NF-κB/IκBα signaling pathways are essential for resistance to heat stress-induced ROS production in pulmonary microvascular endothelial cells

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Abstract. The results of a previous study demonstrated that heat stress (HS) triggered oxidative stress, which in turn induced the apoptosis of epithelial cells. These results uncovered a novel mechanism underlying the activation of NF-κB in primary human umbilical vein endothelial cells. The present study aimed to further investigate the role of NF-κB/IκBα signaling pathways in the inhibition of HS-induced reactive oxygen species (ROS) generation and cytotoxicity in endothelial cells. The results of the present study demonstrated that HS triggered a significant amount of NF-κB and IκBα nuclear translocation without IκBα degradation in a time-dependent manner. Mutant constructs of IκBα phosphorylation sites (Ser32, Ser36) were employed in rat pulmonary microvascular endothelial cells (PMVECs). Cell Counting Kit-8 assays demonstrated that both the small interfering (si)RNA-mediated knockdown of p65 and IκBα mutant constructs significantly decreased cell viability and aggravated ROS accumulation in HS-induced rat PMVECs compared with the control. Additionally, western blot analysis revealed that p65 siRNA attenuated the protein expression of IκBα. However, IκBα mutant constructs failed to attenuate NF-κB activation and nuclear translocation, indicating that IκBα-independent pathways contributed to NF-κB activity and nucleus translocation in a time-dependent manner following HS. Collectively, the results of the present study suggested that the NF-κB/IκBα pathway was essential for resistance to HS-induced ROS production and cytotoxicity in rat PMVECs, and that it could be a potential therapeutic target to reduce the mortality and morbidity of heat stroke.

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Introduction

Heat stroke is a life threatening illness characterized by extreme elevations in core temperature reaching >40.5˚C and damage to multiple organ systems (1). Previous studies have suggested that inflammatory injury and severe hypoxemia induced by heat stroke lead to acute lung injury and respiratory failure (2,3). Clinical and experimental evidence suggested that endothelial cell injury served a vital role in the early acute phase of heat stroke (4). Heat stress (HS) refers to conditions involving increased core body temperature above the normal level due to thermoregulatory failure, which leads to heat stroke (5). However, the molecular mechanisms underlying the endothelial cell injury in heat stroke-induced acute lung injury remain to be elucidated. Thus, further investigation into the molecular mechanisms underlying tissue injury caused by direct HS are required to develop clinical strategies for heat stroke.

HS induces endothelial cell injury through oxidative stress, which stimulates reactive oxygen species (ROS) generation, lipid peroxidation and DNA damage (6). ROS are by-products of oxygen metabolism, including superoxide anions, hydrogen peroxide and hydroxyl radicals (7). ROS target proteins, polysaccharides, DNA and lipids and increase the rate of cell damage (8). Furthermore, results of our previous study demonstrated that NF-κB activation decreased HS-induced oxidative stress in HUVECs (9). However, the potential mechanisms underlying NF-κB in HS-induced oxidative stress remain to be elucidated.

The results of previous studies have demonstrated that NF-κB serves a critical role in regulating cell proliferation, inflammatory responses, survival and apoptosis (10,11). When bound, NF-κB and IκB remain inactive in the cytoplasm (12). A main component of IκB is IκBα, the first protein described for the IκB family and the most extensively studied IκB protein to date (13,14). NF-κB-activating stimuli activate IκB kinase, which induces the phosphorylation at Ser32 and Ser36 on IκBα (15). NF-κB is subsequently activated and translocated into the nucleus to control transcription (16). As a dominant

regulatory kinase in the classic NF-κB signaling pathway, IκBα serves a prominent role in apoptosis (17). In addition, the IκBα/NF-κB signaling pathway is one of the most popular antitumor targets (18). The results of the present study revealed a novel mechanism for HS-induced NF-κB activation in rat pulmonary microvascular endothelial cells (PMVECs) without IκBα degradation, which differs from the classic NF-κB signaling pathway. However, the precise molecular mechanism underlying the generation of ROS by NF-κB without IκBα degradation and phosphorylation remains to be elucidated.

The present study aimed to determine whether the NF-κB/IκBα pathway inhibited HS-induced ROS generation and cell death in PMVECs. The association between HS-induced NF-κB activation and the phosphorylation of upstream IκBα was also investigated.

Materials and methods

Animals. Male Sprague Dawley (SD) rats (n=50; body weight, 125-155 g; age, 7 weeks) were purchased from the Experimental Animal Center of Southern Medical University. Rats were housed at a constant temperature of 22-25˚C and 50-60% relative humidity under a controlled 16-h light/8-h dark cycle for at ≥5 days; they had free access to rat chow and water. Experimental protocols involving animals followed the guidelines approved by the Chinese Association of Laboratory Animal Care and approved by the Institutional Animal Care and Use Committee of Nanfang Hospital (approval no. NFYY-2019-176; Guangzhou, China).

