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NO/cGMP signaling increases mitochondrial membrane potential and affects androgenesis in Leydig cells of adult rats

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Summary. Mitochondria are a key control point for the regulation of steroid hormone biosynthesis in all steroid-producing cells, including androgen-producing Leydig cells. The first step of steroid biosynthesis is mobilization of cholesterol to the mitochondrial inner membrane, which is facilitated by the steroidogenic acute regulatory (StAR) protein. Previous studies on Leydig cells indicated the importance of mitochondrial membrane potential ($\Delta\psi_\mu$) on hormone stimulated androgen production. Since nitric oxide (NO) could regulate androgen production, it was of interest to explore the effects of NO-cGMP-dependent signaling on $\Delta\psi_\mu$ and the steroidogenic activity of rat Leydig cells. The results showed that treatment of purified Leydig cells with NO-donor causes a dose-dependent increase in both $\Delta\psi_\mu$ and cGMP production without effecting cAMP intracellular levels. In the same cells, a biphasic effect of NO-donor on testosterone production was observed; stimulatory in the dose range ($0\text{-}10^{-5}$ M) and inhibitory at 10^{-3} M. Levels of mature StAR protein also exhibit a similar biphasic effect. Furthermore, NO-donor dose dependently stimulates cGMP production and $\Delta\psi_\mu$ even in cells stimulated with a supra-maximal dose of human chorionic gonadotropin (hCG, 50 ng/ml). The hCG-treatment alone slightly increased cGMP production, and this effect was followed by a small but significant increase in $\Delta\psi_\mu$. Together, results from all experimental approaches stimulating cGMP production significantly correlated with $\Delta\psi_\mu$ values, suggesting implication of cGMP signaling in the control of mitochondrial membrane polarity. In summary, our results support a positive role for cGMP signaling in the maintenance of mitochondrial membrane polarity through increased $\Delta\psi_\mu$, followed by stimulated androgen production.

Key words: androgenesis, cGMP, Leydig cells, mitochondrial membrane potential.

Introduction

Testosterone is primarily secreted by Leydig cells in male testes and female ovaries, although smaller amounts are produced by the adrenal gland in both sexes (Wang et al. 2009). Leydig cells, like all other steroid-producing cells, synthesize steroid hormones from a common precursor, cholesterol. Transport of cholesterol from intracellular sources into the mitochondria is a rate-limiting and hormone-sensitive process that requires the presence of a specific complex of transport carrier proteins, including the steroidogenic acute regulatory (StAR) protein: operating as components of a larger "cholesterol transfer" complex, recently named the transduceosome (Stocco et al. 2005; Liu et al. 2006; Jefcoate et al. 2011). Once delivered to the inner mitochondrial membrane, cholesterol is converted to pregnenolone, which is further metabolized to progesterone in mitochondria or microsomes. In Leydig cells, maturation of progesterone occurs via androstenedione to testosterone, and all steps in steroidogenesis are catalyzed by numerous enzymes (Payne and Hales 2004; Manna and Stocco 2011). The steroidogenic function of Leydig cells is predominantly regulated by pituitary luteinizing hormone (LH) or its placental counterpart human chorionic gonadotropin (hCG). Activation of LH/hCG receptors leads to stimulation of adenylyl cyclase, accumulation of cAMP intracellular levels and the concomitant activation of cAMP-dependent kinase (PRKA). Phosphodiesterases terminate cAMP signaling and have a regulatory function in Leydig cells (Catt and Dufau 1973; Dufau 1998). Regulation of Leydig cell steroidogenesis is a

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multi-compartmental process, which, in addition to LH, includes complex neural, endocrine, paracrine and autocrine signaling pathways (Saez 1994; Payne and Hales 2004; Lavoie and King 2009; Martinez-Arguelles and Papadopoulos 2010; Manna and Stocco 2011), and also involves NO-cGMP signaling (Andric et al. 2007; Andric et al. 2010a, 2010b; Kostic et al. 2010).

