Sestrin2 Restricts Endothelial-to-Mesenchymal Transition Induced by Lipopolysaccharide via Autophagy

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Abstract

Objectives: Endothelial-to-mesenchymal transition (EndoMT) is a significant biological phenomenon wherein endothelial cells undergo a loss of their endothelial traits and progressively acquire mesenchymal characteristics. Consequently, this transformation leads to both a compromised ability to maintain lumen permeability and alterations in vascular structure, which hampers the preservation of blood-brain barrier integrity. This study aimed to investigate inflammation-induced EndoMT and its etiology, with the goal of impeding the infiltration of peripheral inflammation into the central nervous system. Materials and Methods: Lipopolysaccharide (LPS) was administered intraperitoneally to mice several times to establish a chronic inflammatory model. A cellular inflammatory model was established by LPS in human brain microvascular endothelial cells (HBMECs). The mRNA expressions of inflammatory cytokines interleukin-1β (IL-1β) and IL-6 were detected by real-time polymerase chain reaction (PCR). Immunofluorescence staining of platelet endothelial cell adhesion molecule-1 (CD31) and alpha smooth muscle actin (α-SMA) was conducted to assess the level of EndoMT. The expression levels of Occludin, zona occludens protein 1 (ZO-1), Sestrin2, microtubule-associated protein1 light chain 3 (LC3) and inducible nitric oxide synthase (iNOS) were detected by western blotting. Results: LPS treatment induced the downregulation of ZO-1 and Occludin, which was accompanied by the elevated expressions of iNOS, α-SMA, Sestrin2 and LC3-II in the mouse cortex and HBMECs. Mechanistically, the knockdown of Sestrin2 in HBMECs exacerbated the EndoMT induced by LPS treatment, while the overexpression of Sestrin2 inhibited this process. Moreover, the induction of autophagy by rapamycin rescued the EndoMT induced by Sestrin2 knockdown. Conclusion: This study revealed that Sestrin2 inhibited endothelial inflammation and EndoMT via enhanced autophagy, which may provide a potential drug target for cerebrovascular inflammatory injury.

Keywords: EndoMT; Sestrin2; autophagy; inflammation

1. Introduction

The brain blood vessels serve as a vital interface for the exchange of nutrients, metabolites and gases between the peripheral and central nervous system (CNS), as well as a protective barrier against exogenous substances and neurotoxic components in blood [1]. Vascular endothelial cells, which form a flattened epithelial layer along the internal surfaces of the heart, blood vessels, and lymphatics, exhibit phagocytic capabilities towards foreign bodies, bacteria, necrotic and senescent tissues, and actively participate in the immune response of an organism [2]. When blood is contaminated with harmful substances, such as endotoxins and inflammatory factors, endothelial cells are initially subjected to abnormal blood environments, leading to impaired structure and function of these cells. Previous research has demonstrated that under the influence of various stimuli, endothelial cells undergo a transformation, losing their original characteristics and becoming deficient in tight junction proteins, thereby undergoing an endothelial-to-mesenchymal transition (EndoMT). This transition is detrimental to maintaining blood-brain barrier homeostasis and has the potential to facilitate the infiltration of peripheral inflammation into the CNS. Inhibition of EndoMT has significant alleviating effect on CNS disease [3–5]. However, the majority of reports about EndoMT are focused on peripheral vascular and organ injuries, and EndoMT in the CNS requires further exploration.

