Sustained in vivo blockade of α₁-adrenergic receptors prevented some of stress-triggered effects on steroidogenic machinery in Leydig cells

Natasa J. Stojkov, Marija M. Janjic, Aleksandar Z. Baburski, Aleksandar I. Mihajlovic, Dragana M. Drljaca, Srđjan J. Sokanovic, Maja M. Bjelic, Tatjana S. Kostic, and Silvana A. Andric
Reproductive Endocrinology and Signaling Group, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia

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Sustained in vivo blockade of α₁-adrenergic receptors prevented some of stress-triggered effects on steroidogenic machinery in Leydig cells. Am J Physiol Endocrinol Metab 305: E194–E204, 2013. First published May 14, 2013; doi:10.1152/ajpendo.00100.2013.—This study was designed to systematically analyze and evaluate the effects of in vivo blockade of α₁-adrenergic receptors (α₁-ADRs) on testosterone production and steroidogenic expression; and blockade additionally stimulated stress-increased transcription profiles of ADRs and oxidases with high affinity to inactivate glucocorticoids. Results showed that sustained blockade of α₁-ADRs prevented stress-induced 1) decrease of the transcripts/proteins for main steroidogenic CYPs (CYP11A1, CYP17A1); 2) decrease of Scarb1 and Hsd3b1 transcripts; 3) decrease of transcript for Nur77, one of the main activator of the steroidogenic expression; and 4) increase of Dax1 and Arr19, the main steroidogenic repressors in Leydig cells. In the same cells, the expression of steroidogenic stimulatory factor Creb1, STAR, and androgen receptor increased. In this signaling scenario, stress-induced stimulation of Adra1a/Adra1b/Adrb1 and Hsd11b2 (the unidirectional oxidative with high affinity to inactivate glucocorticoids) was not changed. Blockade additionally stimulated stress-increased transcription of the most abundantly expressed ADRs Adra1d/Adrb1/Adrb2 in Leydig cells. In the same cells, stress-decreased testosterone production, the main marker of Leydig cells functionality, was completely prevented, while reduction of cAMP, the main regulator of androgenesis, was partially prevented. Accordingly, the presented data provide a new molecular/transcriptional base for “fight/adaptation” of steroidogenic cells and new molecular insights into the role of α₁-ADRs in stress-impaired Leydig cell steroidogenesis. The results are important in term of wide use of α₁-ADR selective antagonists, alone/in combination, to treat high blood pressure, nightmares associated with posttraumatic stress disorder, and disrupted sexual health.

testosterone; stress; α₁-adrenergic receptors; steroidogenic machinery; doxazosin

TESTOSTERONE-PRODUCING LEYDIG CELLS, like all other steroid-producing cells, synthesize steroid hormones from common precursor cholesterol using the steroidogenic machinery (Fig. 1). This complex machinery is comprised of cholesterol transporters (50, 56, 71), steroidogenic enzymes (51), and many regulatory molecules (17, 18, 50, 51, 56, 71) as well as numerous transcriptional factors controlling the expression of steroidogenic gene expression (33, 40, 69). The steroidogenic function of Leydig cells is predominantly regulated by pituitary luteinizing hormone (LH) or its placental counterpart, human chorionic gonadotropin (hCG). LH/hCG receptor activation leads to stimulation of adenyl cyclase (AC), accumulation of cAMP, and activation of the cAMP-dependent kinase (PKA). The phosphodiesterases (PDEs) terminate cAMP/cGMP signaling and have regulatory function in Leydig cells (9, 17, 18, 76, 77). Although Leydig cell steroidogenesis is mainly activated through LH/hCG receptors, regulation itself is a multicompartamental process comprised of neuronal (61) and complex endocrine, paracrine, and autocrine signaling pathways (22, 51, 59) including cGMP (4) and adrenergic signaling (3). All the aforementioned elements of steroidogenic machinery might be involved in the regulation of testicular steroidogenesis, providing an adaptive mechanism by which testicular structures, including Leydig cells, recover from disturbed homeostasis, such as stress, or any other situation disturbing testosterone homeostasis.

It is well established that the organism responds to stress, a state of perceived threat to homeostasis, by activating the sympathetic nervous system, secreting catecholamines and glucocorticoids in the “fight-or-flight” response (7, 8, 20, 24, 39, 55, 60). The ability of stress to inhibit reproductive functions is well documented (21, 23, 24, 36, 37, 49, 55), and the mechanisms mediating these effects depend on the type, duration, and frequency of the stimulus (24, 39, 55) as well as on the influence of the steroid milieu on adrenergic and opiate components that have an impact on the hypothalamo-pituitary-gonadal axis (21, 23, 24, 36, 37, 49, 55). Obviously, each of stress mediators (CRF, POMC-derived peptides, corticosteroids, catecholamines) could play a role in mediating the effect of stress (23, 39, 55). There is evidence for the involvement of the sympathetic nervous system in the regulation of the hypothalamo-pituitary-gonadal axis at all levels, but precise mechanisms are still missing (23, 39, 55). Stressors activate norepinephrine neurons in the cortex, limbic system, and reticular formation and through sympathetic nerves reduce the frequency of pulsatile GnRH/LH release and the sensitivity of the gonadotrophs to GnRH stimulation. (23, 55). Postganglionic sympathetic nerve fibers also secrete CRF, among other substances, whereas catecholamines stimulate interleukin-6 release by immune cells and other peripheral cells (23, 39, 55), making the mechanisms of stress-induced sympathetic-mediated effects even more complicated. A sharp decline of serum testosterone is, beside stress hormones (adrenaline, glucocorticoids) one of the first signs of immobilization stress (IMO). Acute stress-induced reduction of testosterone production appears to be related to the inhibition of the activities of testicular steroidogenic enzymes (49, 70). On the molecular level, we
ADRs in Leydig cells were studied.

