Heat stress combined with lipopolysaccharide induces pulmonary microvascular endothelial cell glycocalyx inflammatory damage in vitro

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Abstract

Heat stroke is a life-threatening disease with high mortality rate and unexpected complications. Vascular endothelial glycocalyx is essential for maintaining endothelial cell structure and function as well as preventing adhesion of inflammatory cells. Potential relationship that underlays the imbalance in inflammation and coagulation remains elusive. Moreover, the role of endothelial glycocalyx in heat stroke-induced organ injury remained unclear. Heat stress and lipopolysaccharide (LPS) are employed to construct in vitro models to study the change of glycocalyx structure and function in human pulmonary microvascular endothelial cells (HPMEC), as well as levels of heparansulfate proteoglycan (HSPG), syndecans-1, heparansulfate, TNF-α, IL-6, vWF, ET-1, Occludin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and reactive oxygen species (ROS). Here, we showed that heat stress and LPS devastated endothelial glycocalyx structure, activated endothelial glycocalyx degradation, and triggered oxidative damage in addition to apoptosis in HPMEC. Stimulation of heat stress and LPS increased HSPG, syndecans-1 (SDC-1), and heparansulfate levels, and promoted the ability to produce and release pro-inflammation cytokines (TNF-α, IL-6,) and coagulative factor (vWF, ET-1) in HPMEC. Furthermore, E-selectin, VCAM-1, and ROS expression were upregulated in contrast to Occludin downregulation. These changes could be deteriorated by Heparanase, whereas could be ameliorated by unfractionated heparin. This study highlights that heat stroke-induced endothelial glycocalyx degradation can trigger oxidative and apoptosis in HPMEC, in addition to dysfunction of inhibition of inflammatory response and protection in vascular permeability.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author's contributions

Y.C. and H.T. designed the research study, and contributed essential reagents or tools. J.D., C. D, and J. C. performed the research and analyzed the data. J.D. wrote the paper. All authors have read and approved the final manuscript.

Abstract

Heat stroke is a life-threatening disease with high mortality rate and unexpected complications. Vascular endothelial glycocalyx is essential for maintaining endothelial cell structure and function as well as preventing adhesion of inflammatory cells. Potential relationship that underlays the imbalance in inflammation and coagulation remains elusive. Moreover, the role of endothelial glycocalyx in heat stroke-induced organ injury remained unclear. Heat stress and lipopolysaccharide (LPS) are employed to construct *in vitro* models to study the change of glycocalyx structure and function in human pulmonary microvascular endothelial cells (HPMEC), as well as levels of heparansulfate proteoglycan (HSPG), syndecans-1, heparansulfate, TNF-α, IL-6, vWF, ET-1, Occludin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and reactive oxygen species (ROS). Here, we showed that heat stress and LPS devastated endothelial glycocalyx structure, activated endothelial glycocalyx degradation, and triggered oxidative damage in addition to apoptosis in HPMEC. Stimulation of heat stress and LPS increased HSPG, syndecans-1 (SDC-1), and heparansulfate levels, and promoted the ability to produce and release pro-inflammation cytokines (TNF-α, IL-6,) and coagulative factor (vWF, ET-1) in HPMEC. Furthermore, E-selectin, VCAM-1, and ROS expression were upregulated in contrast to Occludin downregulation. These changes could be deteriorated by Heparanase, whereas could be ameliorated by unfractionated heparin. This study highlights that heat stroke-induced endothelial glycocalyx degradation can trigger oxidative and apoptosis in HPMEC, in addition to dysfunction of inhibition of inflammatory response and protection in vascular permeability.

Keywords: heat stroke, human pulmonary microvascular endothelial cells, glycocalyx

Abbreviations

Acute Lung Injury, ALI; Adult Respiratory Distress Syndrome, ARDS; Control, CON; Disseminated Intravascular Coagulation, DIC; Endothelin-1, ET-1; Endothelial Cell Medium, ECM; Enzyme-Linked Immune

Sorbent Assay, ELISA; heat stroke, HS; Human Pulmonary Microvascular Endothelial Cells, HPMEC; Heparan Sulfate Proteoglycan, HSPG; Heparanase, HPSE; Intercellular Adhesion Molecules, ICAM; Interleukin-6, IL-6; Lipopolysaccharide, LPS; Multiple Organ Dysfunction Syndrome, MODS; Reactive Oxygen Species, ROS; Systemic Inflammatory Response Syndrome, SIRS; Syndecans-1, SDC-1; Tumor Necrosis Factor-α, TNF - α· Unfractionated Heparin, UFH; Vascular Cell Adhesion Molecule-1, VCAM-1; Von Willebrand factor, vWF

