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Heat shock protein 27 inhibits HMGB1 translocation by regulating CBP acetyltransferase activity and ubiquitination

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ABSTRACT

Heat-shock protein 27 (Hsp27) is a member of the small heat shock protein family that has been reported to protect cells against pro-inflammatory stresses. High mobility group box 1 (HMGB1) is a proinflammatory cytokine associated with death from sepsis and other inflammatory diseases. After being acetylated by CREB-binding protein (CBP), the transcriptional adaptor and acetyltransferase, HMGB1 translocates from the nucleus to the cytoplasm. In the present study, we investigated the effects of Hsp27 on HMGB1 translocation from the nucleus to the cytoplasm in THP-1 cells. We found that Hsp27 phosphorylation decreased LPS-induced HMGB1 acetylation and translocation from the nucleus to the cytoplasm, as well as its release from THP-1 cells. The study further showed that cytosolic non-phosphorylated Hsp27 enhanced CBP ubiquitination and degradation in LPS-unstimulated cells, which suggested that Hsp27 maintained suitable CBP levels under normal physiological conditions. After LPS stimulation, Hsp27 was phosphorylated at serine residues 15/78 and translocated from the cytoplasm into the nucleus. Consequently, LPS stimulation increased CBP levels and promoted its translocation into the nucleus. In the nucleus, Hsp27 bound to CBP and suppressed CBP acetyltransferase activity and the subsequent CBP-dependent acetylation of HMGB1. Taken together, our data demonstrated that cytosolic non-phosphorylated Hsp27 enhanced the ubiquitin-mediated degradation of CBP, while phosphorylated Hsp27 inhibited CBP acetyltransferase activity in the nucleus. By regulating CBP, Hsp27 maintained cell homeostasis and inhibited excessive inflammatory response.

1. Introduction

As a transcriptional co-activator and histone acetyltransferase (HAT), CREB-binding protein (CBP) and its homolog p300 participate in a multitude of cellular functions including DNA repair, cell growth, apoptosis and inflammatory response (Goodman and Smolik, 2000; Liu et al., 2008; Matt, 2002). The acetyltransferase function of CBP/p300 involves not only the acetylation of histones leading to changes in chromatin structure (Kouzarides, 2000), but also that of transcription factors (Giuseppe Marzio and Kristian Helin, 2000; Kawai et al., 2001). For example, CBP/p300 mediates the assembly of multi-protein complexes that serve as molecular scaffolds or bridges (H M and N B, 2001). It is worth mentioning that high mobility group box 1 (HMGB1), a key late proinflammatory mediator associated with death from sepsis (Chen et al., 2013; Lotze and Tracey, 2005), can be acetylated by CBP (Wu et al., 2012). In the nucleus, HMGB1 is among the most important chromatin proteins, and interacts with nucleosomes, transcription factors, and histones (Bianchi and Agresti, 2005). HMGB1 supports the transcription of many genes through interactions with many transcription factors. Posttranslational modifications are important for HMGB1 functions. In macrophages, when HMGB1 is not acetylated, it stays in the nucleus, but hyperacetylation on lysine residues causes it to translocate into the cytoplasm and be released from cells as a pro-inflammatory cytokine. Some actions of HMGB1 are mediated by toll-like receptors (TLRs) (van Beijnum et al., 2008). The interaction between HMGB1 and TLR4 results in upregulation of NF-κB, which leads to the increased production and release of cytokines. HMGB1 is also able to interact with TLR4 on neutrophils to stimulate the production of

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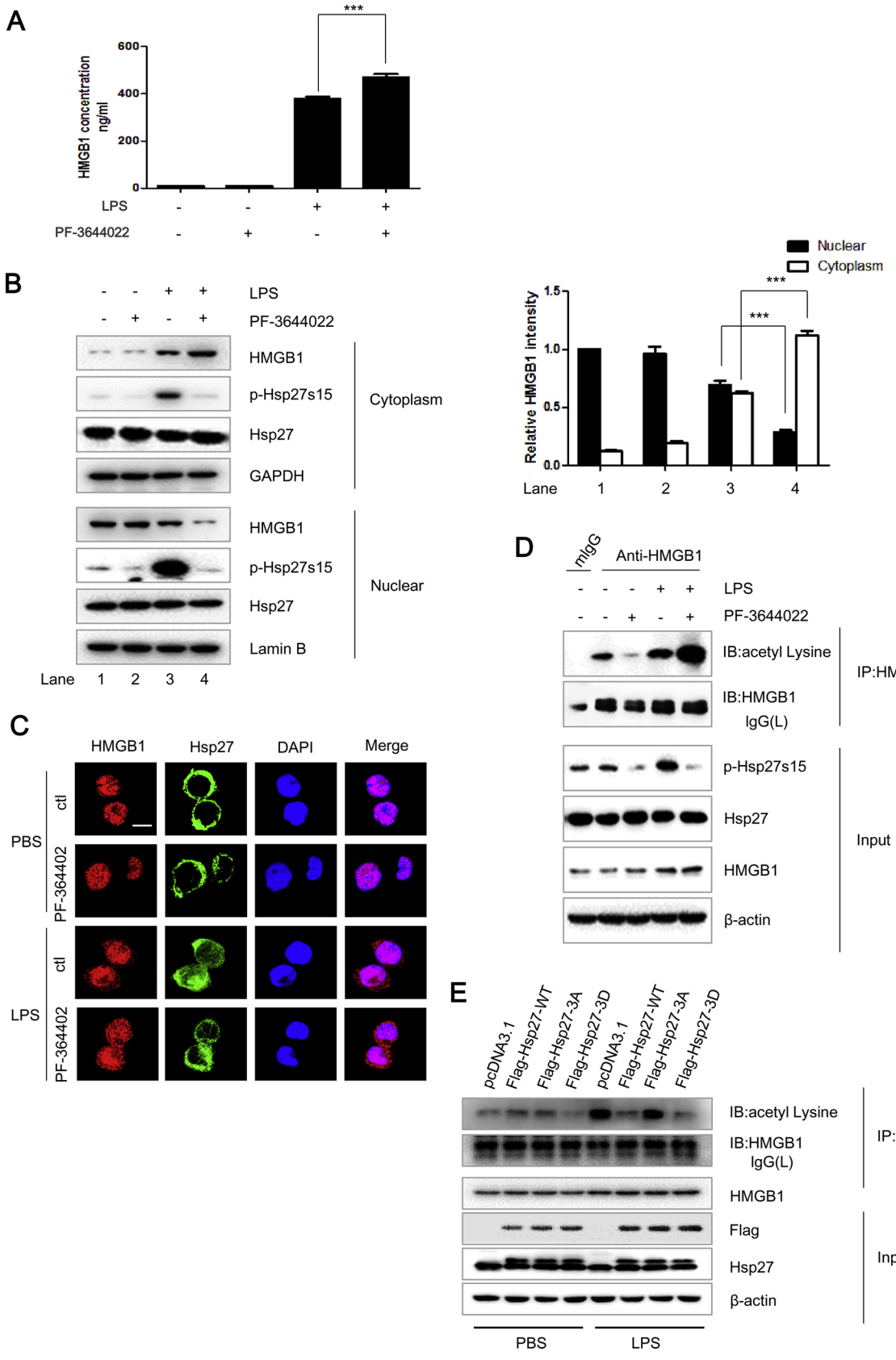

