

# NF- $\kappa$ B regulates HSF1 and c-Jun activation in heat stress-induced intestinal epithelial cell apoptosis

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**Abstract.** Heat stress may induce intestinal epithelial cell apoptosis; however, the molecular mechanisms have not yet been identified. The present study used IEC-6 rat small intestinal epithelial cells to investigate heat stress-induced production of reactive oxygen species (ROS), which may be involved in nuclear factor (NF)- $\kappa$ B activation during heat stress. IEC-6 cells were transfected with NF- $\kappa$ B p65-specific small interfering RNA (siRNA), and observed a significant increase in cell apoptosis and caspase-3 cleavage; however, in cells transfected with adenovirus that constitutively overexpressed p65, the opposite results were obtained. Furthermore, p65 knockdown increased the heat stress-induced expression and activity of heat shock transcription factor 1 (HSF1); conversely, p65 overexpression slightly decreased HSF1 activity. The levels of heat stress-induced c-Jun phosphorylation were also examined: Knockdown of p65 resulted in a reduction of c-Jun phosphorylation, whereas p65 overexpression resulted in increased phosphorylation. Furthermore, siRNA-mediated knockdown of HSF1 in IEC-6 cells significantly increased heat stress-induced apoptosis. Cells pretreated with c-Jun peptide, an inhibitor of c-Jun activation, exhibited a significant reduction in apoptosis. These findings indicated that heat stress stimulation in IEC-6 cells induced the pro-apoptotic role of NF- $\kappa$ B by regulating HSF1 and c-Jun activation.

## Introduction

Intestinal epithelial cells are crucial components of the intestinal mucosal barrier, and damage to these cells increases the permeability of this barrier, which may lead to increased translocation of gut-derived bacterial endotoxins (1). Previous studies on the effects of heat stress on these cells have reported a significant level of apoptosis in the rat small intestine (2,3). Indeed, apoptosis in the small intestine was demonstrated to serve a major role in the pathogenesis of heat stroke (2,3).

Heat stress may induce the production of reactive oxygen species (ROS), which may lead to cellular dysfunction and cell death (3-5). Nuclear factor (NF)- $\kappa$ B was previously reported to be induced by a multitude of stimuli, such as cytokines, oxidative stress or thermal stress (6). A previous study demonstrated that NF- $\kappa$ B is activated in response to heat stress in HeLa human cervical cancer cells (6). Many inducers of NF- $\kappa$ B expression may be inhibited by antioxidants, which suggested that ROS may modulate the signal transduction pathway leading to NF- $\kappa$ B activation (7,8). However, the role of ROS heat stress-induced NF- $\kappa$ B activation in IEC-6 rat small intestinal epithelial cells remains unclear. Cultured IEC-6 cells are similar to the mature intestinal epithelium (3), and were used as a model to investigate the mechanisms of intestinal epithelial cell survival and apoptosis.

The heterodimeric NF- $\kappa$ B complex comprises two DNA binding subunits, p50 and p65, which may form either homo- or heterodimers (9). NF- $\kappa$ B signaling is a transcriptional regulator of several genes that are involved in the inflammatory response, cell growth, cell survival and apoptosis (9,10). A number of studies have reported that NF- $\kappa$ B activation may downregulate proapoptotic signaling and thus prevent apoptosis in many cells types (10,11). By contrast, other studies have demonstrated that NF- $\kappa$ B activation may be an inducer of apoptosis (12,13). However, the mechanisms underlying heat stress-induced apoptosis in IEC-6 cells and the involvement of NF- $\kappa$ B activation are still unknown.

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Heat shock transcription factor 1 (HSF1) is a master regulator of the genes that encode molecular chaperones and serves a role in the attenuation of apoptosis induced by multiple factors (14,15). Upon heat shock, HSF1 rapidly translocates into the nucleus and exhibits the properties of a stable trimer which correlates with the acquisition of DNA binding activity; furthermore, the transcriptionally active form of HSF1 becomes inducibly phosphorylated (16). Previous studies have reported interactions between HSF1 and NF- $\kappa$ B, which serve opposite roles in cytoprotection and cell injury (17).

The signal-transducing transcription factor c-Jun, also known as activating protein 1 (AP1), has previously been reported to serve a role in cell cycle progression, differentiation and transformation, as well as apoptosis (18,19). c-Jun protein activity is regulated by phosphorylation at specific sites; for example, Ser63 phosphorylation in the transactivation domain leads to an increased ability of c-Jun to activate the transcription of target genes (20). Previous studies have demonstrated that c-Jun is able to physically interact with NF- $\kappa$ B p65 through the Rel homology domain (21). Nevertheless, whether NF- $\kappa$ B can interact with HSF1 and c-Jun, and thereby influence IEC-6 cell apoptosis, still remains unknown.

Results from the present study demonstrated that heat stress-induced increases of ROS levels may lead to NF- $\kappa$ B activation, which in turn may activate caspase-3 and, thus, apoptosis in IEC-6 cells. In addition, a putative role for NF- $\kappa$ B in the regulation of HSF1 and c-Jun activation induced by heat stress treatment was investigated. This study also aimed to examine whether HSF1 might prevent apoptosis and c-Jun activation-induced apoptosis in the same experimental settings.

## Materials and methods

**Cell culture and treatments.** Approximately  $2 \times 10^6$  IEC-6 cells (ATCC, Manassas, VA, USA) were grown as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. To induce heat stress, culture dishes were placed into a circulating water bath at 43 $\pm$ 0.5°C for indicated times; control cells were held at 37 $\pm$ 0.5°C. Following heat-stress culture, the media was replaced and cells were further incubated at 37°C for 0, 2, 6 and 12 h.

