Activation of PPARβ/δ by DHA induces VEGF-A expression to promote angiogenesis in the cerebral ischemia penumbra

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Abstract

The ischemia penumbra is an area of brain tissue where is damaged but not yet dead after ischemic stroke,it has the potential for functional recovery provided if local blood flow can be reestablished.Docosahexaenoic acid (DHA) is protective after experimental ischemic stroke.To explore DHA promote angiogenesis underlying mechanisms of angiogenesis in the cerebral ischemia penumbra, Male Sprague-Dawley rats underwent 2h of middle cerebral artery occlusion (MCAo) and treated with DHA (5mg/kg, IV) 1h after.Neuro-behavioral assessments and 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining was used to assess the cerebral infarction volume on hours 6, 12, 24,48 and 72, PPARβ/δ、VEGF-A、VEGFR2 and P-VEGFR2 expression were measured by Western blotting、brain tissue expressions of TNF-α, IL-1β and NF-κB were assessed using ELISA, NeuN、CD31、α-sma cells in the ischemia penumbra were analyzed using immunofluorescenceon hour 72 after onset of stroke. Post-treatment with DHA ameliorated neurological defects, diminished cerebral infarction volume, enhanced neuronal survival, downregulated inflammation、promoted the expression of angiogenesis related proteins and protected ischemic penumbra by increasing CD31、α-sma cells and vessels .On the contrary, this promote angiogenesis in the cerebral ischemia penumbraprotective effect was reversed after DHA cotreatment with GSK0660, a selective antagonist of the PPARβ/δ.Finally, our experiment clearly demonstrating DHA protect the rat cerebral ischemic injury (MCAO) model, more importantly, DHA was first demonstrated to promote ischemic penumbra angiogenesis through the PPARβ/δ pathway.

Introduction

Acute ischemic stroke (AIS) has a high morbidity, mortality and disability rate. It is estimated that AIS causes 6.5 million deaths per year worldwide and is expected to result in the loss of more than 200 million people per year by 2030, which places a heavy burden on the world economy[1].In the development of AIS the ischemic core is irreversibly damaged tissue distal to the occluded vessel, characterized by < 20% baseline blood flow levels, depletion of ATP stores, and failure of energy metabolism, while the cerebral ischemia penumbra represents damaged but viable brain tissue located distal to the occluded vessel[2].Currently, intravenous recombinant tissue plasminogen activator (t-PA) is an effective treatment for acute ischemic stroke.In addition, endovascular mechanical embolization is also an effective route for AIS treatment. The former is limited by the narrow time window of administration and more contraindications; while the latter is constrained from the level of technology, economic conditions, and regional differences, which reduces the number of beneficiaries[3–4].Peroxisome proliferator-activated receptor (PPAR) is a ligand-regulated transcription factor with three nuclear receptor isoforms encoded by different genes, PPAR γ, PPAR α, and PPARβ/δ. Among them, PPARβ/δ is expressed in all brain cells and seems to have the highest expression in neurons, and PPAR-β/δ plays an important role in the pathology of central nervous system hypoxia-ischemia, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis (MS) [5].Different from PPARα or PPARγ in inhibiting angiogenesis, PPARβ/δ is a major pro-angiogenic signaling molecule[6]. Pharmacological activation of PPARβ/δ in rat corneal endothelial cells has been

shown to increase angiogenesis in these cells. Activated PPARβ/δ promotes neointima formation mainly by upregulating VEGF-A expression, at the same time decreases tumor necrosis factor TNF-α, interleukin-1β (IL-1β) and NF-κB[7–8].Meanwhile, other related studies have shown that PPARβ/δ induces VEGF-A in bladder, breast and prostate cancers[9–10]. The vascular endothelial-derived growth factor VEGF-A and its major receptor VEGFR-2 increase angiogenesis not only under normal physiological conditions, but also under most pathological conditions[11].However, in acute ischemic stroke, whether activated PPARβ/δ can mediate VEGF-A is unclear. Docosahexaenoic acid (DHA; 22:6, n = 3) is a member of the essential omega-3 fatty acid family, an important component of the cell membranes of the central nervous system and a natural activator of PPARβ/δ[12].Recent studies have demonstrated that docosahexaenoic acid treatment after cerebral ischemia in rats improved neurological function, reduced infarct volume, attenuated BBB permeability, and protected ischemic cells[13–14].Although DHA administration intervention, in terms of postischemic neuroprotective effects, is well established, its effect on cerebral ischemia penumbra angiogenesis and its underlying mechanisms remain unclear. Therefore, we hypothesized that DHA promotes ischemic penumbra angiogenesis after acute ischemic stroke through activation of PPARβ/δ, possibly via the VEGF-A pathway.

Materials And Methods

Animals

All experiments were conducted in accordance with the guidelines of the China Institutional Animal Care and Use Committee and approved by the Ethics Committee for the Use of Laboratory Animals of Weifang Medical College. SPF-grade 8-week-old healthy Sprague-Dawley male rats (270 to 300 g; number SCXK (Lu) 2019-0003, Jinan Pengyue Animal Experimental Breeding Co.)The rearing environment was maintained at 12 h/12 h light/dark cycle, 21 ± 2°C, standard food and ad libitum water.

Establishment of the Middle Cerebral Artery Occlusion/Reperfusion (MCAO/R) Rat Model□Drug administration and experimental grouping

The SD rats were randomized into the following four groups: Sham-operated + vehicle, middle cerebral artery occlusion/reperfusion (MCAO/R) + vehicle, MCAO/R + DHA + vehicle and MCAO/R + DHA + GSK0660 + vehicle and treated accordingly. DHA (Sigma, St. Louis, USA) was dissolved in 2% DMSO ((Sigma, St. Louis, USA) and administered at (5 mg/kg,iv) to MCAO/R + DHA + DMSO group rats at 1 hour of reperfusion.GSK0660 (MCE,USA) dissolved in 2% DMSO was administered (1 mg/kg,ip) to rats in the MCAO/R + DHA + GSK0660 + DMSO (1 hour earlier than DHA). The rats in the remaining groups were given equal volumes of equal concentrations of DMSO at the same time points and in the same manner of administration.Fasting overnight prior to the procedure with free access to water. Anesthesia was induced by inhalation of a mixture of 70% nitrous oxide and 30% oxygen in 3% isoflurane. During the procedure, isoflurane was kept at 1.5% and the ratio of nitrous oxide to oxygen was kept constant.The MCAO/R model was established as described[15].Briefly, the right common carotid artery (CCA), right internal carotid artery (ICA), and right external carotid artery (ECA) were exposed through a midline carotid

incision, and the branches of the ECA were ligated and disconnected. A nylon monofilament with a rounded tip (Guangzhou Jialing Biotechnology Co., Ltd., China) was inserted along the stump of the ECA into the ICA until a slight resistance was detected. 2 hours after MCAO, the nylon monofilament was removed, and then rats at different observation time points completed reperfusion at 6 h, 12 h, 24 h, 48 h and 72 h after MCAO. In the sham-operated group of rats, the surgical procedure was identical except that no nylon monofilament were inserted.