Cell culture and treatment. Primary PMVECs were isolated from SD rats as previously described (19). Briefly, after anesthesia with pentobarbital sodium (50 mg/kg, i.p.), the distal lung tissues (diameter, 3-5 mm) were resected from rat lungs, cut into small pieces and digested with type I collagenase (2 mg/ml) and neutral protease (0.6 U/ml) for 1 h. The mixture was centrifuged at 800 x g for 10 min at 37˚C. Subsequently, cells were resuspended and plated with Dynabeads (Invitrogen; Thermo Fisher Scientific, Inc.) and rat anti-mouse platelet endothelial adhesion molecule-1 antibody (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h. The cell-bead mixture was separated using a magnetic particle concentrator (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated in EGM-2 culture media containing 10% FBS with growth factor bullet kit (Lonza Group, Ltd.). For HS treatment, the bottom of each culture dish was placed into a circulating water bath maintained at 43±0.5˚C for 2 h and PMVECs were further incubated at 37˚C for periods as indicated.

Cell Counting Kit-8 (CCK-8) assay. Cells were seeded in a 96-well plate at a density of $1x10^5$/ml and incubated at 37˚C overnight. Subsequently, cells were cultured at 43˚C for 2 h or treated with p65 small interfering (si)RNA or IκBα mutant construct, mixed with 100 μl CCK-8 solution (Dojindo Molecular Technologies, Inc.) and incubated for 1 h. Cell viability was assessed following the manufacturer's protocol.

Apoptosis assay. Cell apoptosis was detected using an Annexin V-FITC apoptosis detection kit and flow cytometry according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc). A total of $1x10^6$ cells were collected and washed three times in PBS. Subsequently, cells were incubated in binding buffer containing Annexin V-FITC for at 4˚C 15 min, the buffer was removed by centrifugation at 1,000 x g for 5 min at 4˚C and the cells were resuspended in buffer containing PI solution. After 5 min, apoptotic cells were detected using a FACSCalibur cytometer (Becton, Dickinson and Company). The data were analyzed using Flowing version 2.5.3 software (Turku Bioscience) and Origin version 7 software (OriginLab). The results of a previous study have suggested that Annexin V-positive cells in the right quadrant are apoptotic, including the early and late apoptotic cells (20).

Florescence confocal assay of intracellular ROS. Cells were treated with 2'-7'-dichlorofluorescin diacetate (Beyotime Institute of Biotechnology) at 37˚C for 20 min in the dark as previously described (21). For live imaging, DNA was stained with 0.2 mg/ml Hoechst 33342 (Thermo Fisher Scientific, Inc.) at 37˚C for 10 min and PMVECs were washed with DMEM Fluoro Brite (Gibco; Thermo Fisher Scientific, Inc.). Cells were analyzed using a confocal fluorescent microscope (magnification, x400; LI-COR Biosciences, Inc.).

Western blot analysis. Cells were exposed to HS treatment and a nuclear extraction kit (Active Motif, Inc.) was used to obtain cytoplasmic and nuclear protein extracts, according to the manufacturer's protocol. Total protein was quantified using an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). Western blotting was performed as previously described (22), the proteins (40 μg per lane) were separated via 12% SDS-PAGE and further transferred onto PVDF membranes. Then, the membranes were blocked with 5% skimmed milk for 1 h at room temperature, and cultured with the following primary antibodies: p65 (cat. no. ab16502; 1:1,000; Abcam), IκBα (cat. no. 4812; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (p)-IκBα (cat. no. 2859; 1:1,000; Cell Signaling Technology, Inc.), p-p65 (cat. no. 3303; 1:1,000; Cell Signaling Technology, Inc.), Lamin B (cat. no. 13435; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174; 1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C. Following primary incubation, membranes were incubated with HRP-conjugated anti-rabbit IgG antibody (cat. no. ZB-2301; 1:5,000; OriGene Technologies, Inc.) at room temperature for 1 h. Protein bands were visualized using ECL reagents (Pierce; Thermo Fisher Scientific, Inc.) with GAPDH and Lamin B as the internal control. ImageJ (version 6.0; National Institutes of Health) was used for the semi-quantification of protein expression.

NF-κB dual-luciferase assay. The luciferase activity was determined using the Dual-Glo luciferase assay system (cat. no. E2980; Promega Corporation) following the manufacturer's protocol. Briefly, plasmids were purified from bacterial cultures using the QIAGENP plasmid Midi kit (Qiagen GmbH). Rat PMVECs were transfected with the vector constructs using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the pGL3-Basic plasmid without promoter as the negative control. The pGl4.74 vector (Promega Corporation) was used as an internal control to determine the efficiency of transfection.