**Measurement of ROS levels.** Levels of intracellular ROS were assessed using a ROS assay kit (Beyotime Institute of Biotechnology, Haimen, China). Dichlorofluorescein diacetate (DCFH-DA; Molecular Probes; Thermo Fisher Scientific, Inc.) enters the cells and reacts with ROS, producing the fluorophore DCF. Briefly, cells were either kept untreated or incubated at 43°C for 20 (short-term heat stress), 40, 60 (moderate-term heat stress) and 80 min (long-term heat stress), followed by an additional incubation at 37°C for 6 h. IEC-6 cells ( $3 \times 10^5$ ) with the antioxidant glutathione (GSH; 100  $\mu$ mol/l) for 1 h, followed by exposure to heat stress at 43°C for 60 min. Control cells were always incubated at 37°C. Cells ( $3 \times 10^5$ ) were harvested, washed with serum-free DMEM culture medium, and stained with

10  $\mu$ M DCFH-DA for 30 min at 37°C in the dark. Following this, the cells were harvested by trypsin, the supernatants was removed by centrifugation (1,000  $\times$  g for 3 min at room temperature) and resuspended in serum-free DMEM culture medium three times. The fluorescence intensity was determined using a flow cytometer (FACSCanto™ II; BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software version 9.0 (FlowJo LLC, Ashland, OR, USA).

**Flow cytometric analysis of cell apoptosis.** Cell apoptosis was analyzed by flow cytometry with annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, cells were either kept untreated or incubated at 43°C for 20, 40, 60, 80 min, followed by an additional incubation at 37°C for 6 h. Control cells were always incubated at 37°C. IEC-6 cells ( $1 \times 10^6$ ) were collected, washed in ice cold PBS and resuspended in the binding buffer containing 5  $\mu$ l annexin V-FITC (5  $\mu$ g/ml). Following incubation at room temperature for 10 min, the buffer was removed by centrifugation (1,000  $\times$  g, 3 min, room temperature) and cells were resuspended in reaction buffer containing 10  $\mu$ l propidium iodide (PI; 5  $\mu$ g/ml) for 10 min at room temperature. Flow cytometric analysis was immediately performed to detect apoptosis (FACSCanto™ II; BD Biosciences). The combination of Annexin V-FITC and propidium iodide allows for the distinction between early apoptotic cells (Annexin V-FITC positive), late apoptotic and/or necrotic cells (Annexin V-FITC and propidium iodide positive), and viable cells (unstained). The fluorescence intensity was analyzed using FlowJo software version 9.0 (FlowJo LLC, Ashland, OR, USA).

**Small interfering (si)RNA transfection.** siRNAs for NF- $\kappa$ B p65 and HSF1 were designed and synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). The sequence of each siRNA and the negative control (non-targeting siRNA) are shown in Table I. Prior to transfection,  $1 \times 10^5$  IEC-6 cells were plated onto a 6-well plate (Nest Biotechnology Co., Ltd., Wuxi, China) and incubated for 24 h to 30–50% confluence at 37°C. Cells were transfected with 1  $\mu$ M siRNA (all siRNAs were used at this concentration) using siRNAMate Transfection Reagent (Shanghai GenePharma Co. Ltd.) and incubated for 12 h at 37°C, according to the manufacturer's protocol. Cells were incubated following 48–72 h at 37°C for further experiments.

**Adenoviral infection.** Adenoviruses (Ad) that constitutively overexpressed p65 (Ad-p65) or empty construct (Ad-empty) were constructed by Vigene Biosciences (Jinan, China). Cells were infected with the adenoviruses in serum-free DMEM for 6 h and then the media was replaced with DMEM supplemented with 10% FBS. Cells ( $1 \times 10^5$ /well) were infected with 100 MOI Ad in serum-free DMEM for 6 h at 37°C, according to the manufacturer's protocol, following which the media was replaced with DMEM supplemented with 10% FBS, cells were incubated following 48–72 h at 37°C for further experiments.

**Western blot analysis.** IEC-6 cells ( $\sim 1 \times 10^6$ ) were kept at 37°C or 43°C for 60 min, and further incubated for 2, 6 or 12 h

Table I. Small interfering RNA oligonucleotide sequences.

Gene	Sequence (5'→3')
p65	Sense: GCCCUAUGCCUUUACGUCATT Antisense: UGACGUAAAGGGAUAGGGCTT
HSF1	Sense: GGAAAGUGGUCCACAUCGATT Antisense: UCGAUGUGGACCACUUUCCTT
Negative control	Sense: UUCUCCGAACGUGUCACGUTT Antisense: ACGUGACACGUUCGGAGAATT

HSF1, heat shock transcription factor 1.

at 37°C. For cytoplasmic and nuclear protein of p65 extraction, cells were lysed in NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The IEC-6 cells were homogenized in radioimmunoprecipitation buffer with phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Following centrifugation at 14,000 × g at 4°C for 10 min, the supernatants were used for western blot analysis. Protein concentration was determined using a Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (20 µg/well) were separated by SDS-PAGE using 10% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with blocking solution (5% skimmed milk diluted with PBS) at room temperature for 2 h, followed by incubation with primary antibodies overnight at 4°C. The following rabbit primary antibodies were used at a 1:2,000 dilution: p65 (cat. no. Ab16502; Abcam, Cambridge, MA, USA), HSF1 (cat. no. Ab59963; Abcam), phosphorylated (p)-HSF1 (cat. no. Ab52757; Abcam), Lamin-B1 (cat. no. Ab16048; Abcam), c-Jun (rabbit antibodies; cat. no. 9165p; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-c-Jun (rabbit antibodies; cat. no. 8222S; Cell Signaling Technology, Inc.), caspase-3 (rabbit antibodies; cat. no. 14220S; Cell Signaling Technology, Inc.), cleaved caspase-3 (rabbit antibodies; cat. no. 9654S; Cell Signaling Technology, Inc.) and GAPDH (rabbit antibodies; cat. no. ab70699; Abcam). An anti-rabbit horseradish peroxidase-conjugated immunoglobulin G antibody (cat. no. TA130023; 1:5,000; OriGene Technologies, Inc., Beijing, China) was used as the secondary antibody for incubation for 2 h at room temperature. Antibodies were detected with Enhanced Chemiluminescence Western Blot Detection reagent (Pierce; Thermo Fisher Scientific, Inc.). Membranes were exposed to light-sensitive film and quantified using ImageJ software (version 1.3.4.67; National Institutes of Health, Bethesda, MD, USA).

