RESEARCH ARTICLE

1,25(OH)₂ vitamin D₃ signalling on immature rat Sertoli cells: gamma-glutamyl transpeptidase and glucose metabolism

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Received: 2 September 2016 / Accepted: 22 November 2016 / Published online: 3 February 2017
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Abstract 1 α ,25-Dihydroxyvitamin D₃ (1,25-D₃) is critical for the maintenance of normal male reproduction since reduced fertility is observed in vitamin D-deficient rats. Gamma-glutamyl transpeptidase (GGT) is a membrane-bound enzyme that is localized on Sertoli cells and catalyses the transfer of the gamma-glutamyl residues to an amino acid or peptide acceptor. Sertoli cells are also responsible for providing nutrients, as lactate, to the development of germ cells. The aim of this study was to investigate the effect and the mechanism of action of 1,25-D₃ on GGT on Sertoli cell functions from 30-day-old immature rat testis. Results demonstrated that 1,25-D₃ stimulates GGT activity at Sertoli cells plasma membrane through a PKA-dependent mechanism of action, which was not dependent of active de novo protein synthesis. The hormone increases glucose uptake, as well as lactate production and release by Sertoli cells without altering the reactive oxygen species (ROS) generation. In addition, 1,25-D₃ did not change reduced glutathione (GSH) amount or oxygen consumption, and diminished Sertoli cell death. These findings demonstrate that 1,25-D₃ stimulatory effect on GGT activity, glucose uptake, LDH activity and lactate production seem to be an important contribution of Sertoli cells for germ cells nutrition and for a full and active ongoing spermatogenesis.

Keywords Gamma-glutamyl transpeptidase · Glucose · Lactate · Sertoli cells · Testis · Vitamin D₃

Abbreviations

BDC	Bisdemethoxycurcumin
CM	Curcumin
1,25-D ₃	1 α ,25-dihydroxyvitamin D ₃
¹⁴ C–DG	[U- ¹⁴ C]-2-deoxy-D-glucose
GGT	Gamma-glutamyl transpeptidase
KRb	Krebs Ringer-bicarbonate buffer
LDH	Lactate dehydrogenase
GSH	Reduced glutathione
VDR	Vitamin D receptor
VDR-AP	Vitamin D alternative pocket
VDRnuc	Vitamin D nuclear receptor
VDRmem	Vitamin D membrane receptor
CH	Cycloheximide
ST	Stearyl carnitine chloride

Introduction

1 α ,25-dihydroxyvitamin D₃ (1,25-D₃), the active form of vitamin D₃, has biological effects in 38 tissues, including rodent and human testis (Norman et al. 2001b; Blomberg Jensen et al. 2010; Zanatta et al. 2011a; b). The classical mechanism of action of 1,25-D₃ is through a nuclear receptor (VDRnuc), where the hormone interacts with its receptor and induces VDRnuc heterodimerization with retinoid receptor X, resulting in the regulation of gene expression (Bouillon et al. 1995). On the other hand, several studies demonstrate that 1,25-D₃ also acts through a plasma membrane receptor (VDRmem) and triggers signal transduction pathways

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mediated by ionic channels and/or protein kinases, known as rapid responses (Norman et al. 2002; Menegaz et al. 2009).

Additionally to the classical regulation of 1,25-D₃ on calcium and phosphorus homeostasis, several findings indicate that the hormone has an important role on male reproduction and fertility (Menegaz et al. 2009; Blomberg Jensen et al. 2010; Silva 2014). These findings were validated since vitamin D-deficient male rats show reduced fertility. In addition, in VDR null mutant mice sperm count and motility are significantly reduced (Kwiecinski et al. 1989; Kinuta et al. 2000). Recently it was verified the presence of 1,25-D₃ metabolizing enzymes in the male reproductive system, which indicates that 1,25-D₃ may have a local and direct effect on spermatogenesis (Jensen 2014), reinforcing the functional importance of 1,25-D₃ in the testis (Silva 2014).

A marker of Sertoli cell activity in the testis is the gamma-glutamyl transpeptidase (GGT). It is a plasma membrane bound enzyme related to glutathione homeostasis (Schteingart et al. 1988; Mareš et al. 2005) and with amino acid transport for the synthesis of specific proteins known to be secreted by Sertoli cells (Tate and Meister 1981). GGT is widely distributed in mammalian tissues, and in the testis, its activity is attributed to the Sertoli cell function (Hodgen and Sherins 1973; Lu and Steinberger 1977; Caston and Sanborn 1988). Studies show that GGT activity is important to reproductive function, since GGT-deficient mice are infertile, with diminished testis size, oligospermy and accessory glands hyperplasia (Harding et al. 1997; Kumar et al. 2000).