In addition to its central role in cholesterol transport and metabolism in steroidogenic cells, mitochondria are best known for their role in the synthesis of ATP. In many cells, mitochondrial ATP synthesis provides the bulk of cellular ATP through oxidative phosphorylation, a process in which electrons flow from electron donors (NADH and FADH2) generated by mitochondrial metabolic processes to the terminal electron acceptor, oxygen. Electron transfer occurs along a series of mitochondrial polypeptide complexes—complex I (NADH dehydrogenase), complex III (cytochrome c reductase), and complex IV (cytochrome c oxidase) — and is electrochemically coupled to the translocation of protons across the inner mitochondrial membrane, generating a proton-motive force composed of an electrical gradient ($\Delta\psi_m$) and an H^+ gradient (ΔpH). The mitochondrial membrane potential ($\Delta\psi_m$) is utilized by mitochondria for numerous processes, including powering mitochondrial ATP synthase, and steroid hormones biosynthesis (Allen et al. 2006; Wittig and Schägger 2009; Kadenbach et al. 2010; Midzak et al. 2011).

It has been demonstrated that mitochondrial disruption inhibits steroid biosynthesis at multiple steps in the steroidogenic pathway (for references please see Allen et al. 2006; Midzak et al. 2011). Numerous studies indicate that disrupting mitochondria results in posttranscriptional changes in StAR, and that mitochondria must be energized, polarized, and actively respiring in order to support cAMP-stimulated Leydig cell steroidogenesis. It has also been reported that primary Leydig cell ATP levels are highly sensitive to $\Delta\psi_m$ disruption, and highly dependent on mitochondrial ATP for their steroidogenic function (for references please see Allen et al. 2006; Midzak et al. 2011). However, these studies did not address the relative contributions of mitochondrial membrane potential $\Delta\psi_m$ to cGMP-dependent control of steroidogenesis. Knowledge of these contributions is important for our mechanistic understanding of steroid synthesis and metabolism.

A major objective of the present study was to compare the relationship between activation of NO-cGMP-signaling and mitochondrial membrane potential ($\Delta\psi_m$) and testosterone synthesis in Leydig cell primary culture. To this end, relationships among $\Delta\psi_m$, cellular cAMP/cGMP levels and testosterone were analyzed in primary Leydig cells, which were freshly isolated from rat testes.

Materials and methods

Antisera for StAR protein were generous gifts from Professor Douglas Stocco (Clark et al. 1994), while the actin (ACTB) detection kit was obtained from Oncogene Research Products (www.emdbiosciences.com). The anti-rabbit secondary antibodies linked to horseradish peroxidase were obtained from Kirkegaard & Pery Labs (www.kpl.com). The anti-testosterone-11-BSA serum №250 was kindly supplied by Gordon D. Niswender. The (1,2,6,73H(N)) labeled testosterone was obtained from Perkin-Elmer Life Sciences (www.PerkinElmer.com). Human CG (hCG; Pregnyl, 3000 IU/mg), Medium 199 containing Earle's salt and L-glutamine (M199), DMEM/Nutrient Mixture F-12 Ham With L-glutamine and 15 mM HEPES (DMEM/F12), HEPES, penicillin, streptomycin, EDTA, Percoll, BSA fraction V, collagenase type IA, testosterone, trypan blue, phosphoric acid β-glycerophosphate, tergitol (Niaproof 4, type 4), dithiothreitol, leupeptin, aprotinin were from Sigma (www.sigmaaldrich.com). Sodium nitrite and 3,3'-(hydroxynitro-sohydrazino] bis-1-propanami-ne (DPTA) were obtained from Alexis Biochemicals (www.enzolifesciences.com/alexis/). The tetramethylrhodamine ethylester (TMRE) was purchased from Fluka (www.fluca.com).

Ethical approval

All the experimental protocols were approved by the local Ethical Committee on Animal Care and Use at the University of Novi Sad, operating under the rules of the National Council for Animal Welfare and following statements of the National Law for Animal Welfare (copyright March 2009). All experiments were performed and conducted in accordance with the National Research Council (NRC) publication Guide

for the Care and Use of Laboratory Animals (copyright 1996, National Academy of Sciences, Washington D.C.) and NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23, revised 1996, 7th edition). All experiments were carried out in the Laboratory for Reproductive Endocrinology and Signaling, DBE, Faculty of Sciences at University of Novi Sad.

Animals

Adult (3 months old, 250-270 g) male Wistar rats, bred and raised in the Animal Facility of the Department of Biology and Ecology (Faculty of Sciences, University of Novi Sad, Serbia), were used for the experiments. The animals were raised in controlled environmental conditions (22 ± 2°C; 12 hours light/dark cycle, lights on at 0700 h) with food and water *ad libitum*.

