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In vitro blockade of α1-adrenergic receptors (α1-ADRs) affects testosterone production in Leydig cells of adult rats

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Summary. Testosterone, the main male reproductive hormone, is produced exclusively in testicular Leydig cells. Regulation of testosterone biosynthesis, i.e. testicular steroidogenesis, is a multi-compartmental process comprised of neuronal and complex endocrine, paracrine and autocrine signaling pathways, including adrenergic receptor signaling. It was shown recently that systemic *in vivo* blockade of α1-adrenergic receptors (α1-ADRs) disturbed testicular steroidogenesis. However, it is not clear whether the effects on Leydig cells are direct or caused by other organs/structures *in vivo*, and that Leydig cells are only indirectly affected by other mediators. The present study was designed to evaluate the relationship between α1-ADRs and testosterone synthesis in primary cultures of Leydig cells. To do this, prazosin, a potent and widely used antagonist of α1-ADRs was applied *in vitro* to treat primary cultures of Leydig cells freshly isolated from adult rat testes. The relationships between blockade of α1-ADRs receptors and cAMP and testosterone production by Leydig cells were analyzed. Results show that blockade of α1-ADRs in Leydig cells increases cAMP levels, but reduces the steroidogenic capacity of Leydig cells to produce testosterone. In the same cells, testosterone production was affected only at the highest concentration of prazosin. Application of adrenaline (a natural and potent agonist of all α/β-ADRs) during blockade overcame this reduction of testosterone production, but was not able to abolish the inhibitory effects of α1-ADRs blockade on the steroidogenic capacity of Leydig cells, suggesting that this effect is caused through α1-ADRs. The present data provide a new role for α1-ADRs in the regulation of testosterone biosynthesis in Leydig cells and new molecular insight into the relationship between α1-blockers and mammalian reproduction. The results are important in terms of human reproductive health and the widespread pharmacological application of prazosin, an important drug that has been used clinically for decades to treat hypertension, prostate hyperplasia, posttraumatic stress disorder, and scorpion stings, and holds promise as a pharmacologic treatment for alcohol dependence.

Keywords: α1-adrenergic blockade, androgenesis, prazosin, cAMP, Leydig cells.

INTRODUCTION

Although the testes are not essential for individual survival, these endocrine and exocrine tissues are vital for the survival of the species, because of their crucial role in mammalian reproduction through production of sperm and sex-steroid hormones. Testosterone, the main male reproductive hormone, is produced exclusively in testicular Leydig cells. Like all other steroid-producing cells, Leydig cells synthesize testosterone from a common precursor, cholesterol, using the steroidogenic machinery (Fig. 1) comprised of cholesterol transporters (Stocco et al. 2005; Papadopoulos and Miller 2012; Rone et al. 2012), steroidogenic enzymes (Payne and Hales 2004) and many regulatory molecules (Dufau 1993, 1998; Payne and Hales 2004; Stocco et al. 2005; Papadopoulos and Miller 2012; Rone et al. 2012). 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

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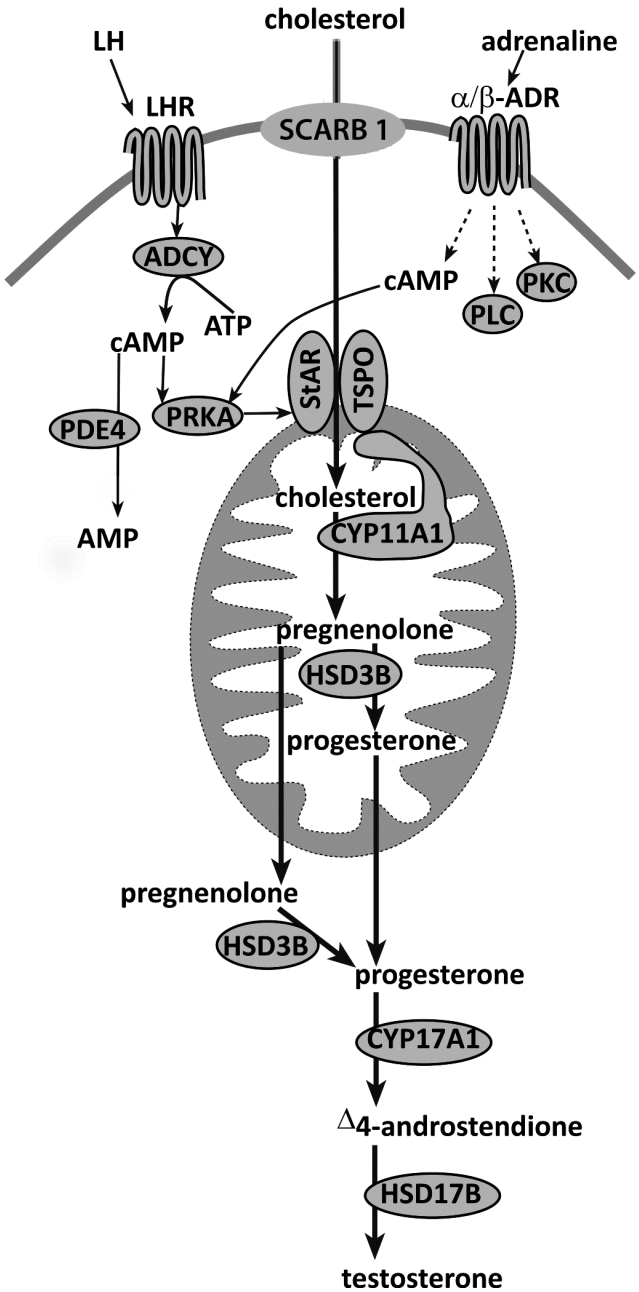

