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Neuroprotective Effect of TRPA1 Activation in Focal Cerebral Ischemia: Insights from Wild-Type and Knockout Mice

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Abstract Hypothermia and focal brain cooling (FBC) demonstrate neuroprotective effects in ischemic stroke, but their invasiveness limits clinical use. We explored transient receptor potential (TRP) channels as an alternative, focusing on TRP Ankyrin 1 (TRPA1), which operates within the temperature range of FBC. Activation of TRPA1 has been reported to offer neuroprotection, suggesting it may contribute to the effects seen with FBC. We hypothesized that pharmacological activation of TRPA1 could replicate the neuroprotective effects of FBC, providing a less invasive treatment for cerebral infarction. We examined the effects of a TRPA1 agonist and FBC in focal cerebral ischemia induced by photochemically triggered thrombosis in wild-type (WT) and TRPA1 knockout (KO) mice. In WT mice, intracerebroventricular administration of the TRPA1 agonist allyl isothiocyanate reduced infarct size by approximately half, comparable to FBC. TRPA1 KO mice had larger infarcts than WT, but FBC significantly reduced infarct size in both groups. Furthermore, Evans blue extravasation, used to assess the extent of blood-brain barrier disruption, was approximately twice as high in TRPA1KO mice compared to WT mice. These findings underscore the neuroprotective potential of TRPA1 agonists and the increased vulnerability against ischemia with TRPA1 deficiency. However, the neuroprotective effects of TRPA1 activation are likely mediated by a mechanism distinct from that of FBC. Our study suggests TRPA1 channels are crucial for ischemic stroke protection and may offer a novel therapeutic approach.

Key words: ischemic stroke, transient receptor potential ankyrin 1, knockout mice, photochemically induced thrombosis, neuroprotection

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Introduction

Stroke is the leading cause of death and disability worldwide, with ischemic stroke accounting for the majority of cases.[1,2] Due to population growth and aging, the incidence of ischemic stroke is expected to rise significantly in the coming decades.[3] The only approved pharmacological treatments for acute ischemic stroke are intravenous thrombolysis with alteplase and tenecteplase, a recombinant tissue plasminogen activator. For large vessel occlusion, endovascular treatment with mechanical thrombectomy can be combined. However, no effective treatment exists for infarctions that these therapies cannot salvage. The neuroprotective effects of hypothermia have long been established, but the use of systemic hypothermia for the therapy of neuronal injuries, including ischemic stroke, was discontinued due to severe side effects such as cardiac arrhythmia, shivering, infections, and coagulation disorders.[4,5] To minimize these complications, selective brain cooling methods have been explored.[6-8] Selective brain cooling can be categorized into noninvasive and invasive cooling.[9] Invasive cooling offers more direct and targeted focal cooling compared to noninvasive methods like surface or intranasal cooling. Our department previously developed a focal brain cooling (FBC) device and demonstrated its anti-ischemia effects.[10] However, despite its proven efficacy, the high risks associated with invasive procedures present significant hurdles to clinical use. Whether systemic or selective, the neuroprotective effect of cooling is currently associated with a risk of complications.

Transient receptor potential (TRP) channels are membrane proteins, and the mammalian TRP superfamily is grouped into six subfamilies based on amino acid sequence homology. Some TRP channels are activated within specific temperature ranges. In recent years, therapeutic approaches leveraging the temperature-dependent activation of TRP channels have been explored. For example, cooling is known to suppress epileptiform discharges[11], and we previously demonstrated that pharmacological activation of the TRP melastatin 8 channel, which is activated by temperatures between 10-26 ℃, suppresses these discharges without cooling.[12,13] Therefore, we hypothesized that pharmacological activation of TRP channels could mimic the neuroprotective effects of hypothermia, offering a less invasive treatment for cerebral infarction.

