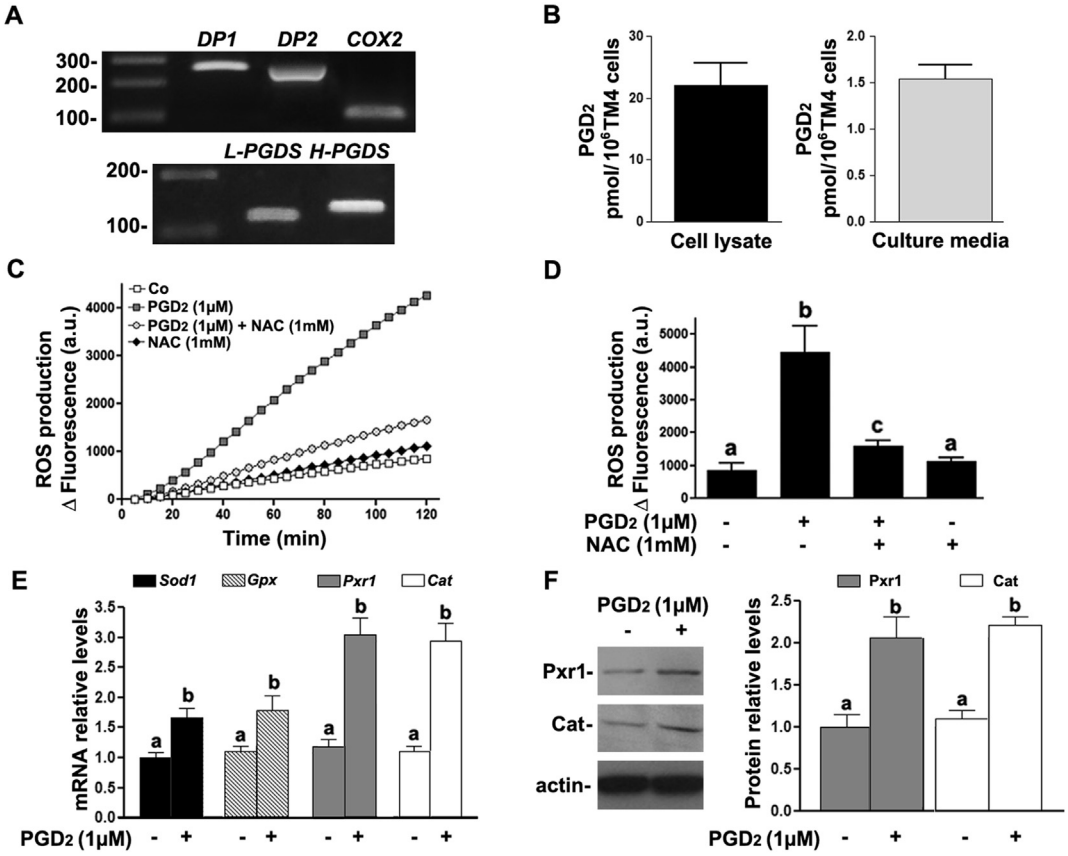

Fig. 2. *Identification of a PGD_2 system in TM4 Sertoli cells: PGD_2-induced ROS generation and antioxidant enzymes expression.* (A) mRNA expression of the PGD_2 receptors *DP1* (275 bp) and *DP2* (262 bp), as well as of the PGD_2 biosynthetic enzymes *COX2* (111 bp), *L-PGDS* (118 bp) and *H-PGDS* (136 bp) was detected by RT-PCR in TM4 Sertoli cells. (B) TM4 Sertoli cells were incubated in basal conditions for 3 h. Intracellular PGD_2 concentrations and PGD_2 levels secreted to the incubation media from TM4 Sertoli cells were determined by enzyme immunoassay. Bar plot graphs represent the mean ± SEM (n = 5). (C) ROS generation was determined over time by a fluorometric assay in TM4 Sertoli cells incubated in the presence or absence of PGD_2 (1 μM) with or without NAC (1 mM). This graph is representative of three independent experiments performed in different cell preparations that showed comparable results. Co, cells incubated in basal conditions. (D) Bar plot graph illustrates the endpoint fluorescence values after 120 min incubation. This graph is representative of three independent experiments performed in different cell preparations that showed comparable results. Bar plot graph represents the mean ± SEM (n = 8 replicate wells per treatment). Different letters denote a statistically significant difference between groups. $p < 0.05$. Student-Newman–Keuls test. (E) TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) for 3 h. The relative levels of mRNA *Sod1*, *Gpx*, *Pxr1* and *Cat* expression were determined by RT-qPCR using *Gapdh* as the housekeeping gene. The mRNA antioxidant enzymes expression levels obtained in three independent experiments were analyzed using the mathematical model of Pfaffl. Bar plot graph represents the mean ± SEM. Different letters denote a statistically significant difference between groups. $p < 0.05$. Student's *t*-test. (F) TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) for 5 h. Pxr1 (22 kDa), Cat (60 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. These immunoblots are representative of three experiments performed in different cell preparations that showed comparable results. Bar plot graph represents the mean ± SEM and depicts the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (cells incubated in basal condition) which was assigned a value of 1 and normalized to actin. Different letters denote a statistically significant difference between groups. $p < 0.05$. Student's *t*-test.

