**In vivo blockade of α1-adrenergic receptors mitigates stress-disturbed cAMP and cGMP signaling in Leydig cells**

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**ABSTRACT:** The molecular mechanism of stress-associated reproductive dysfunction is complex and largely unknown. This study was designed to systematically analyze molecular effects of systemic in vivo blockade of α1-adrenergic receptors (α1-ADRs) on stress-induced disturbance of cAMP/cGMP signaling in testosterone-producing Leydig cells using the following parameters (i) level of circulating stress hormones, LH and testosterone; (ii) level of main molecular markers of Leydig cell functionality (testosterone, Insl3, cAMP); (iii) expression of cAMP signaling (cAMP ‘producers’/’effectors’/’removers’) and (iv) expression of NO-cGMP signaling (NO-cGMP ‘producers’/’effectors’/’removers’). The results showed that oral administration of α1-ADR blocker before stress increased cGMP and diminished stress-reduced cAMP production in Leydig cells. In the same cells, stress-induced effects on cAMP/cGMP signaling pathways elements were changed. Sustained in vivo α1-ADR blockade completely abolished stress-increased transcription of most abundantly expressed phosphodiesterase that remove cAMP (Pde4b) and potentiated stress-increased expression of PRKA, the main stimulator of Leydig cell steroidogenesis. In the same Leydig cells, stress-decreased NOS3 expression was abolished, while stress-increased GUCY1 (cGMP ‘producer’) and PRKG1 (cGMP ‘effector’) were potentiated. It is possible that all molecules mentioned could be involved in the regulation of testosterone production. Regardless of whether the effects of α1-blocker + stress are direct or indirect, the results are important in terms of human reproductive health and the wide use of α1-ADR antagonists, alone or in combination, to treat post-traumatic stress disorders, hypertension, benign prostatic hyperplasia symptoms and potential drugs for prostate cancer prevention/treatment.

**Key words:** cAMP/cGMP signaling / Leydig cells / testosterone / stress / doxazosin

**Introduction:** 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) comprising 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 et al., 1993; Dufau, 1998; Payne and Hales, 2004; Stocco et al., 2005; Papadopoulos and Miller, 2012; Rone et al., 2012). The steroidogenic function of Leydig cell is predominantly regulated by pituitary LH and its receptor through stimulation of adenylyl cyclase (ADCY), accumulation in cAMP and activation of the cAMP-dependent kinase (PRKA). Through cGMP-dependent activation of protein kinase G (PRKG1), cGMP signaling also stimulate testosterone production (Valenti et al., 1999; Andric et al., 2007, 2010). The phosphodiesterases (PDEs) terminate cAMP/cGMP signaling and have regulatory function in Leydig cells (Catt and Dufau, 1973; Dufau et al., 1993; Dufau, 1998; Tsai and Beavo, 2011; Tsai and Beavo, 2012). Although Leydig cell steroidogenesis is mainly activated through LH receptors, regulation itself is multi-compartmental process comprises of neural (Selvage et al., 2006) and complex endocrine, paracrine and autocrine signaling pathways (reviewed in Saez, 1994; Gnassi et al., 1997; Payne and Hales, 2004) including adrenergic signaling (Anakwe et al., 1985). All mentioned molecules could be involved in the regulation of testosterone production, providing a molecular adaptive mechanism by which testicular structures, including Leydig cells, recover from disturbed homeostasis such as stress.

The ability of stress to disturb reproduction and Leydig cell function is well documented (reviewed in Rivier and Rivest, 1991; Hardy et al.,...
but molecular events and mechanisms are still missing. A sharp decline of serum testosterone is, besides rise in stress hormones (adrenocorticoid, glucocorticoids), one of the first signs of immobilization stress (IMO). Acute stress-reduced testosterone production appears to be related to the inhibition of the activities of testicular steroidogenic enzymes (Srivastava et al., 1993; Orr et al., 1994). On the molecular level, the disturbance of steroidogenic machinery and decrease of cAMP in Leydig cells from IMO rats were coupled with sustained up-regulation of mRNA expression for several adenylyl cyclases (ADCYs) and PDE subtypes.

It is well established that stress affects the expression of receptors for stress hormones (Flügge et al., 2004; Schutsky et al., 2011). Stress and glucocorticoids rapidly increase alpha1-adrenergic receptors α1a-ADRs in vivo in rat dentate gyrus (Campeau et al., 2010) and in vitro experiments showed that glucocorticoids can up-regulate α1a-ADR mRNA expression (Rouppe van der Voort et al., 1999). It is well known that ADRs play an important role in stimulation of androgen production in rat (Anakwe and Moger, 1986; Stojkov et al., 2012) and hamster (Mayerhofer et al., 1993) and that testicular α1-ADR activate antiapoptotic signaling in 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 of stressed rats (Stojkov et al., 2012) and this effect was even more pronounced with sustained blockade of α1-ADRs in vivo (Stojkov et al., 2013a). Accordingly, giving the importance of cAMP and cGMP signaling for regulation of steroidogenesis, we speculated that α1-ADRs may also contribute to the stress-induced disturbance of cAMP and cGMP signaling in Leydig cells.

