

ORIGINAL ARTICLE

Heat-shock pretreatment reduces expression and release of TSLP from keratinocytes under Th2 environment

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Abstract

Background: Atopic dermatitis is a chronic, relapsing inflammatory disease of the skin. Current therapy is not curative, and recalcitrant disease is a big stress and challenge for parents and physicians. This study explored the potential role of heat-shock protein 70 (HSP-70) and its anti-inflammatory effects on keratinocyte under TH2 environment.

Methods: Human keratinocyte cell line (HaCa T) was stimulated with IL-4, IL-13, and TNF- α to synthesize and secrete thymic stromal lymphopoietin (TSLP), an important cytokine of immunopathogenesis in atopic dermatitis. Heat shock was performed by immersing the cell-contained flask into a water bath of 45°C for 20 min. Cell viability, TSLP expression, and secretion of HaCa T cells were measured and compared. Possible regulatory mechanisms influencing the expression of TSLP, such as the STAT6 and NF- κ B signal pathways, were investigated.

Results: Heat-shock treatment induced intracellular HSP-70 expression in HaCa T cells without affecting cell viability. The induced expression and secretion of TSLP in HaCa T cells were suppressed by heat shock. The NF- κ B signal pathway was inhibited by heat shock, leading to decreased TSLP expression and secretion.

Conclusion: Heat stress-induced HSPs can significantly reduce the production and secretion of TSLP from HaCaT cells under Th2 environment. Thus, the evidence highlights the potential role of HSP-70 for atopic dermatitis in the future.

Atopic dermatitis (AD) is a common inflammatory disease of the skin that often precedes asthma and allergic disorders. High prevalence rates in a number of other countries reveal that AD is a major problem in developing and developed countries (1). In Taiwan, the prevalence is 6.7% (2), with 40% of patients aged <20 years old. It occurs most often in infants (22.4%) and decreases with age, reaching 6.1% in 14-year-olds.

The major features of AD include intense pruritus and chronic or relapsing dermatitis, which may cause secondary

skin infection. In severe cases, AD can lead to school absenteeism, occupational disability, and emotional stress. Unfortunately, the current treatment is limited to relieving the severity of symptoms. A new direction of treatment is essential to improve the quality of life of patients and can decrease its burden on patients and families.

Before AD goes into the chronic stage, its pathogenesis is predominantly Th2 immune response. The skin itself actually plays an important role in its immunopathogenesis. When the

skin as a barrier is damaged by allergen or viral infection, epidermal keratinocytes (KCs) are activated to release of cytokines, chemokines, and antimicrobial peptides. Among them, thymic stromal lymphopoietin (TSLP), an IL-7-like chemokine/cytokine released mainly by epithelial cells and KCs, has been well discussed.

In animal models, skin-restricted overexpression of TSLP in transgenic mice results in an AD-like phenotype (3). The skin epidermis of patients with AD highly expresses TSLP (4–6), which plays a key role in Th2 immune response of AD through several ways. It can stimulate local network of dendritic cells to secrete chemokines such as eotaxin and IL-8 to recruit eosinophils and neutrophils, and provoke response on other types of cells such as basophils or mast cells (6–8). Furthermore, TSLP influences dendritic cells to prime naïve T cells into Th2 cell differentiation, then producing IL-4 and IL-13. TSLP increases the severity of AD via positive feedback loop; however, attention in reducing TSLP synthesis or release as a therapeutic strategy is limited.

The network of heat-shock proteins (HSPs) represents an emerging paradigm to provide protection from and to facilitate cellular recovery after exposure to damaging stimuli (9). The HSPs are a highly conserved family of intracellular proteins that are constitutively expressed in virtually all nucleated cells. Their synthesis is enhanced by exposure to a variety of stressful conditions, including heat, toxins, oxidative stress, and glucose deprivation (10). They are categorized into various families depending on their molecular weights and functional attributes. Increasing evidence suggests that HSPs may play important roles in both innate and adaptive immunities by acting as chaperones for immunologically important molecules such as MHC class I, immunoglobulins, TCRs, and TLRs. HSPs are reported as anti-inflammatory molecules. The intracellular overexpression of HSP-70 has been shown to reduce production of NO and iNOS expression in astrocytes (11). In experimental stroke or sepsis, overexpression of HSP-70 is also associated with the downregulation of expression of pro-inflammatory genes (TNF- α and IL-1 β) (12). Several HSPs, including HSP-70, are constitutively expressed in the skin and can be upregulated as a result of exposure to stresses (13–15).

This study aimed to explore the potential role of HSPs in reducing the TSLP expression and secretion of keratinocytes under Th2 environment, and the possible mechanism was discussed.