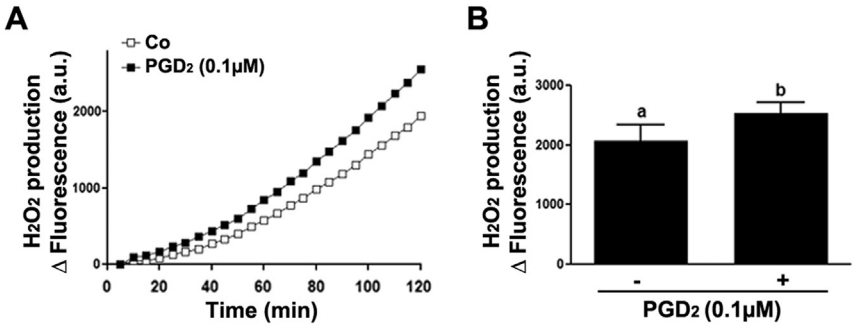

Fig. 3. *Stimulatory effect of PGD_2 on H_2O_2 production in TM4 Sertoli cells.* (A) H_2O_2 production was determined over time by a fluorometric assay in TM4 Sertoli cells incubated in the presence or absence of PGD_2 (0.1 μM). This graph is representative of three independent experiments performed in different cell preparations that showed comparable results. (B) Bar plot graph illustrates the endpoint fluorescence values after 120 min incubation. This graph is representative of three independent experiments performed in different cell preparations that showed comparable results. Bar plot graph represents the mean ± SEM (n = 8 replicate wells per treatment). Different letters denote a statistically significant difference between groups. $p < 0.05$. Student's *t*-test.

3.2. Identification of a PGD_2 system in TM4 Sertoli cells involved in the induction of ROS generation and antioxidant enzymes expression

The existence of a PGD_2 system in TM4 Sertoli cells was investigated by RT-PCR assays. Studies revealed the expression of the PGD_2 receptors *DP1* and *DP2*, as well as the PGD_2 biosynthetic enzymes, *COX2*, *L-PGDS* and *H-PGDs* in TM4 Sertoli cells (Fig. 2A). In addition, TM4 Sertoli cells produce and release PGD_2 into the incubation media (Fig. 2B).

In order to analyze a potential effect of PGD_2 on cellular oxidative status, ROS generation was investigated. TM4 Sertoli cells were incubated in the presence or absence of 1 μM PGD_2 with or without the antioxidant NAC (1 mM). After 120 min incubation, the levels of ROS were significantly higher than those detected under control conditions. This effect was prevented by the antioxidant NAC (Fig. 2C and D).

To investigate whether antioxidant mechanisms leading to cell survival are activated after PGD_2 treatment, the expression of

