Extracellular histones induce inflammation and senescence of vascular smooth muscle cells by activating the AMPK/FOXO4 signaling pathway

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Received: 25 March 2022 / Revised: 25 March 2022 / Accepted: 18 July 2022 / Published online: 1 August 2022
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Abstract
Background Sepsis is an abnormal immune-inflammatory response that is mainly caused by infection. It can lead to life-threatening organ dysfunction and death. Severely damaged tissue cells will release intracellular histones into the circulation as damage-related molecular patterns (DAMPs) to accelerate the systemic immune response. Although various histone-related cytotoxicity mechanisms have been explored, those that affect extracellular histones involved in vascular smooth muscle cell (VSMC) dysfunction are yet to be determined.

Methods Mouse aortic vascular smooth muscle cells (VSMCs) were stimulated with different concentrations of histones, and cell viability was detected by CCK-8 assay. Cellular senescence was assessed by SA β-gal staining. C57BL/6 mice were treated with histones with or without BML-275 treatment. RT-qPCR was performed to determine the expression of inflammatory cytokines. Western blotting was used to analyze the expression of NLRP3, ASC and caspase-1 inflammasome proteins. The interaction of NLRP3 and ASC was detected by CoIP and immunofluorescence staining.

Results In this study, we found that extracellular histones induced senescence and inflammatory response in a dose-dependent manner in cultured VSMCs. Histone treatment significantly promoted apoptosis-associated speck-like protein containing CARD (ASC) as well as NACHT, LRR and PYD domains-containing protein 3 (NLRP3) interaction of inflammasomes in VSMCs. Forkhead box protein O4 (FOXO4), which is a downstream effector molecule of extracellular histones, was found to be involved in histone-regulated VSMC inflammatory response and senescence. Furthermore, the 5′-AMP-activated protein kinase (AMPK) signaling pathway was confirmed to mediate extracellular histone-induced FOXO4 expression, and blocking this signaling pathway with an inhibitor can suppress vascular inflammation induced by extracellular histones in vivo and in vitro.

Conclusion Extracellular histones induce inflammation and senescence in VSMCs, and blocking the AMPK/FOXO4 pathway is a potential target for the treatment of histonemediated organ injury.

Keywords Inflammatory response · Senescence · VSMC · Extracellular histones · Organ injury

Abbreviations
ASC Apoptosis-associated speck-like protein containing CARD
NLRP3 NACHT, LRR and PYD domains-containing protein 3
FOXO4 Forkhead box protein O4
LPS Lipopolysaccharide
AMPK 5′-AMP-activated protein kinase
VSMC Vascular smooth muscle cell
**Introduction**

Sepsis is described as an abnormal immune-inflammatory response that is caused primarily by infection. This condition can lead to life-threatening multiple organ dysfunction syndrome and death [1]. More than 31.5 million people worldwide are threatened with sepsis annually; of them, more than 5.3 million die [2]. In China, more than 20% of patients in the intensive care unit (ICU) develop sepsis, and the mortality rate among these patients is as high as 36% [3]. Despite tremendous efforts made for decades, there is no specific treatment for sepsis [4]. Traditional therapy often ignores the immunopathological nature of sepsis, making it difficult to improve the survival of patients with severe sepsis and septic shock [5]. Vascular smooth muscle cells (VSMCs) are present in the media layer of blood vessels and regulate the tension and contraction of blood vessels [6]. Unlike vascular endothelial cells, the role of VSMCs in sepsis is often overlooked [7]. The increased permeability of endothelial cells in sepsis allows direct contact of VSMCs with inflammatory mediators in the blood, which could lead to disruption of the autoregulation of normal blood vessels [8]. Vascular dysregulation and the toxicity of inflammatory mediators may be important factors for organ dysfunction [9]. Therefore, controlling the dysregulated immune response and balancing the function of VSMCs should be considered to reduce the incidence of death due to sepsis.