**Measurement of p65 and HSF1 DNA-binding capacity by ELISA.** Nuclear extracts were prepared from treated and control cells (~5 × 10<sup>6</sup> cells) using a Nuclear Extract kit (Active Motif, Shanghai, China) according to the manufacturer's protocol. The ability of p65 and HSF1 to bind the DNA consensus sequence was assessed using an ELISA-based TransAM NF-κB kit (cat. no. 40096; Active Motif, Carlsbad, CA, USA) and an ELISA-based TransAM HSF1 kit (cat. no. 47096; Active

Motif), according to the manufacturer's protocol, quantitative analysis was performed by spectrophotometry at 450 nm using an automatic microplate reader (SpectraMax® M5; Molecular Devices, LLC, Sunnyvale, CA, USA).

**Statistical analysis.** All data were analyzed for statistical significance using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were expressed as the mean ± standard deviation from at least three independent experiments performed in duplicate. One-way analysis of variance was performed followed by Fisher's least significant difference post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Heat stress increases ROS generation and apoptosis in IEC-6 cells.** As ROS generation serves an important role in heat stress (3-5), the effects of heat stress on ROS accumulation was examined in IEC-6 cells. IEC-6 cells were exposed to heat stress (43°C) for different time intervals (20, 40, 60 and 80 min), followed by an additional incubation at 37°C for 6 h in fresh media. Intracellular ROS levels increased in a time-dependent manner (Fig. 1A). In addition, apoptotic rates were quantitative using flow cytometry. Cells were exposed to different durations of heat stress, and the results demonstrate that the number of early apoptotic cells gradually increased and reached a peak of 32.37% in IEC-6 cells exposed incubated at 43°C for 80 min (Fig. 1B).

**Role of ROS in activating p65 in heat stress-treated IEC-6 cells.** Compared with the untreated control cells, the capacity of p65 to bind to DNA in IEC-6 cells was not modified at 20 min of heat stress at 43°C (Fig. 2A). By contrast, at 40 min heat stress, the DNA-binding capacity of p65 increased and continued to increase significantly at 60 and 80 min (Fig. 2A). Additionally, a time course of p65 activation was analyzed in heat stressed IEC-6 cells at 43°C for 60 min followed by a recovery period at 37°C for 0, 6, or 12 h. When nuclear and cytoplasmic extracts of these timepoints were collected and analyzed by western blot, nuclear levels of p65 increased as the recovery time increased (Fig. 2B). Collectively, these data indicated that heat stress may induce the activation of NF-κB p65, but this event seems not to occur during 20 min of heat stress at 43°C (short-term heat stress).

The role of heat stress-induced ROS generation in p65 activation was examined by pretreating IEC-6 cells with the antioxidant glutathione (GSH; 100 µmol/l) for 1 h, followed by exposure to heat stress at 43°C for 60 min. Cells pretreated with GSH significantly decreased the heat stress-mediated translocation of p65 to nucleus and the binding capacity of p65 to DNA (Fig. 2C and D, respectively). These data suggested that heat stress treatment of IEC-6 cells activated the NF-κB p65 pathway through the release of ROS.

**p65 and HSF1 activation is involved in heat stress-induced apoptosis in IEC-6 cells.** To clarify the role of NF-κB p65 and HSF1 in heat stress-induced apoptosis in IEC-6 cells, the effects of p65-siRNA and HSF1-siRNA knockdown, as well as Ad-p65 overexpression were examined (Fig. 3A-C).

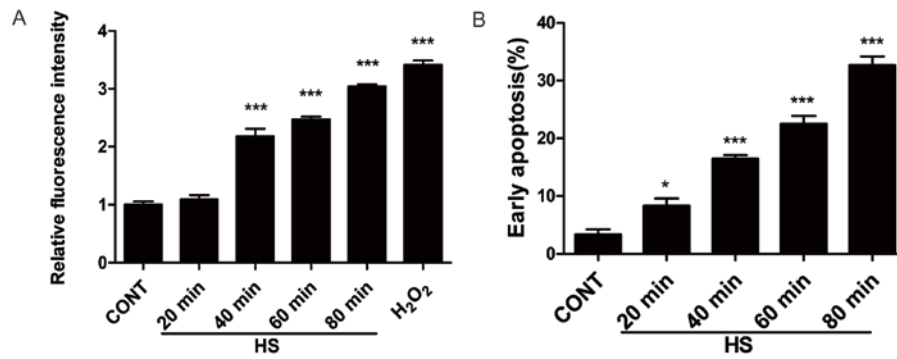


Figure 1. Effects of heat stress on ROS production and apoptosis in IEC-6 cells. Cells were left untreated or exposed to 43°C for the indicated times, followed by a 6 h incubation at 37°C. (A) ROS levels were assayed by dichlorofluorescein diacetate staining. (B) Apoptosis was analyzed by flow cytometry using annexin V-fluorescein isothiocyanate/propidium iodide staining. Data are presented as the mean  $\pm$  standard deviation of three independent experiments; \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. CONT. CONT, untreated control; HS, heat stress.