Cerebral blood flow measurement

To evaluate the success of the MCAO model, cerebral blood flow (CBF) was measured by the RFLSI III system (RWD, China), a laser scattering system. Rats were anesthetized with the protocol described above. Laser scanning imaging measurements were performed on the skull after dental drill polishing. Real-time CBF changes were recorded every 1 minute using the RFLSI III system placed approximately 10 cm above the brain. From the obtained measurements, relative ipsilateral:contralateral CBF ratios were calculated[16].

Neurological assessment

Neurological function was assessed using the modified neurological severity scale (mNSS) at 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours after reperfusion. Neurological function is scored on a 0–18 scale (normal score 0, maximum deficit score 18).

Infarct Volume Measurement

At the same five time points mentioned above, rats were euthanized and dissected rapidly to obtain brain tissue, which was frozen at -20°C for 20 min and then cut into 2-mm-thick coronal sections. Brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, USA) in PBS for 20 min at 37°C. Normal brain tissue was stained dark red, and infarcted brain tissue was white. Then it was fixed in 4% paraformaldehyde for 2 hours. Digital camera photographs were taken and calculated using ImageJ software (USA), ischemic area volume ratio = (sum of area of white ischemic area in each section)/(sum of area of brain slices in each section) x 100%.

Hematoxylin-Eosin Staining

After 72 hours of reperfusion, rats were anesthetized in the same manner as described above, perfused with 0.9% cold saline via the heart, and then the whole brain was rapidly removed from the skull, fixed in 4% paraformaldehyde, paraffin-embedded, and cut into 2-μm-thick coronal sections in the 2-mm area behind the fontanelle, and the brain sections were stained with hematoxylin-eosin (HE; Sigma, St. Louis, USA)). Six random fields of view were captured in the ischemic rim region of each tissue sample using light microscopy. Images of the injured cortex were observed under high-power fields (magnification, ×200, ×400).

Western Blotting

After 72 hours of reperfusion, rats were anesthetized in the same way as described above, perfused with 0.9% cold saline solution via the heart, brains were removed, and peri-ischemic brain tissue was dissected out and homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors and centrifuged at 15,000 rpm for 20 minutes at 4°C to extract peri-ischemic cortical proteins.

Protein concentrations were measured using a BCA assay kit (Thermo Fisher Scientific, USA). Equal amounts (50 μg) of each sample were separated by SDS-PAGE before transferring to PVDF membranes (Immobilon, USA), then the membranes were blocked with 5% nonfat dry milk 90 minutes. The membranes were mixed with anti-PPARβ/δ (1:1,000, Abcam, USA), VEGF-A (1:1,000, Abcam USA), p-VEGF-A (1:1,000, Abcam, USA), anti-β-actin (1:,5000, Abcam,USA), incubated overnight at 4°C. After washing with TBST and incubation with secondary antibody, immunoreactive bands were observed by ECL chemiluminescence. Specific bands were analyzed using ImageJ software.

Measurement of NF-κB、IL-1β and TNF-α levels

After 72 h of reperfusion, rats were anesthetized in the same manner as described above, perfused transcardially with 0.9% cold saline solution, and the ischemic brain tissue was immediately dissected out on a cooled ice pack (-20°C),and then analyzed for NF-κB, IL-1β, and TNF-α activity according to the manufacturer's instructions (MEIMIAN, China).

Immunofluorescent Staining

Paraffin sections were prepared by reperfusion for 3 days.Briefly, brain tissues were obtained by cold saline perfusion of the heart, reperfused with 4% paraformaldehyde, and the intact brain tissue was removed and fixed with 4% paraformaldehyde for 2 days and transferred to 30% sucrose solution. Subsequently, the tissues were frozen and cut into frozen sections (2-μm thick in the 2–5 mm area behind the fontanelle) at -20°C. Slices were washed with 0.01 M PBS for 5 min, incubated overnight at 4°C with anti-blocking protein (1:100, Abcam, USA), and then incubated with NeuN (1:100, Thermo Fisher Scientific, USA) antibody; After washing with PBS, tissues were incubated with secondary antibodies; CD31 and α-smooth muscle actin (α-SMA) immunofluorescent double staining, slices were incubated overnight at 4°C with anti-blocking protein (1:100, Abcam, USA), and then incubated with CD31 (1:100, Thermo Fisher Scientific, USA) antibody; After rinsed with PBS, the sections were incubated with secondary antibody for 2 hours without light exposure and then with α-SMA (1:100, Thermo Fisher Scientific, USA) antibody overnight at 4°C after rinsed again, and all sections were photographed with a 4',6-diamidino-2-phenylindole (DAPI, Beyotime, China) compound-stained, autofluorescence microscope (PANNORAMIC DESK/MIDI/250/1000, 3DHISTECH, Hungary). The angiogenesis was evaluated by new vessels expressed by CD31/α-SMA double-positive cells.The number of immunoreactive cells in predefined areas was quantified using CaseViewer2.4 software (3DHISTECH, Hungary) and Image-Pro Plus 6.0 software(Media Cybemetics,U.S.A). Cells were counted in six different fields of view for each rat in each group of five rats.Images of the cerebral ischemia penumbra were observed under high-power fields (magnification, ×400).

Statistical Analysis

All data were expressed as Mean ± SEM. The Student's t-test was used to compare the two groups, and a one-way analysis of variance (ANOVA) was used for multiple comparisons. All statistical analyses were conducted using a GraphPad Prism 8. P-values < 0.05 were considered statistically significant.