It is well known that the antioxidant system is highly active in the testis as demonstrated in several reports (Zamoner et al. 2007; Zamoner et al. 2008; de Liz Oliveira Cavalli et al. 2013). Reduced glutathione (GSH) can contribute for the redox potential in the testis and GSH content can be replenished by GGT activity (Zamoner et al. 2007; Aprioku 2013; de Liz Oliveira Cavalli et al. 2013).

Sertoli cells are the main structural and nutritional element on seminiferous epithelium, responsible for maintaining the distinguished microenvironment needed for germ cell development (Sharpe et al. 2003). Germ cells have specific metabolic needs, and use preferentially lactate as substrate for ATP production. Glucose is transported into the Sertoli cells via glucose transporters (GLUTs) and metabolized mainly to increase lactate (Galarido et al. 2008; Rato et al. 2012). In fact, the regulation of carbohydrates, lipids and amino acids metabolism in Sertoli cells is crucial for germ cells development (Bajpai et al. 1998; Riera et al. 2001; Rato et al. 2012). Although Sertoli cells require high energy levels to function correctly, the cellular balance of reduction-oxidation (redox cycle) is active and able to maintain the ROS generation within physiological levels (Aprioku 2013; de Liz Oliveira Cavalli et al. 2013). So, the aim of this study was to investigate the mechanism of action of 1,25-D₃ stimulatory effect on GGT activity in Sertoli cells from 30-day-old rat testis, as well as to

highlight the effects triggered by 1,25-D₃ at plasma membrane to regulate Sertoli cell metabolism.

Materials and methods

Chemicals

1 α ,25-dihydroxvitamin D₃ (1,25-D₃), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), KT 5720 (KT), dibutyryl cAMP (dibAMPc), cycloheximide (CH), stearyl carnitine chloride (ST), Ro31-0432 (Ro), ethylenediamine tetraacetic acid (EDTA), curcumin (CM), phorbol 12-myristate 13-acetate (PMA), Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, penicillin, streptomycin, kanamycin and amphotericin B, Serum Replacement 3, bovine pancreas deoxyribonuclease (DNase type I), hyaluronidase (type I-S), trypsin, soybean trypsin inhibitor, sodium pyruvate, D-glucose, sodium bicarbonate, oligomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), rotenone and antimycin A were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase-dispase and bovine serum albumin (BSA) were from Roche Diagnostics (Indianapolis, IN). [U-¹⁴C]-2-Deoxy-D-glucose (¹⁴C-DG), specific activity 9.25 GBq/mmol and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Waltham, MA). All other chemicals were of analytical grade.

Animals

Male Wistar rats (30-day-old) were bred in an animal facility and housed in an air-conditioned room (21 $\pm$ 2 °C) with controlled lighting (12 h/12 h light/dark cycle). Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available ad libitum. All the animals were carefully monitored and maintained in accordance with the local Ethical Committee for Animal Use (Protocol CEUA-UFSC #PP00418).

Sertoli cells primary culture and germ cells isolation

Sertoli cells were obtained from 30-day-old Wistar rats. Rats were killed by decapitation, and testes were removed and decapsulated. Sertoli cells were obtained by sequential enzymatic digestion as previously described by Dorrington et al. (Dorrington et al. 1975). Sertoli cells were seeded at a concentration of 650,000 cells/cm² in 6 and 24-wells culture plates in Ham's F12/ DMEM (1:1) medium supplemented with 2% Serum Replacement 3, 2.2 g/L. sodium bicarbonate, antibiotics (50,000 IU/L. penicillin, 50 mg/L. streptomycin, and 50 mg/L. kanamycin), and a fungicide (0.25 mg/L.

amphotericin B), in a humidified atmosphere of 5% CO₂ and 95% air at 34 °C. Three days after being plated, residual germ cells were removed by a hypotonic treatment using 20 mM Tris-HCl (pH 7.2) for 150 s (Galdieri et al. 1981). Cells were washed with PBS, and fresh Ham's F12/DMEM (1:1) medium without serum was added 24 h before treatment. Five days after being plated, cells were incubated in the presence or absence of treatment in medium devoid of serum replacement 3 or in Krebs Ringer-bicarbonate buffer (KRb) (122 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, and 25 mM NaHCO₃). Germ cells were obtained during Sertoli cell isolation process, constituting a germ cell rich fraction.