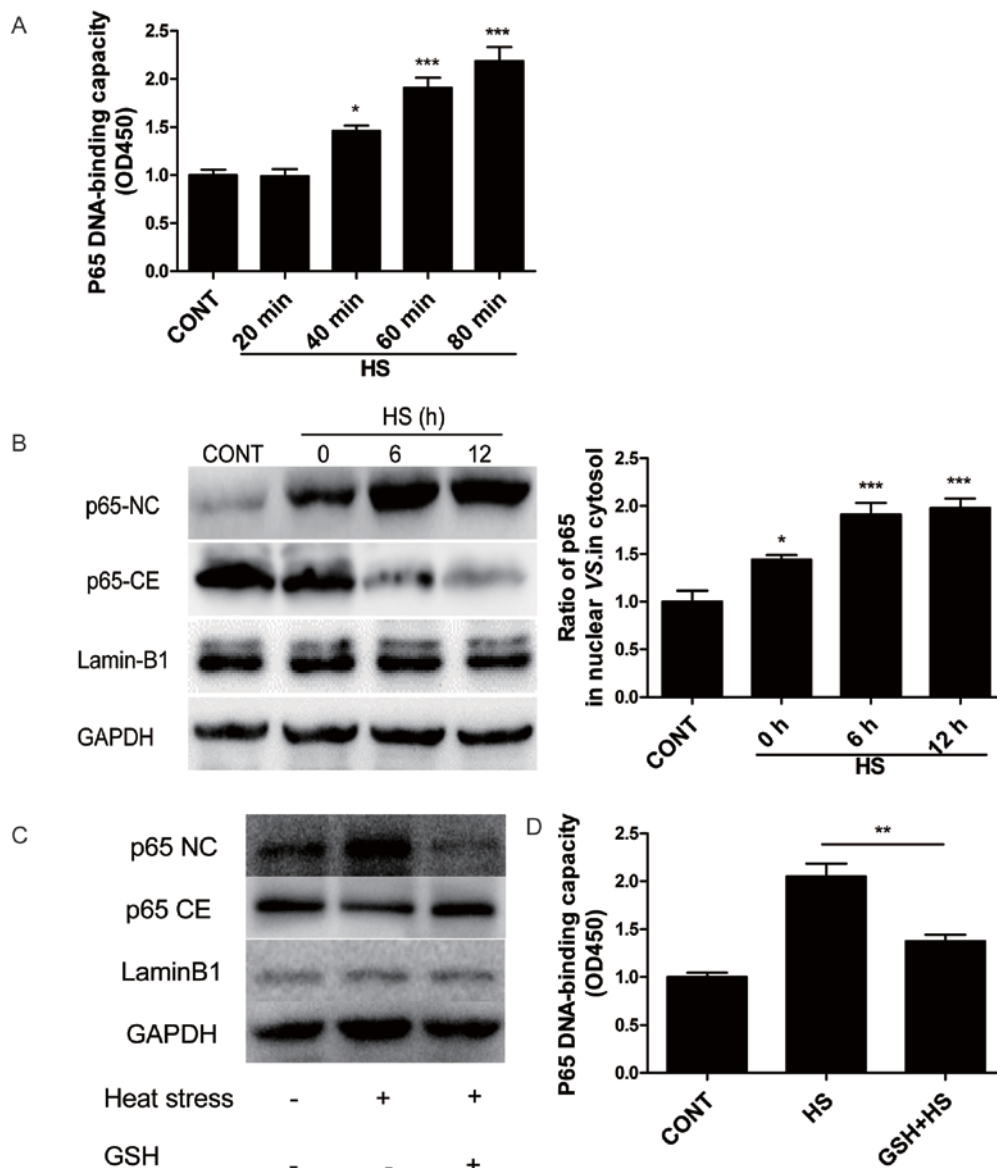


Figure 2. Reactive oxygen species may be involved in heat stress-induced p53 activation. (A) IEC-6 cells were either left untreated or exposed to 43°C for indicated times, followed by a 6 h incubation at 37°C. (B) IEC-6 cells were either left untreated or exposed to 43°C for 60 min and were further incubated for indicated times. (C) Cells were pretreated with or without GSH (100  $\mu$ mol/l) for 1 h, then incubated at 43°C for 60 min, and further incubated for 6 h at 37°C. p53-CE and p53-NC expression levels were detected by western blotting. (D) p53 DNA-binding capacity was quantified by TransAM NF- $\kappa$ B kit; Data are presented as the mean  $\pm$  standard deviation of three separate experiments; \* $P$ <0.05 and \*\*\* $P$ <0.001 vs. CONT. \*\* $P$ <0.01 vs. HS group. GSH, glutathione; BHA, butylhydroxyanisole; CE, cytoplasmic extract; CONT, untreated control; NC, nuclear extract; NF, nuclear factor.