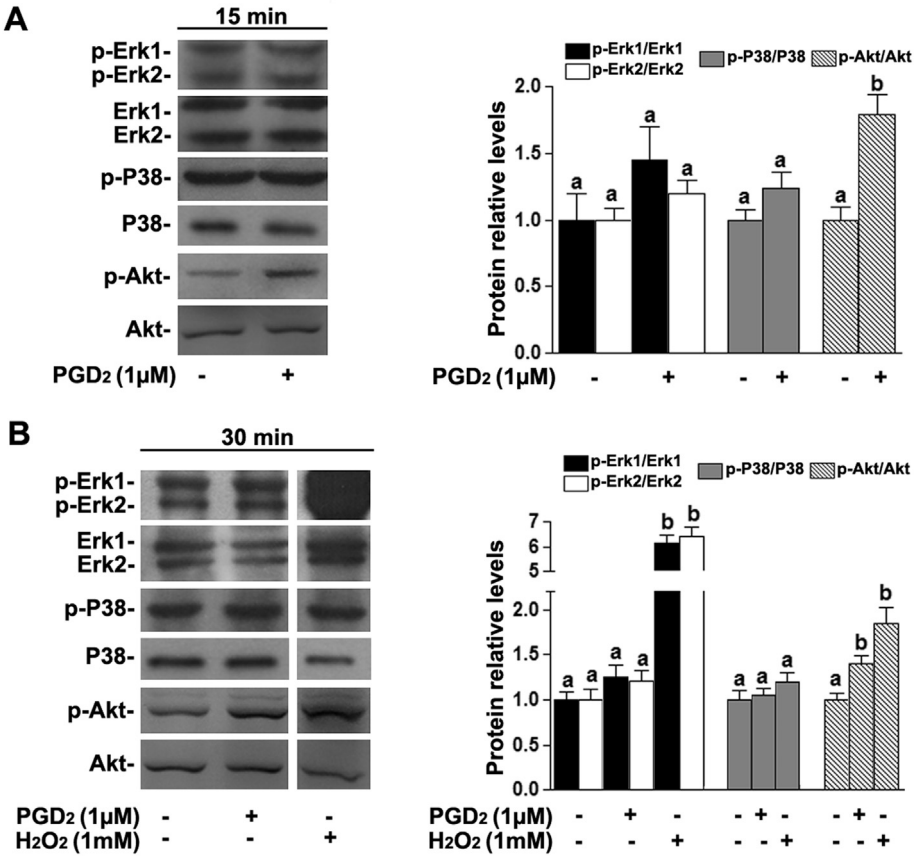

Fig. 4. *Participation of Akt in the signaling pathway triggered by PGD_2 in TM4 Sertoli cells.* (A) TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) for 15 min (B). TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) or H_2O_2 (1 mM) for 30 min. Total and phosphorylated levels of Erk1/2 (44-42 kDa), P38 (38 kDa), and Akt (60 kDa) were determined by immunoblotting. These immunoblots are representative of three experiments performed in different cell preparations that showed comparable results. Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (cells incubated in basal condition), which was assigned a value of 1, and normalized to total Erk1/2, P38 or Akt, respectively. Different letters denote a statistically significant difference between groups. $p < 0.05$. Student's *t*-test.

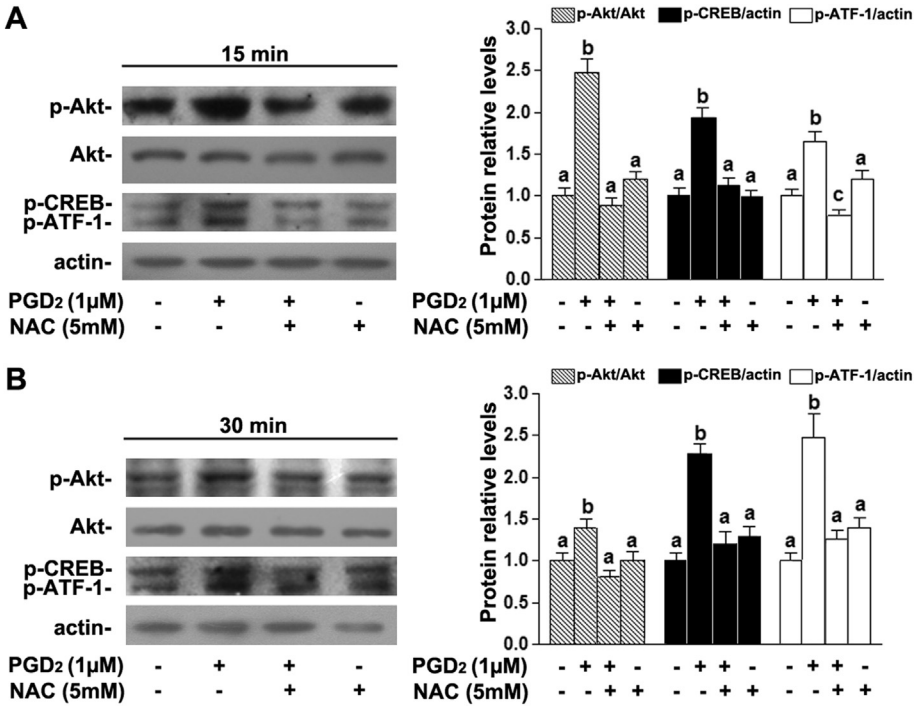

Fig. 5. *Inhibitory effect of the antioxidant NAC on the PGD_2-dependent phosphorylation of Akt/CREB/ATF-1 in TM4 Sertoli cells.* (A) and (B) TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) for 15 min and 30 min, respectively, with or without NAC (5 mM). The levels of total and phosphorylated Akt (60 kDa), phospho-CREB (p-CREB; 43 kDa), phospho-ATF-1 (p-ATF-1; 38 kDa) and actin (42 kDa) were determined by immunoblotting. These immunoblots are representative of three experiments performed in different cell preparations that showed comparable results. Bar plot graphs represent the mean ± SEM and depict the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (cells incubated in basal condition), which was assigned a value of 1, and normalized to total Akt or actin. Different letters denote a statistically significant difference between groups. $p < 0.05$; Student-Newman–Keuls test.

antioxidant enzymes was evaluated by RT-qPCR and immunoblotting. In addition, cell viability and morphology were assessed by a colorimetric assay and live cell imaging studies, respectively.

PGD_2 (1 μM) induced a significant increase in *Sod1*, *Gpx*, *Pxr1* and *Cat* mRNA expression (Fig. 2E), as well as on Pxr1 and Cat protein expression (Fig. 2F).

After 24 h incubation in the presence or absence of PGD_2 (1 μM, 10 nM and 0.1 nM), no significant differences were seen in cell viability between groups (data not shown).

Throughout 24 h incubation, live cell imaging showed no changes in the morphology of TM4 Sertoli cells incubated in the presence or absence of 1 μM PGD_2 (data not shown).

3.3. Identification of the main compounds of the signaling pathway triggered by PGD_2 in TM4 Sertoli cells

Taking into account that among all ROS and other oxygen-derived free radicals, H_2O_2 has been recently suggested to act as a central player in signal transduction pathways (Hossain et al., 2015), we evaluated H_2O_2 generation by a fluorometric assay in TM4 Sertoli cells incubated in the presence or absence of PGD_2.

