Dihydrocapsaicin-induced angiogenesis and improved functional recovery after cerebral ischemia and reperfusion in a rat model

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Abstract
This study investigated the long-term effects of dihydrocapsaicin (DHC)-induced angiogenesis and improved functional outcomes in cerebral ischemia and reperfusion (I/R) rats. Middle cerebral artery occlusion was induced in I/R rats for 2 h, followed by reperfusion. The animals were divided into three groups: sham, I/R + vehicle, and I/R + DHC (10 mg/kg body weight). Fourteen days after I/R injury, the DHC-treated I/R rats had decreased neurological deficit scores, infarct volume, and brain morphology changes. DHC-induced angiogenesis significantly increased the expression of angiogenic factor proteins, such as hypoxia inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), and matrix metalloprotease 9 (MMP-9), at 3 d and 14 d following I/R and also increased the expression of angiogenic inhibitors, such as angiopoietin 1 (Ang-1) and its receptor tyrosine kinase (Tie-2), at 14 d following reperfusion. DHC-mediated angiogenesis was confirmed by a significant increase in positive BrdU labeling that co-localized with the von Willebrand factor (an endothelial cell marker) at 14 d after I/R. Furthermore, rotarod and pole tests demonstrated that DHC promoted functional recovery when compared with the vehicle group. Thus, the results reveal that DHC mediates angiogenesis and functional recovery after an ischemic stroke.

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1. Introduction

Ischemia-reperfusion (I/R) injury contributes the basis of tissue damage, long-term disability and cognitive impairment. Angiogenesis has an important role in brain recovery after an injury. Angiogenesis is the formation of new blood vessels from pre-existing vasculature in response to stimuli such as tissue hypoxia or trauma. I/R injury causes blood—brain barrier (BBB) breakdown and releases reactive oxygen species (ROS) that initiate a morphological change in the astrocytes, and the reactive astrocytes modify the extracellular matrix (ECM), which leads to the migration of endothelial cells to form new blood vessels. In the ischemic core, the activation of hypoxia inducible factor (HIF-1α) correlates with VEGF expression at 24–48 h after the onset of ischemia. The formation of new blood vessels after a stroke is initiated at the border of the infarcted area in 2 d–7 d. The new vessels in the penumbra area develop and sprout to the ischemic core during the period from 2 d to 14 d. Moreover, vascular permeability has beneficial effects on angiogenesis, but excessive vascular leakage leads to circulatory collapse. Angiopoietin-1 (Ang-1), a ligand for the endothelial tyrosine kinase Tie-2 receptor that promotes vascular stability is an essential factor in angiogenesis. Restoring neurovascular function through the angiogenesis has garnered interest as a potential treatment for ischemic stroke.

Dihydrocapsaicin (DHC, 8-methyl-N-vanillylnonanamide; N-[4-hydroxy-3-methoxybenzyl]-8-methylnonanamide) is a...
capsaicinoids compound mostly found in chili peppers. Recent studies have shown that DHC has multiple pharmacological and physiological effects.\textsuperscript{11–15} Our previous study suggested that DHC can be neuroprotective against acute cerebral ischemia and reperfusion in rat models via attenuating cerebral and BBB damage through oxidative stress and the inflammatory pathway.\textsuperscript{16} In this study, the long-term effects of DHC-induced angiogenesis and improved functional recovery after cerebral ischemia and reperfusion in a rat model were investigated. The results of the study revealed that DHC induced functional angiogenesis without BBB leakage and improves functional recovery at 14 d after I/R.

2. Materials and methods

2.1. Animals and drug administration

Male Wistar rats (280–300 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand. The animals were housed in a temperature-controlled environment (25 ± 1 °C) with a 12 h–12 h light–dark cycle and had free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University and performed in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. The 54 rats were randomized and divided into three groups: (1) sham group; (2) I/R + vehicle; and (3) I/R + 10 mg/kg BW DHC. In the DHC treatment and vehicle groups, DHC and vehicle were injected by intraperitoneal administration 15 min before reperfusion\textsuperscript{16} and daily for 14 d (except the sham group). DHC (Anyang General International, Henan, China) was dissolved in DMSO and freshly diluted in 0.5% 2-hydroxyl cellulose in normal saline before injection (final concentration of DMSO was 1%).

