ERK1/2 regulates heat stress-induced lactate production via enhancing the expression of HSP70 in immature boar Sertoli cells

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Abstract

Lactate produced by Sertoli cells plays an important role in spermatogenesis, and heat stress induces lactate production in immature boar Sertoli cells. Extracellular signaling regulated kinase 1 and 2 (ERK1/2) participates in heat stress response. However, the effect of ERK1/2 on heat stress-induced lactate production is unclear. In the present study, Sertoli cells were isolated from immature boar testis and cultured at 32 °C. Heat stress was induced in a 43 °C incubator for 30 min. Proteins and RNAs were detected by western blotting and RT-PCR, respectively. Lactate production and lactate dehydrogenase (LDH) activity were detected using commercial kits. Heat stress promoted ERK1/2 phosphorylation, showing a reducing trend with increasing recovery time. In addition, heat stress increased heat shock protein 70 (HSP70), glucose transporter 3 (GLUT3), and lactate dehydrogenase A (LDHA) expressions, enhanced LDH activity and lactate production at 2-h post-heat stress. Pretreatment with U0126 (1×10^{-6} mol/L), a highly selective inhibitor of ERK1/2 phosphorylation, reduced HSP70, GLUT3, and LDHA expressions and decreased LDH activity and lactate production. Meanwhile, *ERK2 siRNA1* reduced the mRNA level of *ERK2* and weakened ERK1/2 phosphorylation. Additionally, *ERK2 siRNA1* reduced HSP70, GLUT3, and LDHA expressions decreased LDH activity and lactate production. Furthermore, *HSP70 siRNA3* downregulated GLUT3 and LDHA expressions and decreased LDH activity and lactate production. These results show that activated ERK1/2 increases heat stress-induced lactate production by enhancing HSP70 expression to promote the expressions of molecules related to lactate production (GLUT3 and LDHA). Our study reveals a new insight in reducing the negative effect of heat stress in boars.

Keywords GLUT3 · LDHA · siRNA · U0126

Introduction

Developing germ cells prefer using lactate as substrate for ATP production (Boussouar and Benahmed 2004; Rato et al. 2012). Sertoli cells actively metabolize glucose but the majority of it is converted to lactate (Regueira et al. 2015a). In seminiferous tubules, the majority of lactate utilized by germ cells comes from Sertoli cells (Boussouar and Benahmed 2004). Sertoli cells' abnormal proliferation and maturation

are associated with impaired spermatogenesis, male infertility, and testicular tumorigenesis (Oliveira et al. 2015). In addition to its energetic function, lactate enhances RNA and protein synthesis, and reduces the apoptosis of germ cells (Erkkila et al. 2002; Grootegeed et al. 1984; Oliveira et al. 2015). Thus, lactate may be a potential compound for optimizing in vitro methods involving male germ cells for assisted reproduction (Regueira et al. 2015a). Heat stress disturbs spermatogenesis, which results in impaired fertility (Fan et al. 2017; Liang et al. 2013). Emerging evidence shows that spermatogenesis can recover to normal after the withdrawal of mild heat stress (Liu 2010), and that lactate reduces excessive apoptosis of germ cells (Boussouar and Benahmed 2004). Therefore, the mechanisms that regulate lactate production post-heat stress is a key to restore normal spermatogenesis and male fertility.

The process of lactate production mainly includes the transport of glucose from extracellular to intracellular compartments, and the conversion of pyruvic acid to lactate (Schrade et al. 2016). Glucose transporters (GLUTs) and

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lactate dehydrogenase (LDH) participate in the transport of glucose and the conversion of pyruvic acid to lactate, respectively (Riera et al. 2002, 2009). Our previous results showed that GLUT3 and LDHA are the main molecules associated with lactate production in immature boar Sertoli cells (Bao et al. 2017).

Heat stress and growth factors can activate extracellular signal regulated kinase 1 and 2 (ERK1/2) (Nadeau and Landry 2007). Testicular heat stress significantly elicits ERK1/2 phosphorylation to prevent excessive apoptosis of mouse spermatocytes (Liang et al. 2013). ERK1/2 was activated and led to dedifferentiation of Sertoli cells by increasing their number in the cryptorchid testis of the rhesus monkey (Zhang et al. 2006a). These results infer that ERK1/2 plays a protective role during heat stress. However, it is unclear whether ERK1/2 regulates lactate production in immature boar Sertoli cells.