Preparation of purified Leydig cell and ex vivo hormones; cAMP/cGMP and NO production

Primary cultures of purified Leydig cells were individually obtained from male rats, and isolated/purified as described previously by our group (for references please see Andric et al. 2010a, b; Kostic et al. 2010; Kostic et al. 2011). Primary Leydig cell cultures were used to follow ex vivo progesterone and androgens (T+DHT), cAMP/cGMP and NO production, the mitochondrial membrane potential ($\Delta\psi_m$), and mature StAR protein expression. Primary cultures of purified Leydig cells were prepared from suspensions of interstitial cells. Interstitial cell suspensions were prepared according to Anakwe et al. (1985) with some modifications described previously by our group (for references please see Andric et al. 2010a, b; Kostic et al. 2010; Kostic et al. 2011). Briefly, testes were quickly removed, decapsulated and placed in a 50-ml plastic tube (2 testes per tube) containing 3 ml of collagenase solution (1.25 mg/ml collagenase Type I; 1.5% BSA; 20 mM HEPES in DMEM/F12) and incubated for 15 min at 34°C in a shaking water bath oscillating at 120 cycles/min. The dissociated cells were diluted in 20 ml cold M199-0.5% BSA and placed on ice for 5 min to allow the seminiferous tubules to settle before filtering the supernatant through Mesh №100 (Sigma Inc). The resulting cell suspension was centrifuged at 160xg for 5 min at room temperature, and the cell pellet was washed twice and resuspended in a corresponding amount (5 ml per testis) of DMEM/F12-0.1% BSA. The 0.2% trypan blue dye exclusion test (Sigma Inc) was used to determine cell viability and to ensure that greater than 95% of the cells were viable. Suspensions of interstitial cells (Klinefelter et al. 1987) were used to prepare primary cultures of purified Leydig cells (for references please see Andric et al. 2010a, b; Kostic et al. 2011) by centrifugation on a Percoll gradient consisting of four 2 ml layers of Percoll with densities of 1.090, 1.080, 1.065, and 1.045 g/ml (formed by mixing isotonic Percoll consisting of 10x concentrated DMEM/F12 enriched with 3% of BSA and the corresponding amount of Percoll and distilled water). A crude suspension of interstitial cells (approximately 35-40 x 10^6 cells), containing (in addition to Leydig cells) macrophages and endothelial cells (Klinefelter et al. 1987), was applied to each Percoll gradient and centrifuged at 500xg for 28 min at room temperature. Fractions containing Leydig cells were collected from the 1.080/1.065 g/ml and 1.065/1.045 g/ml interfaces, washed in 50 ml M199-0.1% BSA and centrifuged at 200xg for 5 min at room temperature. Cells were resuspended in a corresponding volume (2.5 ml per testis) of culture medium (DMEM/F12 supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin) and counted. The proportion of Leydig cells present in culture was determined by staining for 3β-HSD activity (Payne et al. 1980), and was found to be 95.3 ± 2.7%, while cell viability was > 90%. The steroidogenic capacity of Leydig cells (estimated by dose-dependent stimulation with hCG) and the activity of steroidogenic enzymes (estimated by incubating cells with increasing concentrations of steroid substrates), were in line with those previously published by others (Akinbami et al. 1994) and our group (for references please see Kostic et al. 2011). Purified Leydig cells obtained individually from 4 rats were pooled, plated in 90 mm Petri dishes (5x10^6 cells in 5 ml culture medium per dish) and placed in a CO_2 incubator (34°C) for 3 h to attach and recover. Three to five replicates from each pool/group were cultured for further analysis of ex vivo testosterone production,

cAMP/cGMP, mitochondrial membrane potential and StAR expression. Using the same pool of Leydig cells for these measurements enabled comparison of these results with secretion analysis.

Hormones, cAMP/cGMP and NO measurement

Androgens (T+DHT) were measured by radioimmunoassay (for references please see Andric et al. 2010a, b; Kostic et al. 2011). Levels of androgens are referred to as testosterone+dihydrotes tosterone (T+DHT), because anti-testosterone serum №250 showed 100% cross-reactivity with DHT (for references please see Andric et al. 2010a, b; Kostic et al. 2010; Kostic et al. 2011). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5-8%). Levels of cAMP in the medium of purified Leydig cells were measured using a cAMP EIA Kit that permits cAMP measurements with a limit of quantification of 0.1 pmol/mL (at 80% B/B0) and an IC50 of approximately 0.5 pmol/mL for acetylated cAMP samples (for references please see Andric et al. 2010b; Kostic et al. 2011). Levels of cGMP in the medium of purified Leydig cells were measured by a cGMP EIA Kit which permits cGMP measurements typically with the limit of quantification of 0.07 pmol/ml (at 80% B/B0) for acetylated cGMP (Andric et al. 2007; Andric et al. 2010a, b; Kostic et al. 2010). For measurement of nitrite (a stabile metabolic product of NO) levels in the medium, sample aliquots were mixed with an equal volume of Griess reagent and absorbance was measured at 546 nm (Green 1986). Nitrites concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite (Andric et al. 2010a, b; Kostic et al. 2010).