**Glossary**

- **ACTB**: β-actin
- **Adr**: Gene for adrenergic receptor (ADR)
- **Adrbk1**: Gene for β-adrenergic receptor kinase-1 (ADRBK1)
- **Ar**: Gene for androgen receptor (AR)
- **Arr19**: Gene for androgen receptor corepressor-19 kDa
- **CORT**: Corticosterone
- **Cyp11a1**: Gene for cholesterol side-chain cleavage enzyme (CYP11A1 or P450scC)
- **Cyp17a1**: Gene for enzyme 17α-hydroxylase/17,20-lyase (CYP17A1 or P450c17)
- **Dax1**: Gene for dosage-sensitive sex reversal adrenal hypoplasia critical region on chromosome X gene
- **DHT**: Dihydrotestosterone
- **Doxa**: Doxazosin
- **Esr1**: Gene for estrogen receptor 1 (alpha)
- **hCG**: Human chorionic gonadotropin
- **Hsd11b**: Gene for 11β-hydroxysteroid dehydrogenase (HSD11B or 11β-HSD)
- **Hsd17b**: Gene for 17β-HSD (HSD17B or 17β-HSD)
- **Hsd3b**: Gene for 3β-HSD (HSD3B or 3β-HSD)
- **Lhr**: Gene for LH/hCG receptor
- **Nur77**: Gene for nerve growth factor (NR4A1, nuclear receptor subfamily 4 group A member 1)
- **Scarb1**: Gene for scavenger receptor class B, member 1 (SCARB1)

(72) recently showed that disturbance of steroidogenic machinery and decrease of cAMP in Leydig cells from IMO rats was coupled with sustained upregulation of mRNA expression for several adenylyl cyclases and phosphodiesterase (PDE) subtypes. Our study also revealed the strong stimulation of transcription of all adrenergic receptors (ADRs) expressed in Leydig cells.

It is well established that ADRs play an important role in stimulation of androgen production in rat (3, 72) and hamster (47) and that α1-ADRs in Leydig cells activate antiapoptotic signaling in Leydig cells of stressed animals (5), we speculated that α1-ADRs may also contribute to the stress-induced disturbance of steroidogenesis. It is well established that stress accelerates turnover of brain norepinephrine and epinephrine, and the resulting changes in their blood concentrations depend on the nature, intensity, and duration of a stressor. It has also been documented that stress affects the expression of receptors for these stress hormones (20, 60). In addition, stress and glucocorticoids rapidly increase α1d-ADRs in vivo in rat dentate gyrus (7), and in vitro experiments have shown that glucocorticoids can upregulate α1d-ADR mRNA expression (58).

Here, we studied the role of α1-ARs in stress-disturbed testicular androgenesis. IMO was chosen as a typical and frequently used model of psychophysiological stress (5, 36, 37, 39, 49, 72). The IMO sessions, established and justified previously (5, 36, 37, 39, 72), include the acute (1×IMO) and repeated stress without (2×IMO) or with (10×IMO) partial recovery of circulating T levels. The focus in our study was on the role of α1-ADRs in stress-induced disturbance and/or recovery of Leydig cell steroidogenic machinery. To do this, doxazosin, a potent antagonist of α1-ADRs described previously (32, 73) was applied per os, and serum hormonal profiles, testicular and Leydig cell testosterone homeostasis, the status of steroidogenic machinery elements, transcription factors, and ADRs in Leydig cells were studied.
The methodology for the studies included in this article is briefly outlined here, but it was previously reported by our group in more detail (72, 73).

**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; 12:12-h light-dark cycle, lights on at 0700) with food and water ad libitum. All the experimental protocols were approved by the local Ethics Committee on Animal Care and Use of the University of Novi Sad operating under the rules of the 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* (NIH Publications no. 80 23, revised 1996, 7th edition). All the experiments adhered 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-wk period of acclimation before experiments. Immobilization stress (IMO) was performed in the morning (from 0800 to 1000) by the method of Kvetnansky (39) described previously (5, 36, 37, 72). Briefly, IMO rats were bound in the supine position by fixing the rat’s limbs by thread to a wooden board, but head motion was not limited. Freely moving rats served as controls. In vivo blockade of α1-ADRs during IMO was performed to mimic the most likely route of human exposure to doxazosin (Doxa), a selective α1-ADR antagonist. Groups of rats received Doxa in pharmacological dose for rat model (5 mg/kg body wt po) as described previously (32, 73) or water prior to stress. Due to pharmacokinetic properties of Doxa (32), oral gavage was performed 1.5 h before IMO, and rats were killed immediately after the first, second, or tenth session. At the end of experiments, all animals were quickly decapitated without anesthesia, and 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.