Heat stress induces the expression of heat shock protein 70 (HSP70) to regulate glucose metabolism in diverse cells and tissues (Gupte et al. 2011; Kavanagh et al. 2011; Kavanagh et al. 2009). Increment of HSP70 in old monkeys promoted glucose metabolism (Kavanagh et al. 2016). However, whether HSP70 enhances lactate production from glucose via regulating the expression of lactate production-related genes is unclear. Although both HSP70 and ERK1/2 play vital roles in heat stress, the relationship between the expression of HSP70 and heat stress-induced ERK1/2 is inconsistent in previously published studies (Chen et al. 2008; Ding et al. 2014; Stankiewicz et al. 2005). However, cadmium-induced activation of ERK1/2 led to the phosphorylation and activation of HSF1, which in turn induced HSP70 expression in rat brain tumor cells (Hung et al. 1998). Therefore, we hypothesize that heat stress-induced phosphorylation of ERK1/2 increases the expression of HSP70, which enhances the expressions of molecules related to lactate production, resulting in an increase of lactate production.

In the present study, our objective was to investigate the role of activated ERK1/2 signaling pathway in heat stress-induced lactate production, as well as the relationship between activated ERK1/2 signaling pathway and the expression of HSP70.

Methods

Animals used in this research

Animals used in the study were 3-week-old immature healthy boars ($n = 3-5$ in every experiment) from a local farm in Beibei, Chongqing, China. Experimental procedures involved were conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China) and approved by

the Institutional Animal Care and Use Committee in Southwest University (China).

Sertoli cell isolation and culture

Sertoli cell isolation and culture were performed according to previous procedures in our laboratory (Wang et al. 2015). In brief, testes were collected in a sterile manner from immature boars housed at a local farm. After excising the epididymides and fat, the testes were washed three times with 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4) [8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 1.44 g sodium hydrogen phosphate (Na_2HPO_4), and 0.24 g dipotassium phosphate (K_2HPO_4) per liter] and then cut into pieces of approximately 1 mm³. The pieces were homogenized with PBS and centrifuged at 2000×g for 5 min. Tissues were digested with collagenase IV (0.3%; Gibco, Grand Island, NY, USA) for 40 min at 32 °C and centrifuged at 2000×g for 5 min. Tissue pellets were digested with trypsin (0.25%; Solarbio Science & Technology Co., Ltd., Beijing, China) for 20 min at 32 °C and centrifuged at 2000×g for 5 min. Tissue pellets were resuspended with Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1; Gibco, Grand Island, NY, USA), and the suspension was filtered through stainless steel sieves with 0.2- and 0.038-mm apertures, respectively. Cells were collected, centrifuged at 2000×g for 5 min, resuspended with DMEM/F-12, counted with a hemocytometer, transferred into either 25-cm² tissue culture flasks (1×10^6 cells/flask) or 96 multiwell plates (5×10^3 cells/well) (Thermo Scientific Technology, Rockford, IL, USA), and cultured in DMEM/F-12 containing 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% (v/v) penicillin/streptomycin (10,000 U/mL; Gibco, Grand Island, NY, USA) at 32 °C in a humidified atmosphere containing 5% CO₂. After 24 h, medium was replaced. Cells were utilized in the following experiments until 70–80% confluences were reached.

Protein extracts and western blotting

Cells were lysed with cell lysis buffer [0.02 mol/L Tris (pH 7.5), 0.15 mol/L NaCl, 1% Triton X-100, 2.5×10^{-3} mol/L sodium pyrophosphate, 1×10^{-3} mol/L EDTA, 1% Na_3VO_4 , 0.5 µg/mL leupeptin, and 1×10^{-3} mol/L phenylmethanesulfonyl fluoride (PMSF)] (Beyotime, Shanghai, China). Lysates were centrifuged (12,000×g for 10 min) and supernatant was collected. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China), according to the manufacturer's instructions. Approximately 25 µg of total proteins per lane was separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide-bisacrylamide for the resolving gel and 5% acrylamide-bisacrylamide for the stacking gel),

products of which were electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with blocking buffer [PBS-Tween containing 5% dried skim milk or 3% bull serum albumin (BSA, Solarbio Science & Technology Co., Ltd., Beijing, China)] for 2 h at room temperature and probed with primary antibodies that recognized pERK1/2 (8544 s, rabbit, 1:1000; Cell Signaling Technology, Beverly, MA, USA), ERK1/2 (RLT1625, rabbit, 1:300; Ruiying, Suzhou, Jiangsu, China), HSP70 (RLM3042, mouse, 1:300; Ruiying, Suzhou, Jiangsu, China), GLUT3 (bs-1207R, rabbit, 1:300; Bioss, Beijing, China), LDHA (bs-1810R, rabbit, 1:300; Bioss, Beijing, China), and beta-actin (bs-0061R, rabbit, 1:1000; Bioss, Beijing, China) at 4 °C overnight. Membranes were then incubated with either goat anti-rabbit or goat anti-mouse immunoglobulin G coupled to horseradish peroxidase (1:1000; Beyotime, Shanghai, China) for 2 h at room temperature. Immunoreactive proteins were visualized with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific Technology, Rockford, IL, USA) on a chemiluminescent imager (Bio-Rad, Hercules, CA, USA). Membranes were stripped and probed again with other primary antibodies including against beta-actin serving at the loading control. The protein band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). The densitometric value of pERK1/2 was normalized to that of total ERK1/2 in the same membrane. The densitometric values of HSP70, GLUT3, and LDHA were normalized to that of beta-actin in the same membrane.