This study was designed to examine the role of α1-ADRs in stress-disturbed cAMP and NO-cGMP signaling system in Leydig cells. IMO was chosen as a typical and frequently used model of psychophysiological stress (Kvetnansky et al., 1970; Orr et al., 1994; Kostic et al., 2008, 2010; Stojkov et al., 2012, 2013a; Andric et al., 2013). IMO sessions include the acute (1 × IMO) and repeated stress without (2 × IMO) or with (10 × IMO) partial recovery of circulating testosterone levels. The focus in our study was on α1-ADR-mediated alterations in cAMP and NO-cGMP signaling system in Leydig cells from stressed rats. To do this, doxazosin (Doxa), a potent and widely used antagonist of α1-ADRs (Kaye et al., 1986; Andersson and Gratzke, 2007) was applied per os (p.o.) and molecular markers of Leydig cell functionality, as well as cAMP and NO-cGMP signaling in Leydig cells were studied.

**Materials and Methods**

**Materials**

The anti-mouse, anti-rabbit and anti-chicken secondary antibodies linked to the horse-radish peroxidase were obtained from Kirkegaard and Pery Labs.
Doxa mitigates stress-disturbed cAMP/cGMP signaling

(Daughtersburg, MD, USA). The antibodies against catalytic (Cat. No. 61980) and regulatory (Cat. No. 610165) subunits of PRK A, as well as for NOS3 (Cat. No. 610296) were purchased from BD Transduction Laboratory (Lexington, KY, USA). The anti-GUCY1a/β (Cat. No. 371716) and anti-PRKG1 (Cat. No. 370661) were obtained from Calbiochem (Darmstadt, Germany), while anti-ACTIN (Cat. No. CP01, Oncogene) was from EMB Bioscience (La Jolla, CA). The anti-testosterone-I-1-BSA serum No250 was kindly supplied by Gordon D. Niswender. The (1,2,6,7H(N)) labeled testosterone was from Perkin-Elmer Life Sciences (Waltham, MA, USA), ALPCO Diagnostic-LH (Rat) RIA from ALPCO (Salem, NH, USA), CAMP EIA Kit, cGMP EIA Kit and corticosterone (CORT) EIA Kit were purchased from Cayman (Ann Arbor, MI, USA), whereas adrenaline research ELISA Kit was from Labor Diagnostika Nord (Nordhorn, Germany). The RNAeasy kit for total RNA isolation was purchased from Qiagen Co., GmbH, (Qiagen, Valencia, CA, USA) while Superscript III kit for cDNA preparation was obtained from Invitrogen (Grand Island, NY, USA). TaqMan Low Density Rat Endogenous Control Panel, TaqMan Low Density Rat Phosphodiesterase Panel, TaqMan Universal PCR Master Mix and Power SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA), while primers for real-time RQ-PCR were obtained from Integrated DNA Technologies (Munich, Germany). 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, acid β-glycerophosphate, tergitol (type 4), dithiothreitol, leupeptin, aprotinin were purchased from Sigma Aldrich (Saint Louis, MO, USA). Doxa, selective α1-ADR antagonist (in a form of doxazosin mesylate) was obtained from Zdravlje AD (Zdravlje, Leskovac, Serbia). All other reagents were of analytical grade and previously reported by our group (for references please see Stojkov et al., 2012).

Methodology for the studies included in this article is briefly outlined here, but it was previously reported by our group in more details (for all references please see Stojkov et al., 2012, 2013a, b).

Animals

Three-month old (250–270 g) male Wistar rats, bred and raised in the Animal Facility of Faculty of Sciences, University of Novi Sad, Serbia were used for the experiments. Animals were raised in controlled environmental conditions (22 ± 2 °C; 12 h light/dark cycle, lights on at 07:00 h) with food and water ad libitum. All the experimental protocols were approved by the local Ethical Committee on Animal Care and Use of the University of Novi Sad operating under the rules of National Council for Animal Welfare and the National Law for Animal Welfare (copyright March 2009), and in accordance with the National Research Council publication Guide for the Care and Use of Laboratory Animals (copyright 1996; National Academy of Sciences, Washington DC) and Council (NRC) publication Guide for the Care and Use of Laboratory Animals (copyright 1996; National Academy of Sciences, Washington DC) and NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 23, revised 1996, 7th edition; www.nap.edu/readingroom/books/labrats). All the experiments adheres to APS’s Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training and were carried out in the Laboratory for Reproductive Endocrinology and Signaling, DBE, Faculty of Sciences at University of Novi Sad.

