Hypothermia Protects Against Ischemic Stroke Through Peroxisome-proliferator-activated-receptor Gamma

Feng Jia (✉ rjjiafeng@163.com)

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Shuai Shao

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Yanlin Chen

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Jiansong Zhang

Shanghai Changzheng Hospital

Dilimulati Dilirebati

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Kui Chen

Tongji University Affiliated East Hospital: Shanghai East Hospital

Xiaohua Zhang

Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Research

Keywords: hypothermia, ischemic stroke, peroxisome-proliferator-activated-receptor gamma, neuroinflammation

Posted Date: August 3rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-754337/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: IS (ischemic stroke) remains to be a global public health burden and urgently demands novel strategies. Hypothermia plays a beneficial role in central nervous system diseases. However, the function of hypothermia in IS has not been elucidated. Here we demonstrated the role of hypothermia in IS and explore the mechanism.

Methods: IS phenotype was detected by infarct size and infarct volume as well as brain edema in mice. The neuroinflammation was evaluated by activation of microglial cells and expression of inflammatory genes after ischemia/reperfusion (I/R) and oxygen-glucose deprivation/reperfusion (OGD/R). The apoptosis of neuronal cells was assessed by Tunnel staining, expression of Cleaved Caspase-3 and Bax/Bcl-2, cell viability, and LDH release after I/R and OGD/R. Blood-brain-barrier (BBB) permeability was calculated by Evans blue extravasation, the expression of tight junction proteins and MMP-9, cell viability, and LDH release after I/R and OGD/R. The expression of peroxisome-proliferator-activated-receptor gamma (PPAR γ) was detected by western blotting after I/R and OGD/R.

Results: Hypothermia significantly reduced the infarct size and infarct volume as well as brain edema after ischemia/reperfusion. Consistently, hypothermia induced attenuated neuroinflammation, apoptosis of neuronal cells, and BBB disruption after I/R and OGD/R. Mechanistic studies revealed that hypothermia protected against IS by upregulating the expression of PPAR γ in microglial cells, the effect of hypothermia was reversed by GW9662, a PPAR γ inhibitor.

Conclusions: Our data showed that hypothermia inhibited the activation of microglial cells and microglial cell-mediated neuroinflammation by upregulating the expression of PPAR γ in microglial cells. Targeting hypothermia may be a feasible approach for IS treatment.

Introduction

Stroke remains the leading cause of mortality and morbidity worldwide ¹, 70% of the strokes are caused by ischemic stroke (IS) ². Despite advancements in clinical treatments, FDA-approved treatments to IS only include endovascular thrombectomy and intravenous thrombolysis with tissue plasminogen activator (tPA) ³⁻⁴. Due to the limitation of the time window and the hemorrhagic risk of tPA, only a small number of stroke patients received effective treatments ⁵. Therefore, strategies which benefit more stroke patients are urgently needed

Neuroinflammation is the majority pathophysiological process that contributes to the development of brain injury after IS. Previous studies have shown that anti-inflammatory strategies are beneficial for IS ⁶⁻⁷. Microglial cells are the innate immune cells in the central nervous system (CNS) and are widely activated following IS ⁸. Studies have reported that activated microglial cells release pro-inflammatory cytokines and aggravate neuroinflammation ⁹⁻¹⁰. The drastic neuroinflammation enhances apoptosis of

the neuronal cell and exacerbates the disruption of the tight junction between the endothelial cells ¹¹. Thus, targeting microglia-mediated neuroinflammation provides a feasible strategy to treat IS.

Previous studies have demonstrated the involvement of hypothermia in a variety of diseases, including stroke, traumatic brain injury, and hypoxic-ischemic encephalopathy ^{12–14}. Growing evidence from animal experiments and clinical researches show that therapeutic hypothermia prevents brain injury through suppression of oxidative stress, inflammatory responses, and cell death ¹⁵. Thus, hypothermia is an attractive treatment for ischemic stroke. However, the protective mechanism remains unclear. Peroxisome proliferator-activated receptor gamma (PPAR γ) has been reported to modulate macrophage/microglia polarization and microglia-mediated neuroinflammation after IS ¹⁶. It has not been reported whether hypothermia plays an important role in regulating PPAR γ in microglial cells after middle cerebral artery occlusion (MCAO) or oxygen-glucose deprivation/reperfusion (OGD/R).

In this study, we investigated the effect of hypothermia in IS, including infarct size, infarct volume, brain edema, neuroinflammation in vivo and vitro, neuronal cell apoptosis in vivo and vitro, and BBB disruption in vivo and vitro by MCAO model and OGD/R model. We also explored the cellular mechanisms and studied how hypothermia protects against ischemia and hypoxia through PPAR γ in microglial cells after MCAO and OGD/R.

Methods

Animals and experimental design

Adult male C57BL/6 mice (8–10 weeks, 22–25g) were used in this study. The mice were housed in 12 hours light and 12 hours dark cycle in the standard cages. Food and water were freely available. All animal experiments were approved by the Animal Care and Experimental Committee of the School of Medicine of Shanghai Jiao Tong University.

The mice were randomly divided into four groups: sham injury with normothermia group (SNG; 37°C), sham injury with hypothermia (SHG; 33°C), MCAO with normothermia group (MNG; 37°C), and MCAO with hypothermia group (MHG; 33°C). The investigators were blinded to group assignments during all results assessments.