**Materials and methods.**

**Materials.** The antisera for StAR protein was the generous gift from Prof. Doug Stocco (10), and the purified rabbit polyclonal antibody against HSD3B was the generous gift from Prof. Ian Masson (1, 6). The goat polyclonal antibody raised against a peptide mapping within an internal region of CYP11A and a rabbit polyclonal antibody raised against the COOH terminus of CYP17A1 were obtained from Santa Cruz Biototechnology (www.scbt.com). Actin detection kit was purchased from Oncogene Research Product (www.emdbiosciences.com). The secondary antibodies linked to horseradish peroxidase were obtained from Kirkegaard and Perry Labs (www.kpl.com). The anti-testosterone-11-BSA serum no. 250 was kindly supplied by Gordon D. Niswender. [1,2,6,7-3H(N)]T was obtained from PerkinElmer Life Sciences (www.perkinelmer.com). Doxazosin (Doxa), a selective α1-ADR antagonist (in a form of doxazosin mesylate), was obtained from Zdravlje (www.zdravlje.rs). All other reagents were of analytical grade and previously reported by our group (72, 73).

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 × 10⁶ cells in 5 ml of culture medium per dish) for analysis or the ex vivo testosterone production in medium, while cells were used for extraction of nucleotides (72, 73).

**Hormones and cAMP measurement.** For serum LH levels, all samples were measured in duplicate, in one assay (sensitivity less than 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 was assayed at 0.14 ng/ml (72, 73). Levels of androgens in serum, medium, or extracts are referred to as T+DHT, because the anti-testosterone serum no. 250 showed 100% cross-reactivity with DHT (72, 73). All samples were measured in duplicate in one assay (sensitivity: 6 pg per tube; intra-assay coefficient of variation 5–8%). For serum corticosterone (CORT) levels, all samples were measured in duplicate in one assay with a CORT EIA kit (www.caymanchem.com) that typically displays an IC₅₀ value (50% B/B₀) of ~150 pg/ml and detection limit (80% B/B₀) of ~30 pg/ml. Serum epinephrine levels were also determined in duplicates (standard range of 0.45–45 ng/ml and detection limit of 3.9 pg/ml) using an epinephrine research ELISA kit (www.ldn.de). The levels of cAMP in medium or in cell content of scraped purified Leydig cells were measured by cAMP EIA kit (www.caymanchem.com), which permits cAMP measurement with a limit of quantification of 0.1 pmol/ml (at 80% B/B₀) and IC₅₀ of ~0.5 pmol/ml for acetylated cAMP samples (72, 73).

**RNA isolation and cDNA synthesis.** Total RNA from purified rat Leydig cells was isolated using an RNeasy 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 was checked using primers for β16, GAPDH, and β-actin, as described previously by our group (72, 73).

**Real-time PCR and relative quantification.** The relative expression of the genes was quantified by PCR using SYBR Green-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 calculated on starting RNA) and specific primers. The primer sequences used for real-time PCR analysis, including GenBank accession codes for full gene sequences (www.ncbi.nlm.nih.gov/sites/entrez) were published previ-
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onitoring of stress is a complex process involving the release of stress hormones such as epinephrine and corticosterone. In vivo and in vitro studies have shown that stress can lead to alterations in the production of androgens by Leydig cells. The effects of stress on Leydig cell functionality have been the subject of numerous studies, and the importance of understanding these effects cannot be overstated.

**Results**

To block the effects of stress mediated by α1-ADRs, Doxa, the widely used selective α1-ADR antagonist, was administrated per os before IMO. To mimic the most likely route of human exposure to selective α1-ADR blockers, rats were subjected to administration of Doxa in pharmacological dose [5 mg/kg body wt po (32, 73)], once (1×IMO+Doxa), twice (2×Doxa+2×IMO), or for 10 consecutive days (10×IMO+Doxa). 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 on stress-disturbed circulating stress hormones (epinephrine, CORT), LH, and androgens. In vivo α1-ADR blockade was not able to change the IMO-elevated serum epinephrine (Fig. 2A) and CORT (Fig. 2B). The levels of CORT in serum from 1×Doxa+1×IMO (49.55 ± 3.2 pmol/ml) and 2×Doxa+2×IMO (61.2 ± 6.5 pmol/ml) groups were significantly reduced compared with 1×IMO (65.57 ± 0.75 pmol/ml) or 2×IMO (102.45 ± 6.1 pmol/ml) (Fig. 2B). After Doxa application, the level of circulating LH was reduced in all IMO groups (Fig. 2C), whereas 10×Doxa application abolished a partial recovery of androgenesis observed after 10×IMO (0.88 ± 0.24 vs. 4.13 ± 0.27 ng/ml; Fig. 2D).

