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Hsp105,70 and 60 in monkey Sertoli cells
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·*Original Article*·

Effect of 43°C treatment on expression of heat shock proteins 105, 70 and 60 in cultured monkey Sertoli cells

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Abstract

Aim: To examine the possible effect of heat treatment on expression of heat shock proteins (Hsps) 105, 70, and 60 in primary monkey Sertoli cells and to evaluate the possible signal pathways. **Methods:** Western blot analysis, real-time polymerase chain reaction (PCR), and confocal immunohistochemistry were used to analyze mRNA and protein levels of the Hsps in response to 43ºC treatment of Sertoli cells isolated from pubertal monkey testes. **Results:** Staining with Hoechst 33342 indicated Sertoli cells did not undergo apoptosis after heat treatment. Hsp105 was expressed in cytoplasm of untreated Sertoli cells. Both Hsp105 mRNA and protein levels were increased approximately 20-fold compared to those of the untreated controls at 12 h after heat treatment. Untreated Sertoli cells did not express Hsp70, but heat stress induced its expression in the cell cytoplasm. The time-course of changes in Hsp70 was similar to that of Hsp105. In contrast to Hsp105 and Hsp70, the change in Hsp60 expression was much less obvious. The protein level between 12 h and 48 h after heat treatment was only approximately 1.5-fold that of the untreated control. Extracellular regulated kinase (ERK) 1/2 inhibitor (U0126) or phosphoinositide kinase-3 (PI3K) inhibitor (LY294002) could partially block the response of Hsp105 and Hsp70 induced by heat treatment. **Conclusion:** These results indicate that the heat-induced expression of the three types of Hsp in monkey Sertoli cells might be regulated by ERK and/or PI3K signal pathways, but the profile of their expression is different, suggesting that they might have different regulatory functions in Sertoli cells. *(Asian J Androl 2008 May; 10: 474–485)*

Keywords: Hsp105; Hsp70; Hsp60; Sertoli cells; heat treatment; monkey

1 Introduction

Mild hyperthermia leads to cessation of spermatogenesis [1]. Experimental cryptorchidism or local testicular heat treatment with 43ºC water induced reversible oligospermia or azoospermia in rodents and monkeys by increased germ cell apoptosis [2–4]. Sertoli cells are the only somatic cells in seminiferous tubules that surround spermatogenic cells at various stages of development. It is well known that spermatogenic cells rely on Sertoli cells for structural and nutritional support [5, 6]. Unlike germ cells, the population of Sertoli cells is not affected by heat treatment and the protective mechanism is not known [2].

Physiological stress such as heat shock, hypoxia,

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ischemia and heavy metals can result in the synthesis of a group of highly conserved proteins, termed heat shock proteins (Hsps), in prokaryotic and eukaryotic organisms [7, 8]. Hsps are capable of protecting cells from harmful effects of stress by repairing denatured proteins or promoting damaged protein degradation, and they are implicated to function at key regulatory points in the control of cell apoptosis [9]. Mammalian cell Hsps have been classified into six major families according to their molecular size: small stress proteins; Hsp40; Hsp60; Hsp70; Hsp90; and Hsp110 families [10]. Evidence has shown that most Hsps are expressed in normal unstressed cells as molecular chaperones assisting in the folding/unfolding, assembly/disassembly, and transport of various proteins [11]. In testis, different members of Hsp families, such as the Hsp110, Hsp70, and Hsp60 families, are found to be expressed, and might play important roles in spermatogenesis. Three members of the Hsp110 family, ATP and peptide-binding protein in germ cells (Apg)-1, Apg-2 and Hsp105 (Hsp110), were observed to be expressed in adult human testis at high, moderate and low levels, respectively [12]. Hsp70-2 and Hsp70t, two members of the Hsp70 family, were constitutively expressed in germ cells and regulated developmentally. Hsp70-2 is specifically expressed in late primary spermatocyte (from pachytene stage onward) and early spermiogenesis (steps 1–7) [13, 14], whereas Hsp70t is mainly expressed in early round spermatids [15]. Hsp60 is expressed in Sertoli cells, Leydig cells, spermatogonia, leptotene and zygotene spermatocytes in testis of rat [16], human and macaque monkey [17]. It has been shown that decrease in expression of Hsp60 [18] or Hsp70-2 [19] in germ cells is consistent with male infertility.

Expression of most Hsps in germ cells is uninducible in response to heat stress. Our early *in vivo* studies showed that Hsp105 [20] or Hsp70-2 [21, 22] expression in germ cells declined after 43ºC local warming of testes or experimental cryptorchidism in monkeys and rats. Inducible Hsp70, another member of the Hsp70 family, was found to be unaffected by elevated temperature in germ cells [23–26]. Although Hsp60 expression was upregulated in spermatogonia, unlike other types of germ cells, spermatogonia did not undergo apoptosis after heat treatment [20]. The uninducibility of Hsp expression in most germ cells might be closely related to the susceptibility of germ cells after heat [23, 27]. In contrast to germ cells, Sertoli cells survive after heat stress. Whether Sertoli cells protect themselves against heat stress by upregulating Hsp expression is not clear. No evidence of expression and regulation of Hsps in primary Sertoli cells in response to heat treatment has been reported. There are only a few *in vitro* studies using Sertoli cell lines [28, 29]. Considering the high homology and similarity of rhesus monkey to human in physiology and biochemistry, we used pubertal rhesus monkey testes for primary Sertoli cell preparation. We examined the effect of the well-defined 43ºC treatment on the expression of three representative Hsps, Hsp105, inducible Hsp70 and Hsp60, which belong to three different Hsp families, and also evaluated the possible involvement of signal pathways on their regulation.