2.2. Induction of a middle cerebral artery occlusion (MCAO) model

MCAO rats were prepared by surgery as described previously.\textsuperscript{16} Briefly, rats were anesthetized by intraperitoneal injection of zoletil (30 mg/kg) and xylazine (10 mg/kg). An intraluminal monofilament (Doccol Corp., Sharon, MA, USA) was introduced past the ECA stump into the ICA for 2 h to block the origin of the MCA. Occlusion was confirmed by a laser-Doppler flowmeter, as previously described.\textsuperscript{16} After 2 h of ischemia, the suture was withdrawn, and the wound was stitched. Rats in the sham operation group were treated in the same manner as in the above procedure, except that the monofilament was not inserted.

Fig. 1. Effects of DHC on animal models. (A) Representative rat weight at 14 d after I/R, (B) survival rate at 14 d after I/R, and (C) representative neurological deficit scores at 1 d and 3 d after I/R. IgG staining at 14 d after I/R. (D and E) the pictures represent IgG-stained cerebral cortex of the penumbra area. The images were visualized using a light microscope (20X) (scale bar = 50 μm). The data are the mean ± SD from six independent experiments (**P < 0.01 in comparison with the vehicle group).
2.3. Neurological evaluation

Behavioral tests were performed at 1 d and 3 d after I/R using the method of Longa et al., 1989. The five-point scale was as follows: 0 = no neurological deficits, 1 = failure to extend contralateral forepaw fully, 2 = circling to the ipsilateral side when held by the tail, 3 = falling to the contralateral side, and 4 = did not walk spontaneously and had depressed level of consciousness.

2.4. Determination of motor coordination and balance by rotarod test

Three days before MCAO, all animals were trained for the rotarod test, by performing three trials per day for three days. Briefly, the animals were placed on the apparatus for 30 s with no rotation and, then after 2 min, at a constant low speed (4 rpm). This procedure was performed only on the first day of training. After a 10-min rest, the animals received a single baseline trial on the accelerating rotarod, in which the spindle increased in speed from 4 rpm to 40 rpm. The test was performed at different times from 1 d to 13 d after I/R. The duration for which the animals were able to walk on the rotarod before falling was measured (the duration on day 0 was considered 100%).

2.5. Determination of gross sensorimotor function by pole test

The animals were placed head upward on the top of a vertical wooden rough-surfaced pole (diameter, 2 cm; height, 100 cm). The animals were trained on the day before I/R and then allowed to descend 5 times in a single session. The total time until the animals reached the floor with their four paws (time to reach the floor) and the time needed to turn the head completely downward (time to turn) were recorded.

2.6. Detection of infarct volume by 2,3,5-triphenyltetrazolium chloride (TTC) staining

At 14 d after I/R, the rat brain tissues were sectioned into six slices (2-mm thick) in the coronal plane. The brain slices were stained with 2% TTC (Sigma, St. Louis, USA). Images of the brain slices were taken and quantified by ImageJ®. The relative infarct volume was calculated as $100\% \times \frac{\text{contralateral hemisphere volume} - \text{non-infarct ipsilateral hemisphere volume}}{\text{contralateral hemisphere volume}}$. 
2.7. Histology analysis by H&E staining

At 14 d after I/R, the rat brain tissues were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Coronal sections (20 μm thickness) were sliced and stained with H&E to observe pathological changes under a light microscope (Olympus AX70, Tokyo, Japan).

2.8. IgG staining

At 14 d after I/R, the coronal sections of rat brain were deparaffinized and rehydrated. The immunohistochemistry was performed VECTASTAIN® ABC Kit. Endogenous peroxidase activity and non-specific staining were blocked and incubated with primary IgG antibody for 30 min, diluted biotinylated secondary antibody for 30 min. Sections were colour-developed using a diaminobenzidine (DAB) solution. The slices were observe the IgG leakage under a light microscope (Olympus AX70, Tokyo, Japan).

2.9. Determination of endothelial cell proliferation by BrdU assay

BrdU (Abcam, Cambridge, UK) (50 mg/kg) was intraperitoneally injected at 6 d–10 d after I/R. At 14 d after I/R, the brain tissues were fixed in 4% paraformaldehyde, sectioned (20 μm) and processed for immunofluorescence by anti-BrdU (Merck, Darmstadt, Germany) co-localization with the anti-Von-Willebrand factor (Abcam, Cambridge, UK), a marker of endothelial cells. The images were observed under a fluorescence microscope (Olympus AX70, Tokyo, Japan). The BrdU+/Von-Willebrand+ positive cells were counted from 5 fields of the ischemic penumbra area. The data are presented as BrdU+/Von-Willebrand+/mm2.

