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Effects and Mechanisms of CARM1 on Cerebrovascular Endothelial Dysfunction After Subarachnoid hemorrhage

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Abstract

Cerebral endothelial cell dysfunction plays a critical role in the pathophysiology of vascular injury subsequent to subarachnoid hemorrhage (SAH), yet the precise molecular mechanism remains largely speculative. Inflammation stands out as a pivotal contributor to an unfavourable prognosis post-SAH, with nuclear factor-κB (NF-κB) pathways being initiated and ultimately leading to inflammation activation and pro-inflammatory cytokine release following SAH. In this study, we explored the impact of the Coactivator-associated arginine methyl transferase 1 (CARM1) inhibitor TP-064 on inflammation in an in vitro SAH model. Exposure of endothelial cells to TP-064 resulted in a significant reduction in CAMR1 and NF-κB expression upon hemoglobin exposure. Similarly, endothelial cells treated with TP064 following hemoglobin incubation exhibited decreased expression levels of intercellular adhesion molecule-1 (ICAM1), myeloperoxidase (MPO), and cytokine production including interleukin-1β (IL1β), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α) in response to hemoglobin exposure. Moreover, subsequent investigations demonstrated that CARM1 transcriptionally regulates NF-κB via methylation. Additionally, TP-064 notably mitigated endothelial dysfunction. Collectively, our findings identify TP-064 as a CARM1 inhibitor targeting inflammation and neutrophil infiltration, offering new insights into therapeutic strategies for addressing endothelial cell dysfunction following SAH.

Keywords: CARM1; inflammation; endothelial dysfunction; Methylation; subarachnoid hemorrhage

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a cerebrovascular ailment characterized by elevated morbidity and mortality rates(Lawton & Vates, 2017). Impaired cerebral autoregulation

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emerges as a significant contributor to unfavorable prognoses in SAH patients, encompassing vasospasm and delayed cerebral ischemia (DCI)(Otite et al., 2014). Endothelial cells (ECs), integral components of cerebral vasculature, play pivotal roles in upholding the blood-brain barrier (BBB) and modulating vascular tone and hemodynamics, thereby exerting influence on vascular function and homeostasis(Komarova et al., 2017; Obermeier et al., 2013). Previous research demonstrates the presence of inflammatory cytokines within the endothelium of spastic arteries, indicating the potential contribution of inflammatory factors to ECs damage(de Oliveira Manoel & Macdonald, 2018; Liu Liu et al., 2019).

Nuclear factor-κB(NF-κB) is a key factor in the regulation of the expression of pro-inflammatory cytokines, adhesion molecules and enzymes(Lawrence, 2009). NF-κB-mediated proinflammatory signaling in endothelial cells and leukocytes induces the chronic inflammatory pathology atherosclerosis(Baker et al., 2011).Coactivator-associated arginine methyltransferase1(CARM1) is an arginine methyltransferase that controls gene transcription, also known as protein arginine methyl transferase 4 (PRMT4) is one of nine members of the protein arginine methyl transferase (PRMT) family ,which is known to enhance transcriptional activation by nuclear receptors(Suresh et al., 2021). Recently, accumulative evidence showed that CARM1 can act as a coactivator for the transcription factor NF-κB and enhance its activity [10, 11]. Increased expression of CARM1 is associated with elevated levels of proinflammatory mediators in atherosclerosis-related cardiovascular disease(Liu et al., 2014). Thus, inhibition of CARM1 activity might be a promising strategy to reduce the pro-inflammatory response. However, it remains unclear whether CARM1 participate in the endothelial cell dysfunction after SAH. In this study, we established cell models of SAH to observe the effects of CARM1 on the endothelial cell dysfunction, we also apply TP-064 as CARM1 inhibitor to study the effects of TP-064 treatment on inflammation to explore the potential mechanisms in this pathophysiological process.