2 Materials and methods

2.1 Antibodies and reagents

The polyclonal antibodies against Hsp105 (sc-6242), Hsp70 (sc-1060), and Hsp60 (sc-1052) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibody against β-actin was from Sigma (St. Louis, MO, USA). Protein kinase A (PKA) inhibitor (H89), extracellular regulated kinase (ERK)1/2 inhibitor (U0126), p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580), phosphoinositide kinase-3 (PI3K) inhibitor (LY294002), protein kinase C (PKC) activator (phorbol myristate acetate, PMA), collagenase type IV, Dnase I, and trypsin were purchased from Sigma (St. Louis, MO, USA). Brilliant SYBR Green QPCR Master Mix was purchased from Stratagene (La Jolla, CA, USA).

2.2 Animals

The male pubertal (4–5-year-old) rhesus monkeys used for preparation and culture of the Sertoli cells were from Beijing Tiantan Biological Products Corporation LTD. (Beijing, China). They were healthy and killed for preparation of the special biological reagents. The use of these monkeys was approved by the Institutional Committee on Animal Care and Use of the Ministry of Health of China. All the protocols had the approval of the Institutional Committee on Animal Care and Use.

2.3 Preparation of rhesus monkey Sertoli cells

After anesthesia with pentobarbital sodium (30 mg/kg body weight, i.v.), testes were obtained from three pubertal rhesus monkeys (4–5 years old) each time using an aseptic technique. A total of nine monkeys were used for this experiment. For preparation of the Sertoli cells,

after being washed in cold (0–4ºC) phosphate-buffered saline (PBS) three or four times, the individual testis was taken back in cold PBS (pH 7.4, containing 100 U/mL penicillin and 100 μg/mL streptomycin sulfate). Isolation and purification of the Sertoli cells was complete 2–3 h after the castration.

Sertoli cells were prepared as described [30] with modifications. Briefly, the individual testis was washed several times and decapsulated prior to being minced into approximately 2 mm pieces. The minced tissues were suspended in cold PBS and shaken vigorously by hand. Seminiferous tubules were recovered after sedimentation at unit gravity for 5 min at 4ºC. The wash and sedimentation were repeated another three times to remove red blood cells and Leydig cells. The pellets were then incubated in a PBS solution of 10-fold volume containing 1 mg/mL collagenase IV and 75 U/mL Dnase I at 33ºC in a shaking water bath (160 oscillations/min) for 30 min and monitored closely to limit clumping of tissue that can result from overdigestion. After digestion, if there were still undigested seminiferous tubules, they were removed to another tube(s) for digestion again with the same enzymes for less than 20 min. After the tissue clusters resulting from the enzyme digestion were discarded, the suspensions were centrifuged for 4 min at 90 × *g* and the pellets were washed with Dulbecco's Modified Eagle's Medium(DMEM)/F12 two or three times. The second digestion was carried out in a PBS solution containing 0.25% trypsin and 75 U/mL Dnase I for less than 8 min at room temperature. On completion of digestion, fetal bovine serum (FBS) was added to the suspension to terminate the digestion. The suspensions were filtered through an 80 mesh stainless steel filter before being centrifuged for 5 min at 180 × *g*. The cells were washed twice with DMEM/F12, suspended in DMEM/F12 supplemented with 10% FBS, and cultured at 33ºC in a CO_2 incubator (5% CO_2 : 95% air). After 40 h of culture, the medium was replaced to remove most unattached germ cells. The cells were digested with trypsin (0.05%) after an additional 12–24 h. The isolated cells from each testis were frozen in liquid nitrogen for use of later primary culture.

2.4 Primary monkey Sertoli cell cultures

After they were thawed, the cells were plated in 35 mm dishes in DMEM/F12 with 10% FBS. The density was $0.2 \times 10^6/cm^2$ for protein and RNA extractions. For confocal immunohistochemistry, the cells were seeded onto 24 mm × 24 mm coverslips placed in 35 mm dishes at a density of $0.07 \times 10^6/cm^2$. Twenty-four hours later, the cells were hypotonically treated with 20 mmol Tris (pH 7.4, 22ºC) for 3 min to lyse the residual amount of germ cells. The cells were then incubated with DMEM/F12 without FBS for 24 h before treatments.

For the cell heat treatment, the dishes containing the cells were sealed with paraffin membrane and put in a sterile 43ºC water bath for 30 min. Then the dishes were immediately put back into the 33ºC CO_2 incubator. The time immediately after completing the 30 min heat treatment was designated as 0 min. At various time points between 30 min and 5 days (5D), the cell cultures were terminated and analyzed.

To analyze the effects of various protein kinase inhibitors or activator on heat-induced Hsp expression, the Sertoli cells were pretreated with PKA inhibitor H89 (10 μmol), ERK1/2 inhibitor U0126 (10 μmol), p38 MAPK inhibitor SB203580 (20 μmol), PI3K inhibitor LY294002 (20 μmol), or PKC activator PMA (10^{-7} mol) for 30 min before the 43ºC treatment and the cells were collected 24 h after heat stress. The doses used here were according to the results of our previous experiments in granulosa cells [31, 32] and Sertoli cells [33, 34].

2.5 Detection of cell apoptosis with Hoechst 33342 staining

Untreated Sertoli cells or heat-treated cells (at 24 h after terminating the 30-min 43ºC warming) were collected for analysis of apoptosis with Hoechst 33342 staining. After two washes with PBS, the Sertoli cells were fixed in freshly prepared mixture of methanol and acetone (1:1) for 15 min at room temperature, followed by three washes with PBS, and incubation in 100 μg/mL Hoechst 33342 at room temperature in the dark for 15 min. After being washed in PBS, the cells were examined by confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, USA).

