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HSP27 phosphorylation protects against endothelial barrier dysfunction under burn serum challenge

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ABSTRACT

F-actin rearrangement is an early event in burn-induced endothelial barrier dysfunction. HSP27, a target of p38 MAPK/MK2 pathway, plays an important role in actin dynamics through phosphorylation. The question of whether HSP27 participates in burn-related endothelial barrier dysfunction has not been identified yet. Here, we showed that burn serum induced a temporal appearance of central F-actin stress fibers followed by a formation of irregular dense peripheral F-actin in pulmonary endothelial monolayer, concomitant with a transient increase of HSP27 phosphorylation that conflicted with the persistent activation of p38 MAPK/MK2 unexpectedly. The appearance of F-actin stress fibers and transient increase of HSP27 phosphorylation occurred prior to the burn serum-induced endothelial hyperpermeability. Overexpressing phospho-mimicking HSP27 (HSP27(Asp)) reversed the burn serum-induced peripheral F-actin rearrangement with the augmentation of central F-actin stress fibers, and more importantly, attenuated the burn serum-induced endothelial hyperpermeability; such effects were not observed by HSP27(Ala), a non-phosphorylated mutant of HSP27. HSP27(Asp) overexpression also rendered the monolayer more resistant to barrier disruption caused by Cytochalasin D, a chemical reagent that depolymerizes F-actin specifically. Further study showed that phosphatases and sumoylation-inhibited MK2 activity contributed to the blunting of HSP27 phosphorylation during the burn serum-induced endothelial hyperpermeability. Our study identifies HSP27 phosphorylation as a protective response against burn serum-induced endothelial barrier dysfunction, and suggests that targeting HSP27 wound be a promising therapeutic strategy in ameliorating burn-induced lung edema and shock development.

1. Introduction

Microvascular barrier dysfunction imposes a life-threatening problem to severe burn patients owing to the consequences of plasma loss, organ edema and even shock [1]. F-actin Rearrangement is an early event in the initiation of endothelial contraction and thus endothelial barrier dysfunction under various pathophysiological conditions [2–5]. It is believed that the central F-actin stress fibers might generate contractile force through F-actin/myosin interaction, tending to pull the connected cells apart [6]. In our previous study, we found that the typical F-actin stress fibers were induced temporally in burn serum-treated endothelial cells (ECs); by contrast, a dense peripheral F-actin was formed when the cell was further challenged by burn serum, associated with the endothelial hyperpermeability [7]. Interestingly, recent studies have shown that the peripheral F-actin also links to the generation of contractile force [8], and more importantly, the transcellular F-actin stress fibers might be in favor of endothelial barrier function [9]. The question of whether or how the F-actin rearrangement is implicated in burn serum-induced endothelial barrier dysfunction remains unclear.

Heat shock protein 27 (HSP27) is required for the induction of F-actin stress fibers in ECs responded to various stimuli such as cytokines, hypoxia and lipopolysaccharide [10–12]. HSP27 normally prevents actin polymerization by binding to G-actin monomers; when phosphorylated, it loses the binding function leading to polymerization of F-actin. The phosphorylation of HSP27 is determined by the balance between the activities of phosphorylation

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and dephosphorylation. For example, HSP27 is known to be able to phosphorylated by p38 mitogen-activated protein kinase (p38 MAPK)/MAPK-activated protein kinase 2 (MK2) signaling [13,14]; meanwhile, its dephosphorylation could be carried out by the Ser/Thr specific protein phosphatases such as phosphatase 2A (PP2A) or PP1 [15,16].

Activation of p38 MAPK signaling is one of the mechanisms by which cells respond to environmental stress. While phosphorylation is the most common posttranslational modification determining the kinase activity, other modifications such as sumoylation also comes into focus as an important regulator of signal transduction [17,18]. Sumoylation is a unique posttranslational modification akin to ubiquitination that conjugates small, ubiquitin-like proteins called SUMO to target proteins. It affects more than 60 proteins involving in transcription, signal transduction and stress response [18]. Recently, sumoylation of MK2 has been observed in ECs, which inhibits the MK2 kinase activity significantly [19]. Deconjugation of SUMO from the target proteins is carried out by SUMO proteases, of which sentrin-specific protease 1 (SENP1) has the highest endopeptidase and isopeptidase activity [20,21].

