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# Anti-ischemic Effects of Focal Brain Cooling are Mediated by Modulation of Transient Receptor Potential Vanilloid 4 Channels in Mice

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**Abstract** Focal brain cooling (FBC) at 15℃ and transient receptor potential vanilloid 4 (TRPV4) deficiency relieve brain infarction. TRPV4 channels are inactivated by cooling (< 27℃), suggesting that the anti-ischemic effects of FBC include those of TRPV4 inactivation. However, the extent to which TRPV4 inactivation contributes to the anti-ischemic, anti- blood-brain barrier (BBB) disruption, and anti-apoptosis effects of FBC on cerebral infarction remains unclear. We investigated the contribution and mechanisms of RN1734, a TRPV4 antagonist, in FBC for cerebral infarction using TRPV4 knockout and wild-type mice. Focal cerebral infarction was induced by photochemically induced thrombosis. Infarct volume, BBB disruption, and number of apoptotic cells were evaluated. The TRPV4 antagonist or deficiency showed similar anti-ischemic and anti-BBB disruptive effects to those of FBC. Intracerebroventricular injection of RN1734 showed a similar reduction in the number of apoptotic cells to that of FBC. These anti-ischemic and -apoptotic effects were completely inhibited with injection of GSK1016790A, a TRPV4 agonist, immediately before FBC. Our results showed that TRPV4 modulation is the primary factor contributing to the anti-ischemic effects of FBC, and TRPV4 channel inactivation relieve focal ischemic infarction by relieving BBB disruption and preventing apoptosis. Therefore, FBC treatment improves ischemic stroke through the modulation of TRPV4 channels.

*Key words:* cerebral infarction, transient receptor potential vanilloid 4, knockout mice, photochemically induced thrombosis, blood-brain barrier, apoptosis

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## Introduction

Cerebral infarction has high morbidity and mortality,[1-3] although recanalization with recombinant tissue-type plasminogen activators and thrombus retrieval have enabled rapid and effective functional restoration. After ischemic stroke, protection of blood-brain barrier (BBB) is important because BBB disruption injuries brain neurons and may result in a poor prognosis.[4] Focal brain cooling (FBC) in rodents and selective brain hypothermia in patients with acute ischemic stroke are reported to induce neuroprotection.[5-7] These anti-ischemic effects suggest that FBC relieves cerebral infarction; however, the mechanisms underlying its anti-ischemic effects remain unclear.

Considering the anti-ischemic effects of FBC, we focused on transient receptor potential (TRP) channels. The physiological functions of TRP channels depend on their activation within a specific temperature range.[8] TRP vanilloid 4 (TRPV4) is expressed in neurons in the cerebral cortex, thalamus, hippocampus, and cerebellum, among others,[9] and is inactivated by cooling (< 27℃) and HC-067047, a TRPV4 antagonist.[10,11]

FBC at 15℃, TRPV4 antagonist administration, and TRPV4 deficiency relieve brain infarction.[6,12,13] TRPV4 channels are inactivated by cooling at 15℃,[10] suggesting that anti-ischemic effects of FBC include those of TRPV4 inactivation. However, the extent to which TRPV4 inactivation contributes to the anti-ischemic effects of FBC on cerebral infarction remains unclear. Attenuation of activation of the apoptotic signaling pathway and prevention of BBB disruption by TRPV4 antagonists and deficiency are mechanisms of anti-ischemia.[12-15] These results indicated that FBC and TRPV4 antagonist treatment relieved BBB disruption and apoptosis. However, the extent to which TRPV4 inactivation contributed to the effects of FBC on BBB disruption and apoptosis remains unclear.

In this study, we compared the anti-ischemic effects of FBC and TRPV4 antagonist administration in cerebral ischemic stroke model mice to clarify the extent to which TRPV4 inactivation contributes to the anti-ischemic, anti-BBB disruption, and anti-apoptosis effects of FBC on cerebral infarction. In addition, we compared the results between wild-type (WT) mice and TRPV4 knockout (TRPV4KO) mice to clarify the extent to which TRPV4 deficiency contributes to the anti-ischemic and anti-BBB disruption effects of FBC on cerebral infarction.

## Methods

### Animals

We used only male mice because of the influence of biological sex on cerebrovascular health in hypertension.[16] Male C57BL/6 mice aged 10-15 weeks and weighing 25-30 g (Japan SLC, Fukuoka, Japan) and TRPV4KO mice (Prof. Makoto Tominaga, Thermal Biology Group, Exploratory Research Center on Life and Living Systems, Okazaki, Japan) were housed in groups of five mice per cage under standard laboratory conditions in a temperature- and humidity-controlled room (25 ± 2℃, 55% ± 5%, respectively) on a 12-h light/dark cycle (lights on at 8:00 a.m.). The animals had ad libitum access to food and water. Animal care and experimental procedures were approved by the Experimental Animal Care and Use Committee of the Yamaguchi University School of Medicine (J18030 and J23053), Japan, and the experiments were performed in accordance with the guidelines of the Japan Association for Laboratory Animal Facilities of National University Corporations. The study reported in accordance with ARRIVE guidelines.