1 Introduction

Heat stroke is identified as multi-organ dysfunction syndrome (MODS) secondary to systemic inflammatory response (SIRS) triggered by hyperthermia,[1, 2] with mortality reached 63.2% under intensive care. [3] Recent studies indicate that the host inflammatory response and coagulatory disorder secondary to heat stroke are associated with MODS. [4] A large number of proinflammatory factors and cytokines are released as a result of the excessive activation of leukocytes and endothelial cells, which led to continuous and intense inflammatory injury. [5, 6] On the other hand, microcirculation of tissues and organs secondary to vascular endothelial injury contributed to ischemia, hypoxia and metabolic disorders of tissues and organs, and further worsen and exacerbated inflammatory response. [7, 8] Taken together, it is suggested that vascular endothelial injury, circulatory dysfunction and SIRS are closely related, which drives severe heat stroke to MODS development.

Endothelial glycocalyx, an important structure of vascular endothelial surface, maintained the structural and functional stability of endothelium effectively and prevented the adhesion of inflammatory cells. [9] Imbalance between synthesis and degradation of glycocalyx caused structural and functional damages, and further aggravated vascular endothelial and tissue injury via mediating adhesion between inflammatory and endothelial cells. Shreds of evidence have demonstrated that vascular endothelium dysfunction induced by glycocalyx injury was the key factor that magnified the inflammatory response in sepsis and promoted SIRS to MODS. [10, 11] Additionally, glycocalyx is known to induce acute lung injury (ALI) or adult respiratory distress syndrome via accelerating leukocyte adhesion.[12, 13]

Hyperthermia may facilitate the leakage of lipopolysaccharide (LPS) from the intestine to the systemic circulation early, which excessively activates endothelial cells to exaggerate the inflammatory and coagulation responses. [1] Our previous studies suggested that mesenteric lymph played critical roles in the pathogenesis of endothelium injury in heats stroke rats.[7] Several evidence indicated that high LPS levels were investigated in the portal vein and systemic circulation in patients with heat stroke and animal models. [14] Collectively, in the present study, Human pulmonary microvascular endothelial cell (HPMEC) was explored to observe the injury characteristic of vascular endothelial glycocalyx under heat stress combined with LPS double hits*in vitro* .

2 Materials and methods

2.1 Preparation of HPMEC

HPMEC was purchased from Guangzhou Dewei Biological Technology Co. Ltd, China. HPMEC was cultured in the endothelial cell medium with 10% fetal bovine serum (ScienCell, America) at 37 °C in a humidified incubator with 5% CO_2. All experiments were carried out during the logarithmic phase of cell growth. The account of cells was adjusted to $1x10^6$ /ml by serum-free medium before culture, inoculating into six-well plates, and incubated at 5% CO_2 and 37 °C.

2.2 Induction of the HPMEC under heat stress combined with LPS in vitro

Pretreatment before molding Heat stress and lipopolysaccharide (HS+LPS) were employed to construct the *in vitro* models of heat stroke in our previous study. [15]. All the HPMEC were categorized into 4 groups with 8 wells in each group: control group (CON group), heat stroke group (HS group), heparanase group (HPSE group), and unfractionated heparin group (UFH group). HPSE was known to aggravate endothelial glycocalyx degradation as the only endonuclease.[16, 17] UFH was known to attenuate glycocalyx degradation via non-covalent binding with glycocalyx.[13] After conventional culture for 72 hours, the cell in the CON

group was cultured in normal conditions, without receiving heat stress and LPS (Sigma, America). No pretreatment was performed on the HS group before modeling. The cell in the HPSE group was subjected to a 0.2 U/ ml Heparanase (Sigma, America) culture medium and incubated at 37 °C for 30 minutes. The UFH group was subjected to a medium containing 10 U/ ml unfractionated heparin (Sigma, America) and incubated 12 hours before modeling. [18, 1920]

Modeling Vitro Briefly, except for CON group, HPMEC in HS, HPSE, and UFH groups were subjected to heat stress at 43 °C for 30 minutes, and then moved to a 5% CO_2, 37 °C incubators, stimulated with LPS at a concentration of 500 ng/ml for 12 hours.