RNA extracts and quantitative RT-PCR

Total RNA was extracted with RNeasy Pure Cell/Bacteria Kit (Qiagen Biotech, Beijing, China), following the manufacturer's instructions. The integrity of RNA was identified by 1% agarose gel electrophoresis. The complementary DNA (cDNA) was obtained with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). In a first-strand cDNA synthesis

reaction system, total volume of per reaction was 20 µL, including 4 µL total RNA (0.5 µg/µL), 4 µL 5× iScript reaction mix, 1 µL iScript reverse transcriptase, and 11 µL nuclease-free water. Reverse transcription was performed at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. The cDNA was used for quantitative real-time polymerase chain reaction (RT-PCR), performed with 5 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 3.5 µL RNase-free water, 0.5 µL forward primer, 0.5 µL reverse primer, and 0.5 µL cDNA using a PerkinElmer multi-wall. Initial DNA denaturation was at 95 °C for 30 s, annealing was at 95 °C for 10 s, extension and plate read was at 60 °C for 30 s, and 40 cycles were conducted. Melting-curve analysis was performed between 65 and 95 °C, collecting the cycle threshold (CT) value in increments of 0.5 °C every 5 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method using *beta-actin* as the house-keeping gene. The sequences of primers were shown in Table 1. All primers were designed and synthesized by Sangon Biotech Company (Shanghai, China).

Cell transfection

When Sertoli cells reached 70–80% confluence, the cells were transfected with small interfering RNA (siRNAs; Invitrogen, Carlsbad, CA, USA) or negative control (NC; Invitrogen, Carlsbad, CA, USA) using cationic liposomes (Lipofectamine 2000 reagent; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 9 µL siRNAs or 9 µL NCs were prediluted in 500 µL Opti-minimum essential media (MEM) reduced serum medium (Gibco, Grand Island, NY, USA). Subsequently, 50 µL Lipofectamine 2000 was prediluted in 500 µL Opti-MEM reduced serum medium and then combined with the siRNA or NC diluted solution. The mixture was incubated for 5 min at room temperature to form transfection complexes and added to the Sertoli cells, which were then incubated at 32 °C in a humidified atmosphere

Table 1 Primer sequences

Gene	Sequence number	Product length	Annealing temperature	Sequences
<i>Beta-actin</i>	XM_003357928.1	130 bp	62.5	F 5'CTAGTTACACACACGCGGCTCT3' R 5'CATGAATACCCTGCACAGATCG3'
<i>ERK1</i>	XM_013991188.1	146 bp	59.9	F 5'CAGTCTCTGCCCTCCAAGA3' R 5'AGGTAAGGATGAGCCAGTGC3'
<i>ERK2</i>	NM_001198922.1	121 bp	57.8	F 5'CCCCATCACAGGAAGACCT3' R 5'GCTTTGGAGTCAGCATTTGG3'
<i>SLC2A3</i>	XM_003355585.3	105 bp	59.8	F 5'AAGGAGAGACCAAGGGAACC3' R 5'CGGGAGGCAGGATTAGAAC3'
<i>LDHA</i>	NM_001172363.2	126 bp	60.0	F 5'CGTCAGCAAGAGGGAGAAAG3' R 5'CACTGGATTGGAAGCAACAA3'
<i>HSP70.2</i>	NM_001123127.1	368 bp	60.0	F 5'TGTCGTCCCCTCTTCTCCA3' R 5'CTTACACTCCCGCACCTGA3'

containing 5% CO₂ for 6 h. Thereafter, the medium was replaced with fresh DMEM/F-12 without penicillin/streptomycin. The cells were incubated for more than 48 h and harvested at the indicated time for further analysis. The sequences of *ERK2* and *HSP70* siRNAs are shown in Table 2.

Cell viability assay

Sertoli cells (5×10^3 cells/well) were cultured in 96-multiwell plates. Cells pretreated with U0126 (Sigma-Aldrich, St. Louis, MO, USA), a highly selective inhibitor of ERK1/2 phosphorylation (Bironaite et al. 2013), were incubated for 2 h at 43 °C in a humidified atmosphere containing 5% CO₂ for 30 min and recovered at 32 °C for 2 h. Cell viability was assayed, using enhanced Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China). In brief, CCK-8 solution (20 µL/well) was added and cells were incubated for 1 h. Then, absorbance was measured at 450 nm using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was calculated by the formula as follows: cell viability (%) = (absorbance of treatment group – absorbance of blank group) / (absorbance of control group – absorbance of blank group) × 100%.