Materials and methods

Chemicals

Cell culture media (DMEM), trypsin, FBS, and related reagents (glutamine, penicillin, streptomycin) were purchased from Gibco, Invitrogen Ltd, Paisley, UK. Anti-HSP-70 antibody, and recombinant HSP-70 were purchased from Enzo. Anti- β -actin mouse monoclonal antibody, recombinant human TNF- α , IL-4, and IL-13 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-NF- κ B(p65) antibody, anti-

phospho-NF- κ B(p65) antibody, and anti-phospho-I κ B α antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-TSLP antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies for Western blot analysis were purchased from Genetex (Irvine, CA, USA). Anti-phospho-STAT6 antibody was supplied by Merck millipore, while anti-STAT6 antibody was obtained from BD Biosciences (Bedford, MA, USA).

Cell cultures

Human keratinocyte cell line, HaCa T cells, was obtained from the American Type Culture Collection and maintained at 37°C in culture medium composed of DMEM with 4.5 mg/ml glucose, 1% penicillin (100 u/ml), 1% streptomycin (100 u/ml; Gibco), 4 mM glutamine, and 25 mM HEPES in pH 7.4, supplemented with heat-inactivated 10% FBS. Culture medium was changed every 2 days.

The cells were seeded at 1×10^4 cells/well in flat-bottomed, 96-well microculture plates and were further cultivated for 24 h, then heated by immersion for 20 min in a water bath at 45°C before stimulation with 20 ng/ml TNF- α , 100 ng/ml IL-4, and 100 ng/ml IL-13 (Sigma).

Cell viability

In the test of the effect of heat shock on cell viability, the cells were seeded at 1×10^4 cells/well in flat-bottomed, 96-well microculture plates and further cultivated for 24 h. The cells were then heated by immersion for 20 min in a water bath at 45°C. The medium was renewed immediately after heat shock.

Cell viability was determined by MTT assays. Briefly, PBS containing 0.5 mg/ml MTT was added to each well. The plates were incubated for 1 h in the absence of light, before the medium was removed and precipitates resuspended in 100 μ l DMSO. Absorbance at 570 nm was measured using a plate reader. The experiments were performed in triplicate.

Western blotting analysis

For analysis of total protein levels of HSP-70 and TSLP, phospho-NF- κ B(p65), NF- κ B(p65), anti-phospho-I κ B α , phospho-STAT6, and STAT6-stimulated cells (4×10^5 cells, cultivated in 35-mm culture dishes) were rinsed twice with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.4, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2% NP-40, and 25% Glycerol). The cell lysates were centrifuged at 20817 g for 15 min at 4°C. The supernatant (25 μ g/each lane) was mixed with equal volumes of 4 $\times$ SDS sample buffer and boiled for 5 min. Equal amounts of protein extracts were separated through 10% SDS-PAGE gels.

After electrophoresis, the proteins were transferred to PVDF electrophoretically for 1 h (100V). The membranes were blocked in 5% skim milk (1 h), rinsed, and incubated with primary antibodies in TBST overnight at 4°C. Excess primary antibody was then removed by washing the membranes four times in TBST. The membrane was incubated with 0.1 μ g/ml

peroxidase-labeled secondary antibodies for 1 h. After three washes in PBST, bands were visualized by electrochemiluminescence and exposed to X-ray film. The antigens were quantitated by densitometry with the values calculated after normalization to the amount of β -actin.

Measurement of TSLP in the supernatant

Concentrations of TSLP in the supernatant were measured using specific enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA, 88-7497) according to the manufacturer's protocol. Absorbance was read at 450 and 570 nm with the microplate reader (Thermo Multiskan SPECTRUM, Thermo Fisher Scientific Inc, MA, USA). The sensitivity of the assay was 7.81 pg/ml.

RNA extraction, reverse transcription, and real-time quantitative PCR of TSLP

Total RNA was isolated from at least 4×10^5 cells using the Quick RNA MiniPre kit (Zymo research, Irvine, CA, USA) according to the recommended protocol. One microgram of total RNA was reverse-transcribed in a 20- μ l volume using a high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). Quantitative polymerase chain reaction (qPCR) was performed on an ABI7300 Real-Time PCR System (Applied Biosystems).

The expression of TSLP mRNA was normalized to RNA loading for each sample using human glyceraldehyde dehydrogenase (GAPDH) mRNA as an internal standard. The TSLP primers (sense, 5'-GCCATGAAACTAAGGCTGC-3'; antisense, 5'-CGCCACAATCCTTGTAATTG-3') and GAPDH primers (sense, 5'-ACCACAGTCCATGCCATCAC-3'; antisense, 5'-TCCACCACCCTGTTGCTGTA-3') were chosen to amplify a 152- and 452-bp fragment, respectively.

Statistical analysis

The TSLP concentrations in the culture media and TSLP mRNA levels in the cultured HaCa T cells were analyzed by ANOVA followed by post hoc analysis. Statistical significance was set at $p < 0.05$.

Results

Heat shock increased intracellular production of Hsp-70 from HaCa T cells without affecting cell viability

Culture dishes of HaCa T cells were sealed with parafilm and heated by immersion into a water bath at the temperature 45°C for 20 min as described before (16) and then replaced with fresh medium. For the indicated recovery, the cells were placed into the incubator. Non-heated cells were sealed for 20 min but remained in the incubator. Heat shock did not affect cell viability measured by MTT test (Fig. S1). The intracellular expression of HSP-70 determined by Western blot analysis increased gradually and peaked at 8 h after heat shock and then decreased (Fig. 1a).