Effects of in vivo α1-ADR blockade on stress-disturbed testosterone homeostasis in testis. Androgens levels in circulation are the outcome of the “interplay” between LH/LHR interaction, testicular microcirculation, TIF volume/content, as well as steroidogenic machinery homeostasis within the Leydig cell influenced by the plethora of signals. To try to dissociate the direct effects of Doxa treatment from the paracrine and/or autocrine, we followed the TIF volume and androgen contents in TIF and testicular tissue, as well as produced in medium by testicular preparation. Results showed that α1-ADR blockade was not able to change IMO-reduced TIF volume (12.41 ± 2.3 vs. 32.25 ± 2.3 μl; Fig. 3A) or levels of androgens in TIF (1.5 ± 0.32 vs. 7.2 ± 0.29 ng/total TIF; Fig. 3B) or extracted from testes (0.027 ± 0.012 vs. 0.092 ± 0.013 μg/g; Fig. 3C) or produced by testes in the absence (0.028 ± 0.012 vs. 0.078 ± 0.009 μg/g) or presence (0.14 ± 0.018 vs. 0.22 ± 0.011 μg/g) of hCG (Fig. 3D). In contrast, α1-ADR blockade diminished 10×IMO-induced recovery of TIF volume (Fig. 3A) and levels of androgens in TIF (Fig. 3B), extracted from testes (Fig. 3C), or produced by testes in the absence or presence of hCG (Fig. 3D). Since Leydig cells are the exclusive producers of androgens, the functionality of these cells was followed.

Effects of in vivo α1-ADR blockade on stress-induced disturbance of Leydig cell functionality. The functionality of isolated and purified Leydig cells obtained from all rats was examined by the 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 Ins13 (the main marker of Leydig cell). As was expected and shown previously (37, 72), all types of IMO decreased testosterone (Fig. 4A) and cAMP (Fig. 4B) production, whereas a partial
recovery of androgenesis was observed after 10×IMO (Fig. 4A). *Ins*3 transcription was also attenuated after 1×IMO and 2×IMO but was normalized after 10×IMO (Fig. 4C). Doxa po administration before IMO partially prevented IMO-induced decrease in ex vivo androgen production by Leydig cells from the 1×Doxa+1×IMO group (28.4 ± 1.8 vs. 10.2 ± 1.6 ng/well), whereas complete prevention was registered in 2×Doxa+2×IMO (38.8 ± 1.6 vs. 11.4 ± 3.2 ng/well) and 10×Doxa+10×IMO (45.15 ± 1.9 vs. 27.2 ± 1.5 ng/well) groups (Fig. 4A). The stress-induced reduction of cAMP in Leydig cell content was partially prevented in all groups that received Doxa before stress compared with corresponding stress group (Fig. 4B). The level of *Ins*3 transcript in Leydig cells from all Doxa+IMO groups remained unchanged compared with corresponding IMO group (Fig. 4C).

Since all types of Doxa po administration before IMO diminished the stress-induced decrease in androgen production, an expression analysis of elements involved and/or related with steroidogenic function of Leydig cells were performed.

*Effects of in vivo α1-ADR blockade on stress-induced disturbances of steroidogenic machinery in Leydig cells.* The purified Leydig cells obtained from controls, IMO, and Doxa+IMO rats were sources of mRNA for transcriptional analysis and proteins for Western blot analysis.

Results showed that sustained blockade of α1-ADRs prevented the IMO-induced decrease of the transcripts/proteins for main steroidogenic CYPs (CYP11A1, CYP17A1) in Leydig cells, as well as Sca*rb*1 and Hsd3b1 transcripts, whereas Ts*po* and StaR increased. In the same cells, Hsd3b5, already stimulated by 10×IMO, was additionally stimulated by α1-ADR blockade compared with the 10×IMO group, whereas the level of HSD3B protein remained unchanged (Fig. 5A). All types of Doxa administration before IMO increased the level of transcripts for steroidogenic stimulatory factor, Cre*bl*, and Ar compared with the corresponding IMO group (Fig. 5B). In the same cells, the stress-reduced transcription of Nur77, a main activator of steroidogenic expression, was abolished, whereas Gata4 and Sp1 remained unchanged by IMO or Doxa+IMO (Fig. 5B). The sustained blockade of α1-ADRs reduced stress-stimulated transcription of Dax1 (1.61 ± 0.11 vs. 2.28 ± 0.12 in relative units) and Arr19 (1.92 ± 0.24 vs. 3.22 ± 0.2 in relative units), the main steroidogenic repressors in Leydig cells, whereas Pparg1α transcription factor, positively regulated by CREB, was not changed by α1-ADR blockade (Fig. 5B). The physiological significance of the present results was proven by testosterone production in Leydig cells (Fig. 4A).

*Effects of sustained in vivo α1-ADR blockade on stress-induced transcriptional “signature” of ADRs in Leydig cells.* Since an elevated level of circulating epinephrine is one of the stress mediators and Doxa is a selective α1-ADR antagonist, it was interesting to analyze the effects of immobilization stress on the transcription profile of α- and β-ADRs in Leydig cells, especially because some of them activate cAMP signaling. A rather unexpected and interesting finding was that sustained blockade of α1-ADRs before/during stress additionally increased transcription of Adra1d/Adrb1/Adrb2 (Fig. 6, A and B). The IMO-induced increase of Adra1a/Adra1b/Adrb1 transcripts in Leydig cells was not changed (Fig. 6, A and C). Finally, α1-ADR blockade was not able to change the level of Hsd11b1 transcript (Fig. 6D) nor the 10×IMO-induced increase of transcript for the unidirectional oxidase with high affinity to inactivate glucocorticoid Hsd11b2 (Fig. 6D), confirming that this transcription reflects activation of GRs (21, 23, 24).
In vitro blockade of $\alpha_1$-ADRs was not able to change the 2×IMO-induced sustained increase of circulating epinephrine, whereas it diminished transient elevation of CORT. Earlier studies showed that microinjections of $\alpha_1$-ADR antagonists directly into the medial amygdala dose-dependently and significantly attenuated the ACTH response to acute stress (42). In addition, $\alpha_1$-ADR antagonists (including Doxa, used in our study) decreased the output of cortisol and deoxycortisols by porcine adrenocortical cells in vitro (29). The ability of stress to inhibit