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Fig. 1. Phosphorylated Hsp27 suppresses the acetylation of HMGB1. (A) THP-1 cells were pre-incubated with PF-3644022 (5 μM) for 2 h, and then were treated with LPS (500 ng/ml) for 18 h, HMGB1 protein levels in the culture medium was measured by ELISA. **(B)** Cells treated the same as in A were harvested for extracting nuclear and cytoplasmic fractions. Western blot analysis was performed by using HMGB1, p-Hsp27s15 or Hsp27 antibodies. Lamin B was used as loading control for nuclear protein, and GAPDH for cytoplasmic protein. **(C)** Cells treated the same as in A were incubated with rabbit anti-Hsp27 and mouse anti-HMGB1 primary antibodies and then visualized using Alexa flour 488-conjugated anti-rabbit (green) and Alexa flour 555-conjugated anti-mouse (red) secondary antibodies. The nuclei were counterstained with DAPI (blue). Scale bar: 10 μm. **(D)** Cells treated the same as in A were subjected to immunoblotting with the indicated antibodies. The cell lysates were immunoprecipitated with anti-HMGB1 antibody, followed by immunoblotting with anti-acetyl lysine and anti-HMGB1 antibodies. **(E)** THP-1 cells were transiently transfected with Flag-Hsp27-WT, Flag-Hsp27-3 A, Flag-Hsp27-3D or pcDNA3.1, and 48 h after transfection, the cells were treated with LPS (500 ng/ml) for an additional 18 h. After immunoprecipitation using HMGB1 antibody, the degree of HMGB1 acetylation was detected using an anti-acetyl lysine antibody. The data shown are representative of three independent experiments. Error bars indicate the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

reactive oxygen species through NADPH oxidase (Fan et al., 2007). The HMGB1-LPS complex activates TLR4and promotes adaptor protein (MyD88 and others) binding, leading to signal transduction and the activation of various signaling cascades. The downstream effects of this signaling are the activation of MAPK and NF-κB, and thus the production of inflammatory molecules such as cytokines (He et al., 2013).

Heat shock protein 27 (Hsp27) is a small and highly conserved heat shock protein, that functions as a molecular chaperone, a free radical scavenger, and an anti-apoptotic factor (Mymrikov et al., 2011). Hsp27 is phosphorylated at serine residues15, 78 and 82 (15 and 86 in rodent Hsp25) by MAPKAP kinase 2/3 via activation of the p38 MAPK pathway in response to stresses (Rogalla et al., 1999). Following phosphorylation, Hsp27 forms small oligomers, often dimers and tetramers, and interacts with other proteins (Vidyasagar et al., 2012). It has been reported that Hsp27 regulates proinflammatory mediator release in keratinocytes by modulating NF-κB signaling (Sur et al., 2008). Our previous study indicated that Hsp27 regulates lipopolysaccharide-induced inflammatory response in THP-1 cells by interacting with TRAF6 (Liu et al., 2010). Our research also demonstrated that phosphorylated Hsp27 activates ATM-dependent p53 signaling in MCF-7 cells (Xu et al., 2013). P53 is a DNA-binding transcription factor that controls cellular proliferation by inducing DNA repair, cell cycle arrest, or apoptosis during a multitude of stress conditions (Prost, 1998). Stress-induced site-specific post-translational modifications, such as phosphorylation, acetylation, and ubiquitination, are essential for regulating transcriptional activities of p53 (Mujtaba et al., 2006). Acetylation of p53 by the HAT domain is a hallmark of CBP/p300 epigenetic function. During genotoxic stress, CBP HAT-mediated acetylation is essential for the activation of p53 to transcriptionally govern target genes, which control cellular responses. Additionally, CBP/p300 has also been shown to associate with p53 in vitro and in vivo to regulate p53 responses to prolonged genotoxic stress (Grossman, 2001; Lambert et al., 1998; Lill et al., 1997). However, it is unclear whether Hsp27 can interact with CBP.

In this study, we explored the effect of Hsp27 on CBP and analyzed whether Hsp27 could regulated HMGB1 release by affecting CBP. Our results showed that non-phosphorylated Hsp27 contributed to CBP ubiquitination. Hsp27 was phosphorylated and translocated into the nucleus after THP-1 cells stimulation by LPS. In the nucleus, Hsp27 decreased CBP acetylation activity to prevent HMGB1 translocation into the cytoplasm. Our study provides novel insights into the roles of Hsp27 and CBP in regulating inflammatory signaling processes.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against Flag-tag, GAPDH, Lamin B, β-actin, Hsp27 (source: rabbit), and phospho-Hsp27 (S15, S78 and S82) were purchased from Bioworld Technology (Minneapolis, MN, USA). The antibody against HMGB1 was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against Hsp27 (source: mouse), ubiquitin, and histone H3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibody against acetyl-histone H3 was obtained from Millipore (Burlington, MA, USA). Antibodies against CBP, and acetyl lysine were purchased from Abcam (Cambridge, UK). Normal mouse IgG and rabbit IgG were obtained from Calbiochem (San Diego, CA, USA). The antibody against HA-tag, and HRP-conjugated secondary antibodies against mouse or rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). DAPI, Alexa Fluor 488 and 555 donkey anti-mouse IgG, and Alexa Fluor 488 and 555 donkey anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA, USA). The MK2 inhibitor PF-3644022 was obtained from Tocris Bioscience (Avonmouth, Bristol, UK). The CBP inhibitor SGC-CBP30 and protease inhibitor MG-132 were purchased from Selleck Chemicals (Houston, TX, USA). LPS (from *Escherichia coli O111:B4*) and X-tremeGENE HP DNA Transfection Reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plasmids

The pcDNA3.0-Flag-Hsp27-WT plasmid for wild type Hsp27 (Hsp27-WT) and the plasmids for the Hsp27 mutants, including Flag-Hsp27-3A (in which serine 15/78/82 were mutated to alanine, phospho-defective) and Flag-Hsp27-3D (in which serine 15/78/82 were mutated to aspartate, phospho-mimetic), were constructed as previously described (Shen et al., 2016; Xu et al., 2013). Small hairpin RNA (shRNA) constructs against HSP27 were purchased from Origene (Rockville, MD, USA).

2.3. Cell culture and transfection

THP-1 cells were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, PR China) and were cultured in RPMI 1640 (Wisent, Quebec, Canada) supplemented with 15% (v/v) fetal bovine serum (Wisent), 100 U/ml penicillin and 100 μg/ml streptomycin (Wisent) at 37 °C in a 5% CO_2 atmosphere.

Transient transfection was performed using the X-tremeGENE HP DNA Transfection Reagent from Sigma-Aldrich following the manufacturer's instructions. In all cases, the total amount of DNA was normalized by the empty vector control.