Experimental models

Rats were handled daily during a 3-week period of acclimation before experiments. IMO stress was performed in the morning (from 08:00 h to 10:00 h) by method of Kvetnansky et al. (1970) described before by our group (Kostic et al., 2008, 2010; Stojkov et al., 2012; Andric et al., 2013). Briefly, IMO rats were bound in the supine position to a wooden board by fixing the rat limbs by thread to the wooden board, but head motion was not limited. Freely moving rats served as a control. In vivo blockade of α1-ADRs before and during IMO was performed in order to mimic the most likely route of human exposure to Doxa, selective α1-ADR antagonist. Groups of rats received Doxa in pharmacologically relevant dose p.o. for rat model (5 mg/kg BW) described before (Kaye et al., 1986; Stojkov et al., 2013a, b) or water prior to stress. Due to pharmacokinetic properties of Doxa (Kaye et al., 1986), the oral gavage was performed 1.5 h before IMO, and rats were sacrificed immediately after first, second or tenth sessions. At the end of experiments all animals were quickly decapitated without anesthesia and the trunk blood was collected. Individual serum samples were stored at −80°C until assayed for hormone levels. All groups consisted of four animals. All experiments were repeated three to five times.

Preparation of purified Leydig cells and ex vivo androgens production

The primary cultures of purified Leydig cells were obtained from all experimental groups as described previously (for references please see Stojkov et al., 2012, 2013a, b). The proportion of Leydig cells present in culture was 95.3 ± 1.7% as determined by staining for HSD3B activity. Purified Leydig cells were plated in 90 mm Petri dishes (5 × 10^6 cells in 5 ml culture medium per dish) for analyses of the ex vivo testosterone and NO production in medium, while cells were used for extraction of cyclic nucleotides or as a source of mRNA or proteins (for references please see Stojkov et al., 2012, 2013a, b).

Hormones, cAMP/cGMP and nitrite measurement

For serum LH levels, all samples were measured in duplicate, in one assay (sensitivity < 1 ng/ml; intra-assay coefficient of variation 4.2%), by RIA according to the manufacturer’s protocol [ALPCO Diagnostic-LH (Rat) RIA] and the minimum detectable concentration has been assayed at 0.14 ng/ml (Stojkov et al., 2012, 2013a, b). Levels of androgens in serum, medium or extracts are referred to as T + DHT, because the testosterone serum No. 250 showed 100% cross reactivity with DHT (Stojkov et al., 2012, 2013a, b). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5–8%). For serum corticosterone levels (Stojkov et al., 2012), all samples were measured in duplicate, in one assay by the corticosterone EIA Kit (www.caymanchem.com) with 30 pg/ml as a lowest standard significantly different from blank. Serum adrenaline levels (Stojkov et al., 2013a) were also determined in duplicates (standard range of 0.45–45 ng/ml and detection limit of 3.9 pg/ml) using the adrenaline research ELISA Kit (www.ldn.de). The levels of cAMP in medium or in cell content of scraped purified Leydig cells were measured by the cAMP EIA Kit (www.caymanchem.com) that permits cAMP measurement with a limit of quantification of 0.1 pmol/ml as a lowest standard significantly different from blank (Stojkov et al., 2012, 2013a, b). The cGMP levels in content were measured by the cGMP EIA Kit (www.caymanchem.com) with a limit of quantification of 0.07 pmol/ml as a lowest standard significantly different from blank (Stojkov et al., 2012, 2013b). For measurement of nitrite levels, the stable metabolic product of NO, in serum and culture medium, sample aliquots were mixed with an equal volume of Griess reagent and the absorbance was measured at 546 nm (Stojkov et al., 2012, 2013b).

RNA isolation and cDNA synthesis

Total RNA from purified rat Leydig cells was isolated using RNAasy kit reagent following a protocol recommended by the manufacturer (www.qiagen.com). Following DNase-I treatment, first-strand cDNA was synthesized according
to the manufacturer’s instructions (www.invitrogen.com). Negative controls consisting of non-reverse transcribed samples were included in each set of reactions. Quality of RNA and DNA integrity were checked by using primers for RSI6, GAPDH and ACTIN, as described before by our group (Stojkov et al., 2012, 2013a, b).

Real-time polymerase chain reaction and relative quantification

The relative expression of the genes was quantified by PCR using the SYBR<sup>®</sup>Gene-based chemistry from Applied Biosystems (www.appliedbiosystems.com) in the presence of an aliquot of 5 μl of the RT reaction product (25 ng RNA calculating on starting RNA) and specific primers. The primer sequences used for real-time PCR analysis (Andric et al., 2010; Stojkov et al., 2012, 2013a, b), including GenBank accession codes for full genes sequences (www.ncbi.nlm.nih.gov/sites/entrez) and Ct values in Leydig cells as well as tests were published before by our group (Stojkov et al., 2013b). Gapdh was also measured in the same samples and used to correct variations in RNA content among samples. Relative quantification of each gene was done in duplicate, three times for each gene and twice for each of three independent in vivo experiments. The expression of the genes for PDEs in Leydig cells obtained from group of rats was analyzed in relative quantification real-time PCR by using the TaqMan<sup>®</sup> Low Density Array (TLDA) Rat PDEs Panel Assay from Applied Biosystems (www.appliedbiosystems.com) as described previously (Andric et al., 2010; Stojkov et al., 2012, 2013b). The Gapdh was used as endogenous controls and quantitated in the same real-time PCR as a part of TLDA, and then used to correct for variations in RNA contents. Each sample was run in duplicate, for each in vivo experiment.