Sestrins, a group of stress-induced proteins, exhibit a high degree of conservation across various organisms and are integral to cellular adaptation in response to stress [6]. The diverse functions of Sestrins are observed in different organs and tissues. Sestrin2, a homologous protein to Sestrin1, is a remarkably conserved antioxidant protein initially recognized as hypoxia-inducing gene 95. Research has demonstrated that Sestrin2 accumulates in cells subjected to stress and assumes a crucial function in suppressing the generation of reactive oxygen species (ROS), consequently safeguarding cells against oxidative harm [7]. As a stress-induced metabolic regulator, Sestrin2 restrains oxidative stress and pro-inflammatory signaling pathways through mechanisms reliant on adenosine

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5′-monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) complex 1 [8,9]. Previous research has demonstrated the protective role of Sestrin2 in cardiovascular-related diseases, wherein it activates AMPK and inhibits mTOR to preserve redox equilibrium [10,11]. Inflammatory response, a characteristic feature of cardiovascular disease, is closely associated with oxidative stress, which results in endothelial dysfunction. Further research has shown that Sestrin2 alleviates oxidative stress and apoptosis of endothelial cells subjected to drug-induced endoplasmic reticulum stress [12]. However, whether Sestrin2 involves in EndoMT and its molecular mechanism are still uncovered. In this study, endothelial inflammation and EndoMT was induced by lipopolysaccharide (LPS) in vivo and in vitro. Here, it is shown that Sestrin2 inhibited endothelial inflammation and EndoMT via enhanced cell autophagy. Our results may provide a potential drug target for cerebrovascular inflammatory injury.

2. Materials and Methods

2.1 Animal Model

Male C57BL/6J mice (6–8-week-old) were obtained from the Experimental Animal Center of Nantong University. For the duration of the experiment, the mice were housed in a controlled environment that ensured pathogen-free conditions, consistent temperature and relative humidity. Treated mice were injected with 1 mg/kg/day LPS (100 ng/mL) for 24 h. Control mice were injected intraperitoneally with an equivalent volume of physiological saline for the same duration. The mice were subjected to anesthesia by isoflurane (R510-22-10, Rayward, Shenzhen, Guangdong, China) and underwent heart perfusion using physiological saline. Brains samples were gathered for further analysis. It is important to note that the animal experiments conducted in this study received approval and strictly adhered to the regulations established by the Animal Ethics Committee of Nantong University (S20230315-005).

2.2 Cell Culture

Human brain microvascular endothelial cells (HBMECs) were obtained from YingBioTech (Shanghai, China) with certification by short tandem repeat (STR) analysis and tested to be free of mycoplasma. HBMECs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (A1049101, Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS, Z7186FBS, Zeta Life, CA, USA) and 1% penicillin-streptomycin (C125C5, NCM Biotech, Suzhou, Jiangsu, China) at 37 °C in a 5% CO2 environment. HBMECs were exposed to 100 ng/mL LPS for 24 h.

2.3 Small Interfering RNA (siRNA) Transfection

GenPharma (Shanghai, China) synthesized the siRNAs for Sestrin2, which were transfected into HBMECs for 48 h with GP-transfect-Mate (G04008, GenePharma, Shanghai, China). Sestrin2 siRNA sequences were presented as below. Sestrin2 siRNA 1, 5′-GCGCAGAGCUCAAGGCUACC-3′; Sestrin2 siRNA 2, 5′-CAGUGUUAAGUGCAAGAAUC-3′; Sestrin2 siRNA 3, 5′-GGCAUGUGAUGACUGUAAUG-3′.

2.4 Lentivirus Infection

Sestrin2 overexpressive lentivirus was constructed by GenePharma (Shanghai, China). HBMECs were plated in a 6-well plate at the confluence of 50% and infected by Sestrin2 overexpressive lentivirus and control lentivirus with polybrene for 48 h. Then, HBMECs were exposed to LPS (100 ng/mL) for 24 h.

