Original Paper

Distinct Testicular Steroidogenic Response Mechanisms Between Neonatal and Adult Heat-Acclimated Male Rats

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Key Words

Adult heat acclimation • Neonatal heat acclimation • Steroidogenesis • Testes • Rat • Steroidogenic enzyme pathways

Abstract

Background: In comparison to short-term gonad heat exposure, little is known about the molecular mechanisms that regulate testicular steroidogenesis during long-term whole body heat acclimation. **Material and Methods:** Testicular slices from neonatal (NHA) and adult (AHA) heat-acclimated Wistar rats were analysed *in vitro* to assess the mRNA expression and enzymatic activity of steroidogenic enzymes under basal and luteinising hormone (LH) or prolactin (PRL) stimulated conditions compared with control rats (CR). Furthermore, a de-acclimated group (DA) was created by transferring adult NHA rats to control conditions. **Results:** Heat acclimation significantly increased plasma LH levels in the AHA group and LH and PRL in the NHA group compared with the CR group; however, after heat acclimation, the T and E2 levels did not differ from the control levels. All heat-acclimated groups showed high basal intra-testicular steroid production *in vitro*. Moreover, basal *Cyp11a1* and *Hsd3b1* levels were upregulated *in vitro* in the NHA and DA groups versus the CR group. LH *in vitro* stimulation upregulated *Cyp11a1* expression in the NHA and AHA groups and PRL stimulation upregulated *Cyp17a1* levels in the NHA and DA groups compared with the basal expression levels. In the AHA group, decreased basal *Star* and CYP11A activities but increased HSD3B1 and CYP17A1 activities were found. **Conclusion:** Our data revealed that despite the similar steroid levels in plasma and secreted *in vitro* by neonatal and adult heat-acclimated rat testicular slices, the molecular mechanisms underlying the steroidogenic response to heat acclimation during these different developmental stages were distinct.

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Introduction

Environmental factors influence reproductive performance in males primarily through two mechanisms of action. The first mechanism, known as the acclimatory response, involves impairment of reproductive functions due to heat exposure at adult age. The second mechanism of response is known as developmental plasticity, which is defined as individual ontogenetic lability with consequences for adult physiology [1]. Environmental modification of adult physiology by developmental plasticity has been shown to affect the development of the visual cortex [2], thermoregulatory effectors [3] and the male reproductive system. For example, neonatal raloxifene treatment exerted an estrogenic action on the hypothalamo-pituitary structures that control reproductive function in rats [4]. Testicular steroidogenic pathway adaptations to high ambient temperature exposure in neonates have not yet been identified; therefore, we focused on elucidating the mechanisms underlying this process in our present study.

Ample data are available in the literature regarding testicular acute heat shock treatment (reviewed in [5]); however, data regarding the ability of the testes to adapt to the increased temperature is lacking. Obtaining knowledge on the possible mechanisms underlying testicular adaptation to increased temperature is important because we do not know whether the thermal conditions caused by human lifestyle choices during neonatal or adult life influence deteriorating sperm counts in men [5]. Elevated gonadal temperature is also suggested as a factor involved in the pathogenesis of human reproductive tract disorders such as fever [6], cryptorchidism [7] or varicocele [8]. The long-term effects of elevated ambient temperature on testicular steroidogenesis were primarily reported from the 1970s to the 1990s [9-12]. By contrast, recent transcriptome data obtained from the heat-acclimated rat hypothalami [13] and hearts [14] showed that short-term heat acclimation (two days) resembled a stress response, whereas long-term heat acclimation activated different sets of gene expression profiles and was characterised by the resumption of pre-acclimation stress response-related gene transcript levels.

Androgen and oestrogen production in adult male rats primarily occurs in Leydig cells [15]. Development of rat Leydig cells from their foetal to mature state during postnatal life [15-17] predisposes these cells to developmental plastic adaptation in response to endocrine-disrupting compounds such as bisphenol A [18] and triclosan [19] or increased testicular temperature [12]. The effects of these conditions may differentially affect testicular steroidogenesis compared with adult-treated males; however, the molecular mechanisms underlying the effects of high ambient temperature applied to males prior to puberty are not yet known.

Luteinising hormone (LH) [20], prolactin (PRL) [21], and testosterone [22, 23] have been reported as predominant factors that regulate the expression and activity of steroidogenic enzymes in adult Leydig cells. The maturity of the Leydig cells correlates with the number of LH, PRL and androgen receptors (AR) present in the cells [17, 24]. Thus, the steroidogenic ability of Leydig cells is also influenced by the activities of LH (LHCGR), PRL (PRLR) and androgen (AR) receptors. However, the effects of chronic exposure to increased environmental temperature, either before or after puberty, on the expression of LHCGR, PRLR and AR in rat testicular tissue remains largely unknown.

Previously, we reported increased PRL and oestradiol-17 β (E2) plasma levels and decreased androstenedione (A4) levels in male rats housed at a high ambient temperature from birth to adulthood [25]. Moreover, isolated Leydig cells from neonatal and adult heat-acclimated rat testes secreted less testosterone (T) compared with control rats [25].