Drug administration and temperature measurement

The body temperature was monitored with a digital electronic thermometer (Omega Engineering, USA). The drug administration was performed as previously described ¹⁷. Briefly, HPI-201 was dissolved in saline and injected intraperitoneally (i.p.). For MNG mice and MHG mice, the first injection (2 mg/kg) was given 30 min after MCAO. The body temperature cooled from 37°C to 33°C in around 30 min. The additional injections (1 mg/kg) were given 3 hours and 5 hours after MCAO to maintain constant mild hypothermia at 33°C for 6 hours. The body temperature was then gradually returned to normothermia. For

SNG mice and SHG mice, equivalent saline was given at the same time points and the body temperature was maintained at the normothermia.

Brain I/R model

The I/R model was performed as previously described¹⁸. Briefly, the mice were anesthesia with 2% avertin (Sigma-Aldrich, USA). Depilatory cream was used to remove hair from the neck skin. Cutting neck skin alongside the midline and separated the left common carotid artery, the internal carotid artery, and the external carotid artery. The left middle cerebral artery was blocked with a nylon suture (0622, Yushun, China). The rectal temperature was maintained at $37 \pm 0.5^{\circ}\text{C}$ with a constant temperature heating pad during the operation. After 60 min of occlusion, the nylon suture was removed. Mice in sham groups underwent the same procedures, but the nylon suture was not inserted into the middle cerebral artery. Mice were housed separately with free availability of food and water after the operations.

2,3,5- triphenyl tetrazolium hydrochloride (TTC) staining

Experimental mice were euthanized using overdose isoflurane 24 hours after I/R. Brains were quickly removed and frozen at -20°C for 30 mins. The brains were coronally cut into 2 mm-thick sections and incubated with 2% TTC (Sigma-Aldrich, USA) at 37°C for 15 minutes. Infarct size and volume were calculated by ImageJ software (National Institutes of Health, USA).

Brain water content

Experimental mice were euthanized using overdose isoflurane 24 hours after I/R. Brains were quickly removed and divided into the contralateral hemisphere, ipsilateral hemisphere, and cerebellum. The ipsilateral hemisphere was weighed (wet weight) and placed in an oven at 105°C for 72 hours for dry weight. The brain water content was measured as follows: $(\text{wet weight} - \text{dry weight} / \text{wet weight}) \times 100\%$.

Neurological score

The neurological score was assessed at 24 hours after I/R, using the Garcia scores system as previously described¹⁹. The Garcia scores include vibrissae touch, touch of the trunk, climbing wall of the wire cage, movements of forelimbs, spontaneous movements of all limbs, and spontaneous activity. All results were assessed by an investigator blinded from the group allocation.

Immunofluorescence

Experimental mice were euthanized using overdose isoflurane 24 hours after I/R. Brains were quickly removed and fixed with 4% paraformaldehyde for 24 hours. Then the samples were dehydrated and paraffin-embedded. After antigen retrieval treatment, coronal sections were incubated with a blocking buffer at 37°C for 1 hour, and the coronal sections were incubated with primary antibodies (anti-Iba1, 1:200, Abcam, USA; anti-NeuN, 1:200, Abcam, USA) at 4°C overnight. Secondary antibodies of Fluorochrome-conjugated were incubated (Thermo Fisher Scientific, USA) at room temperature for 2 hours 24 hours later. Finally, the coronal sections were counterstained with DAPI (Thermo Fisher Scientific, USA). Images were captured with a fluorescence microscope (Leica, German).

Cell culture and OGD/R model

BV2 microglial cells were cultured in DMEM/F12 (Thermo Fisher Scientific, USA) with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin (PS, Thermo Fisher Scientific, USA). HT-22 neuronal cells were cultured in DMEM with 10% FBS and 1% PS. bEnd.3 endothelial cells were cultured in DMEM with 10% FBS and 1% PS. For OGD treatment, the media were replaced with glucose-free EBSS (Thermo Fisher Scientific, USA), and the cell plates were placed in a hypoxia incubator (Sigma-Aldrich, USA) at 37°C with 95% N₂ and 5% CO₂. Cells were exposed to the OGD stimulation at 37°C for 3 hours, 6 hours, and 12 h, respectively. For the control treatment, the media were replaced with EBSS (Thermo Fisher Scientific, USA) supplemented with glucose in a humidified incubator at 37°C with 5% CO₂ and 95% air for the same time. After OGD treatment and control treatment, the media was replaced with normal media, and the cell plates were placed in a humidified incubator at 37°C with 5% CO₂ and 95% air for 24 hours reperfusion.

Pro-inflammatory microglia cell model

Conditioned medium (CM) collected from HT-22 neuronal cells for 6 hours OGD and bEnd.3 endothelial cells for 12-hour-OGD were used to stimulate BV2 microglial cells. Control BV2 microglial cells were treated with medium collected from HT-22 neuronal cells and bEnd.3 endothelial cells without OGD. During the stimulation, hypothermia (33°C) and GW9662 (20 µM) were treated respectively.

Microglial cells-neuronal cells and microglial cells-endothelial cells co-culture

Cells were co-cultured by transwell cell culture inserts (Corning, USA). BV2 microglial cells growing on culture inserts were treated with vehicle placed in normothermia (37°C), CM placed in normothermia (37°C), CM placed in hypothermia (33°C), CM supplemented with GW9662 (20 µM) placed in hypothermia (33°C) for 12 hours, respectively. HT-22 neuronal cells were exposed to OGD for 6 hours and bEnd.3 endothelial cells were exposed to OGD for 12 hours. Then the media were replaced with normal media. Cells co-cultured systems were generated by adding the BV2 microglial cells inserts on top of HT-22 neuronal cells and bEnd.3 endothelial cells. The co-culture systems were sustained for 24 hours before the BV2 microglial cells inserts were removed. The survival was analyzed by MTT kit (Roche, Switzerland) and the cell death was evaluated by lactate dehydrogenase (LDH) release according to the manufacturer's instructions (Beyotime, China).