Results

Effects of MCAO/R on local cerebral blood flow

To assess the hemodynamic effects of MCAO/R, we used the RFLSI III system (RWD Life Science Co., Ltd.) to analyze local CBF in ipsilateral and contralateral tissues. We used the ipsilateral: contralateral rCBF ratio to assess the effect of reduced blood flow before, during, and after stroke. As shown in figure (A - B) 1 h after MCAO, P < 0.001 Vs 1 hour before MCAO, and figure (B-C) 1 h after Ischemia-reperfusion, &&&P < 0.001 Vs 1 hour after MCAO.significant differences were observed at pre-, during, and post-MCAO, while indicated that the cerebral ischemia animal model was established successfully.

DHA improves neurological deficits

To determine the effect of DHA on neurological deficits, neurological scores of rats were assessed using the mNSS at 6 h, 12 h, 24 h, 48h and 72h after reperfusion. mCAO/R rats had poorer neurological function compared to the sham-operated group; DHA treatment significantly improved neurological function at all time points after reperfusion compared to the MCAO/R group, while GSK0660 reversed the protective effect (as shown, *** P < 0.001, ###P < 0.01, &P < 0.05). In conclusion, DHA ameliorated neurological deficits in MCAO/R rats.

DHA treatment reduced infarct volume

Infarct volumes were measured by TTC staining at different time points after reperfusion (as shown, *** p < 0.001, ### P < 0.01, &&&P < 0.05), which indicated that the sham-operated group did not have any cerebral ischemic infarction, but the MCAO/R had significant ischemic injury in the model group.DHA treatment significantly reduced infarct volumes, and GSK0660 reversed this situation. Thus, DHA treatment prevented cerebral MCAO/R injury.

Hematoxylin-Eosin Staining

HE staining showed that the cortical neurons of Sham group were arranged regularly with complete morphology, and the nuclei were large and round with clear outline and obvious nucleoli. In the MCAO group, the ischemic cortical neurons showed atrophic morphology, accompanied by nuclear pyknosis, fragmentation and dissolution. Compared with MCAO group, the ischemic cerebral cortex in DHA group had intact histopathological profile, obvious nucleoli, and a few neurons showed atrophic morphology and nuclear pyknosis. Compared with DHA group, GSK0660 group had more pathological changes in ischemic cerebral cortex.

DHA potentiated PPARβ/δ/VEGF-A/ Vegfr2 signaling pathway activation after stroke

DHA activating PPARβ/δ significantly ,in addition to increased the levels of VEGF-A, Vegfr2, and p-Vegfr2 in MCAO/R rats. Furthermore, GSK0660 reversed these effects of DHA (*** $P < 0.05$, ### $P < 0.001$, and &&&$P < 0.01$). Thus, DHA exerts on MCAO/R rats by modulating the PPARβ/δ/VEGF-A/Vegfr2 axis and increasing the level of p-Vegfr2 expression.

DHA inhibits MCAO/R-induced inflammatory response

DHA significantly reduced the levels of inflammatory cytokines TNF-α, IL-1β and pro-inflammatory transcription factor NF-κB in MCAO/R rats, in addition to activating PPARβ/δ. Thus, DHA exerts anti-inflammatory effects in MCAO/R rats.In addition, GSK0660 did not significantly reverse the anti-inflammatory effects of DHA in the study.

DHA promoted angiogenesis in the ischemic penumbra of MCAO rats

DHA promotes endothelial regeneration and protects neurons

Double-labelling immunofluorescence assay indicated that the numbers of CD31 and α-SMA double-positive cells increased slightly in MCAO compared with sham group,meanwhile the numbers of CD31 and α-SMA double-positive cells were increased in ischaemic cerebral cortex tissues of the DHA group, which was high than those in the MCAO group.Single-labelling immunofluorescence assay indicated thatThe NeuN + cells of MCAO group decreased significantly after stroke;and DHA protects NeuN + cells compared to MCAO group.However, GSK0660 reversed these effects of DHA.

Discussion

After ischaemic stroke, brain damage can be curtailed by rescuing the 'cerebral ischemia penumbra' - that is, the severely hypoperfused, at-risk but not yet infarcted tissue[17]. The presence of an cerebral ischemia penumbra forming an irregular rim around the ischemic core and a decrease in local cerebral blood flow LCBF is the main factor causing necrotizing injury, usually this area (LCBF) decreases to 20–40% of the control group[18].If local blood flow can be re-established, the cerebral ischemia penumbra has the potential for functional recovery, but without adequate reperfusion, irreversible damage will occur, depending on the interaction of ischemic severity and duration, and timely and effective protection of the cerebral ischemia penumbra is thus possible for a specific phase of treatment strategy[19].Cerebral ischemia-reperfusion increases intracellular calcium and activates several degradative enyzmes including phospholipases A2, resulting in the rapid accumulation of free docosahexaenoic acid (DHA; 22:6, n-3) .DHA is involved with brain development, aging, memory, synaptic membrane function, photoreceptor function, and neuroprotection,Treatment with DHA improves behavioral outcomes, reduces infarct volumes, and decreases mortality in focal cerebral ischemia in rats when administered within five hours of stroke onset[20].This is consistent with our experimental results that application of DHA significantly reduced neurological function scores, infarct size, and neuronal damage.Although related experiments

confirm that DHA promotes Penumbra protection and angiogenesis, and neurobehavioral recovery After Experimental Ischemic Stroke[14].However, the specific molecular mechanism of angiogenesis promotion is unclear, and our experiment confirmed the specific mechanism of DHA promote angiogenesis in the cerebral ischemia penumbra.Improving the perfusion of cerebral ischemia penumbra tissues is an important strategy to intervene in acute ischemic stroke because potentially surviving tissues may remain viable for up to 48 hours after the onset of ischemic stroke [21]. In previous rodent experiments, neovascularization occurred in the cerebral ischemia penumbra as early as 12 hours after ischemic stroke, and angiogenesis continued for more than 21 days after this time, the number of replicating endothelial cells peaked at 3 days of reperfusion [22–23],so the present experiment used day 3 after stroke as the time point for observing vascular proliferation.