MATERIALS AND METHODS

Cell culture

Human brain microvascular endothelial cells (Otwo Biotech, China) were cultured in dulbecco modified eagle medium and ham's F-12 medium (DMEM/DF12, Gibco, USA, 1:1) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in 5% CO2 at 37 °C,with medium renewal every 48 hours. To simulate the pathophysiological conditions of subarachnoid hemorrhage (SAH) and determine the optimal stimulating concentration, cells were exposed to hemoglobin (Sigma, USA) at concentrations ranging from 5 μM to 20 μM for 24 hours prior to subsequent assays (the concentration and duration of Hb were set to the appropriate conditions obtained in our previous experiments)(Z. Zhang et al., 2021). Following hemoglobin exposure, western blot analysis was employed to assess the expression of CARM1 across different concentration groups. Cells were treated with 10 μM TP-064 dissolved in an equivalent concentration of dimethyl sulfoxide (DMSO) for 24 hours. Subsequently, cells were stimulated with hemoglobin for an

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additional 24 hours. Following stimulation, cells and supernatants were harvested and stored at −20°C until further analysis of gene expression or cytokine levels.

Western blotting.

Endothelial cells (ECs) cultured in T-25 flasks following the aforementioned treatments were subjected to triple washing, and the proteins were extracted using RIPA buffer supplemented with protease inhibitors (Biotime, China). The total protein concentration was determined using BCA Protein Assay Reagent (Beyotime, China). Subsequently, the protein samples were denatured by boiling and separated (20 μg) via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene difluoride membranes (PVDF, Bio-Rad, USA). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour, and incubated overnight at 4°C with specific antibodies: CARM1 (Abcam, United Kingdom, 1:200), MPO (Abcam, United Kingdom, 1:500), ICAM1 (Abcam, United Kingdom, 1:200), NF-κB (Abcam, United Kingdom, 1:400), GAPDH (ProteinTech Group, China, 1:1000), and Histone (1:1000, Proteintech). Blots were subsequently incubated with the appropriate horseradish peroxidase-conjugated IgG for 1 hour at 37°C and visualized using the Chemiluminescence Kit (Beyotime, China) with X-ray film. The optical densities of these bands were quantified using Quantity One software 4.6.2. GAPDH and Histone were employed as loading controls for whole cell and nuclear proteins.

ELISA.

The concentration of pro-inflammatory cytokines was analyzed in the medium of endothelial cells using the ELISA protocols provided by BD Biosciences and Biolegend (San Jose and San Diego, CA, USA). Absorbance measurements were conducted at 450 nm and 570 nm.

Immunofluorescence.

ECs were cultured on glass coverslips placed in 6-well plates at 65% confluence and preincubated TP-064. For immunostaining, cells were washed three times with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.01% Triton X-100 in PBS, and blocked with 10% goat serum for 30 minutes at 37 °C. The cells were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti–CARM1 (Abcam, United Kingdom, 1:200), mouse-CD31(Abcam, United Kingdom, 1:200), rabbit anti–MPO (Abcam, United Kingdom, 1:200) followed by appropriate fluorescein isothiocyanate-conjugated and tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies (Abbkine, USA, 1:200) for 1hours at room temperature. Subsequently, all cells were incubated with DAPI for 15minutes. Coverslips were mounted in antifade regent (Beyotime, China) and visualized by a fluorescence microscopy timely and effectively (Leica, Germany).

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Gene expression analysis.

Total RNA was extracted from cells following the standard protocol outlined by Chomczynski and Sacchi(Chomczynski & Sacchi, 2006). RNA was reverse transcribed into cDNA using Maxima H Minus Reverse Transcriptase. The PCR cycle

threshold (Ct) values were then determined after incorporation of SensiMix SYBR low-ROX mix, utilizing the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The average Ct values of acidic ribosomal phosphoprotein P0 (36b4), hypoxanthine guanine phosphoribosyl transferase (Hprt), and ribosomal

protein L27 (Rpl27) were employed as housekeeping genes.