We and others have shown that burn serum activates p38 MAPK in ECs, which acts as an important participant in burn serum-induced endothelial barrier dysfunction [7,22]. It is not clear, however, whether HSP27, a downstream target of p38 MAPK/MK2, is involved in this process through the regulation of actin dynamics. Here, we showed that burn serum induces a temporal appearance of central F-actin stress fibers associated with an unexpected transient increase of HSP27 phosphorylation prior to the occurrence of barrier dysfunction in pulmonary endothelial monolayer, which function as responses of barrier protection rather than disruption. Both phosphatases and MK2 sumoylation contribute to the blunting of HSP27 phosphorylation during the burn serum induced-monolayer barrier disruption.

2. Materials and methods

2.1. Cell culture

Rat pulmonary microvascular endothelial cells (PriCells company, China) were cultured with DMEM containing 10% fetal bovine serum (FBS, Gibco) in cell incubator (37 °C, 5% CO_2). Cells were cultured to 70–80% confluence, and then digested by 0.25% trypsin and passaged at a ratio of 1:3.

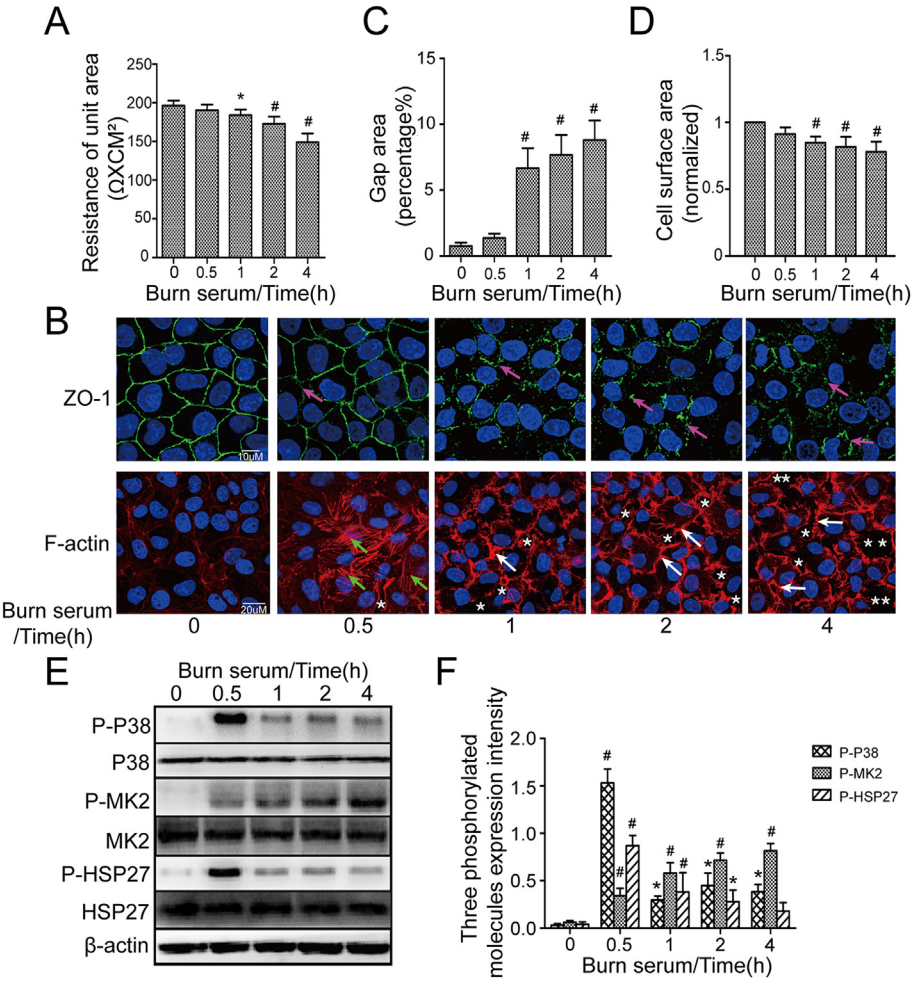

Fig. 1. Endothelial monolayer permeability, F-actin rearrangement and phosphorylation of p38 MAKP/MK2/HSP27. (A) Detection of TEER cross endothelial monolayers after burn serum treatment; (B) Immunofluorescence staining of ZO-1 (pink arrow, left panel), central F-actin stress fibers (green arrow, right panel), and peripheral F-actin (white arrow, right panel) in monolayers after burn serum treatment. White asterisks (right panel) indicate the intercellular gaps. (C, D) Quantification of intercellular gap area (C) and cell surface area (D). (E, F) Western blots results (E) or quantitative analysis (F) of phosphorylation of p38 MAPK, MK2 and HSP27 in monolayers after burn serum treatment. *, $P < 0.05$; #, $P < 0.01$ *vs.* nontreated control (0 h). n = 5 (A) or 3 (B–F) independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Burn serum collection