Gamma-glutamyl transpeptidase assay

GGT activity was measured by the modified technique described previously by Orlowski and Meister (Orlowski and Meister 1963) using L- c-glutamyl *p*-nitroanilide as the substrate and glycylglycine as the acceptor molecule. Since our previous studies showed that 1 nM 1,25-D₃ has a positive effect on GGT activity in rat testis (Zanatta et al. 2011a), the same concentration was chosen to carry out these experiments in isolated Sertoli cells. Sertoli cells in either control or treated group were incubated in serum free medium with or without 1,25-D₃ (1 nM) or curcumin (1 nM) or PMA (100 nM) for 1, 3, 6 and 24 h. In some experiments inhibitors (CM, CH, H-89, KT, dibAMPc, ST, Ro) were added 30 min before the incubation time and maintained for the whole incubation period. In all cases, the inhibitors' concentrations used were those that have previously resulted in inhibition in similar assays (Menegaz et al. 2009, Menegaz et al. 2010, Menegaz et al. 2011; Zanatta et al. 2011a, Zanatta et al. 2013; Rosso et al. 2012).

After incubation, the cells were homogenized in cold 0.1 M Tris buffer, pH 8.5 and adequate aliquots were saved for total protein determinations and 30 uL were incubated with the enzyme substrate. The reaction was allowed to proceed for 60 min at 34 °C and the enzymatic reaction was stopped by acetic acid addition. The absorbance of the samples was determined in a plate reader (Tecan Infinite 200 PRO, LAMEB I/CCB/UFSC) at 530 nm. The results were calculated as U/L./μg protein expressed as % of control (Zanatta et al. 2011a).

Lactate determination

Sertoli cells were incubated for 1, 3, 6 and 24 h either in the presence or absence of 1,25-D₃ (1 nM) in serum free medium and the incubation medium was collected for lactate measurements. The lactate levels were estimated by the lactate oxidase method (Hutchesson et al. 1997) and the results were

calculated as mg of lactate/μg of protein expressed as % of control (Zanatta et al. 2011a).

Lactate dehydrogenase activity measurements

For the measurements of intracellular lactate dehydrogenase (LDH) activity, Sertoli cells were incubated in serum free medium in the presence or absence of 1,25-D₃ (1 nM) for 6 h. After incubation, the cells were homogenized in cold 0.1 M Tris buffer, pH 7.3 and adequate aliquots were saved for total protein determinations and LDH activity. For the extracellular LDH activity, after cell incubation in the presence or absence of 1,25-D₃ (1 nM) for 1, 3, 6 and 24 h, the incubation medium was collected for determination of LDH activity. The enzyme activity was measured by a spectrophotometric method. Enzymatic activity estimation was carried out by measuring NADH oxidation (Nehar et al. 1997) and the results were calculated as U/L./μg of protein expressed as % of control (Zanatta et al. 2011a).

Reduced glutathione assay

For the GSH assay, Sertoli cells were incubated in serum free medium in the presence or absence of 1,25-D₃ (1 nM) for 6 h. After incubation, the cells were homogenized in 12% trichloroacetic acid (TCA). GSH was determined according to Beutler et al. (Beutler et al. 1963) using the reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). An aliquot was added to 2.5 mM DTNB in 0.2 M sodium phosphate buffer, pH 8.0, and the formation of the yellow thiolate anion was immediately measured at 412 nm. Determinations were expressed in % of control (de Liz Oliveira Cavalli et al. 2013).