(ADCY), accumulation of cAMP and concomitant activation of cAMP-dependent kinase (PRKA), stimulating steroidogenesis. Through cGMP-dependent activation of protein kinase G (PRKG1), cGMP signaling also stimulates testosterone production (Valenti et al. 1999; Andric et al. 2007, 2012). Phosphodiesterases (PDEs) terminate cAMP/cGMP signalling and have regulatory functions in Leydig cells (Catt and Dufau 1973; Dufau 1993, 1998; Tsai and Beavo 2011, 2012). Although Leydig cell steroidogenesis is mainly activated through LH receptors, regulation itself is a multi-compartmental process comprised of neural (Selvage et al. 2006) and complex endocrine, paracrine and autocrine signaling pathways (reviewed in Saez 1994; Gnessi et al. 1997; Payne and Hales 2004), including adrenergic signaling (Anakwe et al. 1985).

It is well known that ADRs play an important role in the stimulation of androgen production in rat (Anakwe and Moger 1986) and hamster (Mayerhofer et al. 1993) and that testicular α1-ADRs activate antiapoptotic signaling in the Leydig cells of stressed animals (Andric et al. 2013). Our study also revealed the strong stimulation of transcription of all ADRs expressed in Leydig cells from stressed rats (Stojkov et al. 2012) and this effect was even more pronounced with sustained blockade of α1-ADRs *in vivo* (Stojkov, Janjic, Baburski et al. 2013). In addition, it was shown recently that systemic *in vivo* blockade of α1-ADRs disturbed testicular steroidogenesis in adult rats (Stojkov, Janjic, Kostic et al. 2013). However, in none of these studies was it clear whether the effects of activation/blockade of ADRs are direct on Leydig cells or caused by other organs/structures *in vivo*, meaning that Leydig cells are only indirectly affected by other mediators.

Accordingly, this study was designed to evaluate the relationship between α1-ADRs and testosterone synthesis in primary cultures of Leydig cells. To do this, prazosin, a potent and widely used antagonist of α1-ADRs (D'Armiento et al. 1980; Kaye et al. 1986; Zhang et al. 2012) was applied *in vitro* to treat primary cultures of Leydig cells freshly isolated from adult rat testes. The relationships between blockade of α1-ADRs receptors, cAMP levels and testosterone production by Leydig cells were analyzed.

Figure 1. Biosynthesis of testosterone in testicular Leydig cells – testicular steroidogenesis.

Leydig cells, as other steroidogenic cells, synthesize steroid hormones from a common precursor, cholesterol. Transport of cholesterol from intracellular sources into the mitochondria is a rate-limiting process that requires the presence of a specific complex of transport carrier proteins, including the translocator protein (TSPO) and the steroidogenic acute regulatory (StAR) protein (Stocco et al. 2005; Papadopoulos and Miller 2012; Rone et al. 2012). On the inner mitochondrial membrane, cholesterol is converted to pregnenolone by cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), and further metabolized to progesterone by mitochondrial/microsomal 3β-hydroxysteroid dehydrogenases (HSD3B). In Leydig cells, maturation of progesterone to androstenedione is catalyzed by 17α-hydroxylase/C17–20 lyase (CYP17A1) (Payne and Hales 2004). The steroidogenic function of Leydig cell is predominantly regulated by pituitary luteinizing hormone (LH) or its placental counterpart human chorionic gonadotropin (hCG). LH/hCG receptor activation leads to stimulation of adenylyl cyclases (ADCYs), accumulation in cAMP and activation of the cAMP-dependent kinase (PRKA). Although Leydig cell steroidogenesis is mainly activated through LH/hCG receptors, regulation itself is a multi-compartmental process comprises of neural and complex endocrine, paracrine and autocrine signaling pathways (Valenti et al. 1999; Andric et al. 2007, 2012; Stojkov, Janjic, Kositc et al. 2013). The phosphodiesterases (PDEs) terminate cAMP signalling and have regulatory function in Leydig cells (Catt and Dufau 1973; Dufau 1998; Tsai and Beavo 2011).