In this study, we hypothesised that high ambient temperature during neonatal and adult life in rats may differentially influence testicular steroidogenesis. Consequently, we aimed to identify these two putatively distinct mechanisms that may control testicular steroidogenesis in rats at neonatal and adult ages exposed to high ambient temperature. Furthermore, we characterised the consequences of neonatal and adult heat acclimation on rat testicular steroid production.

Material and Methods

Experimental animals

Twenty male Wistar rats were separated into the following 4 groups: 1) control males (CR, n=5) were born and housed until the 90th day of life at an ambient temperature of 20±1 °C; 2) adult heat-acclimated males (AHA, n=5) were born at 20±1 °C, and after reaching puberty (44.5th day), they were housed at 34±1 °C for the next 44.5 days; 3) neonatal heat-acclimated males (NHA, n=5) were born and housed at 34±1 °C for 90 days; and 4) de-acclimated males (DA, n=5) were housed at 34±1 °C from birth to the 44.5th day and then later were housed at 20±1 °C for the next 44.5 days. The experimental rat pups, from 3 different litters per group, stayed with the dams until the 30th postnatal day at the designated temperature (20 or 34 °C). The 5 rats/group were never chosen from the same litter. Both the humidity and the light:dark cycle (12:12 hours) were controlled. The rats were provided access to tap water and chow pellets *ad libitum* (Labofeed H; Feeds and Concentrates Production Plant, Kcynia, Poland). The local Ethics Committee for Animal Experimentation of the University of Warmia and Mazury in Olsztyn approved all animal experiments (approval no 46/2008/N).

Tissue collection and in vitro incubation of testicular slices

On postnatal day 90, the rats were weighed and sacrificed by cervical dislocation. Blood samples and whole testes were collected. Blood samples were collected in heparinised tubes, and plasma was isolated by centrifugation (1200 x *g* at 4°C for 15 min) (Beckman Coulter, Brea, CA, USA) and stored at -20°C for future hormonal analysis. The gonado-somatic index (GSI) was also calculated as total testicular weight/body weight x 100 [26]. Both testes from each rat were weighed, and were aseptically cut into 6 equal slices (~2.1 mm thick and 250±10 mg of weight/slice). Three slices per rat were used to isolate mRNA or protein for Western blotting; the other 3 slices from each rat were used to isolate mitochondrial and microsomal proteins. Each slice was incubated separately in a six-well plate (BD Labware, Europe) in 3 ml of F-12 medium (pH=7.4; AppliChem, Germany) supplemented with 1% BSA (AppliChem, Germany). We used 2 slices from each rat for each treatment group. After a one-hour preincubation in a shaking water bath at 34°C in a 95% O₂ + 5% CO₂ atmosphere, the medium was changed, and incubation was performed for the next eight hours in medium without (basal treatment) or stimulated with LH (100 ng/ml, NIDDK NIH, Bethesda, MD, USA) or PRL (100 ng/ml, NIDDK NIH, USA) [25]. After the 8-h incubation, the media were collected and frozen at -20 °C for hormone analysis, and the tissues were stored at -80°C for further analysis.

To verify the effects of high ambient temperature on the testicular interstitial tissue morphology, 7 µm paraffin rat testicular sections were de-waxed, stained with haematoxylin and eosin (POCH, Gliwice, Poland) and DPX (POCH, Gliwice, Poland) embedded. Next, we calculated the percentage of interstitial tissue using the dissector method and interstitial cell numbers using calibrated photomicrographs (Olympus, Tokyo, Japan) of interstitial tissue and picture analysis software (AnalySis, Olympus). This procedure was repeated to obtain 3-4 measurements from different cross-sections/one testis.

Steroid hormone concentrations in blood plasma and media

Total A4, T and E2 concentrations were measured by radio-immunoassay (RIA) in blood plasma after diethyl ether extraction (POCH, Gliwice, Poland) [27], and the steroids were measured in media without extraction [28, 29]. The intra- and inter-assay coefficients of variance were as follows: 2% and 13.3%, respectively, for A4; 2.1% and 5.1%, respectively, for T; and 1.4% and 10.6%, respectively, for E2. The assay sensitivity for A4 and T was 2 pg/tube and was 1 pg/tube for E2.

Plasma LH, FSH and PRL concentrations

The plasma concentrations of LH, FSH and PRL were measured using commercially available rat LH (RK-552), FSH (RK-550) and PRL (RK-553) RIA kits (Izotop, Budapest, Hungary) according to the manufacturer's instructions. The assay sensitivity for FSH and LH was 0.09 ng/tube and was 0.07 ng/tube for PRL. Intra-assay variations were: 1.6%, 1.2% and 7.9% for the LH, FSH and PRL assays, respectively.