DISCUSSION

In this study, we have demonstrated, to our best knowledge for the first time, that sustained in vivo blockade of $\alpha_1$-ADRs prevented stress-induced 1) decrease of the transcripts/proteins for main steroidogenic CYPs (CYP11A1, CYP17A1), 2) decrease of Scarb1 and Hsd3b1 transcripts, 3) decrease of transcript for Nur77, one of the main activators of steroidogenic expression, and 4) increase of Dax1 and Arr19, main steroidogenic repressors in Leydig cells. In the same cells, transcription of steroidogenic stimulatory factor CREB (Crebl) and androgen receptor (Ar) were increased. In this signaling scenario, stress-induced stimulation of Adra1a/Adra1b/Adrbk1 and Hsd11b2 (the unidirectional oxidase with high affinity to inactivate glucocorticoids) was not changed, while transcription of the most abundantly expressed ADRs Adra1d/Adrb1/Adrb2 in Leydig cells additionally increased.

An emerging body of evidence has established over many years epinephrine and glucocorticoids (CORT in rats) as main stress mediators (55). In this study, irrespective of duration, in

Fig. 4. In vivo $\alpha_1$-ADR blockade prevented stress-induced disturbance of Leydig cell androgen (T+DHT) production. A: ex vivo basal production of androgens by Leydig cells (5×10^6 cells per well) isolated from control, IMO, or Doxa+IMO rats. B: cAMP in content of Leydig cells isolated from control, IMO, or Doxa+IMO rats. C: insl3 transcript in Leydig cells isolated from control, IMO, or Doxa+IMO rats. Leydig cells were isolated and incubated (5×10^6 cells/ml well) for 2 h. Androgens in medium were measured by RIA, cAMP in content by EIA; insl3 was determined by RQ-PCR (for details please see materials and methods). Data points are mean ± SE values of 3–5 independent in vivo experiments. Statistical significance at level $P < 0.05$: * vs. control group; † vs. 1×IMO group, †† vs. corresponding IMO group.