MATERIAL AND METHODS

The anti-testosterone-11-BSA serum №250 and anti-progesterone-11-BSA №337 were kindly supplied by Gordon D. Niswender (Colorado State University, Fort Collins, CO, USA). (1,2,6,7^{3}H(N)) labeled testosterone and [1,2,6,7^{3}H(N)]-progesterone were obtained from Perkin-Elmer Life Sciences (www.PerkinElmer.com). The 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, progesterone, testosterone, trypan blue, prazosin·HCl were from Sigma (www.sigmaaldrich.com). All other chemicals were of analytical grade. Adrenaline·HCl, 1:1000 injectable solutions (1 mg/ 1 ml) was from Jugoremedija (http://www.jugoremedija.rs).

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 National Council for Animal Welfare and following statements of 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) and 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 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 hormones and cAMP productions

To follow *ex vivo* production of progesterone and androgens (T+DHT), as well as cAMP level, we used primary cultures of purified Leydig cells obtained individually from male rats and isolated/purified as described previously by our group (Andric et al. 2007; Kostic et al. 2010; Stojkov, Janjic, Baburski et al. 2013; Stojkov, Janjic, Kostic et al. 2013). Primary cultures of purified Leydig cells were prepared from suspensions of interstitial cells. Suspensions of interstitial cells were prepared according to Anakwe et al. (1985) with some modifications described previously by our group (Andric et al. 2007; Kostic et al. 2010; Stojkov, Janjic, Baburski et al. 2013). Briefly, the 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 160*xg* for 5 min at room temperature, and then 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 total cell counts and to ensure that greater than 95% of the cells were viable. This suspensions of interstitial cells (Klinefelter et al. 1987) was used to prepare primary cultures of purified Leydig cells (Andric et al. 2007; Kostic et al. 2010; Stojkov, Janjic, Baburski et al. 2013; Stojkov, Janjic, Kostic et al. 2013) 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 Leydig cells, macrophages and endothelial cells (Klinefelter et al. 1987), was applied to each Percoll gradient and centrifuged at 500*xg* 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 200*xg* for 5 min at room temperature. The cells were resuspended in a corresponding amount (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 HSD3B activity (Payne et al. 1980), and was found to be 95.3% ± 2.7%, while cell viability was more than 90%. Purified Leydig cells obtained individually from undisturbed rats were pooled and plated in 96-well plates (5x10^4 cells / 0.2 ml/ per well) in eight replicates for each experimental group and placed in a CO_2 incubator (34 °C) for three hours to attach and recover. At the end of the recovery period, the culture medium was discarded and cells were challenged with hCG (50 ng/ml) to estimate steroidogenic capacity, in the absence/ presence of adrenaline (nonselective ADRs activator) or prazosin (selective α1-ADRs blocker) alone or in combination. After 2 hours, cell-free media was collected and stored at -80 °C prior to the measurement of cAMP, progesterone and androgens (T+DHT) levels.

Hormones and cAMP measurement

Androgens (T+DHT) were measured by radioimmunoassay and referred to as testosterone+dihydrotestosterone (T+DHT), because anti-testosterone serum №250 shows 100% cross-reactivity with DHT (Andric et al. 2007, 2012; Kostic et al. 2010; Stojkov, Janjic, Baburski et al. 2013; Stojkov, Janjic, Kostic et al. 2013). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5-8%). **Progesterone** measurements were assayed in duplicate, by RIA (the sensitivity: 6 pg per tube; the intra-assay coefficient of variation: 6.8%; the inter-assay coefficient of variation: 8.7%) using the anti-progesterone serum №337 (Stojkov, Janjic, Kostic et al. 2013). Level of cAMP in the medium of purified Leydig cells was measured by the cAMP EIA Kit that permits cAMP measurements with a limit of quantification of 0.1 pmol/ml (at 80% B/B_0) and IC_{50} of approximately 0.5 pmol/ml for acetylated cAMP samples (Andric et al. 2012; Stojkov, Janjic, Baburski et al. 2013; Stojkov, Janjic, Kostic et al. 2013).

Statistical analysis

The results represent group means ± SEM values from three to five independent *in vitro* experiments (8 replicates per group in each experiment). The results from each experiment were analyzed by one-way ANOVA, followed by the Student-Newman-Keuls multiple range test.