2.5 Immunofluorescence Staining

Mouse brain sections of 15 μm containing cortical regions were obtained by freezing section. Then, permeability of cell membrane was increased by 0.3% Triton (P0096, Beyotime, Shanghai, China) in phosphate buffer saline (PBS). Brain sections were blocked in 10% sheep serum (ZLI-9056, ZSGB-BIO, Beijing, China) at 4 °C overnight. The next day, brain sections were incubated in Alexa Fluor 594-conjugated anti-rabbit IgG (1:500; A-11037, Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 488-conjugated anti-mouse IgG (1:50, 14395-1-AP, Proteintech, Wuhan, Hubei, China) at 4 °C overnight. Cell nuclei were visualized using ProLong gold anti-fade reagent containing 4′,6-diamidino-2-phenylindole (DAPI, 0100-20, Southern Biotech, Homewood, AL, USA). Immunofluorescence photos were captured by a fluorescence microscopy (BX53, Olympus, Tokyo, Japan).

2.6 Western Blotting

A radio-immunoprecipitation assay buffer (P0013C, Beyotime, Shanghai, China) was used to lyse mouse cortex tissues and HBMECs followed by the measurement of protein concentration using a bicinchoninic acid (BCA) analysis kit (P0012S, Beyotime). After separation on 10% polyacrylamide gels, proteins were transferred to polyvinylidene fluoride membrane (PVDF) (IPVH00010, Millipore, Boston, MA, USA) membranes, which were then incubated with primary antibodies overnight at 4 °C after blocking in 5% nonfat milk. The next day, PVDF membranes were incubated in goat anti-rabbit secondary antibody (1:2000, SA00001-2, Proteintech) and goat antimouse secondary antibody (1:2000, SA00001-1, Proteintech) labeled by horseradish peroxidase at room temperature for 1 h. Blot images were detected using a chemiluminescence system and the band signal was analyzed using ImageJ software 1.51j8 (National Institutes of Health, USA).
2.7 Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using Trizol reagent (T9108, Takara, Tokyo, Japan). First, RNA was purified with gDNA wiper solution and reverse transcribed into cDNA with a HiScript III 1st Strand cDNA Synthesis Kit (R312-01, Vazyme, Nanjing, China). Then, target mRNA was detected by real-time polymerase chain reaction (PCR) with Taq Pro Universal SYBR qPCR Master Mix (Q712-02, Vazyme, Nanjing, China). The PCR conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Target RNA levels were normalized to GAPDH. The quantitative expression level was analyzed using the 2^{−ΔΔCT} method. Details of primer sequence were provided as below. Mouse IL-1β forward primer: 5′-GAAATGCCACCTTTTGACAGTG-3′; reverse primer: 5′-TGATGCTCTCATCAGGACAG-3′. Mouse IL-6 forward primer: 5′-TAGTCCCTTCTACCCATAATCC-3′; reverse primer: 5′-GGGCTGGTGTGACGATTG-3′; reverse primer: 5′-GGGGTCGGAGGACCGAACA-3′.

2.8 Transmission Electron Microscope

HBMECs were digested with 0.25% trypsin (C100C1, NCIM Biotech, Suzhou, Jiangsu, China) containing ethylene diamine tetraacetic acid (EDTA) followed by centrifugation at 1200 rpm for 5 min. Cell pellets were resuspended with fixation fluid for electron microscope (G1102, Servicebio, Wuhan, Hubei, China) after discarding supernatant. Astrocytes were washed using sucrose-sodium cacodylate buffer and soaked in osmium tetroxide-sodium cacodylate for 2 h. Then, astrocytes were stained using 2% aqueous uranyl acetate after washing in water. Subsequently, samples were dehydrated in an ethanol series and embedded in Epon 812 (90529-77-4, SPI Science, West Chester, PA, USA). Ultrathin sections of 80 nm were obtained using a ultramicrotome with a diamond knife. Grids were poststained with 2% saturated uranyl acetate in 1% lead citrate and 50% ethanol. Photographs were captured using an electron microscope camera (TECNAI G2 20 TWIN, Thermo fisher, Waltham, MA, USA).

2.9 Statistical Analysis

The statistical analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). A Student’s t-test was used to compare the difference between two groups and one-way analyses of variance (ANOVA) was used to compare the differences among multiple groups. The statistical difference was considered significant if \( p < 0.05 \). At least three replicates of each experiment were conducted, and all data were presented as mean ± standard deviation (SD).