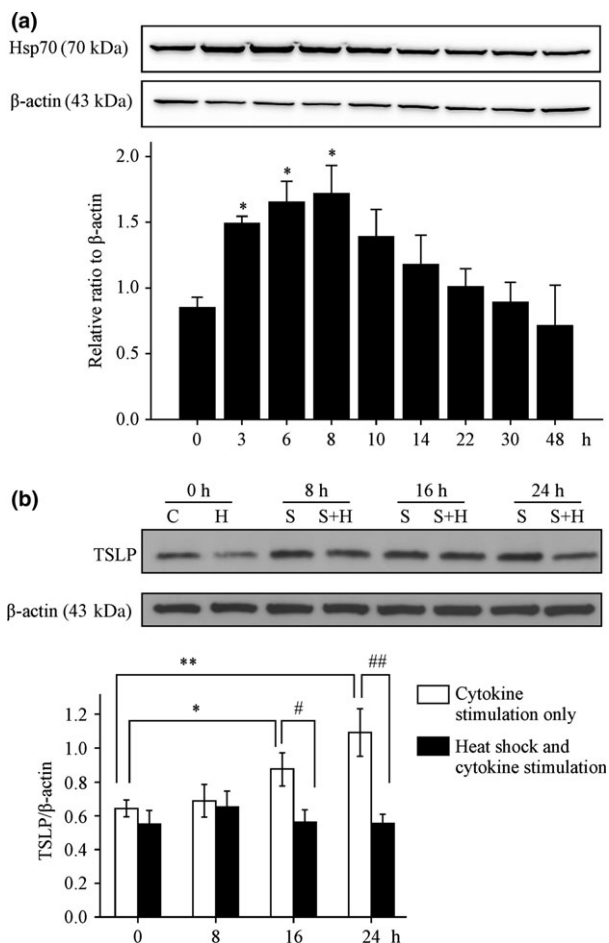


Figure 1 Heat shock increased HSP-70 expression and suppressed the increased intracellular TSLP after stimulation (a) Time-course effect of heat shock on Hsp-70 protein expression in HaCa T cells. Hsp-70 protein expressions were determined by Western blot analysis. The exposure to heat stress produced a progressive increase in Hsp-70 protein expression until the peak at 8 h and then decreased. β -Actin served as an internal control ($n = 6$). Representative of Western blot from experiments was shown. The data are presented as the mean $\pm$ SEM, * $p < 0.05$. (b) Heated and non-heated HaCa T cells were stimulated with TNF- α , IL-4, and IL-13 to induce TSLP expression. Intracellular TSLP levels were measured at 0, 8, 16, and 24 h after three combined cytokine stimulations. Non-heated, cytokine-stimulated HaCa T cells increased TSLP expression at time-dependent manner and heat stress prevented TSLP production via cytokines stimulation ($n = 6$). The data are presented as the mean $\pm$ SEM (significant compared with the control, * $p < 0.05$, ** $p < 0.01$; significant compared with non-heated, # $p < 0.05$, ## $p < 0.01$). C, control (non-cytokine stimulation and non-heat shock); H, heat shock; S, cytokine stimulation.

Heat shock in HaCa T cells suppressed the intracellular production and secretion of TSLP

Several cytokines, such as TGF- β , IFN- β , and, in particular, a combination of TNF- α and IL-4 or IL-13, were reported to enhance TSLP expression (17–19). Cultured in the incubator

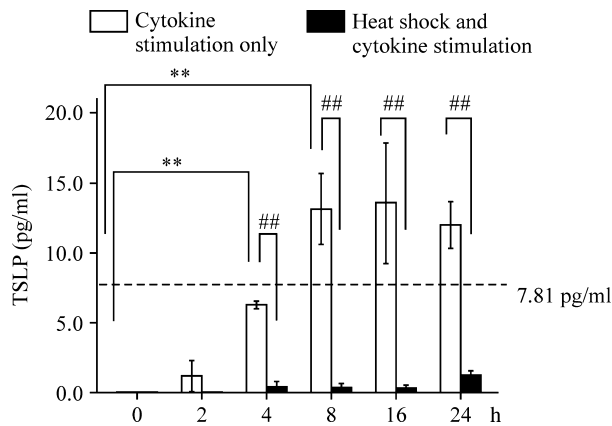


Figure 2 Thymic stromal lymphopoietin (TSLP) was secreted from HaCa T cells after stimulation and suppressed significantly by heat shock. Heated and non-heated HaCa T cells were stimulated with TNF- α , IL-4, and IL-13 to induce TSLP expression. The level of TSLP protein secreted into the supernatant from HaCa T cell after stimulation was measured by ELISA at 2, 4, 8, 16, and 24 h. Data are presented as the mean $\pm$ SEM ($n = 6$) (significant compared with the beginning, * $p < 0.05$, ** $p < 0.01$; significant compared with non-heated, # $p < 0.05$, ## $p < 0.01$). H, heat shock; S, cytokine stimulation.

for 6 h after heat shock, pre-heated and non-heated HaCa T cells were stimulated with TNF- α (20 ng/ml), IL-4 (100 ng/ml), and IL-13 (100 ng/ml) to induce TSLP secretion. The cytokine used to stimulate non-heated cells did not affect the intracellular level of HSP-70 (Fig. S2). Non-heated HaCa T cells progressively raised TSLP expression from 4 to 6 h after cytokine stimulation. However, pre-heated HaCa T cells did not show increased intracellular levels of TSLP.