Histones are highly cationic nuclear proteins that are mainly present in the nucleus and participate in chromatin assembly and regulation of gene expression [10]. When cells are exposed to harsh conditions, histones are released into the circulation and serve as damage-related molecular patterns [11]. Circulating histones interact with phospholipids in the cell membrane to mediate distant organ damage [12]. Histones also mediate inflammation, organ damage, and death by activating the Toll-like receptor (TLR) and NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome pathways [13–15]. Emerging research has shown that extracellular histones play a role in multiple organ damage and death in sepsis [16]. Shi et al. found that lipopolysaccharide (LPS)-induced extracellular histones can cause septic pyrolysis via the NOD2 and VSG4/NLRP3 pathways [17]. Extracellular histones were found to induce autophagy and apoptosis of human endothelial cells via the mTOR signaling pathway [13]. Circulating histones can cause cerebrovascular damage or brain dysfunction by altering the blood–brain barrier [18]. Although VSMCs play a key role in sepsis [19, 20], the effect of extracellular histones on VSMCs in organ injury remains unclear.

Previous research found that extracellular histones are cytotoxic to endothelial cells [13]. However, if extracellular histones are also cytotoxic to VSMCs, they may lead to cell responses that might worsen the disease. Thus, elucidating the mechanism underlying histone-mediated cytotoxicity could aid in understanding the complex pathogenesis of organ injury. In this study, we found that extracellular histones facilitate VSMC senescence and inflammation in a dose-dependent manner. In addition, Forkhead box protein O4 (FOXO4), a downstream histone regulator, was found to be involved in histone-regulated VSMC inflammation and senescence. Mechanically, the AMPK signaling pathway mediates extracellular histone-induced FOXO4 expression. Targeting AMPK/FOXO4 might be a potential method for treating histone-mediated organ injury.

**Results**

**Extracellular histones facilitate VSMC senescence and inflammation**

A previous study reported that extracellular histones induce apoptosis of human endothelial cells [13]. However, the damage caused by extracellular histones in VSMCs remains unclear. In this study, VSMCs were treated with various concentrations of extracellular histones, and cell viability was examined using flow cytometry. As shown in Fig. 1a, cell viability was hardly reduced at 25 μg/mL; however, the reduction in the number of cells was more pronounced when the cells were incubated with 50–100 μg/mL histones. However, the number of cells incubated with 150 μg/mL histones did not change significantly compared with that incubated with 100 μg/mL histones. After 6 h of treatment with 100 μg/mL histones, the number of cells began to significantly reduce, which was not significantly different from that at 12 and 24 h (Fig. 1B). To examine whether histones facilitate VSMC senescence, we performed SA β-gal staining. The results showed that as the concentration of histones increased, the number of SA β-gal-positive cells also increased (Fig. 1c, d). Western blotting analysis showed similar results through assessment of senescence marker genes (Fig. 1e, f). Next, we investigated the expressions of inflammatory cytokines after VSMC treatment with varying concentrations of extracellular histones. As expected, histone treatment significantly increased the mRNA expressions of IL-β, TNF-α, and IL-18 in a dose-dependent manner (Fig. 1G–I). These data suggest a function of extracellular histones in VSMC senescence and inflammation in organ injury.

**Extracellular histones promote inflammasome assembly**

To explore how extracellular histones exert their functions, the expression of inflammasome molecules in VSMCs...
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As indicated in Fig. 2a, histone treatment markedly elevated NLRP3, apoptosis associated speck-like protein containing CARD (ASC), and caspase-1 protein levels in VSMCs. RT-qPCR showed the same results (Fig. 2c). Next, we performed double immunofluorescence staining and found that as the number of histones increased, ASC and NLRP3 expression increased and were co-located in the cytoplasm (Fig. 2d, e). To investigate whether histones affect inflammasome assembly, co-immunoprecipitation (CoIP) assay was performed. The results indicated that histone treatment significantly increased the interaction of ASC and NLRP3 in VSMCs (Fig. 2f). Collectively, these data support the role of histones in inflammasome assembly regulation.