Measurement of the mitochondrial membrane potential ($\Delta\psi_m$) of Leydig cells

To monitor mitochondrial membrane potential ($\Delta\psi_m$), Leydig cells were loaded with TMRE. TMRE is a membrane-permeable cationic fluorophore that accumulates electrophoretically in mitochondria in response to their negative potential (Allen et al. 2006). Purified Leydig cells were loaded with 100 nM TMRE (Tetramethylrhodamine ethyl ester) in M199-0.1% BSA at 34^{0}C for 20 min, according to methods described previously (Allen et al. 2006). Loading buffer was changed with PBS-0.1%BSA, and fluorescence readings were recorded on a fluorimeter (Fluoroskan, Ascent FL, Thermo Labsystems; Waltham, MA,) with an excitation wavelength of 550 nm and emission wavelength of 590 nm.

Protein extraction and Western Blot Analysis

After incubation, Leydig cells ($5x10^6$ per well) were washed twice with ice-cold PBS and lysed in 1 ml of buffer containing 20 mM HEPES, 10 mM EDTA, 40 mM β-glycerophosphate, 1% tergitol, 2.5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM 4-(aminoethyl)-benzenesulfonyl fluoride hydrochloride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and a cocktail of phosphatase inhibitors (0.05 mM (-)-P-bromotetramisol oxalate, 10 μM cantharidin, and 10 nM microcystinLR; pH 7.5) (for references please see Andric et al. 2010a, 2010b; Kostic et al. 2010; Kostic et al. 2011). Protein concentrations were estimated by the Bradford method using BSA as a standard (Bradford 1976). For Western blot analysis, an equal amount of cell lysate proteins was mixed (1:1 v/v) with SDS-PAGE loading buffer, denatured for 5 min at 95ºC, and loaded onto 16% SDS-PAGE gels. All gels were analyzed by one-dimensional SDS-PAGE, using a discontinuous buffer system and proteins were transferred to a PVDF membrane, Immobilon-P (www.millipore.com), using wet transfer, according to the manufacturer's recommendations. Immunodetection of StAR was performed by using StAR protein antisera (kindly provided by Professor Douglas Stocco) (Clark et al. 1996). Actin was detected using an Actin detection kit (www.emdbiosciences.com). Immunoreactive bands were analyzed as two-dimensional images using the Image J (version 1.32; http://rsbweb.nih.gov/ij/download.html). The OD of the images was expressed as volume (OD x area) adjusted for background, giving arbitrary units of adjusted volume (for references please see Andric et al. 2010a, 2010b; Kostic et al. 2010; Kostic et al. 2011).