Burn serum was collected from burn patients (2 women and 8 men aged from 25 to 53 years) with second and third-degree burn over 50% of their body surface area.10 mL of venous blood from each patient was drawn within 24 h postburn and centrifuged at 3,000 g for 15 min. Serum was removed, heated at 56 °C for 30 min to inactivate the complement, and then stored at −70 °C until use. Before experiments, all the burn serums were mixed together to form one large sample to reduce any variation caused by donor differences. Blood were obtained and used according to the protocols reviewed and approved by the ethics committee of Third Military Medical University.

2.3. Transfection of recombinant adenovirus

Adenovirus vectors of HSP27 (WT), HSP27 (S15/78/82A) or HSP27(Ala), and HSP27 (S15/78/82D) or HSP27(Asp) were constructed by Genechem Corporation (China). Adenovirus vectors of SENP1 were the kind gifts from Dr. Zhang (Department of Hematology, Southwest Hospital, Third Military Medical University). To examine the efficiency of adenovirus transfection, cells were observed under fluorescence microscope or analyzed by Western blots 48 h later after transfection.

2.4. Assessment of endothelial monolayer permeability

Monolayer permeability was assessed by the measurement of trans-endothelial electrical resistance (TEER) [5]. Briefly, non-transfected or transfected cells were seeded on polyethylene terephthalate cell culture inserts with 0.4 μm pore size (Greiner, Germany) precoated with rat tail collagen (Roche Diagnostics GmbH, Mannheim, Germany) in 24-well plates. When cells were cultured to confluence, the culture medium was replaced to DMEM containing 20% burn serum or 2 μM Cytochalasin D (Cyt-D, sigma, USA). TEER was measured at indicated time points using Millicell ERS-2 equipment.

2.5. Western blots, MK2 sumoylation and immunoprecipitation

For Western blots or MK2 sumoylation, cells were lysed, and the protein extracts were separated by SDS PAGE and transferred onto a PVDF membrane respectively. After blocking with 5% nonfat dried milk, the membrane was incubated at 4 °C overnight with primary antibodies and secondary antibodies conjugated with horseradish peroxidase (Proteintech, USA) insequence. Specific protein bands were detected using WesternBright ECL HRP substrate or WesternBright Sirius HRP substrate kit (Advansta, USA). Antibodies used in this study included: (1) rabbit polyclonal anti-p-p38 (Thr180/Tyr182), anti-p38, anti-p-MK2(Thr334), anti-MK2 and anti-p-