Incubations carried out with 1 μM PGD_2 saturated H_2O_2 measurement in seconds (data not shown). Thus, experiments were repeated in the presence of 0.1 μM PGD_2 to evaluate H_2O_2 production throughout 120 min incubation (Fig. 3A). H_2O_2 production was significantly higher in TM4 Sertoli cells incubated with 0.1 μM PGD_2 than in cells kept under basal conditions (Fig. 3A and B).

Since it has been reported that the mechanism by which ROS act as signaling molecules involves several transduction pathways (Ji et al., 2010; Loh et al., 2009; Tai and Ascoli, 2011), immunoblotting experiments for detection of protein phosphorylation were conducted. PGD_2 significantly increased the phosphorylation levels of Akt in TM4 Sertoli cells after 15 and 30 min incubation (Fig. 4A and B, respectively). This stimulatory effect of PGD_2 on phospho-Akt was reverted by the antioxidant NAC after 15 and 30 min incubation (Fig. 5A and B, respectively). In contrast, phospho-Erk1/2 and phospho-P38 levels were not affected by PGD_2 (Fig. 4A and B). When TM4 Sertoli cells were treated with 1 mM H_2O_2, phosphorylation levels of Erk1/2 and Akt were markedly increased, but phospho-P38 levels remained unchanged (Fig. 4B).

After 15 and 30 min incubation, PGD_2 also exerted a stimulatory effect on the phosphorylation levels of CREB and ATF-1 (nuclear transcription factors associated to Akt kinase) that was prevented by NAC (Fig. 5A and B).

3.4. Identification of the signaling pathway by which PGD_2 regulates LDH expression/activity

In order to investigate the participation of PGD_2 in the regulation of LDH expression/activity, TM4 Sertoli cells were incubated in the presence or absence of 1 μM PGD_2. RT-qPCR and LDH activity assays showed a stimulatory effect of PGD_2 on both *Ldh a* mRNA expression and LDH activity (Fig. 6A–D).

Ldh a mRNA expression and LDH activity were also significantly increased by 1 mM H_2O_2 (Fig. 6A and B).

To further evaluate the involvement of ROS as well as the participation of the PI3K/Akt pathway in the PGD_2-dependent regulation of LDH, the antioxidant NAC (100 μM and 5 mM) or the PI3K/Akt inhibitor LY 294002 (10 μM) were added to the incubation media. NAC and LY 294002 prevented the stimulatory effect of PGD_2 on *Ldh a* mRNA expression (Fig. 6A and C). Furthermore, NAC also blocked the positive regulation exerted by PGD_2 on LDH activity, but LY 294002 had no effect (Fig. 6B and D, respectively).