RESULTS

Adrenaline, nonselective agonist of ADRs increased while α1-ADRs blocker prazosin reduced testosterone production by Leydig cells

In order to monitor the effects of activation of all ADRs on Leydig cells steroidogenic activity and capacity, primary Leydig cells were challenged *in vitro* with increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) of adrenaline (natural potent agonist of ADRs) in the absence or presence of hCG (50 ng/ml). As expected, and as shown previously (Anakwe et al. 1985; Anakwe and Moger 1986), only the highest concentrations of adrenaline (10^{-6} & 10^{-5} M) stimulated basal androgen (T+DHT) production (Fig. 2A). In the presence of hCG, stimulation of testosterone production was registered with a 100x lower concentration of adrenaline than in the absence of hCG (Fig. 2A), suggesting that Leydig cell response to adrenalin is more "sensitive" when LH/hCG receptors are activated.

To monitor the effect of α1-ADRs blockade on Leydig cells steroidogenic activity and capacity, primary Leydig cells were challenged *in vitro* with increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) of prazosin (a potent and widely used α1-ADRs blocker), in the absence or presence of hCG (50 ng/ml). Prazosin reduced basal testosterone production

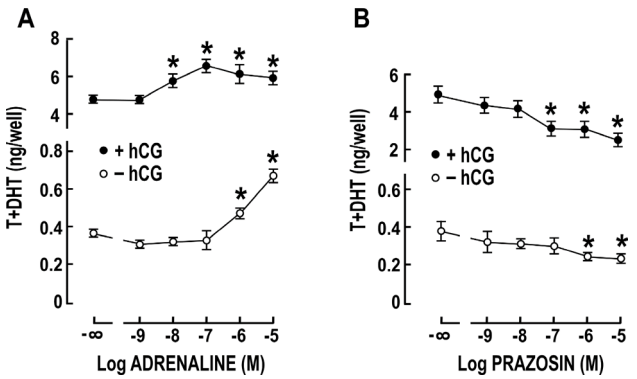

Figure 2. Nonselective agonist of ADRs stimulated, while α1-ADRs blocker reduced steroidogenic capacity and testosterone production by Leydig cells.
A) Adrenaline-dependent activation of ADRs increased basal and hCG-stimulated (reflects steroidogenic capacity) testosterone production by Leydig cells.
B) Prazosin-dependent blockade of α1-ADRs reduced basal and hCG-stimulated (reflects steroidogenic capacity) testosterone production by Leydig cells.
For this and the following figures, Leydig cells were isolated from 90 day old male *Wistar* rats using the procedure described previously (please see Material and methods). Purified Leydig cells were plated in 96-well plates ($5x10^4$/0.2ml/well) and placed in a CO_2 incubator (34 °C) for three hours to attach and recover. At the end of recovery period, the culture medium was discarded and cells were challenged for 2 h with increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) of either adrenaline (non-selective, natural potent activator of ADRs) or prazosin (potent selective α1-ADRs blocker) in the absence (basal) or presence of saturating concentration (50 ng/ml) of hCG (potent agonist of LH receptor and activator of steroidogenesis). At the end of stimulation medium was used to measure androgen (T+DHT) production using RIA (for all details please see Material and methods).
Data bars are group means ± SEM values of three independent experiments. Statistical significance at level $p < 0.05$: *vs. control group.

when applied at higher concentrations (Fig. 2B). Testosterone production by Leydig cells in response to hCG was more sensitive to α1-ADRs blockade and reduced in the presence of 10x lower concentrations of prazosin than in the absence of hCG (Fig. 2B), suggesting that Leydig cell response to prazosin is more "sensitive" when LH/hCG receptors are activated.

Adrenaline, nonselective agonist of ADRs, overcame the effect of α1-ADRs blockade on basal, but not hCG-stimulated testosterone production by Leydig cells

In order to determine whether the nonselective ADRs agonist, adrenaline, could overcome the inhibitory effects of α1-ADRs blockade on testosterone production, primary Leydig cells were challenged *in vitro* with increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) of prazosin in the presence of two doses of adrenaline (10^{-9} & 10^{-6} M) under basal and hCG stimulated conditions. Results showed that adrenalin abolished the inhibitory effects of α1-ADRs blockade

on basal testosterone production by Leydig cells (Fig. 3A). However, adrenalin was not able to overcome the inhibitory effect of α1-ADRs blockade on hCG-stimulated testosterone production (Fig. 3A), suggesting that reduced testosterone production in the presence of activated LH/hCG receptor is a consequence of specific α1-ADRs blockade.

To examine the possible involvement of α1-ADRs in adrenaline-associated stimulation of testosterone production, primary Leydig cells were challenged *in vitro* with prazosin (10^{-6} M) in the presence of increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) of adrenaline under basal and hCG stimulated conditions. Results showed that *in vitro* blockade of α1-ADRs with prazosin completely prevented adrenaline-induced stimulation of basal testosterone production by Leydig cells (Fig. 3B). Under the condition of occupied LH/hCG receptors, prazosin not only prevented adrenalin-induced stimulation of testosterone production, but also caused a reduction of hCG-stimulated testosterone production (Fig. 3B), confirming dissociation of adrenaline-associated stimulation and prazosin-associated inhibition of Leydig cells steroidogenesis when LH/hCG receptors are occupied.