2.4. Co-immunoprecipitation and immunoblotting

Cell lysates were prepared with lysis buffer (Beyotime, Shanghai, China) supplemented with protease inhibitor cocktail and phosphatase inhibitors. Protein concentrations were measured with a Modified BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For immunoblotting, 50 μg of each sample were electrophoresed via SDS-PAGE and transferred to PVDF membrane. After blocking with 5% non-fatty milk in TBST, the membrane was then incubated with the indicated primary antibodies at 4 °C overnight. HRP-conjugated secondary antibodies were used against the respective primary antibodies. The antibody-antigen complexes were visualized by chemiluminescence using the ECL immunoblotting system from Tanon (Shanghai, China). Western blotting signal intensity was quantified using the ImageJ software (National Institutes of Health (NIH): Bethesda, MA,

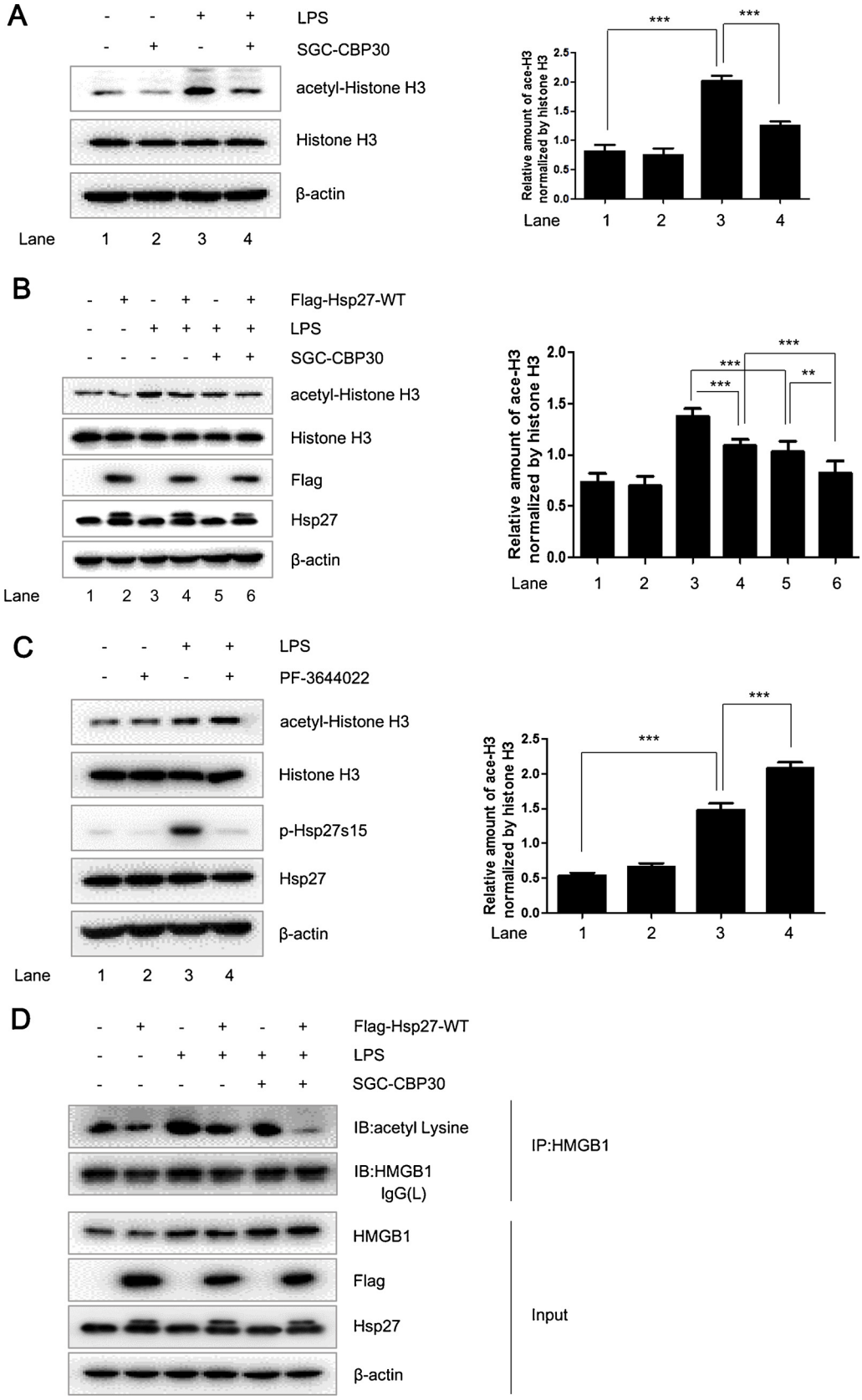

Fig. 2. Phosphorylated Hsp27 inhibits CBP-mediated acetylation of histone H3 and HMGB1. (A) THP-1 cells were treated with LPS (500 ng/ml) for 8 h and then incubated with SGC-CBP30 (4 μM) for another 10 h. The levels of acetyl-histone H3, histone H3, and β-actin in the whole cell lysates were measured by immunoblotting. **(B)** THP-1 cells were transiently transfected with Flag-Hsp27-WT or pcDNA3.1. After 28 h, the cells were incubated with LPS (500 ng/ml) for 8 h and then treated with SGC-CBP30 for another 10 h. Histone H3 acetylation was measured using an anti-acetyl-histone H3 antibody. **(C)** THP-1 cells were pre-incubated with PF-3644022 (5 μM) for 2 h and then treated with LPS (500 ng/ml) for another 10 h. The cell lysates were subjected to immunoblotting using the indicated antibodies. **(D)** Cells treated the same as in B were immunoprecipitated with the anti-HMGB1 antibody and immunoblotted with anti-acetyl lysine antibody. The data shown are representative of three independent experiments. Error bars indicate the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

USA). For immunoprecipitation, equal amounts of proteins (500 μg) were immunoprecipitated overnight with the indicated antibodies (0.5 μg). Then, 40 μl of precleared protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were incubated with the immunocomplexes for an additional 3 h at 4 °C and then washed four times with cold lysis buffer. After the final wash, the supernatant was discarded and the pellet was resuspended with 40 μl electrophoresis buffer. The samples were subsequently boiled and analyzed by Western blotting. In the co-