Bearing in mind that increased LDH activity could result not only

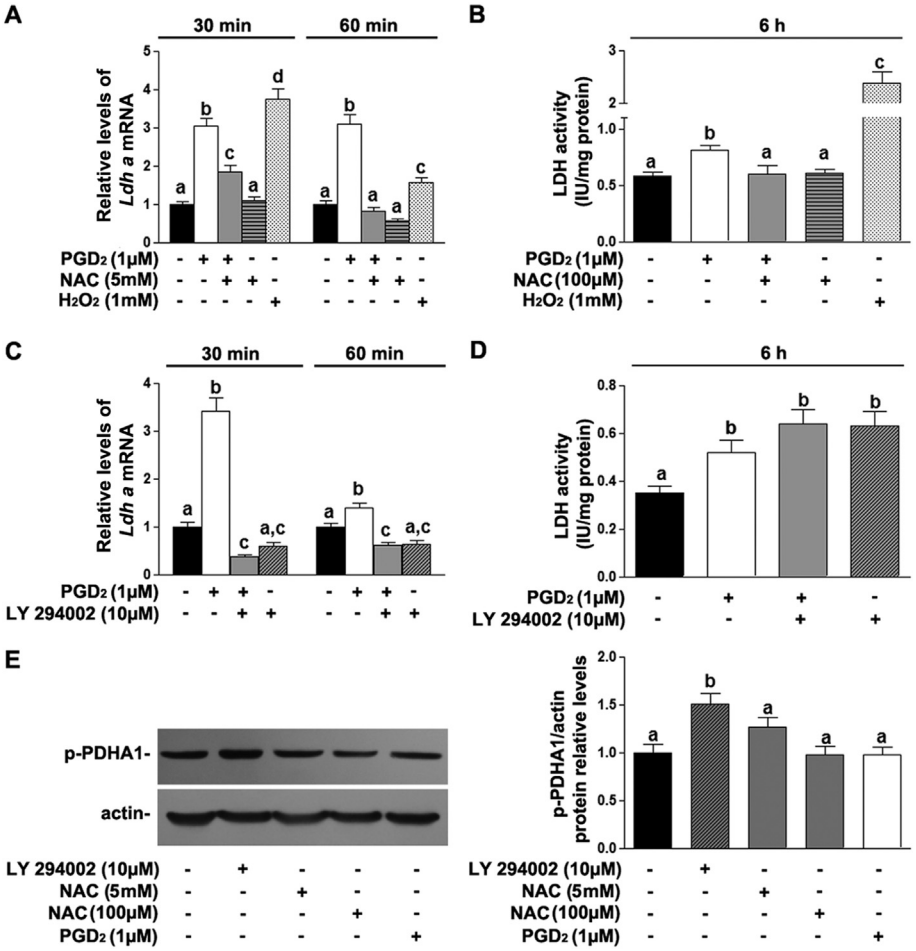

Fig. 6. *Stimulatory effect of PGD_2 on LDH expression/activity in TM4 Sertoli cells*: *participation of ROS and the PI3K/Akt pathway.* (A) and (B) TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) for 30 min and 60 min (left panel) as well as for 6 h (right panel) with or without NAC (100 μM or 5 mM). Cells were also incubated in the presence or absence of 1 mM H_2O_2. (C) and (D) TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) for 30 min and 60 min (left panel) as well as for 6 h (right panel) with or without the PI3K/Akt inhibitor LY 294002 (10 μM). Cell extracts were used for RT-qPCR assays and determination of LDH activity using the LDH-P unitest commercial assay. The relative levels of mRNA *Ldh a* expression were determined using *Gapdh* as the housekeeping gene in three independent experiments and analyzed by the mathematical model of Pfaffl. Bar plot graphs represent the mean ± SEM. Different letters denote a statistically significant difference between groups. $p < 0.05$. Student-Newman–Keuls test. LDH activity was determined in three independent experiments performed in different cell preparations that showed comparable results. Bar plot graphs represent the mean ± SEM. (n = 3 replicates per treatment). Different letters denote a statistically significant difference between groups. $p < 0.05$. Student-Newman–Keuls test. (E) TM4 Sertoli cells were incubated in the presence or absence of PI3K/Akt inhibitor LY 294002 (10 μM), NAC (100 μM and 5 mM) or PGD_2 (1 μM) for 90 min. Phospho-PDHA1 (p-PDHA1; 43 kDa) and actin (42 kDa) protein levels were determined by immunoblotting. These immunoblots are representative of three experiments performed in different cell preparations that showed comparable results. Bar plot graph represents the mean ± SEM and depicts the quantification by densitometry of the bands obtained in three independent experiments. Results are expressed as fold change relative to the control (cells incubated in basal condition), which was assigned a value of 1, and normalized to actin. Different letters denote a statistically significant difference between groups. $p < 0.05$; Student-Newman–Keuls test.

from higher *Ldh a* mRNA levels but also from increased substrate availability (Spriet et al., 2000), we investigated the regulation of the pyruvate dehydrogenase complex (PDC). We found that PDHA1 is phosphorylated by 10 μM LY 294002 (Fig. 6E) and therefore its activity is inhibited. Consequently, higher concentrations of pyruvate might remain available for its conversion to lactate and explain why LY 294002 failed to block the positive modulation exerted by PGD_2 on LDH activity while abolishing the stimulatory effect of this PG on *Ldh a* gene expression. In contrast, no significant changes were observed in phospho-PDHA1 when TM4 Sertoli cells were incubated in the presence or absence of NAC (100 μM and 5 mM) or PGD_2 (1 μM) (Fig. 6E).

3.5. The stimulatory effect of PGD_2 on Ldh a mRNA expression and LDH activity is reverted by specific DP receptor antagonists

In order to analyze the participation of DP receptors in the stimulatory effect exerted by PGD_2 on *Ldh a* mRNA expression and LDH activity, TM4 Sertoli cells were incubated in the presence or absence of 1 μM PGD_2 with or without BW A686C and CAY 10471 (1 μM and 10 μM), specific antagonists of DP1 and DP2 receptors, respectively. The stimulatory effect of PGD_2 on the relative levels of *Ldh a* mRNA expression was fully blocked by CAY 10471 (Fig. 7B). After 30 min incubation, BW A686C reverted the positive modulation of PGD_2 on *Ldh a* mRNA expression but its effect was less profound after 60 min incubation (Fig. 7A).

Both DP receptor antagonists reverted the PGD_2-dependent stimulation of LDH activity (Fig. 7C and D).

3.6. Identification of the main components of the PGD_2 system in the human testis

To examine whether this newly described system is present in human Sertoli cells, we studied testicular biopsies from men