At 16 h after stimulation, the gap of intracellular TSLP between the pre-heated and non-heated groups reached statistically significant difference (Fig. 1b). The secretion of TSLP measured by ELISA gradually increased from non-heated cells 4 h after stimulation and reached a peak at 16 h. The secretions of pre-heated cells were strongly and persistently suppressed very early time after heat shock (4 h after stimulation) (Fig. 2).

Heat shock suppressed TSLP transcription through the NF- κ B signal pathway

The total RNA of stimulated HaCa T cells was extracted at 4 and 8 h after stimulation and reverse-transcribed. The messenger RNA level of TSLP was measured and analyzed by real-time PCR. After stimulation, the mRNA level of non-heated cells was elevated and peaked at 4 h, before being reduced. In contrast, the transcription of TSLP in pre-heated HaCa T cells was inhibited soon after heat shock and the effects of inhibition declined slowly. The two groups had a significant difference in mRNA level at 4 h but no difference after 8 h (Fig. 3a).

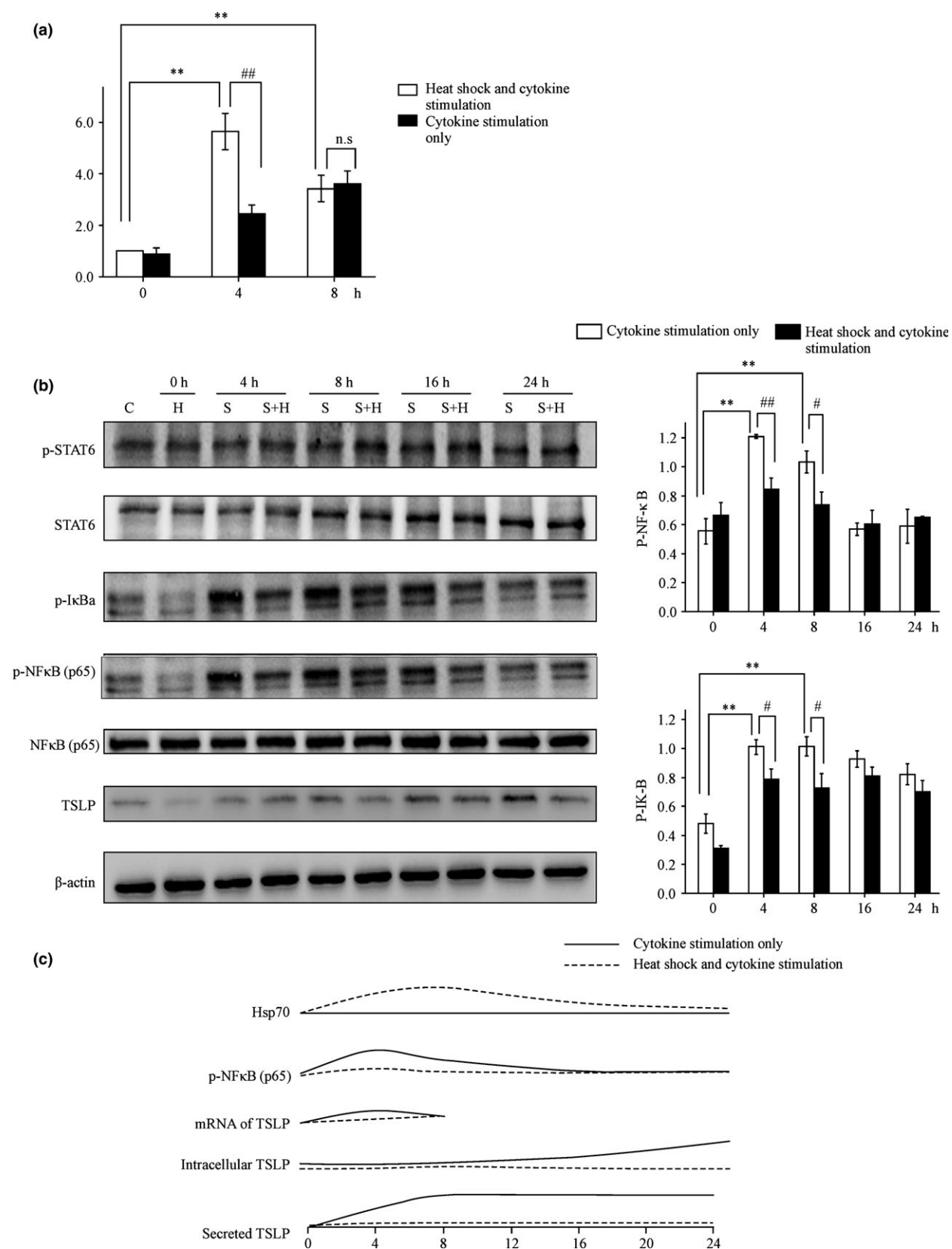
Heat-shock protein was previously reported to inhibit NF- κ B activation in a dose- and time-dependent manner (20), and HSP-70 was also reported being regulated by reactive oxygen species via the JAK/STAT pathway (21). So, it is possible that the inhibitory effect of heat shock on TSLP transcription in HaCa was via interaction with either NF- κ B or JAK/STAT pathway.