TRP Ankyrin 1 (A1) channels, broadly expressed in the central nervous system, particularly in astrocytes, brain capillary endothelial cells, dorsal root ganglia, and trigeminal ganglia, are activated by low temperatures around 17 ℃.[14-18] Activation of TRPA1 channels has been reported to be neuroprotective against cerebral ischemia[19,20] suggesting that TRPA1 channels, which operate within the temperature range used in FBC, may contribute to the anti-ischemic effects observed with FBC. FBC has been reported to exert neuroprotective effects against focal cerebral ischemia by preventing blood-brain barrier (BBB) disruption.[21] Since TRPA1 is expressed in capillary endothelial cells and astrocytes that constitute the BBB, we also considered that the prevention of BBB disruption through TRPA1 activation might contribute to the neuroprotective effects mediated by TRPA1. However, no studies have directly demonstrated the role and mechanism of TRPA1 channels in the neuroprotective effects of hypothermia. In this study, we utilized TRPA1 knockout (KO) mice to investigate whether TRPA1 channels are involved in the neuroprotective mechanisms of brain cooling.

Materials and methods

Animals

Male C57BL/6 mice aged 10-15 weeks, weighing 25-30 g (Japan SLC Inc. Fukuoka, Japan), and TRPA1 homozygous KO (TRPA1KO) mice (Prof. Makoto Tominaga, Thermal Biology Group, Exploratory Research Center on Life and Living Systems, Okazaki, Japan) were housed in groups of five per cage and kept under standard laboratory conditions in a temperature- and humidity-controlled room (25 ± 2 ℃, 55% ± 5%, respectively) with a 12 h light/dark cycle (lights on at 8:00 a.m.). The animals had free access to food and water. All animal care and experimental procedures were approved by the Experimental Animal Care and Use Committee of Yamaguchi University School

of Medicine (J18030 and J23053), Japan, and conducted in accordance with the guidelines of the Japan Association for Laboratory Animal Facilities of National University Corporations.

Photochemically induced thrombosis (PIT) method

Focal cerebral ischemia and FBC of the primary sensorimotor cortex in the right hemisphere were induced using the PIT method and cooling treatment, as previously reported.[10] All mice were anesthetized with 3% isoflurane (Pfizer, New York, USA) for induction and 1.5% for maintenance in an 80%/20% mixture of oxygen and nitrous oxide delivered via a ventilator (A.D.S. 1000, Engler Engineering Corporation, Florida, USA). During the procedure, rectal temperature was monitored and maintained at 37 ± 1 ℃ using a temperature-controlled heating pad (NS-TC10, Neuroscience Inc., Tokyo, Japan). A scalp incision was made, and the pericranial tissue was dissected to expose the bregma. A craniotomy (3 mm diameter) was performed at coordinates relative to the bregma: 1 mm anterior and 2 mm lateral to the right side (Fig. 1). Rose bengal 0.25 mg (0.1 mL of a 10 mg/kg solution in saline; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was injected via the left jugular vein. Five minutes after injection, the craniotomy site was illuminated for 10 min with a fiber optic bundle consisting of a light emitting diode light source (dimming; 30%, KL 2500 LED; Schott, Mainz, Germany).

Measurement of the infarct area

Animals were euthanized 24 h after Rose bengal injection and transcardially perfused with phosphate-buffered saline. Whole brains were immediately removed and sectioned into 1 mm slices. These slices were stained with a 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution and incubated in a dark chamber at 37 ℃ for 10 min. Four sections were selected, and infarct area was measured. In addition, the areas of the ipsilateral and contralateral hemispheres for each coronal section were quantified. All measurements were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). During the acute phase of cerebral infarction, brain edema can lead to an overestimation of infarct volume. To correct for the effects of edema, we followed a previously established method and calculated the edema index by dividing the area of the contralateral hemisphere by the area of the ipsilateral hemisphere. The infarct area was then adjusted by dividing the measured infarct area by the edema index.[22,23] The total corrected infarct area was then compared between groups.

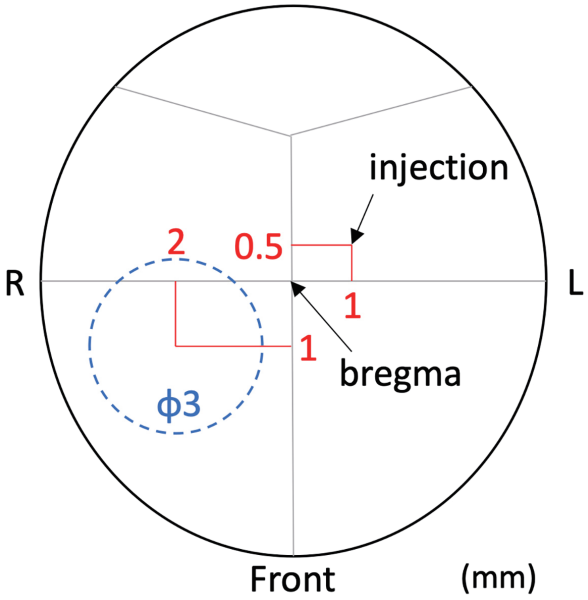

Fig. 1 Schematic diagram of the craniotomy area and the burr hole for intracerebroventricular injection.

