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Stress triggers mitochondrial biogenesis to preserve steroidogenesis in Leydig cells

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ABSTRACT

Adaptability to stress is a fundamental prerequisite for survival. Mitochondria are a key component of the stress response in all cells. For steroid-hormones-producing cells, including also Leydig cells of testes, the mitochondria are a key control point for the steroid biosynthesis and regulation. However, the mitochondrial biogenesis in steroidogenic cells has never been explored. Here we show that increased mitochondrial biogenesis is the adaptive response of testosterone-producing Leydig cells from stressed rats. All markers of mitochondrial biogenesis together with transcription factors and related kinases are up-regulated in Leydig cells from rats exposed to repeated psychophysical stress. This is followed with increased mitochondrial mass. The expression of PGC1, master regulator of mitochondrial biogenesis and integrator of environmental signals, is stimulated by cAMP-PRKA, cGMP, and β-adrenergic receptors. Accordingly, stress-triggered mitochondrial biogenesis represents an adaptive mechanism and does not only correlate with but also is an essential for testosterone production, being both events depend on the same regulators. Here we propose that all events induced by acute stress, the most common stress in human society, provoke adaptive response of testosterone-producing Leydig cells and activate PGC1, a protein required to make new mitochondria but also protector against the oxidative damage. Given the importance of mitochondria for steroid hormones production and stress response, as well as the role of steroid hormones in stress response and metabolic syndrome, we anticipate our result to be a starting point for more investigations since stress is a constant factor in life and has become one of the most significant health problems in modern societies.

1. Introduction

Stress was introduced and popularized as a medical and scientific idea by Selye: "Without stress, there would be no life" [1,2]. "Adaptability and resistance to stress are fundamental prerequisites for life, and every vital organ and function participates in them" [1,2]. Nowadays stress is established as a unifying concept to understand the interaction of organism with the environment and occurs when homeostasis is threatened or perceived to be so [3]. During stress, an orchestrated adaptive compensatory specific response of the organism (so-called "fight and flight" response) is activated to sustain homeostasis. This includes activation of the sympathoadrenal, the sympathoneuronal systems, and the hypothalamo–pituitary–adrenocortical (HPA) axis, but suppression of hypothalamic–pituitary–gonadal (HPG) axis. The main hallmarks of stress are increased levels of circulating stress mediators/hormones, including corticotropin-releasing hormone (CRH), adrenocorticotropin (ACTH), glucocorticoids (GCs), and the catecholamines (adrenaline, noradrenaline) [3–9] as well as decrease of circulating testosterone in males [10–16]. It is well recognized that stress is a major contributor to the wide variety of psychosocial and pathological conditions in humans [3,4,9].

At cellular level, stressors are able to cause deleterious effects on cellular infrastructure and to disturb cellular homeostasis. As a consequence, organisms have developed the capacity to initiate a number of adaptive cellular response pathways that attempt to reduce damage and maintain or reestablish cellular homeostasis. Many aspects are not stressor specific because cells monitor stress based on macromolecular damage regardless of the type of stress that causes such damage [3,7]. Epidemiological studies strongly indicate that stress-induced DNA damage may promote ageing, tumorigenesis, neuropsychiatric conditions and that a stress response pathway regulates DNA damage through β2-adrenergic receptors (β2-ADRs) [17]. For all our cells, mitochondria are a key component of the stress response since they are primarily responsible for meeting the enormous energy demands of the "fight

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and flight response" by oxidizing the large amounts of substrates that are made available by stress hormone-induced mobilization from energy storages [3,7].

Specifically for steroid-hormones-producing cells, such as testosterone-producing Leydig cells of testes, the mitochondria are a key control point for the steroid biosynthesis and regulation [18–25]. The key enzymes of the steroidogenic machinery which are located in mitochondria, together with mitochondrial protein-import systems, enable steroidogenic cells to initiate and terminate massive levels of steroidogenesis within a few minutes, permitting the rapid regulation of circulating steroid hormone concentrations [19]. In addition, energized, polarized, and actively respiring mitochondria are required for acute Leydig cell steroidogenesis [18,20,21]. The complex mitochondrial protein-import machinery [26] and mitochondrial fusion [27,28] are essential for steroid biosynthesis. Despite crucial importance of mitochondria for steroid hormones production, there is no published evidence about mitochondrial biogenesis in any steroid-producing cell. Mitochondrial biogenesis is a complex sophisticated and multistep interplay of cellular and molecular processes that cells use to renew, adapt, or expand its mitochondrial population during episodes of damage or periods of intensified energy demand [29–35]. The spatio-temporal regulation of mitochondrial biogenesis is achieved by the nucleo-mitochondrial interactions dependent on the interplay between transcription factors (NRF1, NRF2, PPARs, ERRα, CREB, and others) and members of the PGC1 family of regulated coactivators (PGC1α, PGC1β, PRC). Consequently, many genes that specify the respiratory chain, the mitochondrial transcription–translation–replication machinery, and protein import-assembly apparatus are activated [29–38]. These processes are controlled by an array of signaling pathways [29–38] which convey environmental signals including temperature [29–37,39,40], energy deprivation [35,41,42], availability of nutrients [44,44,45], and growth factors [46].

Here we hypothesize that the stress alters the signaling pathways and molecules responsible for mitochondrial biogenesis in testosterone-producing Leydig cells with consequences for the mitochondrial-located-steroid-biosynthesis machinery and testosterone production. The immobilization stress (IMO) was chosen as a typical and frequently used model of psychophysiological stress [6,10–13,15,16]. The IMO sessions, established and justified before [10–13,15], include the acute (1 × IMO) and repeated stress without (2 × IMO) and with (10 ×IMO) partial recovery of circulating testosterone levels [15,16,47]. The focus of present study was on the effect of stress on mitochondrial biogenesis and on potential signaling pathways responsible for regulation of this process in testosterone-producing Leydig cells. Specifically, we studied serum hormonal profiles, Leydig cells functionality, as well as mitochondrial biogenesis after IMO was applied once (1 × IMO), twice (2 × IMO), and 10 times (10 × IMO). Our results are the first to show the contribution of mitochondrial biogenesis in establishing the new adaptive homeostasis of steroid-producing cells during psychophysiological stress. All markers of mitochondrial biogenesis and related kinases are up-regulated in Leydig cells from rats exposed to 10 × IMO. In addition, we observed that mitochondrial biogenesis in Leydig cells depends on the cAMP-PRKA and cGMP signaling as well as activation of β-ADRs but not α1-ADRs. Accordingly, mitochondrial biogenesis does not only correlate with testosterone biosynthesis but also represents an essential step in testosterone production, since both events depend on the same regulators.

2. Methods

Most of the methods employed for the experiments included in the present study were previously reported by our group in more detail (for all references please see [12,13,15,16,47] as well as in *Supplemental Material and Methods*), and are outlined briefly here.

2.1. Animals and ethical issue

Three-month-old (250–270 g) male Wistar rats, bred and raised in the Animal Facility of the Faculty of Sciences, University of Novi Sad, Serbia, were used for 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 experimental protocols were approved (statement no. 01-201/3) by the local Ethical Committee on Animal Care and Use of the University of Novi Sad operating in accordance with the National Research Council publication *Guide for the Care and Use of Laboratory Animals* 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).

2.2. Model of psychophysical stress by immobilization

Rats were handled daily during a 3-week period of acclimation before experiments. Immobilization stress (IMO) was performed in the morning (from 08^{00} h to 10^{00} h) by the method of Kvetnansky [6] described previously by our group [12,13,15,16]. Rats were divided into the following groups consisting of 4 animals each: (i) control group—freely moving (unstressed, C) rats; (ii) 1 × IMO—rats subjected to IMO for 2 h; (iii) 2 × IMO—rats subjected to repeated 2 h IMO for 2 consecutive days; (iv) 10 × IMO—rats subjected to repeated 2 h IMO for 10 consecutive days. At the end of IMO period, all animals were quickly decapitated without anesthesia and trunk blood was collected. Individual serum samples were stored at −80 °C until they were assayed for adrenaline (ADR), corticosterone (CORT), LH, and androgen (testosterone + dihydrotestosterone, T + DHT) levels. All experiments were repeated five times.