Chromatin immunoprecipitation assay (ChIP). The ChIP assay was conducted using a commercially available kit (Millipore) following the manufacturer's instructions. Briefly, cells were crosslinked with 1% formaldehyde for 10 minutes, followed by quenching with glycine (0.125 M). Subsequently, the cell pellets were lysed in lysis buffer and sonicated for 5 minutes.

The lysates were centrifuged, and the supernatants were incubated with specified antibodies overnight at 4 °C. Immunocomplexes were captured using 30 µl of protein A/G sepharose on a rotator at 4 °C for 2 hours. Following four times of washing, protein

complex attached to the beads was dissolved in the SDS sample buffer and subsequently resolved in 7% SDSPAGE gel. All experiments were performed in three biological replicates.

Statistical Analysis.

Statistical analysis was carried out using SPSS 20.0 and GraphPad Prism 9.5. All values were expressed as mean ± standard deviation. Multigroup comparisons were conducted through oneway analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. Each experiment was repeated a minimum of three times. A significance level of $P < 0.05$ was deemed statistically significant.

RESULTS.

Hemoglobin Induced increased CARM1 in Cultured Cerebral ECs.

The cells were treated with varying concentrations of hemoglobin, and the expression of CARM1 and NF-κB were examined by

western blot analysis after 24 hours. As depicted in Fig. 1A, elevated levels of CARM1 and NF-κB were detected within cultured ECs following hemoglobin treatment. The expression of both proteins reached maximal levels with a hemoglobin concentration of 10 µM. However, excessive hemoglobin dosage led to a decrease in the expression of both proteins (Fig.1a, b, c). Based on the preliminary data, a concentration of 10 µM (optimal stimulating concentration) was chosen for subsequent experiments. Following a 24-hour incubation with hemoglobin, a

significant increase in the expression of CARM1 and NF-κB was observed compared to the control group. Similarly, an enhanced immunoreactivity of CARM1 was observed within cultured ECs after hemoglobin treatment (Fig. 1d).

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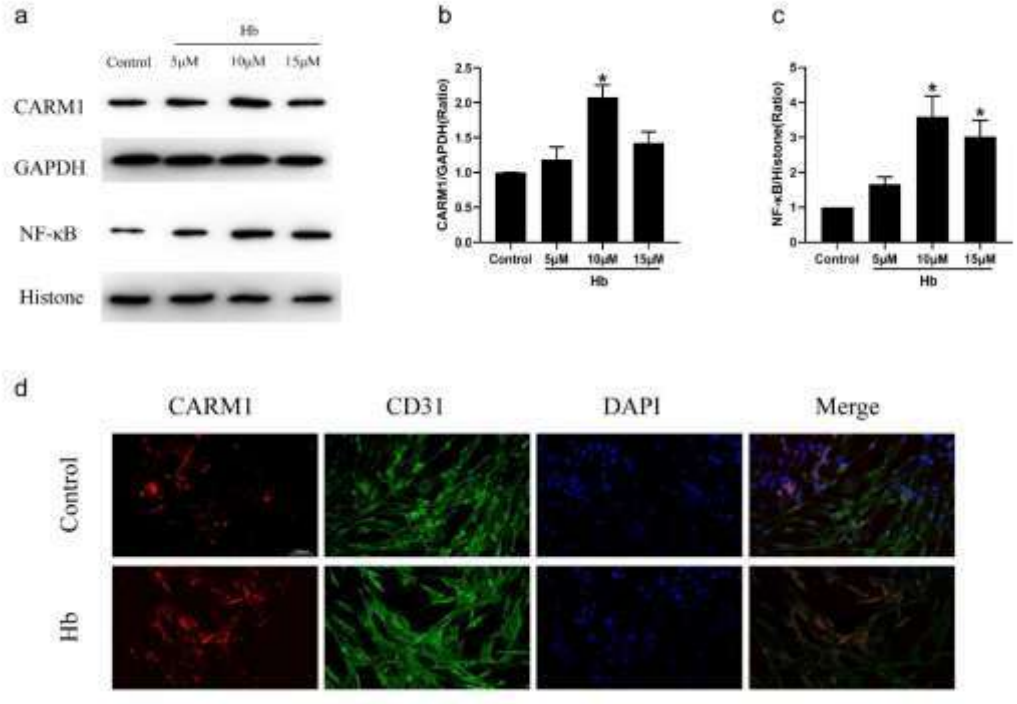