The downstream of JAK/SAT and NF- κ B signal pathways was investigated to reveal the possible inhibitory mechanism of heat shock. The results showed that pNF- κ B and pIKB were suppressed in the pre-heated cells, while pSTAT6 was not affected by heat shock. The decreased phosphorylation of I κ B- α , the major cytosolic inhibitor of NF- κ B, avoid IKB being degraded by proteasome degradation. Therefore, un-degraded cytosolic I κ B- α downregulated the NF- κ B phosphorylations, the activation of NF- κ B, and reduced the induction of NF- κ B target genes (Fig. 3b). To demonstrate that HSP-70 reduces TSLP by inhibiting NF- κ B activation, HaCa T cells were pre-treated recombinant HSP-70 (rHSP-70) (0.1 μ g/ml) for 2 h, and then washed out and stimulated with TNF- α (20 ng/ml), IL-4 (100 ng/ml), and IL-13 (100 ng/ml) to induce TSLP secretion. Although not so strongly as the phenomenon noted in heat shock, extragenous rHSP-70 also had a significant inhibition effect on TSLP secretion with a dose-dependent manner (Fig. 4a) and again suppressed the NF- κ B signal pathway (Fig. 4b).

Discussion

Atopic dermatitis is a complicated syndrome initiated by the interaction between genetic cofactors and environmental factors. Mutant genes such as filaggrin result in a defective, dry

Figure 3 The increased transcription of thymic stromal lymphopoietin (TSLP) after stimulation was suppressed 4 h after heat shock via inhibition of the NF- κ B signal pathway. Heated and non-heated HaCa T cells were stimulated with TNF- α , IL-4, and IL-13 to induce TSLP expression. (a) The TSLP mRNA expression was measured by real-time reverse-transcriptase PCR (RT-PCR) analysis at 0, 4, and 8 h after three combined cytokine stimulations. The results were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Data are presented as the mean $\pm$ SEM ($n = 6$) (significant compared with the beginning, * $p < 0.05$, ** $p < 0.01$; significant compared with non-heated, # $p < 0.05$, ## $p < 0.01$; NS, not significant). (b) Cell lysates at 0, 4, 8, 16, and 24 h were Western-blotted with anti-phospho-STAT6, anti-STAT6, anti-phospho-I κ B α , anti-phospho-NF- κ B(p65), and anti-NF- κ B(p65) ($n > 5$). Representative of Western blot from experiments was shown. The cell lysate of only heated group was collected 6 h after heat shock (0 h before cytokine stimulation). Data of p-I κ B α and p-NF- κ B (p65) are presented as the mean $\pm$ SEM (significant compared with the 0 h, * $p < 0.05$, ** $p < 0.01$; significant compared with non-heated, # $p < 0.05$, ## $p < 0.01$). C, control (non-cytokine stimulation and non-heat shock); H, heat shock; S, cytokine stimulation. (c) Time courses of transcription factor p-NF- κ B, TSLP mRNA, protein of TSLP, Hsp-70 (Western blot), and TSLP secretion were analyzed from 0 to 24 h after HaCa T cells were stimulated with TNF- α , IL-4, and IL-13 in the presence or absence of heat shock.