Protein extraction and western blot analysis

After incubation, Leydig cells (5 × 10<sup>6</sup> per well) were washed twice with ice-cold PBS, lysed and western blot analysis was performed as described previously (Andric et al., 2010; Stojkov et al., 2012, 2013a, b). The immuno-detection of the catalytic and regulatory subunits of PRKA, as well as NOS3 proteins was performed by commercial antibodies from BD Transduction Laboratories (wwwbdbiosciences.com/home.jsp), while anti-GUCY1α/β and anti-PRKG were from Calbiochem (www.calbiochem.com). The actin was detected by using actin detection kit (www.embdbiosciences.com). The immunoreactive bands were analyzed as two-dimensional images using the Image J (version 1.32; http://rsbweb.nih.gov/ij/download.html). The optical density of images is expressed as volume used for the background, which gives arbitrary units of adjusted volume group (Andric et al., 2010; Stojkov et al., 2012, 2013a, b).

Statistical analysis

For in vivo studies the results represent group means ± SEM values of individual variation from three to five independent experiments (four rats per group per experiment). For ex vivo measurement data represent mean ± SEM from three to five independent replicates. The results from each experiment were analyzed by Mann–Whitney’s unpaired non-parametric two-tailed test (for two point data experiments), or by one-way ANOVA for group comparison, followed by Student–Newman–Keuls multiple range test. Linear correlations were calculated using KaleidaGraph program (Synergy Software, Reading, PA, USA).

Results

In order to block the effects of stress mediated by α<sub>1</sub>-ADRs, Doxa, the widely used selective α<sub>1</sub>-ADR antagonist was administrated p.o. before IMO. To mimic the most likely route of human exposure to selective α<sub>1</sub>-ADR blockers, rats were subjected to p.o. administration of Doxa in pharmacologically relevant dose (5 mg/kg BW, Kaye et al., 1986; Stojkov et al., 2013a, b), for 1 (1 × Doxa + 1 × IMO), 2 (2 × Doxa + 2 × IMO) or 10 (10 × Doxa + 10 × IMO) consecutive days (Stojkov et al., 2013a). Groups of rats were treated with distilled water in the same manner and exposed to stress (1 × IMO, 2 × IMO, 10 × IMO) or were left undisturbed (control).

Effects of Doxa, an α<sub>1</sub>-ADR antagonist, on stress-disturbed circulating stress hormones (adrenaline, corticosterone), LH and androgens

In agreement with recently published results (Stojkov et al., 2013a) systemic in vivo α<sub>1</sub>-ADR blockade did not change the IMO-elevated serum adrenaline (Fig. 2A), while CORT levels were significantly reduced in 1 × Doxa + 1 × IMO and 2 × Doxa + 2 × IMO groups comparing with corresponding stress groups (Fig. 2B). The circulating LH was reduced comparing with the IMO group only when Doxa was applied once (Fig. 2C), whereas 10 × Doxa administration abolished a partial recovery of androgenesis observed after 10 × IMO (Fig. 2D).
In vivo α1-ADR blockade prevented stress-induced disturbance of Leydig cell functionality and increased cGMP production

The functionality of isolated and purified Leydig cells obtained from all rats was examined by ability of primary culture of Leydig cells to produce androgens (T + DHT) and cAMP (the main regulator of androgenesis) ex vivo, as well as by level of transcript for insulin-like factor-3 (Insl3), the main marker of Leydig cell.

As it was expected and shown before (Stojkov et al., 2012, 2013a) all types of IMO decreased testosterone production, whereas a partial recovery of androgenesis was observed after 10 × IMO (Fig. 3A). In parallel, Insl3 transcription was also attenuated after 1 × IMO and 2 × IMO, but was normalized after 10 × IMO (Fig. 3B). The positive linear correlation (R = 0.99) between Insl3 expression and T + DHT release confirmed the validity of Insl3 as an additional indicator of the Leydig cell function. Doxa p.o. administration before IMO partially recovered ex vivo androgens production in Leydig cells from 1 × Doxa + 1 × IMO group comparing with corresponding IMO groups. In the same cells the effect of 1 × IMO decreased cAMP content in Leydig cells, while Doxa p.o. administration before stress diminished effect of IMO (Fig. 3C). The level of cGMP, another nucleotide that stimulates androgenesis in Leydig cells (Valenti et al., 1999; Andric et al., 2007, 2010), was not changed by IMO, while α1-ADR blockade/during IMO increased cGMP levels comparing with corresponding stress groups (Fig. 3D). In line with the previous study (Stojkov et al., 2013a) the level of Insl3 transcript in Leydig cells from all Doxa + IMO groups remained unchanged comparing with corresponding IMO group (Fig. 3B).