### Photochemically Induced Thrombosis (PIT) method and FBC

Focal cerebral ischemia of the primary sensorimotor (SI-MI) cortex of the right hemisphere was induced using the PIT method, and FBC treatment was administered, according to a previously reported method.[6] The protocol is illustrated in Figure 1A. All mice were anesthetized by inhalation of isoflurane (3% for induction and 1.5% for maintenance; Pfizer, New York, NY) in an 80%/20% mixture of oxygen and nitrogen delivered via a ventilator (ADS 1000; Engler Engineering Corporation, Hialeah, FL). The rectal temperature was monitored and kept constant at

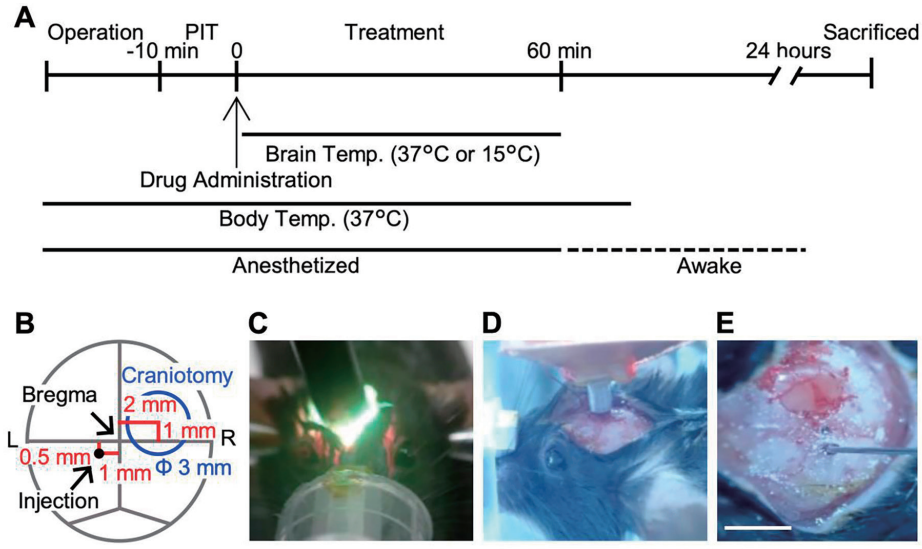


Fig. 1 The experimental protocol and images of each operation.
(A) Protocols for photochemically induced thrombosis (PIT), focal brain cooling, and drug treatments. (B) Schematic diagrams of the craniotomy area and the burr hole for intracerebroventricular injection. (C) Image causing a photochemically induced thrombosis. (D) Image of the thin thermo-sensor and Peltier device placed on the brain surface at the craniotomy site. (E) Image of the brain surface at the craniotomy location with a 30-G stainless steel needle inserted into the burr hole. The scale bar is 3 mm.

37 ± 1℃ by a temperature-controlled heating pad (NS-TC10, NeuroScience Inc., Osceola, WI) to maintain a constant body temperature during the procedure. A scalp incision was made, and the bregma was exposed after dissection of the pericranial tissue. A craniotomy (3 mm diameter) was performed at the center of the following coordinates relative to the bregma: 1 mm anterior and 2 mm lateral to the right side (Fig. 1B). Rose Bengal (0.1 mL) 10 mg/kg solution in saline (Wako, Glostrup, Denmark) was injected via the left jugular vein. Five min after injection, the craniotomy site was illuminated for 10 min using a fiber-optic bundle constituting an LED light source (KL 2500 LED; Schott, Mainz, Germany) (Fig. 1C). Referring to a previous study,[17] a Peltier device based on air-cooling was made. The Peltier device with a thin thermosensor (IT-24; Physitemp, Clifton, NJ) was placed at the craniotomy site (Fig. 1D). Cooling was performed above the dura mater at the craniotomy site at 15℃ for 60 min (cooling group). A brain temperature of 37℃ was maintained for 60 min in the control group. The body temperature spontaneously recovered immediately after cooling.

## Drug treatment

All drugs and solvents were injected intracerebroventricularly, as previously reported.[12] A small burr hole was made 0.5 mm posterior and 1.0 mm lateral to the left side of the bregma after craniotomy. A 30-G stainless steel needle (TSK Laboratory Japan, Tochigi, Japan) was inserted into the burr hole at a depth of 2.5 mm for intracerebroventricular injection of drugs immediately after photothrombosis (Fig. 1E). The TRPV4 agonist, GSK1016790A, and TRPV4 antagonist, RN1734, were dissolved in 1% dimethyl sulfoxide (DMSO; Merck, Rahway, NJ) in saline. GSK1016790A (10 μM) and RN1734 (1 mM) were administered for 15 min at a rate of 0.5 μL/min using a microinjection pump and a 10-μL Hamilton syringe (1701RN-7758-02; Hamilton, Reno, NV). The cortical temperature was controlled for 60 min during intracerebroventricular injection using a Peltier device. The 30-G stainless steel needle was left in place to prevent drug solution leakage until maintenance of the brain surface

temperature at 15℃ or 37℃ was completed (60 min). The vehicle group was injected with 1% DMSO. The animals were randomly assigned to the following experimental groups: cortex temperature controlled at 37℃, intracerebroventricular injection of 1% DMSO (DMSO + non-cooling group); FBC at 15℃, intracerebroventricular injection of 1% DMSO (DMSO + cooling group); cortex temperature controlled at 37℃, intracerebroventricular injection of RN1734 (RN1734 + non-cooling group); and FBC at 15℃, intracerebroventricular injection of GSK1016790A (GSK1016790A + cooling group). In TRPV4KO mice, the cortical temperature was controlled at 37℃, and 1% DMSO was injected intracerebroventricularly (TRPV4KO group).