Quantitative RT-qPCR

Experimental mice were euthanized using overdose isoflurane 24 hours after I/R. TRIzol (Thermo Fisher Scientific, USA) was used to extract total RNA from the ipsilateral hemisphere, and cDNA was synthesized using reverse transcription kits (Takara, Japan) following the manufacturer's instructions. RT-qPCR was conducted using SYBR Green Mix (Thermo Fisher Scientific, USA) on a LightCycler 480 II (Roche, Switzerland). Gene expression was normalized by GAPDH. Primer sequences were as follows (Table 1):

Table 1
Primer sequence for RT-qPCR

Gene	Sequences	species
TNF- α F	5'-GAACTAAGGACAGGAAGGTACAGGG-3'	Mouse
TNF- α R	5'-GCTACGACGTGGGCTACAG-3'	
IL-1 β F	5'-GCAACTGTTCTGAACTCAACT-3'	Mouse
IL-1 β R	5'-ATCTTTTGGGGTCCGTCAACT-3'	
IL-6 F	5'-TAGTCCTTCCTACCCCAATTTCC-3'	Mouse
IL-6 R	5'-TTGGTCCTTAGCCACTCCTTC-3'	
CXCL1 F	5'-CTGGGATTACCTCAAGAACATC-3'	Mouse
CXCL1 R	5'-CAGGGTCAAGGCAAGCCTC-3'	
iNOS F	5'-GTTCTCAGCCCAACAATACAAGA-3'	Mouse
iNOS R	5'-GTGGACGGGTGCATGTAC-3'	
Arg-1 F	5'-CTCCAAGCCAAAGTCCTTAGAG-3'	Mouse
Arg-1 R	5'-AGGAGCTGTCATTAGGGACATC-3'	
Fizz-1 F	5'-CCAATCCAGCTAACTATCCCTCC-3'	Mouse
Fizz-1 R	5'-ACCCAGTAGCAGTCATCCCA-3'	
Ym-1 F	5'-CAGGTCTGGCAATTCTTCTGAA-3'	Mouse
Ym-1 R	5'-GTCTTGCTCATGTGTGTAAGTGA-3'	
CD206 F	5'-CTCTGTTTCAGCTATTGGACGC-3'	Mouse
CD206 R	5'-CGGAATTTCTGGGATTCAGCTTC-3'	
GAPDH F	5'-AGGTCGGTGTGAACGGATTTG-3'	Mouse
GAPDH R	5'-TGTAGACCATGTAGTTGAGGTCA-3'	

Western blotting analysis

Experimental mice were euthanized using overdose isoflurane 24 hours after I/R. The ipsilateral hemisphere was collected and lysed in lysis buffer containing 1 mM PMSF and protease inhibitors cocktail. The proteins were separated by 8% or 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with blocking buffer for 1 hour, then incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies for 2 hours at room temperature 12 hours later. The membranes were detected with chemiluminescence substrates (Thermo Fisher Scientific, USA). The gray values of immunoreactive bands were calculated with ImageJ software. Proteins level were

normalized by α -tubulin or GAPDH. The following primary antibodies were used: anti-Iba1 (1:1000, Abcam, USA), anti-Cleaved Caspase-3 (1:1000, Abcam, USA), anti-Bcl-2 (1:1000, Abcam, USA), anti-Bax (1:1000, Abcam, USA), anti-MMP-9 (1:1000, Proteintech, China), anti-ZO-1 (1:500, Abcam, USA), anti-Occludin (1:500, Santa Cruz, USA), anti-PPAR γ (1:1000, Abcam, USA), anti- α -tubulin (1:5000, Sigma-Aldrich, USA) and anti-GAPDH (1:1000, Cell Signaling Technology, USA).

Measurement of EB extravasation

EB (2%, Sigma-Aldrich, USA) was intravenously injected (5 ml/kg) 23 hours after I/R. The dye was circulated for 1 hour. Collected and weighed the ipsilateral hemisphere, then homogenized samples in 1 ml ice-cold PBS. The supernatants were harvested and an equal volume of 100% trichloroacetic acid (Thermo Fisher Scientific, USA) was added. The mixture was incubated at 4°C overnight. The supernatants were measured at a wavelength of 610 nm by spectrophotometry. The concentration of EB extravasation in the ipsilateral hemisphere was calculated using a standard curve.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical analysis was performed using Prism 8.0 (GraphPad Software, USA). Statistical differences were assessed by Student's t-test for two-group comparisons or ANOVA followed by Tukey's test for multiple comparisons among more than two groups. $P \leq 0.05$ was considered statistically significant.

Results

Hypothermia protects against I/R in mice

To examine the effect of hypothermia in IS, we generated SHG mice and MHG mice by using the NTR1 agonist HPI-201. Animals and experimental designs were showed above (Fig. 1A). Mice in different groups were subjected to 60-minute I/R or sham operation. Infarct size, infarct volume, and brain water content for every group were analyzed in the next 24 hours. Measurements of infarct size and infarct volume by TTC staining manifested that MCAO induced infarction size and infarction volume in both groups but significantly less in MHG mice compared to MNG mice (Fig. 1B and 1C and 1D). Consistently, brain water content measurements showed that brain edema caused by MCAO was significantly reduced in MHG mice compared to MNG mice (Fig. 1E). These results together suggested that hypothermia protects against IS.

Hypothermia inhibits activation of microglial cells and neuroinflammation after I/R in mice.

