High temperature suppressed the steroidogenesis in a mouse tumor Leydig cells with a involvement of Bmal1 and autophagy

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Abstract

Although high temperature induced disorder of testosterone secretion in males is well illustrated across species, its recovery process has not been analyzed, especially in Leydig cells. Here we investigated changes of progesterone, expressions of steroidogenesis-related genes and circadian gene, autophagy-related proteins in Leydig cells treated with heat exposure. The results showed reduced progesterone and cAMP concentration after heat exposure, and both of them surged at 4h in the recovery period. Although immediately suppressed after the heat exposure, Star gene showed rapid increase in the protein and gene levels. hCG supplementation dramatically enhanced gene and protein expressions of Bmal1, which displayed a gradually decrease in the recovery period. Cell autophagy also involved in the steroidogenesis, as identified by the cellular accumulation of p62 and LC3 II proteins, and the proteins were reduced in the recovery period after heat exposure. The results suggest that circadian gene and cell autophagy are involved in the steroidogenesis and suppressed by heat exposure, indicating an absence of them in the process of the rapid increased capacity of hormone synthesis in the recovery period.

Key words: steroidogenesis, high temperature, Bmal1, autophagy

1. Introduction

Mammalian spermatogenesis rely on a robust amount of testosterone, which is mainly produced by Leydig cells in the testis (Awoniyi et al., 1989; Chen et al., 1994; Ewing and Zirkin, 1983). The hypothalamic-pituitary axis is integrally involved in the regulation of testosterone reproduction (Zirkin and Papadopoulos). GnRH, secreted by the hypothalamic, binds to the receptor on the pituitary and stimulates the synthesis and release of luteinizing hormone (LH) to the blood (Kaprara and Huhtaniemi, 2017; Plant, 2015). LH initiates the intracellular signaling cascades of testosterone biosynthesis by binding to the LH receptors on the Leydig cell and bursting the cAMP signaling. Then the cholesterol is transferred the inner mitochondrial membrane through the steroidogenic acute regulatory (StAR) protein and converted to pregnenolone by Cytochrome P450 Family 11 Subfamily A1 (Cyp11a1) (Manna and Stocco, 2005). After delivered to the endoplasmic reticulum, pregnenolone was catalyzed to progesterone by the 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD), which was finally metabolized to testosterone by 17α-hydroxylase (CYP17) and 17β-hydroxysteroid dehydrogenase (17β-HSD).

Mammalian spermatogenesis in the testis is thermosensitive, which is more susceptible to hyperthermia than the other organs (Setchell and B., 1998). In additional to spermatogenesis, high ambient temperature also disrupts the steroidogenesis in Leydig cells. Previously, our study in mice observed an advanced acrophase of serum testosterone under heat treatment (Li et al.). Short time heat exposure reduced serum testosterone concentration in mouse and rat (Li et al., 2016; Lin et al., 2016). In Leydig cells, heat treatment suppressed the steroidogenesis via a reduced expression of Star protein or endoplasmic reticulum stress (2016; Liu and Stocco, 1997; Murphy et al., 2001). Here we investigated the steroidogenesis-pathway in the recovery phase upon the termination of heat exposure to further reveal the mechanism of the blocked steroidogenesis capacity by high ambient temperature.

2. Materials and methods

2.1 Cell culture

The MLTC-1 cell line was purchased from Procell (Wuhan, China), and cultured at 5% CO_2 and 37°C. The ingredient of the 50ml culture medium were 44.5ml RPMI 1640 medium (Gibco, Waltham, MA, USA), 5ml fetal bovine serum (ScienCell, Carlsbad, CA, USA) and 500µl penicillin/streptomycin (ScienCell, Carlsbad, CA, USA). The protocol of this study was approved by the committee of Animal Research Institute (Certification No. SYXK (Su) 2011-0036), Nanjing Agricultural University, China.