As it was expected and shown before (Stojkov et al., 2013a) all types of IMO decreased cAMP content in Leydig cells, while Doxa p.o. administration before stress diminished effect of IMO (Fig. 3C). The level of cGMP, another nucleotide that stimulates androgenesis in Leydig cells (Valenti et al., 1999; Andric et al., 2007, 2010), was not changed by IMO, while α1-ADR blockade/during IMO increased cGMP levels comparing with corresponding stress groups (Fig. 3D). In the same cells the level of NO increased by all types of Doxa administration before stress comparing with the corresponding IMO group, with most prominent effect of 1 × Doxa (Fig. 3E). The negative linear correlation (R = −0.94) between nitrite and androgen concentration in Leydig cells from Doxa + IMO groups supports the hypothesis that elevated NO inhibits androgen production independently of the status of cyclic nucleotide production (Valenti et al., 1999; Andric et al., 2007, 2010).

Since all types of Doxa p.o. administration before stress changed IMO-induced effects on cAMP, cGMP and NO production in Leydig cells, an expression analysis of cAMP and NO-cGMP signaling elements in Leydig cells were performed.

Effects of in vivo α1-ADR blockade on stress-induced disturbance of cAMP signaling in Leydig cells

Due to the necessity and essential importance of cAMP-PRKA signaling pathway for steroidogenesis (reviewed in Dufau, 1998; Hansson et al., 2000; Payne and Hales, 2004; Tsai and Beavo, 2011), the transcriptional and western blot analyses for elements of this signaling in Leydig cells from all animals were performed.

Results showed that blockade of α1-ARDs before/during IMO did not change IMO-induced increase in transcription of Adcy7, Adcy9, Adcy10 in Leydig cells (Fig. 4A).

The expression of cAMP ‘effector’ and main stimulator of androgenesis, PRKA was stimulated in Leydig cells from Doxa + IMO comparing
with the corresponding IMO group (Fig. 4B). Sustained blockade of α1-ADRs increased level of transcripts for PRKA catalytic subunits (Prkaca, Prkacb), while the level of PRKAC protein in Leydig cells increased by both, acute and sustained α1-ADR blockade (Fig. 4B, insert). In the same cells, Prkar1a, Prkar2a transcripts for regulatory PRKA were stimulated, while Prkar2b was not changed. The protein for most abundantly expressed isoform of regulatory subunit of PRKA (PRKAR1) increased only in Leydig cells from 10 × Doxa + 10 × IMO group. Accordingly, catalytic PRKA subunit was more stimulated than the regulatory subunit in Leydig cells from 10 × Doxa + 10 × IMO rats (Fig. 4B, insert).

Transcription of cAMP ‘remover’ the most abundantly expressed in Leydig cells, Pde4b decreased in cells from animals ten times treated with Doxa before IMO comparing with corresponding stress group. Blockade of α1-ADRs before/during IMO did not change IMO-induced increase in the transcription of other cAMP specific PDEs expressed in Leydig cells, Pde4a, Pde4d, Pde7a, Pde8a (Fig. 4C).

The product of ADCYs and PDEs ‘interplay’, cAMP, significantly increased in Leydig cells from rats exposed to Doxa + IMO comparing with the corresponding IMO group (Fig. 3C) suggesting that α1-ADR blockade diminished IMO-reduced cAMP in Leydig cells.

Effects of in vivo α1-ADR blockade on stress-induced disturbance of cGMP signaling in Leydig cells

Considering the importance of ‘cross talk’ between cAMP and cGMP signaling pathways for cell homeostasis and the role of cGMP-PRKG-PDEs signaling in steroidogenesis (reviewed in Tsai and Beavo, 2011), the transcriptional and western blot analyses for elements of NO-cGMP signaling in Leydig cells were performed.

Results suggested that blockade of α1-ADRs before/during stress significantly increased the level of transcript/protein for most abundantly expressed NOS in Leydig cells (CtNos1 = 34.8; CtNos2 = 26.9; CtNos3 = 24.8), endothelial NOS (Nos3/NOS3). Oppositely, IMO-increased level of transcript for neural NOS (Nos1) was reduced by sustained α1-ADR blockade before/during IMO.

Figure 4 Effects of in vivo α1-ADR blockade on stress-induced disturbance of cAMP signaling elements in Leydig cells. (A) The quantitative genes expression of cAMP ‘makers’ (Adcy7, Adcy9, Adcy10) in Leydig cells. (B) The quantitative genes expression of cAMP ‘effector’ subunits (Prkaca, Prkacb, Prkar1a, Prkar2a, Prkar2b) and protein for most abundantly expressed PRKA subunits in Leydig cells. Inserts are representing the western blots for PRKA catalytic (PRKAC) and regulatory (PRKAR1) subunits in Leydig cells. ACTIN was used as internal control; the same loading control is appropriate and is presented on the bottom of the panels since all of them were obtained from the same membrane. The representative blots were shown on panels, while pooled data from scanning densitometry normalized on ACTIN values are shown as bars on the top of the blots. Western blot analysis was performed using commercial antibodies specific for PRKA catalytic or regulatory subunits and ACTIN (for details please see Materials and methods). (C) The quantitative genes expression of cAMP ‘removers’, i.e. cAMP specific PDEs (Pde4a, Pde4b, Pde4d, Pde7a, Pde8a) in Leydig cells. For all genes RQ-PCR analysis was performed by SYBR Green technology. RQ-PCR analysis for PDEs was performed by TaqMan technology using TaqMan Low Density Rat Phosphodiesterase Panel. For Pde4a/b (the most abundantly expressed cAMP-specific PDEs in Leydig cells) RQ-PCR reactions were also performed by SYBR Green technology (for details please see section Materials and methods). Data bars are group means ± SEM values. Statistical significance at level P < 0.05: * versus control group; ** versus corresponding IMO group.
blockade compared with the 10 x IMO group, but still increased compared with the undisturbed control (Fig. 5A). In the same cells, IMO-induced decrease of transcript for inducible NOS (Nos2) was not changed (Fig. 5A). The expression analysis was supported by secretion results showing increased NO level in Leydig cells from rats exposed to α1-ADR blockade before/during stress compared with the corresponding stress group (Fig. 3E).