Table I. Sequences of oligonucleotide primers.

Gene	Direction	Sequence
p65	Forward	5'-GCCCUAUCCCUUUACGUCATT-3'
	Reverse	5'-UGACGUAAAGGGAUAGGGCTT-3'
Negative control	Forward	5'-UUCUCCGAACGUGUCACGUTT-3'
	Reverse	5'-ACGUGACACGUUCGGAGAATT-3'

After 24 h of transfection, cells were washed with PBS and resuspended in Passive Lysis Buffer (Promega Corporation). Cells were treated with HS at 43˚C for 2 h, followed by a recovery period at 37˚C. Activity was measured using a Centro LB 960 Luminometer (Titertek-Berthold).

Transfection of plasmids. The pcDNA3.1-IκBα-mutant (M) and pcDNA3.1-IκBα-wild-type (wt) were synthesized by Cyagen Biosciences, Inc. Cells were transfected with the corresponding vectors according to the Lipofectamine® 2000 reagent procedure (Thermo Fisher Scientific, Inc.) and transfected cells were incubated for 48-72 h at 37˚C before further experiments.

Transfection of siRNA. siRNA targeting p65 was designed and synthesized by Shanghai GenePharma Co., Ltd. The siRNA sequences for each gene and their corresponding negative controls are demonstrated in Table I. PMVECs were seeded into 6-well plates (Wuxi NEST Biotechnology, Co., Ltd.) at 30-50% confluence for transfection. After 24 h, PMVECs were incubated with TurboFect siRNA Transfection reagent (Shanghai GenePharma Co., Ltd.) and 10 nM siRNAs for 6 h at 37˚C according to the manufacturer's protocol. Transfected cells were incubated for 24 h at 37˚C before further experiments.

Co-immunoprecipitation (Co-IP). Cells were subjected to HS treatment at 43˚C for 2 h, followed by an additional incubation period at 37˚C, or exposed to 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) for the times indicated. Co-IP assays were subsequently performed using the Co-IP kit (Pierce; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Cells were lysed with immunol precipitation lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice, and the buffer was removed via centrifugation at 16,000 x g for 10 min at 4˚C. The cell lysates were then immunoprecipitated at 4˚C overnight using anti-IκBα (cat. no. 4812; 1:1,000; Cell Signaling Technology, Inc.) and normal IgG complexes. Then, 40 μl protein A+G agarose beads were used and spun at 4˚C for 3 h. After centrifugation at 250 x g for 5 min at 4˚C, the supernatant was discarded. Finally, 1X loading buffer was added and boiled for 5 min for western blot analysis. Protein expression levels were semi-quantified using ImageJ (version 6.0; National Institutes of Health).

Statistical analysis. All data were analyzed for statistical significance using SPSS V.23.0 software (IBM Corp.). Data are presented as the mean ± standard deviation from at least three independent experiments, each performed in duplicate. The comparisons of multiple groups were analyzed using the Kruskal-Wallis test followed by a Dunn's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Influence of NF-κB p65 on HS-induced ROS accumulation and cytotoxicity in rat PMVECs. As demonstrated in Fig. 1A, nuclear and cytosolic fractions were isolated from rat PMVECs and PMVECs were exposed to HS for 2 h followed by further incubation for 0, 6 and 12 h. The results of the present study indicated a significant amount of NF-κB p65 and IκBα nuclear translocation in a time-dependent manner. As shown in Fig. S1A, the infection efficiency of p65siRNA was observed with p65 expression. Knockdown of p65 significantly decreased the viability of PMVECs, as demonstrated by CCK-8 assays (Fig. 1B). Furthermore, fluorescence confocal assays revealed that siRNA knockdown of NF-κB p65 significantly aggravated ROS accumulation in HS-induced rat PMVECs compared with the control (Fig. 1C). Collectively, the results of the present study demonstrated that NF-κB p65 served a key role in HS-induced cytotoxicity and ROS production.

NF-κB mediates the HS-induced expression of IκBα during the recovery period following HS. IκB is an inhibitor of NF-κB, which interacts with the nuclear localization sequences of the Rel homology domain. IκBα was the first protein described in the IκB protein family (23). To investigate the interaction between NF-κB p65 and IκBα, Co-IP experiments of NF-κB p65 and IκBα were performed. As demonstrated in Fig. 2A, Co-IP assays revealed that NF-κB p65 co-immunoprecipitated with IκBα following HS treatment. Furthermore, treatment with LPS lead to a decrease in the co-immunoprecipitation of IκBα and NF-κB p65. The results of the present study demonstrated that IκBα had the ability to bind to NF-κB and IκBα was not degraded or dissociated from the NF-κB complex during the recovery period following HS. To further investigate the direct interaction between NF-κB p65 and IκBα in HS-induced PMVECs, western blot analysis was used. Western blotting revealed that protein expression of IκBα was significantly reduced following knockdown of NF-κB p65 during the recovery period following HS (Fig. 2B and C). Collectively, the findings of the present study suggested that upstream NF-κB p65 mediated the HS-induced expression of IκBα in HS-induced rat PMVECs.