Determination of lactate concentration

For determination of the concentration of lactate produced by Sertoli cells, a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) was used. Both the intracellular and extracellular lactate content were measured. After the end of treatment, the Sertoli cells' culture medium was collected for measurement of the extracellular lactate content. Cells were lysed with cell lysis buffer and lysates were centrifuged (12,000×g for 10 min). The cell supernatant was collected for measurement of the intracellular lactate content. Absorbance was measured at 530 nm using a Bio-Rad 680 microplate reader. The lactate solution was diluted twice using 0.9% NaCl, and ddH₂O served as a blank group. The coefficient of variation (CV) for lactate assays was 1.7%. Lactate concentration was calculated by the formula in our previous study (Bao et al. 2017).

Determination of LDH activity

Sertoli cells were lysed with cell lysis buffer and lysates were centrifuged (12,000×g for 10 min). The cell supernatant was collected for measurement of the intracellular LDH activity. LDH activity was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), according to the manufacturer's instructions. Absorbance was measured at 440 nm using a Bio-Rad 680 microplate reader. LDH activity was calculated by the formula as follows:

$$A_x = (OD_x - OD_b) / (OD_s - OD_b) \times C_s \times C_p$$

where A_x is the LDH activity, OD_x is the absorbance value of the sample, OD_b is the absorbance value of the blank control, OD_s is the absorbance value of the standard, C_s is the standard concentration ($C_s = 2 \times 10^{-3}$ mol/L), and C_p is the total concentration of proteins.

Statistical analysis

All data are represented as mean ± standard error (SR) of three times in repeated trials, and each trial included three samples. One-way analysis of variance (ANOVA) was performed using GraphPad InStat version 6.02 (GraphPad Software, San Diego, CA, USA). Tukey's multiple comparison test was applied to determine treatment differences. Probabilities < 0.01–0.05 were considered statistically significant. GraphPad InStat version 6.02 and Adobe Illustrator CS6 were used to create the figures.

Results

Changes of pERK1/2, HSP70 expression, and lactate production at various periods of time post-heat stress

Heat stress increased pERK1/2 (phosphorylated ERK1/2) at the 0- to 2-h time points compared with control (cells

Table 2 siRNA sequences

	siRNA1	siRNA2	siRNA3
<i>ERK2</i>	5'-AAAC	5'-GCCA	5'-CAGCAUUUCCACUUGUACCA
	AGAUCUCUACAAGCUCUUGAA-3'	GGAUACAGAUUUUUUUUGU-3'	UUUUAU-3'
	5'-UUCAAGAG	5'-ACAA	5'-AUAAAUGGUACAAGUGGAAA
	CUUGUAGAGAUCUGUUU-3'	AUUUAGAUCUGUAUCCUGGC-3'	UGCUG-3'
<i>HSP70</i>	5'-AAAC	5'-ACUUCUCAAUGGCCGGGAA	5'-AGAC
	ACGGUAUUCUGCGGAUUCAGG-3'	CUGAA-3'	CUUACAGUCACAGCUGACUUG-3'
	5'-CCUGAAUCCGCAGAAUACCG	5'-UUCAGUUCGCCGCC	5'-CAAG
	UGUUU-3'	AUUGAAGAAGU-3'	UCAGCUGUGACUGUAAGGUCU-3'

without treatment) ($P < 0.05$ and 0.01 , respectively; Fig. 1a). ERK1/2 phosphorylation was the peak at 0-h post-heat stress ($P < 0.01$). With the extension of time post-heat stress, pERK1/2 decreased gradually, and was 87% of the control at 6-h post-heat stress. HSP70 protein level was enhanced from 1- to 6-h post-heat stress ($P < 0.05$ and 0.01 , respectively), and increased 350% of the control at 2-h post-heat stress ($P < 0.01$; Fig. 1b). Total lactate production showed no significant change during 0- to 1-h post-heat stress ($P > 0.05$). However, lactate increased by 18% of the control at 2-h post-heat stress and significantly increased in a time-dependent manner from 2 to 6 h compared with control ($P < 0.01$; Fig. 1c).

Effect of U0126 treatment on cell viability and heat stress-induced pERK1/2

The inhibitory effect of U0126 on cell viability gradually increased, accompanying the increasing concentrations of U0126. U0126 (1×10^{-6} mol/L) reduced cell viability by 4%, but there was no significant difference compared with control ($P > 0.05$; Fig. 2a), whereas higher concentrations of U0126 significantly reduced cell viability by 46, 51, 63, and 87%, respectively ($P < 0.05$ and 0.01 , respectively; Fig. 2a). In addition, pERK1/2 was reduced by 71% using 1×10^{-6} mol/L U0126 compared with control ($P < 0.01$; Fig. 2b).