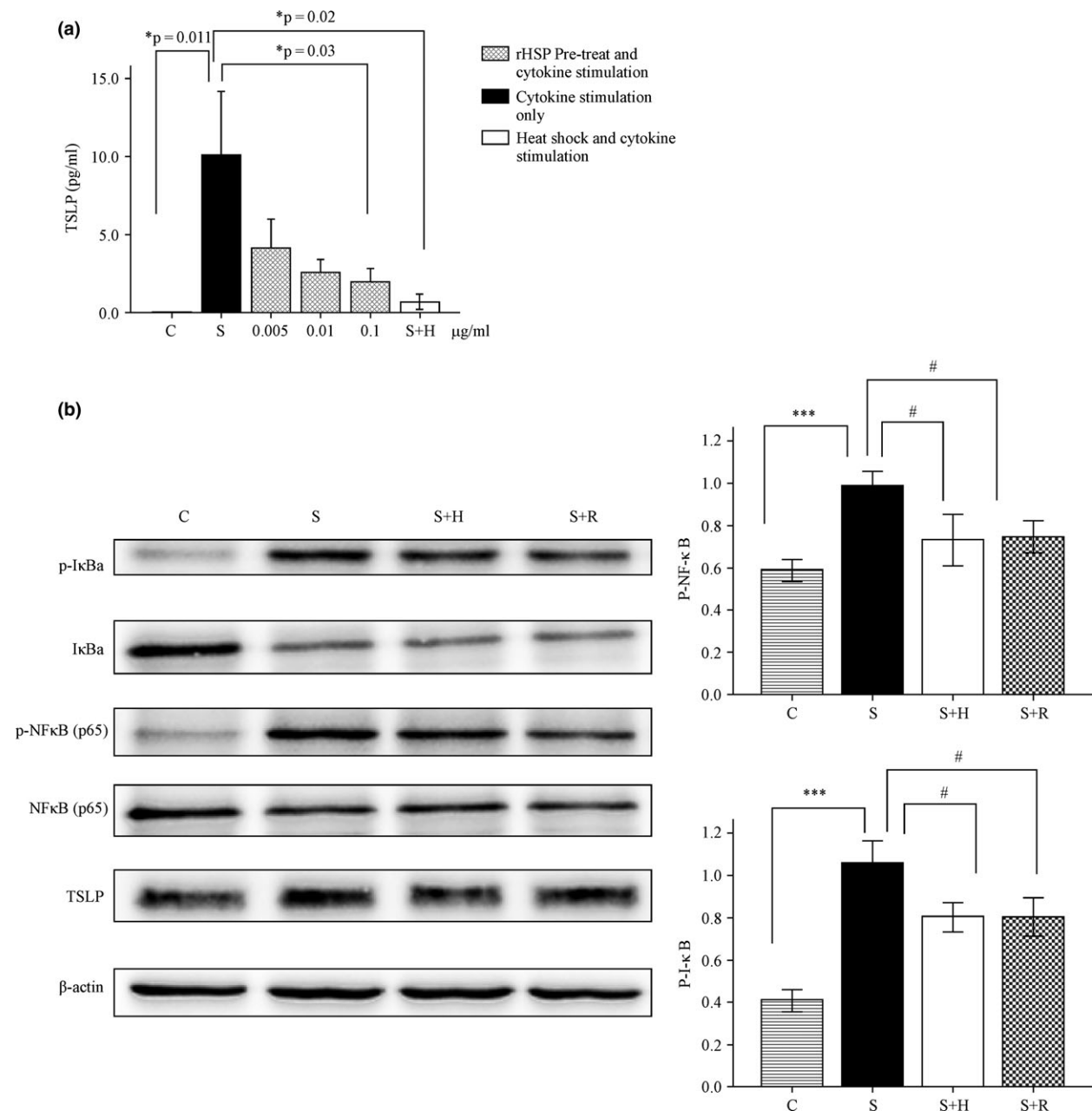


Figure 4 Extragenous Recombinant HSP-70 also suppressed the secretion of thymic stromal lymphopoietin (TSLP) and NF-κB signal pathway. (a) HaCa T cells were pre-treated with rHSP-70 (0.005, 0.01, and 0.1 μg/ml) for 2 h and then washed out and stimulated with TNF-α, IL-4, and IL-13 for 24 h. The effect of rHSP-70 on TSLP secreted into supernatant from HaCa T cell after stimulation was measured by ELISA. All data are presented as mean±SE. (n = 6) (significant compared with the control, *p < 0.05, **p < 0.01; significant compared with non-heated, #p < 0.05, ###p < 0.01). C, control (non-cytokine stimulation and non-rHSP-70). (b) HaCa T cells were pre-treated with rHSP-70 (0.1 μg/ml) for 2 h and then washed out and stimulated with TNF-α, IL-4, and IL-13 for 8 h. Cell lysates were Western-blotted with anti-phospho-IκBα, anti-phospho-NF-κB (p65), and anti-NF-κB(p65). Representative of Western blot from experiments was shown. Data of p-IκBα and p-NF-κB (p65) are presented as the mean±SEM (significant compared with control, *p < 0.05, **p < 0.01, ***p < 0.001; significant compared with only cytokines stimulation, #p < 0.05, ##p < 0.01, ###p < 0.001) (n = 5). C, control (non-cytokine stimulation, non-heat shock and non-rHSP-70 pre-related); H, heat shock; S, cytokine stimulation. R= rHSP-70 pre-treated.

itchy skin barrier (22). This allows the entry of antigens via the skin and creates a milieu that shapes the immune response to these antigens. In addition, the allergic Th2 responses are now

considered important in promoting the development of AD. Among the pro-Th2 cytokines that are important contributors to AD development, TSLP is regarded as an initiation factor.

The elevated levels of TSLP were observed in the skin of lesion of AD but not in contact allergic dermatitis (23) and the animal model by skin damage via tape stripping also revealed increased skin levels of TSLP and TSLP-dependent Th2 responses (24).