2.3 Detection of changes of endothelial glycocalyx structure and functional activity

Flow cytometry To investigate HPMEC apoptosis and heparansulfate proteoglycan (HSPG) concentration, flow cytometry was performed in each group according to our previous methods. [15] Cells in each group were washed with PBS for 15 minutes, centrifuged, and resuspended with 500 μL AnnexinV-FITC (UElandy, China) binding buffer per tube. The cells were further stained with 5 μL AnnexinV-FITC, then mixed and incubated in the dark at 4 °C for 15 minutes. Lastly, 10 μL PI staining solution was added, and the flow cytometry (Becton, Dickinson and Company, American) was performed to detect after incubated in the dark for 5 min. Data compensation and analysis was performed using FlowJo version 7.6.1.

Measurements of reactive oxygen species (ROS) To examine the intracellular ROS levels, a ROS assay kit that sets DCFH-DA as the probe was used. HPMEC supernatant was withdrawn after modeling from each group, and DCFH-DA was diluted to 10 μmol/L by 1:1000 in a serum-free blank medium. Cells were stained with 2, 7-dichloride-hydrofluorescein diacetate (DCFH-DA, Nanjing Jiancheng, China) in a 37 cell incubator for 20 minutes. The cells were washed 3 times with a blank medium, observed and photographed by Olympus positive fluorescence system microscope (Olympus Corporation, Japan), and analyzed by Image J software.

Immunofluorescent Staining Cells from each group were washed with PBS for 3 minutes, permeabilized in precooled 0.25% Triton X-100 solution for 10 minutes on ice, and then were blocked in 1% bovine serum albumin (BSA, Sigma, American) for 1 h. Furthermore, rabbit anti-HSPG (Affinity Biosciences, American) was majorly used, which was diluted in PBS containing 1% BSA. Primary antibody incubation was performed at 4 °C for 2 h and washed with PBS; secondary antibody incubation was performed in the dark at room temperature for 1 h. Finally, the cells were incubated with DAPI in the dark for 5 minutes and then were observed in a fluorescence microscope.

Enzyme-linked immunosorbent assay test The level of major degraded products of endothelial glycocalyx, including Syndecans-1 (SDC-1) and heparin sulfate (HS), was measured by enzyme-linked immunosorbent assay (ELISA) based on an established procedure. Moreover, ELISA was also performed to detect the concentrations of Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6), Von Willebrand factor (vWF), and Endothelin-1 (ET-1).

Western blot The expression level of E-selectin, VCAM-1, and Occludin in HPMEC were investigated via western blot. Cells were collected and lysed in RIPA lysis buffer (LEAGENE, Beijing, China) which added with PMSF for 30 minutes on ice. The protein samples were determined using BCA Protein Assay Kit (Cat#: 23227, Pierce, USA), separated on 12% sulfate-polyacrylamide gel electrophoresis and were then transferred to PVDF membranes (BIO-RAD, American). The membranes were further blocked with 5% nonfat dry milk with 100 ml Tris-buffered saline with 0.05% Tween 20 (TBST) at room temperature and incubated with rabbit anti-E-selectin (SAB, American), rabbit anti-Occludin (SAB, American), rabbit anti-VCAM1 (SAB, American) primary antibodies at 4 °C overnight. TBST was used for rinsing for 10 minutes and shaking at room temperatures. Peroxidase-conjugated Affinipure Goat Anti-rabbit IgG/FITC and Goat Anti-Mouse IgG/PE were used for incubating in the dark at room temperatures for 1 h. The immunoblots were investigated by enhanced chemiluminescence (ECL), and the protein expression levels were analyzed by Image J software.

2.4 Detection of HPMEC ultrastructure, function and cell activity

Transmission electron microscopy (TEM) Cells were fixed in 1% osmium tetroxide (Sinopharm Chemical Reagent Co., Ltd. China) for 2 h, evaporated with a scalar series of acetone washes, infiltrated with propylene epoxied, and embedded in Epon 812. Further, 60–80-nm-thick sections were performed, and uranium acetate saturated with alcohol solution and lead citrates were dying respectively for 15 minutes. TEM (HT7700, HITACHI) was used to observe and image ultrastructures of HPMEC.