Mitochondrial oxygen consumption

For the mitochondrial oxygen consumption assay, Sertoli cells were incubated with serum free medium in the presence or absence of 1,25-D₃ (1 nM) for 1 h. Afterwards, cells were washed with 0.2 M phosphate buffer pH 7.3 and trypsinized with 0.05% Trypsin/ 0.53 mM EDTA solution. The suspension of 350,000 cells/mL was centrifuged, suspended in a 2 mL chamber and the respiration was measured at 34 °C by high-resolution respirometry using the Oroboros® oxygraph (Remor et al. 2011; de Paula Martins et al. 2013). DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition (1 or 2 s intervals) and analysis. The experimental regime was started with routine respiration, which is defined as respiration without additional substrates or effectors. After observing steady-state respiratory flux in the time interval between 15 and 30 min,

the ATP synthase activity was inhibited with oligomycin (1 $\mu\text{g/mL}$), followed by uncoupling of oxidative phosphorylation by stepwise titration of FCCP (0.6 μM for maximal respiration). Finally, respiration was inhibited by sequential addition of rotenone (0.5 μM ; to test for the effect of inhibiting complex I activity) and antimycin A (2.5 μM ; inhibiting complex III) (Remor et al. 2011).

Glucose uptake

Five days after being plated, Sertoli cells were preincubated and incubated in KRB in a Dubnoff metabolic incubator at 34 °C (pH 7.4) and gassed with an O_2/CO_2 mixture (95:5, v/v). 1 nM 1,25- D_3 was added to the preincubation (30 min) and incubation (60 min) medium. ^{14}C -DG (0.1 $\mu\text{Ci/mL}$) was added to each sample during the incubation period. After incubation, the cells were rinsed twice with cold KRB to remove the unincorporated ^{14}C -DG and were homogenised with a 0.5 M NaOH solution. Also, germ cells were assayed in the presence of ^{14}C -DG (0.1 $\mu\text{Ci/mL}$) for 1 h of incubation, in similar experimental conditions. Radioactivity was measured by liquid scintillation using a LKB rack beta liquid scintillation spectrometer (model LS 6500, Multi- Purpose Scintillation Counter, Beckman Coulter, Boston, MA). The protein concentrations were determined by the Lowry method (Lowry et al. 1951) and the results calculated as mg glucose/ μg of protein expressed as % of control (Cazarolli et al. 2009).

Statistical analysis

The results were expressed as mean $\pm$ S.E.M. or % of control of 3 independent experiments, with three samples in each group. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or unpaired Student's *t*-test were used to identify significant differences between groups. Differences were considered to be significant at $p \leq 0.05$.

Results

1,25- D_3 time-course on GGT activity in Sertoli and germ cells

Sertoli cells were isolated as described previously by our group, where the VDR receptor was identified in these cells, as well as the characterization of calcium influx stimulated by 1,25- D_3 (Zanatta et al. 2011b). To study the hypothesis that GGT, an enzyme localized at plasma membrane, is a target for 1,25- D_3 in the testis, the enzyme activity was assayed in Sertoli cells primary culture. Figure 1 shows the time-course of GGT activity in

Fig. 1 Time-course of 1,25- D_3 (1 nM) on GGT activity in 30-day-old rat Sertoli cells (Time-course). Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as U/L/ μg protein and expressed as % of control. ** $p < 0.01$ compared to control group

response to 1 nM 1,25- D_3 in Sertoli cells. A stimulatory effect of the hormone on GGT activity was observed after 6 h of exposure. This increment in enzymatic activity was about 30% compared with the control group. On the other hand, GGT activity on germ cell-rich fraction was not detected (data not shown).

Effect of natural agonist curcumin and cycloheximide in the stimulatory action of 1,25- D_3 on GGT activity in Sertoli cells

Figure 2A shows the ability of a natural VDR agonist (curcumin) to stimulate the GGT activity in Sertoli cells within 6 h comparing with 1,25- D_3 . As it can be seen, 1 nM curcumin was able to increase GGT activity as efficiently as 1,25- D_3 . In addition, when both were co-incubated, curcumin did not potentiate the stimulatory effect of 1,25- D_3 on GGT activity. The Fig. 2B shows that cycloheximide (0.35 mM), a de novo

Fig. 2 Influence of natural VDR ligand curcumin (CM) and cycloheximide (CH) on the stimulatory effect of 1,25- D_3 (1 nM) on GGT activity in 30-day-old rat Sertoli cells. Curcumin (1 nM) and cycloheximide (0.35 mM) were added 30 min before the hormone. Incubation time: 6 h. Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as U/L/ μg protein and expressed as % of control. * $P < 0.05$; ** $p < 0.01$ compared to control group

protein synthesis inhibitor did not alter 1,25-D₃ effect on GGT activity.

PKA pathway participates in 1,25-D₃ stimulatory effect on GGT activity in Sertoli cells

To assert 1,25-D₃ mechanism of action on GGT activity, PMA was used as a GGT activator. PMA in combination with 1,25-D₃ had a synergic effect on GGT activity, as can be seen in Fig. 3.