Peroxisome proliferator-activated receptors (PPAR) belong to a family of hormone- and lipid-activated nuclear receptors, PPARβ/δ are expressed in cells from all regions of the brain and appear to be most highly expressed in neurons [5]. PPARα and PPARγ have been identified as mediating anti-angiogenic processes, and conversely, PPARβ/δ appear as pro-angiogenic nuclear receptors [24].There is evidence that PPAR-β/δ is protective against a variety of neurological diseases, such as Parkinson's disease (PD) [25], Huntington's disease [26] and Alzheimer's disease (AD) [27].NF-κB pathways are known to trigger inflammatory responses, and PPARβ/δ inhibit NF-κB activation [28–29]. DHA is agonist for PPARβ/δ, PPARβ/δ activated by DHA further inhibited inflammatory responses in NF-κB pathways. It has become increasingly clear that the brain injury caused by an ischemic stroke is closely related to inflammatory responses, in the early phase of ischemia-reperfusion injury, except NF-κB pathways mediate inflammation,TNF-α is released by neurons, glial cells and endothelial cells, and IL-1β is also produced by macrophages/microglia and monocytes after inflammatory body formation [30]. Literature suggests that TNF-α and IL-1β infiltrate the infarct and peri-infarct,this information is discussed in the context of suggestions that neuronal sensitivity to ischemia may be modulated by cytokines. The fact that TNF-α and IL-1β are produced by microglia within the therapeutic window place these cells centrally in potential future stroke therapy[31].Our finding that DHA reduced NF-κB activation in the MCAO/Rep rat model, in addition, DHA also downregulated TNF-α and IL-1β, two important inflammatory markers in ischemic stroke. However, in the ELISA assay of INHIBITING PPARβ/δ by GSK0660, NF-κB, TNF-α and IL-1β were still significantly decreased after DHA intervention, which was not statistically significant compared with the DHA group without GSK0660, suggesting that in addition to PPARβ/δ pathway expect, DHA may also have anti-inflammatory effects on NF-κB and other inflammatory factors through other pathways.

Since VEGF-A is a known PPARβ/δ target gene[32]. VEGFR2 is one of the most important receptors for VEGF-A and is expressed mainly in microvascular endothelial cells and endothelial progenitor cells, with subsequent phosphorylation activation initiating intracellular signaling cascades that mediate various signal transduction, biological responses, and angiogenesis [33].PPAR-β/δ is not only a lipid sensor, but also a regulator of mitochondrial function, and may influence oxidative stress and inflammation in brain cells as well as proliferation and angiogenesis in vascular endothelial cells. There is evidence that PPAR-β/δ regulates vascular function by enhancing VEGFR expression, and subsequently regulating endothelial NO production and reducing ROS and inflammation[34].VEGFR-2 is mainly distributed in vascular

endothelial cells, lymphatic endothelial cells, and embryonic precursor cells, and can bind to VEGF-A. By binding and activating VEGFR-2, VEGF-A mediates endothelial cell proliferation, invasion and migration, and survival, and increases vascular permeability and neovascularization.The VEGF/VEGFR system is an important target for anti-angiogenic therapy in cancer and for pro-angiogenic therapy in neuronal degeneration and ischemic diseases.Finally, VEGFR-2 phosphorylated, and then the initiation of the intracellular signaling cascades[35].Our experiments confirmed that DHA promoted the expression of VEGF-A, VEGFR2, and P-VEGFR2 in brain tissue in the ischemic penumbra region in the drug-treated group through activation of PPARβ/δ, while the GSK0660 group was decreased significantly expression of related angiogenic proteins. It is consistent with the parenchymal angiogenesis observed by immunofluorescence in this experiment.

NeuN has been considered to be a reliable marker of mature neurons [36].Mature neurons were believed to be permanent cells t. A mature neuron would execute special cellular functions depending on its specific proteins. Neuroglial cells also express specific proteins. Some of the specific proteins expressed by neural cells can be used as their markers. In particular, NeuN was widely accepted as the “specific” cell marker for neurons [37].In the rats after MCAO/R surgery, the number of neurons decreased, treatment with DHA increased the number of NeuN + cells in the cerebral ischemia penumbra, GSK0660 redud the protective effect of DHA on NeuN + cells. In combination with Hematoxylin-Eosin Staining, the ischemic cortical neurons showed atrophic morphologcey, accompanied by nuclear pyknosis, fragmentation and dissolution. Compared with MCAO group, the ischemic cerebral cortex in DHA group had intact histopathological profile, Compared with DHA group, GSK0660 group had more pathological changes in ischemic cerebral cortex. Our results show that DHA can effectively ameliorate neuronal damage in the context of ischemic stroke. The Neurological protection of DHA was also proved in Neurological assessment and TTC staining. There is growing evidence that the establishment of effective collateral circulation is important to avoid the expansion of ischemic penumbra, which is a key therapeutic approach in the early stages of ischemic stroke, and that the proliferation of vascular endothelial cells and smooth muscle cells after ischemic infarction determines the establishment of collateral circulation [38–39].

In this study, we selected CD31 and α-SMA immunofluorescence double staining to label neovascularization in the penumbra region of cerebral ischemia cortex [40].CD31 and α-SMA are markers of vascular endothelial cells and smooth muscle cells, respectively, and are favorable evidence for tube formation and migration into arteries [41]. We found that compared with MCAO group, the number of new blood vessels in DHA group was significantly increased, while the number of new blood vessels in GSK0660 inhibitor group was significantly decreased compared with DHA group (all $P < 0.05$). It is interesting to note there were fewer neovascularization in the sham operation group, whereas there were numerous neovascularization (CD31/α-SMA double positive) around the lesion in the MCAO group. But this is most likely due to pathological angiogenesis due to hypoxia.Our results suggest that DHA promotes vascular neovascularization by activating the anti-inflammatory PPARβ/δ VEGF-A related pathway, thus protecting rats from MCAO/R injury. However, The extent to which reperfusion improves

collateral circulation, and the proportion of pathological vessels induced by hypoxia to neovascularization deserves further study.