### Measurement of the infarct area by 2,3,5-triphenyltetrazolium chloride (TTC) staining

TTC is enzymatically reduced to red 1,3,5-triphenylformazan in living tissues and remains white in dead tissues with cell death. Therefore, TTC staining is commonly used as an indicator of cell death. Animals were sacrificed 24 h after ischemia and transcardially perfused with bubbled phosphate-buffered saline (PBS). Whole brains were immediately removed and cut into 1-mm sections. Slices were stained with a 2% TTC solution and incubated in a dark chamber maintained at 37℃ for 10 min. Four brain sections were selected from the anterior and posterior regions of the infarcted area. Infarct area was determined as a percentage of the ischemic lesion relative to the contralateral hemisphere area and was the mean of 4 slices. Infarct area was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

### Evans blue (EB) injection

BBB disruption is an important pathophysiological process of acute ischemic stroke,[18] resulting in devastating malignant brain edema. Brain edema is associated with 80% of ischemic infarcts and intracerebral hemorrhage (ICH),[19,20] therefore, protection of BBB disruption is crucial. The leakage of EB, a high-molecular-weight blue pigment, into the ipsilateral hemisphere was measured to assess BBB function. BBB permeability was evaluated 2 h after PIT by measuring EB extravasation (Sigma-Aldrich, St. Louis, MO). A 2% EB solution (4 mL/kg) was injected intravenously 1 h after PIT, and the mice were transcardially perfused with bubbled PBS. Whole brains were removed and cut into 1 mm-thick sections. Slices were promptly weighed and homogenized in 50% trichloroacetic acid. The homogenate was centrifuged (12,000 g, 20 min), and the supernatant was diluted with ethanol (1:3). The EB content in the cerebral hemispheres was quantified at 620 nm using a microplate reader (Bio-Rad 550; Bio-Rad, Hercules, CA). EB leakage was quantified using a standard curve and expressed as the amount per gram of brain tissue.

### Measurement of the infarct area using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

The animals were sacrificed 24 h after ischemia and transcardially perfused with bubbled PBS followed by 4% paraformaldehyde. Whole brains were removed, fixed overnight in 4% paraformaldehyde, and embedded in paraffin before being cut into 5-μm sections. The brain sections located around the central infarct area were selected. Propidium iodide (Sigma-aldrich, St. Louis, MO) staining was used for cell detection (red). Apoptotic cells were detected in each section using TUNEL (Promega, Madison, WI). The number of TUNEL-positive cells (green) in the entire ipsilateral hemisphere was counted using a fluorescence microscope (BZ-9000; KEYENCE, Tokyo, Japan).

### Statistical analyses

All results are expressed as the mean ± standard error of the mean (SEM). Statistical analyses of the infarct area and the amount of EB leakage in multiple groups were performed using Tukey's test. The number of TUNEL-positive cells was assessed using one-way ANOVA followed by Tukey's or Dunnett's test. All tests were two-tailed, and statistical significance was set at $p\text{-value} < 0.05$. Statistical analysis was performed using JMP Pro 16.1.0 for MAC (SAS Institute, Cary, NC).

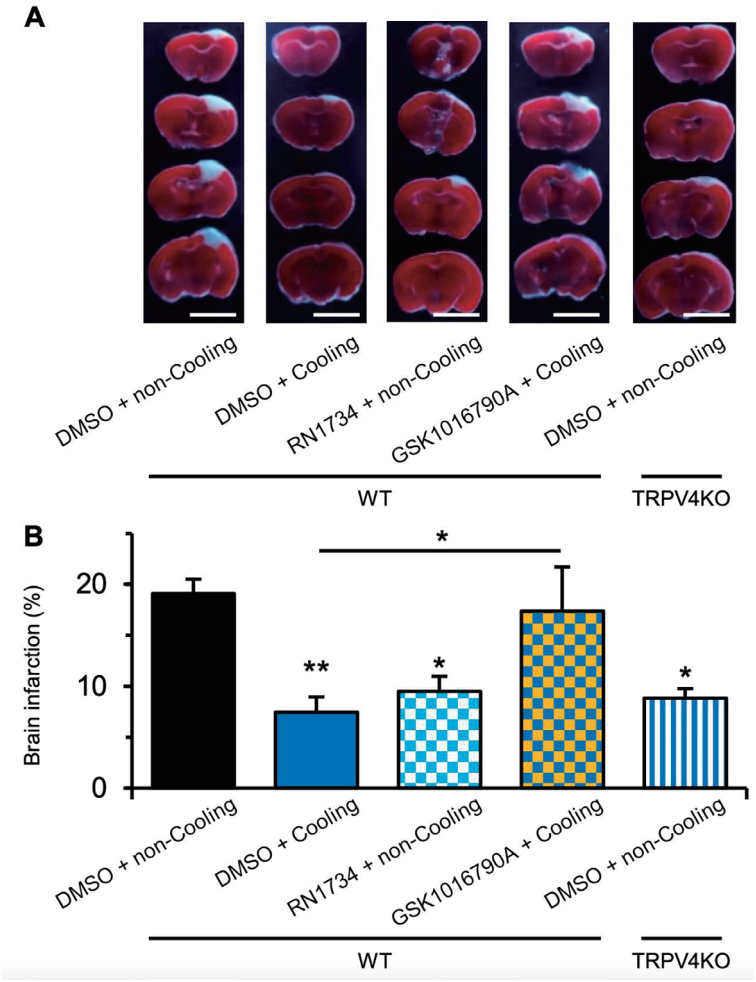


Fig. 2 Effects of focal brain cooling, TRPV4 antagonist, TRPV4 agonist with focal brain cooling, and TRPV4 deficiency on focal ischemic stroke by photochemically induced thrombosis.