Isolation of total RNA and quantitative real-time PCR (qPCR)

For each treatment group, half of one testicular slice (~125 mg) per animal was homogenised, and the total RNA was isolated using TRIzol (Life Technologies, Carlsbad, CA, USA). The quantity and quality of the isolated

Table 1. Primers used for the qPCR reactions

Gene	NCBI Accession No.	Primer sequence	mRNA region spanned by the primer	Product length (b)
<i>Star</i>	NM_031558.3	F: cagtgaccaggagctgtctctaca	320-342	145
		R: cacacctggcaccaccttactta	464-442	
<i>Cyp11a1</i>	NM_017286.2	F: ctggaaggtgtagctcaggacttc	505-528	129
		R: aacctgggtgatggactcaagg	633-611	
<i>Hsd3b1</i>	M38178.1	F: tgccacttggtcacactgtca	1121-1140	148
		R: ccctgtgctgtccactagtgt	1267-1246	
<i>Cyp17a1</i>	NM_012753.2	F: ggtgcacaatcctgaggtgaag	981-1002	137
		R: tgatagcagcacttctcgata	1117-1095	
<i>Hsd17b3</i>	NM_054007.1	F: ctggcctctgtatagcctgtactca	582-606	168
		R: gcttggtcacctgctggtat	749-728	
<i>Cyp19a1</i>	NM_017085.2	F: ctctcctgattcggaattgtg	715-736	131
		R: ttgtagtagttgcaggcacttcca	845-822	
<i>Lhcgr</i>	NM_012978.1	F: gcatccgaaccttccagat	456-475	124
		R: tcgttattcatccctggaaagc	579-557	
<i>Prlr1</i>	NM_001034111	F: tgattatggctggcagtg	930-950	127
		R: agctctcagacttgcctcttc	1056-1034	
<i>Prlr2</i>	NM_012630	F: gcagtggcttgaagggtat	954-974	121
		R: ccactgtatttcttgaggagaa	1074-1052	
<i>Actb</i>	NM_031144	F: accatgtacccaggcattgc	988-1007	131
		R: gccaggatagagccaccaatc	1118-1098	

RNA was determined using the NanoDrop (Thermo Scientific, Waltham, MA, USA) and by gel electrophoresis. In total, 1 µg of RNA was reverse transcribed using a Qiagen kit (Qiagen Corp., USA) according to manufacturer's instructions, and the cDNA was stored at -20°C until used for qPCR. We analysed the expression levels of the following genes: *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Cyp19a1*, *Lhcgr*, *Prlr1* and *Prlr2*. *Actb* was used as a housekeeping reference gene. The primers (Table 1) were designed using Primer Express software (Applied Biosystems, Waltham, MA, USA). The qPCR experiments were performed using a SYBR Green Mix (Applied Biosystems, USA). The reaction parameters were as follows: 95°C for 10 min, then 40 cycles at 95°C for 15 s, followed by 60°C (for *Lhcgr*, *Prlr1*, *Prlr2*, *Star*, *Cyp19a1*, and *Actb*) or 61°C (for *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, and *Hsd17b3*) for 30 s, and 72°C for 5 min. The dissociation curve parameters were: 65°C for 1 s, followed by 95°C for 5 s. Serial dilutions of the appropriate cDNA reaction products were used as standard curves for DNA quantification. The expression levels of all the investigated genes were normalised to the housekeeping gene *Actb*. All of the reaction products were separated and verified by sequencing analysis.

Western blot to detect STAR protein

The remaining half (~125 mg) of one testicular slice/animal was homogenised in RIPA buffer (150 mM sodium chloride, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). The protein concentrations were estimated using the Bradford method [30]. The protein samples (20 µg) were subjected to electrophoresis in 12.5% sodium dodecyl sulphate-polyacrylamide gels, followed by transfer onto nitrocellulose membranes (Whatman, Little Chalfont, England). After blocking, the membranes were incubated with either a primary monoclonal mouse anti-human STAR antibody (1:400, 2 µg/ml; Sigma-Aldrich, USA) or a polyclonal rabbit anti-ACTB antibody (1:200; Sigma-Aldrich, USA). Then, the membranes were incubated in alkaline phosphatase-linked anti-mouse (cat. No sc-2008, Santa Cruz Biotechnology, Dallas, TX, USA) or anti-rabbit IgG (cat. No sc-2057, Santa Cruz Biotechnology, USA), both diluted to 1:2500. Antibody binding was visualised using the BCIP/NBT colour development system (Promega, Madison, WI, USA). The intensity of the specific bands was quantified, and the densitometric data were analysed using GelScan software (Kucharczyk, Gdansk, Poland) [31], normalised to the ACTB signal, and expressed as arbitrary units.

Isolation of mitochondrial and microsomal protein fractions from testicular slices

The second series of testicular slices (3 from each rat) was homogenised in Tris-HCl-sucrose (pH=7.4) buffer [32] supplemented with a protease inhibitor cocktail (Sigma-Aldrich, USA). The mitochondrial and microsomal fractions were obtained *via* sequential centrifugation of the homogenates (9000 × *g* twice for

the mitochondrial fraction and $100000 \times g$ for the microsomal fraction) according to Slominski et al. [33] and McVey et al. [32]. These fractions were re-suspended in the same buffer and stored at -80°C for further analysis.