2.6 Western blot analysis

After two washes with PBS, the Sertoli cells were lysed in cold lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] supplemented with 100 μg/mL phenylmethylsulfonyl fluoride and 1 μg/mL aprotinin). After centrifugation at 13 000 × *g* for 20 min, the supernatants were collected and the total protein concentrations were determined by spectrophotometer, using bovine

serum albumin as a standard. Fifty micrograms of total protein per lane was separated by 10% SDS-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to the nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking in 5% non-fat milk in 0.09% NaCl, 0.05% Tween-20, and 100 mmol/L Tris-HCl (pH 7.5) for 1 h at room temperature, the membranes were incubated with the primary antibodies Hsp105 (1:800), Hsp70 (1:400), Hsp60 (1:800), and β-actin (1:5 000) in blocking solution for 2 h at room temperature or at 4ºC overnight. The membranes were washed three times then incubated with the corresponding peroxidase-conjugated secondary antibodies (1:2 500) for 1 h at room temperature. Reactive bands were visualized by Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and the membranes were then subjected to X-ray autoradiography. Band intensities were determined by Quantity One software (Bio-Rad).

2.7 Confocal immunohistochemistry

After two washes with PBS, the Sertoli cells were fixed in a freshly prepared mixture of methanol and acetone (1:1) for 15 min at room temperature, followed by three washes with PBS, and incubation in 3% bovine serum albumin for 1 h. Then the cells were immunolabeled with the primary antibody (1:150) for 2 h, and the corresponding fluorescein isothiocyanate-conjugated immunoglobulin G (1:200) for 1 h at room temperature. After three washes in PBS, propidium iodide (PI) incubation for 10 min was used to dye the nuclei. Finally, the cells were analyzed by confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, USA). For the negative controls, the cells were processed without the primary antibodies, replaced with the normal immunoglobulin G.

2.8 Real-time polymerase chain reaction (PCR)

A two-step, real-time reverse transcription-PCR was used to measure the expression of the candidate genes. Total cell RNA was isolated with TRIzol reagent (Invitrogen, CA, USA) according to the instructions of the manufacturer and quantified by measuring absorbance at 260 nm. Total RNA (2 μg) was reverse-transcribed into cDNA in a 20 μL reaction containing Superscript III reverse transcriptase (Invitrogen, San Diego, CA, USA), oligo(dT), dNTPs and RNase inhibitor, followed by dilution with RNase-free water in the ratio of 1:4. The primers specific to the candidate genes were designed using Primer 3 software. The primer pairs were designed as follows (5′–3′): Hsp105 sense, AAAGTTGACCAGCCTCCAGA and antisense, TGGTCCACACAGCTTGTCTC (228 bp); Hsp70 sense, CGACCTGAACAAGAGCATCA and antisense, AAGATCTGCGTCTGCTTGGT (213 bp); Hsp60 sense, CATTCCAGCCTTGGACTCAT and antisense, TCACAACCTTTGTTGGGTCA (236 bp); and ribosomal protein L32 (rpl32) sense, GCCCAAGATCGTCAAAAAGA and antisense, GTTGCACATCAGCAGCACTT (250 bp). Rpl32 encodes a ribosomal protein that is a component of the 60S subunit, and this gene is one of the most stably expressed genes in most cells. Real-time PCR was carried out in a 96-well plate using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The previously synthesized cDNA was used as the template. Reactions for each time point were carried out in triplicate and in a 20 μL reaction mixture containing 2 μL cDNA, 12.5 μL 2 × SYBR Green Master Mix, and 150 nmol of each reverse and forward primer specific for the candidate genes. Reactions were run for 40 cycles (95ºC for 30 s, 58ºC for 1 min, 72ºC for 1 min) following an initial 10 min step at 95ºC. The threshold cycle (C_T), which indicates the relative abundance of a particular transcript, was calculated for each reaction by the ABI Prism 7000 sequence detection system (Applied Biosystems). The C_T values of rpl32 were used as endogenous controls. The relative concentration of the candidate gene expression was calculated using the formula $2^{-\triangle\triangle CT}$ as described in the SYBR Green user manual, thus the concentration of the control sample was 1. Real-time PCR quantification of gene expression levels in each sample was the mean of triplicate real-time PCR experiments. For each time point, values are presented as the mean ± SEM of triplicate independent experiments. All gene expression levels were normalized to rpl32 expression levels.

2.9 Data analysis and statistics

All monkey Sertoli cell culture experiments were repeated at least three times by using three different monkey cell preparations. The quantitative results were represented as the means ± SEM. Statistical analysis was carried out with SPSS (version 13.0; SPSS, Chicago, IL, USA), and one-way ANOVA was used for analyzing the data in different groups. $P < 0.05$ were considered as significant. For the confocal immunohistochemistry data, one representative picture of three similar results from three

separate experiments is presented.

3 Results

3.1 Purity of primary monkey Sertoli cell preparations

Purity of the Sertoli cells was evaluated by confocal immunohistochemistry of Wilms' tumor gene 1 (WT1). Transcription factor WT1 is specifically expressed in Sertoli cells in testis. It is a stable marker of Sertoli cells and its expression is switched on from early fetal life and maintained throughout the whole of life [35].

As shown in Figure 1A, WT1 was specifically expressed in the nuclei of monkey Sertoli cells. The purity of Sertoli cell preparations was 91.400% ± 0.021, calculated from three different experiments using three different Sertoli cell preparations.