Fig 1. Expression of CARM1 and NF-κB in cerebral endothelial cell after treatment with Hb. a-c Representative image and quantitative analysis of CAMR1 and NF-κB expression in cerebral ECs at 24hours after incubation with various concentrations of hemoglobin respectively (n=3 per group). d Immunohistochemical staining for CARM1 (red), CD31 (green), and DAPI (blue) in cerebral endothelial cells at 24 hours following hemoglobin incubation. All quantitative data were presented as mean±standard deviation. Scale Bar=100μm. *P<0.05 vs Control group.

TP-064 treatment inhibited the expression of CARM1 and attenuated the hemoglobin-induced pro-inflammatory response of endothelial cells (ECs) in vitro. To investigate the effects of CARM1 on the pro-inflammatory response of ECs, we sought to inhibit CARM1 expression using TP-064. As illustrated in Fig.2a,b, immunoblot results demonstrated a significant increase in CARM1 expression following hemoglobin incubation, which was reversed by TP-064 treatment. Additionally, TP-064 markedly reduced the expression of NF-κB, ICAM1, and MPO (Fig. 2a, c, d, e). Similarly, ELISA results revealed an elevation in the expression of inflammatory cytokines (TNF-α, IL-1β, and IL-12) induced by hemoglobin, which was mitigated by TP-064 treatment (Fig. 3d, e, f). Immunofluorescence analysis showed co-localization of the neutrophil marker (MPO) with a specific marker of endothelial cells (CD31) in ECs. These findings indicate that TP-064 treatment significantly suppressed neutrophil infiltration and the inflammatory response (Fig. 2f).

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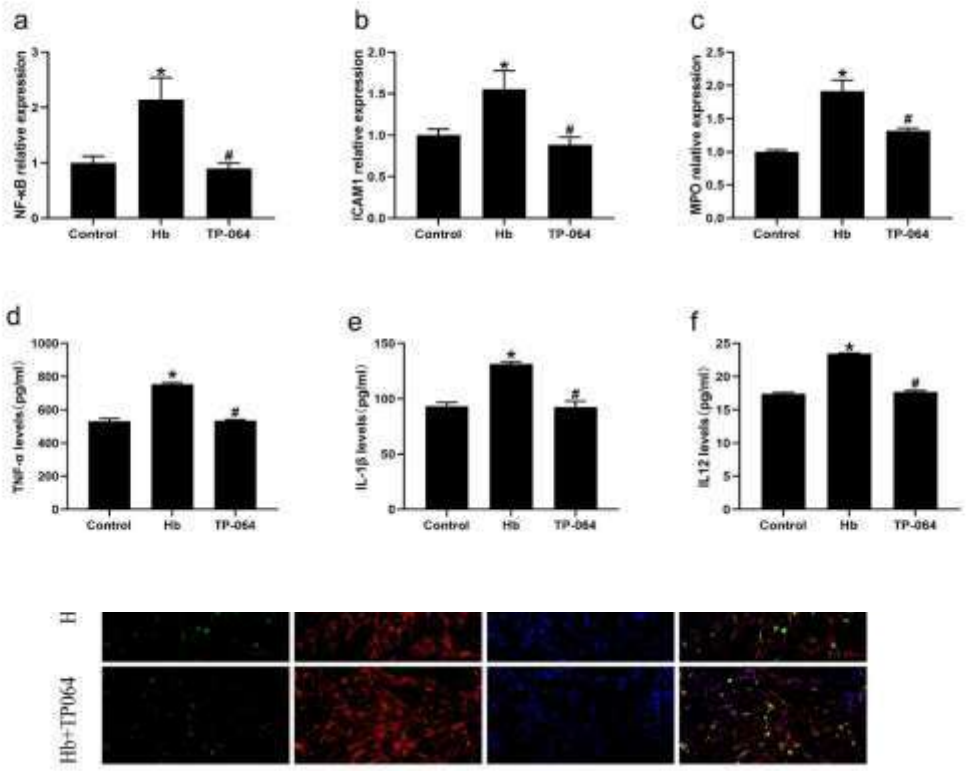