2.2 Heat exposure and progesterone detection

Cells were treated with 1% FBS in RPMI 1640 medium for 12h when the cells grew to 70-80% confluency in each of the 12 wells. Afterwards, cells were treated with pure RPMI 1640 medium containing 0.1IU/ml hCG (Sigma-Aldrich, St. Louis, MO, USA) for 2h, followed by an incubation at 40°C in a 5% CO2 incubator for 2h. After the heat exposure, cells were returned to an incubator at 37°C and collected at 0h, 1h, 2h, 4h, 10h and 16h. The supernatant were detected for progesterone

concentration using a mouse enzyme linked immunosorbent assay ELISA kit (Meimian, Jiangsu, China). Leydig cells were lysed with RIPA protein lysis buffer (beyotime, Shanghai, China) for 30min on ice. The lysates were centrifuged at 12,000g and the supernatant were collected. Protein concentration was measured by a BCA protein assay kit (Takara, Dalian, China).

2.3 Heat exposure and cAMP detection

Cells were treated with 1% FBS in RPMI 1640 medium for 12h when the cells grew to 70-80% confluency in each of the 6 wells. Then cells were divided into two parts: one for heat exposure and the other one in normal culture. The procedure of heat treatment was same as 1.2 and cells were collected at 0h, 1h, 2h, 4h, 10h and 16h. In normal cultured cells, one group was cultured with pure RPMI 1640 medium for 4h and defined as C-; another group was cultured with pure RPMI 1640 medium containing 0.1IU/ml hCG and defined as C+. Cells were treated with 125μm/l IMBX (3-Isobutyl-1-methylxanthine, Sigma-Aldrich, St. Louis, MO, USA) for 30min, then the IMBX was discarded and cAMP was extracted by ethanol with a alternate freezing and thawing from -80°C to 37°C for 3-4 times, which lasted for 30min during each time. The ethanol was collected and dried in cold wind. cAMP was dissolved in 0.01mol/L acetate buffer (pH 6.2) and the concentration was determined by an ELISA kit (Meimian, Jiangsu, China). Protein concentration was detected as described in 1.2.

2.4 Western blotting

Quantification of protein expression, such as Star (1:800), Cyp11a1 (1:1000, CST, Danvers, IL, USA), LC3 (1:1000), Bmal1 (1:800, Sigma-Aldrich, St. Louis, MO, USA), p62 (1:500, Santa Cruz, CA, USA) and β-actin (Beyotime, Shanghai, China) were determined by western blot. The total cellular protein (50μg) was loaded in each lane of 12% PAGE with a Miniprotean Tera System (BioRad, Hercules, CA, USA) using Precision Plus Protein molecular weight standards (BioRad). The proteins

were transferred to nitrocellulose membranes (EMD Millipore Corporation, Billerica, MA, USA). Membranes were incubated with 5% defatted milk powder which was dissolved in PBS for 1 h at room temperature to block non-specific binding. The membranes were incubated with the primary antibodies overnight at 4°C, then washed and incubated with HRP-labelled goat anti-rabbit immunoglobulin G (IgG, Beyotime, Shanghai, China). Finally, the membranes were incubated with enhanced chemiluminescence (ECL) detection reagents and scanned in a computer by a Scion Image v. 4.0.2 (Scion Corporation, Frederick, USA). The band densitometry was quantified by Image-J and β-actin was used as the cytosolic control.

2.5 Immunofluorescence

MLTC-1 cells (250000 cells) were plated on glass coverslip dishes in 6 well plate. When cells grew to 80% confluency, the plates were treated with hCG and heat exposure. Coverslip dishes were collected at 0h, 2h and 8h after the termination of heat exposure. Cells were fixed in 4% paraformaldehyde for 1h. Then dishes were cleaned with PBS and permeabilized with 0.1% Triton X-100 for 10min. Then cells were blocked with 1% bovine serum albumin (BSA) for 1h with agitation. After discard of BSA, Star was detected with a rabbit anti-Star antiserum (1:100) in PBS + 1% BSA overnight at 4°C with agitation. The next day, cells were washed with PBS and incubated with a goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Beyotime, Shanghai, China) in PBS + 1% BSA for 1h at room temperature. Then cells were washed and incubated with 3μg/ml 4',6-diamidino-2-phenylindole (DAPI, a DNA fluorescent dye) in PBS at room temperature for 10min in dark area. Finally cells were washed and the coverslips dishes were put on a microscope slide with fluorescence anti-fade reagent (Solarbio, Beijing, China). Images were obtained using a laser confocal microscope (Thermo Fisher, Waltham, MA, USA) and the Image-Pro Plus software (Carl Zeiss, Jena, Germany).