2.3. Preparation of purified Leydig cell and ex vivo androgens (T + DHT) production

Purified Leydig cells were prepared as described previously by our group [12,13,15,16,47]. The proportion of Leydig cells present in culture was determined by staining for HSD3B activity and was found to be 95.3 ± 1.5%. The viability was tested by using the 0.2% Trypan blue dye exclusion test (Sigma Inc) which determined total cell counts and ensured that greater than 90% of the cells were viable. Purified Leydig cells obtained individually from 4 rats were plated (1×10^6 cells in 1 ml culture medium per dish) and placed in CO_2 incubator (34 °C) for 3 h to attach and recover. Three to five replicates of each pool/group were cultured for *ex vivo* secretion and expression analysis. After 2 h, cell-free media was collected and stored at −80 °C prior to the measurement of cAMP and androgens levels. In some experiments cAMP and androgens were also measured in cell content. For monitoring of mitochondrial membrane potential ($\Delta\Psi_m$), Leydig cells were plated (1×10^5 cells in 0.2 ml culture medium per well) in black 96-well plates. For quantitative monitoring of mitochondrial biogenesis with MitoTracker-Green-FM, Leydig cells were plated (0.5×10^5 cells in 0.2 ml culture medium per well) in black 96-well plates. For fluorescence imaging with MitoTracker-Green-FM, Leydig cells were plated (2×10^6 in 2 ml culture medium in 35 × 10 mm Petri dishes) or were incubated in suspension (0.5×10^6 cells in 500 μl culture medium per test tube).

2.4. In vitro experiments—treatment of Leydig cells isolated from undisturbed rats

In order to obtain information about the role of LHCGR-cAMP-PRKA signaling (the main regulator of steroidogenesis [48–50], NO-cGMP signaling (known activator of mitochondrial biogenesis [37,38], and regulator of steroidogenesis [13]), β-ADRs (known stress mediators [3,7,8], activators of mitochondrial biogenesis [29,51] and regulators of steroidogenesis [15] and α1-ADRs (known stress mediators [3,7,8]

involved in Leydig cells apoptosis [47] in regulation of PGC1 expression and mitochondrial biogenesis, *in vitro* experiments were performed using the primary cultures of purified Leydig cells isolated from undisturbed rats. Leydig cells were incubated for 24 h without (Basal) or with different chemicals to activate different signaling pathways including hCG (50 ng/ml) alone or in the combination with PRKA inhibitor H89 (10 μM), NO donor DPTA (10 μM) alone or in the combination with GUCY1 inhibitor NS2028 (10 μM), adrenaline (AD, 1 μM) alone or in the combination with β-ADRs blocker propranolol (PROP, 1 μM) or α1-ADRs blocker prazosin (PRA, 1 μM). All *in vitro* experiments were repeated for three times.

2.5. Hormones, cAMP/cGMP, and nitrite 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 has been assayed at 0.14 ng/ml [12,13,15,16,47]. Levels of **androgens** in serum, medium, or extracts are referred to as T + DHT, because the antitestosterone serum №250 showed 100% cross-reactivity with DHT [12,13,15,16,47]. 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 [15,16,47], all samples were measured in duplicate, in one assay by the corticosterone EIA Kit (www.caymanchem.com) with 30 pg/ml as the lowest standard significantly different from blank. Serum **adrenaline** levels [15,16] 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 cell content of scraped purified Leydig cells were measured by using a cAMP EIA Kit (www.caymanchem.com) that permits cAMP measurement with a quantification limit of 0.1 pmol/ml as the lowest standard significantly different from blank [12,15,16]. 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 the lowest standard significantly different from blank [13]. The measurement of **nitrite** levels, the stabile metabolic product of NO was perform with Griess reagent as described previously by our group [11,13].

2.6. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

To monitor mitochondrial membrane potential ($\Delta\Psi_m$), Leydig cells were loaded with TMRE as described previously [18,47].

2.7. Quantitative MitoTracker-Green-FM assay and fluorescence imaging with MitoTracker-Green-FM

The MitoTracker-Green-FM dye, which has high affinity and specificity for lipid membranes in mitochondria and produces monofluorescent green images, was employed to examine mitochondrial mass. MitoTracker-Green-FM is green-fluorescent selective mitochondria-specific fluorophore which appears to localize to mitochondria regardless of mitochondrial membrane potential. The dye will stain live cells but is not well retained after aldehyde fixation. To determine if there is an apparent case for mitochondria biogenesis, the Leydig cells were loaded with MitoTracker-Green-FM fluorescent dye (#7514) following a protocol recommended by the Molecular Probes (www.lifetechnologies.com/order/catalog/product/M7514) described previously [37].

For quantitative fluorescence measurement, purified Leydig cells were plated in black 96-well plate (0.5×10^5 cells per well) in eight replicates for each group. At the end of treatment, the medium was discarded and cells were loaded with 200 nM MitoTracker-Green-FM in M199 at 34 °C for 20 min. Loading buffer was changed with PBS-0.1%BSA and fluorescence readings were recorded using a fluorimeter (Fluoroskan, Ascent FL, Thermo Labsystems; Waltham, MA,) with an excitation wavelength of 490 nm and an emission wavelength of 516 nm.

For accurate fluorescence imaging and relative intensity of fluorescence (RIF) quantification, purified Leydig cells isolated from control or stressed rats were incubated with 200 nM MitoTracker-Green-FM for 20 min in plate (2×10^6 in 2 ml culture medium in 35 mm Petri dishes) as well as in suspension (0.5×10^6 cells in 500 μl culture medium per test tube). Leydig cells were plated in 5 plates for each groups and placed in CO_2 incubator (34 °C) for 2 h to attach and recover. After that, cells were treated with 200 nM MitoTracker-Green-FM for 20 min. At the end of incubation, cells were washed with 1 × PBS and imaging was performed in the same buffer. Leydig cells in suspension were incubated with 200 nM MitoTracker-Green-FM green fluorescent dye in culture medium for 20 min at 34 °C (water bath). After that, suspension was centrifuged (500 *g* for 5 min) and the pellet was washed with 1 × PBS. Subsequently, the precipitate was resuspended in 50 μl 1 × PBS and placed on glass microscope slides (8 slides per experimental group). Relative fluorescence imaging and analysis of MitoTracker-Green-FM fluorescence over mitochondrial regions was carried out as described previously [52] with some modifications. Fluorescence staining was detected by *Axio ImagerA2* (#038-16035, Zeiss) fluorescence microscope using 490 nm excitation and 516 nm emission filter. Ten images were acquired per Petri dish as well as slide using both ×40 and ×63-objective. Relative quantification of fluorescence intensity was done by fitting a region of interests and background near to this region using drawing/selection tools of *Image J* (NIH) after conversion of all images to grayscale in *Corel PHOTO – PAINT X5*. Relative fluorescence intensity was calculated for 100 cells per experimental group using the formula: RIF = ID – (CA × MB), where RIF is relative intensity of fluorescence, ID is integrated density, CA is area of selected cell and MB is mean fluorescence of background readings.

2.8. Transmission electron microscopy of Leydig cells

The suspensions of Leydig cells obtained from control and stressed rats were centrifuged and pellets placed in Agar blocs fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. After fixation, the cells were postfixed in 1% osmium tetroxide in the same buffer, dehydrated in a series of ethanol, and then embedded in Araldite (Fluka, Germany). Thin sections were cut with a diamond knife (Diatome, Switzerland) on a Leica UC6 (Leica Microsystems, Germany), mounted on a copper grids, contrasted in uranyl acetate and lead citrate using Leica EM STAIN (Leica Microsystems), and viewed with an transmission electron microscope (Philips/FEI, The Netherlands) equipped with iTEMMegaView CCD. To quantify the mitochondria, for each group, 10 micrographs taken from 10 Leydig cells from four rats from each group were counted in blinded fashion.