Effect of U0126 on heat stress-induced lactate production and related molecules

U0126 with heat stress inhibited pERK1/2 by 35% compared with cells exposed to heat stress alone ($P < 0.05$; Fig. 3a), whereas only U0126 did not suppress pERK1/2 ($P > 0.05$; Fig. 3a). In addition, U0126 significantly reduced protein expression levels of HSP70, GLUT3, and LDHA by 40, 19, and 74%, respectively, compared with heat stress alone ($P < 0.01$; Fig. 3b, d). U0126 also down-regulated the effect of heat stress on *HSP70* and *LDHA* messenger RNA (mRNA) transcript levels by 41 and 51%, respectively ($P < 0.01$; Fig. 3c, e). However, U0126 displayed no significant effect on the mRNA transcript level of *SLC2A3* (13%, $P > 0.05$; Fig. 3e). In addition, treatment with U0126 reduced lactate production and LDH activity by 13 and 13%, respectively, compared with heat stress alone ($P < 0.01$; Fig. 3f, g).

Effect of ERK2 siRNAs on mRNA transcript and phosphorylation levels of ERK1/2

Three siRNAs were used to reduce the mRNA transcript level of *ERK2*. Compared with the control group, *ERK2* siRNA1 significantly reduced the mRNA transcript level of *ERK2* and the efficiency of inhibition was 52% ($P < 0.01$;

Fig. 1 Changes of pERK1/2, HSP70 expression, and lactate production in variable periods of time post-heat stress. Time (0, 1, 2, 4, and 6 h) post-heat stress (HS; 43 °C, 30 min) for Sertoli cells is shown on the x axis. **a**, **b** Cell extracts were utilized for western blotting to detect the expressions of phosphorylated ERK1/2 (pERK1/2), total ERK1/2 (ERK1/2), HSP70, and beta-actin. **c** Intracellular and extracellular lactate were collected and detected using a commercial kit. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control, # $P < 0.05$ compared with HS, ## $P < 0.01$ compared with HS

Fig. 2 Effect of differing concentrations of U0126 on cell viability and pERK1/2. Sertoli cells were treated with differing concentrations of U0126 (0, 1×10^{-6} , 5×10^{-6} , 1×10^{-5} , 1.5×10^{-5} , and 2×10^{-5} mol/L) for 2 h before heat stress (HS; 43 °C, 30 min) and incubated for another 2-h post-heat stress. **a** Cell viability was detected using CCK-8. **b** Cell extracts were utilized for western blotting to detect the expressions of pERK1/2, ERK1/2, and beta-actin. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control, # $P < 0.05$ compared with HS, ## $P < 0.01$ compared with HS

Fig. 4a). *ERK2 siRNA1* decreased *ERK2* mRNA by 51% compared with cells exposed to heat stress alone ($P < 0.01$; Fig. 4b). Furthermore, *ERK2 siRNA1* inhibited pERK1/2 by 18% compared with heat stress alone ($P < 0.01$; Fig. 4c). Thus, *ERK2 siRNA1* was used in subsequent experiments.

Effect of *ERK2 siRNA1* on heat stress-induced lactate production and related molecules

ERK2 siRNA1 and heat stress reduced the protein expressions of HSP70 (54%), GLUT3 (38%), and LDHA (16%) compared with heat stress alone ($P < 0.05$ and 0.01; Fig. 5a, c). Additionally, pretreatment with *ERK2 siRNA1* decreased heat stress-induced mRNA transcript levels of *HSP70*,

SLC2A3, and *LDHA* ($P < 0.01$; Fig. 5b, d). In addition, *ERK2* knockdown (*ERK2 siRNA1*) decreased heat stress-induced lactate production and LDH activity by 9 and 16%, respectively ($P < 0.01$; Fig. 5e, f).

Effect of *HSP70 siRNA3* on heat stress-induced lactate production and related molecules

All three *HSP70* siRNAs inhibited *HSP70* mRNA transcript levels, with *siRNA3* displaying the most significant inhibitory effect, and the efficiency of inhibition was 84% ($P < 0.01$; Fig. 6a). Thus, *HSP70 siRNA3* was used for further experiments. Compared with cells exposed to heat stress alone, *HSP70 siRNA3* significantly inhibited the protein expression of HSP70 in heat stress-treated cells ($P < 0.01$; Fig. 6b). *HSP70* knockdown (*HSP70 siRNA3*) reduced GLUT3 (31%) and LDHA (31%) protein expressions ($P < 0.01$; Fig. 6c), decreased mRNA transcript levels of *SLC2A3* and *LDHA* by 30 and 33%, respectively ($P < 0.01$; Fig. 6d), and inhibited lactate production and LDH activity by 62 and 25%, respectively ($P < 0.01$; Fig. 6e, f), compared with heat stress alone.