Experiment. 1 Administration of TRPA1 agonist

Allyl isothiocyanate (AITC: Sigma-Aldreich, Saint Louis, USA), a TRPA1 agonist, was administered intracerebroventricularly to WT and TRPA1KO mice. The dose of AITC was adjusted and determined in the pre-experiments with reference to previous reports.[24] In the control group, 1% dimethyl sulfoxide (DMSO: Merck KGaA, Darmstadt, Germany) was administered intracerebroventricularly. The animals were assigned into the following four experimental groups (n=5 in each): intracerebroventricular injection of 1% DMSO in WT mice (WT + 1% DMSO group); intracerebroventricular injection of 10 mM AITC in WT mice (WT + AITC group); intracerebroventricular injection of 1%

DMSO in TRPA1KO mice (A1KO + 1% DMSO group); and intracerebroventricular injection of 10 mM AITC in TRPA1KO mice (A1KO + AITC group). Animals were randomly assigned to experimental groups. For the intracerebroventricular injections, a small burr hole was made 0.5 mm posterior to and 1.0 mm lateral on the left side to the bregma after craniotomy (Fig. 1). Immediately after photothrombosis, a 30G stainless steel needle (TSK Laboratory Japan, Tochigi, Japan) was inserted to a depth of 2.5 mm into the burr hole. 10 mM AITC, dissolved in 1% DMSO in saline, was administered for 15 min at a rate of 0.5 μl/min (total 7.5 μl) using a microinjection pump and a 10 μl Hamilton syringe (1701RN-7758-02; Hamilton, Reno, NV). To prevent leakage, the needle was left in place for 45 min. For the vehicle group, the same amount (7.5 μl) of 1% DMSO was injected. After the procedure was completed, the devices were removed, and skull was covered with a skin flap. After recovering from anesthesia, the mice were housed in cages with free access to food and drink.

Experiment 2. Focal brain cooling

The effects of focal brain cooling were evaluated in both WT and A1KO mice. The animals were divided into four experimental groups (n=5 in each): WT mice with brain cortex temperature controlled at 37 ℃ (WT non-cooling group); WT mice undergoing FBC at 15 ℃ (WT cooling group); TPA1KO mice with brain cortex temperature controlled at 37 ℃ (A1KO non-cooling group); TPA1KO mice undergoing FBC at 15 ℃ (A1KO cooling group). Animals were randomly assigned to experimental groups. For focal brain cooling, a peltier device with a thin thermo-sensor was placed on the craniotomy site. Cooling treatment commenced immediately after photothrombosis, with the device cooling the brain surface for 60 min at 15 ℃ above the dura mater at the craniotomy site (cooling group). In the control group, the peltier device was used to maintain a brain temperature of 37 ℃ for 60 min. Body temperature control was maintained until the mice recovered from anesthesia after the cooling or control (non-cooling) period. Following the procedure, the cooling devices were removed, and the skull was covered with a skin flap. Once the mice awoke from anesthesia, they were placed back in cages with free access to food and drink.

Experiment 3. Evans blue (EB) Injection

BBB permeability was assessed using EB (Sigma Aldrich, Saint Louis, USA) in both WT and TRPA1KO mice. The animals were assigned into the following five experimental groups (n=4 in each); brain cortex temperature controlled at 37 ℃ in WT mice (WT non-cooling group); FBC at 15 ℃ in WT mice (WT cooling group); intracerebroventricular injection of 10 mM AITC in WT mice (WT + AITC group); brain cortex temperature controlled at 37 ℃ in TRPA1KO mice (A1KO non-cooling group); FBC at 15 ℃ in TRPA1KO mice (A1KO cooling group). Animals were randomly assigned to experimental groups. A 2% EB solution (4 mL/kg) was injected intravenously 1 h after photothrombosis and intracerebroventricular injection of DMSO or AITC. One hour later, the mice were euthanized and transcardially perfused with phosphate-buffered saline. The whole brain was removed and weighted. Furthermore, the brain with EB leakage was removed with a margin. The brain with EB leakage was promptly weighed and homogenized in 50% trichloroacetic acid (200 μL). The homogenate was centrifuged (12,000 × g for 20 min), the supernatant (150 μL) was diluted with ethanol (1:3), and the EB content in the cerebral hemispheres was quantified at 620 nm using a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Hercules, USA). The EB leakage was quantified using a standard curve and expressed as the amount per gram of brain tissue.

Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). Statistical analyses of the infarct area and EB leakage across multiple groups were conducted using one-way ANOVA followed by Tukey's test. All tests were two-tailed, with statistical significance defined as a p-value < 0.05. Statistical analysis was performed using JMP Pro 16.1.0 for MAC (SAS Institute Inc., USA).

Results

1. Neuroprotective effect of TRPA1 agonist

The results of Experiment 1 are illustrated in Fig. 2. Due to technical failures during the procedure, one animal was excluded from the analysis (A1KO + AITC group). A one-way ANOVA revealed a significant difference among the groups ($F(3, 15) = 9.088$, $p = 0.0011$, partial $\eta 2 = 0.645$). Subsequent pairwise comparisons showed that in WT mice, intraventricular administration of AITC significantly reduced the infarct area by approximately half (11.61 ± 1.81 vs. 4.40 ± 1.19 mm^2, $p = 0.0157$). In contrast, in TRPA1KO mice, intraventricular administration of AITC did not reduce the infarct volume.

2. Neuroprotective effect of FBC in TRPA1 deficiency

The results of Experiment 2 are shown in Fig. 3. A one-way ANOVA showed a significant difference among the groups ($F(3, 16) = 17.824$, $p < .0001$, partial $\eta 2 = 0.770$). In WT mice, FBC reduced the infarct area to less than half (12.04 ± 1.37 vs. 4.34 ± 0.65 mm^2, $p = 0.0013$). One animal from the A1KO non-cooling group died within 24 hours after the procedure, and was excluded from the further analysis. Interestingly, FBC also reduced the infarct area in TRPA1KO mice by about half (17.20 ± 1.22 vs. 8.95 ± 1.34 mm^2, $p = 0.0018$). Additionally, the infarct area in TRPA1KO mice was larger than that in WT mice (12.04 ± 1.37 vs. 17.20 ± 1.22 mm^2, $p = 0.048$).