2.6 RNA extraction and RT-PCR

Cells were collected at 0h, 2h and 8h after heat exposure. NT groups were normal culture and HT groups were heat treated. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, US) and treated with DNase I (RNase-free) (TaKaRa, Dalian, China) to remove genomic DNA. The RNA concentration and purity were determined spectrophotometrically at 260 and 280nm with a Nanodrop 8000 (Thermo Fisher, Waltham, MA, USA). For each sample, 1μg of total RNA was reversely transcribed to cDNA with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (TaKaRa, Dalian, China) and oligo-nucleotide primers.

Target genes and the housekeeping gene β-actin were quantified by real-time PCR on an ABI 7300 system using a commercial kit (SYBR Premix Ex Taq, TaKaRa, Dalian, China). The gene-specific primers were designed based on the corresponding mRNA sequences with Primer Version 5.0 (Table 1). PCR reactions (consisting of SYBR Premix Ex Taq, ROX Reference Dye, 200nM primer, and 100ng cDNA template) were run in triplicates in a 20μl total reaction volume. The relative mRNA concentration was calculated using the 2-ΔΔCt method. The values were normalized using β-actin as the endogenous standard. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between treated and control groups.

2.7 Statistical analysis

All the measurements were made in triplicate, and all the values are presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis were performed using GraphPad Prism Version 5.0 soft-ware program (GraphPad Software, San Diego, CA, USA): for progesterone concentration in medium, t-test was used to compare the difference between groups at the same time points; for gene expressions, t-test was used to compare difference between C- and NT-0h as well as groups at the same time points, and then one way analysis of variance (ANOVA) was used the

compare difference between groups with the same treatment; for protein expressions, t-test was used to compare difference between C- and C+ group and ANOVA was used to compare difference between groups except for C- group. $P < 0.05$ was considered significant.

3. Results

3.1 Effects of heat treatment on progesterone production in MLTC-1 cells

Progesterone is the main steroid metabolite of MLTC-1 (Rebois, 1982; Zirkin and Papadopoulos), so we monitored the progesterone production after heat exposure. Progesterone secretion was significantly reduced immediately after heat exposure (Fig.1 $p < 0.01$). From 0h to 2h, progesterone secretion capacity was gradually decreased, while it was sharply raised from 2h to 4h for the two groups. From 4h to 16h, the secretion capacity was slowly decreasing. The integrated variation of progesterone secretion was same for the two groups.

3.2 Effects of heat treatment on cellular cAMP level

hCG supplementation significantly increased cellular cAMP levels in MLTC-1 (Fig.2 $p < 0.01$). In the recovery period after heat treatment, cellular cAMP production was reduced and the differences were significant at 1h, 2h, 8h and 16h ($p < 0.01$). The variation of cAMP production in heat exposed cells was similar to that of the progesterone secretion.

3.3 Effects of heat treatment on Star and Cyp11a1 protein expression

hCG supplementation significantly enhanced Star protein expression (Fig.3B, $p < 0.01$) and heat treatment significantly suppressed the expression of Star protein ($p < 0.05$). In the recovery period after heat exposure, Star protein expression was gradually improved from 0h to 16h (Fig.3B), and the expression at 10h was significant higher than that in C+ (Fig.3B, $p < 0.01$). Immunofluorescence of Star protein also observed none immune-positive staining cells after heat exposure at 0h

(Fig.4a). From 2h to 8h after heat exposure, the amount of immune-positive cells showed a rapid expansion (Fig.4b, c).

Cyp11a1 protein expression was also significantly increased after hCG stimulation and it was significantly reduced immediately after heat exposure (Fig.3C, $p < 0.05$). In the recovery period after heat exposure, Cyp11a1 protein showed a consistently low level expression.