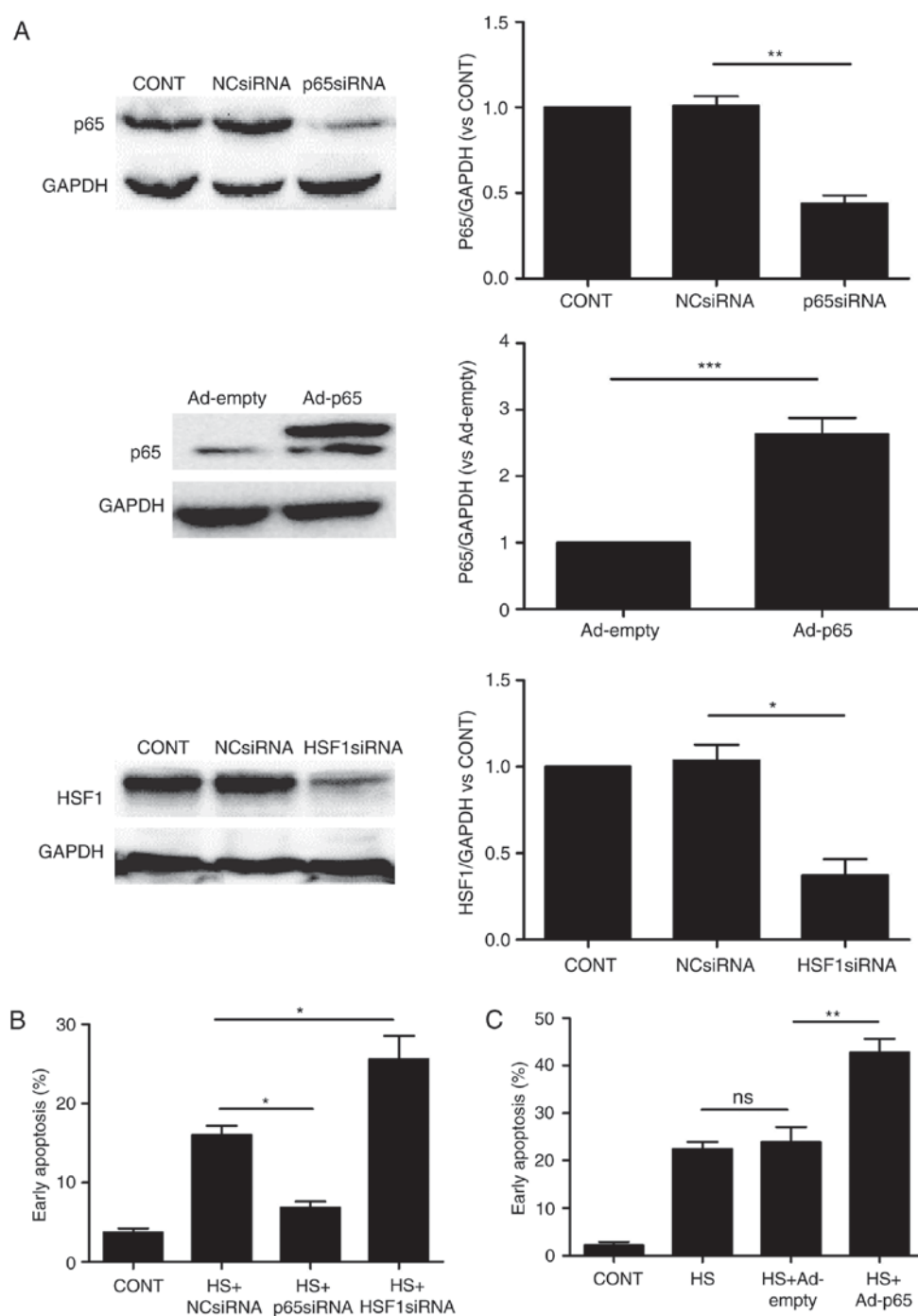


Figure 3. Role of p53 and HSF1 in heat stress-induced cell apoptosis in IEC-6 cells. Cells were transfected with NC-siRNA, p65-siRNA or HSF1-siRNA, as well as Ad-empty or Ad-p65 for 48 h. Western blot analysis was used to detect the effectiveness of transfection for (A-C) p65-siRNA knockdown, Ad-p65 overexpression and HSF1-siRNA knockdown expression. (B and C) Cells were exposed to heat stress for 60, followed by a 6 h incubation at 37°C, and apoptosis was analyzed by flow cytometry using Annexin V-fluorescein isothiocyanate/propidium iodide staining. Data are presented as the mean  $\pm$  standard deviation of three separate experiments; \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001. Ad, adenovirus; CONT, untreated control; HSF1, heat shock transcription factor 1; NC, negative control; ns, not significant; siRNA, small interfering RNA.

Following p65-siRNA mediated knockdown, apoptosis was significantly reduced in heat stress-treated IEC-6 cells (Fig. 3D). By contrast, apoptosis was markedly increased in heat stress-treated cells that were co-treated with Ad-p65 compared with the same cells transfected with Ad-empty control (Fig. 3E). These observations implied that activated p65 may be able to mediate heat stress-induced apoptosis in IEC-6 cells. In addition, heat stress-induced IEC-6 cells co-treated with HSF1-siRNA exhibited a significant increase

in apoptosis, which demonstrated that HSF1 may serve an antiapoptotic role in IEC-6 cells (Fig. 3C and D).