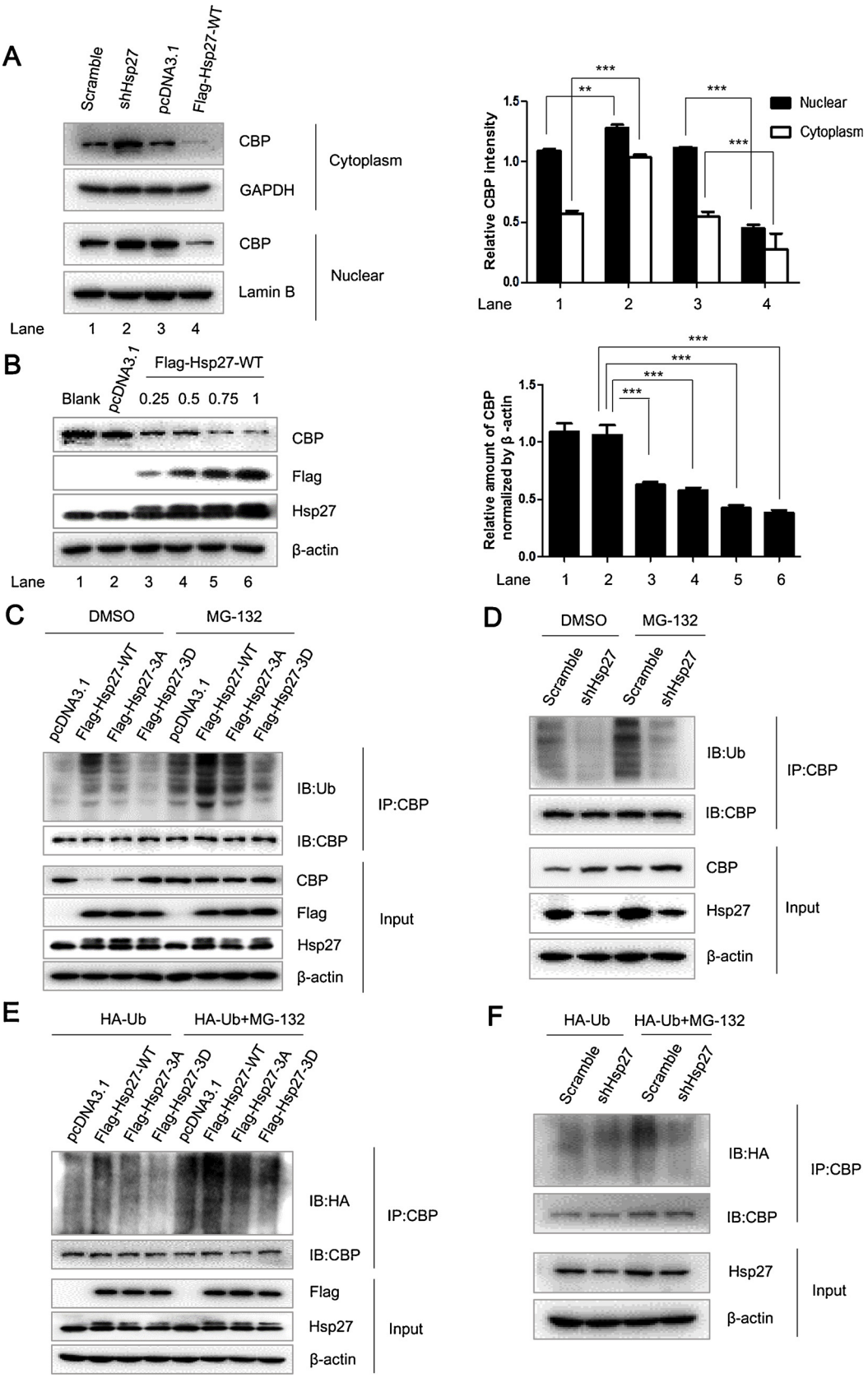

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Fig. 3. Hsp27 promotes the ubiquitin-mediated degradation of CBP. (A) THP-1 cells were transfected with Hsp27 shRNA or Flag-Hsp27-WT, and 72 h after transfection, the cells subjected to extraction of their nuclear and cytoplasmic fractions, followed by immunoblotting using the indicated antibodies. **(B)** THP-1 cells were transfected with the indicated amounts of Flag-Hsp27-WT (0.25–1 μg) or pcDNA3.1, and the cell lysates were subjected to immunoblotting using the indicated antibodies 48 h after transfection. **(C)** THP-1 cells were transfected with Flag-Hsp27-WT, Flag-Hsp27-3 A, Flag-Hsp27-3D or pcDNA3.1, 24 h after transfection, the cells were treated with MG-132 (5 μM) for another 24 h. The cell lysates were immunoprecipitated with anti-CBP antibody followed by immunoblotting with anti-ubiquitin (Ub) and anti-CBP antibodies. **(D)** THP-1 cells were transfected with Hsp27 shRNA plasmids or matched scramble sequence (Scramble), 48 h after transfection, the cells were treated with MG-132 (5 μM) for another 24 h. The lysates were immunoprecipitated with an anti-CBP antibody, followed by immunoblotting with an anti-Ub antibody. **(E)** THP-1 cells were co-transfected with HA-Ub and Flag-Hsp27-WT, Flag-Hsp27-3 A, Flag-Hsp27-3D or pcDNA3.1. After 24 h, the cells were treated with MG-132 (5 μM) for another 24 h. Lysates were immunoprecipitated using an anti-CBP antibody, followed by immunoblotting using an anti-HA antibody. **(F)** Cells were co-transfected with HA-Ub and Hsp27 shRNA plasmids or a matched scramble sequence (Scramble). After 48 h, the cells were treated with MG-132 (5 μM) for another 24 h. The lysates were immunoprecipitated using an anti-CBP antibody and followed by immunoblotting using an anti-HA antibody. The data shown are representative of three independent experiments. Error bars indicate the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

immunoprecipitation assays, aliquots from the same lysates were used as input to determine the internal expression of related proteins, and normal IgG was used as negative control.

2.5. Measurement of HMGB1 release by ELISA

The HMGB1 concentration in the medium was measured using an ELISA kit from Westang Bio-Tech (Shanghai, China), according to the instructions manufacturer's instructions.

2.6. Preparation of cytoplasmic and nuclear extracts

Cells were harvested by centrifuging at 500 *g* for 5 min, followed by washing with ice-cold PBS. The cell pellets were processed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, ice-cold CER I was added to each tube, and the tubes were vigorously vortexed on the highest setting for 15 s to fully resuspend the cell pellet, followed by incubating the tubes on ice for 10 min. After adding ice-cold CER II to the tubes, the samples were vortexed for 5 s on the highest setting and incubated on ice for 1 min. The cytoplasmic component was extracted by centrifuging for 5 min at 16,000 *g*. The insoluble fraction was resuspended in ice cold NER and vortexed for 15 s every 10 min, for a total of 40 min, followed by centrifugation for 10 min at 16,000 *g* to extract the nuclear fraction.

2.7. Confocal laser-scanning microscopy

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized in 0.5% Triton X-100 for 5 min. Then, the cells were then blocked in 5% bovine serum albumin for 30 min, before being incubated separately with primary antibodies at 4 °C overnight, followed by 1 h of incubation with Alexa Fluor secondary antibody at room temperature. The cell nuclei were stained with DAPI for 5 min. Images were captured using a Nikon A1 microscope (Nikon, Tokyo, Japan). Each sample was examined under the same fluorescence detection parameters for comparison of the signal accumulation.