3.4 Effects of heat treatment on Star and Cyp11a1 gene expression

Star gene expression was significantly increased in hCG stimulated MLTC-1 cells (Fig.5A, $p < 0.05$) and the expressions gradually attenuated in the recovery phase at 2h and 8h ($p < 0.01$). In HT groups, Star gene also attenuated in the recovery phase ($p < 0.01$, $p < 0.05$), while the expressions were significantly higher than that in the corresponding NT groups at the same time point ($p < 0.01$). Cyp11a1 gene expressions in NT groups were significantly enhanced at 2h and 8h in the recovery phase than that at 0h (Fig.5B, $p < 0.01$) and there were significant lower expressions in HT groups at 2h and 8h than these in the NT groups at the corresponding time points ($p < 0.01$).

3.5 Effects of heat treatment on Bmal1 protein and gene expression

Cellular Bmal1 protein level was significantly improved in hCG stimulated cells (Fig.6B, $p < 0.05$). In the recovery period after heat exposure, Bmal1 protein expression was gradually attenuated. As to the gene expression, hCG stimulation significantly enhanced the mRNA levels in MLTC-1 cells (Fig.5C, $p < 0.01$). In NT groups, a significantly increase was observed from 0h to 2h in the recovery period ($p < 0.05$). 2h and 8h after heat exposure, the Bmal1 gene expressions were significantly lower than that at 0h ($p < 0.01$) and also lower than that in the NT group at the same time points, respectively ($p < 0.01$).

3.6 Effects of heat treatment on autophagy-related proteins and gene expression

P62 and LC3 I/II protein are involved in the formation and degradation of autophagosome. Here we found that hCG stimulation significantly increased the expression of p62 protein and the ratio of LC3 II to LC3I (Fig.7B, C, $p < 0.05$, $p < 0.01$). In the recovery period after heat exposure, p62 protein showed a stable and low level expression, and the ratios of LC3 II to LC3I in cells from 0h to 16h were significantly lower than that in C+ group ($p < 0.01$).

4. Discussion

In males, the steriod hormone testosterone plays an essential role in the spermatogenesis and genital activity, which is mainly synthesized and secreted by the Leydig cells in the testis. Deficient in the production of testosterone would ruin the entire process of spermatogenesis (Sharpe et al., 1990). Previously, we observed a disorder in the daily oscillations of serum testosterone and testicular expression of steroidogenesis-related genes upon high temperature exposure (Li et al.), which indicated an dysfunction of Leydig cells under thermal stress. So here Leydig cells were used to further clarify the effects of heat exposure on steroidogenesis, circadian gene expression and autophagy.

Differ from the Leydig cells in vivo, MLTC-1 and MA-10 cells produce progesterone rather than testosterone (Rebois, 1982; Zirkin and Papadopoulos), which is an intermediate in the process of steroidogenesis. LH-cAMP signaling pathway involved in the hCG-application induced steroid hormone production. In MA-10 cells, acute heat stress for 10mins incubation at 45°C caused a significantly reduced progesterone production cells 3h after a stimulation by cAMP analogues in the recovery phase, while the progesterone normalized to the control group at 6h after the application (Liu and Stocco, 1997). Cellular cAMP concentration in cultured Leydig cells from rats showed a significant increase treated with LH for 30min or 1h(Baburski et al., 2019), and it was usually 4h when cells were collected for the detection of cAMP and progesterone concentrations after LH application (Feng et al., 2018; Xha et al., 2019). In our present study, progesterone secretion capacity was

distinctly suppressed by the heat exposure, and it reached to a peak at 4h in the recover phase for both groups. The fluctuation of the cell progesterone secretion capacity indicated that cells do not constantly conduct the hormone synthesis under LH stimulation. Similar to progesterone, cellular cAMP concentration also showed a slight decrease in the first 2h and reached a peak at 4h in the recover phase. Also the enhanced cAMP content after hCG stimulation verified the activation of LH-cAMP signaling pathway.