Fig 2. TP-064 inhibited CAMR1 expression and suppressed pro-inflammatory response of ECs. a-e Representative image and quantitative analysis of CAMR1,NF-κB ,ICAM1 and MPO in cerebral ECs at 24hours post-incubation with 10μM Hb, with or without TP-064 pretreatment, respectively. f Immunohistochemistry for mpo(green), CD31 (red), and DAPI (blue) in cerebral ECs at 24hours postHb incubation with or without TP064 pretreatment, respectively. All quantitative data were presented as mean±standard deviation. Scale Bar=100μm. *P<0.05 vs Control group; # P<0.05 vs. Hb group.

TP-064 treatment downregulates inflammation-related gene expression.

Consistently, the inhibition of CARM1 activity by TP-064 was associated with a decrease in NFκB mRNA expression levels. Our current in vitro study demonstrated that TP-064-mediated inhibition of CARM1 resulted in decreased expression levels of ICAM1 and MPO in endothelial cells (ECs) (Fig. 3a, b, c).

CARM1 enhances NF-κB activation through Methylation.

As confirmed previously, CARM1 upregulated NF-κB expression in endothelial cells (ECs), suggesting a potential modulation of NF-κB's transcriptional activity by CARM1. As expected,

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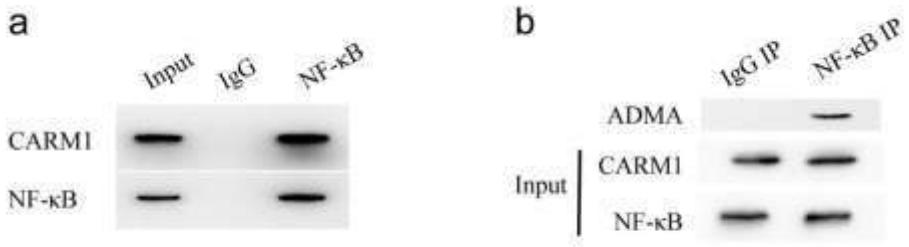
our co-immunoprecipitation (co-IP) assays demonstrated the interaction between CARM1 and NF-κB in ECs (Fig. 4a).



Subsequently, we investigated whether NF-κB served as a substrate

for CARM1. ECs underwent co-IP with immunoglobulin G (IgG) or anti-NF-κB antibodies, followed by immunoblotting with an asymmetric dimethylarginine (ADMA)-specific antibody (Fig. 4b). The presence of methylated NF-κB in the immunoprecipitation (IP) lysates indicated the methylation of NF-κB by CARM1 in vitro.

To assess the impact of TP-064 treatment on endothelial dysfunction levels of endothelin-1 (ET-1) and nitric oxide (NO) were measured using enzyme-linked immunosorbent assay levels increased while NO levels decreased in endothelial cells (ECs). We demonstrated that the CARM1 inhibitor TP-064 effectively reduced ET1 levels and increased NO levels (Fig. 5a, b),

Fig 4. CARM1 transcriptionally controls NF-κB through Methylation. a co-IP assay demonstrated the interaction between CARM1 and NF-κB in endothelial cells (ECs); b Cell lysates from ECs were incubated with anti-IgG or NF-κB antibody, and followed by WB with anti-ADMA.