3. Results

3.1 Multiple Injections of LPS Induced Chronic Inflammation in Mouse Cortex

To investigate the mechanism of EndoMT in the inflammatory response, LPS was administered intraperitoneally to mice several times to establish a chronic inflammatory model. In comparison to the control group, the intraperitoneal injection of LPS significantly enhanced the expressions of iNOS and TNF-α in the mouse cortex (Fig. 1A). LPS treatment also induced the expressions of inflammatory factor IL-1β, IL-6 and IL-18 (Fig. 1B). Additionally, the mRNA expression levels of IL-1β (Fig. 1C) and IL-6 (Fig. 1D) were notably increased in the LPS-injected mice.

3.2 LPS Treatment Induced Endothelial Cell Dysfunction and Sestrin2 Expression in Mouse Cortex

After determining the inflammation induced by LPS, immunofluorescence staining of CD31 and α-SMA was performed to assess the level of EndoMT. As shown in Fig. 2A, a notable increase in the co-localization of CD31 and α-SMA was observed in the cortex of LPS-treated mice. In comparison to the control group, the expression levels of Occludin and ZO-1 were observed to decrease, while the expression level of α-SMA increased in the LPS-treated group (Fig. 2B). Additionally, injection of LPS significantly enhanced the expression of Sestrin2 in the cortex of mice (Fig. 2C). These findings suggested that LPS treatment induced endothelial cell dysfunction and increased protein expression consistent with early EndoMT differentiation, accompanied by upregulated Sestrin2 expression.

3.3 Sestrin2 Overexpression Attenuated EndoMT in HBMECs

Subsequently, HBMECs were subjected to LPS exposure in vitro. This exposure led to an upregulation of iNOS and Sestrin2 expression in HBMECs, as depicted in Fig. 3A. To investigate the impact of Sestrin2 on EndoMT, specific Sestrin2 siRNAs were designed and transfected into HBMECs. Among these siRNAs, Sestrin2 siRNA 1 exhibited the most effective knockdown efficiency in terms of Sestrin2 expression, as shown in Supplementary Fig. 1A. Consequently, Sestrin2 siRNA 1 was employed in subsequent experiments. Notably, knockdown of...
Sestrin2 exacerbated the reduced expression levels of Occludin and ZO-1 in the LPS-treated group (Fig. 3B). Simultaneously, knockdown of Sestrin2 facilitated the enhanced expression of α-SMA in the LPS-exposed group (Fig. 3B). Then, Sestrin2 overexpressive lentivirus was constructed and elevated the expression of Sestrin2 in HBMECs (Supplementary Fig. 1B). Sestrin2 overexpression rescued the decreased expression levels of Occludin and ZO-1 in the LPS-treated group (Fig. 3C). Meanwhile, overexpression of Sestrin2 inhibited the enhanced expression of α-SMA in the LPS-treated group (Fig. 3C). These results suggested that Sestrin2 overexpression attenuated EndoMT in HBMECs.
Fig. 3. Sestrin2 overexpression attenuated EndoMT in human brain microvascular endothelial cells (HBMECs). (A) Representative blots showed iNOS and Sestrin2 expression in the control and LPS-exposed HBMECs. (B) Representative blots showed Occludin, ZO-1 and α-SMA expression in the control and LPS-exposed HBMECs with/without Sestrin2 siRNA treatment. (C) HBMECs infected by Sestrin2 overexpressive lentivirus and the efficiency of Sestrin2 overexpression was detected. Representative blots showed Occludin, ZO-1 and α-SMA expression in the control and LPS-exposed HBMECs with/without Sestrin2 overexpressive lentivirus infection. *p < 0.05, **p < 0.01 and ***p < 0.001. Con, control; OE, overexpression.