3.2 Sertoli cells after 43ºC treatment did not undergo apoptosis

We observed whether Sertoli cells underwent apoptosis with Hoechst 33342 staining. As shown in Figure 1B, the morphological changes of the nuclei, in-

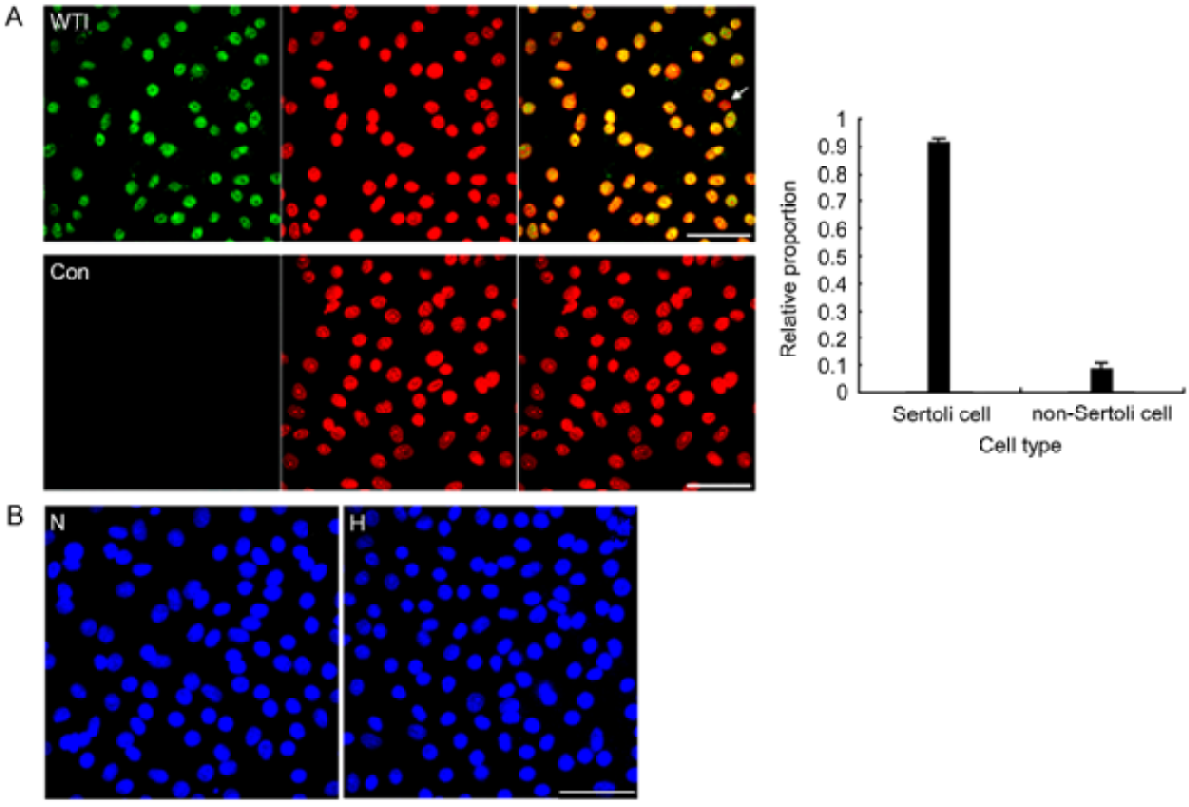

Figure 1. (A): Purity of cultured primary monkey Sertoli cells. The purity of the Sertoli cells was evaluated by confocal immunohistochemistry of Wilms' tumor gene 1 (WT1). The Sertoli cells were isolated from pubertal monkey testes with enzyme digestion. The isolated cells were cultured at 33ºC in a CO_2 incubator (5% CO_2 : 95% air) for 40 h before most unattached germ cells were removed. The Sertoli cells were subsequently frozen in liquid nitrogen and thawed when use. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were processed for confocal immunohistochemistry. Green fluorescence indicates positive staining. The nuclei were stained in red using propidium iodide, and the yellow is the overlap of green and red. Con, the negative control without the primary antibody. WT1 was specifically expressed in Sertoli cell nuclei. The arrow points to a non-Sertoli cell. The relative proportion of Sertoli cells was 91.400% ± 0.021 which was counted from three different experiments using three different Sertoli cell preparations. Bar = 50 μm. (B): Detection of Sertoli cell apoptosis by Hoechst 33342 staining. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were incubated at 43ºC for 30 min. After an additional 24 h, the cultures were terminated and the cells were stained with Hoechst 33342. Blue fluorescence indicates positive staining. No nuclei condensation, ripple, or fragmentation was observed in untreated or heat-treated Sertoli cells. One representative picture of three similar results from three separate experiments is presented. H, heat treatment by 43ºC for 30 min; N, untreated Sertoli cells. Bar = 50 μm.

cluding condensation, ripple, or fragmentation, were not observed, suggesting no obvious cell apoptosis in the heat-treated or untreated Sertoli cells, indicating that 43ºC treatment did not induce Sertoli cell apoptosis.

3.3 Hsp105 mRNA and protein levels increased in primary monkey Sertoli cells after 43ºC treatment

The untreated monkey Sertoli cells expressed Hsp105. After the cells were incubated in 43ºC water for 30 min, as shown in Figure 2A, 2B, both their mRNA and protein increased in a time-dependent manner. The maximums were reached 12 h after terminating the heat treatment, with approximately 20-fold increases compared with the untreated control ($P < 0.01$). Both the mRNA and protein levels then gradually decreased and returned to basal levels at 72 h and 5D, respectively.

The confocal immunohistochemistry showed that Hsp105 was mainly expressed in the cytoplasm of the Sertoli cells. The heat treatment did not affect its localization (Figure 3A). The quantitative change in its expression was similar to that observed with Western blot analysis.

3.4 Hsp70 expression induced in primary monkey Sertoli cells by 43ºC treatment

There was no Hsp70 expression in the untreated Sertoli cells, but heat stress induced its expression. The temporal changes in mRNA and protein resembled those of Hsp105, with the maximum level at 12 h after terminating the heat treatment ($P < 0.01$), as shown in Figure 4A, 4B. The mRNA level then gradually decreased in a time-dependent manner. The protein, however, remained at the high level between 12 h and 72 h, then decreased until 5D when it reached an undetectable level.

No positive staining of Hsp70 in the untreated Sertoli cells was observed, whereas expression clearly appeared in the cytoplasm at 12 h after heat treatment and did not disappear until 5D (Figure 3B).