HS mediates NF-κB activity and nucleus translocation-independent IκBα phosphorylation. The results of our previous study confirmed that HS induced the phosphorylation and

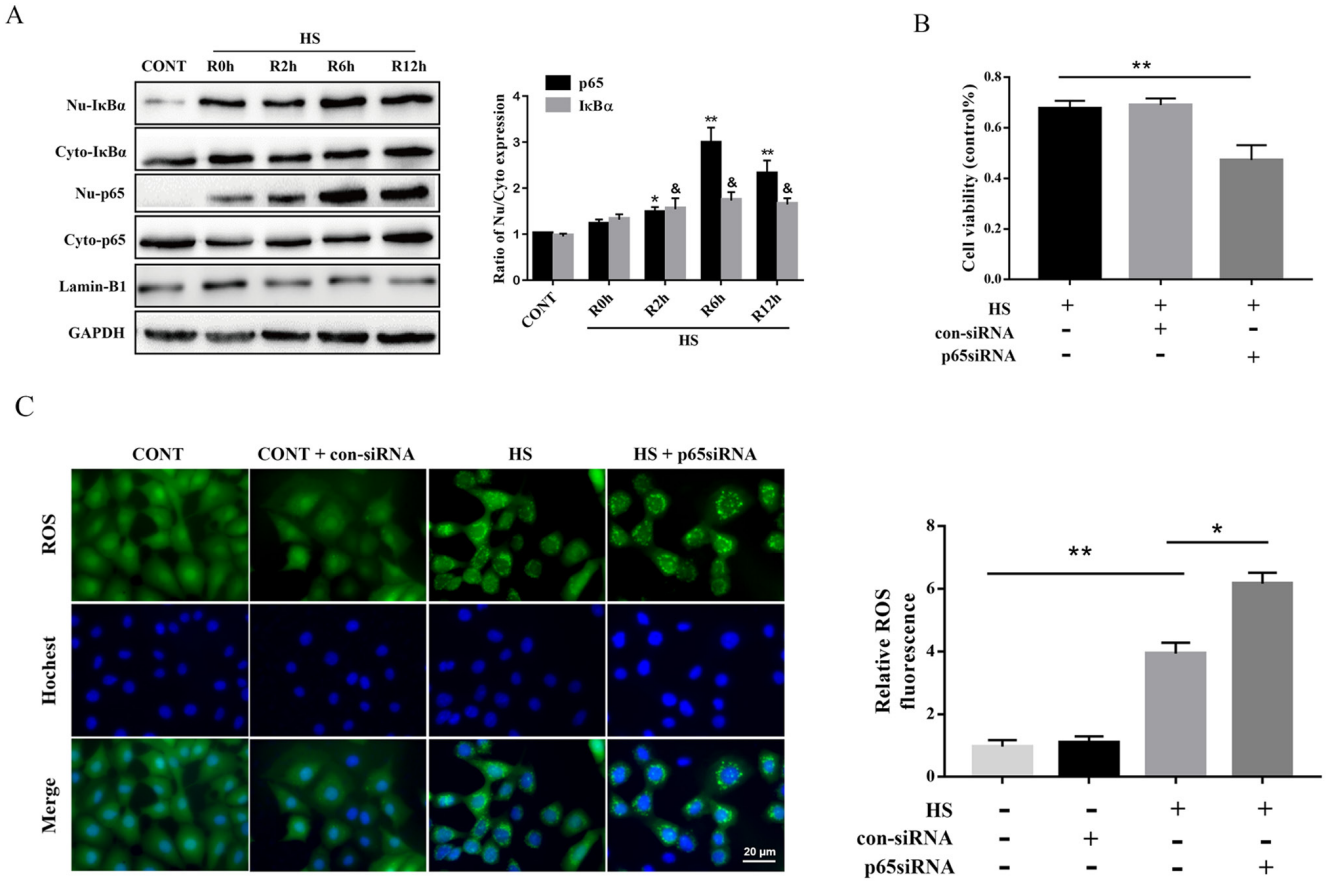

Figure 1. Influence of NF-κB on HS-induced ROS accumulation and cytotoxicity in rat PMVECs. (A) Cells were incubated at 37˚C (CONT) or were subjected to HS treatment at 43˚C for 2 h, followed by a recovery period at 37˚C for 0 (R0h), 2 (R2h), 6 (R6h) or 12 h (R12h). Expression levels of p65 and IκBα were detected in cytoplasmic and nuclear fractions of PMVECs using western blotting. (B) PMVECs were subjected to HS treatment at 43˚C for 2 h and incubated at 37˚C for 12 h before cell proliferation was determined using a Cell Counting Kit-8 assay. (C) Representative images of intracellular ROS visualized using fluorescence microscopy. Sections were co-stained with ROS (green) and Hoechst (blue). Scale bar, 20 μm. Each value represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01, and $^{\&}$P<0.05 vs. the control group. HS, heat stress; ROS, reactive oxygen species; PMVEC, pulmonary microvascular endothelial cells; CONT, control; Nu, nuclear fraction; Cyto, cytoplasmic fraction; si, small interfering.