TP-064 treatment alleviated hemoglobin induced endothelial dysfunction.

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indicating a beneficial effect of CARM1 inhibition on endothelial dysfunction.

(ELISA). Following hemoglobin treatment, ET-1

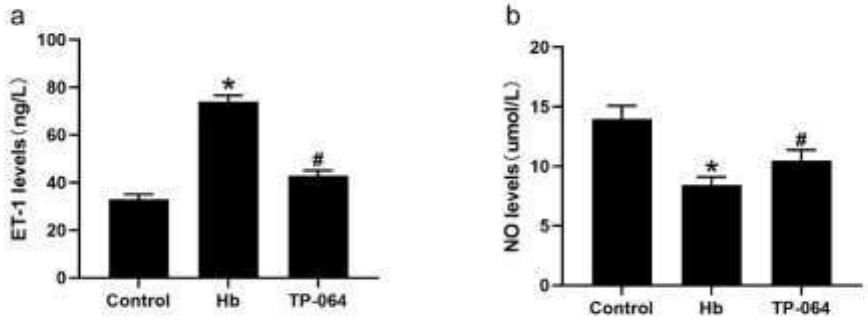

Fig 5. TP-064 treatment alleviated endothelial dysfunction. Representative analysis of effects of TP064 on the expression of ET-1 and NO in cerebral ECs after 10μM Hb incubation. *P<0.05 vs Control group; # P<0.05 vs. Hb group.

DISCUSSION

In the current study, we explored the potential impact of the CARM1 inhibitor TP-064 on endothelial cell dysfunction following SAH in vitro. The primary findings from our study are as follows: 1) CARM1-mediated NF-κB activation through methylation contributes to inflammatory response and endothelial cell dysfunction; 2) Regulation of CARM1 expression by TP-064 in cultured cerebral ECs effectively alleviates inflammation and endothelial cell dysfunction after hemoglobin incubation. Endothelial cells, integral to the neurovascular unit, play a pivotal role in the pathophysiology of subarachnoid hemorrhage, which maintain the blood-brain barrier, regulate vascular tone, modulate inflammatory responses and control blood flow(Peeyush Kumar et al., 2019; Zhang, 2014). In our previous study, we have found that endothelial function was damaged after SAH, subsequently resulting in disturbances of cerebral blood flow autoregulation and the deterioration of cerebral ischemia after SAH(L. Liu et al., 2019). Previous research has described inflammation in blood vessels as involving leukocyte-endothelial cell interactions and the release of inflammatory mediators, resulting in endothelial cell integrity disruption and subsequent dysfunction (Chaichana et al., 2010; Liu Liu et al., 2019). Hemoglobin, a product of blood hemolysis after SAH, oxidizes into various forms, becoming a potent pro-inflammatory and cytotoxic molecule known to activate NF-κB.(Blackburn et al., 2018; Peiró et al., 2003). Hemoglobin-induced endothelial damage and apoptosis, neuroinflammation, and BBB disruption have been reported(Peeyush Kumar et al., 2018). Following hemoglobin incubation with ECs to mimic SAH neuropathology, our results revealed heightened NF-κB expression, which indicating that the NF-κB pathways were initiated.

Coactivator-associated arginine

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methyltransferase 1 (CARM1) acts as a coactivator and a novel transcriptional regulator of NF-κB-mediated inflammatory gene expression(Covic et al., 2005; Hassa et al., 2008).However, the expression level and role of CARM1 in ECs following hemoglobin treatment have yet to be determined. Our study demonstrated a significant increased in both mRNA and protein levels of CARM1 in ECs after hemoglobin treatment compared to controls, with CARM1 also co-localizing with ECs. Our co-IP assay confirmed the interaction between CARM1 and NF-κB, suggesting that CARM1 interacts with NF-κB to catalyze its methylation and restrict its nuclear localization, thereby promoting inflammation. Elevated levels of inflammatory cytokines (TNF-α, IL-1β, and IL-12) correlated with increased expression of CARM1 and NF-κB. Additionally, CARM1 mRNA levels positively correlated with mRNA expression levels of NF-κB, MPO, and ICAM1 in ECs following hemoglobin treatment. To our knowledge, this study is the first to report elevated CARM1 levels in ECs following hemoglobin treatment, further supporting a role for CARM1 in the inflammatory response.