Prazosin, α1-blocker, increased cAMP levels in medium and content of Leydig cells, did not change progesterone but reduced testosterone production

In order to gain better insight into the effects of prazosin on Leydig cell steroidogenesis, levels of cAMP (the main regulator of steroidogenesis) and production of progesterone, a testosterone precursor molecule and the product of HSD3B activity (an enzyme serving as the main marker of Leydig cells) were measured after *in vitro* stimulation of primary Leydig cells with prazosin and adrenaline alone or in combination.

Results showed that prazosin (10^{-6} M) significantly in-

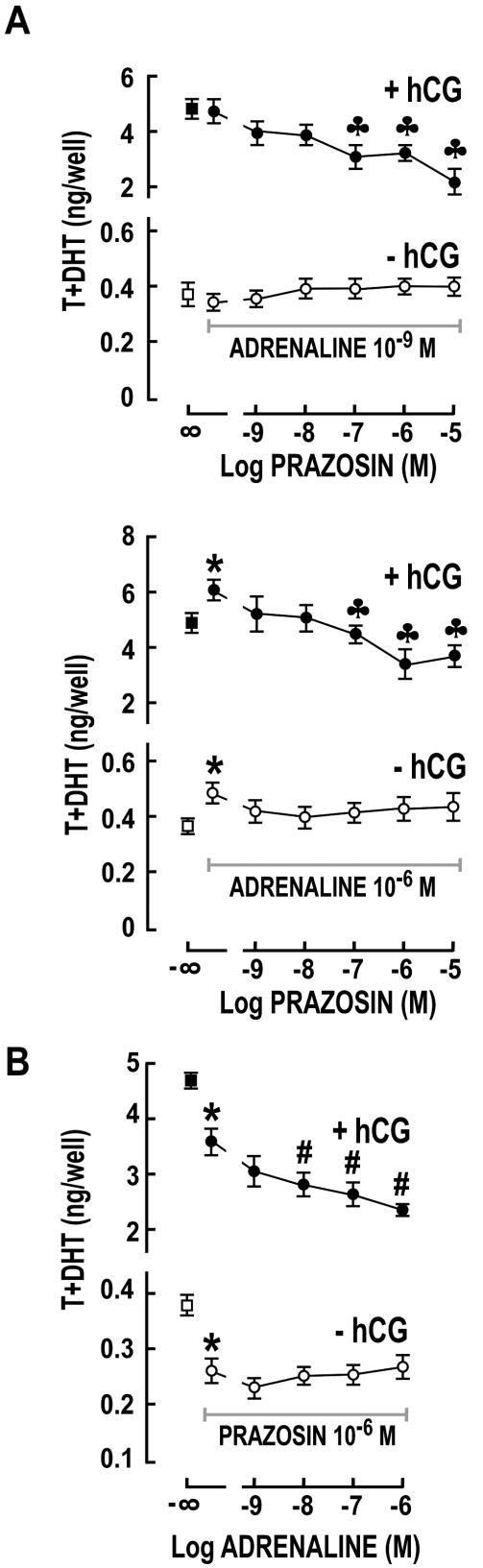

Figure 3. Nonselective agonist of ADRs overcame inhibitory effect of α1-ADRs blockade on basal testosterone production by Leydig cells but not reduction of steroidogenic capacity.

A) Adrenaline-dependent activation of ADRs overcomes effects of α1-ADRs blockade on basal but not hCG-stimulated testosterone production by Leydig cells.

B) Prazosin-dependent α1-ADRs blockade prevented adrenalin-associated stimulation of basal and hCG-induced testosterone production by adrenaline.

At the end of the recovery period, Leydig cells were challenged for 2 h with increasing concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) of either prazosin (potent selective α1-ADRs blocker) or adrenaline (nonselective natural potent activator of ADRs) alone/combined, under basal or hCG-stimulated conditions. At the end of stimulation, medium was used to measure androgens (T+DHT) production using RIA (for all details please see Material and methods).

Data bars are group means ± SEM values of three independent experiments. Statistical significance at level $p < 0.05$: *vs. control group; #vs. prazosin-treated group; ♣vs. corresponding adrenaline-treated group.