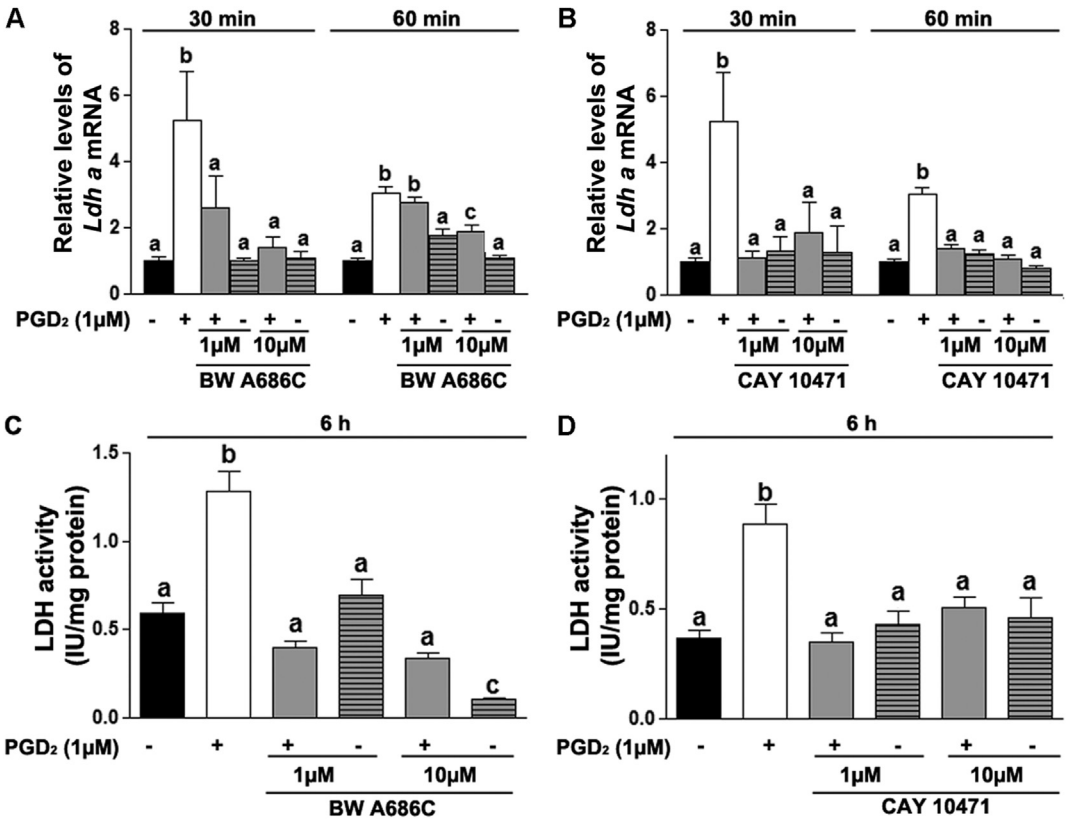

Fig. 7. *Participation of DP1 and DP2 receptors in the PGD_2-triggered stimulation of LDH expression/activity in TM4 Sertoli cells.* TM4 Sertoli cells were incubated in the presence or absence of PGD_2 (1 μM) with or without BW A686C (1 μM and 10 μM) and CAY 10471 (1 μM and 10 μM), specific antagonists of DP1 and DP2 receptors, respectively. (A) and (B) The relative levels of mRNA *Ldh a* expression were determined by RT-qPCR using *Gapdh* as the housekeeping gene. The mRNA *Ldh a* expression levels obtained in three independent experiments were analyzed using the mathematical model of Pfaffl. Bar plot graphs represent the mean ± SEM. Different letters denote a statistically significant difference between groups. $p < 0.05$. Student-Newman–Keuls test. (C) and (D) LDH activity was determined using the LDH-P unitest commercial assay. LDH activity graphs are representative of three independent experiments performed in different cell preparations that showed comparable results. These bar plot graphs represent the mean ± SEM. (n = 3 replicates per treatment). Different letters denote a statistically significant difference between groups. $p < 0.05$. Student-Newman–Keuls test.

presenting hypospermatogenesis or SCO syndrome. PGD_2 testicular concentrations were determined. No significant differences were found between the groups (Fig. 8A). By laser capture microdissection and RT-PCR studies, expression of *DP1* and *DP2* receptors was detected in human Sertoli cells isolated from the testis of patients revealing hypospermatogenesis (data not shown) or SCO syndrome (Fig. 8B and C).

4. Discussion

Sertoli cells are essential for testis formation and spermatogenesis (Griswold, 1998). Hence, morphological and functional alterations of these cells have severe consequences on male fertility. PGs are potential inducers of intracellular oxidative stress (Kondo et al., 2001). Recently, we described the existence of a PGD_2 system in Sertoli cells from reproductively inactive adult hamsters (Matzkin et al., 2012), as well as a marked increase in the production of PGD_2 in testes of short-lived mice undergoing oxidative processes (Matzkin et al., 2016). Consequently, in this study, our investigations attempted to determine the potential impact of PGD_2 on the oxidant-antioxidant status in Sertoli cells.

To our knowledge, functional studies in human Sertoli cells are not possible. In addition, because of germ cell contamination, reports using Sertoli cells purified from reproductively active rodents have been scarce. Therefore, prepubertal/early-pubertal mice and rats or alternatively, adult hamsters with impaired spermatogenesis as a consequence of their exposure to a short-day photoperiod are frequently used for Sertoli cell isolation (Matzkin et al., 2012; Riera et al., 2001, 2009; Yang and Han, 2010). TM4, derived from immature mouse Sertoli cells, is a non-transformed, non-tumorigenic and well characterized cell line that retains the most important aspects of Sertoli cell physiology (Lan et al., 2013; Mather, 1980, Mather et al., 1982; Nakhla et al., 1984; Simon et al., 2007). In addition, the TM4 cell population available in our laboratory expresses significant levels of the Sertoli cell markers AMH, SOX9, *GDNF*, *SHBG*, *CLU* and *NR5A1*. Therefore, in this study, the TM4 cell line was chosen as our experimental model to investigate the participation of PGD_2 in the occurrence of oxidative events in Sertoli cells.