It was previously demonstrated that 1,25-D₃ stimulates GGT activity in whole testis from 30-day-old rats via PKA activation. Figure 4A, B and C show the involvement of PKA in 1,25D₃ mechanism of action on GGT activity in Sertoli cells. When PKA inhibitors were used (H-89, KT) the stimulatory effect of the hormone on GGT activity was prevented. Sertoli cells pretreatment with a cAMP analogue (dibAMPc), a PKA activator, potentiated 1,25-D₃ effect, and the analogue also exhibited a *per se* effect on enzyme activity. The presence of PKA inhibitors did not change PMA effect.

In a similar manner, PKC involvement was also studied on 1,25-D₃ effect on GGT activity (Fig. 5A and B). The co-incubation with PKC inhibitors (ST, Ro) did not change the hormone stimulatory effect on enzyme activity. The inhibitors did not have any *per se* effect. In addition, when co-incubated with PKC inhibitors, PMA was not able to increase GGT activity.

1,25-D₃ effect on GSH content in Sertoli cells

Since GGT has been suggested to play a role on GSH recycling and removal of oxidized substances, 1,25-D₃ effect

Fig. 3 Influence of PKC activator (PMA 100 nM) on the stimulatory effect of 1,25-D₃ (1 nM) on GGT activity in 30-day-old rat Sertoli cells. Incubation time: 6 h. Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as U/L/ μ g protein and expressed as % of control. ** P < 0.01; *** P < 0.001 compared to control group; ### P < 0.001 compared to 1,25-D₃ group and @@@ P < 0.001 when compared to PMA group

Fig. 4 Involvement of PKA pathway on the stimulatory effect of 1,25-D₃ (1 nM) on GGT activity in 30-day-old rat Sertoli cells. 10 μ M H-89 (a), 1 μ M KT5720 (b) and 500 μ M DibAMPc (c) were added 30 min before the hormone. PMA 100 nM, when used, was added at the same time as the hormone. Incubation time: 6 h. Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as U/L/ μ g protein and expressed as % of control. * P < 0.05; ** P < 0.01; *** P < 0.001 compared to control group and # P < 0.05 compared to 1,25-D₃ group

on GSH content in Sertoli cells was also investigated. Figure 6 shows that after 6 h of incubation with 1 nM 1,25-D₃ no change was observed, indicating that the increase in GGT activity stimulated by the hormone does not seem to be related to GSH regeneration.

Fig. 5 Involvement of PKC pathway on GGT activity in 30-day-old rat Sertoli cells. 1 μ M stearylcarbitine (ST) (a) and 20 μ M Ro31-0432 (b) were added 30 min before the hormone. PMA 100 nM, when used, was added at the same time as the hormone. Incubation time: 6 h. Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as U/L/ μ g protein and expressed as % of control. * P < 0.05; ** p < 0.01 compared to control group; @ p < 0.05 when compared to PMA group

1,25-D₃ effect on glucose uptake

Figure 7 shows ¹⁴C-DG uptake in rat Sertoli cells and in germ cells after 1 h of incubation in the presence of 1 nM 1,25-D₃. The results show that the hormone significantly stimulated ¹⁴C-DG uptake in Sertoli cells but not in germ cells.

Fig. 6 Effect of 1 nM 1,25-D₃ on GSH levels in 30-day-old rat Sertoli cells. Incubation time: 6 h. Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, expressed as % of control

Fig. 7 Effect of 1 nM 1,25-D₃ on glucose uptake in 30-day-old rat Sertoli cells and germ cell-rich fraction. Incubation time: 1 h. Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as mg glucose/ μ g of protein and expressed as % of control. * P < 0.05 compared to control group

1,25-D₃ effects on lactate secretion, intracellular LDH activity and LDH release in Sertoli cells

Figure 8A shows the time-course of lactate secretion in culture medium by Sertoli cells stimulated by 1,25-D₃ (1 nM) during 1, 3, 6 and 24 h. These findings demonstrate significant increments in lactate production at 3 and 6 h of incubation in the presence of hormone. Figure 8B shows the data obtained for intracellular LDH activity measurements in rat Sertoli cells stimulated for 6 h with 1 nM 1,25-D₃. A significant increase in enzyme activity in 1,25-D₃-treated cultures corroborates with the increased lactate production. On the other side, Fig. 8C shows LDH release after Sertoli cells exposure to 1,25-D₃ (1, 3, 6 and 24 h). As it can be seen, the hormone diminished LDH release after 1 h and did not alter these values compared with control groups in other incubation times, indicating that the hormone does not exhibits toxicity and also prevents cellular death in Sertoli cells, at least in this concentration and periods studied.