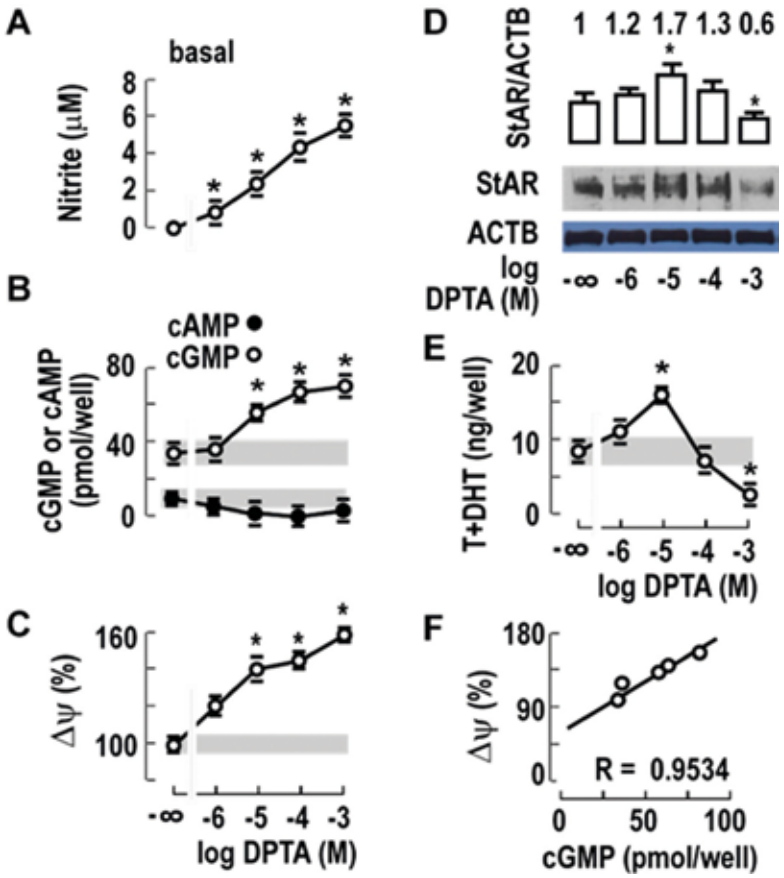

Figure 1. NO-induced positive, dose-dependent, correlation between cGMP and $\Delta\psi_m$ of Leydig cells.
For this and following figures, Leydig cells were isolated from 90 days old male Wistar rats using the procedure described above (please see Material and Methods). Purified Leydig cells were plated ($5x10^6$/5ml/plate or 1x105/0.2ml/well) and placed in a CO_2 incubator (34ºC) for three hours to attach and recover. At the end of the recovery period, the culture medium was discarded and cells were stimulated for 2 h with increasing concentrations of NO-donor, DPTA (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M). At the end of stimulation, medium was used to measure nitrite, cAMP/cGMP and androgens (T+DHT) production, while Leydig cells were used to monitor $\Delta\psi_m$ or StAR protein expression.
A) Nitrite (stabile oxidative product of NO) production in medium was measured using Griess methods (please see Material and methods).
B) cAMP/cGMP production in Leydig cell medium was determined using an EIA assay (for details please see Material and methods).
C) The $\Delta\psi_m$ of Leydig cells was monitored according to a method described previously (for details please see Material and methods).
D) The expression of mature StAR protein in Leydig cells was followed in the lysates of whole cells subjected to antiserum against StAR (Clark et al. 1994). Actin (ACTB) was used as internal control and is presented on the bottom of the panel. The representative blots are shown on the panels, while pooled data from scanning densitometry normalized by ACTB values are shown as bars on the top of the blots, and represent means ± SEM from three independent experiments.
E) Androgens (T+DHT) levels were determined by RIA (for all details please see Material and Methods).
F) Positive, dose-dependent correlation between cGMP production and $\Delta\psi_m$ in Leydig cells.
Data points/bars are group means ± SEM values of 3 independent experiments. Statistical significance at level $p < 0.05$: *vs. control group.