(A) Representative TTC staining 24 h after ischemia in each group: DMSO + non-cooling (n = 8), DMSO + cooling (n = 7), RN1734 + non-cooling (n = 10), and GSK1016790A + cooling (n = 7) in wild-type mice or 1% DMSO solution in TRPV4 knockout mice (n = 7). The scale bar is 5 mm. Ischemic lesion, white; normal brain tissues, red. (B) The percentage of brain infarction in each group. The results are shown as the mean ± SEM. One-way ANOVA test, $F_{(4, 33)} = 6.6211$, $p = 0.0005$. *$p < 0.05$, **$p < 0.01$, Tukey's test.

TRPV4: transient receptor potential vanilloid 4; TTC: 2,3,5-triphenyltetrazolium chloride; SEM: standard error of the mean; WT: wild-type; KO: knockout; RN1734: a TRPV4 antagonist; GSK1016790A: a TRPV4 agonist

## Results

### Anti-ischemic effect of FBC treatment and TRPV4 inactivation

Brain infarction was induced by PIT (Fig. 1) and detected by TTC staining. Representative TTC staining 24 h after PIT in each group is shown in Figure 2A. First, the infarct areas were compared between the non-cooled and cooled groups to clarify the extent to which FBC treatment was effective for cerebral infarction. PIT caused an infarct lesion in 19.10 ± 1.40 percentage of the whole ipsilateral hemisphere area (Fig. 2B). The infarct lesion was reduced by approximately 61% by FBC for 1 h after PIT (7.46% ± 1.49%, $p = 0.006$ vs. DMSO + non-cooling, Tukey's test; Fig. 2B). Second, the infarct area was compared

between FBC and TRPV4 antagonist treatments to clarify the extent to which TRPV4 inactivation contributes to the anti-ischemic effect of FBC. The TRPV4 antagonist reduced the infarct lesion by approximately 50% (9.51% ± 1.47%, $p$ = 0.014 vs. DMSO + non-cooling, Tukey's test; Fig. 2B). These anti-ischemic efficacies of FBC and TRPV4 antagonist were same level ($p$ = 0.827 vs. DMSO + cooling, Tukey's test; Fig. 2B). In contrast, the reduction of the infarct lesion by FBC was completely eliminated by TRPV4 agonist administration (17.39% ± 4.33%, $p$ = 0.016 vs. DMSO + cooling, Tukey's test; Fig. 2B). Third, we confirmed whether the anti-ischemic efficacies of TRPV4 antagonist treatment and TRPV4 deficiency were the same level. TRPV4 deficiency reduced the infarct lesion by approximately 54% (8.82% ± 0.95%, $p$ = 0.016 vs. DMSO + non-cooling, Tukey's test; Fig. 2B) without FBC treatment. The anti-ischemic efficacy of TRPV4 deficiency was same level, compared with TRPV4 antagonist treatment ($p$ = 0.999, Tukey's test; Fig. 2B). The anti-ischemic effect of TRPV4 deficiency supports the results of TRPV4 antagonist treatment in WT mice.

### Protection against BBB disruption by FBC and TRPV4 inactivation

EB leakage was compared among the groups to assess the efficacies of FBC treatment, RN1734, and TRPV4 deficiency on BBB disruption. Fig. 3A shows representative Evans blue leakage into the brain parenchyma in each group. Cerebral infarction increased the amount of EB leakage to 5.39 ± 0.44 μg/g. FBC and TRPV4 antagonist treatment reduced the amount of EB leakage to 2.70 ± 0.60 μg/g and 3.04 ± 0.25 μg/g, respectively ($p$ = 0.018 and $p$ = 0.039 vs. DMSO + non-cooling group, Tukey's test; Fig. 3B). The reduction in EB leakage was similar between the two groups ($p$ = 0.968, Tukey's test; Fig. 3B). TRPV4 deficiency without FBC treatment reduced the EB leakage to 2.88 ± 0.72 μg/g ($p$ = 0.027 vs. DMSO + non-cooling group, Tukey's test; Fig. 3B). The reduction in EB leakage provided by TRPV4 deficiency was similar to that provided by FBC and TRPV4 antagonist treatments ($p$ = 0.994 vs. DMSO + cooling group and $p$ = 0.997, respectively, vs. RN1734 + non-cooling group, Tukey's test; Fig. 3B). The reduction in EB leakage due to TRPV4 deficiency supports the results of TRPV4 antagonist treatment in WT mice.