Environmental factors such as Toll-like receptor ligands, cigarette smoke, and chemicals could trigger TSLP released from epithelial cells or KCs. The present *in vitro* study mimics AD initiation which overexpressed and oversecreted TSLP in keratinocytes. Interestingly, previous heat-shock pre-treatment attenuates TSLP expression and secretion in the study, implying that heat shock, highly possible the synthesis of HSP-70, participates in the immune process of AD.

The HSPs have direct anti-inflammatory and protective effects through refolding denatured proteins, targeting irreversibly denatured proteins for removal, as well as inhibiting apoptosis (25). HSP-70 and HSP-27 are also now reported to suppress inflammation by decreasing NF- κ B activation (26). Furthermore, the NF- κ B-binding site is reportedly located at the proximal region of the TSLP promoter and is critical for TSLP modulation in human intestinal epithelial cells (27). From the results here, both the mRNA and protein expressions of TSLP were inhibited in pre-heated cells, associated with evidence of decreased NF- κ B activation. The presumption that NF- κ B signal pathway was inhibited by HSP-70 was validated by further experiment in our study that recombinant HSP-70 can significantly reduce the production and secretion of TSLP from HaCaT cells under Th2 environment. The extracellular influence of released HSP-70 from heated cells in this study can be excluded because the supernatants of cells in the presence or absence of heat stress do not show any difference (Fig. S3).

Epithelial cells secrete a range of cytokines and chemokines (28) and protein trafficking pathway is dependent on a range of molecular families. The most informative group is a family of SNAP (soluble NSF attachment protein) receptors (SNAREs). SNAREs are intracellular proteins that form the membrane-bridging helix bundles to control intracellular membrane fusion (29, 30). Ashwini reports that HSP-70 stimulates the *in vitro* SNARE complex formation among the ER/Golgi

SNAREs and excessive HSP-70 specifically inhibits ER-to-Golgi transport in permeabilized cells. The results demonstrate that HSP molecular chaperones play a role in regulating steps of the secretory pathway (31). Time courses of transcription factor p-NF- κ B, TSLP mRNA, protein of TSLP, Hsp-70 and TSLP secretion showed that the intracellular level of TSLP was significantly decreased 16 h after heat stress of stimulated HaCaT cells. However, the secretion of TSLP was strongly inhibited at very early heat shock (Fig. 3c). It is supposed that HSPs, highly possible the HSP-70, block the release of TSLP from cells through a mechanism distinct from protein production because it occurs earlier than the level of intracellular protein affected. The results provide evidence, for the first time, that HSPs might interfere the TSLP trafficking pathways before being released.

For the clinical medicine, the finding that extragenous rHSP-70 can significantly reduce the secretion of TSLP from KCs shed new light on the new treatment options for atopic dermatitis. Animal research will be designed and performed further to confirm the role of HSP-70 for the treatment of AD. In addition to atopic dermatitis, many skin diseases such as psoriasis are related to excessive inflammation. The more the understanding about the anti-inflammatory function and immune modulation of HSP, the sooner the HSP can be used in medical application.

The limitation of this study is that intracellular HSP-70 was not knocked down to prove its direct inhibition of the transcription and secretion of TSLP. This limitation needs further research to elucidate the mechanisms of heat shock on TSLP production.

In conclusion, heat shock significantly reduced the production and secretion of TSLP from HaCaT cells under Th2 environment. Modulation in HSPs synthesis, especially HSP-70, might be a new therapy strategy in patients with atopic dermatitis in the future.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Heat shock did not affect cell viability. Effect of heat shock on HaCa T viability. The MTT assay demonstrated that heat shock did not affect cell viability compared to non-heated cells ($n = 6$). * $p < 0.05$.

Figure S2. TH2 cytokines stimulation did not affect intracellular Hsp-70 level. Heated and non-heated HaCa T cells were stimulated with TNF- α , IL-4, and IL-13 to induce TSLP expression. Intracellular HSP-70 protein levels were measured by Western blot analysis at 0, 8, 16, and 24 h after three combined cytokine stimulation ($n = 6$). The stimulation of cytokine

did not change intracellular Hsp-70 protein expression in HaCa T cells. β -actin served as an internal control. Representative of Western blot from experiments was shown. The data are presented as mean $\pm$ SEM. * $p < 0.05$.

Figure S3. Released Hsp-70 in supernatants was the same between non-heated and heated cells. The culture medium of HaCa T cells was renewed immediately after heat shock and then cells were put into incubators. The medium was refreshed after 6 h in incubators as the protocol of inducing TSLP expression by cytokines stimulation (arrow). Supernatants of culture dishes in the presence or absence of heat shock were collected at 0, 3, 6, 8, 10, 14, 22, 30, and 48 h. The level of HSP-70 secreted from HaCa T cells was measured by ELISA. Data was presented as the mean ($n = 5$).