2.9. Immunocytochemistry visualized by fluorescence microscopy

The suspension of Leydig cells in culture medium (100.000 cells) was centrifuged at 500 *g* for 5 min. Supernatant was discarded and pellet was resuspended in 1xPBS and spread on glass microscope slide. Cells were fixed for 10 min with 4% paraformaldehyde. Samples were enlightened during incubation with xylene (3 times for 10 min) and were dehydrated using decreasing concentrations of ethanol (100%–30%). The antigen retrieval step in 10 mM citrate buffer for 20 min, under boiling temperature, was performed. After permeabilization with 0.1 % Triton X − 100 for 20 min, nonspecific antibody binding was prevented by incubation with 3% BSA 0.1% Tween in 1× TBS for 45 min at room temperature. Slides with cells (5 slides for each antibody per group) were overflowed with rabbit anti-PGC1, or anti-TFAM, or anti-ERRα antibody (1:150 in blocking solution, Santa Cruz Biotechnology) and incubated over night at 4 °C. Staining was revealed by using donkey anti-rabbit IgG conjugated with AlexaFluor 488 fluorescence dye (1:400 in blocking buffer). NucBlue™Fixed Cell Stain (#R37606,

Molecular Probes by Life Technologies) for 2 min at room temperature was used for nuclear counterstaining. Post-fixation was performed with 4% paraformaldehyde for 10 min. Immunofluorescence was detected by *Axio ImagerA2* (#038-16035, Zeiss) fluorescence microscope using 490 nm excitation and 516 nm emission filter and ×40 as well as x63 objective. We also tried another way of image acquisition and postprocessing. Immunofluorescence staining images were acquired using a Deltavision Core wide field deconvolution fluorescence microscope (Applied Precision) using an Olympus UPlanSApo40×, air lens. Fluorescence images were taken with a Photometrics Cool Snap HQ2 charge-coupled device (CCD) camera using emission filters for DAPI 435/48 and FITC 540/65. Z-stacks for both channels (DAPI and FITC) comprised of single scans taken at 0.4 μm were acquired using Resolve3D SoftWoRx-Acquire version 4.1.2 release 1 (Applied Precision) with fast acquire settings. The images were then processed using Fiji software, creating maximum intensity projection of z-stacks in focus for each channel acquired separately.

2.10. RNA isolation and cDNA synthesis

Total RNA from purified rat Leydig cells was isolated using RNeasy kit reagent following the protocol recommended by the manufacturer (www.qiagen.com). Following DNase-I treatment, the first strand cDNA was synthesized according to the manufacturer's instructions (www.invitrogen.com). Quality of RNA and DNA integrity were checked using control primers for *Gapdh* as described previously [12,13,15,16,47].

2.11. Real-time polymerase chain reaction and relative quantification

Relative expression of the genes was quantified by PCR using SYBR®Green-based chemistry from Applied Biosystems (www.appliedbiosystems.com) in the presence of specific primers (please see Supplemental Table 1). *Gapdh* was also measured in the same samples and was used to correct for variations in RNA content between samples. Relative quantification of each gene was performed in duplicate, three times for each gene and twice for each of three independent *in vivo* experiments, as described previously by our group [12,13,15,16,47].

2.12. Protein extraction and Western blot analysis

Western blot analysis was performed as described previously by our group [12,13,15,16,47]. Antibodies against PGC1 (sc-13067, sc-5816), NRF1 (sc-33771), ERRα (sc-66882), AMPKα1/2 (sc-74461), mtTFA/TFAM (sc-28200), TFB1M (sc-169583), TFB2M (sc-160858), COX4 (sc-69361) and ACTIN (sc-1616) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), antibodies against pCREB (#9198), CYTC (#4272), pERK1/2 (#9102S), and p38MAPK (#9212S) were from Cell Signaling Technology Inc (Boston, MA, USA), antibody against PRKAc (#610980) was from BS Transduction Laboratories, while antibody against PRKG1 (#370661) was from Calbiochem. The immunoreactive bands were analyzed as two-dimensional images using Image J (version 1.48; http://rsbweb.nih.gov/ij/download.html). The integrated optical density of images was used to quantify both area and the intensity of the immunoreactive bands (version 1.48; http://rsbweb.nih.gov/ij/download.html). For details please see Supplemental Table 2 in Supplemental Material and Methods.

2.13. Statistical analysis

Results represent group mean ± SEM values of individual variation from five independent experiments. Results from each experiment were analyzed by Mann–Whitney's unpaired nonparametric 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 the program GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA).

3. Results

To mimic psychophysiological stress, the most common stress in human society, adult male rats were exposed to the well-established model of immobilization stress (IMO) [12,13,15,16,47].

The effects of the acute as well as repeated IMO treatments were confirmed by measurement of the concentrations of stress hormones (corticosterone and adrenaline) as well as androgens (T + DHT) and LH (the main regulator of steroidogenesis) in serum obtained from undisturbed controls and stressed animals. In agreement with previous studies [12–16], IMO was effective as a stressor (Supp. Fig. 1), elevating serum adrenaline (A) and CORT (B) levels in all stressed groups compared to undisturbed controls. Serum LH was reduced in rats exposed to repeated IMO (C), while circulating androgens (T + DHT) were reduced in all stressed rats, but a partial recovery was observed in serum from 10 × IMO group (D).

Analysis of biochemical parameters in serum showed that IMO significantly affected most of the biochemical parameters: 12 out of 13 parameters were changed (Supp. Fig. 2). As expected, all types of IMO significantly increased (3–3.5 times compared to the control) level of glucose in serum. In agreement with literature [53], activities of serum transaminases (ALT and AST) were significantly increased in rats exposed to acute (1 × IMO) and repeated 2 × IMO. In the same groups, activity of GGT in serum declined. ALP activity decreased in serum of rats exposed to both types of repeated IMO. Protein levels remained unchanged. Only 1 × IMO increased level of urea in serum, while all types of IMO significantly increased level of uric acid in serum. Level of cholesterol increased in serum from rats exposed to 2 × IMO, while both types of repeated IMO (2 × IMO/10 × IMO) increased cholesterol-HDL about 5 times compared to the control. Levels of triglycerides in serum increased in rats exposed to acute (1 × IMO) and repeated 2 × IMO, while bilirubin and creatinine levels were increased only in rats exposed to acute stress (Supp. Fig. 2).

3.1. Ten times repeated stress restored Leydig cells functionality and increased mitochondrial mass

The functionality of isolated and purified Leydig cells obtained from control and stressed rats was examined by monitoring the ability of primary cultures of Leydig cells to produce androgens (T + DHT) *ex vivo*, as well as by measuring the changes in the level of transcripts for *Insl3* (a Leydig cell differentiation marker) and mitochondrial membrane potential ($\Delta\Psi_m$) and as expected and shown previously [47] levels of androgens produced by Leydig cells were reduced after 1 × IMO and 2 × IMO, but partial recovery was registered in Leydig cells from 10 × IMO rats (Fig. 1A). In parallel with $\Delta\Psi_m$, the level of *Insl3* transcript also decreased in Leydig cells from 1 × IMO and 2 × IMO groups, while restored in 10 × IMO group. A linear positive correlation (R = 0.95) between levels of *Insl3* and androgens was observed (Fig. 1B). Mitochondria are the control point for the regulation of steroid hormone biosynthesis because the rate-limiting step of the steroidogenic pathway takes place in these organelles [18–22]. Since only energized, polarized, and actively respiring mitochondria are required for acute Leydig cell steroidogenesis, it is important to examine mitochondrial membrane potential ($\Delta\Psi_m$) [18–22]. To detect changes in the electrochemical gradient, $\Delta\Psi_m$ was measured by quantification of TMRE fluorescence, because TMRE fluorescence values are proportional to the magnitude of $\Delta\Psi_m$ [18]. Obtained results indicated reduced $\Delta\Psi_m$ in Leydig cells derived from 1 × IMO and 2 × IMO, while $\Delta\Psi_m$ was restored in Leydig cells from 10 × IMO rats. A linear positive correlation (R = 0.94) between levels of $\Delta\Psi_m$ and androgens was observed (Fig. 1C). The MitoTracker-Green-FM dye, which has high affinity and specificity for lipid membranes in mitochondria and produces monofluorescent

green images, was employed to examine mitochondrial membrane morphology. Quantitative analysis of MitoTracker-Green fluorescence showed increased mitochondrial mass in Leydig cells from the 10 × IMO group (Fig. 1D).