creased cAMP production in medium and content of Leydig cells compared to controls (Fig. 4A). In the presence of 1 nM of adrenaline alone, levels of cAMP increased only in content (Fig. 4A, lower panel), while in medium they remained unchanged (Fig. 4A, upper panel). *In vitro* treatment of Leydig cells with a combination of prazosin (10^{-6} M) and adrenaline (10^{-9} M) increased cAMP levels in content significantly higher compared to the control, adrenaline-1nM and prazosin group (Fig. 4A, lower panel), but without changes in medium (Fig. 4A, upper panel). The presence of higher concentrations of adrenaline (10^{-6} M) alone significantly stimulated cAMP production in both the medium and content of Leydig cells compared to the control (Fig. 4A). The stimulatory effect of higher concentrations of adrenaline (10^{-6} M) on cAMP production in Leydig cells was more pronounced in medium than in content and was significantly higher than in cells treated with adrenaline-1nM or prazosin (Fig. 3A, upper panel), while in content cAMP was significantly lower than in adrenaline-1nM (Fig. 4A, lower panel). The level of cAMP in the content of Leydig cells incubated with both prazosin and adrenaline was significantly lower compared to groups treated with prazosin (10^{-6} M) or adrenaline (10^{-6} M) alone (Fig. 4A, lower panel). The opposite effect was observed in medium and the levels of cAMP in the medium of Leydig cells incubated with both prazosin and adrenaline was significantly higher compared to groups treated with prazosin (10^{-6} M) or adrenaline (10^{-6} M) alone (Fig. 4A, upper panel).

Neither lower concentrations of adrenaline (10^{-9} M), nor the α1-ADRs blocker prazosin (10^{-6} M) changed progesterone production by Leydig cells *in vitro*. However, higher concentrations of adrenaline (10^{-6} M) significantly increased progesterone production, but blockade of α1-ADRs prevented this adrenaline stimulatory effect, suggesting involvement of α1-ADRs (Fig. 4B).

Testosterone levels in the same Leydig cells were in agreement with results from dose dependent studies. Prazosin reduced basal, as well as adrenaline associated increases in androgen (T+DHT) production *in vitro* (Fig. 4C).

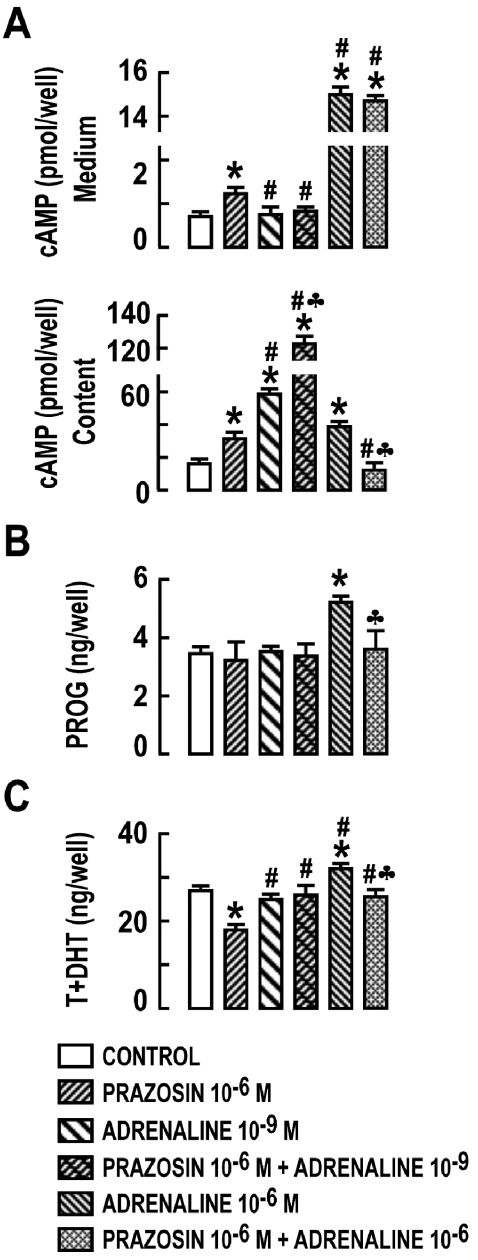

Figure 4. Prazosin, α1-blocker, increased cAMP level in Leydig cells, did not change progesterone but reduced androgens production.
A) *In vitro* effects of prazosin (selective α1-ADRs blocker) and adrenaline (nonselective ADRs agonist), alone or in combination, on cAMP levels in medium (upper panel) and content (lower panel) of Leydig cells.
B, C) *In vitro* effects of prazosin (selective α1-ADRs blocker) and adrenaline (nonselective ADRs agonist), alone or in combination, on progesterone (**B**) and androgens (**C**) production in medium of Leydig cells.
At the end of the recovery period, Leydig cells were challenged for 2 h with either prazosin (potent selective α1-ADRs blocker) or adrenaline (non-selective natural potent activator of ADRs) alone/combined. At the end of treatment medium was used to measure cAMP, progesterone and androgens (T+DHT) production (please see Material and methods). Data bars are group means ± SEM values of three independent experiments. Statistical significance at level p <0.05: *vs. control group; #vs. prazosin-treated group; ♣vs. corresponding adrenaline-treated group.