Determination of steroidogenic enzyme activity

The steroidogenic enzyme activities were determined based on the conversion of tritiated substrates into their appropriate products. The following steroids (100,000 cpm, 20 nM, Hartmann Analytic, Braunschweig, Germany) were used as substrates: $[1,2\text{-}^3\text{H}]$ cholesterol (50-60 Ci/mmol) for CYP11A1 [33], and $[1,2\text{-}^3\text{H}]$ DHEA (50-60 Ci/mmol) for HSD3B1 [32]; $[1,2\text{-}^3\text{H}]$ progesterone (40-60 Ci/mmol) for CYP17A1; $[1,2\text{-}^3\text{H}]$ T (40-60 Ci/mmol) for HSD17B3 [32] and $[^3\text{H}]$ A4 (15-30 Ci/mmol) for CYP19A1 [34].

CYP11A1 activity [33] was measured using 250 μg of the mitochondrial fraction with $[^3\text{H}]$ cholesterol, NADPH (0.5 mM, Sigma-Aldrich, USA) and isocitrate (5 mM, Sigma-Aldrich, USA) throughout a 3 h incubation period in a shaking water bath (34°C). Each reaction was stopped by the addition of excess hexane and 30 μg of each of the following unlabelled standards: cholesterol, pregnenolone (P5), hydroxypregnenolone (P5OH), progesterone (P4) and hydroxyprogesterone (P4OH) (Sigma-Aldrich, USA). The organic phase was separated and subjected to thin layer chromatography (SIL G/UV₂₅₄, Macherey-Nagel Inc., Duren, Germany). This separation was performed twice in a 1:1 solution of hexane:ethyl acetate as a mobile phase. The products were visualised using iodine vapour, scraped into scintillation vials and counted using a beta counter (Beckman-Coulter Inc, Pasadena, CA, USA). The overall enzyme activity was corrected for procedural loss, which was determined by comparing the absolute substrate radioactivity in the probes with no proteins. The recovery of total radioactive substrates in probes without protein was 34.2%, which was used as an index of procedural losses. Inter-assay variation was 11.7%.

HSD3B1, CYP17A1 and HSD17B3 activities were determined during a 1 h incubation on 50 μg of microsomal proteins and 0.25 mM NAD^+ (for HSD3B1 and HSD17B3) or 0.25 mM NADPH (for CYP17A1) in Tris-HCl-sucrose buffer. This procedure was the same as the procedure described for CYP11A1 analysis, except that the unlabelled steroids were as follows: DHEA, T and A4 for HSD3B1 and HSD17B3 and P4, P4OH, A4 and T for CYP17A1. The assay characteristics included 44.3%, 82.4% and 48% recovery and 7.7%, 13.3% and 11.3% inter-assay variation for HSD3B1, CYP17A1 and HSD17B3, respectively.

The CYP19A1 activity was determined based on a $[^3\text{H}]\text{H}_2\text{O}$ release assay [34], in which 50 μg of microsomal protein was incubated for 1 h with $[^3\text{H}]$ A4 and 0.25 mM NADPH in Tris-HCl-sucrose buffer. The reaction was stopped by the addition of excess chloroform. The aqueous phase was separated using dextran-coated charcoal and centrifuged. The supernatant radioactivity was measured using a beta counter. The radioactive substrate recovery was less than 3% in the water phase of the reactive mixture, indicating the low contamination of the radioactive product by the organic phase of the mixture. The inter-assay variation coefficient was 2%.

Statistical analysis

The data are expressed as the means $\pm$ SEM. Significant differences between the means were determined via one-way analysis of variance (ANOVA) for the between groups comparison (Statistica, StatSoft, Tulsa, USA) followed by an LSD post-hoc test. The significant effects of LH or PRL on the testicular slice treatment were determined via Student's t-test. Differences between the means were considered significant when $P < 0.05$.

Results

Neonatal heat acclimation significantly decreased rat body weight at weaning (NHA and DA compared with CR and/or AHA) and at the time of sacrifice (Fig. 1A). Total testicular weight significantly decreased in the NHA group compared with the other groups, and the weight was lower in the DA group compared with the CR group. Heat acclimation did not show any significant effects on the gonado-somatic index (GSI) among the groups (Fig. 1B). Neonatal or adult heat acclimation also did not cause any significant changes in the percentage of interstitial tissue and/or the number of interstitial cells as demonstrated by morphometric analysis on the same testicular cross-section among the studied groups. The mean percentage

Fig. 1. Body (A), testicular weight and gonado-somatic index (GSI) (B) in control (CR), adult heat-acclimated (AHA), neonatal heat-acclimated (NHA), and de-acclimated (DA) rats. (n = 5/group). a, b, c – statistically significant (p<0.05) differences among the groups are indicated by bars with different letters. * – asterisk indicates a statistically significant difference between the NHA/DA and CR/AHA groups in the body weight at 30th postnatal day (weaning time), p<0.05.