1,25D₃ effects on mitochondrial respiration in Sertoli cells

Since oxygen consumption represents the oxidative metabolic activity of a particular cell, this parameter was evaluated after the acute exposure of Sertoli cells to 1,25-D₃ (1 nM 1,25-D₃; 1 h). Figure 9 shows the basal respiration that represents the activity of the mitochondrial electron transfer system coupled to ATP synthesis (without addition of effectors) was not altered by the treatment. This effect was further confirmed by using oligomycin (ATP synthase inhibitor) and FCCP (ionophore uncoupler of mitochondrial respiration), showing no impact on ATP turnover, or in maximal respiration or spare mitochondrial capacity. However, a significant inhibitory effect of 1,25-D₃ was observed after the addition of rotenone plus antimycin (inhibitors of complexes I and III, respectively). The residual activity after the addition of these inhibitors shows non-mitochondrial oxygen consumption, usually represented by partial reduction of oxygen into reactive oxygen

Fig. 8 **a** Time-course of 1,25-D₃ (1 nM) effect on lactate release, **b** intracellular LDH activity and **c** LDH release in 30-day-old rat Sertoli cells. Incubation time: 6 h (**b**). Values are means $\pm$ S.E.M. of three independent experiments, three samples in each group, calculated as (**a**) mg of lactate/ μ g of protein and (**b, c**) U/L/ μ g of protein and expressed as % of control. * $P < 0.05$ and ** $p < 0.01$ compared to control group

species (ROS). In this case, 1,25-D₃ significantly avoided the formation of ROS.

Discussion

It is well known that vitamin D₃ does not have any intrinsic biological activity (Norman 2008) and that the endocrine system of vitamin D is mandatory for the generation of the active hormone and its biological response (Norman and Roberts 1980). 1,25-D₃ effects are mediated through the 1,25-D₃ receptor (VDR), which is expressed in over 38 tissues including rodents and human testis (Habib et al. 1990; Johnson et al.

Fig. 9 Effect of 1,25-D₃ (1 nM) on oxygen consumption by 30-day-old rat Sertoli cells. Incubation time: 1 h. The mitochondrial oxygen consumption under basal respiration, oligomycin, FCCP and rotenone plus antimycin A conditions were assessed as indicated in material and methods. Values are mean $\pm$ S.E.M. of four to five independent experiments (one determination of 350,000 cells per group per experiment). ** $P < 0.01$ compared to control group

1996; Norman et al. 2001b). This hormone acts through its widely distributed nuclear receptor (VDRnuc) to regulate gene transcription (genomic responses) and through a putative membrane receptor (VDRmem) to mediate a variety of rapid or nongenomic responses. The distinguished characteristic that allows 1,25-D₃ to bind both VDRnuc and VDRmem receptors is its conformational flexibility, that can present a bowl-like or planar-like shape and trigger a mechanism by which it can stimulate both genomic and nongenomic cellular responses, respectively (Norman et al. 2001a; Mizwicki and Norman 2009). Herein, we showed for the first time that 1,25-D₃ activates gamma-glutamyl transpeptidase activity in Sertoli cells, an enzyme that is strictly localized at the plasma membrane of these cells in the testis (Tindall et al. 1985).

Numerous studies showed that curcuminoids from turmeric, such as curcumin (CM) and bisdemethoxycurcumin (BDC) are natural ligands for the VDR receptor (Jurutka et al. 2007; Masoumi et al. 2009) and also potential agonists of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels (Berger et al. 2005; Wang et al. 2007; Verkman and Galletta 2009). The planar-like shape of CM, associated with its ability to interact with VDR, are important characteristics that can be useful to determine whether CM and related compounds might also be VDRmem nongenomic agonists. Based on that, Menegaz et al. (Menegaz et al. 2011) provided strong evidence for the functional role of VDRmem in mediating nongenomic responses to vitamin D sterols and curcuminoids through VDR alternative pocket (VDR-AP) binding in TM4 Sertoli cells. The 1,25-D₃-mimetic effect of CM *per se* on GGT activity is in line with the data reported by Menegaz et al. (Menegaz et al. 2011) regarding the action of CM at the plasma membrane to regulate chloride channels in TM4 Sertoli cells. In addition, the equivalent potency on the stimulatory effect on GGT activity observed when both compounds were co-incubated or individually incubated, a fact

that points to a possible common mechanism for these substances.