DISCUSSION

Although testosterone biosynthesis in Leydig cells is mainly activated through LH receptors, regulation itself is a multi-compartmental process comprised of neural (Selvage et al. 2006) and complex endocrine, paracrine and autocrine signaling pathways (reviewed in Saez 1994; Gnessi et al. 1997; Payne and Hales 2004), including adrenergic signaling (Anakwe et al. 1985). It is well known that signaling via ADRs plays an important role in stimulating testicular androgen production in rats (Anakwe et al. 1985; Anakwe and Moger 1986), golden hamsters (Mayerhofer et al. 1989) and Siberian hamsters (Mayerhofer et al. 1989). Recent *in vivo* studies also revealed strong stimulation of transcription of all ADRs expressed in Leydig cells from stressed rats (Stojkov

et al. 2012), and that this effect was even more pronounced with sustained blockade of α1-ADRs *in vivo* (Stojkov, Janjic, Baburski et al. 2013). In addition, testicular α1-ADRs activate antiapoptotic signaling in the Leydig cells of stressed animals (Andric et al. 2013), while systemic *in vivo* blockade of α1-ADRs disturbed testicular steroidogenesis in adult rats (Stojkov, Janjic, Kostic et al. 2013). However, in none of these studies was it clear whether the effects of activation/blockade of α1-ADRs are direct on Leydig cells or caused by other organs/structures *in vivo*, with Leydig cells only indirectly affected by other mediators.

In this study, we have demonstrated, to the best of our knowledge for the first time, that *in vitro* blockade of α1-ADRs on Leydig cells disturbs their functionality. Several lines of evidence support a role for α1-ADRs in Leydig cell steroidogenesis. First, blockade of α1-ADRs in Leydig cells with prazosin decreases basal testosterone production *in vitro*. Second, prazosin reduces the steroidogenic capacity of Leydig cells, i.e. testosterone production, in the presence of a supramaximal hCG dose. Third, prazosin increases cAMP production in Leydig cells. Forth, blockade of α1-ADRs in Leydig cells with prazosin inhibites adrenaline-increased progesterone production *in vitro*.

Measurements of concentrations of norepinephrine and epinephrine in testicular homogenates revealed higher values for these catecholamines than in plasma, implying that catecholamine levels in the interstitial spaces of the testis might be in the range of concentrations effectively stimulating testosterone production *in vitro* (Mayerhofer et al. 1992). Although it has been suggested that rodent Leydig cells possess predominantly β-adrenergic binding sites, the same study pointed out a possible dissociation between receptor recognition and physiologic response (Anakwe et al. 1985). However, it has been shown that in the immature testis of the golden hamster, catecholamines acting through both α-ADRs and β-ADRs as potent physiological stimulators of testosterone production (Mayerhofer et al. 1989, 1992), but that in adult testis steroidogenesis is uncoupled from stimulation of β-ADRs (Mayerhofer et al. 1993). Earlier studies showed that the stimulatory action of norepinephrine on testosterone production was dependent on concentration (Mayerhofer et al. 1992), a finding consistent with data presented in this study, which showed that only the highest concentrations of adrenaline (1, 10 μM) stimulated testosterone production of Leydig cells. In further agreement with studies on testicular pieces (Mayerhofer et al. 1993), our results from incubation of Leydig cells with adrenaline and hCG show an absence of synergistic effects on testosterone release. Our results presented in this study show that prazosin applied *in vitro* on Leydig cells significantly reduces both basal and hCG-stimulated testosterone production (i.e. steroidogenic capacity). This is in line with previous studies on testicular pieces showing that the stimulatory effects of catecholamine on testosterone production by testes of adult golden hamster were prevented by prazosin (α1-ADRs blocker), but not by propranolol (β -ADRs blocker) and the seasonal transition from gonadal activity to quiescence was accompanied by a major increase in the responsiveness of testicular steroidogenesis to catecholamines acting via α1-ADRs (Mayerhofer et al. 1989, 1992). A similar pattern was observed on decapsulated pieces of testes from Siberian hamsters. Catecholamine-induced stimulation of testicular testosterone production was prevented by coincubation with prazosin (α1-ADRs blocker), but not affected by coincubation with propranolol (β-ADRs blocker) indicating that the testicular receptors mediating the action of catecholamines on testicular steroidogenesis in Siberian hamsters are of the α1-subtype (Mayerhofer et al. 1993). In addition, a study on α1-ADRs-KO mice showed reduced steroidogenic capacity in Leydig cells and disrupted Sertoli cell/Leydig cell communication in infertile α1-ADRs-KO mice (Mhaouty-Kodja et al. 2007).