3.5 Hsp60 expression in primary monkey Sertoli cells after 43ºC treatment

Compared with Hsp105 and Hsp70, the changes in Hsp60 expression in response to heat treatment were much less obvious. Its mRNA level was higher than the untreated control between 6 h and 24 h after terminating the heat treatment ($P < 0.05$) (Figure 5A). The Hsp60 protein level only increased approximately 1.5-fold at 12 h after the treatment ($P < 0.05$). This level remained until 48 h, then decreased to the control level at 72 h, earlier than that of Hsp105 and Hsp70 (Figure 5B).

The immunohistochemistry result showed that Hsp60 was not expressed in the nuclei of Sertoli cells. Its location was confined to the area surrounding the nuclei and its expression between 12 and 48 h was slightly higher than the untreated control (Figure 3C).

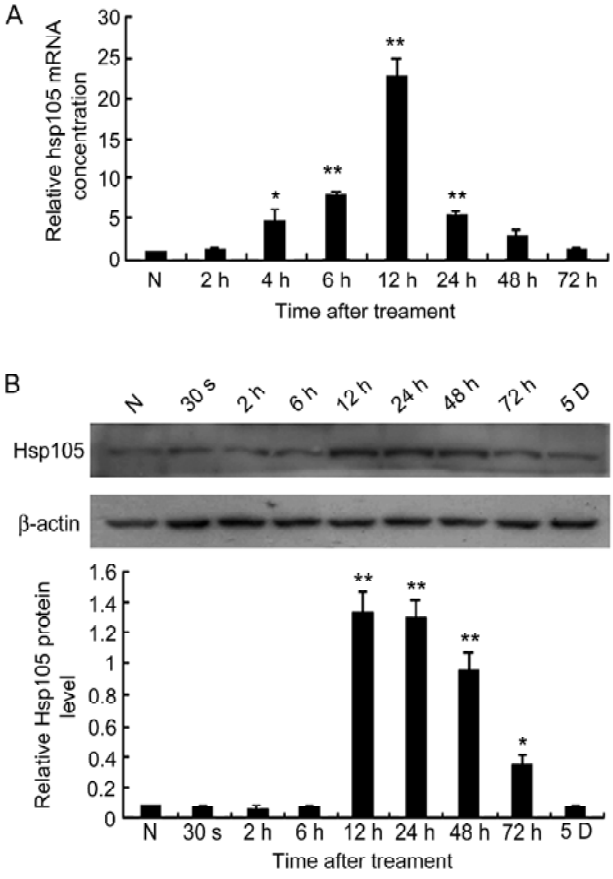

Figure 2. Effect of heat treatment on Hsp105 mRNA and protein expression in Sertoli cells isolated from pubertal monkey testes. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were incubated at 43ºC for 30 min. The time immediately after completing the 30-min heat treatment was designated 0 min. At different time points between 30 min and 5 days (5D), the cells were collected and further processed for measurement. (A): Real-time polymerase chain reaction (PCR) analysis. The relative concentration each point was calculated as $2^{-\triangle\triangle CT}$. The concentration in the untreated control (N) was regarded as 1. (B): Western blot analysis. β-actin was used as an internal control. The relative level at each point was determined by the ratio of heat shock protein 105 (Hsp105) to β-actin, as measured by densitometry. Data are presented as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the untreated control.

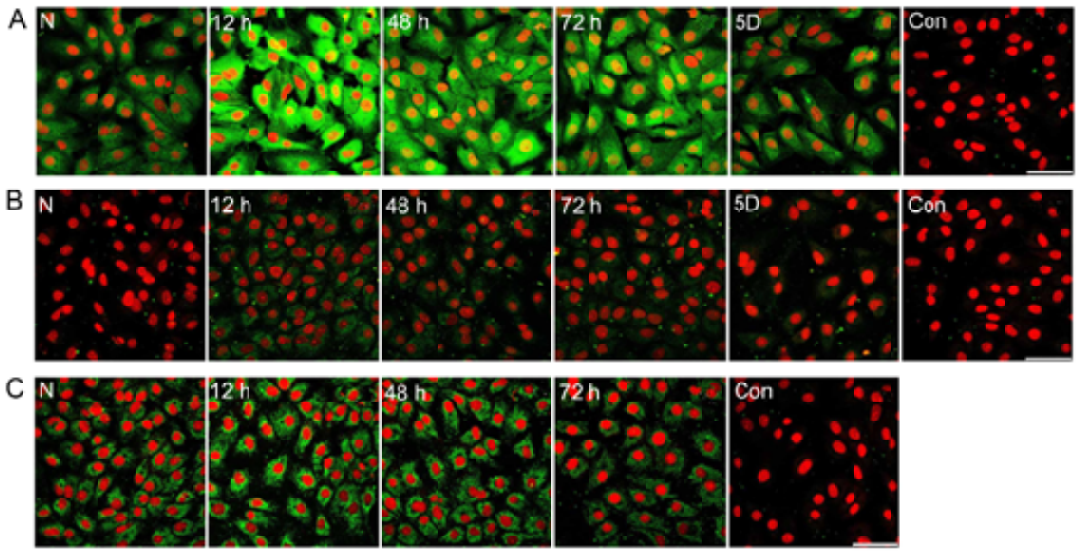

Figure 3. Confocal immunohistochemistry analysis of heat shock protein 105 (Hsp105) (A), Hsp70 (B) and Hsp60 (C) in Sertoli cells isolated from pubertal monkey testes. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were incubated at 43ºC for 30 min. The time immediately after completing the 30-min heat treatment was designated 0 min, and 12 h–5 days (5D) represent the time points after terminating treatment. Green fluorescence indicates positive staining. The nuclei were stained red using propidium iodide. One representative picture of three similar results from three separate experiments is presented. Con, the negative control without the primary antibody. N, untreated control Sertoli cells. Bar = 50 μm.