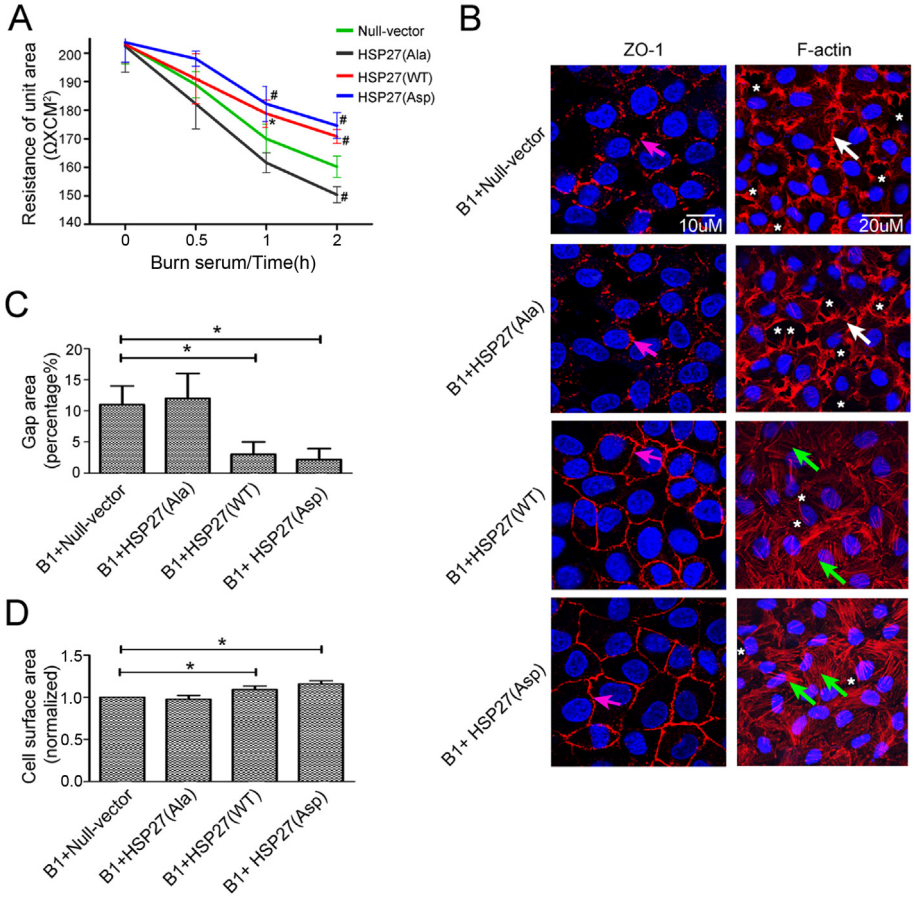

Fig. 2. p-HSP27 protects against endothelial barrier dysfunction in association with the promotion of F-actin stress fibers. (A) Detection of TEER cross monolayers with overexpression of null-vector, HSP27(Ala), HSP27(WT) or HSP27 (Asp) after burn serum treatment. (B) Immunofluorescence staining of ZO-1 (pink arrow, left panel) or F-actin (right panel: peripheral F-actin, white arrow; central F-actin, green arrow) in transfected monolayers at 1-hr after burn serum treatment. Intercellular gaps were marked by white asterisk (right panel); (C, D) Quantitative analysis of intercellular gaps (C) and cell surface area (D) in transfected monolayers at 1-hr after burn serum treatment. *, $P < 0.05$; #, $P < 0.01$ *vs.* null-vector control. n = 5 (A) or 3 (B–D) independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HSP27(Ser82) (Cell Signaling Technology, USA); (2) mouse polyclonal anti-HSP27 and anti-SENP1 antibodies (Abcam, USA); (3) mouse polyclonal anti-β-actin and rabbit polyclonal anti-GAPDH (Proteintech, USA). The level of phosphorylation was quantified by the ratio of band density of the phosphorylated protein to their total protein. Quantification of MK2 sumoylation was determined by the density ratio between the 50–70 kDa band-shift area versus the MK2 area (46 kDa) [19].

2.6. Immunofluorescence and morphometric analysis

Cells were fixed in 4% formaldehyde for 30 min, permeabilized with 0.1% Triton X-100 in PBS and blocked in 10% goat serum. To visualize actin filaments, cells were stained with Rhodamine-Phalloidin (Sigma, USA) for 30 min at room temperature. To observe tight junction, rabbit monoclonal anti-ZO-1 antibodies (Cell Signaling, USA) and secondary antibodies conjugated to Cy3 or FITC (Proteintech, USA) were used. Cells were imaged by confocal microscopy (TCS-NT; Leica, Wetzlar, Germany).

Cell surface area or intracellular gap area was determined according to the methods described previously [7]. Intracellular gap area was expressed as a ratio of the gap area to the area of the whole image. Cell surface area was expressed as the ratio of surface area of the treated cells to control cells, which was normalized as 1.

2.7. Statistical analysis

SPSS software 13.0 was used for statistical analysis. Data were presented as mean ± standard deviation (SD). Statistically significant differences were determined by ANOVA, $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Endothelial barrier dysfunction and F-actin rearrangements after burn serum treatment

TEER was significantly decreased at 1-hr after burn serum treatment, and further decreased with the extension of burn serum challenge (Fig. 1A). TEER decrease induced by burn serum was associated with the alterations of endothelial morphology. In an absence of burn serum treatment, the cells possessed good confluence and clear tight junction (ZO-1) (Fig. 1B, 0 h after burn serum treatment); a significant increase in intercellular gaps,