2.8. Statistical analysis

The data are presented as the mean ± SD. Statistical comparisons were performed using Student's *t*-test and one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Phosphorylated Hsp27 inhibits LPS-triggered acetylation and release of HMGB1

The release of proinflammatory cytokines is associated with the severity of inflammation (Chaves de Souza et al., 2013). High mobility group box 1 (HMGB1) has been considered as a proinflammatory cytokine associated with sepsis and other inflammatory diseases (Huang et al., 2010; Zhou et al., 2014). As shown in Fig. 1A, the MK2 inhibitor PF-3644022 increased LPS-induced HMGB1 release from THP-1 cells. Since MK2 is the upstream kinase of Hsp27, this result suggested the inhibitory effect of phosphorylated Hsp27 on HMGB1 release. Further experiments showed that PF-3644022 increased cytoplasmic HMGB1 levels and reduced nuclear HMGB1 levels (Fig. 1B), suggesting that phosphorylated Hsp27 prevented the translocation of HMGB1 from the nucleus to the cytoplasm. Confocal microscopy imaging of HMGB1 and Hsp27 confirmed the above results. As shown in Fig. 1C, HMGB1 was mainly localized in the nucleus of unstimulated cells but was detected in the cytoplasm 18 h after LPS stimulation. The cytoplasmic levels of HMGB1 increased in cells pre-incubated with PF3644022. As acetylation of HMGB1 is essential for HMGB1 to translocate from the nucleus to the cytoplasm, we evaluated the effect of Hsp27 phosphorylation on HMGB1 acetylation by Western blot using an anti-acetyl lysine antibody after HMGB1 co-immunoprecipitation. The results showed that PF-3644022 significantly increased the acetylation of HMGB1 in THP-1 cells treated with LPS (Fig. 1D). In a subsequent experiment, THP-1 cells were transfected with Hsp27-WT (wild-type), Hsp27-3A (in which serine 15/78/82 were mutated to alanine, phospho-defective) or Hsp27-3D (in which serine 15/78/82 were mutated to aspartate, phospho-mimetic)and then incubated with or without LPS for 18 h. As shown in Fig. 1E, without LPS stimulation only Hsp27-3D decreased the acetylation of HMGB1, whereas after LPS stimulation, both Hsp27-WT and Hsp27-3D inhibited the LPS-induced increase in HMGB1 acetylation. The above results clearly indicate that phosphorylated Hsp27 attenuated LPS-triggered acetylation of HMGB1.

3.2. The inhibitory effect of Hsp27 on HMGB1 acetylation depends on the CBP acetyltransferase activity

Accumulating evidence has demonstrated that CBP functions as an acetyltransferase that acetylates histone H3 via its bromodomain and histone acetyltransferase domain (Bose et al., 2017; Das et al., 2009; Henry et al., 2013). It has also been reported that the acetylation of HMGB1 is closely related to CBP acetyltransferase activity (Ong et al., 2012; Wu et al., 2012). We next explored whether the regulatory effect of phosphorylated Hsp27 on HMGB1 acetylation was dependent on CBP acetyltransferase activity. As shown in Fig. 2A, LPS stimulation increasedhistone H3 acetylation, but the CBP inhibitor SGC-CBP30 (Ariane et al., 2015) significantly diminished histone H3 acetylation, suggesting that LPS-induced acetylation of histone H3 was dependent on CBP acetyltransferase activity. When THP-1 cells were transfected with Hsp27-WT, LPS-induced acetylation of histone H3 was also inhibited. Meanwhile, combining both Hsp27-WT transfection and SGC-CBP30 incubation reduced CBP-dependent histone H3 acetylation levels after LPS stimulationcompared with either Hsp27-WT transfection or SGC-CBP30 incubation alone (Fig. 2B). Because LPS may trigger Hsp27 phosphorylation (Hirano et al., 2004), as expected, similar results were obtained when cells were incubated with PF-3644022 (Fig. 2C). These data strongly suggested that phosphorylated Hsp27 inhibited CBP acetyltransferase activity. We then evaluated whether phosphorylated Hsp27 inhibited LPS-induced HMGB1 acetylation in a