Table 2. Plasma LH, PRL, FSH, A4, T and E2 concentrations (ng/ml) for control (CR), adult heat-acclimated (AHA), neonatal heat-acclimated (NHA), and de-acclimated (DA) rats. (n = 5/group). a, b – different letters indicate significant differences in the examined hormone concentrations among the groups, p<0.05

Hormone	CR	AHA	NHA	DA
LH	1.5±0.2 ^a	2.1±0.2 ^b	2.2±0.2 ^b	1.8±0.2 ^{ab}
PRL	7.5±1.6 ^a	8.4±1.4 ^a	10.5±1.9 ^b	7.5±1.6 ^a
FSH	12.6±0.5 ^{ab}	12.0±0.5 ^a	13.9±0.9 ^b	11.0±1.0 ^a
A4	4.4±0.2 ^{ab}	4.9±0.3 ^a	4.1±0.2 ^b	5.0±0.3 ^a
T	7.5±0.4	8.0±0.3	7.6±0.4	7.7±0.5
E2	0.5±0.04	0.5±0.04	0.5±0.04	0.5±0.03

of interstitial tissue was 4.6 ± 0.4 , 5.2 ± 0.3 , 4.5 ± 0.2 and 4.9 ± 0.2 and the interstitial cell density (cells/100 μm^2 of interstitial tissue) was 0.73 ± 0.02 , 0.76 ± 0.03 , 0.72 ± 0.04 and 0.82 ± 0.03 in the CR, AHA, NHA and DA groups, respectively. This morphometric data supported the hypothesis regarding the equal composition of interstitial cells in all slices.

LH, PRL, FSH, A4, T and E2 plasma levels

The plasma LH concentration was significantly higher in the AHA and NHA groups compared with the CR group (Table 2). The plasma PRL level was higher in the NHA males compared with the other groups. The FSH concentration was higher, while the A4 concentration was lower in the NHA group compared with the AHA and DA groups. The T and E2 plasma concentrations did not differ between the experimental groups.

Intra-testicular basal and LH- or PRL-stimulated Star mRNA and STAR protein levels

In the testicular slices, the basal *Star* mRNA levels were significantly lower in the AHA group than in the CR and DA groups (Fig. 2A). In every group, LH treatment upregulated *Star* mRNA expression compared with the basal levels (Fig. 2A). In the AHA group, LH stimulation increased the *Star* mRNA level by nine-fold; however, PRL stimulation did not affect *Star* expression in any of the experimental groups (Fig. 2A). Neonatal or adult age heat acclimation did not affect the STAR protein basal level. Similarly, the STAR protein levels remained unchanged after *in vitro* treatment with LH and PRL (Fig. 2B).

Fig. 2. Basal and LH- (100 ng/ml) or PRL- (100 ng/ml) stimulated levels of intra-testicular STAR mRNA (A) and protein expression (B) in the testicular slices from control (CR), adult heat-acclimated (AHA) neonatal heat-acclimated (NHA), and de-acclimated (DA) rats. (n = 5/group). ^{a, b} – statistically significant (p<0.05) differences among the groups are indicated by bars with different letters. * – asterisk indicates a statistically significant difference between the basal and LH- or PRL-stimulated levels, p<0.05.

Basal and LH- and PRL-stimulated steroidogenic enzyme mRNA expression and intra-testicular activity levels in vitro

The *Cyp11a1* basal mRNA level was significantly upregulated in testicular slices from the NHA and DA groups compared with the CR and AHA groups (Fig. 3A). *In vitro* LH treatment upregulated *Cyp11a1* expression in both the AHA and NHA groups, whereas PRL downregulated *Cyp11a1* expression in the DA group compared with the basal levels. The basal CYP11A1 activity level was significantly lower in the AHA rats than in the CR and NHA rats, and a similar difference was detected between the DA and the NHA rats. *In vitro* LH or PRL treatment did not affect CYP11A1 activity (Fig. 3F).

In the NHA and DA groups, the basal intra-testicular *Hsd3b1* mRNA levels were higher compared with the CR group (Fig. 3B), and HSD3B1 enzymatic activity was lower (Fig. 3G) compared with the AHA group. *In vitro* LH treatment did not affect *Hsd3b1* expression in any of the groups, whereas PRL reduced *Hsd3b1* expression in the NHA testicular slices compared with the basal level (Fig. 3B). *In vitro* treatment with LH increased the HSD3B1 activity level in the NHA group, while PRL did not affect HSD3B1 activity in any of the groups compared with the basal activity level (Fig. 3G). The basal *Cyp17a1* expression level was similar in all of the experimental groups, and LH treatment did not affect its expression level (Fig. 3C). PRL treatment significantly upregulated *Cyp17a1* expression in the NHA and DA groups compared with the basal level (Fig. 3C). The basal CYP17A1 activity level was higher in the AHA group and lower in the DA group compared with the other groups (Fig. 3H). LH and PRL treatment significantly increased the CYP17A1 activity level in the NHA and DA groups compared with their corresponding basal activity levels (Fig. 3H). The basal *Hsd17b3* expression and HSD17B3 activity levels were similar in all of the experimental groups

Fig. 3. Basal and LH- (100 ng/ml) or PRL- (100 ng/ml) stimulated levels of intra-testicular mRNA expression (A-E) and enzymatic activity (F-J) of steroidogenic pathway enzymes in testicular slices from control (CR), adult heat-acclimated (AHA) neonatal heat-acclimated (NHA), and de-acclimated (DA) rats. (n = 5/group). ^{a,b,c} – statistically significant (p<0.05) differences among the groups are indicated by bars with different letters. * – asterisk indicates a statistically significant difference between the basal and LH- or PRL-stimulated levels, p<0.05.