The activation of microglial cells and microglial cell-mediated neuroinflammation in ischemic brain tissues for every group were analyzed after I/R or sham operation 24 hours later. Immunofluorescence staining showed that I/R induced more Iba-1⁺ cells in the ipsilateral region in MNG mice and MHG mice compared to SHG mice and SHG mice (Fig. 2A and 2B). There were significantly fewer Iba-1⁺ cells detected in MHG mice compared to MNG mice (Fig. 2A and 2B). Accordingly, western blotting analysis

illustrated a significantly lower protein level of Iba-1 in ipsilateral brain tissues of MHG mice compared to MNG mice (Fig. 2C and 2D). Results of qRT-PCR analysis manifested that drastic neuroinflammation was detected in MNG mice and MHG mice (Fig. 2E and 2F). The expression of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, CXCL1, were substantially lower in MHG mice compared to MNG mice (Fig. 2E). Consistently, the expression of anti-inflammatory cytokines, including Arg-1, Fizz-1, Ym-1, CD206, were higher in MHG mice compared to MNG mice (Fig. 2F). These results together indicated that hypothermia attenuates the activation of microglial cells and microglial cell-mediated neuroinflammation after I/R.

Hypothermia reduces apoptosis of neuronal cells after I/R in mice.

The apoptosis of neuronal cells in ischemic brain tissues for every group was analyzed after I/R or sham operation 24 hours later. Tunnel staining showed that I/R led to more NeuN⁺ Tunnel⁺ cells in the ipsilateral region of MNG mice and MHG mice compared to SNG mice and SHG mice (Fig. 3A and 3B). More importantly, significantly fewer NeuN⁺ Tunnel⁺ cells were detected in MHG mice compared to MNG mice (Fig. 3A and 3B), suggesting reduced apoptosis of neuronal cells in MHG mice. Western blotting analysis showed a significantly higher protein level of Cleaved Caspase-3 and a lower protein ratio of Bcl-2/Bax in ipsilateral brain tissues of MHG mice and MNG mice compared to SNG mice and SHG mice (Fig. 3C, 3D and 3E). Consistently, the protein expression of Cleaved Caspase-3 was lower and the protein ratio of Bcl-2/Bax was higher in MHG mice compared to MNG mice (Fig. 3C, 3D, and 3E). Similarly, MHG mice manifested significantly higher neurological scores compared to MNG mice (Fig. 3F). These results together demonstrated that hypothermia alleviates apoptosis of neuronal cells after I/R.

Hypothermia attenuates BBB disruption after I/R in mice

The disruption of BBB in ischemic brain tissues for every group was analyzed after I/R or sham operation 24 hours later. EB staining manifested that I/R induced EB extravasation in ipsilateral hemispheres of MNG mice and MHG mice but significantly less in MHG mice compared to MNG mice (Fig. 4A and 4B). Western blotting analysis showed a significantly higher protein level of MMP-9 in ipsilateral brain tissues of MHG mice and MNG mice compared to SNG mice and SHG mice, and the expression of MMP-9 was lower in MHG mice than MNG mice (Fig. 4C and 4D). Consistently, higher protein expression of ZO-1 and Occludin were detected in ipsilateral brain tissues of MHG mice compared to MNG mice by western blotting (Fig. 4C, 4E, and 4F). These results together illustrated that hypothermia protects the integrity of tight junction in ipsilateral brain tissues and attenuates BBB disruption after I/R.

Hypothermia enhances the protein level of PPAR γ in mice after I/R and in BV2 microglial cells after OGD/R.

PPAR γ plays a critical role in IS ²⁰. The protein level of PPAR γ in ischemic brain tissues and BV-2 microglial cells for every group were analyzed after I/R or sham operation, and OGD/R or control treatment 24 hours later. Western blotting analysis showed a significantly lower protein level of PPAR γ in

ipsilateral brain tissues of MHG mice and MNG mice compared to SNG mice and SHG mice (Fig. 5A and 5B). The protein expression of PPAR γ was higher in MHG mice than in MNG mice (Fig. 5A and 5B). Similarly, OGD/R induced a lower protein level of PPAR γ in BV2 microglial cells (Fig. 5C and 5D). Consistently, hypothermia increases the protein expression of PPAR γ in BV2 microglial cells after OGD/R (Fig. 5C and 5D). Whereas the GW9662 (20 μ M), an inhibitor of PPAR γ , reversed the effect of hypothermia (Fig. 5C and 5D). These results together proved that hypothermia enhances the expression of PPAR γ after I/R and OGD/R.

Hypothermia promotes microglial cells polarized from pro-inflammatory phenotype to anti-inflammatory phenotype through PPAR γ after OGD/R

PPAR γ plays a vital role in microglia polarization²¹. To further investigate the role of PPAR γ in regulating the polarization of microglial cells after OGD/R, GW9662 was used to inhibit the activation of PPAR γ . Results of the qRT-PCR analysis showed that the pro-inflammatory cytokines, including IL-1 β , TNF- α , iNOS, CXCL1, were substantially increased after OGD/R, and hypothermia reduced the mRNA expressions of pro-inflammatory cytokines after OGD/R, whereas GW9662 reversed the function of hypothermia (Fig. 6A). Results of qRT-PCR analysis manifested that the anti-inflammatory cytokines, including Arg-1, Fizz-1, Ym-1, CD206, were substantially decreased after OGD/R, and hypothermia enhanced the mRNA expressions of anti-inflammatory cytokines after OGD/R (Fig. 6B). Consistently, the effect of hypothermia was converted by GW9662 (Fig. 6B). These results together elucidated that hypothermia shifts the polarization of microglial cells from pro-inflammatory phenotype to anti-inflammatory phenotype in BV2 microglial cells through PPAR γ after OGD/R.