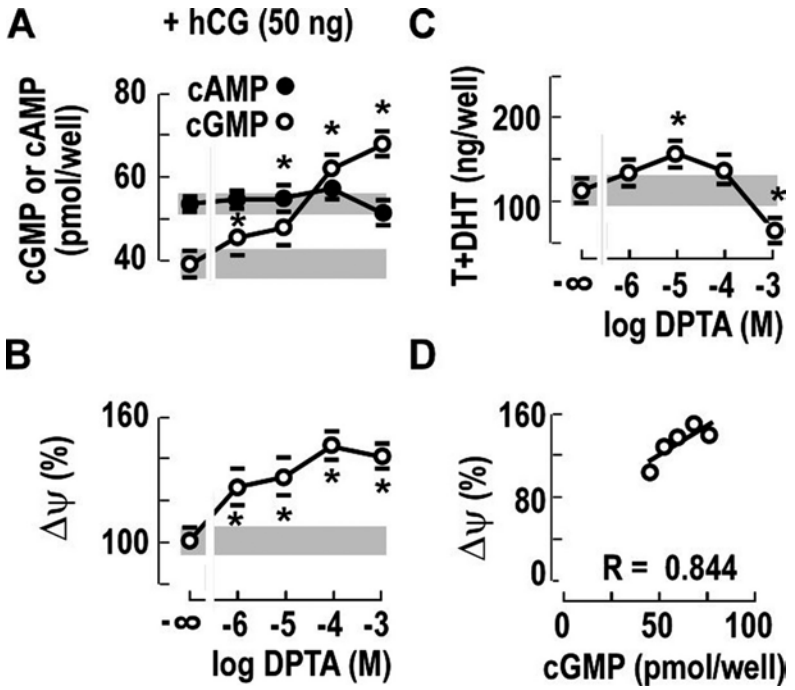

Figure 2. Supra-maximal stimulation of Leydig cell steroidogenic capacity and NO induced positive correlation between cGMP and $\Delta\psi_m$ in Leydig cells.
Purified Leydig cells were plated (5x106/5ml/plate or 1x105/0.2ml/well) and placed in a CO_2 incubator (34ºC) for 3 h to attach and recover. At the end of recovery period, the culture medium was discarded and cells were stimulated for 2 h with increasing concentrations of NO-donor, DPTA (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) in the presence of a supra-maximal dose of hCG (50 ng/ml), and LH receptor activator. At the end of stimulation, medium was used to measure cAMP/cGMP and androgens (T+DHT) production, while Leydig cells were used to monitor $\Delta\psi_m$.
A) cAMP/cGMP production in Leydig cells was determined by EIA assay (for details please see Material and methods).
B) $\Delta\psi_m$ in Leydig cells was monitored according to a method described previously (for details please see Material and-methods).
C) Androgens (T+DHT) levels were determined by RIA (for details please see Material and methods).
D) Positive, dose-dependent correlation between cGMP production and $\Delta\psi_m$ in Leydig cells.
Data points are group means ± SEM values of 3 independent experiments. Statistical significance at level $p < 0.05$: *vs. control group.

Statistical analysis

Data represent the mean ± SEM from 3 to 5 independent replicates. Results from each experiment were analyzed by Mann-Whitney's unpaired nonparametric two-tailed test (for two point data experiments), or, for group comparison, a one-way ANOVA, followed by Student-Newman-Keuls multiple range test. Differences were regarded as statistically significant at $p<0.05$.

Results

In order to monitor the effect of NO on mitochondrial membrane polarity and steroidogenic capacity, Leydig cells were challenged with increasing concentrations of NO donor (DPTA). DPTA spontaneously liberates NO, which was detected as a dose-dependent increase in the levels of nitrites in the incubation medium of purified Leydig cells (Fig. 1A). Such treatment led to a concentration-dependent increase in cGMP production, without affecting intracellular cAMP levels (Fig. 1B). In the same cells, a biphasic effect of NO-donor on testosterone