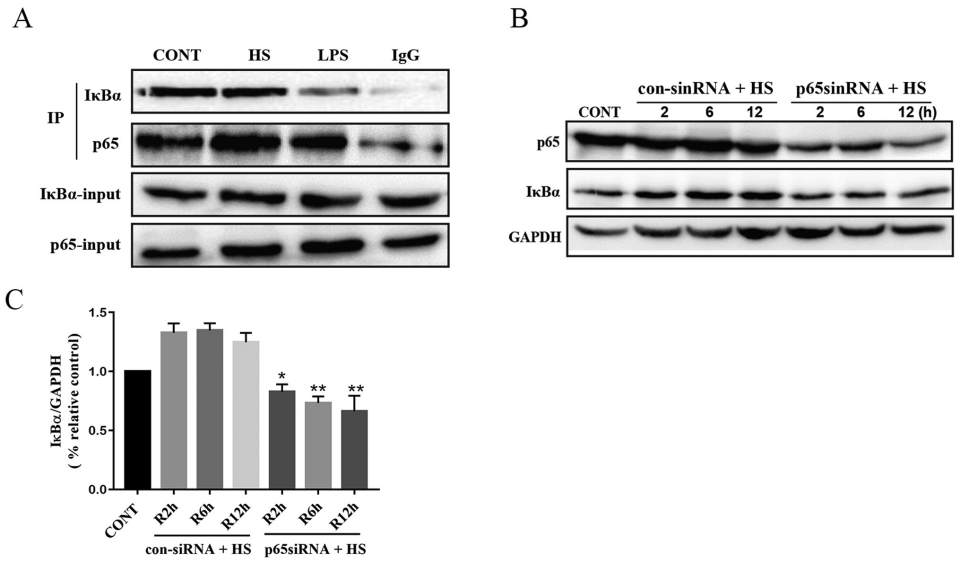

Figure 2. NF-κB mediates the HS-induced expression of IκBα. (A) PMVECs were exposed to HS at 43˚C for 2 h and subsequently incubated at 37˚C for 12 h or treated with 1 μg/ml LPS for 90 min. Cell lysates were normalized to IκBα protein expression levels and subjected to co-immunoprecipitation to analyze the interactions between endogenous IκBα and NF-κB p65. IgG served as the negative control. (B) NF-κB p65 knockdown regulated the expression of IκBα, demonstrated by western blot analysis. (C) Cells were incubated at 37˚C (CONT) or were subjected to HS treatment at 43˚C for 2 h, followed by a recovery period at 37˚C for 0 (R0h), 2 (R2h), 6 (R6h) or 12 h (R12h). Semi-quantification of IκBα expression levels in all groups. All data represents the mean ± SD from four independent experiments. *P<0.05; **P<0.01 vs. the control group. HS, heat stress; PMVEC, pulmonary microvascular endothelial cells; LPS, lipopolysaccharide; CONT, control; si, small interfering.