**FOXO4 is a downstream regulator of histone-treated VSMC**

To investigate how histones regulate inflammation and senescence, we partly examined candidate genes reported with abnormal expression in organ injury [21, 22]. As indicated in Fig. 3a, histone treatment significantly increased the expression of inflammatory cytokines IL-1β, TNF-α, and IL-18. For a, b, d, and f-i, data are from three independent experiments; mean ± SEM; Student’s t test, *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding control.
increased FOXO4, NR1H4, and HOXA9 expression and reduced HMGB1 expression in VSMCs. Next, FOXO4 expression at different concentrations of histone cell treatment was confirmed. The results showed that the mRNA and protein levels of FOXO4 dose-dependently increased (Fig. 3b–d). Consistent with this, immunofluorescence staining indicated similar results (Fig. 3e). Besides, p21 expression increased with FOXO4 in histone-treated VSMCs (Fig. 3e, f). These data suggest that FOXO4 is a downstream effector molecule of histones and may participate in VSMC senescence.
FOXO4 is involved in the histone-induced VSMC inflammatory response and senescence

To study the function of FOXO4 in VSMCs, FOXO4 was knocked down with two shRNAs and effect of the knockdown was confirmed. As indicated in Fig. 4a–c, transfection of shFOXO4-1# or shFOXO4-2# significantly reduced mRNA and protein levels. Next, FOXO4 was knocked down and then treated with histones in VSMCs. We found that histone treatment markedly promoted an SA β-gal-positive cell number, while FOXO4 depletion simultaneously reversed these effects (Fig. 4d, e). In parallel, FOXO4 deletion significantly suppressed the histone-induced promotion of inflammatory cytokine expression in VSMCs (Fig. 4f, g). Collectively, these data establish that FOXO4 regulates histone-induced VSMC inflammatory response and senescence.

The AMPK signaling pathway mediates extracellular histone-upregulated FOXO4 expression

To identify which signaling pathway may regulate FOXO4 expression by extracellular histones, VSMCs were treated with and without histones. Western blotting was used to examine the molecular expression of the signal pathway. The results indicated that histones decreased the protein levels of p-AKT, p-Rb1, and p-mTOR but increased AMPK and
p-AMPK expression (Fig. 5a, b). A previous study reported that Rb-1 was an upstream inhibitor of FOXO4 [23]. Next, an AKT (LY294002) and AMPK (BML-275) inhibitor was used to stimulate histone-treated VSMCs and confirmed that BML-275 could inhibit histone-promoted NLRP3, p21, and FOXO4 expression and increase histone-suppressed p-Rb1 expression (Fig. 5c, d). Furthermore, the expression of inflammatory cytokines in BML-275-treated VSMCs has been examined after shFOXO4 transfection. It was found that FOXO4 depletion significantly downregulated IL-β, andTNF-α expression in VSMCs, while simultaneous BML-275 treatment has further enhanced this effect (Fig. 5e).

Together, these data showed that the AMPK signal pathway is involved in histone-regulated FOXO4 expression and could be a vital regulator in histone-mediated organ injury.
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Blocking the AMPK signal pathway inhibits vascular inflammation induced by extracellular histones in vitro