Recent studies have identified TP-064 as a selective and potent inhibitor of CARM1 function (Y. Zhang, M. de Boer, et al., 2021; Y. Zhang, R. A. F. Verwilligen, et al., 2021). Consistent with previous observations that CARM1 acts as an NFκB coactivator, our research showed that TP-064 reduced pro-inflammatory cytokine secretion(Covic et al., 2005; Miao et al., 2006). Western blot analysis revealed increased expression of MPO and ICAM-1 following hemoglobin treatment, which was significantly reduced by TP-064 administration. The administration of TP-064 significantly decreased the expression of MPO and ICAM-1. Furthermore, MPO is a marker of leukocyte activity(Kitching et al., 2020), and fluorescence microscopy demonstrated MPO co-localization with CD31, indicating leukocyte infiltration into ECs. We also observed that TP-064 suppressed neutrophil infiltration in ECs following hemoglobin treatment. These findings collectively suggest that TP-064 inhibits neutrophil infiltration, possibly through decreased ICAM-1 and MPO expression.

Endothelial cells regulate vascular tone and blood flow through a delicate balance of vasoconstrictors such as endothelin-1 (ET-1) and vasodilators such as nitric oxide (NO)(Sandoo et al., 2010). Animal studies have shown that SAH can induce functional changes in vascular endothelium, resulting in reduced NO levels and increased ET-1 levels(Friedrich et al., 2010; Lin et al., 2001). Endothelial damage may lead to pathological changes in the neurovascular unit, such as abnormal vascular remodeling and angiogenesis, which can disrupt normal brain function and contribute to disease progression (Iuliano et al., 2004; Sandoo et al., 2010). Endothelial cell injury also affects communication between the endothelial cells and adjacent neurons, astrocytes, and pericytes, which can impair the normal regulatory mechanisms of the neurovascular unit and affect overall brain health(Abbott et al., 2006; Schaeffer & Iadecola, 2021; Segarra et al., 2019). Overall, endothelial cell damage within the neurovascular unit can have profound effects on brain function and contribute to the pathology of various

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neurological conditions. In our study, we found TP-064 treatment significantly reduced ET-1 levels and increased NO levels, resulting in significant improvement in endothelial dysfunction following hemoglobin incubation in the TP-064 treatment group.

In conclusion, these findings imply that inhibition of CARM1 function by TP-064 may represent a promising novel therapeutic approach for NF-κBdriven pathologies, a matter of considerable importance in elucidating the molecular mechanisms underlying endothelial dysfunction after SAH. TP-064 may provide a means to address neuro-inflammation. By inhibiting CARM1, which is implicated in inflammatory processes, TP-064 could help mitigate neuroinflammatory responses and protect neuronal health. As with any therapeutic agent, the potential neurotoxicity of TP-064 must be carefully evaluated, as well as its ability to cross the blood-brain barrier. We only conducted in vitro experiments, which is a limitation of our present study. Therefore, it would be very interesting to follow up on our current findings by studying the effects of TP-064 in the treatment of SAH in rats. ***Ethical Statements*** It is not applicable.

Conflicts of interest

The authors declare no competing financial interests.

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Author contributions

Liu Liu conceived and designed the project, Qingtao Zhang executed all experiments and contributed to manuscript preparation. Yidan Liang, Qiang Yang, Min Cui, and Chao Sun assisted in completing specific experiments. Yongbing Deng and Weiduo Zhou conducted data analysis, Lei Xu participated in manuscript revision, particularly focusing on language refinement. All authors critically reviewed and approved the final version of the manuscript. Data availability statement

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

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