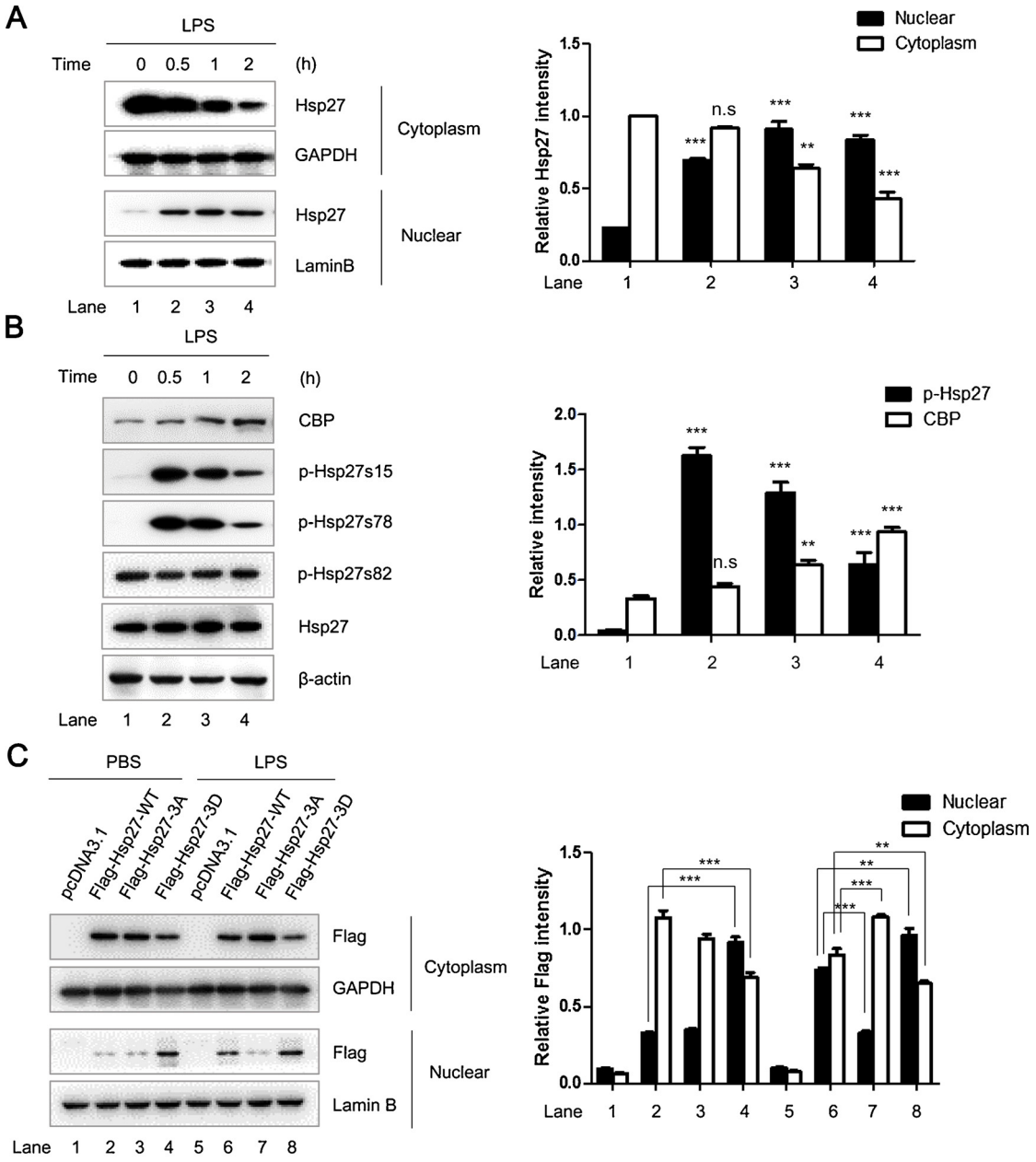

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Fig. 4. LPS stimulation results in Hsp27 phosphorylation and translocation into the nucleus. (A) THP-1 cells were treated with LPS (500 ng/ml) for 0, 0.5, 1 and 2 h. The nuclear and cytoplasmic fractions were extracted and analyzed for Hsp27 by Western blot analysis. **(B)** Cells treated the same as in A were subjected to immunoblotting using the indicated antibodies. **(C)** THP-1 cells were transiently transfected with Flag-Hsp27-WT, Flag-Hsp27-3 A, Flag-Hsp27-3D or pcDNA3.1, 48 h after transfection, the cells were subjected to LPS (500 ng/ml) stimulation for 0.5 h, after which the extracted nuclear and cytoplasmic fractions were subjected to Western blot analysis for Flag. **(D)** THP-1 cells were transiently transfected with Flag-Hsp27-WT, Flag-Hsp27-3 A or Flag-Hsp27-3D, 48 h after transfection, the cells were subjected to LPS (500 ng/ml) stimulation for 0.5 h, incubated with anti-Flag antibody and then incubated with Alexa flour 488-conjugated anti-mouse antibody (green). The nuclei were counterstained with DAPI (blue). The location of Flag was observed under a confocal laser microscope. Scale bar: 10 μm. The data shown are representative of three independent experiments. Error bars indicate the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

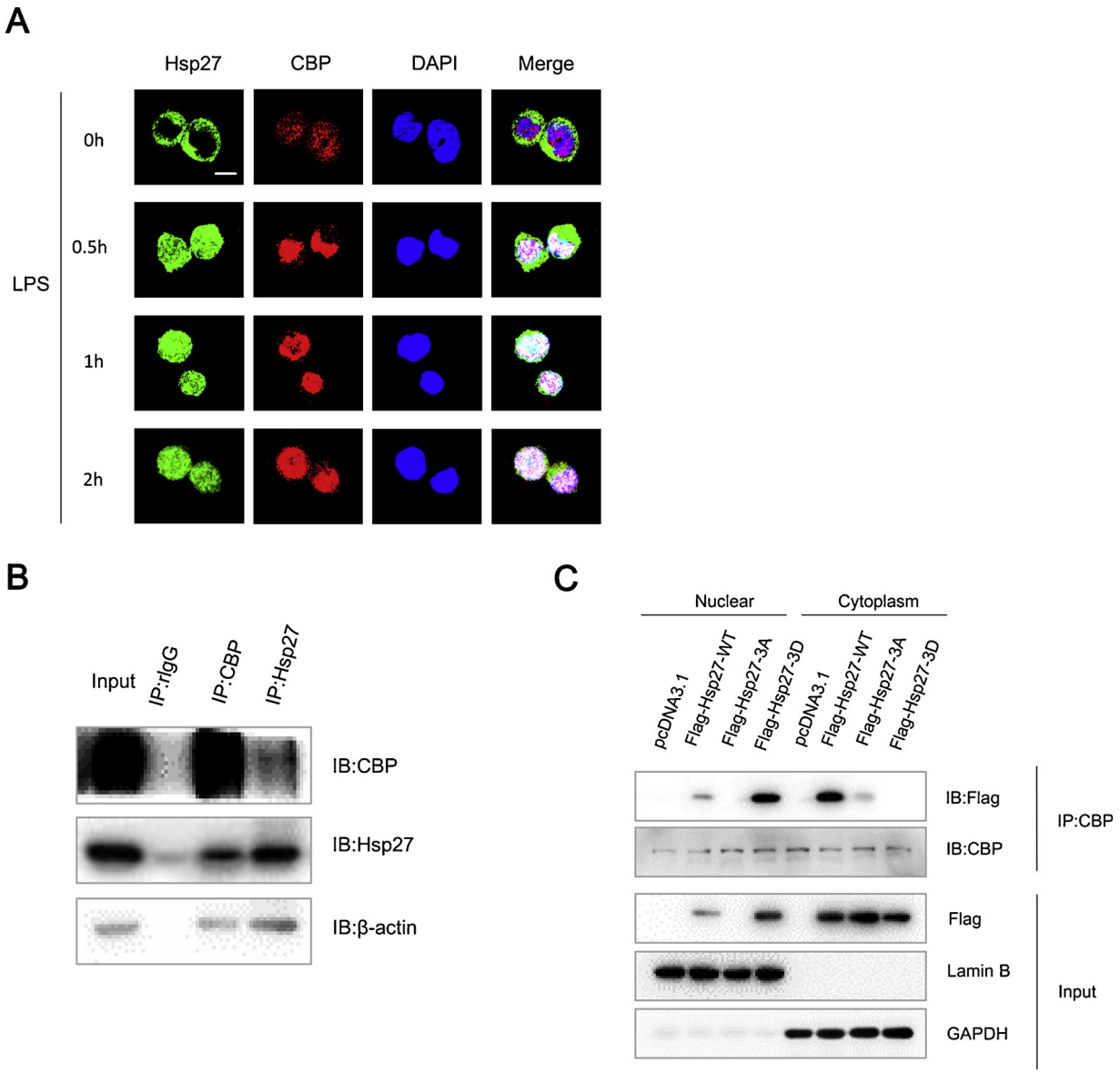

Fig. 5. Phosphorylated Hsp27 enters the nucleus and interacts with CBP in response to LPS stimulation. (A) THP-1 cells were stimulated by LPS (500 ng/ml) for the indicated times, incubated with rabbit anti-CBP and mouse anti-Hsp27 primary antibodies, and then visualized using Alexa flour 488-conjugated anti-mouse (green) and Alexa flour 555-conjugated anti-rabbit (red) secondary antibodies. The nuclei were counterstained with DAPI (blue). Scale bar: 10 μm. **(B)** THP-1 cell lysates were immunoprecipitated with anti-CBP and anti-Hsp27 antibodies. The immunopellets were subjected to immunoblotting using the indicated antibodies. **(C)** THP-1 cells were transiently transfected with Flag-Hsp27-WT, Flag-Hsp27-3 A, Flag-Hsp27-3D or pcDNA3.1, 48 h after transfection, the nuclear and cytoplasmic fractions were subjected to immunoprecipitation with anti-CBP antibody and immunoblotted with anti-CBP or anti-Flag antibody. The data shown are representative of three independent experiments. Error bars indicate the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

CBP-dependent manner. Consistent with a previous report (Wu et al., 2012), LPS-increased acetylation of HMGB1 was significantly inhibited by the CBP inhibitor SGC-CBP30. Overexpression of Hsp27-WT in the cells also inhibited LPS-induced acetylation HMGB1 and promoted the inhibitory effect of SGC-CBP30 on HMGB1 acetylation (Fig. 2D). Taken together, these results demonstrated that phosphorylated Hsp27 inhibited CBP acetyltransferase activity.