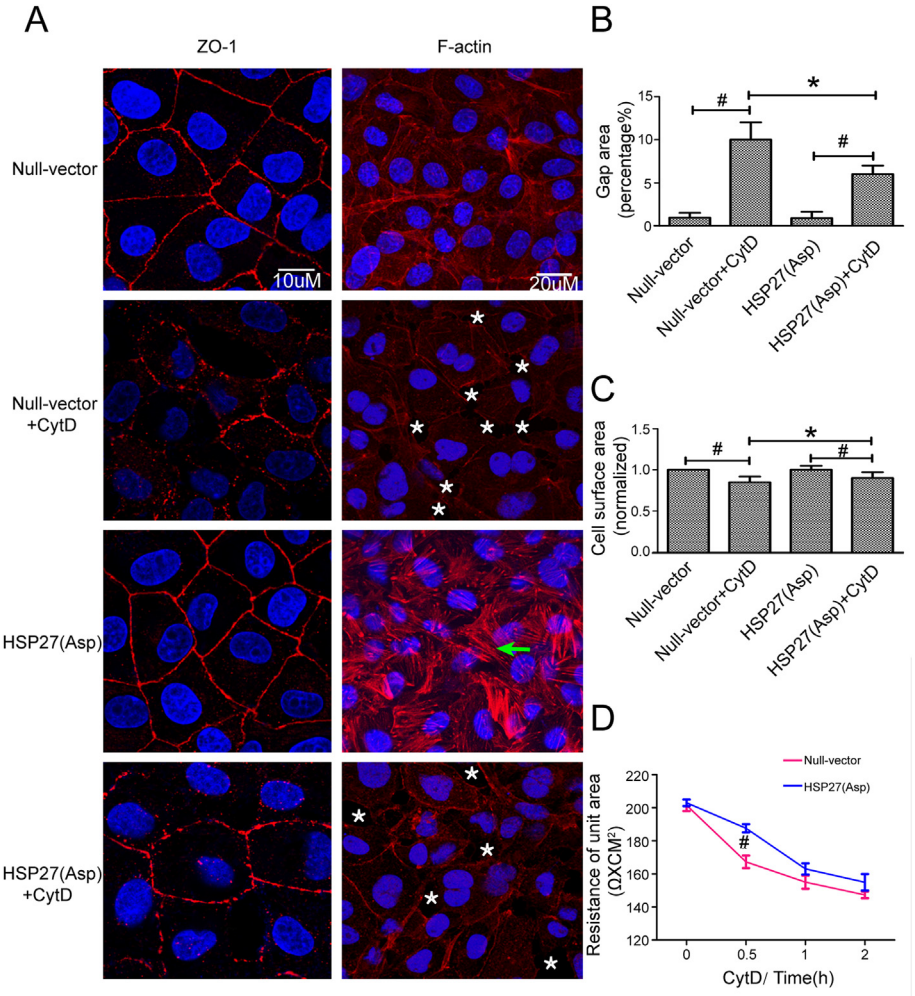

Fig. 3. Depolymerization of F-actin increases the permeability of endothelial monolayer. (A) Immunofluorescence staining of ZO-1 (left panel) or F-actin (right panel) in monolayers with or without HSP27(Asp) overexpression after 30 min of Cyt-D (2 μM) treatments. Central F-actin stress fibers and intercellular gaps were marked by green arrow and white asterisk respectively. (B, C) Quantitative analysis of the intercellular gaps (B) and cell surface area (C). *, $P < 0.05$; #, $P < 0.01$. (D) Detection of TEER cross monolayers with or without HSP27(Asp) overexpression after Cyt-D treatment. #, $P < 0.01$ *vs.* null-vector control. n = 3 (A–C) or 5 (D) independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

however, was observed 1-hr later after burn serum challenge (Fig. 1B and C), concomitant with decrease of cell surface area and disruption of tight junction (Fig. 1B and D). These results concurred with previous observations showing endothelial barrier dysfunction induced by burn serum [7,22].

Exposure to burn serum resulted in a profound rearrangement of F-actin in endothelial monolayer. F-actin was presented as the long, thin fibers in either central or peripheral portion of the cells in the absence of burn serum treatment (Fig. 1B, 0 h after burn serum treatment). At 0.5-hr after burn serum treatment, a remarkable formation of central F-actin bundles was observed (Fig. 1B), indicating the induction of F-actin stress fibers in ECs. The central F-actin, however, got fuzzy, shorter or disappeared at 1-hr and up to 4-hr after burn serum treatment, with an appearance of the irregular dense F-actin in the periphery of cells instead (Fig. 1B). These results suggest a temporal appearance of central F-actin stress fibers in ECs prior to the increase of monolayer permeability by burn serum. It seems that the peripheral, but not central F-actin rearrangement correlates to the burn serum-induced barrier dysfunction.