Fig. 4. Basal and LH- (100 ng/ml) or PRL- (100 ng/ml) stimulated levels of intra-testicular expression of *Lhcgr*, *Prlr1*, and *Prlr2* in testicular slices from control (CR), adult heat-acclimated (AHA), neonatal heat-acclimated (NHA), and de-acclimated (DA) rats. (n = 5/group). ^{a, b} – statistically significant (p<0.05) differences among the groups are indicated by bars with different letters. * - asterisk indicates a statistically significant difference between the basal and LH- or PRL-stimulated levels, p<0.05.

(Fig. 3D, I). LH treatment significantly upregulated *Hsd17b3* expression in the AHA group and HSD17B3 activity in the NHA group compared with the corresponding non-stimulated basal levels (Fig. 3D, I).

The basal *Cyp19a1* expression level was lower in the AHA and NHA groups compared with the CR group (Fig. 3E), whereas *in vitro* LH treatment upregulated *Cyp19a1* expression in the CR, AHA and NHA groups but not in the DA group when compared with the corresponding basal levels (Fig. 3E). PRL downregulated *Cyp19a1* expression levels in the CR and AHA groups. The basal CYP19A1 activity level was higher in the CR group compared with the NHA and DA groups, and the basal CYP19A1 activity level was higher in the AHA and NHA groups compared with the DA group (Fig. 3J). LH treatment decreased the CYP19A1 enzyme activity level in the CR and AHA groups compared with the corresponding basal level. PRL did not affect CYP19A1 activity in any of the groups compared with the corresponding basal enzyme activity level.

Basal and LH- or PRL-stimulated levels of *Lhcgr*, *Prlr1* and *Prlr2* expression

The basal *Lhcgr* expression level was significantly upregulated in the DA group compared with the other groups (Fig. 4A). Incubation of the testicular slices with LH upregulated *Lhcgr* expression in the CR, AHA and NHA groups but downregulated *Lhcgr* expression in the DA rats compared with the corresponding basal levels (Fig. 4A). PRL treatment *in vitro* downregulated *Lhcgr* expression in the DA group compared with the basal level (Fig. 4A). The basal *Prlr1* expression level was not affected by neonatal and adult heat acclimation and

Table 3. *In vitro* secretion of A4, T and E2 (pg/ μ g of microsomal testicular protein) by rat testicular slices from control (CR), adult heat-acclimated (AHA) neonatal heat-acclimated (NHA), and de-acclimated (DA) rats. (n = 5/group). ^{a, b, c} – different letters indicate significant differences in the basal examined hormone secretion among the groups (p<0.05). * - designate significant differences between basal and LH or PRL influenced examined hormone secretion within the experimental group (p<0.05)

Hormone	Treatment group	CR	AHA	NHA	DA
A4	Basal	8.7 $\pm$ 0.3 ^a	8.1 $\pm$ 0.9 ^a	17.9 $\pm$ 2.1 ^b	13.4 $\pm$ 3.7 ^{ab}
	LH (100 ng/ml)	112.9 $\pm$ 13.5*	63.4 $\pm$ 3.0*	102.4 $\pm$ 8.4*	123.6 $\pm$ 6.8*
	PRL (100 ng/ml)	19.6 $\pm$ 1.9	5.7 $\pm$ 2.3	13.2 $\pm$ 1.6	13.3 $\pm$ 6.8
T	Basal	32.2 $\pm$ 3.0 ^a	103.7 $\pm$ 11.9 ^b	170.5 $\pm$ 17.9 ^c	40.9 $\pm$ 14.4 ^a
	LH (100 ng/ml)	365.7 $\pm$ 55.2*	390.3 $\pm$ 74.3*	841.9 $\pm$ 85.5*	465.1 $\pm$ 46.1*
	PRL (100 ng/ml)	31.0 $\pm$ 7.0	55.8 $\pm$ 17.9	192.8 $\pm$ 27.8	19.8 $\pm$ 0.2
E2	Basal	0.2 $\pm$ 0.01 ^a	0.8 $\pm$ 0.03 ^b	0.8 $\pm$ 0.05 ^b	1.3 $\pm$ 0.17 ^b
	LH (100 ng/ml)	0.3 $\pm$ 0.03*	0.7 $\pm$ 0.04	1.4 $\pm$ 0.10*	2.1 $\pm$ 0.42*
	PRL (100 ng/ml)	0.1 $\pm$ 0.01*	0.7 $\pm$ 0.02	1.4 $\pm$ 0.30	1.0 $\pm$ 0.01

LH and PRL treatment, except in the NHA group, in which PRL downregulated *Prhr1* (Fig. 4B). Basal *Prhr2* expression was significantly higher in the testes obtained from the NHA group compared with the CR and DA groups (Fig. 4C). Both LH and PRL *in vitro* treatment downregulated *Prhr2* expression in all of the experimental groups compared with the corresponding basal expression levels.