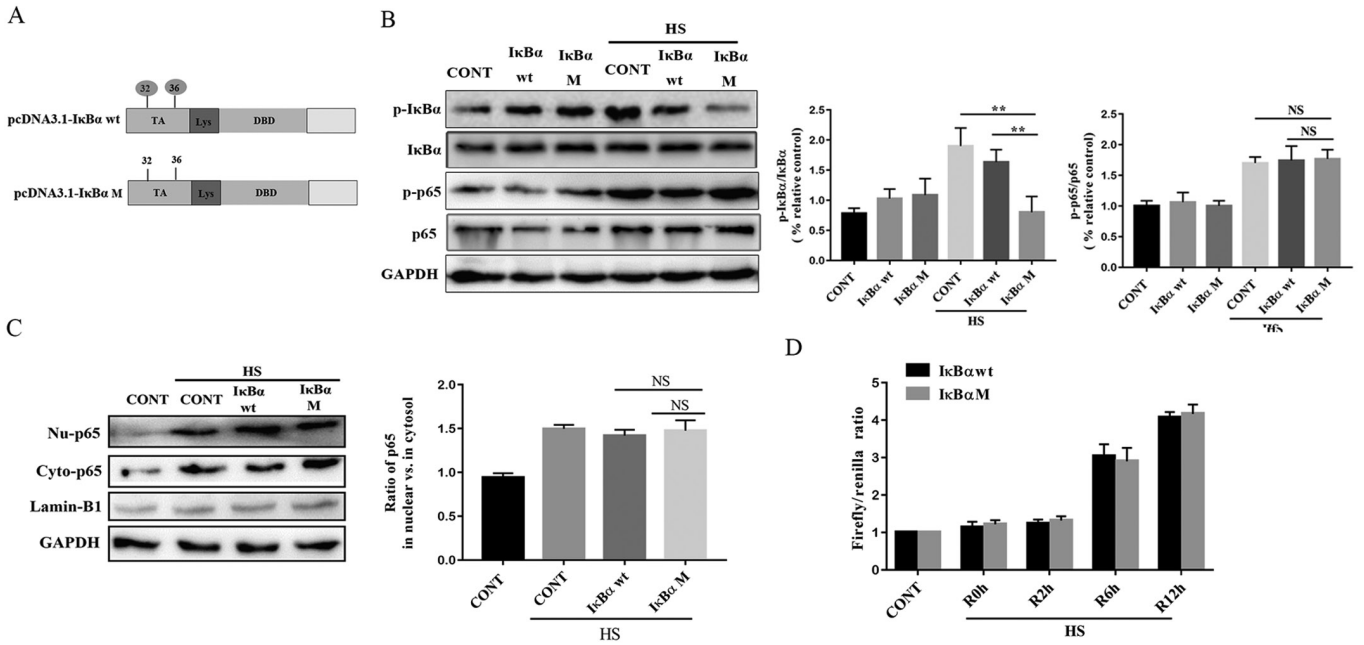

Figure 3. HS mediated NF-κB activity and nucleus translocation-independent IκBα phosphorylation. PMVECs were transfected with the pcDNA3.1-IκBα wt (IκBα wt) or pcDNA3.1-IκBα-M (IκBα M) constructs. After 48 h, cells were exposed to HS at 43˚C for 2 h and were further incubated at 37˚C for 12 h. (A) Schematic of IκBα consensus sites (p-Ser/Ub-Lys). pcDNA3.1-IκBα-M had Ala substitutions at two phosphorylation sites, Ser32 and Ser36. (B) Western blot analysis of the phosphorylation of IκBα and p65. (C) pcDNA3.1-IκBα-M regulated phosphorylation levels of NF-κB p65 in both the cytoplasm and nuclei. (D) Cells were incubated at 37˚C (CONT) or were subjected to HS treatment at 43˚C for 2 h, followed by a recovery period at 37˚C for 0 (R0h), 2 (R2h), 6 (R6h) or 12 h (R12h). Cells were analyzed for NF-κB activation using dual-luciferase assays. Each value represents the mean ± SD from three independent experiments. **P<0.01 vs. the control group. HS, heat stress; PMVEC, pulmonary microvascular endothelial cells; wt, wild-type; M, mutant; NS, not significant; TA, transactivation domain; Lys, lysine enrichment domain; DBD, DNA binding domain; wt, wild-type; M, mutant; Ser, serine; Ala, alanine; CONT, control; p, phosphorylated; Nu, nuclear fraction; Cyto, cytoplasmic fraction.

subsequent activation of IκBα on Ser32 and Ser36 in rat PMVECs (9). To determine whether phosphorylation of Ser32 and Ser36 was responsible for NF-κB activity and nucleus translocation induced by HS, IκBα mutants with substitutions of Ser/Thr with Ala residues at Ser32 and Ser36 were used (pcDNA3.1-IκBα-M; Fig. 3A). The infection efficiency of pcDNA3.1-IκBα-M was observed with levels of phosphorylated IκBα (Fig. S1B). A significant decrease in the phosphorylation of IκBα was observed in the pcDNA3.1-IκBα-M group compared with the pcDNA3.1-IκBα-wt and control groups (Fig. 3B). By contrast, no significant changes in the phosphorylation and nucleus translocation of p65 were observed following transfection with pcDNA3.1-IκBα-M (Fig. 3C). Furthermore, measurement of NF-κB activity indicated that pcDNA3.1-IκBα-M failed to attenuate activation of NF-κB in a time-dependent manner following HS (Fig. 3D). Thus, the results of the present study revealed that HS-mediated NF-κB activity and nucleus translocation are independent of IκBα phosphorylation.

Roles of IκBα phosphorylation in HS-induced apoptosis and ROS accumulation. The role of IκBα phosphorylation on Ser32 and Ser36 in ROS accumulation and cell apoptosis induced by HS was investigated, following transfection of PMVECs with pcDNA3.1-IκBα-M. Compared with the control group, pcDNA3.1-IκBα-M significantly increased the level of apoptosis and significantly decreased the proliferation of PMVECs, as demonstrated by flow cytometry and CCK-8 assays, respectively (Fig. 4A and C). Additionally, the results of the fluorescence confocal assay demonstrated that pcDNA3.1-IκBα-M significantly aggravated ROS generation in HS-induced rat PMVECs (Fig. 4B). Collectively, these findings revealed that IκBα may serve an essential role in HS-induced rat PMVEC apoptosis and ROS generation.