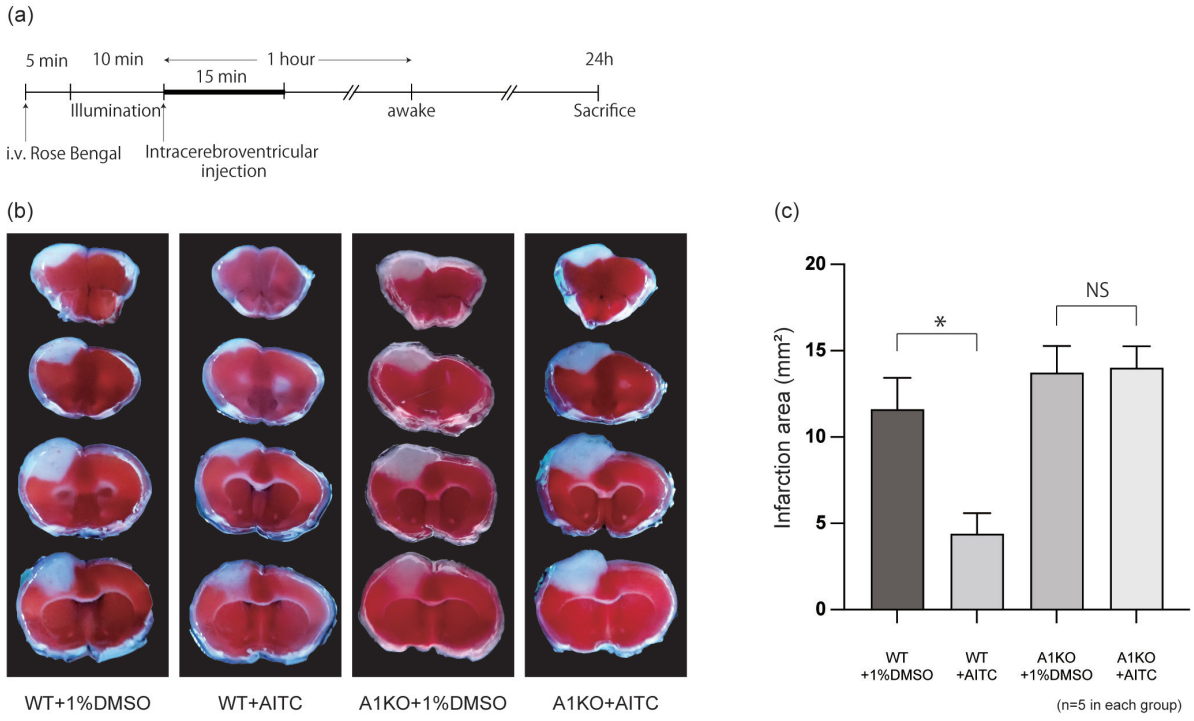

Fig. 2 Effects of transient receptor potential Ankyrin 1 (TRPA1) agonist Allyl isothiocyanate (AITC) on photochemically induced thrombosis-induced focal ischemia in wild-type (WT) mice and TRPA1 homozygous knockout (KO) TRPA1KO mice, using 2,3,5-triphenyltetrazolium chloride (TTC) staining.

(a) Protocols for Experiment 1.

(b) Representative TTC staining 24 h after ischemia in each group; WT + 1% dimethyl sulfoxide (DMSO), WT + AITC, A1KO + 1% DMSO, A1KO + AITC.

(c) The brain infarction in each group (n=5 in each); WT + 1% DMSO; WT + AITC, A1KO + 1% DMSO, A1KO + AITC. AITC reduced infarct area in WT but not in A1KO. The results are shown as the mean ± standard error of the mean (SEM); *$p < 0.05$, Tukey's test.