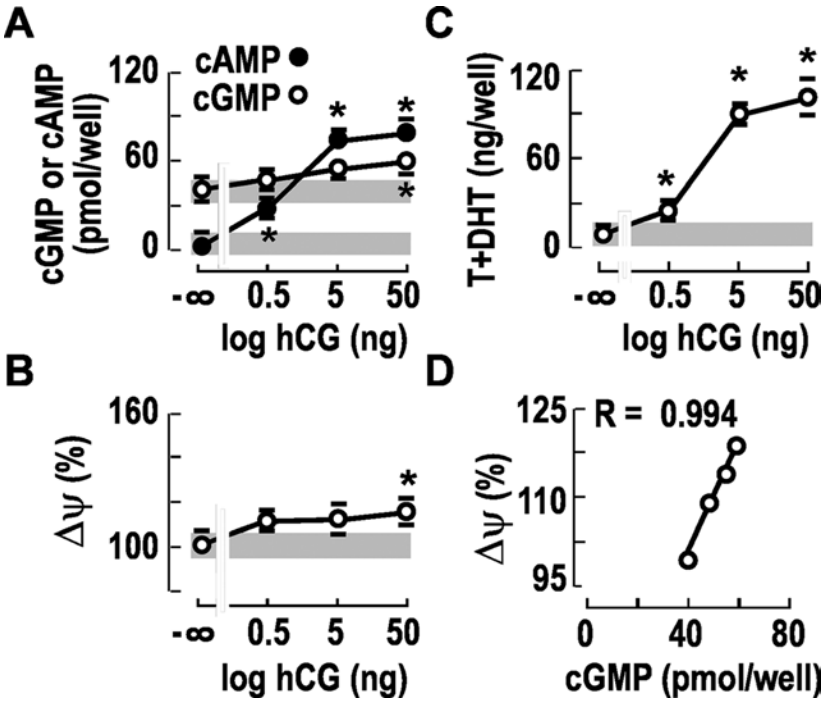

Figure 3. Stimulation of Leydig cells steroidogenic capacity induced positive, dose-dependent, correlation between cGMP and $\Delta\psi_m$ of Leydig cells.
Purify Leydig cells were plated (5x10^6/5ml/plate or 1x10^5/0.2ml/well) and placed in a CO_2 incubator (34^{o}C) for 3 h to attach and recover. At the end of recovery period, the culture medium was discarded and cells were stimulated for 2 h with increasing concentrations of LH receptor activator, hCG (0.5, 5, 50 ng/ml). At the end of stimulation, medium was used to measure cAMP/cGMP and androgens (T+DHT) production, while Leydig cells were used to monitor $\Delta\psi_m$.
A) cAMP/cGMP production in Leydig cells was determined by EIA assay (for details please see Material and methods).
B) $\Delta\psi_m$ in Leydig cells was monitored as described (for details please see Material and methods).
C) Androgens (T+DHT) levels were determined by RIA (for details please see Material and methods).
D) Positive, dose-dependent correlation between cGMP production and $\Delta\psi_m$ in Leydig cells.
Data points are group means ± SEM values from 3 independent experiments. Statistical significance at level p <0.05: *vs. control group.

production was also observed; stimulatory in the dose range (0-10^{-5} M) and inhibitory at 10^{-3} M (Fig. 1E). Since StAR protein is involved in initiation of steroid biosynthesis, by facilitating of cholesterol transport into the inner mitochondrial membrane, it was reasonable to estimate the levels of mature StAR protein. Western blot analysis showed that addition of 10^{-5} M of NO-donor increased the level of mature StAR protein in Leydig cells, while 10^{-3} M of DPTA had the opposite effect (Fig. 1D). To detect changes in the electrochemical gradient, $\Delta\psi_m$ was measured by TMRE fluorescence, because TMRE fluorescence values are proportional to the magnitude of $\Delta\psi_m$. Obtained results indicate gradually increased $\Delta\psi_m$ in Leydig cells treated with NO-donor (Fig. 1C). Finally, a significant correlation between cGMP and $\Delta\psi_m$ (R = 0.9534, p<0.05) was observed (Fig. 1F).

Furthermore, DPTA dose dependently stimulated cGMP production (Fig. 2A) and increased $\Delta\psi_m$ (Fig. 2B), even in cells stimulated with a supra-maximal dose of hCG (50 ng/ml). Again, intracellular cAMP levels were not affected by addition of NO-donor to the Leydig cell incubation medium (Fig. 2A). Moreover, testosterone production showed the same biphasic pattern when Leydig cells were challenged with increasing DPTA concentrations (Fig. 2C). Consequently, our results showed a significant correlation between cGMP production and $\Delta\psi_m$ (R

= 0.844, $p<0.05$) in hCG stimulated Leydig cells (Fig. 2D).

Finally, in order to monitor mitochondrial membrane polarity during hormone supported steroidogenesis, Leydig cells were stimulated with increasing concentrations of hCG. As expected, our results showed a dose dependent increase in cAMP (Fig. 3A) and testosterone (Fig. 3C) production. The hCG-treatment slightly increased cGMP production (Fig. 3A) and this effect was followed by a small but significant increase in $\Delta\psi_m$ (Fig. 3B), which significantly correlated with cGMP levels ($R = 0.994$, $p<0.05$; Fig. 3D). Taking together, results from all experimental approaches stimulating cGMP production significantly correlate with $\Delta\psi_m$ values, suggesting implication of cGMP signaling in the control of mitochondrial membrane polarity.

Discussion

Results from the present study show for the first time the positive correlation between cGMP levels and mitochondrial membrane polarity in steroidogenic cells. Stimulation of Leydig cells with NO increased both cGMP production and $\Delta\psi_m$, which, in the low dose range of NO, overlap with increased testosterone production. These results suggest a new model for the action of cGMP signaling in the regulation of Leydig cell physiology, through modulation of mitochondrial functionality.

It is already known that the NO-cGMP signaling pathway is operative in Leydig cells and its activation modulates testicular androgenesis in accord with systemic hormones and locally produced NO and cGMP (Andric et al. 2007; Andric et al. 2010a, 2010b; Kostic 2010). The NO is produced by NO synthases (NOS), that in Leydig cells exist in two isoforms (Andric et al. 2007; Andric et al. 2010a, 2010b; Kostic 2010): an endothelial (NOS3) and inducible form (NOS2). In addition to Leydig cell NOS activity, NO from neighboring cells significantly contributes to local regulation of Leydig cell function (Weissman et al. 2005). Thus, NO appears to be an important regulator of Leydig cell steroidogenic activity, that, depending on its local concentration, could exert stimulatory or inhibitory actions. At higher concentrations, locally produced NO directly inhibits steroidogenic enzymes by direct inhibition of heme-containing steroidogenic enzymes, cytochrome P450 side-chain cleavage (Del Punta et al. 1996; Drewett et al. 2002) or P450c17 (Pomerantz and Pitelka 1998). At low concentrations, NO stimulates Leydig cells steroidogenesis by activating soluble guanylate cyclase-cGMP signaling (Valenti et al. 1999; Andric et al. 2007), most likely due to activation of cGMP-dependent protein kinase (PRKG) and phosphorylation and maturation of StAR protein (Gambaryan et al. 1999; Andric et al. 2007). The dual effects of NO in experiments presented in the present study are consistent with biphasic modulation of androgen production, stimulatory at small concentrations and inhibitory at high concentrations, clearly confirming previous observations. A similar dual effect of NO on mature StAR protein levels was also observed. Interestingly, the results presented here indicate parallelism between NO levels, cGMP production and mitochondrial membrane polarity in Leydig cells, suggesting a stimulatory effect by this signaling pathway on mitochondria.