To examine the effect of extracellular histones on VSMCs in vivo, mice were treated with histones while giving BML-275 treatment or not. The results showed that histone treatment markedly upregulated ACS and NLRP3 expression in the layer of VSMCs. However, the BML-275 treatment significantly reversed these effects (Fig. 6a, b). In parallel, extracellular histones increased the expression of the inflammatory cytokines IL-β, and TNF-α in VSMCs in vitro, while BML-275 treatment simultaneously depressed their expression (Fig. 6c). To study whether BML-275 has a beneficial effect on decreasing histone-induced organ damage, the levels of cardiac troponin I (cTnl), alanine aminotransferase (ALT), and blood urea nitrogen (BUN) in the serum of mouse models were tested. As indicated in Supplementary Fig. 1, histones-treated mice significantly increase ALT, BUN and cTnl levels in serum. However, BML-275 treatment markedly reduces these protein levels in histones-induced mice. Additionally, double immunofluorescence staining showed that extracellular histones elevated FOXO4 and p21 expression in VSMCs in vitro, but blocking the AMPK signal pathway with BML-275 reversed the expression of these genes (Fig. 6d, e). Additionally, extracellular histones significantly promoted senescence relative marker gene p16, p21, and p53 expression, while BML-275 treatment decreased this promotion of histones (Fig. 6f). To examine whether histones or histones + BML-275 treatment in vivo affected the AMPK/FOXO4 pathway, we detected these proteins level by Western blotting. As indicated in Supplementary Fig. 2, the expression of p-AMPK and FOXO4 was significantly elevated in histones-treated vascular tissue. However, p-AMPK and FOXO4 protein levels were depressed while BML-275 treatment simultaneously. Together,
these results showed that extracellular histones significantly promote inflammation and senescence in VSMCs, and blocking the AMPK signaling pathway by BML-275 would partly reverse these effects.

Discussion

In this study, we explored the role of extracellular histones in regulating the senescence and inflammation of VSMCs via the AMPK/FOXO4 axis. We found that extracellular histones induced senescence and the inflammatory response of VSMCs in a dose-dependent manner. We
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also found that FOXO4, which is a downstream effector molecule of extracellular histones, is involved in histone-regulated VSMC inflammatory response and senescence. Furthermore, the AMPK signaling pathway was found to mediate extracellular histone-induced FOXO4 expression. Disruption of the AMPK signaling pathway by inhibitors obstructed extracellular histone-induced vascular inflammation in vivo and in vitro (Fig. 7).

Endothelial cells play an important role in vascular dysfunction associated with sepsis [24, 25]. However, increasing evidence has demonstrated that VSMCs are involved in sepsis in a manner that is independent of endothelial cells [26, 27]. Because VSMCs are not in direct contact with the bloodstream, it appears that sepsis damages VSMCs after the improvement of acute illness. In the early and late stages of sepsis, the contractile function of VSMCs is impaired [28]. This shows that VSMCs might be involved in the entire sepsis process. Macrophages treated with LPS release a large number of extracellular histones that interact with target cell receptors (especially TLR4) to promote inflammation [29]. In sepsis, alterations in the normal autoregulation of perfusion and the toxic effects of the media can lead to severe organ dysfunction [7]. Although VSMCs have been shown to play a key role in sepsis [19, 20], the effect of extracellular histones on VSMCs in sepsis is unclear. In this study, we found that extracellular histones induced the senescence and inflammatory response of VSMCs in a dose-dependent manner. FOXO4 is involved in histone-regulated VSMC inflammatory response and senescence. Blocking of the AMPK signaling pathway by inhibitors altered extracellular histones-induced vascular inflammation. We found that histones significantly activated the inflammatory response of VSMCs. Previous studies reported that extracellular histones target TLR2, 4, and 9 in various cell types and activate cellular inflammation and cell damage [30–32]. For example, histones cause glomerular cell damage by activating TLR2 and 4 [33] and hepatic reperfusion injury by activating TLR9 [34]. TLRs are a vital inflammatory response pathway. Additionally, several studies have indicated that TLRs are closely related to AMPK signaling pathways [35–38]. Therefore, we speculated that TLRs are potential receptors for histones and activate the AMPK signaling pathway.