3.4 Sestrin2 Regulated EndoMT Depending on Autophagy in Vitro

Subsequently, we aimed to investigate the downstream effects of Sestrin2 in the LPS-induced EndoMT process. As shown in Fig. 4A, the expression of microtubule-associated protein1 LC3-II was observed to be elevated in the cortex of mice treated with LPS. Similarly, an increase in LC3B-II expression was observed in LPS-exposed HBMECs (Fig. 4B). LPS treatment increased the autolysosomes (black arrows) in HBMECs (Fig. 4C). Overexpression of Sestrin2 facilitated the LC3-II expression in HBMECs (Fig. 4D), whereas knockdown of Sestrin2 suppressed the expression of LC3-II in HBMECs (Fig. 4E). Furthermore, knockdown of Sestrin2 resulted in reduced expression levels of Occludin and ZO-1, which were initially inhibited by treatment with the autophagy inducer rapamycin (Fig. 4F). Additionally, the increased expression of α-SMA in the Sestrin2 siRNA-treated group was inhibited by rapamycin (Fig. 4F). Knockdown of Sestrin2 activated phosphorylation of mTOR, which was inhibited by rapamycin (Supplementary Fig. 2). These results suggested that Sestrin2 regulated EndoMT depending on mTOR-mediated autophagy.

4. Discussion

Vascular disease is thought to begin with endothelial dysfunction, which occurs at a preclinical stage, and is prone to complications [13]. As a result of endothelial maladaptation to mechanical, metabolic, or oxidative stress, it causes impaired vasodilator response, altered angiogenesis, and increased expression of pro-inflammatory and pro-thrombotic factors [14]. EndoMT is a cell phenotypic transition driven by inflammation and an important cytopathological manifestation of endothelial dysfunction, which plays an important role in the occurrence and development of chronic cardiovascular diseases [15]. EndoMT is regulated by several signaling pathways, such as basic fibroblast growth factor/fibroblast growth factor receptor 1, transforming growth factor (TGF)-β, platelet derived growth factor receptor, and Wnt/β-Catenin signaling pathways [16]. In this study, LPS significantly induced inflammatory reaction in mouse cortex and HBMECs, which was accompanied by notable EndoMT. This also suggests that an inflammatory reaction can induce EndoMT. Of course, endothelial cell dysfunction also exacerbates the progression of inflammation, and the two complement each other [17].

Oxidative stress is a prominent determinant of EndoMT, and its significance in the development of vascular pathogenesis has been extensively investigated. Suppression of EndoMT has been shown to diminish renal ROS levels and mitigate renal fibrosis [18]. Sestrin2, a widely recognized antioxidant protein, assumes a critical function in curtailing the generation and buildup of ROS, thereby safeguarding cells against oxidative harm [19]. Consequently, we hypothesized that Sestrin2 might be implicated in the
Fig. 4. Sestrin2 regulated EndoMT depending on autophagy in HBMECs. (A) Representative blots showed the LC3-II expression in the cortex of control and LPS-injected mouse. (B) Representative blots showed the expression of LC3-II in the control and LPS-exposed HBMECs. (C) Transmission electron microscope photograph showed the autolysosomes (black arrows). Scale bar: 5 µm. (D) Representative blots showed the LC3-II expression in the control and LPS-exposed HBMECs with/without Sestrin2 overexpressive lentivirus infection. (E) Representative blots showed the LC3-II expression in the control and LPS-exposed HBMECs with/without Sestrin2 siRNA treatment. (F) Representative blots showed the LC3-II expression in the control and rapamycin-exposed HBMECs with/without Sestrin2 siRNA treatment. *p < 0.05 and **p < 0.01. Con, control; Rapa, rapamycin; LC3, light chain 3.