Hypothermia suppresses the neurotoxic effect of pro-inflammatory BV2 microglial cells on co-cultured HT-22 neuronal cells through PPAR γ .

To further confirm that hypothermia indirectly reduced neuronal cells death through regulating the activation of PPAR γ in microglial cells, a microglia-neuron co-culture system was established (Fig. 7A). The results showed that pro-inflammatory BV2 microglial cells enhanced post-OGD/R HT-22 neuronal cells death, indicated by decreased cell viability and increased LDH release, which was alleviated in hypothermia-treated BV-2 microglial cells, and GW9662 reversed the effect of hypothermia (Fig. 7B and 7C). Western blotting analysis demonstrated a significantly higher protein level of Cleaved Caspase-3 and a lower protein ratio of Bcl-2/Bax after OGD/R (Fig. 7D and 7E and 7F). The protein expression of Cleaved Caspase-3 was lower and the protein ratio of Bcl-2/Bax was higher in hypothermia-treated BV-2 microglial cells, which GW9662 blocked the function of hypothermia (Fig. 7D, 7E, and 7F). These results together illustrated that hypothermia reduces the neurotoxic effect of pro-inflammatory microglial cells through upregulating PPAR γ after OGD/R.

Hypothermia alleviates the disruption of pro-inflammatory BV-2 microglial cells on co-cultured bEnd.3 endothelial cells through PPAR γ .

To explore that hypothermia indirectly alleviates endothelial cells disruption through modulating the activation of PPAR γ in microglial cells, microglia- endotheliocyte co-culture system was used (Fig. 8A). The results showed that pro-inflammatory BV2 microglial cells increased post-OGD/R bEnd.3 endothelial cells death, suggesting by decreased cell viability and increased LDH release, which was attenuated by hypothermia, and GW9662 converted the effect of hypothermia (Fig. 8B and 8C). Western blotting analysis illustrated a significantly lower protein level of ZO-1 and Occludin after OGD/R (Fig. 8D, 8E, and 8F). The protein expression of ZO-1 and Occludin was higher in hypothermia-treated BV-2 microglial cells, which GW9662 inhibited the impact of hypothermia (Fig. 8D, 8E, and 8F). These results together indicated that hypothermia decreases the disruption of endothelial cells by upregulating PPAR γ in microglial cells after OGD/R.

Discussion

Although hypothermia has been reported to play beneficial roles in different diseases, the cellular mechanism can be multifaceted and the underlying cellular mechanism remains unclear. In this study, we demonstrated for the first time that hypothermia significantly protected against I/R and OGD/R through upregulating the activation of PPAR γ in microglial cells.

Previous studies have suggested that hypothermia can be effective for cerebrovascular diseases. For example, it has been reported that hypothermia attenuates neuroinflammation by modulating microglia polarization through upregulating the expression of IRF4 after MCAO ²². Hypothermia inhibits activation of microglial cells by modulating autophagy/apoptosis and the MyD88-dependent TLR4 signaling pathway after traumatic brain injury ²³. Moreover, hypothermia reduces death or disability with moderate-severe hypoxic-ischemic encephalopathy infants in the near term ²⁴. Our results provided new evidence that hypothermia treatment was a feasible strategy for treating IS via the PPAR γ signaling pathway.

Suppression on neuroinflammation may be the fundamental mechanism underlying the protective effects on IS ²⁵. Microglial cells are widely activated by damage-associated molecular patterns released from injured neuronal cells, and subsequently, mediate drastic neuroinflammation during the acute stage of IS ²⁶. Previous studies have reported that deficiency of microglial cells inhibits the microglial cell-mediated neuroinflammation, ultimately leading to the alleviation of IS in mouse models ²⁷. Our data showed that hypothermia suppressed the activation of microglial cells and shifts the polarization of microglial cells from pro-inflammatory phenotype to anti-inflammatory phenotype.

Activated microglial cells produce pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 during the acute phase of IS ²⁸. Apoptosis of neuronal cells and disruption of endothelial cells following ischemia and hypoxia is a major cause of pathological processes in IS ²⁹. The cytokines can cross-talk with neuronal cells and endothelial cells, aggravating the apoptosis of neuronal cells and disruption of endothelial cells ³⁰. Previous studies have illustrated that rosiglitazone inhibits MCAO-induced microglial cell-mediated neuroinflammation and reduces apoptosis of neuronal cells ³¹. Integrin $\alpha 5 \beta 1$ inhibition by

ATN-161 reduces neuroinflammation and attenuates the disruption of BBB, ultimately decreases the infarct size³². Our data showed that PPAR γ was a vital regulator of the connection between microglial cell-mediated neuroinflammation and apoptosis of neuronal cells or endothelial cells in hypothermia. PPAR γ was upregulated in hypothermia and plays an essential role in reducing apoptosis of neuronal cells and disruption of endothelial cells. Studies in more depth are required to determine how PPAR γ inhibits the neuroinflammation in hypothermia after I/R and OGD/R.

Transcriptional regulation is an essential biological process in cells. PPARs are ligand-activated transcription factors that regulate genes essential to various biological processes³³. PPAR γ can inhibit transcription factors, such as Stat 1 and nuclear factor-kB^{34–35}. PPAR γ regulates the activation of microglia/macrophages by increasing the release of anti-inflammatory factors and decreasing the release of pro-inflammatory factors³⁶. Previous studies have suggested that rosiglitazone, an agonist of PPAR γ , alleviates neuroinflammation through PPAR γ -independent mechanisms, ultimately leading to the protection of IS in a mouse model³⁷. In this study, we illustrated that hypothermia protected against IS by activating PPAR γ in microglial cells. Future studies investigating the function of PPAR γ in hypothermia may provide more mechanistic insights on IS treatment.