**Role of p65 in HSF1 activation in heat stress-induced apoptosis in IEC-6 cells.** Whether HSF1 activation was linked with NF- $\kappa$ B signaling in the experimental settings was investigated. p65-siRNA-mediated knockdown significantly increased the heat stress-induced phosphorylation of HSF1 as well as the HSF1 DNA-binding capacity at 2 and 6 h post-heat

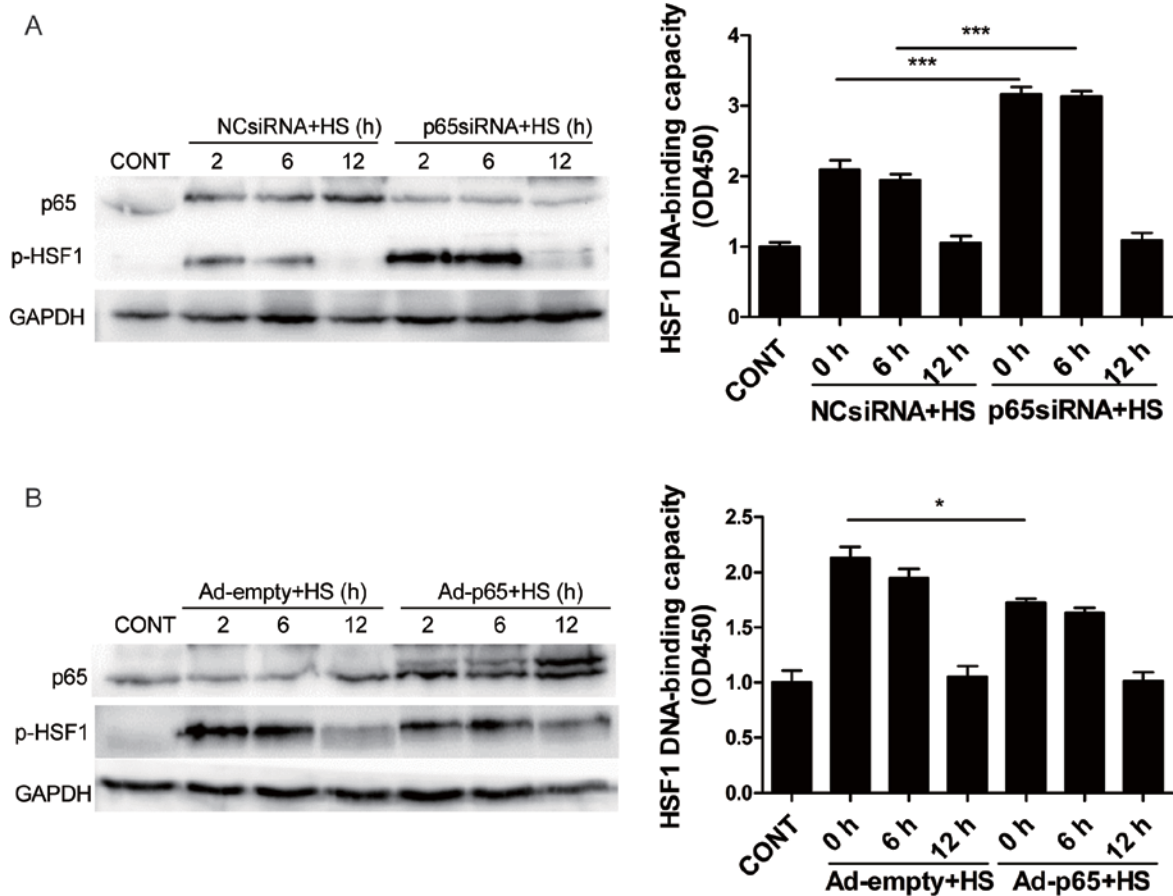


Figure 4. Effects of p65 on HSF1 in heat stress-treated IEC-6 cells. Cells were transfected with NC-siRNA, p65-siRNA, Ad-empty or Ad-p65 for 48 h prior to heat stress treatments. (A) p65 and p-HSF1 protein expressions were determined by western blotting following treatment with p65-siRNA. HSF1 DNA-binding capacity was quantified with the TransAM HSF1 kit following p65-siRNA treatment. (B) p65 and p-HSF1 protein expressions were determined by western blotting following treatment with Ad-p65. HSF1 DNA-binding capacity was quantified with the TransAM HSF1 kit following Ad-p65 treatment. Data are presented as the mean  $\pm$  standard deviation of three separate determinations; \* $P$ <0.05, \*\*\* $P$ <0.001. Ad, adenovirus; CONT, untreated control; HSF1, heat shock transcription factor 1; NC, negative control; p, phosphorylated; siRNA, small interfering.

stress exposure compared with the heat stress-treated cells that were co-treated with the NC-siRNA control, no significant change was observed at 12 h post-heat stress. HSF1 phosphorylation occurred early (0 and 6 h) following heat stress, and HSF1 phosphorylation level returned to normal at 12 h following heat stress, which demonstrated that HSF1 is not constitutively phosphorylated after heat stress. (Fig. 4A and B, respectively). Ad-p65 overexpression led to a slight decrease in HSF1 phosphorylation in heat stress-treated cells, compared with Ad-empty treated IEC-6 cells at 2 h post-heat stress, no significant change was observed at 6 and 12 h post-heat stress (Fig. 4C). Similar results were observed for HSF1 DNA-binding activity (Fig. 4D). These results indicated that p65 activation may have potential inhibitory effects on heat stress-induced HSF1 activation.

**Phosphorylation of c-Jun is necessary for apoptosis in heat-stressed IEC-6 cells.** To examine the effects of heat stress on c-Jun activation and expression, IEC-6 cells were exposed to 43°C heat stress for the indicated times. Heat stress resulted in an increase in the phosphorylation level of c-Jun within 15 min of exposure, reached a peak at 2 h and subsequently began to decline (Fig. 5A); the rapid increase of c-Jun phosphorylation on Ser63 and subsequent accumulation of

c-Jun protein appeared to occur in a time-dependent manner. In addition, heat stress treatment resulted in the increased cleavage of caspase-3 at 2 h of exposure and was expressed at high levels for 12 h, which indicated the activation of apoptosis.

To determine whether c-Jun phosphorylation is necessary for apoptosis, IEC-6 cells were exposed to heat stress in the presence or absence of c-Jun peptide, which effectively acts as c-Jun inhibitor. The inhibition of c-Jun phosphorylation resulted in a significant decrease in the number of apoptotic IEC-6 cells (Fig. 5B). These results suggested that c-Jun phosphorylation may be involved in heat stress induced apoptosis in IEC-6 cells.