Effects of neonatal and adult heat acclimation on steroid secretion by rat testicular slices in vitro

Basal A4 production was higher in the *in vitro*-incubated testicular slices obtained from the NHA group than in the testes obtained from the CR and AHA groups (Table 3). LH treatment significantly increased A4 secretion in all of the groups compared with their basal secretion levels, and PRL treatment was non-effective. *In vitro* testicular T secretion basal levels were higher in the AHA and NHA groups and that of E2 secretion levels were higher in all heat-acclimated groups compared with the CR group. LH treatment significantly increased testicular T and E2 secretion in all of the groups, except in the AHA group testicular slices, compared with the basal secretion levels (Table 3). PRL displayed no effect on testicular T or E2 secretion in any of the groups examined (Table 3).

Discussion

In this study, increased plasma LH levels in all groups compared with the CR group was commonly observed in response to heat acclimation, whereas the increased plasma PRL level was limited to the NHA group. In both heat-acclimated rat groups, despite their plasma tropic hormone changes, no apparent differences in the plasma steroid concentration were found. By contrast, in both heat-acclimated groups and in the de-acclimated group, higher *in vitro* secretion levels of T and E2 or A4 by the testicular slices compared with the CR group was found. These results indicate that increased LH or PRL plasma concentrations were not directly associated with the steroid feedback mechanisms but occurred due to the effects of increased body temperature on pituitary cells as previously shown in heat-acclimated rats [11] or changes in thyrotrophic cell function as observed in farm animals during the summer conditions [35]. Heat acclimation-induced interstitial space enlargement was occasionally shown to be responsible for increased testicular steroid production [10]; however, in our case, no changes in the interstitial tissue morphology and gonado-somatic index (GSI) were found. The discrepancy between the plasma steroid levels and the *in vitro* steroid secretion

ability by the testicular slices may be a result of increased steroid metabolism in adult heat-acclimated rats as previously shown [10]

By analysing the testicular steroidogenic pathway adaptations, we determined that two distinct molecular mechanisms independently regulate intra-testicular steroid production in our heat-acclimated groups. In the neonatal heat-acclimated (NHA and DA) rats, in which the transition from foetal to adult-type Leydig cells [15, 16, 17] occurred under high ambient temperature, upregulated *Cyp11a1* and *Hsd3b1* transcription was detected compared with the CR and AHA groups. *Cyp11a1* was upregulated 1.4-fold, which is similar to the change observed in the hypothalamus of long-term heat-acclimated rats (1.9-fold change) [13]. In the NHA group, an increased basal CYP11A1 activity level was observed compared with the AHA and DA groups, which indicates that in addition to *Cyp11a1* upregulation in DA, the mRNA stability and translation rate of this enzyme may have been affected by subjective cold exposure of de-acclimated rats [36]. *Cyp19a1* downregulation was found in both the NHA and AHA groups, but decreased CYP19A1 activity was observed in both neonatally heat-acclimated groups (NHA and DA) compared with the CR group, which may be due to a synergistic inhibitory effect of increased LH and PRL on CYP19A1 activity [37] in the NHA group. Although we did not detect any increase in the plasma levels of LH or PRL in the DA rats at postnatal day 90, we cannot exclude the possibility of their transient increase during the prepubertal heat exposure period. All of the above mentioned differences in the expression and activity of steroidogenic enzymes in the testes of NHA and DA rats may occur as a result of the impact of high ambient temperature on the function of Leydig cells alone or on both Leydig and Sertoli cells (or peritubular myoid cells) because these cells differentiate during postnatal life and the interaction between these cells strongly affects steroid production [38].

The effect of adult heat acclimation on steroidogenic gene expression in our study remained elusive except regarding *Cyp19a1* downregulation. Increased testicular HSD3B1 activity in adult heat-acclimated rats together with decreased CYP11A1 and increased CYP17A1 activity levels were observed in our AHA group. A previous study [9, 10] showed that increased testicular HSD3B1 activity of adult heat-acclimated rats was accompanied by decreased intra-testicular testosterone production and serum concentration. Thus, the activity of steroidogenic enzymes without significant changes in their transcription rate may act as a molecular starting point for higher *in vitro* steroid production by testicular slices of adult heat-acclimated rats.

Decreased LH binding to Leydig cell membranes was detected in heat-acclimated rats in an earlier report [39]; therefore, we analysed testicular LH receptor expression. Heat acclimation did not affect testicular *Lhcgr* expression compared with the CR group, but de-acclimation of neonatally heat-treated rats (DA group) significantly upregulated *Lhcgr* expression. By contrast, *in vitro* LH treatment upregulated *Lhcgr* in the CR, AHA and NHA groups but was downregulated in the DA testicular slices compared with their respective basal levels. In this study, we used a rather high supraphysiological dose of LH, in line with an earlier publication [25], to confirm that LH diffused into all the cells of an entire slice. LH has been shown to suppress/desensitise its own receptor expression [40]; however, in human chorionic gonadotropin (hCG) over-expressing transgenic mice, constitutively increased bioactive hCG plasma levels did not affect testicular *Lhcgr* expression [41]. Thus, the exact effects of LH on the expression and activity of its own receptor in heat-acclimated rats may require further studies.