In agreement with literature [15,16], results from RQ-PCR analysis confirmed reduced expression of *Cyp11a1* and *Cyp17a1* in Leydig cells of rats exposed to acute and both types of repeated IMO. The level of *Hsd3b1* transcript decreased, but *Hsd3b5* transcript increased in Leydig cells from 10 × IMO rats. All types of stress decreased *Hsd17b3* transcription in Leydig cells, while it did not change *Hsd17b4* (Supp. Fig. 3).

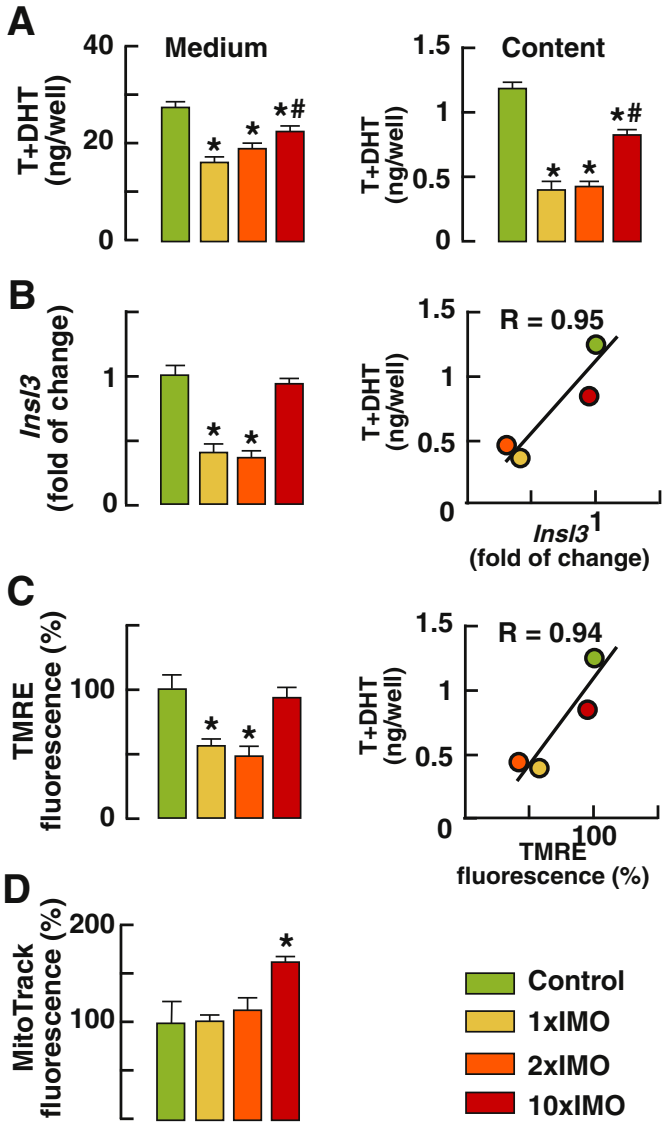


Fig. 1. Stress disturbed Leydig cells functionality and increased mitochondrial mass. Stress transiently reduced level of androgens (***A***), mitochondrial membrane potential (***B***), and transcription of *Insl3* in Leydig cells (***C***). Please notice recovery in the 10 × IMO group compared to the 1 × IMO group and positive correlation between androgens production and mitochondrial membrane potential or *Insl3* transcript, as well as increased mitochondrial mass (***D***). In this and following figures, rats were subjected to 1 × IMO, 2 × IMO, or 10 × IMO, or were left undisturbed. At the end of the IMO period, serum was collected for hormonal analysis. Leydig cells were isolated for secretion and expression analyses. Androgens (T + DHT) levels were measured by RIA, mitochondrial membrane potential by TMRE, *Insl3* was determined by RQ-PCR, while mitochondrial mass was detected by using MitoTracker Green and fluorimeter (for all details, please see *Supp. Methods*). Data bars are mean ± SEM values from five independent *in vivo* experiments. Statistical significance was set at level $P < 0.05$: *vs. control group; # vs. 1 × IMO group.

3.2. Acute and two times repeated stress alters mitochondrial cristae in Leydig cells, while ten times repeated stress increases number of mitochondria in Leydig cells

Because mitochondrial mass quantification by Mito-Tracker-Green-FM revealed increase of mitochondrial mass in Leydig cells from the 10 × IMO group (please see Fig. 1D) in order to determine by imaging if stress affects mitochondrial number in Leydig cells, transmission electron microscopy (TEM) and fluorescent microscopy was employed on Leydig cells isolated from control and stressed rats (Fig. 2). Representative TEM photomicrographs are shown in Fig. 2A and Supp. Fig. 4. Although in the present study, we planned to explore the mitochondrial biogenesis in stressed rats, unexpectedly, however, we found that stress disturbed mitochondrial architecture in Leydig cells. Most mitochondria in the Leydig cells from undisturbed rats (control) presented as bean-shaped structures with numerous transversely orientated cristae enveloped by an intact outer membrane (Fig. 2A and Supp. Fig. 4). In striking contrast, we observed a higher frequency of morphological alterations in Leydig cell mitochondria from acutely stressed rats (1 × IMO) and 2× repeatedly stressed rats (2 × IMO). Oppositely, recovery of mitochondrial cristae and clustering of mitochondria were evident in Leydig cells isolated from rats exposed to 10 × IMO. Higher magnification views of boxed regions revealed recovery of architecture, number of cristae, and clustering of mitochondrial (Fig. 2A and Supp. Fig. 4). Relative quantification of mitochondria revealed significant increase in the number of mitochondrial in Leydig cells isolated from rats exposed to 10 × IMO (Fig. 2A).

Representative images from fluorescence emission intensity from a primary culture of Leydig cells that were labeled with MitoTracker-Green targeting mitochondria and quantification of relative intensity of fluorescence (RIF) are shown in Fig. 2B and supported results obtained by MitoTracker-Green quantitative measurement. Namely, results revealed significant increase in MitoTrack-Green fluorescence emission in Leydig cells isolated from 10 × IMO rats comparing to control (Fig. 2B).

3.3. Stress increases expression of mitochondrial biogenesis markers and kinases related to steroidogenesis and PGC1 activation in Leydig cells

To determine whether mitochondrial biogenesis is involved in adaptive response of Leydig cells, we examined expression of transcripts and proteins for all mitochondrial biogenesis markers as well as for the main kinases involved in mitochondrial biogenesis. Results showed (Fig. 4) that stress induced a significant increase in the expression of PGC1, the master regulator involved in transcriptional control of all the processes related to mitochondrial homeostasis and integrator of environmental signals [29,41]. All types of IMO increased level of *Ppargc1b* transcript in Leydig cells, while *Ppargc1a* increased only after 10 × IMO (Fig. 4). In parallel, repeated stress increased expression of *Nrf1*/NRF1 and *Nrf2a*, PGC1-down-stream-targets that act on the genes for subunits of the oxidative phosphorylation (OXPHOS) encoded by nuclear genome [29,34,35]. In the same cells, increased expressions of TFAM (mitochondrial transcription factor A) and TFB2M (mitochondrial transcription factor B type 2), a downstream targets of both NRF1 and NRF2 were registered. In addition, increased expression of NRF1/NRF2-target CytC (cytochrome C) was detected in Leydig cells from the 10 × IMO group; while another NRF1/NRF2-target COX4 (mitochondrial complex IV cytochrome C oxidase) protein, and *Cox4i2* increased in Leydig cells from 1 × IMO and 10 × IMO groups. Only 10 × IMO treatment was able to increase transcript of *Pparb/d* and *Cox4i1* in Leydig cells, while *Ppara* and *Pparg* remained unchanged. In addition, repeated stress significantly increased transcription of *Ucp1* and *Ucp3*, the mediators of regulated proton leak and controllers of the production of superoxide and other downstream reactive oxygen species [54]. In parallel with PGC1/*Ppargc1b* expression, increased transcription of *Sirt1* (known activator of PGC1-mediated transcription of nuclear and mitochondrial

genes encoding for proteins promoting mitochondrial proliferation, OXPHOS, and energy production) [36,45] was registered in Leydig cells from stressed rats (Fig. 4A).