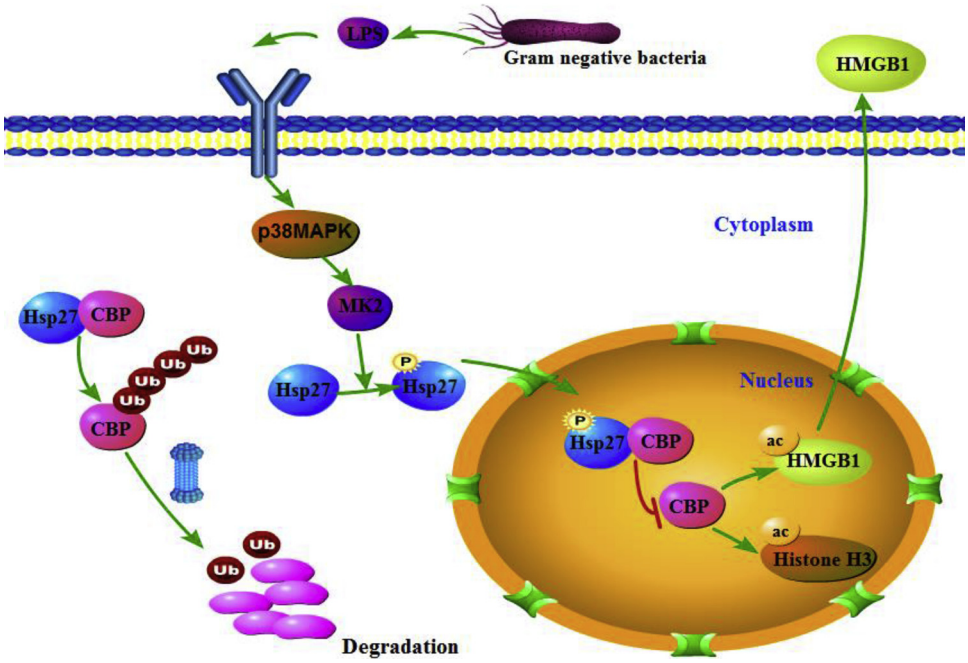

Fig. 6. Schematic diagram of the proposed model illustrating the regulation of CBP acetylation activity and ubiquitin-mediated degradation by phosphorylated and non-phosphorylated Hsp27. In static mononuclear cells, Hsp27 in its non-phosphorylated state interacts with CBP as a native regulator to promote ubiquitin-mediated CBP degradation. After LPS stimulation, Hsp27 is phosphorylated through the p38 MAPK/MK2 signaling pathway and then translocates into the nucleus and binds to CBP to attenuate CBP acetylation activity.

3.3. Cytoplasmic Hsp27 promotes CBP ubiquitination and degradation

We noticed that LPS might promote Hsp27 phosphorylation and translocation from the cytoplasm to the nucleus. Previous studies demonstrated that cytoplasmic Hsp27 is predominantly in a non-phosphorylated state and that phosphorylated Hsp27 plays different roles compared with non-phosphorylated Hsp27 (Leger et al., 2000). We next observed whether cytoplasmic Hsp27 affected CBP levels. As shown in Fig. 3A, when Hsp27 was knocked down by shRNA, cytoplasmic CBP levels increased; in contrast, Hsp27 overexpression significantly attenuated CBP protein levels in the cytoplasm. Furthermore, Hsp27 overexpression reduced CBP protein levels in a dose-dependent manner (Fig. 3B). It has been reported that Hsp27 enhances the catalytic activity of the 26S proteasome to increase the rate of protein ubiquitination and degradation (Parcellier et al., 2003). Coincidentally, CBP is a target for ubiquitination and the 26S proteasome is a key regulator of its activity (St-Germain et al., 2014). We next, transfected THP-1 cells with wild type Hsp27, Hsp27-3A, Hsp27-3D or the empty vector (as a control) and then performed immunoprecipitation assays to detect the ubiquitination levels of CBP. Hsp27-3D transfection did not change CBP ubiquitination compared with the control, and Hsp27-3A transfection showed a detectable increase in CBP ubiquitination; in contrast, Hsp27-WT transfection resulted in a more obvious increase in CBP ubiquitination. Moreover, the proteasome inhibitor MG132 reduced Hsp27-induced CBP degradation (Fig. 3C). We also knocked down cellular Hsp27 using Hsp27-specific shRNA. The result showed that Hsp27 knockdown increased CBP protein levels by reducing ubiquitin-mediated CBP degradation (Fig. 3D). We then co-transfected HA-ubiquitin (HA-Ub) and one kind of Hsp27 plasmid into THP-1 cells and detected CBP ubiquitination and degradation. Consistently, these results confirmed that Hsp27-WT and Hsp27-3 A promoted CBP ubiquitination and degradation (Fig. 3E and F). Collectively, cytoplasmic Hsp27, in its non-phosphorylated state, enhanced CBP ubiquitination and degradation.

3.4. Phosphorylation of Hsp27 is crucial for its nuclear translocation

Accumulating evidence indicates that phosphorylation of Hsp27 is important in response to various stimuli (Qi et al., 2014; Shen et al., 2016; Xu et al., 2013). It has been reported that phosphorylation of Hsp27 may alter its subcellular location (McClaren and Isseroff, 1994). As shown in Fig. 4A, 30 min after THP-1 cells were stimulated with LPS, Hsp27 phosphorylation at Ser15 and Ser78 but not Ser82 was detected, and consequently, CBP levels began to rise. Because Hsp27 phosphorylation will allow it to translocate into the nucleus, we observed the subcellular location of Hsp27 in THP-1 cells after LPS stimulation. The results showed that 2 h after LPS stimulation, nuclear Hsp27 reached a high levels, while cytoplasmic Hsp27 reduced (Fig. 4B). We further transfected Hsp27-WT, Hsp27-3 A or Hsp27-3D into THP-1 cells and extracted nuclear and cytoplasmic fractions 0.5 h after LPS stimulation. Western blot results showed that Hsp27-3D entered the nucleus without stimulation, and LPS triggered wild type Hsp27 (Hsp27-WT) but not Hsp27-3 A to enter the nucleus. Furthermore, the levels of nuclear Hsp27-3D were higher than Hsp27-WT after LPS treatment (Fig. 4C). Similar results were obtained from confocal microscopy detection (Fig. 4D). These data confirmed that phosphorylation was essential for Hsp27 to enter the nucleus.