In the process of cholesterol translocation, Star acts at the mitochondria to trigger cholesterol movement across the membranes, which is the rate-limiting and -determining step in steroidogenesis (Clark, 2016; Miller and Bose, 2011). CYP11A1 is located in the inner mitochondrial membrane, and catalyzes the conversion of cholesterol to pregnenolone, which determines the biosynthetic capacity of the Leydig cells (Payne and Hales). Previously our in vivo study revealed that heat exposure blocked Star protein expression, and it returned to the equivalent level as the control group at 8h after the heat treatment (Li et al.). In MA-10 cells, Star and CYP11A1 protein expressions were significantly reduced at 3h and 6h after an acute heat stress (Liu and Stocco, 1997). The gene expressions of Star, CYP11A1 and 3β-HSD in MA-10 cells were gradually increased from 0h to 6h in the recovery phase after the heat exposure (Murphy et al., 2001). The present study confirmed the rapid increase in Star protein expression after heat exposure, with much higher gene expressions throughout the recovery phase. While protein and gene expressions of CYP11A1 stayed a weak level in the recovery period.

Bmal1 (brain and muscle ARNT-like protein, also known as MOP3 or ARNT3) belongs to the family of the basic helix-loop-helix (bHLH)-PAS domain-containing transcription factors (Lowrey, 2011). In a heterodimer with another member of this family, CLOCK, Bmal1 regulates gene expression through E-box elements in their promoters (Gekakis et al., 1998; Yoo et al., 2005). Study on the autonomous circadian rhythm of glucocorticoid in the adrenal revealed a direct contact of Bmal1 protein

with the E-box elements in the promote of Star gene, which up regulated the gene expression (Son et al., 2008). Mice deficient in Bmal1 gene showed considerably reduced serum testosterone and testicular Star gene expression (Kondratov et al., 2006). The present study observed a significantly increased gene and protein expressions of Bmal1 after the hCG application, which verified the involvement of Bmal1 in steroidogenesis. Heat exposure dramatically blocked the Bmal1 expression both in gene and protein levels, with a continues reduction of protein expression in the 16h recovery phase. This phenotype was contrast with the Star expression levels, which indicated that heat exposure ruined the regulation function of Bmal1 on Star gene.

In Leydig cells, autophagy has been reported to be extremely active (Jing and Tang, 2010; YI et al., 1999). Autophagy is a cellular metabolic process that removes protein aggregates and damaged organelles via lysosomal degradation and provides raw materials as ‘emergency energy’ to maintain cell survival under stress conditions (A. et al., 2015; Schneider and Sanchez, 2016). Studies have shown that autophagy regulates testosterone synthesis by facilitating cholesterol uptake in Leydig cells (Gao et al., 2018). Our present results indicated a intracellular accumulation of p62 and LC3 II proteins after hCG application and the enhanced autophagy was consistent with the previous study. 2h whole body heat exposure caused a reduced p62 and LC3 II protein levels in the testis (Li et al., 2018). Conversion of LC3 II protein was gradually increased in the first 15mins after heat exposure in Hela cells, while it was gradually reduced from 30min to 60min (Kassis et al.). Our results observed a gradually reduced conversion of LC3 II in the first 2h in the recovery phase, which was consistent with the reduced progesterone and Star protein expression.

In conclusion, our results confirm that heat exposure reduced the steroidogenesis via the blocked LH-cAMP signaling pathway and protein expression of Star and Cyp11a1. Additionally, circadian protein Bmal1 and cell autophagy involved in the steroid hormone synthesis, which were both suppressed by heat exposure. Although

315 further studies are needed to elucidate the mechanism of reduced Bmal1 protein
316 expression and autophagy activity with rapidly increased Star protein expression, this
317 work improves our understanding of heat exposure on Leydig cell function in the
318 recovery period.

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322 **Declaration of interests**

323 The authors declare that there is no conflict of interest.

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327 Figure legends

328 Figure1 Progesterone production in MLTC-1 cells treated with heat exposure. NT: neutral
329 treatment, cells were cultured in normal condition; HT: heat treatment, cells were exposed to
330 a 4h 40°C ambient temperature. Results were presented as mean ± S.E.M. of three replicate
331 samples. **$p < 0.01$ versus the HT group at 0h.