3.2. Phosphorylation of p38 MAPK, MK2 and HSP27 in burn serum-treated endothelial monolayer

To determine whether the observed F-actin rearrangement above correlates to activation of p38 MAPK/MK2/HSP27 signaling, phosphorylation of p38 MAPK, MK2 and HSP27 were examined in ECs with or without burn serum treatment. Notably, the p-p38 MAPK showed dual peaks at 0.5 and 2-hr after burn serum treatment. The p-MK2, however, increased progressively in response to burn serum challenge. The p-HSP27, to our surprise, peaked at 0.5-hr, and then turned to a sharp decline significantly (Fig. 1E and F). Burn serum-induced p-HSP27 could be abolished by SB203580 (2 μM), a selective inhibitor of p38 MAPK, or 385880 (10 μM), a selective inhibitor of MK2, confirming that HSP27 is the target of p38 MAPK/MK2 in our study (Fig. S1). These results revealed an unexpected pattern of HSP27 phosphorylation in burn serum-treated ECs that while p38 MAPK or MK2 was persistently activated, the phosphorylation of HSP27 was only transiently increased prior to the occurrence of endothelial barrier dysfunction, and then remarkably declined along with the increase of endothelial permeability.

3.3. p-HSP27 protects against endothelial barrier dysfunction in association with the promotion of F-actin stress fibers

Since phosphorylation of HSP27 was blunted 1-hr later after burn serum treatment, which correlated to the disappearance of central F-actin stress fiber and occurrence of endothelial hyperpermeability, we asked whether there is a cause relationship between the blunting of HSP27 phosphorylation, loss of F-actin stress fibers and increase of endothelial permeability. To test it, two HSP27 mutants were generated that mimicked the phosphorylated or non-phosphorylated form by changing S15, S78 and S82 to aspartic acid (HSP27(Asp)) or alanine (HSP27(Ala)), respectively [23]. Mutant forms of HSP27 (Asp or Ala) or wild-type HSP27 (HSP27 (WT)) were transfected into ECs (Fig. S2A) and expressed at comparable levels (Fig. S2B). Interestingly, although TEER was decreased in all transfectants responded to burn serum, monolayers overexpresssing HSP27(Asp) or HSP27(WT) were more resistant to TEER decrease compared to null-vector control, whereas overexpresssion of HSP27(Ala) rendered the monolayer more sensitive to such TEER decrease (Fig. 2A). Morphologically, HSP27(Asp) or HSP27(WT) significantly inhibited the burn serum-induced formation of intercellular gaps, disorder of tight junctions (ZO-1) and decrease of cell surface area, whereas HSP27(Ala) did not have these effects (Fig. 2B–D). HSP27(Asp) or HSP27(WT), but not HSP27(Ala), largely reversed the burn serum-induced peripheral F-actin rearrangement, with the augmentation of central F-actin bundles (Fig. 2B). These results suggest that HSP27 phosphorylation supports barrier function with the promotion of central F-actin stress fibers in monolayer challenged by burn serum. The rationale of HSP27 (WT)-mediated protective effects may be due to the increased level of total HSP27 that could be partially