2.5 Statistical analysis

All statistical analysis of the data was analyzed by SPSS 27.0 and GraphPad Prism 9. All the data were expressed as mean ± standard deviation (mean ± SD). If the variance is uniform, single-factor descriptive analysis of variance (ANOVA) and least significant difference (LSD) methods were used to analyze comparisons among multiple groups. If not, the Welch test was used. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Heat stress and LPS induced damage and degradation of endothelial glycocalyx

Glycocalyx content Given previous study suggesting the importance of HSPG in accessing the content of endothelial glycocalyx,[21] HPSG expression was firstly investigated in HPMEC among 4 groups under heat stress and LPS. Stimulation of heat stress and LPS decreased HSPG expression compared with those in CON group ($P< 0.05$) (**Figure 1A˜C**), as indicated by immunofluorescent staining and flow cytometry. Interestingly, HSPG expression was higher in HSPG group whereas lower in UFH group than that in HS+LPS group ($P < 0.05$) (**Figure 1A˜C**). These results indicated that heat stroke decreased the content of endothelial glycocalyx, which glycocalyx damage was attenuated by UFH.

Levels of glycocalyx degradation products Considering that SDC-1 and heparin sulfate were identified as the classical degraded products of endothelial glycocalyx, [22] we, therefore, evaluated the expression of them. The results suggested that the expressions of SDC-1 and heparin sulfate were significantly lower in CON group than that in others ($P < 0.05$) (**Figure 1D**). Interestingly, UFH may prevent endothelial glycocalyx from degrading as evidenced by the lower expression in UFH group compared with HS+LPS and HPSE groups ($P < 0.05$). These results revealed that heat stress and LPS may induce endothelial glycocalyx degradation in HPMEC, which showed a positive correlation with the degree of glycocalyx damage.

3.2 Heat stress and LPS triggered HPMEC apoptosis via activating endothelial glycocalyx degradation

Changes in ultrastructure and apoptosis of HPMEC The ultrastructure of HPMEC was next observed to estimate the effects of heat stress and LPS om HPMEC apoptosis (**Figure 2A**). Normal cellular structures were observed in CON group. In model groups, cells tended to generate characteristics of apoptosis such as swollen, broken membranes, abnormal organelles including nucleus, mitochondria and endoplasmic reticulum (**Figure 2A**). Cells assigned to HPSE were more obvious but cells assigned to UFH were milder than HS+LPS group (**Figure 2A**). These results suggested that an altered structure in HPMEC as a result of stimulation of heat stress and LPS.

Evidence suggested that heat stress may lead to cellular apoptosis,[23] we next analyzed HPMEC activities through flow cytometry using Annexin V-FITC/PI staining. As expected, assignment of HPMEC with heat stress and LPS increased apoptotic rates in both HPSE and UFH treatment, compared with CON group ($P < 0.05$) (**Figure 2B,C**). This increase is more pronounced in HPSE group ($P < 0.05$) (**Figure 2B,C**), suggesting potential effects of damaged glycocalyx on HPMEC apoptosis.

3.3 Heat stress and LPS activated vWF, ET-1, E-selectin, VCAM-1, and Occludin released in HPMEC

To further investigate the effect of heat stroke on the function of coagulation factors, the expression of vWF and ET-1 was assessed under stimulation of heat stress and LPS. Exposure to stimulation of heat stress and LPS resulted in a statistically significant increase in the expression of vWF and ET-1 ($P < 0.05$) (**Figure**

3A). HPSE treatment significantly increased generation of vWF and ET-1, but The generation of vWF and ET-1 was significantly increased by HPSE treatment but was reduced by UFH treatment in comparison with HS+LPS treatment alone. These results indicated that heat stress and LPS directly affect glycocalyx degradation as a potential mechanism to induce clotting state in HPMEC.

Given previous studies indicating the importance of VCAM-1 and E-selectin in forecasting cellular inflammation [24] and the critical role of Occludin in preventing endothelial leakage,[25, 26] in this study, we also aimed to examine the expression of VCAM-1, E-selectin, and Occludin. Our results showed that heat stress and LPS significantly enhanced the expression of VCAM-1 and E-selectin but attenuated Occludin expression in comparison with the CON group ($P < 0.05$) (**Figure 3B,C**). Compared with HS+LPS treatment alone, HPSE treatment significantly upregulated the production of VCAM-1 and E-selectin, but downregulated Occludin expression. This data suggested that heat stress and LPS destructed the tight junctions between endothelial cells and aggravated endothelial leakage in HPMEC.