Figure S1

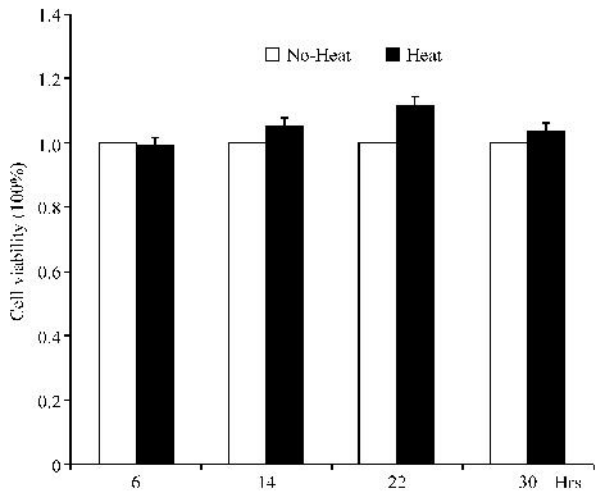


Figure S2

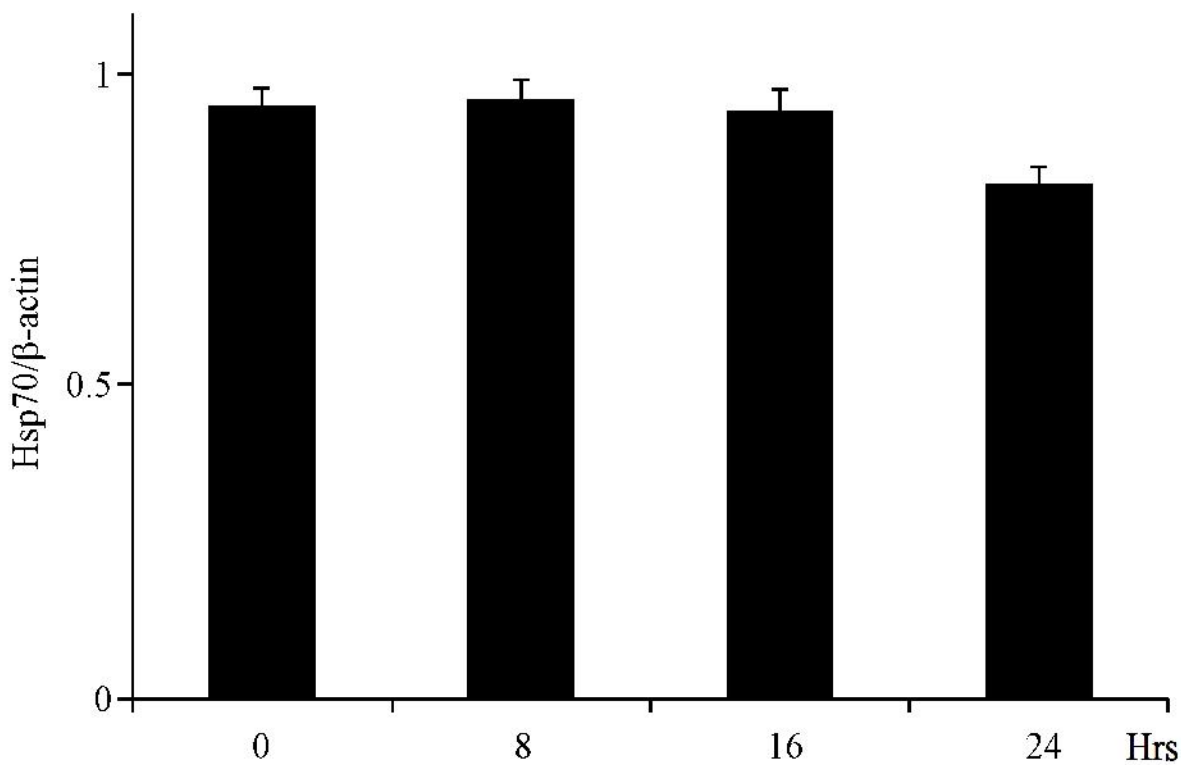
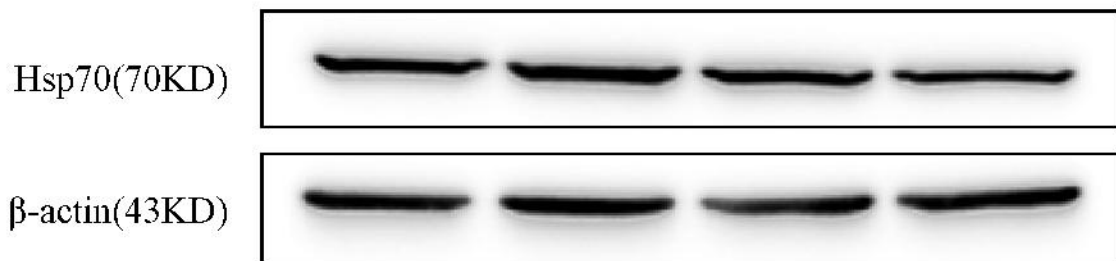


Figure S3