induction of EndoMT through LPS treatment. In our study, it was revealed that Sestrin2 was upregulated in the presence of LPS in the mouse cortex and HBMECs. Sestrin2 siRNA was utilized in the rescue experiment conducted on HBMECs. Surprisingly, the knockdown of Sestrin2 did not suppress the EndoMT induced by LPS; rather, it facilitated its occurrence. Subsequent experiments corroborated that the overexpression of Sestrin2 safeguarded HBMECs against LPS-induced EndoMT. In summary, Sestrin2 exhibited a favorable impact on the preservation of endothelial cell function. The augmentation of Sestrin2 expression in response to LPS stimulation may represent an intrinsic protective mechanism initiated by the cells themselves. Nevertheless, the elevation of endogenous Sestrin2 expression was inadequate to manifest its protective influence. Other studies have found the similar result [20,21]. LPS
treatment significantly induced the upregulation of Sestrin2 in dendritic cells, and Sestrin2 overexpression suppressed the ferroptosis of dendritic cells in sepsis by inhibiting the activating transcription factor 4-CCAAT enhancer binding protein homologous protein signaling pathway [20]. Similarly, Sestrin2 expression was induced in retinal ganglion cells exposed to H2O2, and upregulation of Sestrin2 significantly decreased H2O2-induced apoptosis and ROS generation [21]. The protective role of Sestrin2 in different cells and tissues is hardly controversial. The differential expression of Sestrin2 in different disease models may be related to the degree of cell damage.

Autophagy is a self-degrading process that is crucial for balancing energy sources in response to nutritional stress and during vital periods of development [22]. It serves as a crucial mechanism for cellular maintenance by eliminating misfolded or aggregated proteins, clearing damaged organelles such as mitochondria, endoplasmic reticulum, and peroxisome, and intracellular pathogens. Consequently, autophagy is commonly acknowledged as a survival mechanism, albeit excessive autophagy may also trigger cell death [23]. Beyond its role in removing intracellular damaged or aggregated organelles, autophagy facilitates cell aging and cell surface antigen presentation, and prevents genomic instability and cell necrosis, thereby assuming a pivotal function in various disease processes. The available literatures indicate that autophagy plays a role in the regulation of EndoMT [3]. However, the specific mechanism by which autophagy operates and the means by which it modulates EndoMT remain unclear. In human umbilical vein endothelial cells, the autophagy inhibitor 3-Methyladenine was found to suppress the EndoMT induced by SiO2 and hypoxia [24]. On the contrary, rapamycin counteracted the EndoMT process activated by TGF-β2 via reducing the phosphorylation level of Smad3 in human retinal microvascular endothelial cells [25]. This finding strongly supports the conclusion that autophagy mitigates the EndoMT induced by LPS in HBMECs. As previously mentioned, there exist numerous pathways capable of activating EndoMT, and the induction of EndoMT in various cell models may involve distinct pathways. Furthermore, the roles of both moderate and excessive autophagy in cells differ significantly, which could contribute to the conflicting conclusion.

There is a limitation to our experiment as we were unable to definitively establish that the increased in Sestrin2 and inflammatory response in the mouse cortex induced by LPS were exclusive to endothelial cells. It is possible that these changes are a result of a mixed reaction involving multiple cell types in mouse brain. Subsequent cellular experiment confirmed the increased Sestrin2 and inflammatory response in HBMECs. In conclusion, the findings from both the animal and cell experiments provide support for our conclusion.

5. Conclusion
Our findings demonstrated that Sestrin2 effectively suppressed endothelial inflammation and EndoMT by promoting autophagy, thereby presenting a promising therapeutic target for cerebrovascular inflammatory injury.

Availability of Data and Materials
All data in this study were provided in the manuscript.

Author Contributions
Investigation: RH, KS, and CD. Statistical quantification: RH, LL and KS. Drafting of the paper: RH, LL and KS. Study concept, design, review and editing: CD and ZF. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate
The animal experiments conducted in this study received approval and strictly adhered to the regulations established by the Animal Ethics Committee of Nantong University (S20230315-005).

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Conflict of Interest
The authors declare no conflict of interest.

Supplementary Material
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