What's innovation about this experiment is that we not only verified the angiogenesis promoting effect of DHA from the protein molecular expression level, but also verified the organic structural generation of blood vessels by immunofluorescence technology, moreover, to verify the efficacy of DHA through activating PPARβ/δ, we set up the PPARβ/δ inhibitor GSK0660 intervention group.Finally, we explored the effects of DHA in the cerebral ischemia penumbra by MCAO/R rat model and found that DHA attenuated neurological deficits, reduced infarct volume and protected neurons after MCAO/R, and promoted endothelial neovascularization in the cerebral ischemia penumbra. Mechanistically, DHA activated PPARβ/δ, upregulated VEGF-A、VEGFR2、p-vegfr2 expression, and inhibited pro-inflammatory TNF-α、IL-1β and NF-κB, thereby protecting cerebral ischemia penumbra tissue. In conclusion, the present experiment demonstrated for that DHA promoted cerebral ischemia penumbra angiogenesis through activation of PPARβ/δ-mediated VEGF-A correlated signaling pathway expression.Our experiment is the first one in rodents make it clear to in the cerebral ischemia penumbra of PPARβ/δ in influencing angiogenesis, DHA as PPARβ/δ agonists, DHA has gained a new understanding in the treatment of ischemic stroke, and thus has the potential to become a new clinical drug in the treatment of ischemic stroke.

Declarations

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

Availability of supporting data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

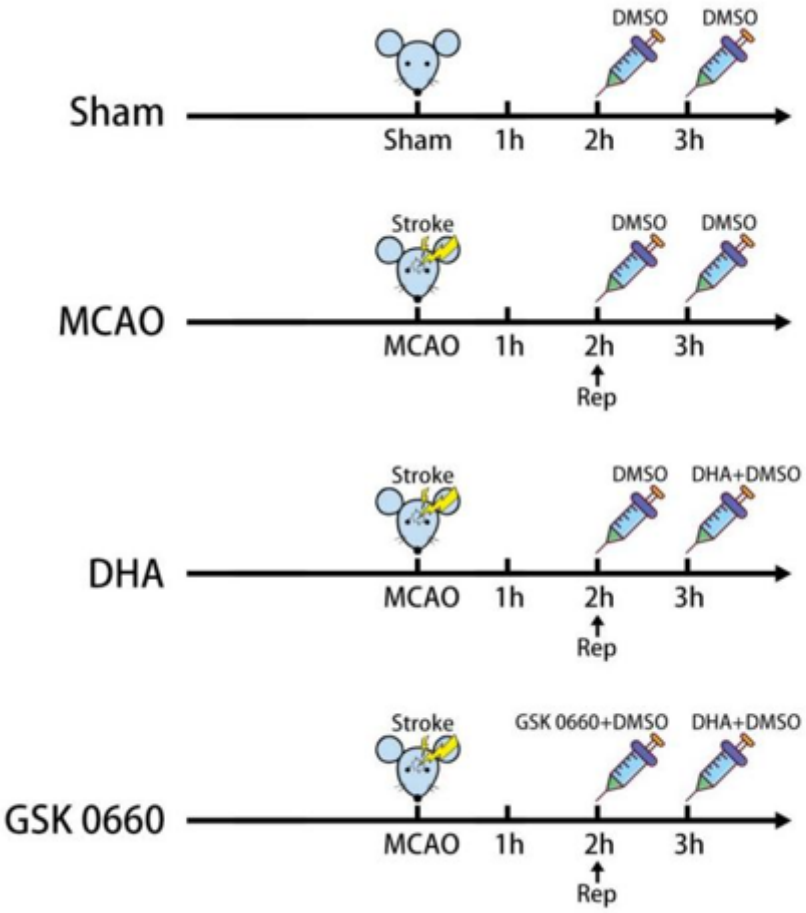

Figure 1

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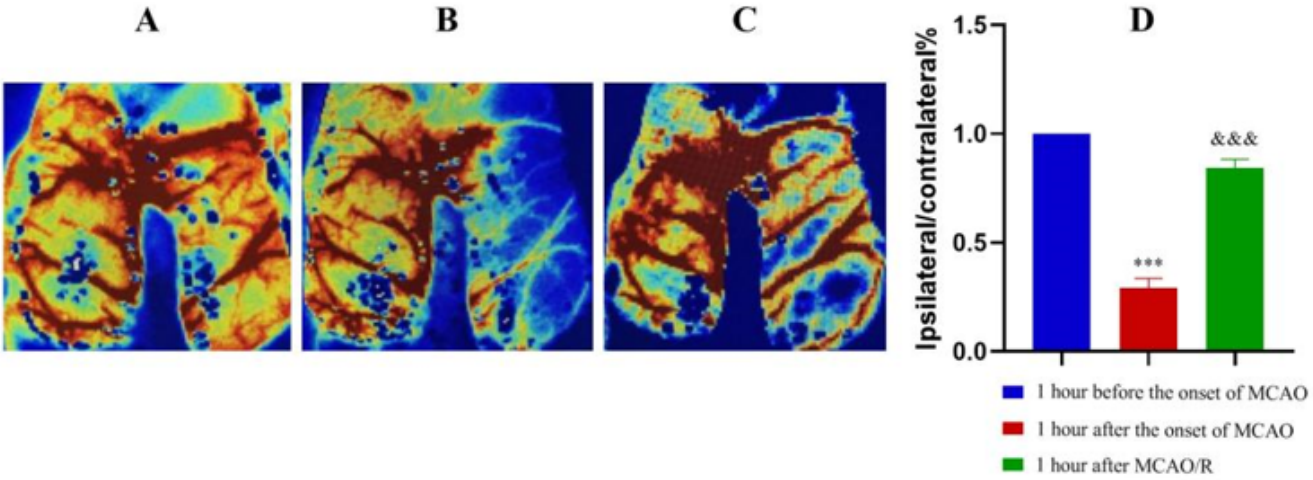
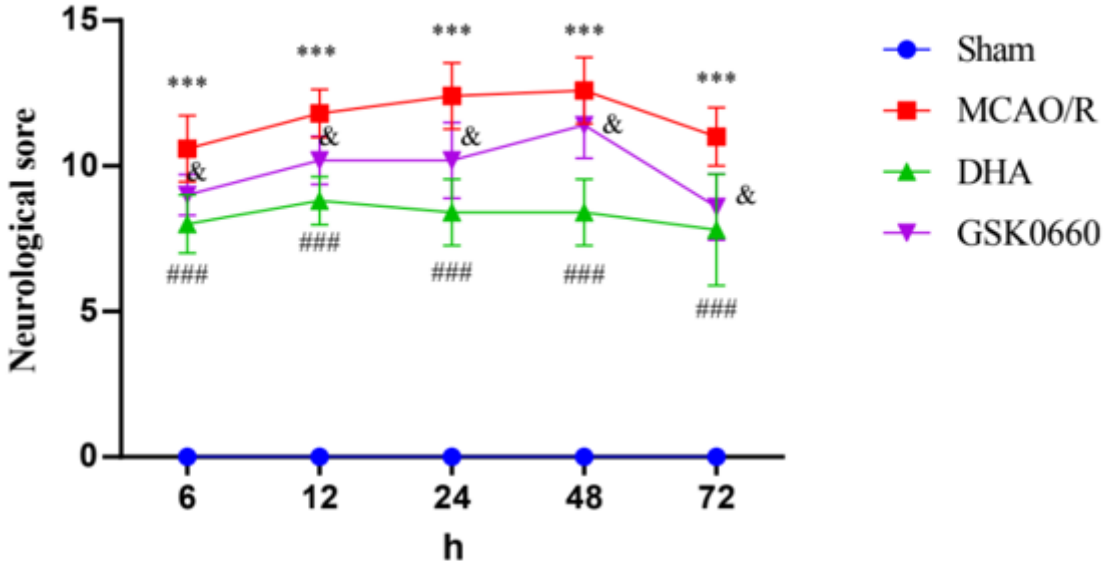