### Anti-apoptotic effects of FBC treatment and intracerebroventricular administration of a TRPV4 antagonist

Apoptosis was detected using TUNEL. Figure 4A shows representative PIT-induced focal cortical ischemia and a representative region of apoptotic cells. The number of PI-positive cells after cerebral infarction was not different among each group (One-way ANOVA test, $F_{(3, 9)}$ = 0.0247, $p$ = 0.994; Fig. 4B). The number of apoptotic cells after cerebral infarction was 513.75 ± 63.47. FBC and TRPV4 antagonist treatments reduced the number of apoptotic cells to 76.50 ± 6.18 and 140.00 ± 33.98, respectively ($p$ = 0.015 and $p$ = 0.034 vs. DMSO + non-cooling, Dunnett's test; Fig. 4C), with similar levels of reduction between the two groups ($p$ = 0.958, Tukey's test; Fig. 4C). The reduction in apoptotic cell death by FBC treatment was completely eliminated by TRPV4 agonist treatment (527.67 ± 220.96, $p$ = 0.032 vs. DMSO + Cooling and $p$ = 1.000 vs. DMSO + non-cooling; Tukey's test; Fig. 4C). These results and representative images of TUNEL-positive cells in Fig. 4D suggest that the anti-apoptotic effect of FBC can be reversed with a TRPV4 agonist.

## Discussion

In this study, we clarified the involvement and mechanisms of TRPV4 inactivation as one of the protective effects of FBC against cerebral infarction. Our study had two major findings. First, the majority of the protective effects of FBC against infarction were caused by TRPV4 channel inactivation. Second, RN1734, the TRPV4 antagonist, TRPV4 deficiency, and FBC treatment relieved BBB disruption and prevented apoptosis after infarction.

We investigated the extent to which TRPV4 inactivation contributed to the protective effect of FBC against cerebral infarction. Our data showed that the PIT-induced ischemic area in WT mice was reduced to approximately half by FBC treatment at 15℃,

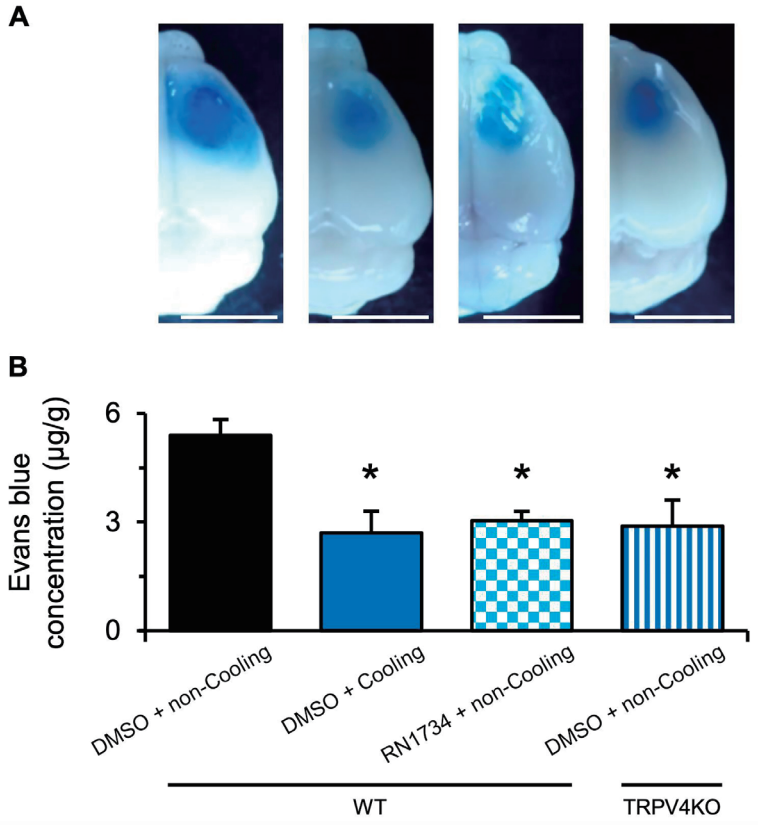


Fig. 3 Effects of focal brain cooling, TRPV4 antagonist, and TRPV4 deficit on photochemically induced thrombosis-induced Evans blue leakage.

(A) Representative examples of Evans blue leakage into the brain parenchyma in each group: DMSO + non-cooling (n = 4), DMSO + cooling (n = 4), and RN1734 + non-cooling (n = 4) in wild-type (WT) mice or 1% DMSO solution in TRPV4 knockout mice (n = 4). The scale bar is 5 mm. (B) The amount of Evans blue leakage into the brain parenchyma. The results are shown as the mean ± SEM. One-way ANOVA test, $F_{(3, 12)} = 5.6215$, $p = 0.0121$. *$p < 0.05$, vs. WT / DMSO+ non-Cooling group, Tukey's test.

TRPV4: transient receptor potential vanilloid 4; SEM: standard error of the mean; WT: wild-type; KO: knockout; RN1734: a TRPV4 antagonist

intracerebroventricular injection of a TRPV4 antagonist, and TRPV4 deficiency (Fig. 2B). The reduction in infarct lesions did not differ significantly among the three groups. The reduction in the infarct lesion by FBC was eliminated by the injection of GSK1016790A, a TRPV4 agonist, and the infarct lesion did not differ from that in the DMSO + non-cooling group (Fig. 2B). The anti-ischemic effects of each treatment and TRPV4 deficiency on ischemia are supported by previous reports. FBC at 15℃ for 5 h reduced the PIT-induced ischemic area, and a TRPV4 antagonist decreased the infarct area in middle cerebral artery occlusion (MCAO) mice.[6,12] The infarct area in MCAO mice was also reduced by TRPV4 deficiency.[13] Therefore, our data indicated that most anti-ischemic effects of FBC were mediated by TRPV4 channel inactivation. Because TRPA1 inactivation also contribute the anti-ischemic effects,[21] further studies using TRPV4KO mice for FBC treatment, TRPV4 agonist, and TRPV4 antagonist may elucidate direct or indirect pathways for the anti-ischemic effects of TRPV4 channel inactivation and additive or synergistic effects between FBC and TRPV4 antagonist.