Fig. 5. Effects of in vivo $\alpha_1$-ADR blockade on stress-induced disturbance of steroidogenic machinery in Leydig cells. A: quantitative gene expressions of Lhr, Scarb1, Star, Tspo, Cyp11a1, Hsd3b1/5, Cyp17a1, and Hsd17b4 in Leydig cells isolated from control, IMO, or Doxa+IMO rats. Insets represent Western blots for STAR, CYP11A1, HSD3B, and CYP17A1 in Leydig cells. Actin was used as internal control and is presented on the bottom. The same internal/loading control is appropriate and was presented for some blots on the bottom, since all were obtained from the same membrane. Representative blots are shown, while pooled data from scanning densitometry normalized on actin values are shown as bars on the top. Values are shown as bars on top of the blots and represent means ± SE for 3 independent experiments. B: quantitative gene expression of Creb1, Nur77, Gata4, Sf1, Dax1, Arr19, Ar, Esr1, and Ppargc1a in Leydig cells isolated from control, IMO, or Doxa+IMO rats. Quantitative gene expressions were followed by RQ-PCR using specific primers (72, 73), while protein expressions were analyzed by Western blotting. Data points/bars are mean ± SE values of 3 independent in vivo experiments. Statistical significance at level $P < 0.05$: * vs. control group; † vs. corresponding IMO group.
Androgen levels in the circulation are the outcome of the complex structural organization of the testes and of the coexistence of multiple regulatory mechanisms that control testicular cells and microvasculature and could provide a degree of redundancy in the maintenance of testicular steroidogenesis. The paraventricular nucleus and the brain stem area containing the locus coeruleus are part of the inhibitory neural pathway that regulates testosterone secretion by central β-ADRs independently of the pituitary (61). Cross-talk between different testicular cell types appears to be required to allow the testis to fulfill its endocrine and exocrine functions. The regulation of Leydig cells must be integrated with that of the other somatic cells and germ cells via a short-loop feedback system (22, 51, 59). The presence of local mechanisms for controlling testosterone levels locally by modulation of capillary permeability and testosterone secretion (66), the interaction between LH and the locally produced factors by macrophages, Sertoli cells, and endothelial and peritubular myoid cells is required (22, 25, 34, 49, 51, 59). The most described local factors are growth factors (IGF-I, TGF-3, EGF and TGFα, FGF, and other growth factors), inhibins/activins, cytokines, GHRH, CRF, and gonadal peptides (17, 22, 51, 59). Additionally, circulating and/or locally secreted catecholamines play a physiological role in the control of testicular function (22, 33, 47, 51, 59). Sertoli cells respond trough β1-ADRs, whereas Leydig cells express β2-ADRs most abundantly, but β1-ADRs and α1d-ADRs are also present and operative (72). The catecholamines can interact with LH or GnRH to regulate Leydig cell function (3). All mentioned molecules and many others could change not only Leydig cells but also functionality of other testicular cells, as well as testicular microvasculature. To try to dissociate the direct effects of Doxa treatment from paracrine and/or autocrine, we followed TIF volume and androgen content in TIF, testicular tissue, and what is produced in medium by testicular preparation and isolated Leydig cells. Results showed that a single Doxa treatment significantly decreased TIF volume compared with 1×IMO, increased it in 2×Doxa+2×IMO vs. 2×IMO group, but dramatically reduced it in the 10×Doxa+10×IMO group compared with the corresponding stress group. The androgen level in TIF and extracted from testicular tissue mirrored Doxa’s effect on TIF volume. Some small discrepancy between androgen levels in TIF/testicular tissue and circulation could be explained, at least in part, by reduced LH in the 1×Doxa+1×IMO group. Additionally, Doxa could exert important vascular effects (16) including the change of blood flow in the testes and affect TIF vascular perfusion (73). This phenomenon would decrease the amount of LH delivered to Leydig cells and impair entrance of androgens from TIF to the testicular microvasculature by restriction in movement of testosterone across the endothelial barrier (62), especially since the rat TIF contains mediators of vasopermeability (74). The picture about in vivo Doxa+IMO effects could be even more complicated, since testicular blood flow exhibits vasomotion, the process sensitive to catecholamines (11, 12). Numerous papers (63–67, 78, 79) showed that measurement of the volume of TIF and the rates of its formation (i.e., capillary permeability) and clearance are valid parameters of testicular vasculature, because not only does this control the rate of transport of LH and other compound to the Leydig cells, but it may also affect the concentration of testosterone in TIF by altering the volume of fluid.
into which the androgen is secreted. Additionally, it is possible that in vivo Doxa application could affect the metabolic rate of testosterone, since Doxa affected vasculature, metabolism, and hepatic vascular resistance (16). Accordingly, all mentioned could be the explanation for the discrepancy between testicular and circulating androgens. Ex vivo production of androgens by testicular preparation showed that only sustained \( \alpha_1 \)-ADR blockade diminished \( 10 \times \text{IMO} \)-induced recovery of androgen production in basal as well as when testes were challenged with hCG to estimate steroidogenic capacity. This result is consistent with testosterone levels in serum, TIF, and testicular tissue extract. Although testosterone is the exclusive product of Leydig cells, secretory products originating from seminiferous tubules and/or Sertoli cells and/or nonsteroidogenic cells in the testicular interstitium (25, 34, 59) could take place in the regulation of TIF characteristics as well as the level of androgens produced ex vivo by primary testicular culture isolated from all rats. In summary, reported changes in TIF and testosterone content in testis could be the consequence of the complex effects of Doxa on microvasculature and/or on secretory contribution of the different cell types, including besides HSD3B-positive Leydig cells, also macrophages, endothelial cells, fibroblasts, peritubular cells, Sertoli cells, etc. Accordingly, as the next step, our study focused on exclusive producers of testosterone, primary culture of purified Leydig cells.

The functionality of purified Leydig cells isolated from control, IMO, or Doxa + IMO rats was investigated by following the ex vivo production of testosterone and cAMP (the main regulator of steroidogenesis), as well as transcript for \( \text{Ins}3 \) (the main marker of Leydig cells). \( \text{INSL3} \) is a major secretory product of testicular Leydig cells, and, although released in a constitutive manner, it is considered in pair with androgens in the evaluation of testicular function and dysfunction (26). Along with androgens, \( \text{Ins}3 \) transcription was also attenuated after \( 1 \times \text{IMO} \) and \( 2 \times \text{IMO} \) but was normalized after \( 10 \times \text{IMO} \) and remained unchanged in Leydig cells from all groups that received Doxa before stress. The most important finding presented in this study is that blockade of \( \alpha_1 \)-ADRs partially prevented IMO-induced decrease in ex vivo androgen production in Leydig cells from the \( 1 \times \text{Doxa} + 1 \times \text{IMO} \) group, whereas complete prevention was registered in the \( 2 \times \text{Doxa} + 2 \times \text{IMO} \) and \( 10 \times \text{Doxa} + 10 \times \text{IMO} \) groups. The IMO-induced reduction of cAMP in Leydig cell content was partially prevented in all groups that received Doxa before stress compared with the corresponding stress group. The effect on cAMP content could be explained, at least in part, by published findings showing increased cAMP in Leydig cells of rats treated in vivo with Doxa (73). In addition, it has been shown that prazosin, another \( \alpha_1 \)-blocker very similar to Doxa, induced marked inhibition of PDE activity in aorta, thus causing an increase in cAMP levels (13). In accord with this, ex vivo treatment of Leydig cells from undisturbed rats with prazosin (10 \( \mu \text{M} \)) increased the cAMP level about threefold (Stojkov NJ, Drljaca DM, Bjeletic MM, Baburski, AZ, Mihajlovic AL, Sokanovic SJ, Janjic MM, Kotic TS, Andric SA unpublished results). Accordingly, restored androgen production in Leydig cells from Doxa + IMO rats could be at least partly explained by cAMP. The discrepancy between levels of androgens in serum, TIF, testes, and that produced ex vivo by Leydig cells could be explained by the fact that isolated Leydig cells are removed from the biologically active inhibitory and/or stimulatory endocrine signals and/or paracrine compounds released from the seminiferous tubules and/or macrophages, endothelial cells, fibroblasts, peritubular cells, and Sertoli cells (25, 34, 59). Since blockade of \( \alpha_1 \)-ADRs before/during IMO recovered androgenesis, an expression analysis of elements involved and/or related to steroidogenic function of Leydig cells was performed.