**Influence of NF- $\kappa$ B signaling on c-Jun phosphorylation and caspase-3 activation.** The effects of p65 on c-Jun phosphorylation in heat stressed IEC-6 cells were also examined. Western blotting results demonstrated that heat stress-induced c-Jun phosphorylation was reduced by knockdown of p65 at 6 and 12 h post-heat stress exposure, whereas it was increased by in cells overexpressing p65 (Fig. 6A and B, respectively). In addition, p65 knockdown caused a decrease in the cleavage of caspase-3, whereas p65 overexpression led to an increase in caspase 3 cleavage (Fig. 6C and D).

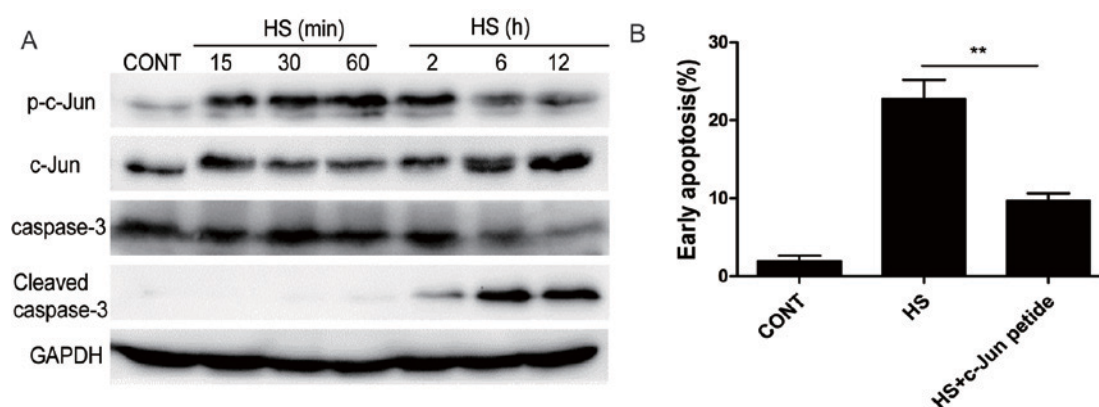


Figure 5. Heat stress induces c-Jun phosphorylation on Ser63, which may be involved in heat stress-induced apoptosis in IEC-6 cells. (A) Cells were exposed to heat stress for the indicated time periods, and the expression levels of p-c-Jun, c-Jun, caspase-3 and cleaved caspase-3 were detected by western blotting. The images are representative of three independent experiments. (B) Cells were pretreated with or without c-Jun peptide prior to heat stress exposure, and apoptosis was analyzed by flow cytometry using Annexin V-fluorescein isothiocyanate/propidium iodide staining. Data are presented as the mean  $\pm$  standard deviation of three separate determinations. \*\* $P < 0.01$ . CONT, untreated control; HS, heat stress; p, phosphorylated.

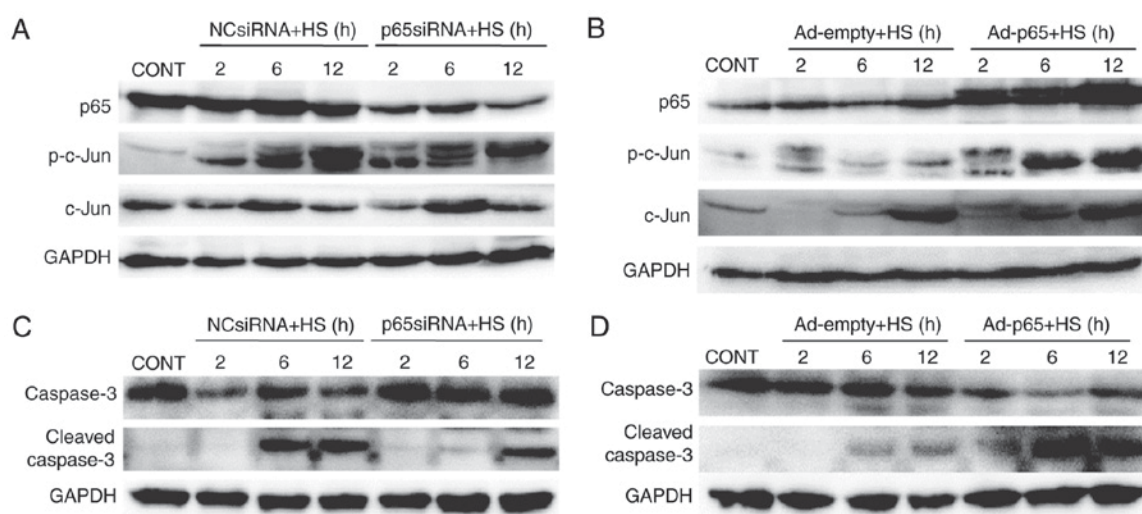


Figure 6. p65 is involved in the c-Jun activation in IEC-6 cells. Cells were transfected with NC-siRNA, p65-siRNA, Ad-empty or Ad-p65, exposed to heat stress for 60 min, and further incubated for indicated times. (A and B) Expression of p-c-Jun and c-Jun were detected by western blotting following treatment with (A) p65-siRNA or (B) Ad-p65. (C and D) Expression of caspase-3 and cleaved caspase-3 was detected by western blotting following treatment with (C) p65-siRNA or (D) Ad-p65. The images are representative of three independent experiments. Ad, adenovirus; CONT, untreated control; HS, heat stress; NC, negative control; p, phosphorylated; siRNA, small interfering RNA.