Discussion

The study presented here showed that heat stress increased pERK1/2, lactate production, LDH activity, and the expressions of HSP70 and molecules related to lactate production 2-h post-heat stress (43 °C, 30 min). Both U0126 and *ERK2 siRNA1* inhibited lactate production, LDH activity, and the expressions of HSP70 and lactate production-related molecules. *HSP70* knockdown (*HSP70 siRNA3*) downregulated lactate production, weakened LDH activity, and decreased the expressions of molecules related to lactate production. Therefore, our findings indicate that activated ERK1/2 by heat stress regulates lactate production by enhancing the expression of HSP70 to promote the activation of molecules related to lactate production in immature boar Sertoli cells.

Heat stress response (HSR) can be viewed as an adaptive or “survival instinct” response for the defense and maintenance of its structural and functional integrity (Verbeke et al. 2001). Increased HSP synthesis and activity is essential for cell survival (Rattan 2004). In the present study, heat stress enhanced the expression of HSP70 in a time-dependent manner. To confirm the effect of HSP70 on heat stress-induced lactate production, *HSP70 siRNA3*-mediated knockdown was performed. Our results showed that *HSP70 siRNA3* reduced the protein expression and mRNA levels of GLUT3 and LDHA, weakened LDH activity, and subsequently inhibited lactate production. Heat stress induces oxidative stress in testis (Li et al. 2013). The expressions of GLUT3

Fig. 3 Effect of U0126 on pERK1/2, HSP70, GLUT3, and LDHA expressions; LDH activity; and lactate production. Sertoli cells were preincubated with U0126 (1×10^{-6} mol/L) for 2 h, exposed to heat stress (HS; 43 °C, 30 min), then incubated for another 2 h. **a, b, d** Cell extracts were utilized for western blotting to detect the expressions of pERK1/2, ERK1/2, HSP70, GLUT3, LDHA, and beta-actin. **c, e** Cell

extracts were utilized for RT-PCR to detect the mRNA levels of *HSP70*, *SLC2A3*, and *LDHA*. **f** Intracellular and extracellular lactate were collected and detected using a commercial kit. **g** Cell extracts were utilized for measurement of intracellular LDH activity using a commercial kit. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control, # $P < 0.05$ compared with HS, ## $P < 0.01$ compared with HS

and LDHA decreased in cells subjected to oxidative stress, but increased when oxidative stress was inhibited (Covarrubias-Pinto et al. 2015; Saberi et al. 2008). Mounting evidence also showed that HSP70 protected cells from oxidative stress (Sable et al. 2018). Thus, heat stress-induced HSP70 promoted the expressions of GLUT3 and LDHA via inhibiting oxidative stress. Our results also showed that the reduction in the expression of HSP70 had no effect on basal LDH production, inferring that HSP70 requires a threshold to promote LDH activity and lactate production. Developing germ cells rely on lactate produced by Sertoli cells to obtain energy because there is a blood testis barrier in seminiferous tubules (Boussouar and Benahmed 2004). In normal physiological situations, Sertoli cells use oxidation of fatty acids (FAs) to yield much of the energy required, and utilize glucose to produce lactate for germ cells via *gluconeogenesis*, despite adequate oxygen availability (Oliveira et al. 2015; Regueira et al. 2015b). Our previous study showed that heat stress could induce autophagy to produce some small molecules as

substrates to obtain energy (Bao et al. 2017). Therefore, HSP70-enhanced lactate production post-heat stress alleviated energy shortage caused by heat stress, provided energy substrate for germ cells, and inhibited germ cell apoptosis causing male infertility.

Although signaling pathways involved in heat stress response are still largely unknown, evidence shows that mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and stress-activated protein kinase 9 (SAPK9) play roles in both survival and death pathways in response to heat stress (Rattan 2004). Recent studies showed that ERK1/2 signaling pathway is activated by heat stress in many cells and tissues (Niederlechner et al. 2013; Yu et al. 2010; Yunoki et al. 2015). In adult monkey Sertoli cells, heat stress caused a transient activation of ERK1/2 that resulted in the dedifferentiation and proliferation of Sertoli cells to resupply the cell population after heat stress (Zhang et al. 2006b). These results are consistent with the fact that activated ERK1/2 showed a protective role against