We initially found that TM4 Sertoli cells express key enzymes in the biosynthesis of PGD_2 namely *COX2* and the two distinct types of PGDS (*L-PGDS* and *H-PGDS)*. In fact, TM4 Sertoli cells produce and secrete PGD_2 and also express *DP1* and *DP2* receptors.

Recent published data from our group indicate that PGD_2 concentrations in mouse testes range between 3000 and 9000 pmol per g tissue (Matzkin et al., 2016). Bearing in mind the testicular tissue density of 1.05 g/cm^3 (Yang et al., 1990; Zhou et al., 2010), in this study we used doses from 1 to 10 μM PGD_2 which can be considered as physiological concentrations.

Two different effects on the oxidant-antioxidant status were observed when TM4 Sertoli cells were incubated with physiological concentrations of PGD_2. Firstly, PGD_2 stimulated overall ROS production and, specifically, H_2O_2 generation. Secondly, PGD_2 increased the expression levels of the antioxidant enzymes *Sod1*,

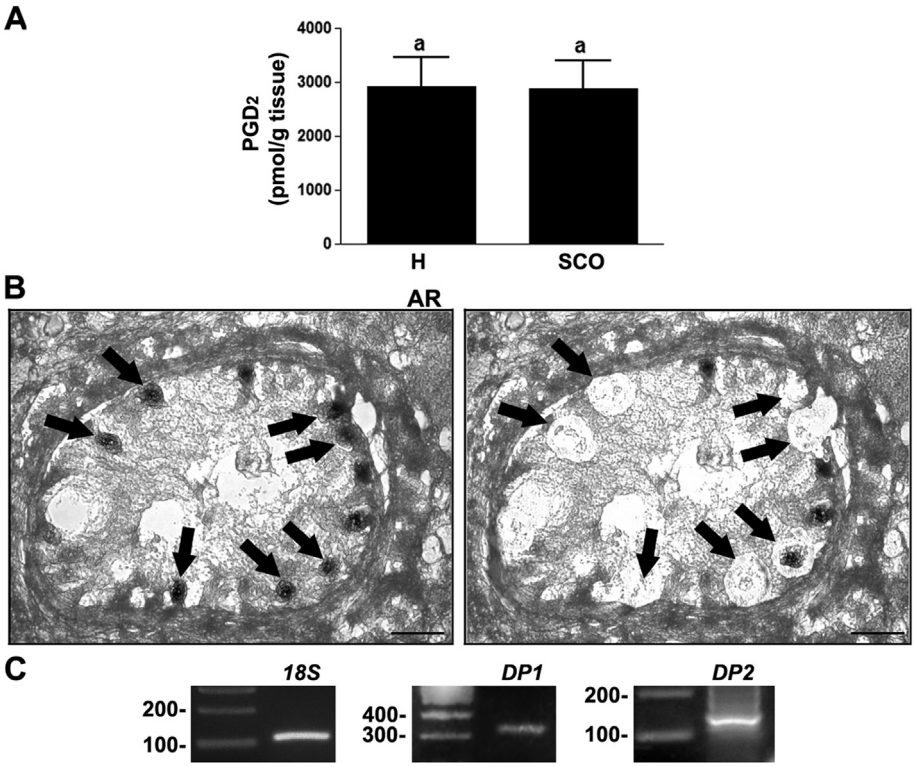

Fig. 8. *Concentrations of PGD_2 and expression of DP receptors in human testes.* (A) PGD_2 concentrations in testicular biopsies of patients suffering from hypospermatogenesis (H) or Sertoli cell only syndrome (SCO) were determined by enzyme immunoassay. Bar plot graph represents the mean ± SEM (n = 4–5 testicular biopsies per group). (B) Using the laser capture microdissection technique, androgen receptor (AR)-immunoreactive Sertoli cells were isolated from the testis of a patient with SCO syndrome and subjected to RT-PCR studies. Bar 40 μm. (C) mRNA expression of *18S* rRNA (109 bp) and the PGD_2 receptors *DP1* (345 bp) and *DP2* (138 bp) was detected by RT-PCR. Similar results were obtained when biopsies from patients suffering from hypospermatogenesis were analyzed (data not shown).

Gpx, *Pxr1* and *Cat*. Furthermore, Sertoli cell viability and morphology were not negatively affected by PGD_2. Depending on the levels of ROS produced, signaling could lead to death program and apoptosis, a gross structural/metabolic damage and necrosis, or to induction of antioxidant mechanisms and cell survival. In this context, under normal physiological states, the antioxidant capacity of the testicular tissue seems to be enough to avoid cellular damage (Turner and Lysiak, 2008). Thus, we conclude that TM4 Sertoli cells activate enzymatic defense mechanisms and prevent a potential cell injury triggered by the PGD_2-dependent ROS generation.