## Discussion

Heat stroke is a life-threatening condition and is the leading cause of morbidity and mortality during heat waves; it is characterized by a rapidly increasing core temperature to  $>40^{\circ}\text{C}$  and multiple organ dysfunction syndrome (1). As global temperatures continue to rise, heat stroke morbidity and mortality rates may also continue to increase (1,22,23). Heat stroke is a condition of complex pathogenesis in which the intestine appears to serve a key role (1,22); for example, gut-derived endotoxemia has been implicated in the pathophysiology of heat stroke (1-3). In addition, studies performed in animal models demonstrated widespread apoptosis in the intestine of animals affected by this type of injury (22,23). Although apoptosis is a normal physiological process, the disruption of intestinal mucosa by apoptosis allows bacterial components or complete microorganisms to enter the

bloodstream, which further complicates the already complex pathophysiology of heat stroke (23). Therefore, an examination of the mechanisms of heat stroke-induced apoptosis in intestinal cell models, such as the IEC-6 cell line used in the present study, is required for a better understanding of heat stroke pathogenesis.

Oxidative stress generated during heat stress, as well as ROS modulation of the NF- $\kappa$ B pathway, have been previously demonstrated in a number of cell types (3-7). Therefore, the present study examined whether ROS was involved in the activation of NF- $\kappa$ B during heat stroke. The biological effects of heat stress on IEC-6 cell apoptosis was investigated, and the results demonstrated that heat stress was able to induce ROS accumulation, which might subsequently lead to NF- $\kappa$ B activation. In addition, the DNA-binding capacity of NF- $\kappa$ B p65 was not modified in response to short-term heat stress exposure. By contrast, during moderate- and long-term heat stress treatments,

the activity of NF- $\kappa$ B increased. These results indicated that short-term heat treatment may not induce notable ROS accumulation, which is necessary for NF- $\kappa$ B activation.

Previous studies have reported that NF- $\kappa$ B binds to DNA in heat-stressed HeLa cells (6). The results of the present study are in line with these previous results; however, the present results, to the best of our knowledge, are the first to establish a link between apoptosis and NF- $\kappa$ B signaling in IEC-6 cells. An increasing number of studies have suggested that NF- $\kappa$ B serves an important role in preventing apoptosis (10,11). By contrast, NF- $\kappa$ B activation has also been reported to induce apoptosis in certain cell types, as demonstrated by the induction of Fas ligand expression in Jurkat T lymphocyte cells treated with the drugs etoposide or teniposide (12). The promotion or inhibition of apoptosis by NF- $\kappa$ B signaling may depend on the cell type and the type of inducer (24). The present study demonstrated that heat stress-induced NF- $\kappa$ B activity may promote cell apoptosis in IEC-6 cells and that NF- $\kappa$ B p65 knockdown increased the heat stress-induced phosphorylation of HSF1 and HSF1 DNA-binding activity. HSF1 is able to block apoptosis at different stages owing to its regulatory role on genes encoding molecular chaperones that are involved in the apoptotic pathway (15). The present study demonstrated that heat stress-induced HSF1 activity may inhibit apoptosis in IEC-6 cells, and that NF- $\kappa$ B p65 activity may be linked to the suppression of HSF1 activation in these cells. A previous study reported that inhibition of NF- $\kappa$ B by prostaglandin A1 may be associated with HSF activation in Jurkat T cells, CEM-SS T lymphoid cells and HeLa cells, the possibility then exists that NF- $\kappa$ B may be affected by the state of transcriptional activation of HSF1, or that simultaneous activation of these transcription factors may be incompatible (25). The inhibition of NF- $\kappa$ B signaling by aspirin has also been associated with HSF activation (26). In addition, it was reported that the inhibition of NF- $\kappa$ B activation was common to several HSF inducers, including heat shock, which suggested that the regulatory pathways of NF- $\kappa$ B and HSF may be linked and offers new avenues in the search for effective NF- $\kappa$ B inhibitors as useful immunosuppressive, anti-inflammatory and anti-apoptotic drugs (25).

A previous study reported that NF- $\kappa$ B interacts directly with c-Jun and enhances the transcription of AP1-responsive genes in HeLa cells (21). In the present study, NF- $\kappa$ B was demonstrated to be involved in the regulation of c-Jun phosphorylation. Previous studies have indicated that c-Jun, a signal-transducing transcription factor of the AP-1 family, is associated with apoptosis (18,19). The present study used c-Jun peptide to inhibit c-Jun phosphorylation and this treatment resulted in a substantially decreased number of IEC-6 cells in early apoptosis after heat stress. These data suggested that a pro-apoptotic pathway may be induced by NF- $\kappa$ B via enhancement of c-Jun phosphorylation and caspase-3 activation. However, additional studies are required to determine the potential mechanism by which NF- $\kappa$ B modulates c-Jun phosphorylation.

In the present study, the effects of three different transcription factors, NF- $\kappa$ B, HSF1 and c-Jun, as well as NF- $\kappa$ B p65-mediated regulation of HSF1 and c-Jun in heat stress-induced IEC-6 apoptosis was examined. The putative interactions between NF- $\kappa$ B,

HSF1 and c-Jun appeared to be important for apoptosis in IEC-6 cells, and the interactive mechanism of these three transcription factors merits further study. In conclusion, an understanding of NF- $\kappa$ B regulation and the mechanism by which NF- $\kappa$ B induces cell apoptosis during heat stroke may lead to the development of novel strategies for treating heat-induced illnesses related to the intestinal mucosa, in which intestinal epithelial cell apoptosis serves a major etiological role.

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