Three lines of evidences implicate cGMP involvement in the regulation of mitochondrial functionality: (1) the NO-induced positive, dose-dependent, correlation between cGMP and $\Delta\psi_m$ in Leydig cells; (2) the NO-induced positive correlation between cGMP production and $\Delta\psi_m$ even in Leydig cells with increased cAMP signaling; and (3) the LH receptor activation induced positive, dose-dependent, correlation between cGMP and $\Delta\psi_m$ in Leydig cells.

Once thought to be merely the center of cellular ATP production, mitochondria are now believed to integrate a large number of signal transduction pathways for a wide variety of biologically active molecules. The mitochondrial $\Delta\psi_m$ is a central component of mitochondrial metabolism, providing a driving force for oxidative phosphorylation and the import of proteins and metabolites (Mokranjac et al. 2008; Wittig et al. 2009; Kadenbach et al. 2010). In steroidogenic cells, hormone biosynthesis is initiated on mobilization of cholesterol from cellular stores to the mitochondrial inner membrane, the site of the cytochrome P450 side chain cleavage enzyme (CYP11A1). This process is driven by cAMP/PRKA activation and phosphorylation of StAR protein as

well as other transduceosome protein (Stocco et al. 2005; Liu et al. 2006; Jefcoate et al. 2011). It has been demonstrated that cAMP, in addition to StAR protein phosphorylation, increases $\Delta\psi_m$ and respiration without affecting total ATP levels in Leydig cells. Disruption of $\Delta\psi_m$ prevents cholesterol transport and StAR function (Allen et al. 2006). To date, the mechanism by which cAMP increases $\Delta\psi_m$ in Leydig cells is unknown. Results obtained in the present study suggest a possible role for cGMP in mediating the effects of an LH-induced rise in cAMP on $\Delta\psi_m$ in Leydig cells. Namely, LH receptor mediated up-regulation of cAMP production increases cGMP production, suggesting that cAMP could translate LH receptor activity into cGMP production in Leydig cells. Further, the parallelisms between up-regulation of cGMP production and levels of $\Delta\psi_m$ further suggests that cGMP could translate the actions of NO into changes in mitochondrial membrane polarity in Leydig cells and enhanced steroidogenesis. Since it is known that a rise in cAMP can enhance NO/cGMP signaling in certain types of cells (Kostic et al. 2004), it is possible to assume a cAMP/cGMP relationship in determination of $\Delta\psi_m$. One possible straightforward interpretation could be that increased intracellular levels of cAMP stimulate soluble guanylyl cyclases to produce cGMP (Kostic et al. 2004), or alternatively activate NOS3, followed by increased cGMP (Butt et al. 2000) production and eventually increased $\Delta\psi_m$ in Leydig cells. The signaling events leading increased $\Delta\psi_m$ following activation of cGMP signaling in steroidogenic cells are completely unknown, but could involve PRKG-dependent activation of different mitochondrial kinases (Manna and Stocco 2011).

Although Leydig cell steroidogenesis is mainly regulated through the LH receptor (Catt and Dufau 1973; Dufau 1998), it also requires local paracrine-autocrine factors to maintain and modulate steroidogenesis. Such regulation is multi-compartmental process and includes several endocrine, paracrine and autocrine signaling pathways (Saez 1994; Payne and Hales 2004). The complexity of this extracellular-intracellular network is a major obstacle to understanding the physiology of Leydig cell function. The results presented here suggest involvement of the NO-cGMP signaling pathway, together with LH receptor signaling, as an important regulator of steroidogenesis at the mitochondrial level in Leydig cells.

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