Results of this study show that IMO-reduced transcription for the most sensitive components of steroidogenic machinery in Leydig cells, the key CYP enzymes (\( \text{Cypl1a1, Cypl7a1} \)), was prevented by sustained \( \alpha_1 \)-ADR blockade. A possible explanation for the restored CYP expression could be, at least in part, partial cAMP recovery, increased transcription of \( \text{Crebl1} \) and \( \text{Nur77} \), the main steroidogenic stimulators (33, 40, 45, 52, 68), and decreased transcription of \( \text{Dax1} \) and \( \text{Arr19} \), the main steroidogenic repressors (27, 33, 40, 54). Additionally, increased transcription of PGC-1\( \alpha \), the SIRT3 activator (35) could be involved in recovery of CYP expression, since it was shown recently that SIRT3 and/or SIRT5 are novel positive regulators of CYP11A1 expression in adrenal cortex (41). Sustained in vivo \( \alpha_1 \)-ADR blockade also prevented a \( 10 \times \text{IMO} \)-induced decrease of \( \text{Hsd3b}1 \) transcript, the most abundantly expressed HSD3B in Leydig cells, while additionally stimulated IMO increased \( \text{Hsd3b}5 \) and \( \text{StAR} \). It is well known that regulation of steroidogenic gene transcription is quite complicated, multifactorial, tissue specific, and cAMP dependent/independent and includes a broad range of different transcription factors and activities/interactions (33, 40, 45, 52, 68, 69). It was demonstrated that optimal steroidogenic capacity is achieved if transcription pressure is applied on all steroidogenic genes (33, 40, 45, 69). In Leydig cells, \( \text{StAR, CYPs, and HSD3B} \), cAMP-regulated promoter activity primarily involves \( \text{Sf1, CREB/CREM, GATA4, and Nur77} \) and is modulated by other transcriptional cofactors as well as repressor such as DAX1 and ARR19 (33, 40, 45, 52, 68, 69).

Here, we show that in vivo \( \alpha_1 \)-ADR blockade increased transcription of \( \text{Crebl1} \) and prevented IMO reduction of \( \text{Nur77} \). CREB could be activated by an increase in the concentration of either calcium or cAMP (2), phosphorylated by a variety of kinases in response to mitogens and stress-dependent signals, and it is a very important regulator of steroidogenic gene expression, including \( \text{StAR, CYPs, HSD3B, Nur77, and GATA4} \) (14, 44, 75). Our results showed parallelism between increased \( \text{Crebl1} \) transcription and recovery of IMO-reduced \( \text{Nur77} \) transcription in Leydig cells from Doxa + IMO rats. This could be explained by findings showing PKA-CREB-mediated induction of NGFI-B/Nur77 gene expression in the mouse tumor Leydig cell line MA-10 (28). Another possible explanation for restored \( \text{Nur77} \) could be the high level of circulating epinephrine and enhanced activation of \( \beta \)-ADRs, since \( \alpha_1 \)-ADRs are blocked by Doxa. Also, it has been shown that the \( \beta \)-ADR-cAMP-PKA pathway is the most potent activator of \( \text{Nur77} \) expression in white (19) and brown (38) adipose tissue, as well as in muscle (46, 53). We were not able to detect significant changes in \( \text{Gata4} \) transcripts, although it is well known that \( \text{Gata4} \) is a downstream effector of recruitment of the cAMP-PKA-CREB pathway (75). The same applies for \( \text{Sf1} \), an essential steroidogenic stimulator (33, 40) negatively regulated with DAX1 (43), suggesting that other transcription factors/signaling pathways are more affected, including the "so-called" antisteroidogenic factors DAX1 and ARR19. The important finding present in this study is that the sustained \( \alpha_1 \)-ADR blockade partially diminished the IMO-induced in-
crease of Dax1 and Arr19, the main repressors of steroidogenic genes (27). One possible explanation for the decreased transcription in Leydig cells of 10×Doxa+10×IMO rats could be that PI3K/Akt signaling, described as a positive regulator of DAX1 expression (48), is less active due to in vivo α1-ADR blockade. Reduction of IMO-induced increase in Arr19 transcript in Leydig cells could be explained by increased cAMP in Doxa+IMO groups compared with the corresponding IMO group, since ARR19 gene expression in testicular Leydig cells is negatively regulated by LH-cAMP signaling (54). ARR19 acts as an AR co-activator in vivo and suppresses Nur77-induced promoter activity of steroidogenic enzyme genes, including StAR, CYP17A1, and HSD3B, in Leydig cells (54), so the decreased level of Arr19 in Leydig cells of 10×Doxa+10×IMO rats could be one more reason for the recovery of CYPs and the increase of StAR. In summary, increases of CYPs/Hsd3b1, StAR, and cAMP positively correlate with transcripts for stimulators (Creb1, Nur77) on the one hand and negatively correlate with repressors (Dax1/Arr19) on the other hand, supporting the hypothesis that the complete prevention of ex vivo testosterone production in Leydig cells of 10×Doxa+10×IMO rats is, at least in part, a consequence of stimulated transcription of steroidogenic machinery elements (Fig. 7).