In summary, hypothermia plays a beneficial role in IS treatment through its downregulation of neuroinflammation, apoptosis of neuronal cells, and disruption of endothelial cells through upregulating PPAR γ in microglial cells. These data have unveiled the effects of hypothermia in the setting of IS and have explored the mechanisms.

Conclusion

In the present study, the microglial cells could be activated by I/R and OGD/R, and hypothermia could inhibit the number of activated microglial cells and microglial cell-mediated neuroinflammation by upregulating PPAR γ . In addition, hypothermia reduced apoptosis of neuronal cells and attenuated BBB disruption. This study preliminarily concluded the possible mechanism for the participation of PPAR γ in the neuroprotective effect of hypothermia and provided a new method for the treatment of IS. Further studies are required to deeply explore the effect of hypothermia on PPAR γ after IS and the underlying mechanism.

Abbreviations

IS, ischemic stroke;

PPAR γ , peroxisome-proliferator-activated-receptor gamma;

I/R, ischemia/reperfusion;

tPA, tissue plasminogen activator;

MCAO, middle cerebral artery occlusion;

OGD/R, oxygen-glucose deprivation/ reperfusion;

SNG, sham injury with normothermia group;

SHG, sham injury with hypothermia group;

MNG, MCAO with normothermia group;

MHG, MCAO with hypothermia group;

TTC, triphenyl tetrazolium chloride;

Iba-1, Ionized calcium binding adaptor molecule-1;

Tunnel, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling;

MMP-9, matrix metalloprotein;

Bax, BCL2-associated X protein;

BBB, blood-brain-barrier;

MMP-9, matrix metalloprotein-9;

ZO-1, zonula occludens 1.

Declarations

Acknowledgements

Not applicable.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81971093), Science and Technology Commission of Shanghai Municipal (21ZR1439000).

Availability of data and materials

All data generated or analyzed during this study are included in the manuscript submission.

Authors' contributions

SS, YLC, and JSZ designed experiments; XHZ and FJ helped design the study; SS, YLC, and DD performed experiments and analyzed data; SS and KC wrote the manuscript; KC, XHZ, and FJ supervised and

coordinated the study.

Ethics approval

Animal protocols were approved by the Animal Care and Experimental Committee of the School of Medicine of Shanghai Jiao Tong University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

Author Details

¹Department of Neurosurgery, Ren-Ji Hospital, School of Medicine, Shanghai Jiao Tong University, No. 160 Pujian Road, Shanghai 200127, People's Republic of China. ²Department of Neurosurgery, Changzheng Hospital, The Second Military Medical University, No. 415 Fengyang Road, Shanghai 200003, People's Republic of China. ³Department of Neurosurgery, Shanghai East Hospital, Tongji University School of Medicine, No. 150 Jimo Road, Shanghai 200120, People's Republic of China.

Contributor Information

Kui Chen, Email: Dr_Chen2017@163.com.

Xiaohua Zhang, Email: zxh1969@aliyun.com.

Feng Jia, Email: projiafeng@163.com.

References

1. Campbell BCV, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, et al. Ischaemic stroke. *Nat Rev Dis Primers*. 2019;10;5(1):70.
2. Feigin VL, Nguyen G, Cercy K, Johnson CO, Alam T, Parmar PG, et al. Global, Regional, and Country-Specific Lifetime Risks of Stroke, 1990 and 2016. *N Engl J Med*. 2018;379(25):2429-2437.
3. Prabhakaran S, Ruff I, Bernstein RA. Acute stroke intervention: a systematic review. *JAMA*. 2015;313(14):1451-62.
4. Tsivgoulis G, Katsanos AH, Schellinger PD, Köhrmann M, Varelas P, Magoufis G, et al. Successful Reperfusion With Intravenous Thrombolysis Preceding Mechanical Thrombectomy in Large-Vessel Occlusions. *Stroke*. 2018;49(1):232-235.