The results with cycloheximide (CH), an mRNA translation inhibitor, indicate that 1,25-D₃ effects on GGT activity could be independent of de novo protein synthesis in a short-term period. Prelot et al. (Prelot et al. 1990; Prelot et al. 1991) also demonstrated that CH had no *per se* effect in 24R,25-(OH)₂ vitamin D₃ and 1,25-D₃ effects on GGT activity in the kidney in similar incubation periods. In another work, Ito et al. (Ito et al. 2014) suggest that 1,25-D₃ effects on GGT activity in kidney epithelial cells may involve changes in the enzyme's turnover, since they did not find any changes in GGT mRNA levels. This could explain 1,25-D₃ effects on GGT activity in Sertoli cells demonstrated in the present work, but further studies are needed to confirm this hypothesis. In a whole, these data strongly point a short-term effect of 1,25-D₃ mediated by VDRmem to regulate GGT activity on Sertoli cells.

To assert 1,25-D₃ mechanism of action on GGT activity, PMA was used as a GGT activator. PMA is a phorbol ester that acts as an unspecific PKC activator, but can also activate

NADPH oxidase enzyme resulting in increased ROS generation and consequently GGT activation (Ravuri et al. 2011; Pandur et al. 2014). The synergistic action observed when PMA was co-incubated with 1,25-D₃ could imply an increased response of 1,25-D₃ over GGT activity in front of the additional ROS generation by PMA through NADPH oxidase activation.

Our group has already demonstrated that PKA acts as mediator on rapid responses stimulated by 1,25-D₃ on amino acid accumulation in whole testis, chloride currents activation in TM4 Sertoli cell line and in calcium influx in Sertoli cells (Menegaz et al. 2009; Menegaz et al. 2010; Zanatta et al. 2011b). Concerning PKA's involvement, specifically on GGT activity on Sertoli cells, our results are in agreement with those described by Zanatta et al. (Zanatta et al. 2011a), who reported that PKA activation by 1,25-D₃ is essential for 1,25-D₃ effect on GGT activity in the testis. Our results are therefore in line with precedent works, which describe the participation of PKA on the stimulatory effect of FSH on GGT in Sertoli cells (Caston and Sanborn 1988) and GGT regulation through protein kinases activity and calcium levels (Meroni et al. 1997).

Fig. 10 Schematic representation of 1,25D₃-induced signalling pathways on Sertoli cells for GGT activity and energetic metabolism. 1,25-D₃ binds to VDRmem on Sertoli cells, activates PKA, which stimulates the plasma membrane GGT enzyme activity. The stimulatory effect of 1,25-D₃ on GGT, glucose uptake, LDH activity and lactate production seems to be the contribution of Sertoli cells for the germ cells nutrition. In addition, the unchanged GSH content and reduced

ROS generation in the presence of 1,25-D₃ should play a key role for a full and active ongoing spermatogenesis. GGT: gamma-glutamyl transpeptidase; VDR: vitamin D receptor; GLUT: glucose transporters; PKA: protein kinase A; LDH: lactate dehydrogenase; ROS: reactive oxygen species; MCT: monocarboxylate transporter. Straight lines are direct pathways; dotted lines represent multi-step pathways

Meroni et al. (Meroni et al. 1997) demonstrated that FSH regulates GGT activity in Sertoli cells through PKC and calcium. When we investigated the participation of PKC in the stimulatory effect of 1,25-D₃ on GGT activity in Sertoli cells, the results suggested no involvement of PKC in this effect. When incubated with PKC inhibitors, PMA effect on GGT activity was inhibited, demonstrating the efficacy of PKC inhibitors used in this work. On the other hand, PKA inhibitors did not change PMA effect on GGT activity, and these facts altogether corroborate to the 1,25-D₃'s mechanism of action on GGT activity suggested in the present work.