3.6 U0126 or LY294002 inhibited heat-induced Hsp105 and Hsp70 expression

PKA inhibitor H89, ERK1/2 inhibitor U0126, p38 MAPK inhibitor SB203580, PI3K inhibitor LY294002, and PKC activator PMA were used to further clarify which signal pathway could be involved in heat-induced Hsp expression in Sertoli cells. In our previous experiments we have examined the possible toxic effect of these compounds and the relevant solvents on granulosa [31, 32] and Sertoli cell functions [33, 34] in the doses used for this experiment. No obvious negative effect could be observed. The results of Western blot analysis (Figure 6A) and confocal immunohistochemistry (Figure 6B, 6C) showed that U0126 significantly inhibited the induction of Hsp105 and Hsp70 expression in response to heat treatment. A partial inhibitory effect of LY294002 on heat-induced Hsp105 and Hsp70 expression was also observed, whereas the protein levels were still higher than the untreated controls (Hsp105, $P < 0.05$; Hsp70, $P < 0.01$). H89, SB203580, and PMA did not have an obvious effect. These results indicate that heat-induced Hsp expression in monkey Sertoli cells might be regulated by the ERK and/or PI3K signal pathways.

4 Discussion

Hsps are among the most conserved proteins known to be expressed in normal cells or induced by various harmful conditions. They serve as molecular chaperones, helping to correct folding of newly synthesized proteins, protein oligomerization, and intracellular translocation. In stress conditions, they are capable of refolding denatured proteins or degrading them [36]. It has been suggested that production of Hsps might be necessary for cell survival against apoptosis and recovery of cells from stress [37, 38].

In this study, we have selectively examined expression of Hsp105, Hsp70, and Hsp60, members of three different Hsp families, in primary monkey Sertoli cells. We showed that Hsp105 and Hsp60 were expressed in untreated control Sertoli cells, whereas Hsp70 was not. The expression of these three Hsps in the untreated Sertoli cells (maintained at 33ºC) was not altered along the same time points as the groups treated by 43ºC (data not shown). The heat treatment significantly induced expression of Hsp105 and Hsp70, but only a slightly increased expression of Hsp60 was observed. In our pre-

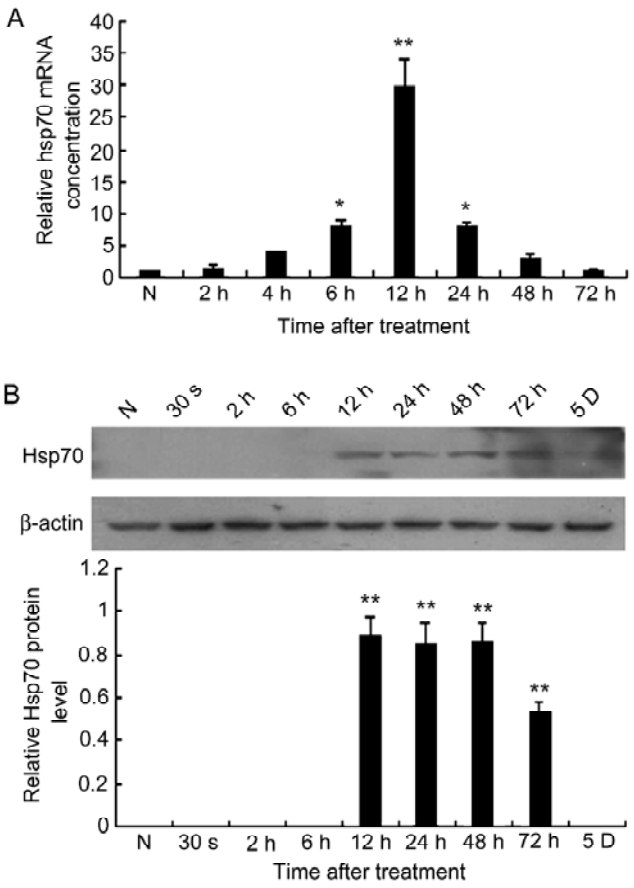

Figure 4. Effect of heat treatment on heat shock protein 70 (Hsp70) mRNA and protein expression in Sertoli cells isolated from pubertal monkey testes. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were incubated at 43ºC for 30 min. The time immediately after completing the 30 min heat treatment was designated 0 min, and 30 min–5 days (5D) represent the time points after terminating treatment. (A): Real-time polymerase chain reaction (PCR) analysis. The relative concentration was calculated as $2^{-\triangle\triangle CT}$. The concentration in the untreated control (N) was regarded as 1. (B): Western blot analysis. β-actin was used as an internal control. The relative level was determined by the ratio of Hsp70 to β-actin as measured by densitometry. Data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the untreated control.