Given the central importance of many different kinases for activation of PGC1 [29,41] as well as activation of mitochondrial proteins [52,55] and steroidogenesis [48–50], we analyzed expression of kinases and some of the transcription factors related to mitochondrial biogenesis in Leydig cells isolated from control or stressed rats (Fig. 3B). Results showed that both types of repeated stress (2 × IMO, 10 × IMO) significantly increased the expression of proteins for catalitic PRKA subunit (PRKAc) and target-transcription factor pCREB, both well known as the essential regulators of steroidogenesis [48–50,56], as well as activators of PGC1 [29,41] and the biogenesis of OXPHOS [51]. In the same Leydig cells, a similar pattern was observed for pP38MAPK, also a well-known component of signalosome involved in regulation of steroid hormones biosynthesis [51,56] as well as critical actor in the mobilization of the transcriptional machinery that promotes mitochondrial proliferation and fatty acid oxidation [29,41]. In addition, same profile was observed in the expression of ERRα, another key partner for PGC1α, providing direct and indirect control of numerous genes involved in mitochondrial homeostasis [29,41], but also mediator of PGC1α-dependent functional induction of CYP11A1 and CYP17A1, steroidogenesis rate limiting enzymes in human hepatic cells [43]. In parallel, results indicated increased expression of protein for AMPK, the PGC1α activator [36] acting as an intracellular energy sensor activated by concomitant increase of AMP/ATP ratio, ROS levels, and oxidative stress [36]. Only 10 × IMO increased level of proteins for pERK1/2 and PRKG (Fig. 3B), also well-known steroidogenesis regulatory kinases [13,47] as well as stress-recovery kinases [47].

Given the importance and exclusivity of adrenal gland for steroid and stress hormone synthesis as well as the fact that hypothalamic–pituitary–adrenal axis is stimulated in stress response, we followed main markers of mitochondrial biogenesis in adrenal tissue from stressed rats. The opposite of testicular Leydig cells from stressed rats, dramatic reduction of *Ppargc1a*, *Ppargc1b*, *Tfam* transcription was registered in adrenal glands (Supplementary Fig. 6), indicating that the changes observed in Leydig cells represent tissue-specific response and adaptation to stress and perturbed conditions.

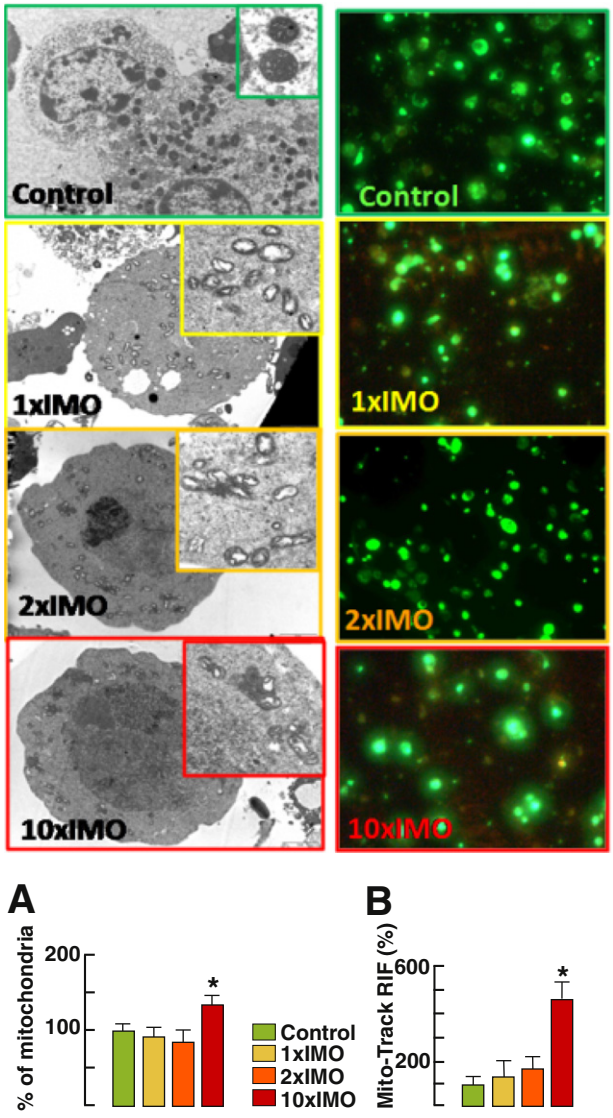


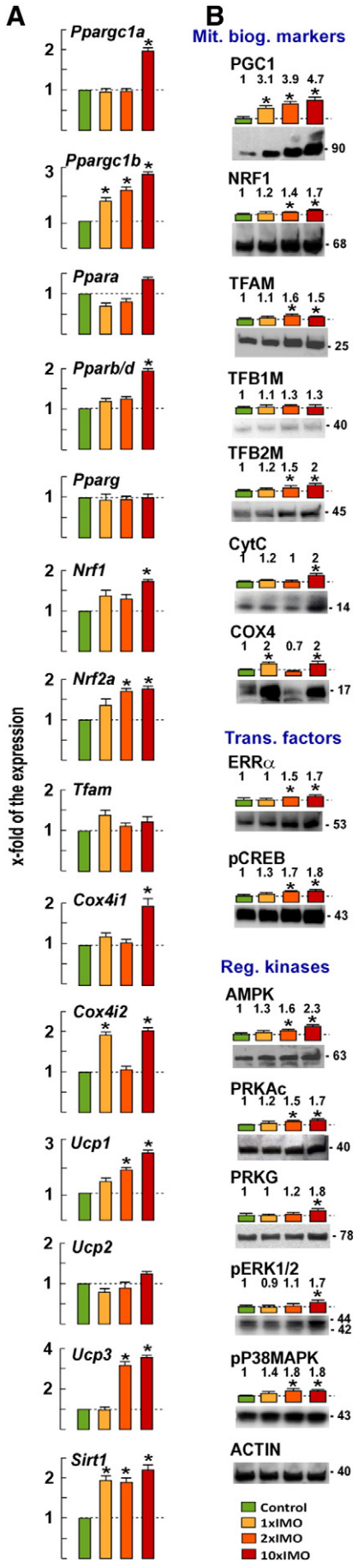
Fig. 2. Acute and two times repeated stress alters mitochondrial cristae in Leydig cells, while ten times repeated stress increases number of mitochondria in Leydig cells. *A:* Representative TEM photomicrographs (please see also Supp. Fig. 4) and relative quantification of mitochondria in Leydig cells from undisturbed (control) and stressed (IMO) rats. *B*: Representative images from fluorescence emission intensity from a primary culture of Leydig cells that were labeled with MitoTracker-Green-FM targeting mitochondria and quantification of relative intensity of fluorescence (RIF). In order to determine by imaging if stress affects mitochondrial number in Leydig cells, transmission electron microscopy (TEM) and fluorescent microscopy were employed on Leydig cells isolated from control and stressed rats. For detailed TEM and fluorescence microscopy methodology, please see the main text of the manuscript as well as *Supp. Material.* Data bars are mean ± SEM values from two independent *in vivo* experiments. Statistical significance was set at level $P < 0.05$: * vs. control group.