Fig. 4 Effect of *ERK2* siRNAs on *ERK2* mRNA level and pERK1/2. Sertoli cells were incubated with *ERK2* siRNAs and negative control (NC) for 6 h. Medium containing *ERK2* siRNAs and NC were replaced with DMEM/F-12 culture medium, and cells were incubated for more than 48 h. Then, cells were harvested directly or subjected to heat stress (HS; 43 °C, 30 min) and harvested at 2-h post-heat stress. **a** Cell extracts were utilized for RT-PCR to measure the inhibitory efficiency of *ERK2* siRNAs on *ERK2* mRNA level. **b** Cell extracts were utilized for RT-PCR to detect the effect of *ERK2* siRNA1 on the mRNA level of *ERK2*. **c** Cell extracts were utilized for western blotting to detect the effect of *ERK2* siRNA1 on the expressions of pERK1/2, ERK1/2, and beta-actin. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control, # $P < 0.05$ compared with HS, ## $P < 0.01$ compared with HS

apoptosis of adult mouse Sertoli cells post-heat stress (Sun et al. 2015). In our study, pERK1/2 peaked at 0-h post-heat stress, while lactate production began to increase at 2-h post-heat stress. Thus, it is possible that pERK1/2 requires a threshold to regulate lactate production. Although pERK1/2 in the HS + *ERK2* siRNA1 group was significantly lower than that of HS group, this value remains higher than that of the control, inferring that ERK2 pathway plays an important role in the heat stress response. These results show that ERK1/2-enhanced lactate production could be a mechanism of preventing cell damage and recovering cell functions.

To elucidate the role that ERK1/2 plays in regulating the expressions of molecules related to lactate production, we investigated the relationship between ERK1/2 and HSP70. Activated ERK1/2 promotes the expression of HSP70 in mesothelioma and human colorectal cells (Chen et al. 2001; Roth et al. 2009). However, HSP70 suppresses ERK1/2 by forming Bag1-HSP70 complexes to inhibit activation of MEK1/2 in an O23 hamster fibroblast cell line (OT cell line) (Song et al. 2001), and can prevent inactivation of mitogen-activated protein kinase phosphatase 3 (MKP3) and MKP1 in Cos-7 cells and human lung fibroblasts (Yaglom et al. 2003). In the present study, we showed that activated ERK1/2 enhanced the expression of HSP70 at both the protein and mRNA levels. U0126

and *ERK2* siRNA1 reduced the expression of HSP70, indicating that activated ERK1/2 regulated the expression of HSP70 induced by heat stress in immature boar Sertoli cells. Heat shock factor 1 (HSF1), the central player in heat stress response, serves to integrate diverse biological and pathological responses (Li et al. 2017). In heat-stressed cells, HSF1 is activated by hyperphosphorylation (Guettouche et al. 2005; Wales et al. 2015). In colorectal cancer cells, the activation of ERK1/2 can phosphorylate HSF1 at Ser326, which results in a fivefold increase in accumulation of HSP70 (Guettouche et al. 2005; Wales et al. 2015). Therefore, activation of ERK1/2 regulates HSP70 expression in heat stress treatment of Sertoli cells. But ERK1/2 is not the only signaling pathway that plays a role in regulation of HSP70 expression. Our unpublished data suggests that heat stress inhibits adenosine monophosphate-activated protein kinase (AMPK) phosphorylation. Inactivation of AMPK under heat stress may contribute to HSP70 protein synthesis (Wang et al. 2010). In addition, heat stress-induced p38 MAPK activation phosphorylated HSF1 at Ser326, which induced HSP70 production (Naidu et al. 2016). This explained why U0126 weakened HSP70 expression but did not inhibit its synthesis totally.

Our results showed that heat stress, U0126, *ERK2* siRNA1, and *HSP70* siRNA3 regulated the expressions of

Fig. 5 Effect of *ERK2* siRNA1 on HSP70, GLUT3, and LDHA expressions; LDH activity; and lactate production. Sertoli cells were incubated with *ERK2* siRNA1 and NC for 6 h. Medium containing *ERK2* siRNA1 and NC were replaced with DMEM/F-12 culture medium, and cells were incubated for more than 48 h before heat stress (HS; 43 °C, 30 min) exposure, followed by another 2-h incubation. **a**, **c** Cell extracts were utilized for western blotting to detect the expressions of