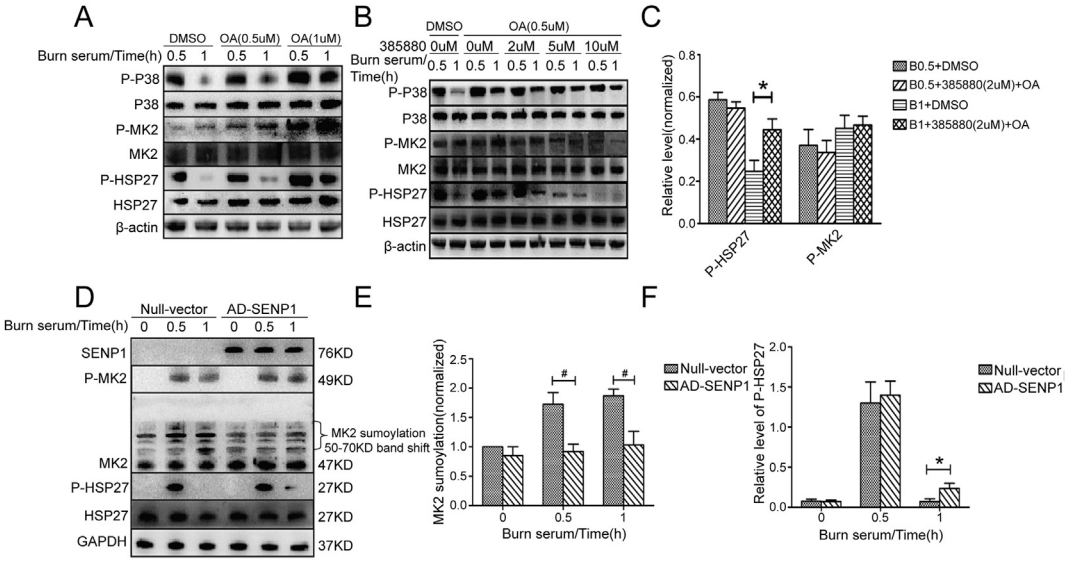

Fig. 4. Regulation of p-HSP27 by phosphatases and MK2 sumoylation in burn serum-treated ECs. (A) Western blots results showing expression of p38 MAPK/p-p38 MAPK, MK2/p-MK2 and HSP27/p-HSP27 in ECs with or without OA pretreatment under burn serum challenge. (B, C) Western blots results (B) or quantitative analysis (C) of the expression of p38 MAPK/p-p38 MAPK, MK2/p-MK2 and HSP27/p-HSP27 in ECs with or without OA and 385880 co-pretreatments under burn serum challenge. (D) Western blots results showing MK2 sumoylation (band shift of 50–70 kDa above MK2) and expression of MK2/p-MK2 as well as HSP27/p-HSP27 in ECs with or without SENP1 overexpression after burn serum treatment. (E, F) Quantification of MK2 sumoylation (E) or p-HSP27 expression (F) in ECs with or without SENP1 overexpression after burn serum treatment. *,$P < 0.05$; #,$P < 0.01$. n = 3 independent experiments.

phosphorylated by burn serum, therefore offering protection against the burn serum-induced barrier dysfunction.

3.4. Depolymerization of F-actin increases endothelial permeability

To verify the relationship between F-actin stress fibers and endothelial permeability directly, endothelial monolayer with or without HSP27(Asp) overexpression were pretreated with Cyt-D (2 μM), an reagent that inhibits actin formation specifically, 30 min prior to the experimental observations. Both ECs pretreated with Cyt-D inhibited F-actin formation, with increased intercellular gaps and smaller cell surface area (Fig. 3A–C). Overexpression of HSP27 (Asp), however, attenuated the Cyt-D-induced endothelial alterations compared with null-vector control (Fig. 3B and C). TEER assay showed that Cyt-D-induced endothelial hyperpermeability was less significant in HSP27 (Asp) transfectants (Fig. 3D). These results, thus, demonstrated a beneficial role of F-actin stress fibers in endothelial barrier function, which in turn indicates that the transient increase of p-HSP27 as well as the temporal formation of central F-actin stress fibers in burn serum-treated monolayers may act as an adaptive and protective response against the burn serum-induced endothelial barrier dysfunction.

3.5. HSP27 dephosphorylation in burn serum-treated ECs

As the transiently increased HSP27 phosphorylation conflicted with the persistently increased MK2 phosphorylation in ECs after burn serum treatment (Fig. 1E), we postulated that HSP27 dephosphorylation through the Ser/Thr specific protein phosphatases may exist accounting for this inconsistency. ECs under burn serum challenge were pretreated with okadaic acid (OA), a specific inhibitor of phosphatase, at final concentration of 0.5 or 1 μM. Unexpectedly, levels of p-HSP27 and p-p38 MAPK/p-MK2 were all increased by OA pretreatment in burn serum-treated ECs (Fig. 4A). To exclude the interference on HSP27 phosphorylation by further activated p38 MAPK/MK2 due to OA pretreatment, ECs were pretreated with OA (0.5 μM)) and MK2 inhibitor, 385880 (2–10 μM) concurrently, and then challenged by burn serum. It was noted that 385880 at 2 μM reduced p-MK2 to a level comparable to that of control; however, a significant increase in p-HSP27 was still detected, particularly at 1-hr after burn serum treatment (Fig. 4B and C). These results indicate that HSP27 dephosphorylation contributes to the observed blunting of HSP27 phosphorylation during burn serum-induced endothelial barrier dysfunction.