Discussion

The incidence of heat stroke is increasing with the proliferation in heatwaves caused by global warming (24). The results of a previous study suggested that systemic and cellular responses to heat stroke include heat cytotoxicity, coagulopathies and systemic inflammatory response syndrome (25). Notably, the primary injury of vascular endothelium induced by HS has been considered as a key factor for multi-organ system dysfunction, which acts as an early target of thermal injury (26). Thus, further investigation into the promotion of HS-induced vascular endothelium damage is required.

The results of a previous study suggest that HS-induced ROS accumulation mediates oxidative damage and disturbance of mitochondrial homeostasis and cellular function (27). Gu *et al* (28) reveal that HS induces the accumulation of intracellular ROS, which ultimately acts as an upstream signal to promote the mitochondrial apoptotic pathways in HUVECs. Yu *et al* (29) also demonstrate that HS increased ROS and malondialdehyde generation and significantly attenuates anti-oxidase activity resulting in intestinal damage. The results of previous studies demonstrate that the NF-κB/IκBα pathway exhibits a potent inhibitory potential against inflammatory

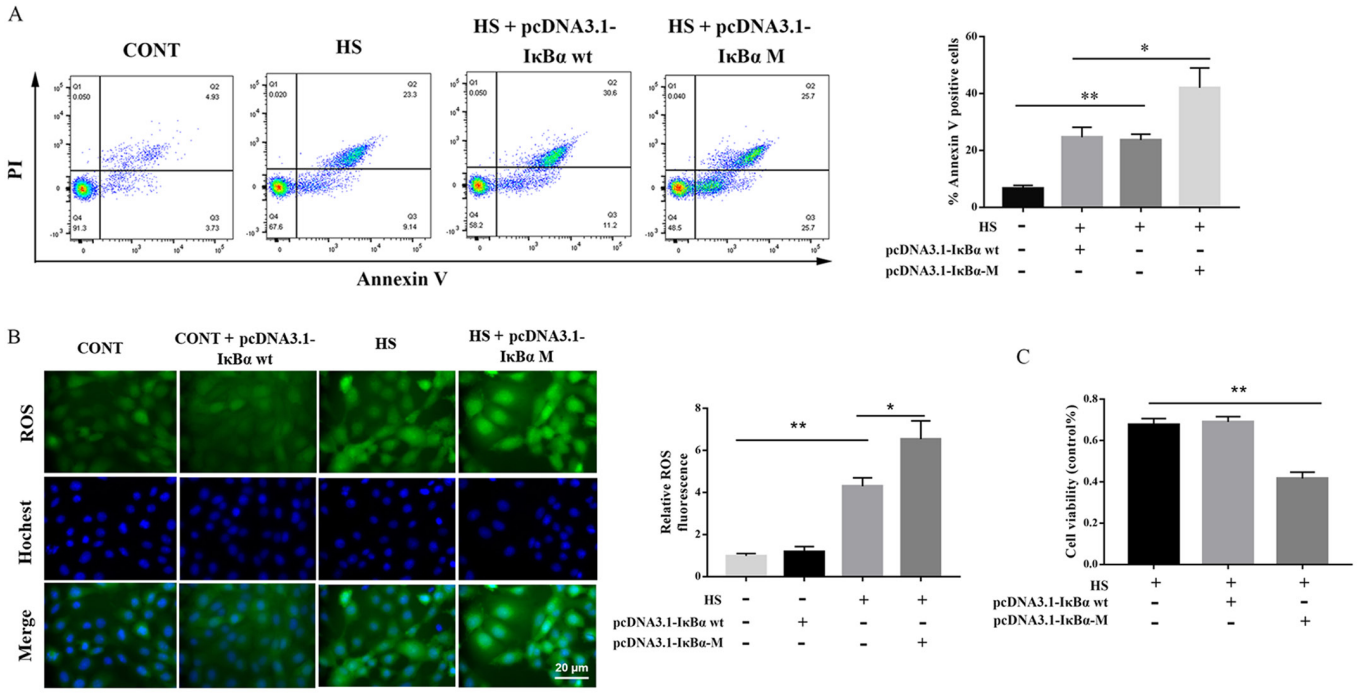

Figure 4. Phosphorylation of IκBα mediates HS-induced apoptosis and ROS accumulation in rat PMVECs. PMVECs were transfected with pcDNA3.1, pcDNA3.1-IκBα wt or pcDNA3.1-IκBα-M constructs for 48 h. Cells were subsequently subjected to HS treatment at 43˚C for 2 h and were further incubated at 37˚C for 12 h. (A) Apoptosis of PMVECs was measured with Annexin V-FITC/PI staining and analyzed by flow cytometry. (B) Representative images of intracellular ROS visualized using fluorescence microscopy and the quantification of intracellular ROS in all groups. Sections were co-stained with ROS (green) and Hoechst (blue). (C) Cell proliferation of PMVECs was determined using Cell Counting Kit-8 assays. Scale bar, 20 μm. Each value represents the mean ± SD from three independent experiments. *P<0.05; **P<0.01. HS, heat stress; ROS, reactive oxygen species; PMVEC, pulmonary microvascular endothelial cells; wt, wild-type; M, mutant; CONT, control.

and oxidative stress (9,15). The present study used PMVECs induced by heat stress to demonstrate the role of NF-κB/IκBα pathway. The findings uncovered an alternative NF-κB/IκBα pathway involving oxidative stress in HS-induced vascular endothelial cell damage.

NF-κB is ubiquitously expressed and regulates the expression of a number of genes involved in immune, inflammatory and apoptotic processes both *in vitro* and *in vivo* (30). The results of our previous study demonstrate that the NF-κB signaling pathway is essential for resistance to HS-induced apoptosis in HUVECs (9). NF-κB is mainly sequestered in the cytoplasm and associated with the IκB inhibitor family (31). When NF-κB-activating stimuli activate IκB kinase, p-IκB proteins are subsequently poly-ubiquitinated and degraded by the 26S proteasome, which in turn activates NF-κB to relocate to the nucleus for transcription (32). We previously revealed that no signs of IκBα degradation are observed in HS-induced HUVECs compared with the control. By contrast, levels of phosphorylated IκBα increase during the recovery period following heat stress (9). However, the results of the present study demonstrated that both IκBα and NF-κB translocated from the cytoplasm into the nucleus in HS-induced PMVECs. Furthermore, the degradation of IκBα and subsequent dissociation from NF-κB did not occur and transfection of PMVECs with pcDNA3.1-IκBα-M failed to inhibit the migration of NF-κB into the nucleus. NF-κB remained transcriptionally active. Thus, the results of the present study suggested that upstream NF-κB mediated the expression of IκBα in HS-induced rat PMVECs.