3.4. Mitochondrial biogenesis in Leydig cells depends on cAMP-PRKA, cGMP, and β-ADRs signaling

To further understand signaling pathways that regulate mitochondrial biogenesis and to try to get information about the role of LHCGR-cAMP-PRKA signaling (the main regulator of steroidogenesis [48–50] induced by stress [12,15], NO-cGMP signaling (known activator of mitochondrial biogenesis [37,38] and regulator of steroidogenesis [13]), β-ADRs (known stress mediators [6], activators of mitochondrial biogenesis [29,51], and regulators of steroidogenesis [16]) and α1-ADRs (involved in Leydig cells apoptosis [47]) in regulation of PGC1 expression, *in vitro* experiments were performed using the primary cultures of purified Leydig cells isolated from undisturbed rats. Results showed that hCG-dependent activation of LHCGR-cAMP signaling stimulates expression of PGC1 through mechanism involving PRKA (Fig. 4A), a known activator of steroidogenesis, as well as PGC1 and the biogenesis of OXPHOS. In the same cells, NO, a known activator of mitochondrial biogenesis, stimulates expression of PGC1 with mechanism involving GUCY1 (soluble guanylyl cyclase)—cGMP (Fig. 4B). Incubation of Leydig cells with adrenaline (AD), a well-known stress hormone, increased transcription of main markers of mitochondrial biogenesis including *Ppargc1a*, *Ppargc1b*, *Nrf1*, and *Nrf2*. Application of propranolol (PROP), a well-known nonselective β-ADRs-blocker, diminished this effect, suggesting involvement of β-ADRs in Leydig cells mitochondrial biogenesis (Fig. 4C). In contrary, the presence of prazosin (PRA), the selective α1-ADRs antagonist, in the combination with adrenaline did not change adrenaline-induced stimulation of *Ppargc1a*, *Ppargc1b*, *Nrf1*, and *Nrf2* transcription, while PRA alone significantly increased level of *Nrf2* transcript in Leydig cells (Fig. 4D).

4. Discussion

The “fight and flight” response is a universal mechanism of extraordinary physiological and pathophysiological significance [3,7–9]. The organism's response to stress is a complex, multifactorial process that involves an elaborate neuroendocrine, cellular, and molecular infrastructure [3,7]. Here we show, according to the best of our knowledge, for the first time, that increased mitochondrial biogenesis is the adaptive response of testosterone-producing Leydig cells from repeatedly stressed rats. Several lines of evidence prove that (Fig. 5) (i) all markers



of mitochondrial biogenesis together with transcription factors and related kinases are up-regulated in Leydig cells from rats exposed to repeated psychophysical stress; (ii) this is followed with increased number of mitochondria, and recovery of disrupted cristae; (iii) the expression of PGC1, the master regulator of mitochondrial biogenesis and integrator of environmental signals [33], is stimulated by cAMP-PRKA and cGMP signaling and β-ADRs, but not α1-ADRs. Altogether, our results demonstrate that stress triggers mitochondrial biogenesis in Leydig cells and that mitochondrial biogenesis does not only correlate with recovery of testosterone biosynthesis in stress (10 × IMO) but also represents an adaptive mechanism and essential step for testosterone production, being both events that depend on the same regulators. By this transcriptional signaling scenario, it is possible that the Leydig cell is trying to preserve basic steroidogenic activity. Physiological significance was proven by recovery of main markers of Leydig cell functionality (androgens production, $\Delta\Psi_m$ and *Insl3*).

Given the crucial role of mitochondria in cell physiology, it is obvious that these organelles are among the first responders to various stressors challenging homeostasis of the cell and organism [3,7]. Growing body of evidence indicates many changes of mitochondrial function in Leydig cells from stressed rats [10–13,15,47]. Acute IMO increased NO [11,13] and ROS [10,11] and disturbed activity of mitochondrial steroidogenic enzymes [12], antioxidative enzymes, and NADPH-P450 reductase [10] in Leydig cells. In parallel, reduced oxygen consumption and the mitochondrial membrane potential coupled with a leak of cytochrome C from mitochondria and GCs-induced mitochondria-dependent apoptosis of Leydig cells was also observed recently [47]. In contrast, repetitive stress, despite still elevated stress hormones, was accompanied with a partial recovery of androgen secretion in Leydig cells, coupled with recovery of oxygen consumption, abolition of mitochondria-related proapoptotic signaling, and normalization of the apoptotic events [47]. Disturbed steroidogenic enzymes activity [10–12] and expression [15] could cause electron leaking, affect the electron flow [10], favoring ROS [11] production and impairing ATP synthesis, which could further exacerbate the prooxidative environment and activate mitochondrial biogenesis. All events can activate mitochondrial biogenesis by means of retrograde signaling from damaged mitochondrion to the nucleus constituting a "mitochondria-specific stress response" [35].

Indeed, results of this study showed that transcription signature of all markers of mitochondrial biogenesis increased in Leydig cells of repeatedly stressed rats. This was accompanied by an increase in protein levels, up-regulation of steroidogenic kinases, and related transcription factors. As a consequence, the number of mitochondria increased. Here we propose that the conditional increase of NO/ROS [11,13], as well as decrease of oxygen consumption [47], and mitochondrial membrane potential and ATP levels (unpublished results of our group) induce divergent activation of several signaling pathways leading to mitochondrial biogenesis (Fig. 5). Activation of AMPK regulates the energy status by stimulating catabolic processes to increase ATP. Also, it initiates a bipartite response that activates PGC1 and enhances SIRT1 activity by consequent phosphorylation and activation of nuclear and cytosolic PGC1 and SIRT1 [36]. A complex of SIRT1–MyoD–PGC1α binds to the promoter of PGC1α, creating a positive feedback and auto-regulatory loop for PGC1α expression. PGC1α activity is increased by both SIRT1-dependent deacetylation

Fig. 3. Stress triggers stimulation of mitochondrial biogenesis signalosome in Leydig cells. Expression of transcripts (***A***) and proteins (***B***) for main mitochondrial biogenesis markers and related signaling pathways increased in Leydig cells from rats exposed to repeated IMO. Relative quantification of the transcription was determined by RQ-PCR and the specific primers (please see Supp. Table 1). Western blot was performed using the specific antibodies (please see Supp. Table 2).The representative blots are shown as panels, while pooled data from scanning densitometry normalized on ACTIN (internal control) values are shown as bars on the top of the blots. Normalized data shown are mean ± SEM from triplicate determination from each of five independent experiments (for all details, please see *Supp. Methods*). Data bars are mean ± SEM values from three to five independent *in vivo* experiments. Statistical significance was set at level $P < 0.05$: * vs. control group.

and AMPK-mediated phosphorylation. In the mitochondria, nucleus, and cytoplasm, PGC1α can be activated by SIRT1, leading respectively to the enhanced transcription of nuclear gene-encoding mitochondrial proteins or TFAM [36]. SIRT1 deacetylates and activates both members of PGC1α-ERRα, and in complexes with SIRT1, phosphorylated nuclear PGC1α can induce its own gene expression and also enhances expression of NRF1/NRF2 that in turn up-regulate the expression of nuclear gene-encoding mitochondrial proteins forming the subunits of the OXPHOS complexes. NRF1 and NRF2 also induce expression of TFAM, which is imported into mitochondrial sub-compartments. Within the mitochondrial matrix, TFAM binds to mtDNA and initiates the expression of mtDNA gene products encoding different subunits of OXPHOS enzyme complexes such as cytochrome C and COX4 [29,34,35]. All mentioned could lead to activation of OXPHOS enzyme complexes, promoting oxidative phosphorylation as well as recovery of oxygen consumption and ATP levels.