Blockade of α1-ADRs before/during stress abolished increase in transcripts for GUCY1 subunits caused by acute stress (1 x IMO), while did not change 2 x IMO/10 x IMO-induced increase in the transcription level of GUCY1. The level of GUCY1 protein, soluble cGMP producer, increased in Leydig cells from animals exposed to all types of Doxa treatment comparing with corresponding IMO groups (Fig. 5B).

The expression of the transcript for soluble form of PRKG, Prkg1, significantly stimulated in Leydig cells from rats exposed to repeated stress (2 x IMO/10 x IMO), was not changed by blockade of α1-ADRs before/during stress (Fig. 5C). The level of transcript for membrane form of PRKG, Prkg2, significantly increased in Leydig cells from 10 x Doxa + 10 x IMO rats comparing with 10 x IMO (Fig. 5C). Since PRKG1 is the main effector of cGMP signaling in Leydig cells (Prkg1 is 32 times more expressed than Prkg2), protein expression analysis was performed. Results showed the significant increase of the PRKG1 protein levels in Leydig cells isolated from all Doxa + IMO rats comparing with the corresponding IMO group (Fig. 5C, Insert).

The transcriptional profile of cGMP ‘removers’ in Leydig cells, cGMP-specific and dual-specific PDEs, was not disturbed in a great fashion by blockade of α1-ADRs before/during stress (Fig. 5D, E). The only change was abolition of IMO-induced level of the transcripts for cGMP-specific Pde6d (Fig. 5D). Stress-induced changes in the transcriptional signature of other cGMP-specific (Pde5a, Pde9a) and dual specific (Pde1a, Pde1c, Pde2a, Pde3a, Pde3b, Pde10a) PDEs were not significantly affected by Doxa p.o. treatments before stress (Fig. 5D, E).

The product of GUCYs and PDEs ‘interplay’, cGMP, significantly increased in Leydig cells from rats exposed to α1-ADR blockade before/during stress comparing with corresponding stress group (Fig. 3D).

**Discussion**

In this study, we have demonstrated, to our best knowledge for the first time, the role of α1-ADRs in stress-disturbed cAMP and cGMP signaling in steroidogenic cells, such as Leydig cells. Several lines of evidence supported the role α1-ADRs in stress-triggered changes of cAMP and cGMP signaling pathway in Leydig cells (Fig. 6). First, blockade of α1-ADRs before/during stress partially abolished stress-reduced cAMP (the main stimulator of steroidogenesis) and completely abolished stress-increased transcription of most abundantly expressed PDE that remove cAMP (Pde4b). Secondly, the expression of PRKA protein (the cAMP ‘effector’ and the main stimulator of steroidogenesis and transcription of steroidogenic genes), was significantly stimulated comparing with corresponding stress groups. Thirdly, the NOS3 protein expression was stimulated and was accompanied with increased NO production in Leydig cells. Fourthly, the expression of GUCY1 (cGMP ‘producer’) was stimulated and cGMP levels increased in Leydig cells from animals exposed to α1-ADR blockade before/during stress comparing with corresponding stress groups. Fifthly, the expression of PRKG1 (cGMP ‘effector’ and stimulator of steroidogenesis) also increased comparing with corresponding stress groups. Accordingly, it is possible that all mentioned molecules (Fig. 6), together with changes in steroidogenic machinery (Stojkov et al., 2013a), could contribute, at least in part, to an increased ex vivo androgen production by purifying Leydig cells from animals exposed to α1-ADR blockade during stress comparing with corresponding stress groups.

It is very well known for many years that adrenaline and glucocorticoids (CORT in rats) are the main stress mediators (Selye, 1936; Rivier and Rivest, 1991). As it was expected and shown before (Stojkov et al., 2013a), irrespective of duration, in vivo blockade of α1-ADRs did not change IMO-induced sustained increase of circulating adrenaline, while the transient elevation of CORT was diminished. The effect on CORT could be explained by earlier studies showed that α1-ADR antagonists (including Doxa used in this study) decreased the output of cortisol by porcine adrenocortical cells in vitro (Jager et al., 1998). The reduced levels of LH in serum of IMO rats exposed to Doxa are in accordance with findings showing that Doxa p.o. application significantly decreased circulating LH (Stojkov et al., 2013a, b). In parallel, the decrease in the levels of circulating androgens in Doxa + IMO-treated rats are in line with findings showing that the in vivo administration of Doxa used the same model like in our study (Stojkov et al., 2013a, b) or with doubled dose for 15 days (de-la-Chica-Rodrı´guez et al., 2008) decreased circulating testosterone.