Considering that BBB disruption exacerbates ischemic stroke, protection of BBB disruption is crucial.[18-20] Our data showed that

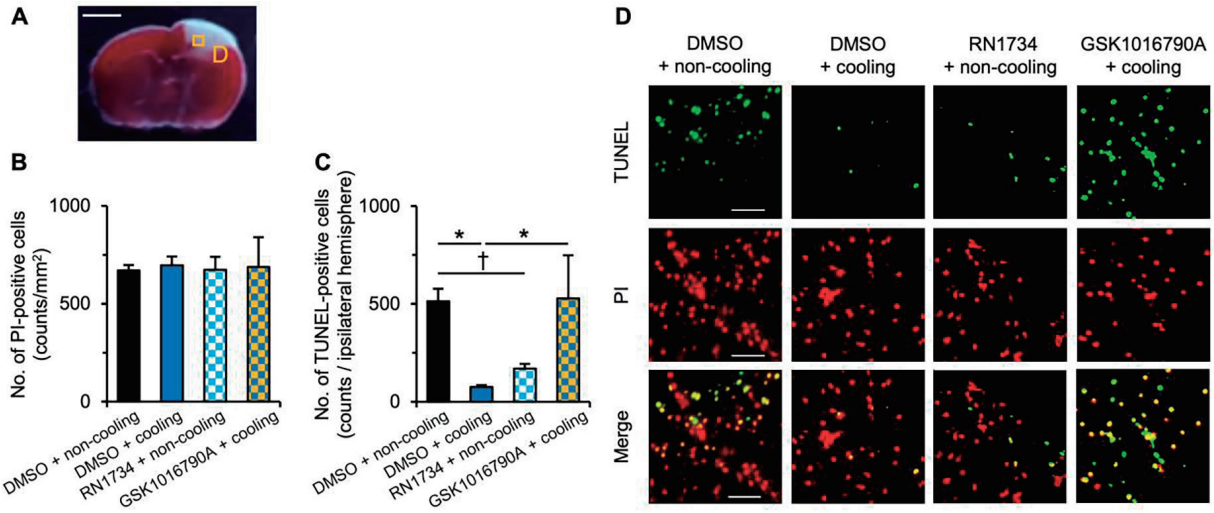


Fig. 4 Effects of focal brain cooling, TRPV4 antagonist, and TRPV4 deficiency on apoptosis induced by PIT.

(A) Representative PIT-induced focal-cortical ischemia. The scale bar is 3 mm. Orange square indicates the representative region of interest for TUNEL staining. (B,C) Number of PI-positive cells and TUNEL-positive cells in the ischemic hemisphere in each group; DMSO + non-cooling (n = 4), DMSO + cooling (n = 4), RN1734 + non-cooling (n = 4), and GSK1016790A + cooling (n = 3) in wild-type mice. (D) Representative images of TUNEL-positive cells, PI staining, and merged images in each group are shown using high-magnification (×20) photomicrographs. The scale bar is 20 μm. The results are shown as the mean ± SEM. One-way ANOVA test, $F_{(3, 11)} = 6.6041$, $p = 0.0082$. $^{*}p < 0.05$, Tukey's test; $^{\dagger}p < 0.05$, vs DMSO + non-cooling, Dunnett's test.

TRPV4: transient receptor potential vanilloid 4; SEM: standard error of the mean; PIT: photochemically induced thrombosis; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PI: propidium iodide; RN1734: a TRPV4 antagonist; GSK1016790A: a TRPV4 agonist

EB leakage was increased by transient focal ischemia and relieved by FBC, intracerebroventricular injection of a TRPV4 antagonist, and TRPV4 deficiency (Fig. 3B). The reduction in EB leakage was similar among the three groups (Fig. 3B). The impact of both treatment and TRPV4 deficiency on BBB disruption closely mirrored findings from prior studies. Pharmacologically induced hypothermia (33℃) protected against BBB disruption after focal cerebral ischemic stroke in mice.[14] ICH increased EB extravasation around the arterioles, and perivascular EB dye leakage was reduced by treatment with a TRPV4 antagonist.[15] The extent of EB leakage was significantly lower in *Trpv4*$^{-/-}$ mice than in WT mice.[13] Therefore, our data indicated that the majority of FBC-induced BBB protection is mediated by TRPV4 channel inactivation.