Stress-induced increase in the expression of steroidogenic-stimulatory kinases (PRKA, pERK1/2, pP38MAPK, PRKG) and sustained increase in circulating adrenaline levels can activate ADR-sensitive signaling pathways and target transcription factors as well as the coactivators PGC1α/β [29,34,35]. Activated transcription factors, such as CREB, NRF1, NRF2, ERRα, regulate the expression of nuclear gene-encoding mitochondrial proteins, as well as the transcription and mRNA expression of PGC1α/β. Once translated, PGC1α/β binds to transcription factors (including NRF1, NRF2, ERRα) to also regulate nuclear gene-encoding mitochondrial proteins. Following transcription and translation, the newly synthesized mitochondrially destined proteins are imported into the organelle via the translocase of the outer and inner membrane complexes and become incorporated into the OXPHOS enzyme complexes once imported into the organelle [29,34,35]. Stress-induced up-regulation of PRKA and pCREB in Leydig cell could be also involved in regulation of OXPHOS enzyme complexes since it was recently shown that PRKA, CREB, and PGC1α are involved in the regulation of OXPHOS in cell transition from the replicating to the quiescent state [51]. Our results are in line with findings that glucocorticoids stimulate mitochondrial biogenesis in skeletal muscle [57] and that stress stimulates brain PPARγ [58]. Since it has been shown that PGC1α is required for the induction of many ROS-detoxifying enzymes [59], we believe that in Leydig cells PGC1 could serve in the same manner. Accordingly, we believe that stress-induced coordinated expression of both the nuclear and mitochondrial genomes via PGC1 contributes to the expansion of the organelle network and enhances the process of stress-induced mitochondrial biogenesis. Increased mitochondrial biogenesis in Leydig cells of stressed rats is a mechanism that contributes to the establishment of a new adaptive response to maintain homeostasis and prevent loss of steroidogenic function.

Although in the present study, we planned to explore the mitochondrial biogenesis in stressed rats, unexpectedly; however, we found that stress disturbed mitochondrial architecture in Leydig cells. We observed a higher frequency of morphological alterations in Leydig cell mitochondria from acutely stressed rats (1 × IMO) and 2× repeatedly stressed rats (2 × IMO). Oppositely, recovery of mitochondrial cristae and clustering of mitochondria were evident in Leydig cells isolated from rats exposed to 10 × IMO. At the present ,we do not have experimental explanation for this, but we will explore in further studies the ultrastructural analysis of mitochondria, including the mitochondrial mean diameter and volume density, as well as mitochondrial cristae volume density. We will also monitor OPA1, MFN1/2, and DMF since they are involved in formation of mitochondrial cristae and decreased number

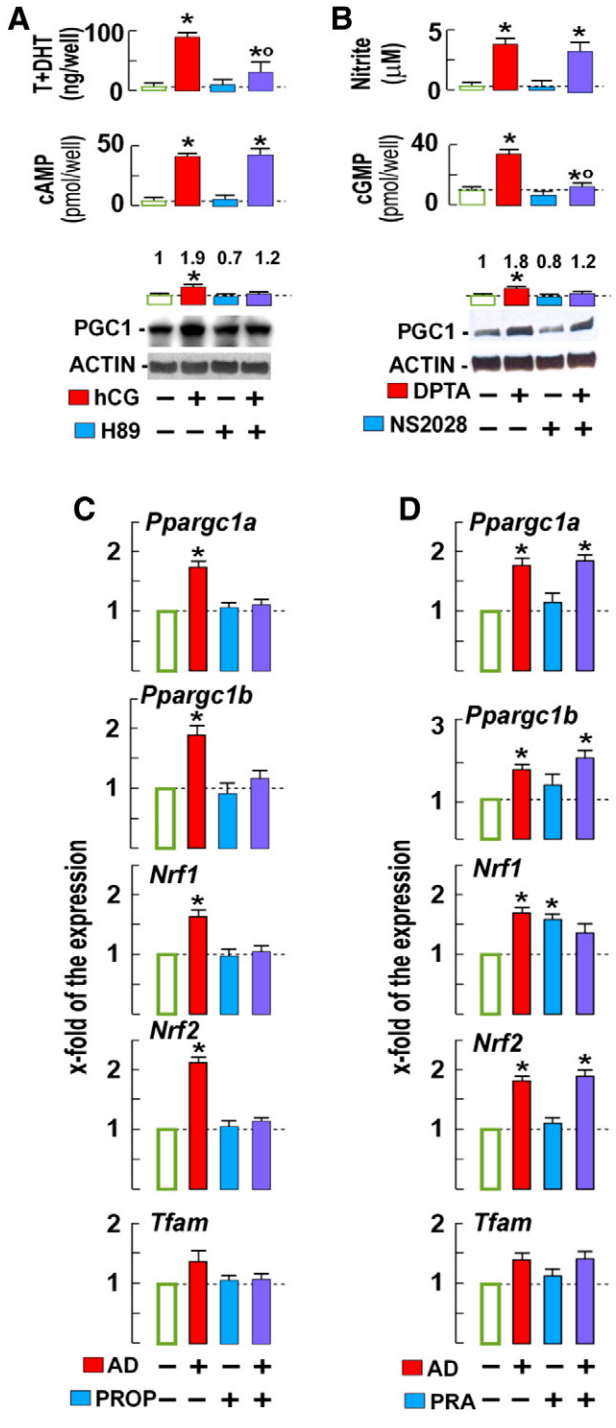


Fig. 4. Multiple signaling pathways in Leydig cells regulates expression of PGC1 the master regulator of mitochondrial biogenesis. The main regulator of steroidogenesis in Leydig cells, LHCGR-cAMP signaling stimulates expression of PGC1 through mechanism involving PRKA (***A***). In the same cells, known activator of mitochondrial biogenesis (NO) stimulates expression of PGC1 with mechanism involving GUCY1 (soluble guanylyl cyclase) – cGMP (***B***). Adrenalin, well known stress hormone stimulates transcription of main markers of mitochondrial biogenesis through adrenergic β (***C***) but not α1 (***D***) receptors. Leydig cells isolated from undisturbed rats were plated ($1x10^6$/well) for monitoring cAMP, NO, cGMP, proteins and gene expression and placed in a CO_2 incubator (34 °C) for 2 h to attach and recover. At the end of the recovery period, culture medium was discarded and Leydig cells were incubated for 24 h with ***A:*** hCG (50 ng/ml) alone or in the combination with PRKA inhibitor H89 (10 μM); ***B:*** with NO donor DPTA (10 μM) alone or in the combination with GUCY1 inhibitor NS2028 (10 μM); ***C, D:*** with adrenaline (AD, 1 μM) alone or in the combination with β-ADRs blocker propranolol (PROP, 1 μM) or α1-ADRs blocker prazosin (PRA, 1 μM). All *in vitro* experiments were repeated for three to four times. The level of cAMP and cGMP in cells content was determined by EIA, NO by Griess reagent, the transcription was determined by RQ-PCR, protein expression with specific antibody (for all details please see *Supp. Methods*). Data bars are mean ± SEM values from five independent *in vivo* experiments. Statistical significance was set at level $P < 0.05$: * vs. untreated cells.