5. Lou M. Can imaging extend the thrombolytic time window after stroke? *Nat Rev Neurol*. 2019;15(9):496-498.
6. Liu ZJ, Ran YY, Qie SY, Gong WJ, Gao FH, Ding ZT, et al. Melatonin protects against ischemic stroke by modulating microglia/macrophage polarization toward anti-inflammatory phenotype through STAT3 pathway. *CNS Neurosci Ther*. 2019;25(12):1353-1362.
7. Kelly PJ, Murphy S, Coveney S, Purroy F, Lemmens R, Tsivgoulis G, et al. Anti-inflammatory approaches to ischaemic stroke prevention. *J Neurol Neurosurg Psychiatry*. 2018;89(2):211-218.
8. Ma Y, Wang J, Wang Y, Yang GY. The biphasic function of microglia in ischemic stroke. *Prog Neurobiol*. 2017;157:247-272.
9. Qin C, Zhou LQ, Ma XT, Hu ZW, Yang S, Chen M, et al. Dual Functions of Microglia in Ischemic Stroke. *Neurosci Bull*. 2019 Oct;35(5):921-933.
10. Xiong XY, Liu L, Yang QW. Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke. *Prog Neurobiol*. 2016;142:23-44.
11. Eldahshan W, Fagan SC, Ergul A. Inflammation within the neurovascular unit: Focus on microglia for stroke injury and recovery. *Pharmacol Res*. 2019;147:104349.
12. Zhao Y, Wei ZZ, Lee JH, Gu X, Sun J, Dix TA, et al. Pharmacological hypothermia induced neurovascular protection after severe stroke of transient middle cerebral artery occlusion in mice. *Exp Neurol*. 2020;325:113133.
13. Lee JH, Wei L, Gu X, Wei Z, Dix TA, Yu SP. Therapeutic effects of pharmacologically induced hypothermia against traumatic brain injury in mice. *J Neurotrauma*. 2014 15;31(16):1417-30.
14. Rocha-Ferreira E, Poupon L, Zelco A, Leverin AL, Nair S, Jonsdotter A, et al. Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy. *Brain*. 2018;141(10):2925-2942.
15. Kurisu K, Yenari MA. Therapeutic hypothermia for ischemic stroke; pathophysiology and future promise. *Neuropharmacology*. 2018;134(Pt B):302-309.
16. Villapol S. Roles of Peroxisome Proliferator-Activated Receptor Gamma on Brain and Peripheral Inflammation. *Cell Mol Neurobiol*. 2018;38(1):121-132.
17. Lee JH, Wei ZZ, Cao W, Won S, Gu X, Winter M, et al. Regulation of therapeutic hypothermia on inflammatory cytokines, microglia polarization, migration and functional recovery after ischemic stroke in mice. *Neurobiol Dis*. 2016;96:248-260.
18. Jiang J, Dai J, Cui H. Vitexin reverses the autophagy dysfunction to attenuate MCAO-induced cerebral ischemic stroke via mTOR/Ulk1 pathway. *Biomed Pharmacother*. 2018;99:583-590.
19. Huang L, Tang H, Sherchan P, Lenahan C, Boling W, Tang J, et al. The Activation of Phosphatidylserine/CD36/TGF- β 1 Pathway prior to Surgical Brain Injury Attenuates Neuroinflammation in Rats. *Oxid Med Cell Longev*. 2020;2020:4921562.
20. Cai W, Yang T, Liu H, Han L, Zhang K, Hu X, et al. Peroxisome proliferator-activated receptor γ (PPAR γ): A master gatekeeper in CNS injury and repair. *Prog Neurobiol*. 2018 Apr-May;163-164:27-58.

21. Zhou D, Ji L, Chen Y. TSP0 Modulates IL-4-Induced Microglia/Macrophage M2 Polarization via PPAR- γ Pathway. *J Mol Neurosci.* 2020;70(4):542-549.
22. Yu X, Feng Y, Liu R, Chen Q. Hypothermia Protects Mice Against Ischemic Stroke by Modulating Macrophage Polarization Through Upregulation of Interferon Regulatory Factor-4. *J Inflamm Res.* 2021;14:1271-1281.
23. Zhang F, Dong H, Lv T, Jin K, Jin Y, Zhang X, et al. Moderate hypothermia inhibits microglial activation after traumatic brain injury by modulating autophagy/apoptosis and the MyD88-dependent TLR4 signaling pathway. *J Neuroinflammation.* 2018;15(1):273.
24. Wassink G, Davidson JO, Dhillon SK, Zhou K, Bennet L, Thoresen M, et al. Therapeutic Hypothermia in Neonatal Hypoxic-Ischemic Encephalopathy. *Curr Neurol Neurosci Rep.* 2019;19(2):2.
25. Jayaraj RL, Azimullah S, Beiram R, Jalal FY, Rosenberg GA. Neuroinflammation: friend and foe for ischemic stroke. *J Neuroinflammation.* 2019;16(1):142.
26. Kim E, Cho S. Microglia and Monocyte-Derived Macrophages in Stroke. *Neurotherapeutics.* 2016;13(4):702-718.
27. Li T, Zhao J, Xie W, Yuan W, Guo J, Pang S, et al. Specific depletion of resident microglia in the early stage of stroke reduces cerebral ischemic damage. *J Neuroinflammation.* 2021;18(1):81.
28. Xu S, Lu J, Shao A, Zhang JH, Zhang J. Glial Cells: Role of the Immune Response in Ischemic Stroke. *Front Immunol.* 2020;11:294.
29. Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol.* 1998 Oct;56(2):149-71.
30. McDonough A, Weinstein JR. Neuroimmune Response in Ischemic Preconditioning. *Neurotherapeutics.* 2016;13(4):748-761.
31. Han L, Cai W, Mao L, Liu J, Li P, Leak RK, et al. Rosiglitazone Promotes White Matter Integrity and Long-Term Functional Recovery After Focal Cerebral Ischemia. *Stroke.* 2015 Sep;46(9):2628-36.
32. Edwards DN, Salmeron K, Lukins DE, Trout AL, Fraser JF, Bix GJ. Integrin $\alpha 5 \beta 1$ inhibition by ATN-161 reduces neuroinflammation and is neuroprotective in ischemic stroke. *J Cereb Blood Flow Metab.* 2020;40(8):1695-1708.
33. Wagner N, Wagner KD. The Role of PPARs in Disease. *Cells.* 2020;9(11):2367.
34. Kotla S, Singh NK, Rao GN. ROS via BTK-p300-STAT1-PPAR γ signaling activation mediates cholesterol crystals-induced CD36 expression and foam cell formation. *Redox Biol.* 2017;11:350-364.
35. Li Q, Sun J, Mohammadtursun N, Wu J, Dong J, Li L. Curcumin inhibits cigarette smoke-induced inflammation via modulating the PPAR γ -NF- κ B signaling pathway. *Food Funct.* 2019;10(12):7983-7994.
36. Bouhlel MA, Derudas B, Rigamonti E, Dièvert R, Brozek J, Haulon S, et al. PPAR γ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* 2007;6(2):137-43.

37. Li Y, Zhu ZY, Lu BW, Huang TT, Zhang YM, Zhou NY, et al. Rosiglitazone ameliorates tissue plasminogen activator-induced brain hemorrhage after stroke. *CNS Neurosci Ther.* 2019;25(12):1343-1352.