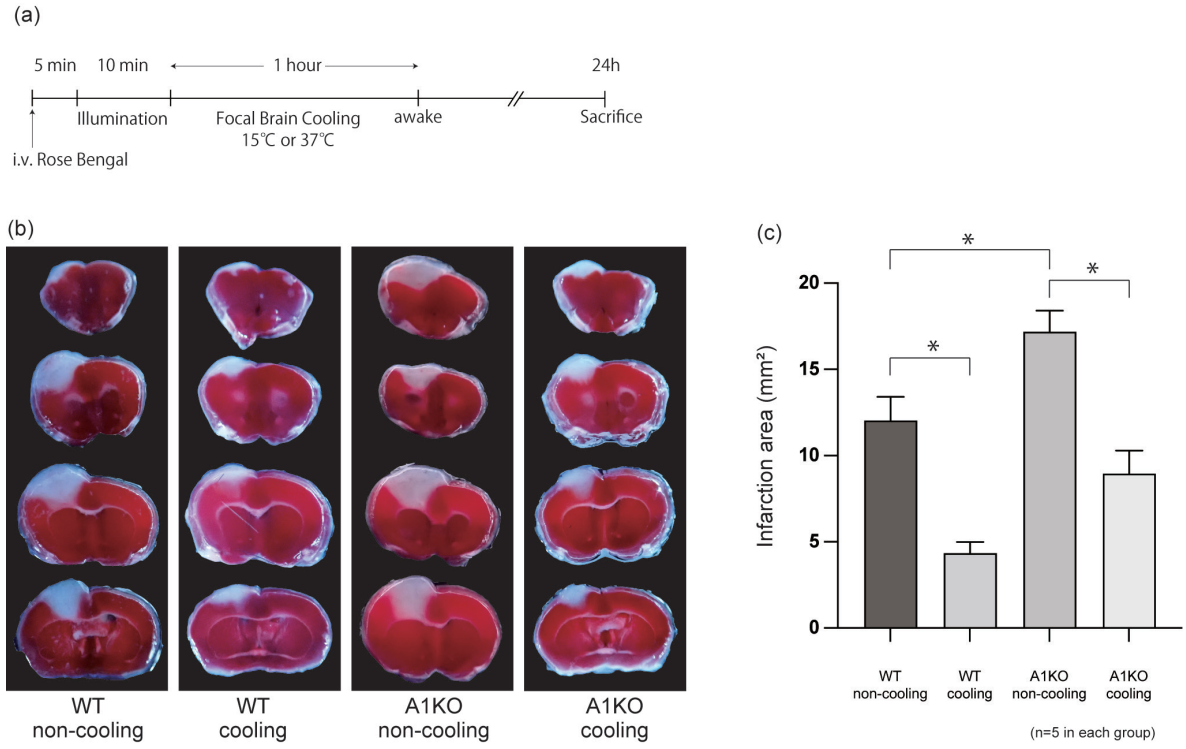

Fig. 3 Effects of focal brain cooling on photochemically induced thrombosis-induced focal ischemia in wild-type (WT) mice and TRPA1 homozygous knockout (KO) TRPA1KO mice, using 2,3,5-triphenyltetrazolium chloride (TTC) staining.
(a) Protocols for Experiment 2.
(b) Representative TTC staining 24 h after ischemia in each group; WT non-cooling, WT cooling, A1KO cooling, A1KO non-cooling.
(c) The brain infarction in each group (n=5 in each); WT non-cooling, WT cooling, A1KO non-cooling, A1KO cooling. Focal brain cooling reduced infarct area in both WT and TRPA1 KO mice. TRPA1 KO mice showed larger infarct area compared to WT. The results are shown as the mean ± SEM; *$p < 0.05$, Tukey's test.

3. Evans blue extravasation

To investigate the effects of FBC, TRPA1 agonist, and TRPA1 deficiency on BBB disruption, EB extravasation was evaluated. The results of Experiment 3 are shown in Fig. 4. Due to technical failures during the procedure, one animal was excluded from the analysis (A1KO cooling group). A one-way ANOVA revealed a significant difference among the groups ($F(4, 14) = 16.124$, $p < .0001$, partial $\eta 2 = 0.822$). Subsequent pairwise comparisons showed that in TRPA1KO mice, the amount of EB leakage after PIT was approximately twice as high as in WT mice (5.39 ± 0.44 vs. 10.72 ± 1.33 μg/g, $p = 0.0039$). In WT mice, both FBC treatment and intraventricular administration of AITC reduced EB leakage, though the difference was not statistically significant ($p = 0.2110$; $p = 0.1714$, respectively). Similarly, in TRPA1KO mice, FBC reduced the amount of EB leakage, but again, the difference was not statistically significant ($p = 0.0681$).

Discussion

In this study, we aimed to clarify whether TRPA1 channel plays a role in the neuroprotective effects of FBC on cerebral infarction. We employed a TRPA1 channel agonist and TRPA1KO mice to compare their effects with those of FBC. Our results revealed that TRPA1 agonist administration reduced the infarct area, as did FBC. Surprisingly, TRPA1KO mice showed larger infarct area following PIT. However, contrary to our

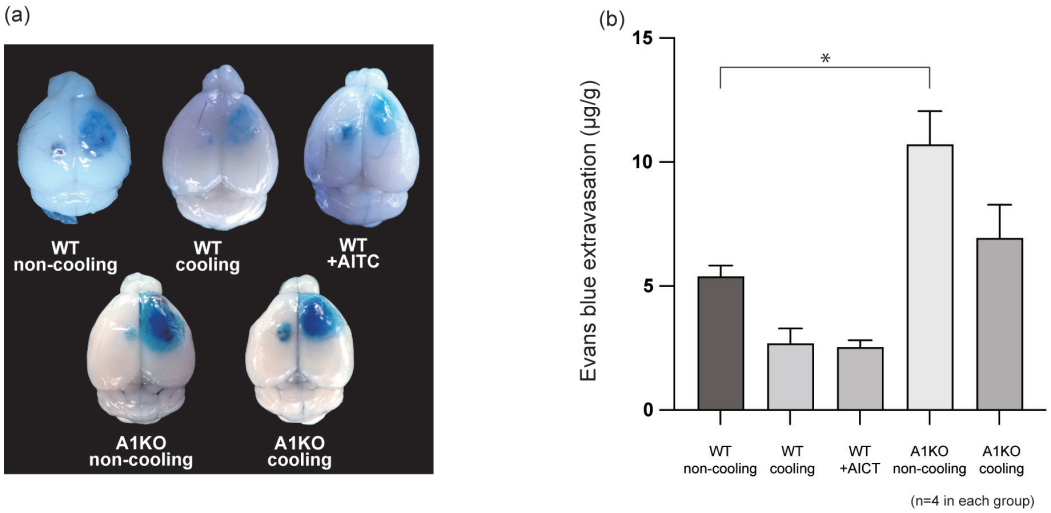

Fig. 4 Effects of focal brain cooling (FBC) and transient receptor potential Ankyrin 1 (TRPA1) agonist Allyl isothiocyanate (AITC) on photochemically induced thrombosis-induced Evans blue leakage in wild-type (WT) mice and TRPA1 homozygous knockout (KO) TRPA1KO mice.