2.10. Determination of protein expression by Western blot analysis

At 1 d, 3 d, and 14 d after I/R, the brain tissues were homogenized in lysis buffer. Western blot analyses were performed as described previously.16 The antibodies used were: anti-VEGF (Merck, Darmstadt, Germany), anti-HIF1 (Merck, Darmstadt, Germany), anti-Ang-1 (Merck, Darmstadt, Germany), anti-Tie-2 (Abcam, Cambridge, UK), anti-MMP-9 (Cell Signaling, MA, USA), and anti-actin (Cell Signaling, MA, USA). Densitometric analysis was performed using a scanning densitometer for films, and the results were normalized to β-actin by ImageJ® analysis.

2.11. Statistical analysis

All values are reported as the mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) for comparison between groups, followed by Dunnett’s post hoc test. P values <0.05 were indicative of a statistically significant difference between the experimental and control groups.

3. Results

3.1. Effects of DHC on body weight, survival rate, neurological deficit scores and BBB leakage by IgG staining

The body weights of the rats in the different groups at 14 d after I/R were not significantly different (Fig. 1A). Compared to the I/R + vehicle group, the survival rate increased at 14 d after I/R in the I/R + DHC group (Fig. 1B). The neurological deficit scores in the I/R + vehicle group and the I/R + DHC group increased significantly at 1 d and 3 d after I/R (P < 0.05). Compared to the I/R + vehicle group, the neurological deficit scores in the I/R + DHC group were significantly reduced at 1 d and 3 d after I/R (Fig. 1C). The IgG staining demonstrated that the rat brains had no BBB leakage at 14 d after I/R (Fig. 1D and E).

3.2. DHC ameliorates the behavioral deficit after I/R

The I/R injury induced a neurological function deficit on the first day, but recovery after the time of the injury is possible. At 13 d after I/R, the rats in both groups were similar to the sham group, but their ability on rotarod and pole tests was observed. The DHC group had a significantly reduced motor deficit after I/R, as demonstrated by the increase in the latency to fall off the rotarod at 24 h compared to the vehicle group (Fig. 2A). At 13 d after I/R, the latency time to fall off the rotarod was not significantly different between

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Fig. 4: DHC treatment improved the histopathological changes at 14 d after I/R. (A) H&E-stained rat brain in the coronal plane. (B) The pictures represent an H&E-stained cerebral cortex of the penumbra area (20×). The images were visualized using a light microscope (scale bar = 50 μm). The red arrow represents viable neurons; the black arrow represents pyknotic nuclei.
the vehicle group and the DHC group, but it was significantly different from the sham group (P < 0.001 and P < 0.01, Fig. 2A). In addition, the strength of the forelimb and the ability of the animals to move along the pole were evaluated by the pole test. The normal rats took less time to turn and reach the floor than the I/R rats. At 13 d after I/R, the DHC group did not show any significant difference in the time to turn compared to the vehicle and sham groups (Fig. 2B). The time to reach the floor for the vehicle rats increased remarkably after I/R + DHC treatment in comparison with the time for the vehicle group (P < 0.01, Fig. 2C).

3.3. DHC attenuates cerebral infarction

At 14 d after I/R, the percentage of infarct volume was reduced in both groups, a result that is consistent with the findings of a previous study at 24 h after reperfusion. However, at 14 d after I/R, the I/R + DHC treatment group had a significantly reduced infarct volume when compared with the vehicle group (Fig. 3A and B).

3.4. DHC improves histological changes in the peri-infarct area

At 14 d after I/R, a peri-infarct area was observed in the cerebral cortex. The pictures showed a loss of neuronal cells at the ischemic core, and pyknotic nuclei (denoted by a black arrow, Fig. 4) were widely present in the vehicle group in both the ischemic core and penumbra area. Treatment with DHC improved the morphological changes and presented more viable neurons (denoted by the red arrow, Fig. 4).