Figures

Figure 1

Hypothermia protects against I/R in mice. (a) Animals and experimental designs. (b) Representative TTC staining in cerebral sections 24 hours after sham operation or I/R for every group. (c) Quantification of infarct size in total brain hemisphere. (d) Quantification of infarct volume in serial coronal sections. (e) Quantification of brain water content in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. Values are presented as Mean $\pm$ SD, n=6 per group. ns: not significant. *P < 0.05, **P < 0.01, ***P<0.001.

Figure 2

Hypothermia inhibits activation of microglial cells and inflammation after I/R in mice. (a) Representative immunofluorescence staining of Iba-1+ in ischemic ipsilateral brain sections 24 hours after sham operation or I/R for every group. Scale bar: 50μm, magnification: 400×. (b) Quantification analyses of the percentage of Iba-1 positive cells in total cells. (c) Representative western blotting analysis of Iba-1 in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. GAPDH was used as a loading control. (d) Quantifications of the expression of Iba-1. (e) qRT-PCR analysis of M1 activated microglial cells markers in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. All gene expression was normalized to GAPDH. (f) qRT-PCR analysis of M2 activated microglial cells markers in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. All gene expression was normalized to GAPDH. Values are presented as Mean ± SD, n=6 per group. ns: not significant. *P < 0.05, **P < 0.01, ***P<0.001.

Figure 3

Hypothermia reduces apoptosis of neuronal cells after I/R in mice. (a) Representative Tunnel staining of neuronal cells in ipsilateral brain sections 24 hours after sham operation or I/R for every group. Magnifications of the boxed areas are shown by the inset images. Scale bar: 50µm. (B) Quantitative analyses of the percentage of NeuN⁺ Tunnel⁺ cells in NeuN⁺ cells. (c) Representative western blotting analysis of Cleaved Caspase-3, Bcl-2, and Bax in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. α -tubulin was used as a loading control. (d) Quantifications of the expression of Cleaved Caspase-3. (e) Quantifications of the expression ratio of Bcl-2/Bax. (f) Neurological scores 24 hours after sham operation or I/R for every group. Values are presented as Mean $\pm$ SD, n=6 per group. ns: not significant. **P < 0.01, ***P<0.001.

Figure 4

Hypothermia attenuates BBB disruption after I/R in mice. (a) Representative photographs of EB extravasation 24 hours after sham operation or I/R for every group. (b) Quantifications of EB extravasation. (c) Representative western blotting analysis of matrix metalloprotein-9 (MMP-9), ZO-1, and Occludin in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. GAPDH was used as a loading control. (d) Quantifications of the expression of MMP-9. (e) Quantifications of the expression of ZO-1. (f) Quantifications of the expression of Occludin. Values are presented as Mean $\pm$ SD, $n=6$ per group. ns: not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5

Hypothermia enhances the expression of PPAR γ in mice after I/R and BV2 microglial cells after OGD/R. (a) Representative western blotting analysis of PPAR γ in ipsilateral brain tissues 24 hours after sham operation or I/R for every group. GAPDH was used as a loading control. (b) Quantifications of the expression of PPAR γ . (c) Representative western blotting analysis of PPAR γ in BV-2 microglial cells 24 hours after with or without 3h-OGD for every group. Hypothermia and GW9662 (20 μ M) were treated in the next 24 hours of reperfusion respectively. GAPDH was used as a loading control. (d) Quantifications of the expression of PPAR γ . n=6 per group for a-b; experiments were repeated for 4 times for c-d. Values are presented as Mean $\pm$ SD. ns: not significant. *P < 0.05, **P < 0.01, ***P<0.001.

Figure 6

Hypothermia promotes microglial cells polarized from pro-inflammatory phenotype to anti-inflammatory phenotype through PPAR γ after OGD/R. (a) qRT-PCR analysis of M1 activated microglial cells markers 24 hours after with or without 3h-OGD for every group. All genes expression was normalized to GAPDH. Hypothermia and GW9662 (20 μ M) were treated in the next 24 hours of reperfusion respectively. (b) qRT-PCR analysis of M2 activated microglial cells markers 24 hours after with or without 3h-OGD for every group. All genes expression was normalized to GAPDH. Hypothermia and GW9662 (20 μ M) were treated in the next 24 hours of reperfusion respectively. Experiments were repeated for 4 times. Values are presented as Mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P<0.001.

Figure 7

Hypothermia suppresses the neurotoxic effect of pro-inflammatory BV2 microglial cells on co-cultured HT-22 neuronal cells through PPAR γ . (a) The strategy of the co-cultured system. (b) Quantifications of cell ability for every group. (c) Quantifications of LDH release ratio for every group. (d) Representative western blotting analysis of Cleaved Caspase-3, Bcl-2, and Bax for every group. GAPDH and α -tubulin were used as the loading control. (e) Quantifications of the expression of Cleaved Caspase-3. (f) Quantifications of

the expression ratio of Bcl-2/Bax. Experiments were repeated for 4 times. Values are presented as Mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P<0.001.

Figure 8

Hypothermia alleviates the disruption of pro-inflammatory BV-2 microglial cells on co-cultured bEnd.3 endothelial cells through PPAR γ . (a) The strategy of the co-cultured system. (b) Quantifications of cell ability for every group. (c) Quantifications of LDH release ratio for every group. (d) Representative western

blotting analysis of ZO-1 and Occludin for every group. GAPDH was used as a loading control. (e) Quantifications of the expression of ZO-1. (f) Quantifications of the expression of Occludin. Experiments were repeated for 4 times. Values are presented as Mean $\pm$ SD. ns: not significant. *P < 0.05, **P < 0.01, ***P<0.001.