3.6. MK2 sumoylation regulates HSP27 phosphorylation in burn serum-treated ECs

While p-HSP27 was elevated by OA pretreatment, the level of which was not recovered to its maximum at 0.5-hr after burn serum treatment (Fig. 4B and C), indicating that other unidentified factors also contribute to the blunting of HSP27 phosphorylation. Since MK2 kinase activity could be inhibited by sumoylation through SUMO protein (19), we examined whether MK2 sumoylation plays a role in the regulation of HSP27 phosphorylation in this study. Band shifts of 50–70 KD above MK2 (47KD) could be detected in ECs that were further increased by burn serum treatment (Fig. 4D and E), suggesting an augmentation of MK2 sumoylation by bur serum in this study. Overexpression of SENP1, a protease that deconjugates SUMO proteins from the targeted proteins [21], suppressed the band shifts (Fig. 4D and E), and increased the level of p-HSP27 significantly at 1-hr after burn serum treatment (Fig. 4D and F). These results identified MK2 sumoylation as another contributor for the blunting of HSP27 phosphorylation during burn serum-induced endothelial barrier dysfunction.

4. Discussion

Reassembly of F-actin from a cortical morphology to transcellular stress fibers is observed in ECs responded to isolated inflammatory mediators [3,5,24]. Our results showed a unique F-actin rearrangement in burn serum-treated ECs that was characterized with a temporal appearance of central F-actin stress fibers followed by a formation of irregular dense peripheral F-actin. Although periphery F-actin also links to cellular contractile force [8], our finding highlighted the differences in F-actin remodeling caused by burn serum comparing to isolated mediators. The reason for this discrepancy is not clear, but may be due to the complexity of burn serum that contains not only the inflammatory mediators, but also other detriments such as oxidants, lipid peroxides and burn toxin [1], which may produce profound effects such as depolymerization or disruption on cytoskeleton. Similar results of burn-induced profound endothelial F-actin rearrangement have been also observed by us and others previously [4,7].

We revealed that HSP27 phosphorylation was only transiently increased in ECs following burn serum challenge, which conflicted with the pattern of p38 MAPK/MK2 activation, but concurred with the temporal appearance of central F-actin stress fibers. By genetic manipulation and chemical inhibition experiments, we demonstrated that HSP27 phosphorylation supports endothelial barrier function through the promotion of central F-actin stress fibers, suggesting HSP27 phosphorylation as a protective response against the burn serum-induced endothelial barrier dysfunction. A beneficial role for p-HSP27 as well as F-actin stress fibers in endothelial barrier function has been also suggested in hypoxia, TGF-β or laminar shear stress-treated endothelial cells through reinforcing the endothelial tethering [9,25]. Since inhibition of p38 MAPK protects against the burn serum-induced endothelial barrier dysfunction [2,7], we speculate that this pathway may play a dual role in endothelial permeability: one leading to increased contractility by phosphorylation of myosin light chain or actin-binding protein *l*-caldesmon [6,7]; the other leading to F-actin stress fibers formation that counteracts the contraction by targeting HSP27.

The phosphorylation of HSP27 is reversible by phosphatases. As we found that the p38 MAPK pathway is also regulated by phosphatases in ECs, we designed the experiments with the pretreatment of OA and MK2 inhibitor concurrently, by which the involvement of HSP27 dephosphorylation in the blunting of p-HSP27 during burn serum-induced endothelial hyper-permeability was determined clearly. OA can inhibit PP2A and PP1 *in vitro*, but has little effect on PP1 in cells up to 1 μM [26,27]. It is also reported that PP2A, but not PP1, is mainly responsible for HSP27 dephosphorylation *in vivo* [28]. We also found an increased interaction of PP2A with HSP27 in ECs under burn serum challenge (Fig. S3). Therefore, PP2A is likely the possible candidate for HSP27 dephosphorylation in our study. Another finding is that we identified sumoylation as an unsuspected posttranslational modification of MK2 in burn serum-treated ECs, which also contributes to the blunting of p-HSP27 through inhibition of MK2 kinase activity. Interestingly, sumoylation as a mechanism to modulate MEK activity has been also identified in ERK pathway recently [29]. Notably, either OA pretreatment or MK2 sumoylation inhibition partially re-elevated p-HSP27 in burn serum-treated ECs when comparing to its peak level at 0.5-hr after burn serum challenge, indicating that the mechanisms underlying the blunting of HSP27 phosphorylation in burn serum-treated ECs are far more complex than expected, which need further investigation. Nevertheless, we have provided the first evidence in this study showing an adaptive protective role of HSP27 phosphorylation in burn serum-induced endothelial barrier dysfunction (Fig. S4), which suggests that

targeting HSP27 would be a promising therapeutic strategy against the lung edema and shock development in burn patients.

Conflicts of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.152.

Transparency document

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