The FOXO family of proteins comprises a series of transcription factors, including FOXO1, FOXO3a, FOXO4, and FOXO6 [39]. According to upstream and downstream gene regulation, FOXO4 can be used as a transcriptional activator and repressor [40, 41]. Several studies have shown that FOXO4 is involved in the regulation of various processes, including cell proliferation, apoptosis, autophagy, cell senescence, inflammation, and energy production [42–46]. Zhang et al. found that GUARDIN serves as a scaffold to stabilize the LRP130/PGC1α heterodimer to promote FOXO4 expression and upregulate the expression of the target gene p21, causing cell senescence [47]. The activation of FOXO4 in melanoma promotes the transcription of p21 and subsequently accelerates cell senescence [48]. Using FOXO4-knockout mice, Zhu et al. found that FoxO4 promotes early inflammatory response in myocardial infarction by regulating Arg1 expression [45]. Blocking the interaction between XBP1u and FoxO4 promoted the nuclear translocation of FoxO4, promoted in vitro proinflammatory activity, and stimulated the formation of aortic aneurysms [49]. In the present study, extracellular histones promoted FOXO4 expression, which is then involved in the histone-regulated VSMC inflammatory response and senescence. Deletion of FOXO4 suppressed the promoting effect of histones on inflammatory cytokine expression and SA β-gal-positive cells in VSMCs.

In conclusion, extracellular histones damaged VSMCs in vivo and in vitro. Extracellular histones induced an
inflammatory response and senescence of VSMCs. FOXO4 expression was mediated by the AMPK signaling pathway in histone-treated VSMCs. Deleting FOXO4 or blocking the AMPK signaling pathway could relieve the extracellular histone-induced inflammatory response and senescence of VSMCs. AMPK/FOXO4 might be potential targets in the treatment of histone-mediated organ injury.

Materials and methods

Cell culture and treatment

Mouse aortic vascular smooth muscle cells (ATCC, No.CRL-2797TM) were routinely cultured in low-glucose Dulbecco’s modified Eagle’s medium (Gibco Life Technologies, Rockville, MD) containing 100 units/ml of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (Gibco) in a humidified incubator at 37 °C with 5% CO2. The cells from passages 3 to 6 were used in all studies. The cells were maintained in 5% CO2 at 37 °C within a humidified atmosphere, and their morphology and α-SMA expression were evaluated. When the cells attained 80% confluence, the media was replaced, and the cells were exposed for 6 h to various concentrations of calf thymus histone (10, 25, 50, or 100 μg/mL prepared in PBS; pH 7.4 [Gibco]) [50]. LY294002 (Selleck) was used as a PI3K/AKT inhibitor, as previously described [51]. The AMPK inhibitor BML-275 (4 μM; Selleck) was used in this study [52].

Western blotting

According to a previous description [4], radioimmunoprecipitation assay lysis buffer was used to extract proteins from cultured VSMCs. The proteins were then separated using 8% or 10% SDS-PAGE and electrotransferred to PVDF membrane (Millipore). After blocking in 5% milk in TBS for 2 h, the membrane was incubated overnight at 4 °C with the primary antibody. The signal was detected using ECL (enhanced chemiluminescence) Fuzon Fx (Vilber Lourmat). The following antibodies were used: anti-p16 (1:1000), anti-p21 (1:1000), anti-p53 (1:1000), anti-NLRP3 (1:1000), anti-ASC (1:500), anti-caspase-1 (1:1000), anti-FOXO4 (1:1000), anti-AKT (1:500), anti-p-AKT (1:1000), anti-ERK (1:1000), anti-p-ERK (1:1000), anti-AMPK (1:1000), anti-p-AMPK (1:1000), anti-Rb1 (1:1000), anti-p-Rb1(1:1000), anti-mTOR (1:1000), anti-p-mTOR (1:1000), and anti-β-actin (1:1000). The images were captured and processed using FusionCapt Advance Fx5 software (Vilber Lourmat). All experiments were conducted in triplicate independently.

RNA extraction and RT-qPCR

The VSMCs were lysed, and total RNA was extracted according to the instructions of the E.Z.N.A.®Total RNA Kit I (R6834-01) manual. A NanoDrop 2000 (Thermo Fisher) spectrophotometer was used to determine the concentration and purity of the RNA. Reverse RNA transcription was then performed using the M-MLV first-strand kit (Life Technologies) for mRNA expression analysis. Then, the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) was used for mRNA RT-qPCR. RT-qPCR was performed on a CFX96™ real-time system (Bio-Rad). The primers used are listed in Supplementary Table 1. The 2^−ΔΔCt method was used to normalize the gene expression of GAPDH.