3.5. DHC upregulates angiogenic factors

To investigate the mechanism of DHC on I/R injury-induced angiogenesis, Western blot analysis was performed to detect the expression of HIF-1α, VEGF, and MMP-9 at 1 d, 3 d, and 14 d after I/R. Compared with the sham group, the expression of HIF-1α in the ischemic penumbra was significantly increased in both the vehicle

![Fig. 5. DHC-treated I/R upregulated the angiogenic factors HIF-1α, VEGF, and MMP-9. (A) Representative western blotting at 1 d, 3 d, and 14 d after I/R and quantitative analysis of protein expression of HIF-1α. (B) VEGF, and (C) MMP-9 normalized to β-actin. The data are the mean ± SD from three independent experiments (**P < 0.01 and ***P < 0.001 in comparison with the sham group; *P < 0.05 and **P < 0.01 in comparison with the vehicle group).](image)

![Fig. 6. DHC treatment promoted the angiogenic inhibitors Ang-1 and Tie-2 receptor in I/R. (A) Representative western blotting at 1 d, 3 d, and 14 d after I/R and quantitative analysis of protein expression of Ang-1 and (B) Tie-2 normalized to β-actin. The data are the mean ± SD from three independent experiments (**P < 0.01 and ***P < 0.001 in comparison with the sham group; *P < 0.05 and **P < 0.01 in comparison with the vehicle group).](image)
group and the DHC group at 1 d and 3 d after I/R (P < 0.05) (Fig. 5A). At 24 h after I/R, no effect on VEGF levels in both the vehicle and DHC groups was noted when compared with the sham group. However, the expression of VEGF in the DHC group increased significantly at 3 d and 14 d after I/R when compared with the vehicle group (P < 0.01 and P < 0.05, Fig. 5B). Compared with the sham group, the expression of MMP-9 increased significantly increased at 1 d, 3 d, and 14 d after I/R in both groups (Fig. 5C). Compared with the vehicle group, the DHC group had a significant decrease in MMP9 expression at 1 d and 14 d after I/R in both groups (Fig. 5C). Compared with the vehicle group, the DHC group had a significant increase in MMP-9 expression at 1 d and 14 d after I/R (P < 0.05, Fig. 5C), and DHC treatment significantly increased the expression of MMP-9 at 3 d of reperfusion when compared with the vehicle group (P < 0.05, Fig. 5C), which correlates with the upregulation of HIF-1α and VEGF at 3 d of reperfusion.

### 3.6. Effects of DHC on angiogenic inhibitors

To investigate the effects of DHC on the inhibition of angiogenesis after I/R injury, Western blot analysis was performed to detect the expression of Ang-1 and Tie-2 at 1 d, 3 d, and 14 d after I/R. The expression of Ang-1 decreased in both the vehicle and DHC groups at 3 d after I/R injury but was significantly decreased in the DHC-treated group when compared with the vehicle group (P < 0.01, Fig. 6A). The results showed that I/R suppressed the expression of Tie-2 receptors in both the vehicle and DHC groups when compared with the sham group, but at 14 d after I/R, the DHC-treated I/R group had significantly increased Tie-2 when compared with the vehicle group (P < 0.05, Fig. 6B).

### 3.7. DHC enhances new microvessels after I/R injury

To investigate the number of new microvessels after I/R injury, a BrdU proliferation assay was performed with co-localization assay with Von-Willebrand factor, an endothelial cell marker. At 4 d after BrdU injection, the number of BrdU-positive cells in the DHC group was markedly higher than the numbers in the vehicle and sham groups (Fig. 7A). Dual-labeling immunofluorescence showed that Von-Willebrand-positive cells expressed BrdU, indicating the presence of new microvessels. The number of new microvessels in the DHC group increased significantly when compared with the vehicle group (P < 0.001, Fig. 7B).

### 4. Discussion

Our previous study demonstrated that DHC can protect against cerebral I/R injury that induces BBB damage via attenuation of oxidative stress and inflammation. The present study shows that DHC can attenuate cerebral infarction, improve functional outcomes, and promote angiogenesis in I/R models at 14 d after I/R injury.
In the acute phase (24–48 h), I/R injury induces cerebral infarction, BBB damage, and neurological deficit via increasing oxidative stress, inflammation, and apoptosis. In the recovery phase, the penumbra area initiates self-repair via angiogenesis, which is effective in improving functional recovery in the recovery phase. Our results are consistent with those of other studies, and the severity of neurological deficit scores and cerebral infarction at 14 d after injury are less than those at 24 h after I/R injury. In addition, DHC helps to reduce the severity of brain damage and improve the functional outcome.

During and after a stroke, the brain vasculature breaks down, which leads to neuronal tissue damage. Then, angiogenesis, or the formation of new microvessels from the existing vasculature, becomes the key process for reconstruction and the improvement of functional outcomes following cerebral ischemia.