Our results showed that apoptosis in the infarct hemisphere due to PIT was reduced by FBC at 15℃ and intracerebroventricular injection of a TRPV4 antagonist (Fig. 4C). The extent of apoptosis was not significantly different between the two groups (Fig. 4C). The anti-apoptotic effect of FBC treatment was eliminated with TRPV4 agonist injection immediately after PIT (Fig. 4C). The effects of FBC and TRPV4 antagonist on apoptosis were consistent with those reported in earlier studies. Pharmacologically induced hypothermia (33℃) decreased the number of TUNEL-positive cells in the penumbra region of mice with focal cerebral ischemic stroke and increased the expression of the anti-apoptotic gene *Bcl2*.[14] ICH caused an increase in apoptosis in the perihematomal area 24 h after their onset, and treatment with a TRPV4

antagonist significantly reduces the number of TUNEL-positive cells and effectively prevents apoptosis.[15] In contrast, TRPV4 agonist induced neuronal death in hippocampal CA1-3 areas in a dose-dependent manner.[12] Apoptosis requires energy and is prominent in neurons in the ischemic penumbral region, where energy production occurs.[21-23] Based on these findings, our data indicated that most FBC-induced anti-apoptotic effects were mediated by TRPV4 channel inactivation. Since TRPV4 channels in neurons, astrocytes, and microglia could have different roles in ischemic stroke,[12,24-27] further studies may clarify cell-specific anti-ischemic effects of TRPV4 channels.

In this study, we evaluated the association and mechanisms of FBC-induced TRPV4 inactivation on the protective effects against cerebral ischemic stroke in mice. Our present data showed that TRPV4 channel inactivation contributed to most of the anti-ischemic effects of FBC. Comparing between male and female mice for ischemic stroke would add valuable findings because of the influence of biological sex on cerebrovascular health in hypertension.[16] Our present data also showed that TRPV4 channel inactivation contributed to most of the relief of FBC treatment on BBB disruption and apoptosis. Because the neuroprotection of the inflammation, reactive oxygen species, and mitochondrial homeostasis also contribute anti-ischemic effects, further studies contribute more understanding of anti-ischemic mechanisms of FBC through the modulation of TRPV4 channels. In conclusion, prevention of BBB disruption and apoptosis in the ischemic penumbral region improves functional outcomes after stroke; therefore, FBC treatment may improve functional outcomes after stroke through the modulation of TRPV4 channels.

## Conclusion

The majority of the protective effects of FBC against infarction were caused by TRPV4 channel inactivation. The protective effects of FBC against infarction mediated by TRPV4 inactivation included the protection of BBB disruption and prevention of apoptosis.

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## Conflict of Interest

The authors declare no conflict of interest.

## References

1. Iadecola C, Anrather J. Stroke research at a crossroad: asking the brain for directions. Nat Neurosci. 2011 Oct 26; 14(11): 1363-8. doi: 10.1038/nn.2953.
2. Kim AS, Cahill E, Cheng NT. Global Stroke Belt: Geographic Variation in Stroke Burden Worldwide. Stroke. 2015 Dec; 46(12): 3564-70. doi: 10.1161/STROKEAHA.115.008226. Epub 2015 Oct 20.
3. Hankey GJ. Stroke. Lancet. 2017 Feb 11; 389(10069): 641-654. doi: 10.1016/S0140-6736(16)30962-X. Epub 2016 Sep 13.
4. Molina CA, Alvarez-Sabín J. Recanalization and reperfusion therapies for acute ischemic stroke. Cerebrovasc Dis. 2009; 27 Suppl 1: 162-7. doi: 10.1159/000200455. Epub 2009 Apr 3.
5. Choi JH, Poli S, Chen M, Nguyen TN, Saver JL, Matouk C, Pile-Spellman J. Selective BrainHypothermia in Acute Ischemic Stroke: Reperfusion Without Reperfusion Injury. Front Neurol. 2020 Nov 13; 11: 594289. doi: 10.3389/fneur.2020.594289.
6. He Y, Fujii M, Inoue T, Nomura S, Maruta Y, Oka F, Shirao S, Owada Y, Kida H, Kunitsugu I, Yamakawa T, Tokiwa T, Yamakawa T, Suzuki M. Neuroprotective effects of focal brain cooling on photochemically-induced cerebral infarction in rats: analysis from a