This study has shown that sustained in vivo α1-ADR blockade was not able to change the IMO-elicited increase in the level of transcript for ADRs and Adrbk1 in Leydig cells. Moreover, the transcription of the most abundantly expressed ADRs (Adrb1, Adrb2) in Leydig cells was significantly stimulated in 10×Doxa+10×IMO compared with the 10×IMO group. According to our best knowledge there is not much evidence on the transcription for ADRs and was not able to change the IMO-elicited increase in the level of CYP17A1, CYP11A1, and HSD3B, in Leydig cells (54), so the decreased level of Arr19 in Leydig cells of 10×Doxa+10×IMO rats could be one more reason for the recovery of CYPs and the increase of StAR. In summary, increases of CYPs/Hsd3b1, StAR, and cAMP positively correlate with transcripts for stimulators (Creb1, Nur77) on the one hand and negatively correlate with repressors (Dax1/Arr19) on the other hand, supporting the hypothesis that the complete prevention of ex vivo testosterone production in Leydig cells of 10×Doxa+10×IMO rats is, at least in part, a consequence of stimulated transcription of steroidogenic machinery elements (Fig. 7).

In conclusion, the results obtained in this study support the important role of cross-talk from α1-ADRs and the steroidogenic machinery of Leydig cells from stressed rats and might provide new insights into the relationship between stress and α1-ADRs in mammalian reproductive function. The data presented provide a new molecular/transcriptional basis for “fight/adaptation” of steroidogenic cells (Fig. 7) and new molecular insights into the role of α1-ADRs in stress-impaired Leydig cells. This study has shown that sustained in vivo α1-ADR blockade was not able to change the IMO-elicited increase in the level of transcript for ADRs and Adrbk1 in Leydig cells. Moreover, the transcription of the most abundantly expressed ADRs (Adrb1, Adrb2) in Leydig cells was significantly stimulated in 10×Doxa+10×IMO compared with the 10×IMO group. According to our best knowledge there is not much evidence on the transcription for ADRs and was not able to change the IMO-elicited increase in the level of CYP17A1, CYP11A1, and HSD3B, in Leydig cells (54), so the decreased level of Arr19 in Leydig cells of 10×Doxa+10×IMO rats could be one more reason for the recovery of CYPs and the increase of StAR. In summary, increases of CYPs/Hsd3b1, StAR, and cAMP positively correlate with transcripts for stimulators (Creb1, Nur77) on the one hand and negatively correlate with repressors (Dax1/Arr19) on the other hand, supporting the hypothesis that the complete prevention of ex vivo testosterone production in Leydig cells of 10×Doxa+10×IMO rats is, at least in part, a consequence of stimulated transcription of steroidogenic machinery elements (Fig. 7).

In conclusion, the results obtained in this study support the important role of cross-talk from α1-ADRs and the steroidogenic machinery of Leydig cells from stressed rats and might provide new insights into the relationship between stress and α1-ADRs in mammalian reproductive function. The data presented provide a new molecular/transcriptional basis for “fight/adaptation” of steroidogenic cells (Fig. 7) and new molecular insights into the role of α1-ADRs in stress-impaired Leydig cells.

![Fig. 7. Effects of in vivo α1-ADR blockade on stress-disturbed Leydig cell homeostasis.](http://ajpendo.physiology.org/)
cell steroidogenesis. The results could be also important in terms of wider use of \( \alpha_1 \)-ADR selective antagonists alone to treat nightmares associated with posttraumatic stress disorder and hypertension, or in combination to treat benign prostatic hyperplasia, the lower urinary tract symptoms, as well as disrupted sexual health, including erectile and ejaculatory dysfunctions. Eventually, this study could be the solid basis for updating and reevaluating doxazosin clinical and pharmacogenomic data in human reproductive health risk assessment.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Kostic TS, Stojkov NJ, Janjic MM, Andric SA.

Klinefelter GR, Hall PF, Ewing LL.

E204 DOXAZOSIN MITIGATES STRESS-DISTURBED LEYDIG CELL STEROIDOGENESIS

Kostic TS, Stojkov NJ, Janjic MM, Andric SA.

Ma S, Morilak DA.

Li D, Dammer EB, Sewer MB.

Manna PR, Dyson MT, Jo Y, Stocco DM.

Martinez-Arguelles DB, Papadopoulos V.

Mayerhofer A, Bartke A, Began T.

Papadopoulos V, Miller WL.

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