DHC promotes angiogenesis, the expression of the angiogenic factor (IFG-1), epidermal growth factor (EGF; initiator of blood coagulation), hepatocyte growth factor (HGF), interleukin hormones and chemokines) that depends on the balance of stimulating or inhibiting actions. First of all, the signaling cascade involving VEGF is now assumed as the critical event in the regulation of angiogenesis.

The numerous of molecules are known that can serve as regulators of angiogenesis (erythropoietin (EPO), insulin-like growth factor (IGF-1), epidermal growth factor (EGF; initiator of blood coagulation), hepatocyte growth factor (HGF), interleukin hormones and chemokines) that depends on the balance of stimulating or inhibiting actions. First of all, the signaling cascade involving VEGF is now assumed as the critical event in the regulation of angiogenesis.

Hypoxic tissues generate HIF-1α, which induces the upregulation of VEGF, a key signaling molecule involved in the formation and stabilization of blood vessels involved in angiogenesis following I/R. After cerebral ischemia, VEGF peaks at 24–48 h after onset. Our previous study demonstrated that I/R injury induces the expression of MMP-9, which results in BBB breakdown, then DHC can reduce the MMP-9 level. The delayed MMP-9 expression interacts with VEGF to provide vascular permeability in the angiogenesis process. However, the expression of MMP-9 should be suppressed at the acute state following I/R to protect against BBB leakage. Our results demonstrated that the expression of HIF-1α was upregulated at 24 h and 3 d after I/R, whereas the VEGF level peaks at 3 d after I/R. At 3 d after I/R, the increase in HIF-1α level induced the expression of VEGF in the I/R + vehicle group. To explore the underlying mechanism by which DHC promotes angiogenesis, the expression of the angiogenic factors HIF-1α, VEGF, and MMP-9 was evaluated. The DHC group had more proliferative vessels and higher expression levels of HIF-1α, VEGF, and MMP-9 in the peri-infarct area at 3 d after I/R. Moreover, DHC attenuates the level of MMP-9 at 1 d and 14 d after I/R, which protects against BBB leakage. The formation of new brain microvessels is initiated from 14 d to 21 d after a stroke.

The number of newly generated vascular endothelial cells was higher after I/R, which significantly increased the number of newly formed vessels in the peri-infarct areas at 14 d after I/R. Tie/Ang signaling system is involved in regulation of complex interactions between endothelium and surrounding cells. Ang-1 and Ang-2 present differently directed action on the Tie-2-associated signaling cascade. Ang-2, an inhibitor of the Tie-2 receptor induces the detachment of smooth muscle cells and pericytes in mature blood vessels. Ang-1/Tie-2 system is essential for normal vessel stability, vascular remodeling, and angiogenesis. The action of Ang-1 is mainly exerted at the expression of the Tie-2 receptor, which is upregulated from 2 d to 21 d following an ischemic stroke.

Our data show that I/R suppressed the expression of Tie-2 in both the vehicle and DHC groups but, at 14 d following I/R, the Tie-2 level was elevated in the DHC group, which may be related to the Ang-1 level. The results indicate that DHC induces angiogenesis and promotes vascular stability. Our previous data found that DHC attenuates the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2 and NOX4) proteins. However, previous evidence indicates that NOX4 inhibits the expression of HIF-1α and VEGF. These effects have been hypothesized to lead to improved functional recovery following an ischemic stroke. Rotarod and pole tests are widely used to measure functional recovery by following the MCAO model. Previous studies revealed that angiogenesis improves functional recovery following an ischemic stroke. These data indicate that I/R injury induces a functional deficit by decreasing the latency to fall off of the rotarod apparatus and increasing the time to turn and the time to reach the floor in the pole test. However, DHC significantly improved the time to reach the floor in the pole test, which corresponds with an increase in the number of angiogenic proteins and newly formed blood vessels. The lack of a significant difference in rotarod time and time to turn in the DHC group compared to I/R + vehicle group may result from assistance from the hindlimb, because the hind limbs are not directly used by MCA. However, in the time to reach the floor, the rats mainly use the forelimb to grasp the pole when descending. Additionally, DHC administration attenuates brain morphology changes in the penumbra area of the cerebral cortex at 14 d after I/R injury.

In conclusion, our data demonstrated that DHC induced functional angiogenesis without enhancing BBB leakage and improves functional recovery at 14 d after I/R. This study provides further therapeutic techniques and clinical trials based on DHC in ischemic stroke patients.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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