HSP70, GLUT3, LDHA, and beta-actin. **b**, **d** Cell extracts were utilized for RT-PCR to detect the mRNA levels of *HSP70*, *SLC2A3*, and *LDHA*. **e** Intracellular and extracellular lactate were collected and detected using a commercial kit. **f** Cell extracts were utilized for measurement of intracellular LDH activity using a commercial kit. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control, # $P < 0.05$ compared with HS, ## $P < 0.01$ compared with HS

molecules related to lactate production. Previous studies have shown that the increment expression of GLUT3 contributes to the increase of glucose uptake (Natalicchio et al. 2009). Activated ERK1/2 by loss of p66^{Shc} protein leads to the alteration of subcellular localization of GLUT1 and GLUT3 to promote glucose uptake in L6 myoblasts (Natalicchio et al. 2009). Therefore, enhanced expression of GLUT3 altered subcellular localization of GLUT3 and promote glucose uptake. Furthermore, an increase in LDH

subunit A (translated from *LDHA*) enhances LDH activity and increases the production of lactate from pyruvic acid (Riera et al. 2007), resulting in the increase of lactate. However, follicle-stimulating hormone (FSH) and basic fibroblast growth factor (bFGF) could enhance the expressions of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) and pyruvate dehydrogenase kinase (PDK) to promote lactate production in Sertoli cells (Regueira et al. 2015a). PFKFB catalyzes both synthesis

Fig. 6 Effect of *HSP70* siRNA3 on *HSP70*, GLUT3, and LDHA expressions; LDH activity; and lactate production. Sertoli cells were incubated with *HSP70* siRNAs and NC for 6 h. Then, medium containing *HSP70* siRNAs and NC were replaced with DMEM/F-12 culture medium and cells were incubated for more than 48 h. Cells were then harvested directly or subjected to heat stress (HS; 43 °C, 30 min) and harvested at 2-h post-heat stress. **a** Cell extracts were utilized for RT-PCR to measure the inhibitory efficiency of *HSP70*

siRNAs on *HSP70* mRNA level. **b, c** Cell extracts were utilized for western blotting to detect the expressions of *HSP70*, GLUT3, LDHA, and beta-actin. **d** Cell extracts were utilized for RT-PCR to detect the mRNA levels of *SLC2A3* and *LDHA*. **e** Intracellular and extracellular lactate were collected and detected using a commercial kit. **f** Cell extracts were utilized for measurement of intracellular LDH activity using a commercial kit. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control, # $P < 0.05$ compared with HS, ## $P < 0.01$ compared with HS

and degradation of fructose 2,6-biphosphate (Fru-2,6-P2). Fru-2,6-P2 is the most potent activator of 6-phosphofructo-1-kinase (PFK1), which catalyzes the major regulatory step in the glycolytic pathway. The pyruvate dehydrogenase complex (PDC) is responsible for the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA. Phosphorylation of PDC by PDK causes inactivation of the complex. It is necessary to study the role of PFKFB and PDK in heat stress-induced lactate production.

Conclusions

In summary, activated ERK1/2 by heat stress regulates lactate production by enhancing the expression of *HSP70* to promote the activation of molecules related to lactate production. Our results showed that enhanced lactate by mild heat stress could reduce germ cell apoptosis in vitro culture system and provide a method for optimizing in vitro methods involving male germ cells for assisted reproduction.

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Authors' contribution Jia-Yao Guan performed most of the experiments, analyzed most of the data, and was a major contributor in writing the manuscript. Ting-Ting Liao performed the experiments of effect of *HSP70 siRNA3* on heat stress-induced lactate production and related molecules and analyzed the data. Chun-Lian Yu and Wei-Rong Yang assisted in cell culture. Hong-Yan Luo participated in the analysis of data and revision of the manuscript. Xian-Zhong Wang designed the experiment, provided the theoretical support, and revised our manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Abbreviations ERK1/2, Extracellular signaling regulated kinase 1 and 2; GLUT, Glucose transporter; HS, Heat stress (heat shock); HSP, Heat shock protein; HSR, Heat stress response; HSF, Heat shock factor; LDH, Lactate dehydrogenase; SCs, Sertoli cells; SiRNA, Small interfering RNA; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F-12; FBS, Fetal bovine serum; PMSF, Phenylmethanesulfonyl fluoride; BCA, Bicinchoninic acid; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, Polyvinylidene fluoride; BSA, Bull serum albumin; RT-PCR, Real-time polymerase chain reaction; CT, Cycle threshold; NC, Negative control; MEM, Minimum essential media; CCK-8, Cell Counting Kit-8; CV, Coefficient of variation; SR, Standard error; ANOVA, Analysis of variance; pERK1/2, Phosphorylated ERK1/2; FA, Fatty acids; MAPK, Mitogen-activated protein kinase; SAPK9, Stress-activated protein kinase 9; MKP, Mitogen-activated protein kinase phosphatase; AMPK, Adenosine monophosphate-activated protein kinase; FSH, Follicle-stimulating hormone; bFGF, Basic fibroblast growth factor; PFKFB, 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase; PDK, Pyruvate dehydrogenase kinase; Fru-2,6-P₂, Fructose 2,6-biphosphate; PFK1, 6-Phosphofructo-1-kinase; PDC, Pyruvate dehydrogenase complex; OT cell line, O23 hamster fibroblasts cell line; SLC2A3, Solute carrier family 2, facilitated glucose transporter member 3

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