3.4 Degradation of endothelial glycocalyx potentially activated oxidative stress injuries in HPMEC

TNF-α and IL-6 were the classical pro-inflammation cytokines to accessing inflammatory activities and prognostic outcomes. We, therefore, evaluated the expression of TNF-α and IL-6 in treated cells. Our results found that heat stress and LPS significantly increased the production of TNF-α and IL-6 in HS, HPSE, and UFH groups, compared with the CON group ($P < 0.05$) (**Figure 4A**). This upregulation was more pronounced in cells treated with HPSE but more modest in those treated with UFH in comparison with those treated with heat stress and LPS alone ($P < 0.05$) (**Figure 4A**). These results indicated that heat stress and LPS promoted inflammatory activities of HPMEC by an endothelial glycocalyx degraded mechanism.

Our previous study demonstrated that generation of ROS was the critical mediator in heat stress-induced apoptosis, [27] which was also known to be a classical marker related to oxidative stress in the tissue. We, thus, detected the changes of ROS level in HPMEC by DCFH-DA marked with green fluorescence, which produces enhanced fluorescence when cells generate ROS. Heat stress and LPS significantly increased the intracellular ROS levels in HPMEC in comparison with the CON group (**Figure 4B**) ($P < 0.05$). In addition, the ROS level was significantly higher in HPSE treated-cells but was lower in UFH treated-cells than HS+LPS-treated cells ($P< 0.05$) (**Figure 4B**). These results suggested that oxidative stress of HPMEC as a result of damaged endothelial glycocalyx induced by heat stroke.

4 Discussions

In this study, the features of vascular endothelial glycocalyx under stimulation of heat stress and LPS corroborated vascular inflammatory injury and coagulation disorder. For the first time, the study showed that heat stroke triggered endothelial glycocalyx degradation and further induced oxidative damage and apoptosis of HPMEC, which impaired its ability of resisting inflammatory injury and maintaining vascular permeability.

HSPG was a major element of glycocalyx, which played important roles in endothelial cell homeostatic signal based on their unique structures and interactions with both the intra- and extracellular environments. Decreased HSPG prompted endothelial glycocalyx degradation and vascular disease. [21] In this study, we detected lower HSPG level in HPMEC under heat stress and LPS compared with controls (**Figure 1A˜C**). Heparn sulfate (HS) and syndecans-1 (SDC-1) were principal components of glycocalyx which documented the erosion of glycocalyx. [28] Previous study reported that increased blood levels of SDC-1 as a signal for glycocalyx degradation. [29] In the present study, upregulated supernatant levels of SDC-1 and HS(**Figure 1D**) derived from cells treated with heat stress and LPS were found, which suggested that glycocalyx was possibly degraded in heat stroke. Interestingly, these changes were more obviously in cells treated with HPSE and UFH, which suggested that greater damage to the glycocalyx resulted in more severe degradation.

Earlier studies demonstrated that heat stress contributed to injury and apoptosis of endothelial cells by

an activation of inflammation, which played an important role in organ injury secondary to heat stroke.[30] Given this timeline, our results showed that heat stress and LPS induced endothelial cells apoptosis morphologically, which were more significantly in HPSE-treated cells (**Figure 2**). A recent study found that endothelium apoptosis may be induced and aggravated by endothelial glycocalyx injury[31]. Collectively, our results suggested that the activation of endothelium apoptosis may be highly associated with endothelial glycocalyx damage as a result of heat stroke.

Inflammation and coagulation played a critical role in pathophysiological basis of tissues/ organs damage secondary to heat stroke. [32] Moreover, abnormal structure and function of endothelium contributed to exacerbate inflammation through increasing vascular permeability and promoting adhesion of inflammatory cells, as well as producing massive inflammatory mediators and procoagulants. However, the development of inflammation and coagulation after heat stroke-induced glycocalyx injury was still to be elucidated. Given this association, it will be essential to explore the relationship between inflammation and glycocalyx damage in addition to endothelium.

TNF-α was known to promote inflammation via upregulating the expression of adhesion factors on the surface of endothelium, such as VCAM-1 and ICAM-1. [33] IL-6 played a promoted role in development of inflammation, which was related to intracellular cascade signaling transduction induced by binding to specific receptors, and further magnified the effects of TNF-α. [34, 35] Moreover, IL-6 played strong roles in promoting inflammation in the pathogenesis of MODS secondary to heat stroke. [35] vWF and ET-1 were associated with the function of promoting coagulation.[36] Endothelium was effective in constricting blood vessels and reducing the leakage via releasing ET-1.[36] In addition, endothelium damage was mediated by inflammatory factors which contributed to enlarging endothelial space and further aggravating inflammatory response. [37]These may explain why the expression of TNF-α, IL-6, vWF and ET-1 showed increased actions in glycocalyx injury in combination stimulation of heat stress and LPS, typically in HPSE-treated cells (**Figure 3**). Taken together, these results might imply that heat stroke-induced glycocalyx degradation enhanced the activation of inflammation and coagulation.