In this study, *Star* mRNA levels were significantly upregulated by LH stimulation *in vitro* in all the treated groups compared with the controls, whereas STAR protein levels remained unchanged. A previous study [42] showed that 8 h LH/hCG treatment of isolated murine Leydig cells upregulates STAR expression at the mRNA and protein levels. A plausible explanation for this discrepancy in STAR protein expression after LH stimulation may be due to the stimulation of testicular slices, and longer stimulation periods may have been needed to upregulate this protein expression in our experimental model. Regarding the gene expression and activity of the steroidogenic enzymes, we observed three distinct response

patterns to LH treatment in heat-acclimated compared with control testes. First, the upregulation of intra-testicular steroidogenic gene expression with no changes in enzymatic activity (*Cyp11a1* in the AHA and NHA, *Hsd17b3* in the AHA vs. basal) was observed in response to *in vitro* LH treatment. Upregulated *Cyp11a1* after 12-24 hours of LH treatment in porcine primary [40] and MA-10 Leydig cells [43] or LH-mediated dose-dependent inhibition of *Hsd17b3* expression and HSD17B3 activity has been previously shown [44]. Heat acclimation appeared to accelerate the response to LH at the level of *Cyp11a1* and *Hsd17b3* transcription by a currently unknown mechanism. Upregulation of *Cyp11a1* and *Hsd17b3* by LH was not accompanied by increased CYP11A1 activity in the NHA group or by increased HSD17B3 activity in the AHA group, which is most likely due to the need of stimulation to translate genes for more than 8 h [43]. After the cessation of high ambient temperature treatment (DA group), LH-induced *Cyp11a1* transcription disappeared, suggesting that this effect is transient.

Second, LH-induced effects were related to increased steroidogenic enzyme activity without respective increases in mRNA transcription. Eight hours of LH treatment increased HSD3B1, CYP17A1 and HSD17B3 activity in the NHA group. Possible mechanisms for this effect involve the posttranslational modification of these enzymes [45] or the ability of these enzymes to interact with their red-ox partner proteins [46]. The third response pattern after LH treatment involves the neonatal heat-acclimated rats and the absence of an effect on CYP19A1 activity in the NHA and DA groups in contrast to the CR and AHA groups. This study demonstrated the increased susceptibility to LH in neonatal (NHA) heat-acclimated testicular slices with respect to steroidogenic gene expression and/or the activity of steroidogenic enzymes. The finding is further supported by the increased intra-testicular androgen production, especially testosterone, observed in the NHA group compared with the basal levels and the control group response.

We selected PRL to stimulate the testicular slices because high plasma PRL levels have been observed in neonatally heat-acclimated rats [25]. High PRL doses have known inhibitory effects on testicular steroid production [47], and the same effect on the increased plasma PRL level in NHA rats may be prevented by upregulated intra-testicular basal *Prlr2* expression because the short form of PRLR has been demonstrated to act as a negative regulator of PRL signal transduction *via* the PRLR long form [48]. PRL treatment of testicular slices *in vitro* also decreased *Prlr1* in NHA and *Prlr2* in all the groups compared with the basal levels. Similar effects of PRL stimulation on *Prlr1* and *Prlr2* expression were previously determined in MA-10 Leydig tumour cells [49]. Intra-testicular *Lhcgr* after PRL treatment was downregulated only in DA males. Previously, dose- and time-dependent effects of PRL-induced testicular *Lhcgr* upregulation was detected in murine Leydig cell primary cultures [47]. Thus, 8 h of testicular slice incubation with a rather high (100 ng/ml) PRL concentration was insufficient to induce *Lhcgr* changes. PRL treatment downregulated intra-testicular *Cyp11a1* in the DA group and *Hsd3b1* in the NHA group but increased *Cyp17a1* and CYP17A1 enzymatic activity in the NHA and DA groups compared with the basal levels. Downregulation of testicular *Hsd3b1* by PRL has been previously shown [21]. *Cyp17a1* regulation by PRL is not known and may be indirect. In our study, opposite effects of PRL on *Hsd3b1* and *Cyp17a1* expression may explain the lack of effect of PRL on the NHA group testicular slice steroid production *in vitro*.

In summary, our present data revealed two individual mechanisms underlying rat heat acclimation for neonatal and adult ages. In the NHA males, transcriptional regulation of the levels of the steroidogenic enzymes was pronounced, whereas in the AHA males, the enzymatic activity was altered. Despite these two distinct mechanisms, the ultimate biological outcome, i.e., steroidogenesis, was similar in both the neonatal and adult heat-acclimated males, which is reflected by the similar plasma concentrations of steroid hormones in the AHA and NHA groups. These data also indicate that heat treatment of rats prior to puberty may induce irreversible changes in the transcription efficacy of particular enzymes in the steroidogenic pathway (e.g., *Cyp11a1*, *Hsd3b1*) that persist after de-acclimation during adulthood.

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