(a) Representative examples of Evans blue leakage into the brain parenchyma in each group; WT non-cooling, WT cooling, WT + AITC, A1KO non-cooling, A1KO cooling.

(b) The amount of Evans blue leakage into the brain parenchyma was measured in the following groups (n=4 in each): WT non-cooling, WT cooling, WT + AITC, A1KO non-cooling, and A1KO cooling. TRPA1 KO mice exhibited more Evans blue leakage compared to WT mice. While focal brain cooling and AITC treatment reduced leakage, the reduction did not reach statistical significance. Results are presented as the mean ± SEM; *$p < 0.05$, Tukey's test.

hypothesis, FBC in TRPA1KO mice reduced cerebral infarction area.

Administration of TRPA1 agonist significantly reduced the infarct area, suggesting that TRPA1 activation may have a neuroprotective effect. The neuroprotective effects of TRPA1 agonists have been previously reported. Pires et al. reported TRPA1 activation with TRPA1 agonist cinnamaldehyde reduced infarct area in WT mice.[19] It has been reported that, under hypoxic conditions, activation of the TRPA1 channel has vasodilatation effects, which improve cerebral blood flow and exert a neuroprotective effect against cerebral ischemia.[19,20,25] Furthermore, anti-inflammatory effects through activation of TRPA1 channels have been reported and may also be involved in the neuroprotective effects.[26] While AITC itself has anti-inflammatory effects, the fact that AITC did not reduce infarct volume in TRPA1KO mice suggests that its neuroprotective effect relies on TRPA1 channel activation.[27] To explore other potential neuroprotective mechanisms, we also examined the effects of BBB disruption. EB leakage was relieved by FBC and TRPA1 agonist administration, although the difference was not statistically significant ($p = 0.2110$; $p = 0.1714$, respectively, Tukey's test).

In Experiment 2, the area of cerebral infarction induced by the PIT method in TRPA1KO mice was larger than in WT mice, suggesting that TRPA1 channels can be activated outside their typical low-temperature range. Hypoxia has been reported to activate TRPA1 channels and induce cerebral vasodilation regardless of the temperature range at which TRPA1 is typically activated. The absence of these effects due to TRPA1 deficiency may contribute to the increased cerebral infarct size observed in TRPA1KO mice.[19,20,25] In contrast to Experiment 2, Experiment 1 showed a trend toward a larger cerebral infarct size in TRPA1KO mice compared to

WT mice; however, this difference was not statistically significant. We presume that the small sample size, differences in experimental methods—such as the intracerebroventricular injection of DMSO in Experiment 1 and the placement of a cooling probe on the brain surface even in the non-cooled group in Experiment 2—may have contributed to the differing results.

EB leakage in TRPA1KO mice was increased compared with WT, suggesting that the TRPA1 channel might play a protective role against BBB disruption. The cerebral infarction was suppressed by FBC, even in TRPA1KO mice. This indicates that the neuroprotective effect of FBC can be exerted without the involvement of TRPA1 channels. Alternatively, its effect is limited, even if TRPA1 channels are involved.

In this study, we demonstrated the neuroprotective effects of a TRPA1 agonist and the vulnerability of focal cerebral ischemia in TRPA1-deficient mice. These findings suggest that TRPA1 channels play an important role in ischemic stroke tolerance or protection. While this study did not clarify if TRPA1 channels are involved in the neuroprotective mechanism of FBC, the reduction in cerebral infarction by TRPA1 agonist administration was comparable with that of FBC, suggesting that TRPA1 agonist administration may be an alternative treatment to FBC. Further studies will clarify TRPA1 channel involvement in ischemic stroke, potentially leading to the development of new, effective, and minimally invasive therapies.

Conclusion

In ischemic stroke, TRPA1 agonist showed neuroprotective potential similar to FBC, and TRPA1 deficiency increased vulnerability.

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Conflict of interest

The authors declare no conflict of interest.

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