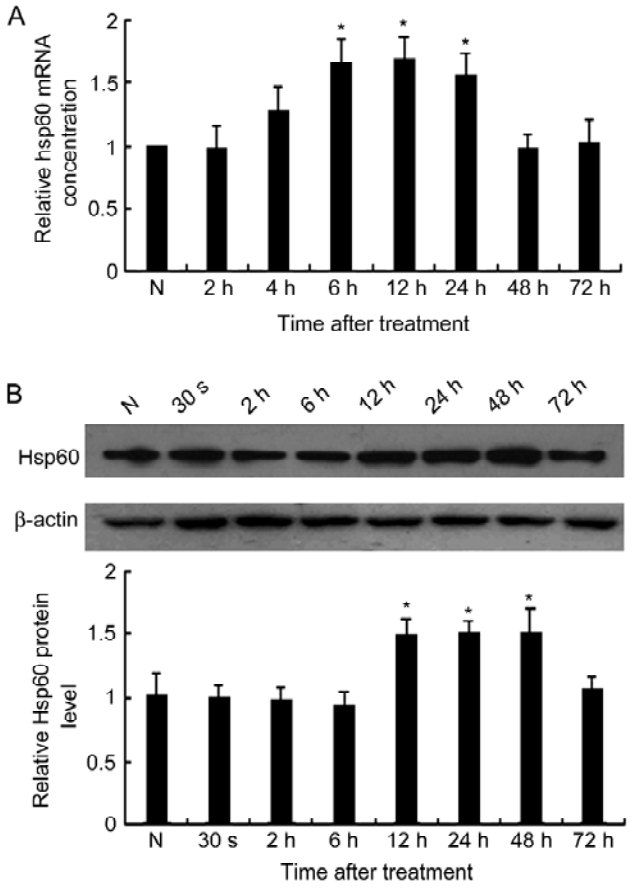

Figure 5. Effect of heat treatment on heat shock protein 60 (Hsp60) mRNA and protein expression in Sertoli cells isolated from pubertal monkey testes. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were incubated at 43ºC for 30 min. The time immediately after completing the 30 min heat treatment was designated 0 min, and 30 min–72 h represent time points after terminating treatment. (A): Real-time polymerase chain reaction analysis. The relative concentration was calculated as $2^{-\triangle\triangle CT}$. The concentration in the untreated control (N) was regarded as 1. (B): Western blot analysis. β-actin was used as an internal control. The relative level was determined by the ratio of Hsp60 to β-actin as measured by densitometry. Data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$, compared with the untreated control.

vious *in vivo* studies on monkey testes, we observed that Hsp105 expression in spermatids decreased dramatically with the loss of spermatids on days 3, 8 and 30 after 43ºC treatment, and returned to the baseline levels on days 84 and 144. In contrast, the expression of Hsp60 increased on days 3, 8 and 30 and was only detected in Sertoli cells and spermatogonia. Temporal changes in the expression pattern of Hsp105 and Hsp60 in relation to germ cell death suggested that they might be involved in the key processes in regulation of germ cell apoptosis [20].

Hsp105 is also denoted as Hsp110 [10]. Hsp105, Apg-1, and Apg-2 constitute the Hsp110 family. Anti-Hsp105 antibody used in our studies detects Hsp105 and also