The combination of a long life span and a small size allows the H_2O_2 molecule to cross through the internal membrane systems of the cell facilitating signaling functions (Hossain et al., 2015). For this reason, among all ROS, H_2O_2 has been recently postulated to act as a central player in signal transduction pathways (Hossain et al., 2015). As mentioned, PGD_2 increased H_2O_2 generation in TM4 Sertoli cells. We also found that PGD_2 induced mRNA expression of *Ldh a*, a key enzyme in lactate production. This stimulatory action of PGD_2 on the mRNA expression levels of *Ldh a* was prevented by the antioxidant NAC. Thus, H_2O_2 might act as transducer of the PGD_2-triggered signaling pathway involved in lactate production in TM4 Sertoli cells. Several hormones and factors through the protein kinase A and C signal pathways and the transcription factor CREB contribute to the regulation of LDH expression and/or activity in Sertoli cells (Boussouar and Benahmed, 1999; Jungmann et al., 1986, 1998; Nehar et al., 1997; Riera et al., 2001; Short et al., 1994). When we analyzed the phosphorylation of various protein kinases and nuclear factors, we found that Erk and P38 phosphorylation were unaffected by PGD_2. In contrast, this eicosanoid significantly increased the phosphorylation levels of Akt kinase and the transcription factors ATF-1 and CREB. It is well known that CREB and ATF-1 are regulatory targets for the protein kinase Akt (Du and Montminy, 1998; Fan et al., 2005; Jia et al., 2006). Interestingly, the antioxidant NAC not only blocked the stimulatory action of PGD_2 on *Ldh a* gene expression in TM4 Sertoli cells but also the PGD_2-dependent phosphorylation of Akt, ATF-1 and CREB. Furthermore, incubations of TM4 Sertoli cells in the presence of LY 294002, an inhibitor of PI3K-dependent Akt phosphorylation and kinase activity, abolished the stimulatory effect of PGD_2 on *Ldh a* gene expression. Taken together, these results let us speculate that PGD_2 increases ROS generation and subsequently Akt phosphorylation. Then, this kinase might phosphorylate and activate the transcription factors ATF-1 and CREB leading to up-regulation of *Ldh a* gene expression in TM4 Sertoli cells. However, this hypothesis awaits further testing.

Behaving as expected, PGD_2 also stimulated LDH activity in TM4 Sertoli cells and this stimulatory effect was prevented by the antioxidant NAC. LY 294002 abolished the stimulatory effect of PGD_2 on *Ldh a* gene expression but it failed to block the positive modulation exerted by this PG on LDH activity. Subsequent experiments showed an increased phosphorylation of PDHA1 by LY 294002 in TM4 Sertoli cells, leading to inactivation of the PDC that converts pyruvate into acetyl-CoA. It has already been established that LDH becomes more active under experimental and physiological conditions which result in an increase of the substrates required for lactate production (Spriet et al., 2000). Thus, our results support the idea that PGD_2 via the Akt/PI3K pathway not only induces *Ldh a* gene expression but also indirectly regulates LDH activity by increasing substrate availability.

Experiments using specific antagonists of DP1 and DP2

receptors, allowed us to conclude that PGD_2 actions on *Ldh a* gene expression and LDH activity in TM4 Sertoli cells are exerted via a direct mechanism which involves PGD_2 interacting with specific G-protein coupled DP receptors. Both DP1 and DP2 receptors have previously been described in the testis (Moniot et al., 2014; Rossitto et al., 2015).

To our knowledge, this is the first time that PGD_2 concentrations are quantified in the human testis. Keeping in mind the tissular levels of PGD_2 as well as the average weight, volume and density of the human testis (Sośnik, 1988; Yang et al., 1990; Zhou et al., 2010), concentrations used in this study ranging from 1 to 10 μM PGD_2 can be considered as physiological concentrations.

Previously, we described the expression of *DP1* receptors in the interstitial compartment of the adult human testis (Schell et al., 2007). In this study, we detected the expression of *DP1* and *DP2* receptors in Sertoli cells isolated from testicular biopsies of patients suffering from hypospermatogenesis or SCO syndrome. However, PGD_2 levels and the expression of DP receptors in testes of fertile men remain unknown as a consequence of ethical and legal considerations. At least, a recent report described a negative impact of non-steroidal anti-inflammatory drugs (NSAIDs) in human testes showing normal spermatogenesis suggesting a physiological role of testicular PGs in adult men (Albert et al., 2013). Thus, our studies performed in mouse TM4 Sertoli cells might be of relevance to the human situation.

In summary, our data reveal the existence of a PGD_2-dependent testicular source of ROS generation. Furthermore, ROS not only act as antioxidants in Sertoli cells but also as signaling molecules involved in the regulation of LDH expression/activity. Sertoli cell lactate production is crucial for the support of germ cell development. Hence, the modulation of LDH by free radicals including H_2O_2 supports the assumption of a stimulatory role of PGD_2 on spermatogenesis taking place through its binding to specific DP receptors located in the Sertoli cell membrane and the subsequent local generation of ROS.

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