In the testis and specifically in Sertoli cells, the antioxidant system is characterized by high activity of both enzymatic and non-enzymatic antioxidant defences (Zamoner et al. 2007; Zamoner et al. 2008; Aly and Khafagy 2011; de Liz Oliveira Cavalli et al. 2013). GSH, a tripeptide of L- γ -glutamyl-L-cysteinyl-glycine, is one of the main protectors against oxidative and electrophilic stresses and radiation in cells (Meister 1988). GSH is able to act either as a substrate in the cytosolic GSH redox cycle or can act directly to inactivate ROS. A myriad of biological functions that requires GSH can lead to variations in the cellular availability of GSH (Hayes and Pulford 1995). Taking into account our data, 1,25-D₃ did not change GSH content on Sertoli cells after 6 h. These data indicate that the increased GGT activity by 1,25-D₃ does not participate directly in the GSH metabolism to keep the redox cycle. On the other hand, the hormone could be assisting the maintenance of GSH content on these cells, since 1,25-D₃ effects on GGT activity did not decrease GSH content.

One of the main roles of Sertoli cells, also known as nurse cells in the testis, is to provide enough energy and nutritional support for germ cells' survival and development. Energy production in germ cells comes mainly from lactate, which is produced by glucose metabolism in Sertoli cells and secreted in the seminiferous tubules compartment (Rato et al. 2012). Once lactate is uptake by germ cells through a monocarboxylate transporter MCT2 (a family of proton-linked plasma membrane transporters), it is used to generate ATP for metabolic processes (Robinson and Fritz 1981; Grootegoed et al. 1986; Rato et al. 2012). Lactate production by Sertoli cells is classically reported to be regulated by FSH, insulin, basic fibroblast growth factor and cytokines (see review by Rato et al. (Rato et al. 2012)). Here we show that 1,25-D₃ stimulates glucose uptake, increases lactate release and LDH activity in Sertoli cells. Altogether, these data show a role of 1,25-D₃ on Sertoli cells' energetic metabolism that is crucial for germ cell development and the ongoing spermatogenesis.

Cellular energy production generally requires glucose catabolism to pyruvate by the enzymes of the glycolytic pathway. This process produces some ATP but the major role of glycolysis is to fuel the Krebs cycle and oxidative phosphorylation on the respiratory chain, where most ATP is produced (Hatefi et al. 1962). Associated with the respiratory capacity

of the cells, mitochondria is a major source of ROS even under physiological conditions, since around 3% of the oxygen consumed is converted to O₂⁻ (Kowaltowski and Vercesi 1999). ROS can induce deleterious effects in cells, like impairing the oxidative phosphorylation, mitochondrial DNA deletions and mitochondrial membrane permeability modifications (Cardoso et al. 1999). Taking into account that 1,25-D₃ stimulated glucose uptake associated with the physiological conditions that favour ROS generation, we observed that in these experimental conditions, 1,25-D₃ neither modified basal respiration nor oxygen consumption. In addition, 1,25-D₃ inhibited ROS production in Sertoli cells and prevented cellular death, reinforcing the protective role of this hormone, also reported on breast cancer cells and in monocytes (Marchionatti et al. 2009; Jain and Micinski 2013). In a whole, the present data suggests that 1,25-D₃ effect on GGT activity in Sertoli cells is mediated by PKA possibly through VDRmem activation of nongenomic responses. In addition, 1,25-D₃ contributes for germ cells nutrition since the hormone activates LDH and enhances lactate secretion, as hypothesized in Fig. 10.

Conclusion

The results demonstrate that 1,25-D₃ stimulates GGT activity at plasma membrane of Sertoli cells possibly through a nongenomic mechanism of action involving protein kinases activation, like PKA. In a whole, these data demonstrated that 1,25-D₃ stimulatory effects on GGT activity, glucose uptake, LDH activity and lactate production seem to enhance Sertoli cells' contribution to germ cells nutrition. These results, together with the unchanged GSH content and decreased ROS generation in the presence of 1,25-D₃ could demonstrate the fundamental role of the hormone for a full and active ongoing spermatogenesis.

Acknowledgments This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico-Brasil (CNPq n° 472071/2013-0) and Coordenação de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Santa Catarina (FAPESC). RG, AR and DLS are registered on the PPG-Biochemistry/UFSC. APZ is registered on the PGFAR/UFSC. FRMBS, AZ and AL are recipient of CNPq productivity fellowship. We thank LAMEB I-CCB/UFSC technicians for their support.

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