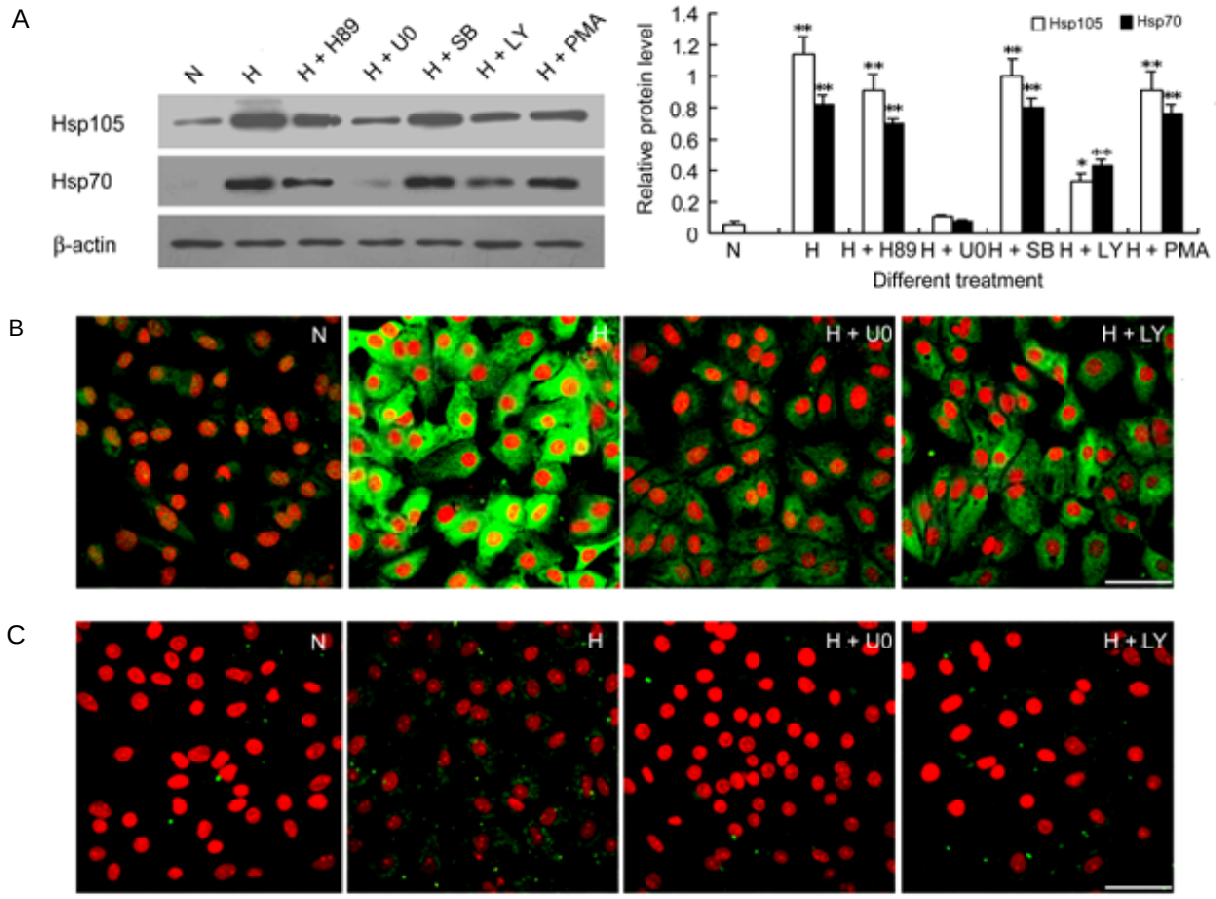

Figure 6. Effects of various protein kinase inhibitors or activator on heat-induced expression of heat shock protein 105 (Hsp105) and Hsp70 in Sertoli cells isolated from pubertal monkey testes. After hypotonic shock and culture in serum-free medium for 24 h, the Sertoli cells were pretreated with protein kinase A (PKA) inhibitor H89 (10 μmol/L), extracellular regulated kinase (ERK) 1/2 inhibitor U0126 (U0) (10 μmol/L), p38 mitogen-activated protein kinase inhibitor SB203580 (SB) (20 μmol/L), phosphoinositide kinase-3 (PI3K) inhibitor LY294002 (LY) (20 μmol/L), or protein kinase C activator phorbol myristate acetate (PMA) (10^{-7} mol) for 30 min before the 43°C treatment, and the cells were collected at 24 h after the heat stress. (A): Western blot analysis of Hsp105 and Hsp70. β-actin was used as an internal control. The relative level was determined by the ratio of Hsp105 or Hsp70 to β-actin as measured by densitometry. Data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the untreated control. (B) and (C): Confocal immunohistochemistry of Hsp105 and Hsp70, respectively. Green fluorescence indicates positive staining. The nuclei were stained red using propidium iodide. One representative picture of three similar results from three separate experiments is presented. Bar = 50 μm. H, heat treatment without preincubation with any inhibitor or activator; N, untreated control Sertoli cells.

cross-reacts, to a lesser extent, with Apg-1 and Apg-2. Apg-2 expression is ubiquitous in various mouse tissues and is most abundant in testis and ovary. Its expression is not induced by heat treatment, suggesting that it might play a role under non-stress conditions [28]. Therefore, the changes in Hsp105 expression observed in the Sertoli cells after heat treatment in our study, to some extent, might be changes in Hsp105 and Apg-1 expression. Apg-1 has been reported expressed in germ cells, but not in testicular somatic cells, in mouse and human. In testis without germ cells (Sertoli-cell-only syndrome) or arrested at spermatogonia, staining of Apg-1 was absent [39]. When the TAMA26 Sertoli cell line was transferred from 32ºC to 39ºC for 2 h, Apg-1 transcript was induced, but the induction did not occur with a temperature shift from 32ºC to 42ºC. The induction of Hsp105 in the TAMA26 Sertoli cell line was similar to Apg-1 [29]. However, in the present study, we observed Hsp105

expression was significantly induced at 12 h after incubating the primary monkey Sertoli cells at 43ºC for 30 min, and its expression at 72 h was still higher than that of untreated control cells. The different heat treatment (42ºC for 2 h *vs*. 43ºC for 30 min), cell collection time (immediately after treatment *vs*. 30 min–5D after treatment), or cell types (mouse cell line vs primary monkey cells) might account for the different results.

The Hsp70 family comprises at least eight highly homologous members that differ in intracellular localization and expression pattern [40]. It is known that Hsp70-2 and Hsp70t are constitutively expressed in testis. They are not inducible in germ cells [41–43]. Hsp70-1A and Hsp70-1B are collectively referred to as Hsp70, often called major stress-inducible Hsp70 [44]. Because of its inducibility, it was chosen for our experiment. Inducible Hsp70 has been found to have multiple roles in cytoprotection against apoptosis by reducing or blocking caspase activation and suppressing mitochondrial damage and nuclear fragmentation [45, 46]. High expression of Hsp70 is also a prerequisite for survival of human cancer cells of various origin [47, 48]. Although Allen *et al*. [49, 50] found that Hsp70 was induced in germ cells by heat stress, more recent studies indicated that Hsp70 could not be induced in germ cells [23–26]. In primary monkey Sertoli cells, however, we showed that Hsp70 was significantly induced by the 43ºC treatment.

Hsp60 has been reported as essential for correcting folding of nuclear-encoded proteins imported to mitochondria [51]. The concentration of Hsp60 has been found to correlate well with mitotic activity of spermatogonia [16]. We observed that, after heat treatment, Hsp60 in Sertoli cells was only approximately 1.5-fold that of the untreated control, which is consistent with an earlier report [43], suggesting that Hsp60 might function more as a chaperone and less as a heat-inducible protein.

Protein kinases and kinase pathways form highly interactive networks to achieve the integrated function of cells. They play important roles in regulation of cell proliferation, differentiation, and survival [52, 53]. Our earlier reports showed that heat-induced cytokeratin (ck)-18 re-expression in adult monkey Sertoli cells could be blocked by a PKA or an ERK1/2 inhibitor. The 43ºC treatment of monkey testes is capable of activating Akt, a key effector of PI3K. However, ck-18 induction in Sertoli cells remained unaltered when the PI3K/Akt pathway was blocked [34]. In the present study, heat-induced Hsp105 and Hsp70 expression could be partially blocked by the inhibitor of ERK1/2 or PI3K, but not by the inhibitor of PKA, suggesting that heat stress regulates the expression of different molecules in Sertoli cells through different pathways.

In conclusion, this study has shown for the first time, to our knowledge, that 43ºC treatment of primary monkey Sertoli cells could induce differential expression of Hsp105, Hsp70, and Hsp60 in the cells, which might be regulated by the ERK and/or PI3K signal pathways. The profiles of Hsp expression in Sertoli cells after heat treatment are different from those in germ cells, as reported earlier. The induced Hsp expression might help Sertoli cells survive the stress condition. However, we have no direct evidence from the present study. Further research, for example, suppressing *Hsp* gene expression with short interfering RNA or blocking their protein functions with specific inhibitors or antibodies, is needed. Our earlier studies showed that 43ºC treatment could induce re-expression of ck-18 and liver receptor homolog-1 (LRH-1) in Sertoli cells, and this might be regarded as a dedifferentiation feature [34, 54]. The induced Hsp expression might also play a role in the processes of regulation, but this also needs further investigation.

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