E-selectin and VCAM-1 were the important symbols to reflect injury of endothelial glycocalyx and endothelium, which also played critical roles in the spread of inflammation via mediating the adhesion between leukocyte and inflammatory cells. [24, 38, 39] In our results, increased expressions of VCAM-1 and E-selectin were significantly observed in endothelium treated with heat stress and LPS, especially in cells suffered from HPSE (**Figure 3C**). These findings were consistent with those revealed a significant upregulation of E-selectin in injured vascular endothelial glycocalyx,[40, 41], which confirmed a dysfunction of endothelial cells inhibiting in inflammation. Occludin was effective in preventing endothelium from leakage, localized in the tight junctions between cellular preferentially. [25, 26] In the present study, western blot analysis revealed that the expression of Occludin was aggravated by injured endothelial glycocalyx in HPMEC (**Figure 3B** ˜C). These data suggested that heat stroke possibly triggered endothelial glycocalyx damage, which resulted in endothelial tight junction injury. Decreased Occludin may be associated with widening gaps between endothelial cells, which aggravated the vascular leakage possibly. Our data indicated that heat stroke-induced glycocalyx damage enhanced adhesion proteins expression, and further led to pathophysiological changes including increased vascular permeability and leukocyte adhesion. [42] It is consequently hypothesized that endothelial glycocalyx damage may be th key pathophysiological basic of dysfunction of endothelium and inflammatory damages induced by heat stroke.

ROS was widely involved in signaling transduction and life process of cells, however, excessive ROS may lead to diseases via inducing oxidative stress in mitochondria. [43] Further studies showed that under heat stroke, increased inflammatory factors mediated iNOS to generate NO, and further generated excessive ROS, which was associated with heat stroke-induced ALI. [44] In the present study, the relationship between intracellular oxidative stress and heat stroke was confirmed by an overproduction of ROS under heat stress, which may be associated with the extent of severity of glycocalyx damage (**Figure 4B**). Our results suggested that heat stroke contributed to severe oxidative stress injury of vascular endothelial cells, which might be activated and aggravated by glycocalyx degradation.

5 Conclusions

Heat stroke possibly triggered glycocalyx degradation and induced oxidative damage and apoptosis in HPMEC, as well as leading to dysfunction that inhibited inflammation and maintained vascular permeability. Future studies are needed to explore the potential signal pathways and mechanism of dysfunction of vascular endothelium induced by endothelial glycocalyx degradation under heat stroke.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. Heat stroke decreased the expression of HSPG and concentration of SDC-1 and HS in HPMEC. (A). Representative immunofluorescence images of HSPG in glycocalyx from each groups at magnification × 100. Green indicated HSPG, and blue indicated nuclear. (B-C). HSPG concentration in each group was detected using flow cytometry. (D). Concentration of SDC-1 and HS in cell culture supernatant in HPMEC from each group. $^{*}P< 0.05$ versus CON, $^{\#}P < 0.05$ versus HS; n = 8.

Figure 2. Heat stroke induced cells apoptosis in HPMEC. (A) Representative morphological images of distinct groups in HPMEC using TEM. (B) The apoptotic rates in each group were measured by flow cytometry. (C). Quantitative analysis of the apoptotic rates in distinct groups. $^{*}P < 0.05$ versus CON,$^{\#}P < 0.05$ versus HS; n = 8.

Figure 3. Heat stroke changed expression of coagulation enhancers (vWF and ET-1), adhesion proteins, and tight proteins. (A). Concentration of vWF and ET-1 were performed using ELISA. (B). Western blot were performed to detect expression of E-selectin, VCAM-1, and occludin, and quantification of the proteins. The data are presented as mean values ± SEM for independent experiments.$^{*}P < 0.05$ versus CON,$^{\#}P < 0.05$ versus HS; n = 8.

Figure 4. Heat stroke exacerbated the inflammatory state and oxidative stress levels. (A). ELISA was performed to detect concentration of TNF and IL-6. (B). The level of intracellular ROS was measured with a DCFH-DA. $^{*}P < 0.05$ versus CON, $^{\#}P < 0.05$ versus HS; n = 8.