Effect of MCAO/R on regional cerebral blood flow. Figure A shows the quantitative analysis of local cerebral blood flow 1 hour before the onset of MCAO. Figure B is the quantitative analysis of local cerebral blood flow 1 hour after the onset of MCAO. Figure C is the quantitative analysis of local cerebral blood flow 1 hour after MCAO/R. Mean±SEM of 5 rats in each group. p<0.001 compared with 1 hour before the onset of MCAO, &&& p<0.001 compared with 1 hour after the onset of MCAO.MCAO, middle cerebral artery occlusion; MCAO/R, middle cerebral artery occlusion/reperfusion.**

Figure 2

See image above for figure legend.



Treatment with DHA ameliorated MCAO/R-induced nerve injury. Modified Nerve Severity Scale (mNSS) scores were assessed in different groups at 6 hours, 12 hours, 24 hours, 2 days and 3 days after reperfusion. Data are expressed as mean ± SEM, n = 5, *** P < 0.001 vs. sham group, ###P < 0.01 vs. MCAO/R group, & P < 0.05 vs. DHA group. mCAO/R, middle cerebral artery occlusion/reperfusion; PPAR-β/δ, peroxisome proliferator-activated receptor-β/δ; DHA , docosahexaenoic acid; GSK0660, PPAR-β/δ inhibitor.

Figure 3

See image above for figure legend.