neurophysiological perspective. Brain Res. 2013 Feb 25; 1497: 53-60. doi: 10.1016/j.brainres.2012.11.041. Epub 2012 Dec 23.
7. Cattaneo G, Schumacher M, Wolfertz J, Jost T, Meckel S. Combined Selective Cerebral Hypothermia and Mechanical Artery Recanalization in Acute Ischemic Stroke: In Vitro Study of Cooling Performance. AJNR Am J Neuroradiol. 2015 Nov; 36(11): 2114-20. doi: 10.3174/ajnr.A4434. Epub 2015 Aug 6.
8. Clapham DE. TRP channels as cellular sensors. Nature. 2003 Dec 4; 426(6966): 517-24. doi: 10.1038/nature02196.
9. Liedtke W, Choe Y, Martí-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell. 2000 Oct 27; 103(3): 525-35. doi: 10.1016/s0092-8674(00)00143-4.
10. Güler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M. Heat-evoked activation of the ion channel, TRPV4. J Neurosci. 2002 Aug 1; 22(15): 6408-14. doi: 10.1523/JNEUROSCI.22-1506408.2002.
11. Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD, Nilius B. Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. J Biol Chem. 2002 Apr 19; 277(16): 13569-77. doi: 10.1074/jbc.M200062200. Epub 2002 Feb 4.
12. Jie P, Lu Z, Hong Z, Li L, Zhou L, Li Y, Zhou R, Zhou Y, Du Y, Chen L, Chen L. Activation of Transient Receptor Potential Vanilloid 4 is Involved in Neuronal Injury in Middle Cerebral Artery Occlusion in Mice. Mol Neurobiol. 2016 Jan; 53(1): 8-17. doi: 10.1007/s12035-014-8992-2. Epub 2014 Nov 18.
13. Tanaka K, Matsumoto S, Yamada T, Yamasaki R, Suzuki M, Kido MA, Kira JI. Reduced Post-ischemic Brain Injury in Transient Receptor Potential Vanilloid 4 Knockout Mice. Front Neurosci. 2020 May 12; 14: 453. doi: 10.3389/fnins.2020.00453.
14. Choi KE, Hall CL, Sun JM, Wei L, Mohamad O, Dix TA, Yu SP. A novel stroke therapy of pharmacologically induced hypothermia after focal cerebral ischemia in mice. FASEB J. 2012 Jul; 26(7): 2799-810. doi: 10.1096/fj.11-201822. Epub 2012 Mar 29.
15. Zhao H, Zhang K, Tang R, Meng H, Zou Y, Wu P, Hu R, Liu X, Feng H, Chen Y. TRPV4 Blockade Preserves the Blood-Brain Barrier by Inhibiting Stress Fiber Formation in a Rat Model of Intracerebral Hemorrhage. Front Mol Neurosci. 2018 Mar 27; 11: 97. doi: 10.3389/fnmol.2018.00097.
16. Chambers LC, Yen M, Jackson WF, Dorrance AM. Female mice are protected from impaired parenchymal arteriolar TRPV4 function and impaired cognition in hypertension. Am J Physiol Heart Circ Physiol. 2023 May 1; 324(5): H581-H597. doi: 10.1152/ajpheart.00481.2022. Epub 2023 Mar 10.
17. Shibasaki K, Yamada K, Miwa H, Yanagawa Y, Suzuki M, Tominaga M, Ishizaki Y. Temperature elevation in epileptogenic foci exacerbates epileptic discharge through TRPV4 activation. Lab Invest. 2020 Feb; 100(2): 274-284. doi: 10.1038/s41374-019-0335-5. Epub 2019 Oct 22.
18. Qian C, Li PC, Jiao Y, Yao HH, Chen YC, Yang J, Ding J, Yang XY, Teng GJ. Precise Characterization of the Penumbra Revealed by MRI: A Modified Photothrombotic Stroke Model Study. PLoS One. 2016 Apr 19; 11(4): e0153756. doi: 10.1371/journal.pone.0153756.
19. Stokum JA, Gerzanich V, Simard JM. Molecular pathophysiology of cerebral edema. J Cereb Blood Flow Metab. 2016 Mar; 36(3): 513-38. doi: 10.1177/0271678X15617172. Epub 2015 Nov 16.
20. Hoshi Y, Okabe K, Shibasaki K, Funatsu T, Matsuki N, Ikegaya Y, Koyama R. Ischemic Brain Injury Leads to Brain Edema via Hyperthermia-Induced TRPV4 Activation. J Neurosci. 2018 Jun 20; 38(25): 5700-5709. doi: 10.1523/JNEUROSCI.2888-17.2018. Epub 2018 May 23.
21. Sabri M, Lass E, Macdonald RL. Early brain injury: a common mechanism in subarachnoid hemorrhage and global cerebral ischemia. Stroke Res Treat. 2013;

2013: 394036. doi: 10.1155/2013/394036. Epub 2013 Feb 28.
22. Mehta SL, Vemuganti R (2014). Mechanisms of stroke induced neuronal death: multiple therapeutic opportunities. Adv. Anim. Vet. Sci. 2 (8): 438-446. doi: http: // dx.doi.org/10.14737/journal.aavs/2014/2.8.438.446
23. Uzdensky AB. Apoptosis regulation in the penumbra after ischemic stroke: expression of pro- and antiapoptotic proteins. Apoptosis. 2019 Oct; 24(9-10): 687-702. doi: 10.1007/s10495-019-01556-6.
24. Dunn KM, Hill-Eubanks DC, Liedtke WB, Nelson MT. TRPV4 channels stimulate Ca2+ induced Ca2+ release in astrocytic endfeet and amplify neurovascular coupling responses. Proc Natl Acad Sci U S A. 2013 Apr 9; 110(15): 6157-62. doi: 10.1073/pnas.1216514110. Epub 2013 Mar 25.
25. Nishimoto R, Derouiche S, Eto K, Deveci A, Kashio M, Kimori Y, Matsuoka Y, Morimatsu H, Nabekura J, Tominaga M. Thermosensitive TRPV4 channels mediate temperature-dependent microglia movement. Proc Natl Acad Sci U S A. 2021 Apr 27; 118(17): e2012894118. doi: 10.1073/pnas.2012894118.
26. Pires PW, Earley S. Neuroprotective effects of TRPA1 channels in the cerebral endothelium following ischemic stroke. Elife. 2018 Sep 21; 7: e35316. doi: 10.7554/eLife.35316.
27. Tureckova J, Hermanova Z, Marchetti V, Anderova M. Astrocytic TRPV4 Channels and Their Role in Brain Ischemia. Int J Mol Sci. 2023 Apr 12; 24(8): 7101. doi: 10.3390/ijms24087101.