SA β-gal staining and quantitative analysis

The cells were stained with SA β-gal to detect cell senescence, as previously described [53]. Briefly, VSMCs were seeded on a 12-well plate and incubated at 37 °C with 5% CO2 for 48 h. Then, the cells were fixed for 15 min, washed with PBS, and incubated with the staining mixture at 37 °C for 18 h. The staining mixture of the SA-β-gal staining kit (Abcam, Inc.) was used. Quantification of the SA β-gal-stained cells was performed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Immunofluorescence staining

The cells were placed on a slide, fixed with 4% formaldehyde for 15 min, and washed with PBS. The slide was incubated with 10% normal goat serum (710 027, KPL) at room temperature for 30 min and later incubated overnight with the corresponding primary antibody at 4 °C. After washing with PBS, the slide was incubated for 2 h with a fluorescent-labeled IgG antibody (021,815 or 031,506, KPL, SeraCare Life Sciences, Inc., USA). Finally, the cell smear was treated with DAPI for 15 min for nuclear counterstaining. The images were acquired using a confocal microscope (DM6000CFS, Leica) and digitized using LAS AF software.

CoIP assay

CoIP was performed as previously described [4]. In brief, the cell lysates were immunoprecipitated with the indicated antibody at 4 °C overnight and then incubated with protein A-agarose at 4 °C for 1 h. Protein A-agarose–antigen–antibody complexes were then collected by centrifugation at 12,000 g for 2 min at 4 °C and washed five times with 1 ml immunoprecipitation-HAT buffer for 20 min at 4 °C. The bound proteins were resolved using SDS-PAGE, followed by western blotting with the corresponding antibody.
Animal experiments

Male C57BL/6 mice (12–16-week old) were reared under a 12-h light cycle with drinking water and a standard laboratory diet provided ad libitum [4]. The mice were anesthetized with isoflurane (2.5%); a single intravenous injection of histone 45 mg/kg (consisting of 7.5% H1, 20.8% H2A, 32.5% H2B, 10.2% H3, and 28.9% H4) [18] and BML-275 (0.5 mg/kg) + histone 45 mg/Kg intravenously. We injected 0.05 mg/kg/ buprenorphine for pain control and saline (50 ml/kg) for liquid supply subcutaneously at 1 and 12 h, and blood samples were collected from the tail veins before and 8 h after injection. The mice were euthanized 24 h after the histone or saline injection, and blood vessels and other tissues were collected and stored. All procedures were performed in accordance with the Research Ethics Committee of Guangdong Provincial People’s Hospital (No.KY-D-2021–018-01).

Cell counting kit-8 assay

VSMCs were seeded in 96-well plates (2 × 10⁴ cells/well) and cultured for 24 h, as previously described [4]. Then, 5, 10, 20, and 40 μM MC were added to the media for 24 h. The viability of VSMCs was determined using Cell Counting Kit-8 (CCK-8) assays. After culture, 10 μL of CCK-8 reagent (Beibo, China) was added to each well, and the plates were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 2.5 h. The absorbance was read at 450 nm on a microplate reader (Thermo Fisher Scientific).

Statistical analysis

All data are presented as means ± standard error of the mean. Between-group differences were analyzed using Student’s t test. Analysis of variance was performed for statistical analysis of multiple groups. Spearman’s correlation was used to determine the correlation between two genes. P values of < 0.05 were considered statistically significant.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00011-022-01618-7.

Author contributions Hang Yang and Yong-Yan Luo conceived and designed the experiments. Hang Yang, Kai-Ran He, and Lue-Tao Zhang performed all the experiments. Kai-Ran He and Hang Yang analyzed the data. Xiao-Jun Lin and Yang Hang wrote the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by Zhuhai Medical Research Fund Project (no. 911292645025).

Declarations

Conflict of interest All authors declare no conflicts of interest.

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