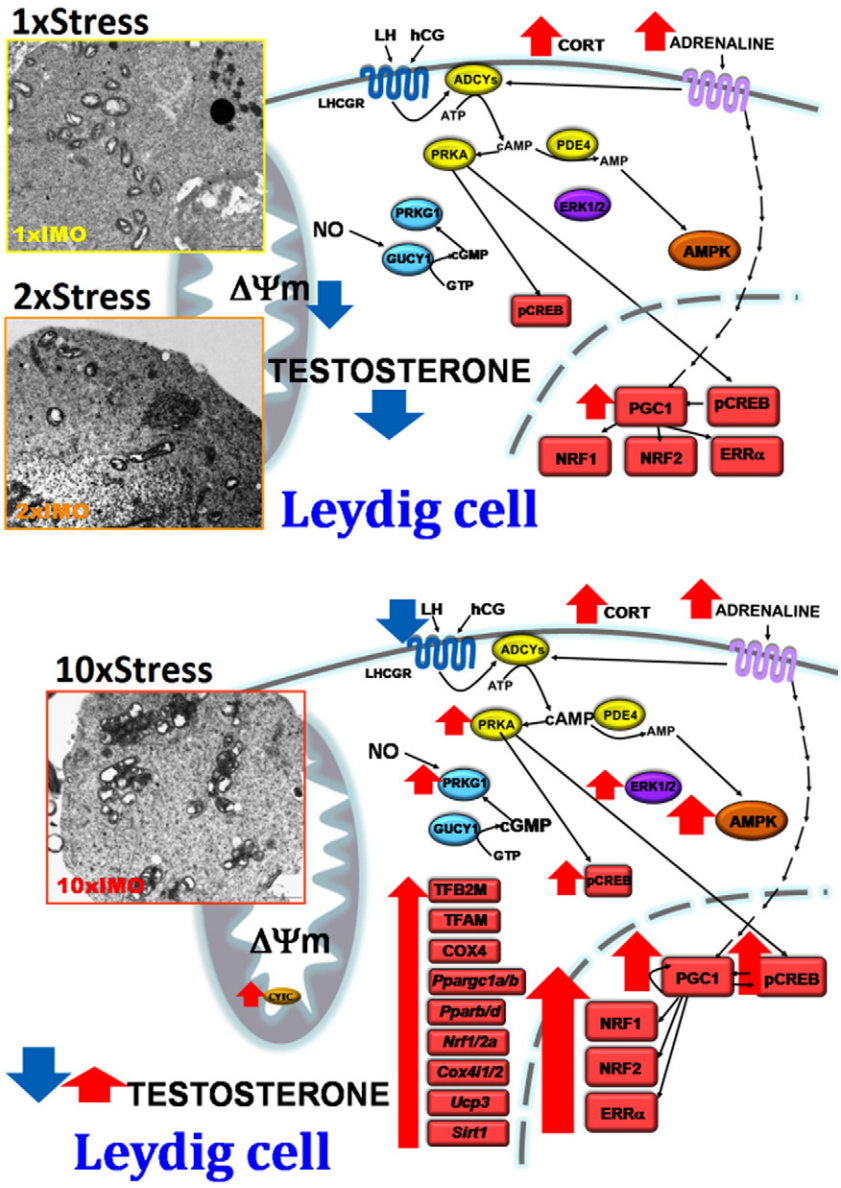


Fig. 5. Stress disturbs functionality of Leydig cell and activates many signaling pathways in order to trigger mitochondrial biogenesis and prevent loss of basal steroidogenic function (model is based on our results presented in this study and previously). *UP:* Adrenaline and corticosterone (CORT) released in acute-stress-response may be available to bind to their receptors and initiates a bipartite response: (1) through GRs can decrease transcription of steroidogenic enzymes; (2) through ADRs activate recovery kinases and mitochondrial biogenesis. 1 × IMO and 2 × IMO alter mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial architecture. *DOWN:* Repeated 10 × IMO recovers mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial architecture. Sustained GRs and ADRs occupation and concomitant activation of many signaling pathways promote (i) ADRs-sensitive signaling pathways, including PRKA, PLC, MAPK; (ii) GRs and ADRs-sensitive PGC1 activation and initiation of mitochondrial biogenesis. AMPK activates PGC1α and enhances SIRT1 leading to deacetylation and activation of both members of PGC1α-ERRα complex. Stress-increased expression of PRKA, PRKG, pERK1/2, pP38MAPK activates PGC1α/β and transcription factors (CREB, NRF1, NRF2, ERRα) and regulates the expression of nuclear gene-encoding mitochondrial proteins, as well as the transcription and mRNA expression of PGC1. Once translated, PGC1 binds to transcription factors (including NRF1, NRF2, ERRα) to also regulate nuclear gene-encoding mitochondrial proteins [29,34,35]. The newly synthesized mitochondrially destined proteins such as TFAM induces mtDNA transcription and increases the expression of proteins encoded by the mitochondrial genome that will join as subunits of complexes in the OXOPHOS such as cytochrome C and COX4. All mentioned could recover $\Delta\Psi_m$ and stimulate mitochondrial cristae biogenesis. The steroidogenic function of Leydig cell is predominantly regulated by pituitary luteinizing hormone (LH) or its placental counterpart, human chorionic gonadotropin (hCG), through LHCG receptors (**dark blue**) through stimulation of cAMP-PRKA signaling pathway (**yellow**). Other signaling pathways, including cGMP signaling (**light blue**), ERK1/2 (**violet**), also stimulate steroidogenesis.

of cristae per unit of mitochondrial surface was demonstrated in Opa1 −/−, Mfn2 −/−, as well as DMF −/− cells [52]. Also, it was demonstrated recently that MAPK cascade regulates mitochondrial shape via MFN1 [55], and this could support our results showing the increased expression of pERK1/2 and pP38MAPK in Leydig cells isolated from 10 × IMO rats.

Here we propose that all events induced by acute stress, the most common stress in human society, provoke adaptive response of Leydig cells in hormesis-dependent fashion. It seems that acute stress beside disturbance of mitochondrial function in Leydig cells also activate the so-called "energy cleaning program," since PGC1, a protein required to produce new mitochondria, also protects against the resulting oxidative damage [60]. By sensing the intracellular energy status, the mitochondrial functional state, and ROS production, the stress network regulates mitochondrial biogenesis in Leydig cells by coordinating information flow along its convergent, divergent, and multiple branched signaling pathways, including many genes/proteins involved in preserving steroidogenesis during stressful conditions.

Given the importance of mitochondria for steroid hormones production and stress response, as well as the role of steroid hormones in stress response and metabolic syndrome, we anticipate our result to be a starting point for more investigations. For example, studies of the

mitochondrial biogenesis in adrenal gland, brown/white adipose tissues, or cardiovascular system of stressed organism since stress is a constant factor in life and has become one of the most significant health problems in modern societies [3,7,9]. Also, studies have shown that psychophysiological stress can alter the cytochrome-P450-catalyzed drug metabolism [61]. Social rank have stressful characteristics [4,62] and the highest-ranking (alpha) males exhibited much higher levels of stress hormones than second-ranking (beta) males, suggesting that being at the very top is stressful [4]. Also, stress and emotional brain networks foster eating behaviors that can lead to obesity. By activating a neural-stress-response network, stressors bias cognition toward increased emotional activity and degraded executive function [63]. On the other hand, several lines of evidence support a primary role of mitochondrial impairment in the pathophysiology of stress-related disorders [3,7,9] and the relation of testosterone with inefficiency of energy utilization and metabolic syndrome [64–66]. Testosterone levels correlate inversely with insulin resistance and positively with maximal aerobic capacity and *OXPHOS* gene expression [65]. Clinical evidence shows that in males, low testosterone levels have been considered a "hallmark" of metabolic syndrome and that there is a strict connection between anatomically and functionally distinct cell types such as white adipocytes and testosterone-producing Leydig cells [66]. Biochemical evidence indicates that testosterone is involved in mitochondrial oxidative phosphorylation, while clinical studies have demonstrated an association between low levels of testosterone and the insulin-resistant states, such as type 2 diabetes mellitus and the metabolic syndrome, the major risk factors for cardiovascular disease [64]. Based on all mentioned, we suggest that pharmacological interventions that activate PGC1 and consequently stimulate mitochondrial biogenesis in Leydig cell might offer a new approach for treating declined testosterone production during hypogonadism or aging and prevent low-testosterone-induced metabolic syndrome.

5. Conclusions

Here we show that increased mitochondrial biogenesis is the adaptive response of testosterone-producing Leydig cells in stressed rats. All markers of mitochondrial biogenesis together with transcription factors and related kinases are up-regulated in Leydig cells from rats exposed to repeated psychophysical stress. This is followed with increased mitochondrial mass. The expression of PGC1, master regulator of mitochondrial biogenesis and integrator of environmental signals, is stimulated by cAMP-PRKA, cGMP and β-adrenergic receptors. Accordingly, stress-triggered mitochondrial biogenesis represents an adaptive mechanism and does not only correlate with but also is an essential for testosterone production, being both events depend on the same regulators.

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Disclosure Statement

The authors have nothing to disclose.

Author contributions

I.A.G., S.M.R., A.R.D., M.M.J., N.J.S-M., T.S.K., and S.A.A. performed the experiments, edited and revised the manuscript, approved the final version of the manuscript; I.A.G., S.M.R., and S.A.A. did most of the experiments, interpreted results of experiments, drafted the manuscript; T.S.K. provided advice on the data analysis and the manuscript; S.A.A. did the conception and design of the research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.05.030.

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