3.5. Nuclear phosphorylated Hsp27 associates with CBP in response to LPS stimulation

Confocal microscopy detection showed that Hsp27 and CBP visibly congregated in the cytoplasm in untreated cells, while the co-localization of Hsp27 and CBP in the nucleus was evident after stimulation with LPS (Fig. 5A), suggesting the interaction of Hsp27 and CBP in the nucleus. In accordance, co-immunoprecipitation assays also showed that there was protein binding between Hsp27 and CBP in THP-1 cells (Fig. 5B). Moreover, we examined the association of CBP with Hsp27 in the nuclear and cytoplasm fractions by immunoblotting analysis. The results showed that Hsp27-3D associated with CBP in the nuclear components, while Hsp27-WT and Hsp27-3A but not Hsp27-3D formed complexes with CBP in the cytoplasm (Fig. 5C). These results, together with those described previously, suggested that cytoplasmic non-phosphorylated Hsp27 interacted with CBP in the cytoplasm, but phosphorylated Hsp27 bound to CBP in the nucleus. Phosphorylated Hsp27 potentially inhibited CBP acetyltransferase activity by binding with CBP bromodomain.

4. Discussion

As an important molecular chaperone and a crucial regulatory protein, Hsp27 affords a variety of functions in maintaining cell homeostasis in response to environmental stresses (Fawzy, 2013; Liang et al., 2018; Vidyasagar et al., 2012; Yang et al., 2013). However, the precise mechanisms involving non-phosphorylated and phosphorylated Hsp27 remain unclear. We initiated our research to investigate the influence of Hsp27 phosphorylation on LPS-triggered excessive inflammatory response. Our research demonstrated that most cytoplasmic Hsp27 is non-phosphorylated in LPS-unstimulated THP-1 cells. Cytoplasmic non-phosphorylated Hsp27 binds to CBP to facilitate CBP ubiquitination and degradation and prevent excessive CBP from entering the nucleus. This finding suggested that, without proinflammatory stimulation, Hsp27 is mainly located in the cytoplasm as a non-phosphorylated form, which is important for monocytes/macrophages to maintain physiological levels of CBP. The ubiquitin-proteasome pathway has been considered a key mode for CBP degradation (St-Germain et al., 2014). Consistently, previous studies have shown that Hsp27 enhanced the catalytic activity of the proteasome and the degradation of ubiquitinated proteins in various cell types in response to stress stimuli (Parcellier et al., 2003). As is commonly known, mono-ubiquitinated proteins are involved in the internalization of plasma membrane proteins and the regulation of histones, whereas poly-ubiquitination is an identification signal for proteasomal degradation (Sadowski and Sarcevic, 2010). Therefore, it was hypothesized that Hsp27 could influence CBP degradation by modulating its poly-ubiquitination. The detailed molecular mechanisms regarding whether Hsp27 regulates only one ubiquitinated form of CBP and which of the E2 conjugates and E3 ligases are involved in this process remain to be elucidated.

In the present study, we also found that Hsp27 was phosphorylated at Ser15/78 and translocated into the nucleus after LPS stimulation. Consequently, CBP levels increased, especially in the nucleus. Our data demonstrated that LPS stimulation not only induced the increase in nuclear CBP, but also promoted phosphorylated Hsp27 to translocate into the nucleus. This leads us to wonder whether nuclear Hsp27 could affect the function of CBP. Previous studies have shown that HMGB1, like histones, is among the most important chromatin proteins. In the nucleus, HMGB1 interacts with nucleosomes, transcription factors, and histones (Bianchi and Agresti, 2005). Upon LPS stimulation, HMGB1 is released from macrophages as a late proinflammatory cytokine and is related with cell death from inflammatory diseases such as sepsis. Acetylation of HMGB1 is vital for its nuclear-cytoplasmic translocation (Yang et al., 2014). Through its intrinsic acetyltransferase activity, CBP plays a pivotal role in the acetylation of histone H3 and HMGB1 (Henry et al., 2013; Ong et al., 2012). Thus, CBP plays an important role in triggering HMGB1 extracellular secretion under LPS stimulation (Wu et al., 2012). We also provide evidence that inhibition of Hsp27 phosphorylation reduced CBP acetyltransferase activity and decreased HMGB1 acetylation, which strongly suggested that LPS stimulation resulted in Hsp27 phosphorylation as well as its cytoplasmic-nuclear translocation, and attenuated HMGB1 acetylation by inhibiting CBP acetyltransferase activity. It is worth noting that in the nucleus, Hsp27 associated with CBP upon LPS treatment, while suppression of Hsp27 phosphorylation reduced Hsp27 nuclear translocation and Hsp27/CBP complex formation in the nucleus. The above findings indicated that under physiological conditions, cytoplasmic Hsp27 plays an important role in maintaining cellular homeostasis, while under proinflammatory stimulation, nuclear Hsp27 protected cells from excessive inflammatory response. Protein post-translational modification is closely related with protein subcellular location (Jensen et al., 2002), implying that one or more post-transcriptional modifications to CBP may cause its translocation to the nucleus. In addition, one mechanism by which CBP activity is regulated in the cells is through post-translational modifications (Karamouzis et al., 2007), such as phosphorylation, acetylation, ubiquitination, methylation and sumoylation. Although the specific binding site between Hsp27 and CBP remains to be elucidated, it can be predicted that in response to LPS stimulation, phosphorylated Hsp27 interacts with post-translational-modified CBP in the nucleus either at the bromodomain to shield “reading” acetylated lysine residues or directly at the HAT domain to decrease its acetyltransferase activity. The detailed molecular mechanisms by which the post-translational modification of CBP exerts its actions on cytoplasmic-nuclear translocation and how phosphorylation of Hsp27 inhibits CBP acetyltransferase activity require further investigation.

Hsp27 has been considered to significantly suppress apoptosis, oxidative stress, and inflammatory responses (Shimada et al., 2014). Stress may induce an increase in Hsp27 expression (after hours) and phosphorylation (after several minutes) (Rogalla et al., 1999). Additionally, there is growing evidence for the cytoprotective effects of phosphorylated Hsp27 against several pathological conditions (Shimada et al., 2014). Our previous study also showed that Hsp27 regulated LPS-induced inflammatory response in THP-1 cells by interacting with TRAF6 (Liu et al., 2010). Consistent with these investigations, our present study also showed the cell protective effects of Hsp27. However, our research first demonstrated a novel role for Hsp27 in regulating CBP protein stability and acetyltransferase activity in THP-1 cells. Further experiments conducted in primary human monocyte-derived macrophages or primary human macrophages will be helpful to understand the role of Hsp27 in regulating CBP activity after LPS stimulation.

Based on the present results, we revealed a novel mechanism by which Hsp27 plays a critical role in the regulation of intracellular inflammatory signaling processes via modulating CBP protein levels and activity (Fig. 6). Through conversion from its non-phosphorylated to phosphorylated form, Hsp27 plays a different role in macrophages in response to LPS stimulation or not. Understanding the mechanism underlying the effects of phosphorylated and non-phosphorylated Hsp27 on CBP will help us to develop novel strategies to control inflammation.

Author contributions

Conceived and designed the experiments: ZY, LL, SL, XB and LS. Performed the experiments: XB, MX, JL, TH, BJ and LS. Analyzed the data: XB, ZY and LL. Wrote the paper: LL, ZY and XB.

Competing interests

The authors declare that they have no competing interests.

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