In order to gain better insight into the effects of prazosin on Leydig cell steroidogenesis and to eventually reveal the specific step(s) affected by prazosin, levels of cAMP (the main regulator of steroidogenesis) and production of progesterone (a testosterone precursor molecule and the product of HSD3B activity – an enzyme serving as the main marker of Leydig cells) were measured after *in vitro* stimulation of primary Leydig cells with prazosin and adrenaline alone or in combination. Results showed that prazosin significantly increased cAMP levels in the medium and content of Leydig cells. These results could be compared with a study showing that in spontaneously hypertensive rats prazosin treatment induced marked inhibition of PDE activity, thus causing an increase in cAMP levels while adenylate cyclase activity remained unaffected (D'Armentio et al. 1980). Although results from our study clearly show that prazosin reduces basal and hCG-stimulated testosterone production, and did not affect production of progesterone, the product of HSD3B activity (an enzyme serving as the main marker of Leydig cells and testosterone precursor molecule). Prazosin prevented only an increase in testosterone production in the presence of 10 μM adrenaline. A possible explanation for these results could be that progesterone is a precursor of testosterone which is more chemically similar to cholesterol, only one CYP enzyme (CYP11A1) is involved in the biosynthetic route to progesterone, and although it is known that prazosin could interfere with enzymes of the aldosterone biogenesis pathway (Jager et al. 1998), there is a possibility that cAMP increases can overcome this inhibition.

How to explain prazosin-induced reduction of testosterone production in an environment of increased cAMP? Certainly, it is difficult to give a clear explanation, but there are several possible explanations. First, our preliminary unpublished results from *in vitro* experiments on Leydig cells show decreased mitochondrial membrane potential in the presence of α1-ADRs blocker. It has been demonstrated that

polarized and energized mitochondria are required for normal steroidogenesis and 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). Accordingly, a prazosin-associated decrease in mitochondrial membrane potential could be one possible reason for this reduction of testosterone production even though cAMP levels are increased. Second, it has been suggested that the development of response to catecholamines by rat Leydig cells is a post-receptor, post-cAMP event, and that catecholamines can interact with LH or GnRH to regulate Leydig cell function (Anakwe and Moger 1986) and that cAMP does not mediate the stimulatory action of norepinephrine on testosterone production by the testis of the golden hamster (Mayerhofer et al. 1991). Third, the selective α1-ADRs antagonist prazosin selectively blocked polyphosphoinositide hydrolysis and inhibited phosphoinositide signaling (Moraru et al. 1995), a very important regulator of steroidogenesis (Manna et al. 2011). Forth, it has been shown that doxazosin, another α1-ADRs blocker, is the first small molecule agonist of a receptor tyrosine kinase that is capable of inhibiting malignant behaviors *in vitro* and *in vivo* (Petty et al. 2012). Accordingly, all of the above mentioned possibilities could paint a "picture" about the *in vitro* effects of prazosin which is even more complicated than first suspected. However, regardless of whether the effects of prazosin are mediated by one or several signaling pathways, steroidogenesis is affected and this in turn can influence Leydig cells homeostasis and testosterone production. Additional studies on possible receptor "cross-talk" and post-receptor events will be required to resolve these puzzling results.

In conclusion, the results obtained in the present study support an important role for α1-ADRs in Leydig cell androgenesis and might provide new insights into the relationship between α1-ADRs and mammalian reproductive function. The presented data could be also important in terms of the current wide use of α1-ADRs selective antagonists, alone or in combination, to treat posttraumatic stress disorders, hypertension, benign prostatic hyperplasia symptoms and disrupted sexual health. Eventually, this study could be a solid basis for evaluating prazosin's clinical and pharmacogenomic data in human reproductive health risk assessments. Prazosin is an important drug that has been used clinically for decades to treat hypertension, prostate hyperplasia, posttraumatic stress disorder, scorpion stings and holds promise as a pharmacologic treatment for alcohol dependence (D'Armiento et al. 1980; Kaye et al. 1986; Zhang et al. 2012). Its primary known mechanism is to antagonize α1-adrenergic receptors, a subfamily of GPCRs (G-protein coupled receptors). GPCR receptor drugs often bind to GPCRs rather than the primary target, and such off-target interactions can play important roles in therapy and toxicity (Rosenbaum et al. 2009). The effects of prazosin on testosterone biosynthesis is interesting clinically, both because testosterone plays important roles in reproduction and is involved in numerous disease-related processes and because the action of prazosin implicates a GPCR from an important family in a basic and general biological regulatory process. Given that many clinically approved drugs target GPCRs, a better understanding of these receptors' biological functions will be critical in developing more efficacious therapeutics (Zhang et al. 2012). Prazosin is the only clinically approved drug that inhibits a specific step of steroidogenesis, and we know from its long history that it is relatively nontoxic. Our findings highlight the increasingly accepted concept that even the most widely used and safe drugs often have unexpected targets and mechanisms. Off-target effects of compounds that have been thoroughly vetted in humans are always of great interest to both understand their efficacy and toxicity in their current indications.

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