332 Figure2 Cellular cAMP level in MLTC-1 cells treated with heat exposure. C-: no hCG
333 treatment; C+: hCG treatment; 0h to 16h: recovery time upon the termination of heat
334 exposure; black line means that cells in C+ group to 16h group were treated with hCG; red
335 line means that cells in 0h group to 16h group were treated with heat exposure. Results were
336 presented as mean ± S.E.M. of three replicate samples. **$p < 0.01$ versus the C- group and
337 ##$p < 0.01$ versus the C+ group.

Figure3 Western blot analysis of Star and Cyp11a1 protein expression in MLTC cells exposed to heat treatment. β-actin was used to assess equal loading. A: representative western blots of Star and Cyp11a1 protein; B, C: the means ± S.E.M. of StAR and Cyp11a1 protein integrated optical density corrected by corresponding β-actin. C-: no hCG treatment; C+: hCG treatment; 0h to 16h: recovery time upon the termination of heat exposure; black line means that cells in C+ group to 16h group were treated with hCG; red line means that cells in 0h group to 16h group were treated with heat exposure. *$p < 0.05$ and **$p < 0.01$ versus the C- group; #$p < 0.05$ and ##$p < 0.01$ versus the C+ group.

Figure4 Immunofluorescence detection of Star protein in MLTC-1 cells exposed to heat treatment. NT: neutral treatment, cells were cultured in normal condition; HT: heat treatment, cells were exposed to a 4h 40°C ambient temperature; 0h, 2h and 8h: recovery time after heat exposure; A, B, D: NT groups at 0h, 2h and 8h in the recovery phase, respectively; a, b, d: HT groups at 0h, 2h and 8h in the recovery phase after heat exposure. Arrows indicated the immune-positive staining sites. Bar = 50 μm.

Figure5 Gene expressions of Star, Cyp11a1, Bmal1 and Atg5 in MLTC-1 cells exposed to heat treatment. A: Star gene; B: Cyp11a1 gene; C: Bmal1 gene; D: Atg5 gene. NT: neutral treatment, cells were cultured in normal condition; HT: heat treatment, cells were exposed to a 4h 40°C ambient temperature; C-: none hCG stimulation; 0h, 2h and 8h: recovery time after heat exposure; # $p < 0.05$ and ## $p < 0.01$ versus the C- group; * $p < 0.05$ and **$p < 0.01$ versus the corresponding groups with the same treatment at 0h, such as NT groups compare to NT group at 0h and HT groups compare to HT group at 0h; && $p < 0.01$ versus the corresponding NT groups at the same time points.

Figure6 Western blot analysis of Bmal1 protein expression in MLTC cells exposed to heat treatment. β-actin was used to assess equal loading. A: representative western blots of Bmal1 protein; B: the means ± S.E.M. of Bmal1 protein integrated optical density corrected by corresponding β-actin. C-: no hCG treatment; C+: hCG treatment; 0h to 16h: recovery time upon the termination of heat exposure; black line means that cells in C+ group to 16h group were treated with hCG; red line means that cells in 0h group to 16h group were treated with

heat exposure. *p < 0.05 versus the C- group.

Figure7 Western blot analysis of p62 and LC3I/II protein expression in MLTC cells exposed to heat treatment. β-actin was used to assess equal loading. A: representative western blots of p62 and LC3I/II protein; B, C: the means ± S.E.M. of p62 and LC3I/II protein integrated optical density corrected by corresponding β-actin. C-: no hCG treatment; C+: hCG treatment; 0h to 16h: recovery time upon the termination of heat exposure; black line means that cells in C+ group to 16h group were treated with hCG; red line means that cells in 0h group to 16h group were treated with heat exposure. *p < 0.05 and **p < 0.01 versus the C- group; #p < 0.05 and ##p < 0.01 versus the C+ group; &p < 0.05 versus the 1h group.

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