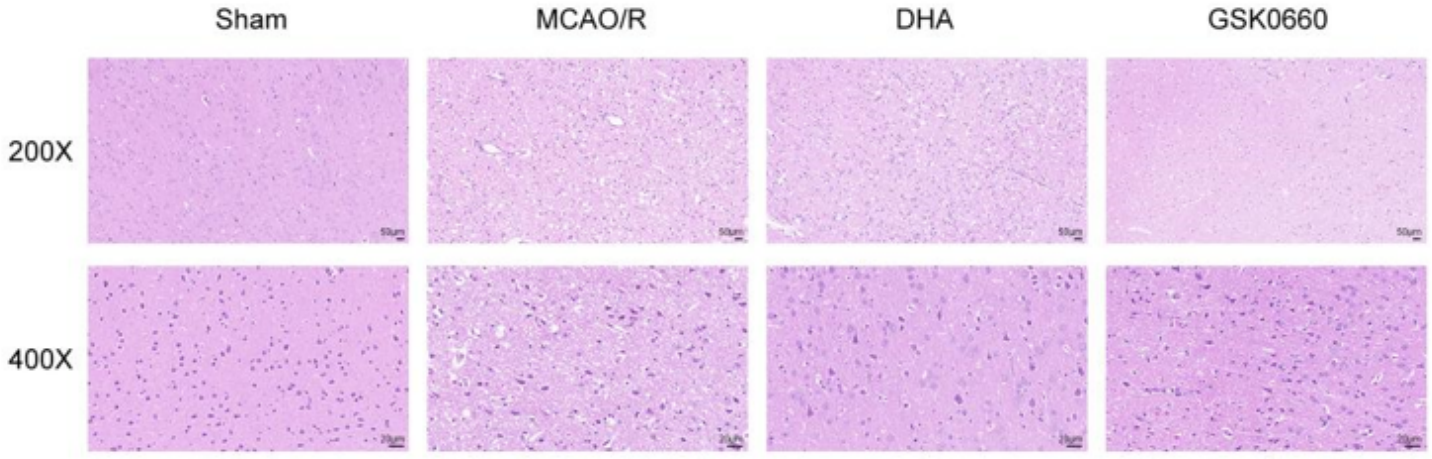


Figure 4

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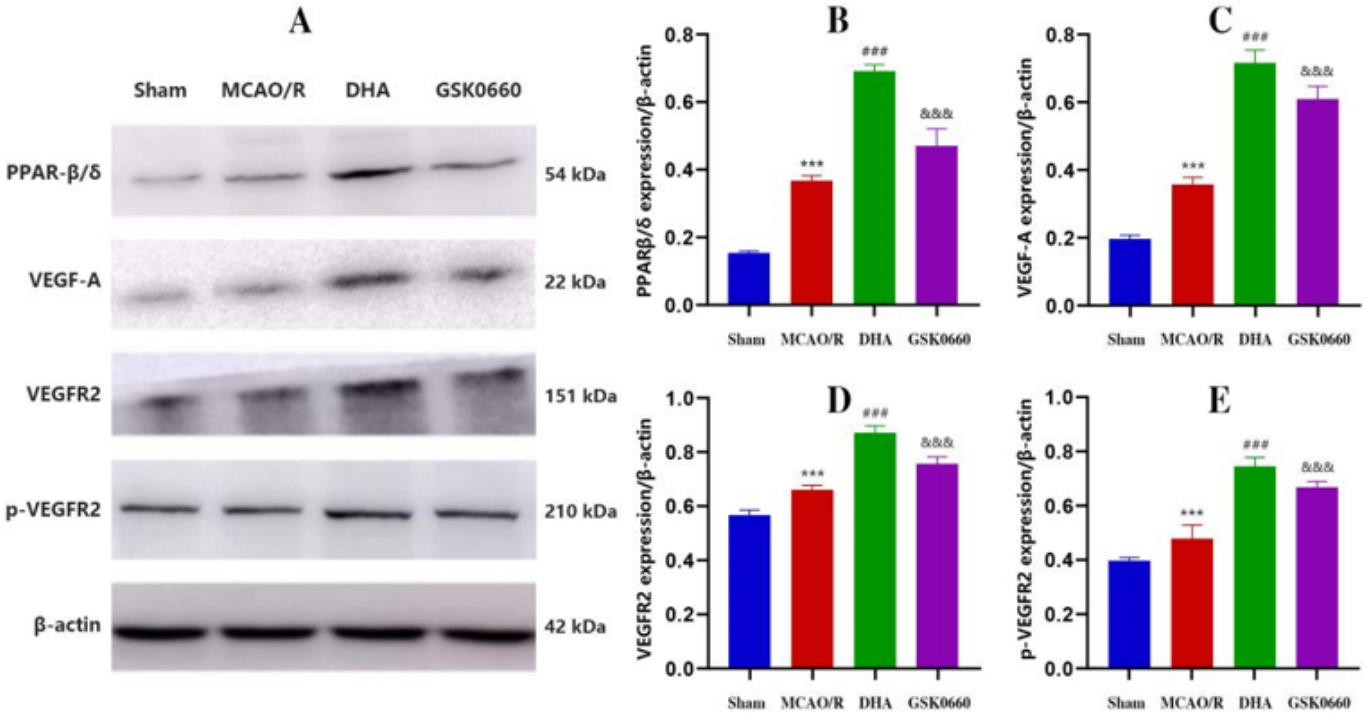
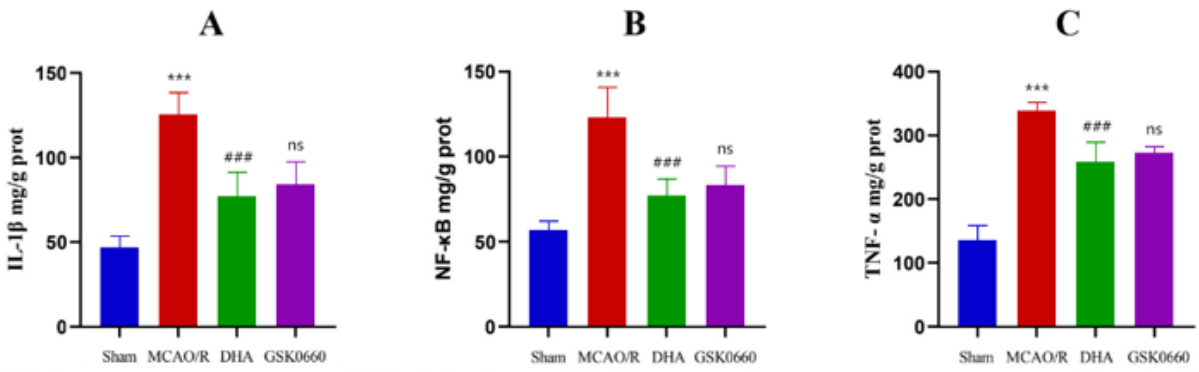


A)Representative images of protein blot analysis of PPARβ/δ,VEGF-A,VEGFR2,andp-VEGFR2.(B-E)Protein blots showing the expression levels of PPARβ/δ,VEGF-A,VEGFR2,and p-VEGFR2 in ischemic corticaltissue.Data are expressed as mean ±SEM, n=5, ***P<0.05vs.sham group, ### P<0.001 vs.MCAO/R group,&&&P < 0.01vs.DHA group.MCAO/R,middle cerebral artery occlusion/reperfusion;DHA, docosahexaenoicacid;PPAR-β/δ,peroxisomeproliferator-activatedreceptor-β/δ; GSK0660,PPAR-β/δ inhibitor.

Figure 5

See image above for figure legend.



DHA inhibited the MCAO/R-induced inflammatory response. (A- C) Pro-inflammatory cytokines were analyzed using enzyme-linked immunosorbent assay (ELISA) methods for IL-1β,NF-κB and TNF-α, respectively. Data points indicate mean±SEM, n=5. *** p < 0.001 compared with Sham group, ### p < 0.01 compared with MCAO group; ns p > 0.05 compared with DHA group;MCAO/R, middle cerebral artery occlusion/reperfusion; DHA , docosahexaenoic acid; GSK0660. PPAR-β/δ inhibitor; IL-1β , interleukin-1β ;NF-κB, nuclear factor kappa-B; TNF-α, tumor necrosis factor-α.

Figure 6

See image above for figure legend.