Mechanistically, a number of potential mechanisms, such as the role of NF-κB in the expression of target genes that mediate cell proliferation and release antimicrobial molecules and cytokines, may explain this phenomenon (33). All IκB proteins, including IκBα, IκBβ, IκBε, p105/IκBγ and p100/IκBδ, are reported as target genes of NF-κB (34). Previous studies have demonstrated that IκBα is a transcriptional target for NF-κB, creating a negative feedback loop (16,35). Thus, it was hypothesized that IκBα was not degraded, as NF-κB regulated the synthesis of new IκBα, which entered the nucleus and inhibited the transcriptional function of NF-κB. However, the potential mechanisms underlying NF-κB activation following HS have yet to be elucidated. Thus, it was hypothesized that heat cytotoxicity or alternate mechanisms may contribute to NF-κB activation.

However, there are limitations of the present study. The intracellular nature of NF-κB may require upstream activation via cytokines and pathogen-associated molecular patterns. By contrast, the results of the present study revealed that HS induced activation of NF-κB independent of the phosphorylation of upstream IκBα. Further investigation into the specific molecular mechanisms underlying the activation of NF-κB without the degradation of IκBα is required. Furthermore, prevention of oxidative stress in patients with heat stroke is a crucial therapeutic intervention. Further experiments are required to uncover the mechanisms underlying oxidative stress and potential therapeutic targets. The results of the present study were also limited by the use of PMVECs *in vitro* and further mechanisms underlying endothelial cell injury *in vivo* remain to be elucidated.

Collectively, the results of the present study suggested that the NF-κB/IκBα pathway was essential for resistance to HS-induced ROS production and cytotoxicity in rat PMVECs. Further investigation will demonstrate the molecular mechanisms underlying the functional role of NF-κB/IκBα proteins in HS. Moreover, the current study suggested roles for NF-κB/IκBα as a potential target to modulate treatment of heat stroke.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WX, WH, YL and SC performed the study and composed this manuscript. WX, WF and YL were responsible for primary data generation and analysis. HC and ZC participated in cell culture and transfection. WH, WX and YL performed the western blot analysis. YL was the principal investigators and corresponding authors for these studies. WX, WH, YL and SC are responsible for confirming the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experimental protocols involving animals followed the guidelines approved by the Chinese Association of Laboratory Animal Care and approved by the Institutional Animal Care and Use Committee of Nanfang Hospital (approval no. NFYY-2019-176; Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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