In line with our previous study (Stojkov et al., 2013a), here we show that oppositely to circulating androgens (Fig. 2D), 1 x Doxa p.o. administration before IMO partially abolished 1 x IMO-reduced ex vivo androgen production in Leydig cells, while complete abolition of reduction was registered in Leydig cells from 2 x Doxa + 2 x IMO and 10 x Doxa + 10 x IMO groups (Fig. 3A). How to explain the huge discrepancy between levels of androgens in serum (Fig. 2D) and produced in vivo by Leydig cells from Doxa + IMO-treated rats (Fig. 3A)? The androgens levels in circulation are outcome of ‘interplay’ between LH/LHR interaction, testicular microcirculation, testicular interstitial fluid (TIF) volume/content, as well as steroidogenic machinery homeostasis within the Leydig cells influenced by plethora neuronal/endocrine/paracrine/autocrine signals. Doxa could exert important vascular effects (Dell’Omo et al., 2005) including the change of blood flow in the testes and affects TIF volume/content (Stojkov et al., 2013a, b). The Leydig cells isolated from in vivo treated rats are removed from the biologically active inhibitory and/or stimulatory paracrine compounds released from the seminiferous tubules and/or macrophages, endothelial cells, fibroblasts, peritubular cells and Sertoli cells (Klinefelter et al., 1987; Saez, 1994; Hutson, 2006).

Our results indicate that cAMP levels in Leydig cells from animals exposed to blockade of α1-ADRs before/during stress were significantly increased comparing with corresponding stress groups (Fig. 3C), which could reflect an increase in ADCY activity or reduced degradation of cAMP by PDEs. Consistent with this hypothesis, we observed significant decrease in the expression of cAMP-specific Pde4b (Fig. 4C) and increase in cAMP in Leydig cells of rats treated with Doxa alone (Stojkov et al., 2013b). In vitro experiments of others have shown that glucocorticoids directly influence many steps in the β-ADR signaling pathway, including super-sensitization through increased β-agonist stimulation of ADCYs, potentiation of high-affinity β-agonist binding and up-regulation of β-ADR genes transcription and receptor expression (Schutsky et al., 2011). Our recent results (Stojkov et al., 2012) showed increase in the level of transcripts for all ADRs and β-ADR kinase-1 (Adrbk1) in Leydig
Figure 5 The effects of *in vivo* α1-ADR blockade on stress-induced disturbance of cGMP signaling elements in Leydig cells. (A) The quantitative genes expression of NO ‘makers’ (*Nos1*, *Nos2*, *Nos3*) and NOS3 protein in Leydig cells. (B) The quantitative genes expression of subunits for soluble cGMP ‘makers’ (*Gucyl-a*, *Gucyl-b*) and GUCY1 protein in Leydig cells. (C) The quantitative genes expression of cytosolic (*Prkg1*) or membrane located (*Prkg2*) cGMP ‘effectors’ and PRKG1 protein in Leydig cells. Inserts are representing the western blots for NOS3, GUCY1 and PRKG1 in Leydig cells. ACTIN was used as internal control; the same loading control is appropriate and was presented on the bottom of the panels since all of them were obtained from the same membrane. The representative blots were shown on panels, while pooled data from scanning densitometry normalized on ACTIN values are shown as bars on the top of the blots. Western blot analyses were performed using commercial antibodies specific for NOS3, GUCY1, PRKG1 and ACTIN in Leydig cells (for details please see section Materials and Methods). (D) The quantitative genes expression of cGMP ‘removers’, i.e. cGMP specific PDEs (*Pde5a, Pde6a, Pde9a*) in Leydig cells. (E) The quantitative gene expression of dual-specific (cAMP/cGMP) PDEs (*Pde1a, Pde1c, Pde2a, Pde3a, Pde3b, de10a*) in Leydig cells. For all genes RQ-PCR analysis was performed by SYBR Green technology. RQ-PCR analysis for *Pde5a* (the most abundantly expressed cGMP-specific PDE in Leydig cells) RQ-PCR reactions were also performed by SYBR Green technology (for details please see section Materials and methods). Data bars are group means ± SEM values. Statistical significance at level *P* < 0.05: * versus control group; † versus corresponding IMO group.
Doxa mitigates stress-disturbed cAMP/cGMP signaling