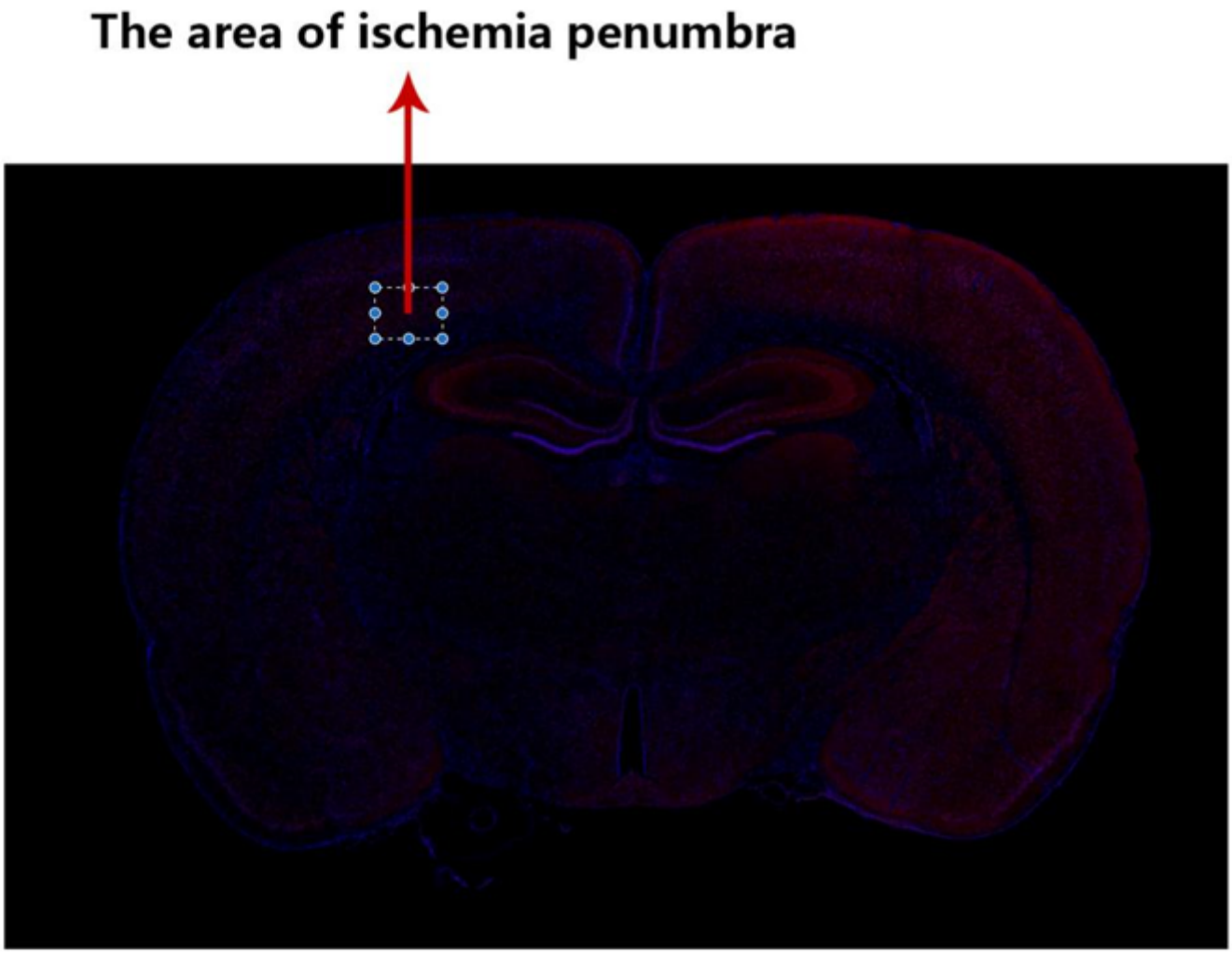


Figure 7

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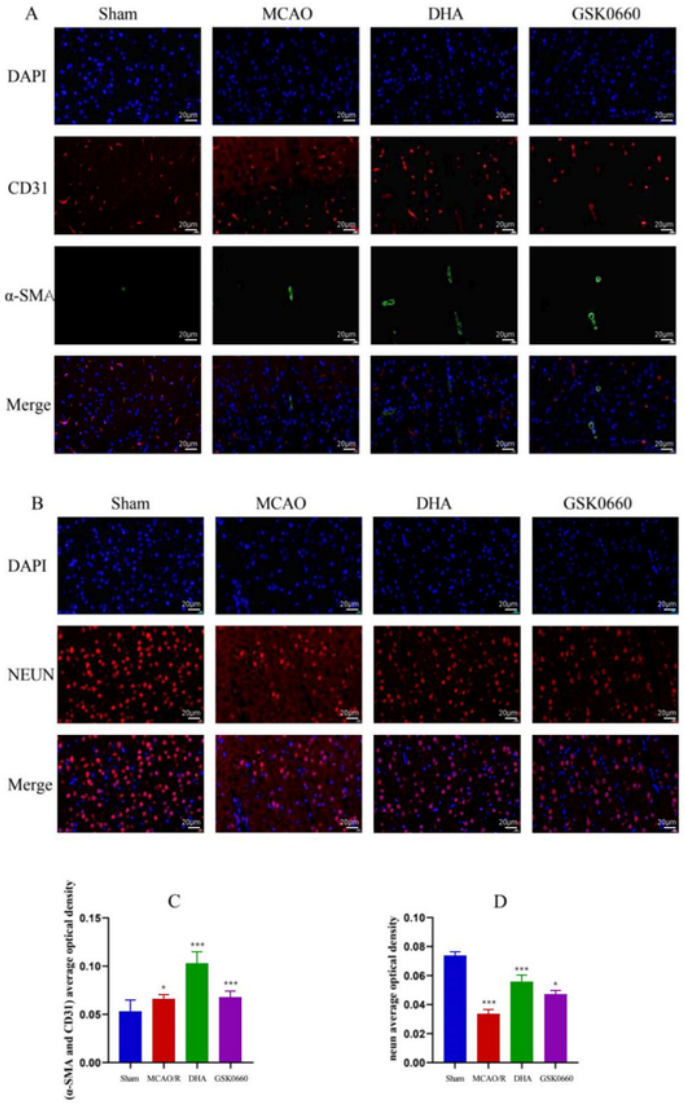

DHA promoted angiogenesis after ischemic stroke. (A) Images showing CD31 and α-smooth muscle actin (α-SMA) immunofluorescent double staining detection in the ischemic penumbra via immunofluorescent staining and counterstaining with DAPI. (B) Images showing NeuN detection in the ischemic penumbra via immunofluorescent staining and counterstaining with DAPI. (C) Quantification of CD31/α-SMA double-positive cells in the ischemic penumbra.(D) Quantification of NeuN-positive cells in the ischemic penumbra. Mean ± SD. n = 5.in figure C,* P <0.05 vs sham-operated group, ***P < 0.01 vs MCAO/R, ***P < 0.01 vs DHA group;in figure D,* P <0.01 vs sham-operated group, ***P < 0.01 vs MCAO/R, *P < 0.05 vs DHA group . mCAO/R, middle cerebral artery occlusion/reperfusion; DHA, docosahexaenoic acid; PPAR-β/δ, peroxisome proliferator activated receptor-β/δ; GSK0660, PPAR-β/δ inhibitor.NeuN,neuron-specific nuclear protein;CD31,Platelet endothelial cell adhesion molecule-1 , PECAM-1;α-SMA, alpha-smooth muscle actin ; DAPI,4',6-diamidino-2-phenylindole.**

Figure 8

See image above for figure legend.