Figure 6 The effects of sustained in vivo α1-ADR blockade on stress-induced disturbance of cAMP and cGMP signaling in Leydig cell. Repeated oral administration of Doxa, a potent α1-ADR antagonist, abolished IMO-induced increase in the transcription of most abundantly expressed PDE that remove cAMP (Pde4b), and potentiated stress-induced stimulation of the expression of PRKA, the main stimulator of Leydig cell steroidogenesis. The expression of Nos3 (constitutive NO ‘producer’, GUCY1 (soluble cGMP ‘producer’) and PRKG1 (cGMP ‘effector’) were stimulated. By this signaling scenario, in the presence of high level of adrenaline, it is possible that Leydig cell homeostasis is more regulated by β2ADRs, the most abundantly expressed ADR in the Leydig cells (Stojkov et al., 2012) and potent activator of cAMP signaling, especially since in vivo α1-ADR blockade potentiated β2ADR expression in Leydig cells from stressed rats (Stojkov et al., 2013a). The physiological significance of the presented results was proved by increased testosterone, cAMP and cGMP production in Leydig cells from Doxa + IMO rats, comparing with IMO groups (please see Fig. 3). Green arrows indicate the effects of 10 × IMO, while violet arrows indicate the effects of in vivo α1-ADRs blockade before/during stress (10 × Doxa + 10 × IMO). Two opposite arrows represent the diminished IMO-associated effects, but not abolition. The absence of arrows indicates that IMO-induced effects were completely abolished.

Collectively, both cAMP and cGMP signaling pathway in Leydig cells may contribute to increased androgens production in Leydig cells from rats exposed to α1ADR blocker + stress comparing with the corresponding stress group. This effects could be mediated by either cAMP-PRKA (Kostic et al., 2008) or cGMP-PRKG1 (Kostic et al., 2010) dependent phosphorylation/activation of StAR protein, or by both (Andric et al., 2010), or by phosphorylation/activation of CREB, the main stimulator of steroidogenic genes transcription (reviewed in Payne and Hales, 2004; King and LaVoie, 2012). The increased NO level in Leydig cells from Doxa + IMO rats comparing with the corresponding IMO group (Fig. 3D). In the same cells, the expression of PRKG1 also increased (Fig. 5C). These results are in line with findings that sustained in vivo administration of Doxa to normal rats caused increased transcription of Nos1, Nos3, Gucy1a/b, Prkg1 in Leydig cells (Stojkov et al., 2013b), while in the spontaneously hypertensive rats caused an up-regulation of Nos3 in the penis (Yono et al., 2007).
testis (Weissman et al., 2007, 2009; Kostic et al., 2010). Certainly, the action of NO in testis of stressed rats is not limited to Leydig cells. The major regulators of NO production originate from seminiferous tubules, Sertoli cells and/or non-steroidogenic cells in the testicular interstitium (Niemi et al., 1986; Klinefelter et al., 1987; Pomerantz and Pitelka, 1998; Hutson, 2006; Weissman et al., 2005, 2007, 2009) and NO is significant paracrine modulator of androgen synthesis in rat Leydig cells (Weissman et al., 2005, 2007, 2009). This could serve as one of the possible explanation for the results of this study showing the opposite profiles of androgens produced in vivo (in serum) and ex vivo (by isolated Leydig cells) in rats exposed to α1-ADR blocker + stress. Certainly, the effects of Doxa + stress on other organs/structures in vivo cannot be excluded and Leydig cells could only be indirectly affected by other mediators. It has been shown that Doxa is the first small molecule agonist of receptor thyrosin kinase that is capable of inhibiting malignant behaviors in vitro and in vivo (Petty et al., 2012). In addition, our preliminary results from in vitro experiments on Leydig cells showed a decreased HCG response and mitochondrial membrane potential in the presence of α1-ADR blocker (Drljaca et al., unpublished results). The possible involvement of all mentioned molecules could make the ‘picture’ regarding Doxa + IMO effects even more complicated. However, regardless of whether the effects of Doxa on cAMP/cGMP signaling are direct or indirect, cAMP and cGMP signaling is affected and this in turn can influence Leydig cell homeostasis and testosterone production. Accordingly, the complex structural organization of the testes and coexistence of multiple regulatory mechanisms that control testicular cells and microvasculature could provide a degree of redundancy in the maintenance of testicular steroidogenesis, a crucial component of the reproductive process. It is also conceivable that this multifactorial system could reflect the gradation from simple to more complex neuroendocrine control systems for regulating hypothalamic-pituitary function and gonadal activity.

In conclusion, the results obtained in this study support the important role α1-ADRs in cAMP and cGMP signaling pathway triggered by stress in Leydig cell androgenesis and might provide new insight into the relationship between stress and the mammalian reproductive function. Presented data provide new molecular base for ‘fight/ adaptation’ of steroidogenic cells (Fig. 6) and could also be important in terms of the wide use of α1-ADR selective antagonists, alone or in combination, to treat post-traumatic stress disorders, hypertension, benign prostatic hyperplasia symptoms and the disrupted sexual health. Eventually, this study could be the solid base for the evaluating Doxa clinical and pharmacogenomic data in human reproductive health risk assessment.

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Authors’ roles

N.J.S., A.Z.B., M.M.B., S.J.S., A.I.M., D.M.D, M.M.J., T.S.K. and S.A.A. performed the experiments; edited and revised the manuscript; approved the final version of the manuscript; N.J.S., A.Z.B., T.S.K. and S.A.A. analyzed the data; interpreted results of experiments; T.S.K. and S.A.A. did the conception and design of the research; N.J.S. and S.A.A. prepared the figures; drafted the manuscript.

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Conflict of interest

The authors have nothing to disclose.

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