Pharmacologically induced hypothermia via TRPV1 channel agonism provides neuroprotection following ischemic stroke when initiated 90 min after reperfusion

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Cao Z, Balasubramanian A, Marrelli SP. Pharmacologically induced hypothermia via TRPV1 channel agonism provides neuroprotection following ischemic stroke when initiated 90 min after reperfusion. Am J Physiol Regul Integr Comp Physiol 306: R149–R156, 2014. First published December 4, 2013; doi:10.1152/ajpregu.00329.2013.—Traditional methods of therapeutic hypothermia show promise for neuroprotection against cerebral ischemia-reperfusion (I/R), however, with limitations. We examined effectiveness and specificity of pharmacological hypothermia (PH) by transient receptor potential vanilloid 1 (TRPV1) channel agonism in the treatment of focal cerebral I/R. Core temperature (Tcore) was measured after subcutaneous infusion of TRPV1 agonist dihydrocapsaicin (DHC) in conscious C57BL/6 WT and TRPV1 knockout (KO) mice. Acute measurements of heart rate (HR), mean arterial pressure (MAP), and cerebral perfusion were measured before and after DHC treatment. Focal cerebral I/R (1 h ischemia + 24 h reperfusion) was induced by distal middle cerebral artery occlusion. Hypothermia (>8°C) was initiated 90 min after start of reperfusion by DHC infusion (osmotic pump). Neurofunction (behavioral testing) and infarct volume (TTC staining) were measured at 24 h. DHC (1.25 mg/kg) produced a measurable effect on HR and cerebral perfusion but produced a slight transient drop in MAP (<6 mmHg). In stroke mice, DHC infusion produced hypothermia, decreased infarct volume by 87%, and improved neurofunctional score. The thermogenic and neuroprotective effects of DHC were absent in TRPV1 KO mice or mice maintained normothermic with heat support. PH via TRPV1 agonist appears to be a well-tolerated and effective method for promoting mild hypothermia in the conscious mouse. Furthermore, TRPV1 agonism produces effective hypothermia in I/R mice and significantly improves outcome when initiated 90 min after start of reperfusion.

STROKE is the second highest cause of death in the world and the main cause of long-term disability in the United States (43). However, current treatment options for stroke are quite limited (51). Thrombolysis is the only approved method for stroke therapy (41), but it can be applied to less than 10% of the patients due to the stringent treatment criteria (26) and in some cases may even produce further reperfusion injury (2). Mild hypothermia (32–34°C) has been demonstrated to reduce stroke injury and improve neurofunctional recovery in animal studies and has shown promise in small-scale clinical trials (20, 25, 37, 49, 50). At present, most clinical therapeutic hypothermia (TH) protocols involve methods of forced cooling such as with cold blankets and ice baths or intravenous methods of cooling (25). Although TH seems to have significant promise in stroke therapy, current protocols are accompanied by many adverse complications, which reduce effectiveness and preclude broad application (21, 50).

One of the major complications encountered with forced cooling protocols is the triggering of the cold-defense mechanisms. When core temperature is challenged, mammals respond by increasing thermogenesis by shivering and nonshivering processes (30). To combat the cold-defense response, current TH protocols typically include strong narcotic sedatives and muscle paralysis. These agents promote respiratory depression and thus necessitate ensuing mechanical ventilation (44). The numerous clinical complications as well as the difficulty in maintaining patients within the therapeutic temperature range present significant barriers to the application of TH for stroke neuroprotection.

Transient receptor potential vanilloid channel 1 (TRPV1) is highly expressed in warm-sensing nerve fibers and is activated by heat, protons, and both endogenous and exogenous agonists (35, 47). This nonselective cation channel plays a significant role in thermoregulation; however, the exact mechanisms have yet to be fully elucidated (28, 30, 35, 46). Studies with intravenously infused dihydrocapsaicin (DHC), a chili pepper-derived TRPV1 agonist, have shown the capacity of this and other capsaicinoids to produce hypothermia in rodents and larger mammals (15). The present study had two goals. The first was to determine the acute cardiovascular consequences of subcutaneous infusion of mild hypothermia producing doses of DHC. The second goal was to demonstrate specificity and effectiveness of pharmacologically induced hypothermia (PH) by prolonged (8 h) activation of TRPV1 to provide neuroprotection following acute cerebral ischemia and reperfusion (I/R) injury when initiated 90 min postreperfusion. These studies provide proof of principle that thermoreceptor targeting could be an effective strategy for stroke treatment in the conscious subject, even when initiated significantly after reperfusion.

MATERIALS AND METHODS

Animals. Experiments were performed with male C57BL/6 mice of 10–16 wk age (24–37 g). In all, 125 mice were used of either wild-type (WT) or TRPV1 knockout (KO) (Jackson Labs). All animal studies were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

Hypothermia mouse model. Mice were anesthetized with isoflurane (5% induction, 2% maintenance in 100% oxygen) during surgery to implant subcutaneous delivery lines and temperature probes. Two methods were employed for the delivery of DHC (Cayman, Ann Arbor, MI). In the syringe pump method, a PE-10 tube (Instech Solomon, San Antonio, TX) was fixed under the back skin roughly...

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even with the kidneys for infusion by syringe pump. In addition, a small thermocouple (40 gauge wire; Omega Engineering, Stamford, CT) was implanted in the abdominal cavity for monitoring the core temperature (Tcore). Data were logged by a digital thermometer (HH806W, Omega Engineering). In the osmotic pump method, a miniature wireless Implantable Programmable Temperature Transponder (IPTT-300, BioMedica Data Systems, Seoul, DE) was implanted under the skin of the left side even with the location of the PE-10 tubing in the first model. Temperature was monitored and logged by a wireless reader. An osmotic pump (Alzet 2001D, Cupertino, CA) was loaded with either saline or DHC and placed under the back skin. Preliminary experiments showed that DHC delivered subcutaneously at 1.25 mg·kg\(^{-1}\)·h\(^{-1}\) by osmotic pump (−6 µl at 33°C) produced hyperthermia in the target temperature range (32–34°C). Age-matched TPRV1 WT and KO mice were treated with 1.25 mg·kg\(^{-1}\)·h\(^{-1}\) DHC (or vehicle) to determine the specificity of the DHC response to TRPV1 receptors.

Measurement of cardiovascular indexes. Mice were anesthetized with 1.5% isoflurane and rectal temperature was maintained at 36.5 ± 0.5°C by a feedback-controlled heating pad (Omega). For direct measurement of mean arterial blood pressure, microethanethane tubing (MRE 025, Braintree Scientific, Braintree, MA) was inserted in the left femoral artery. In separate groups, ECG leads were connected to both front limbs and the right hind limb. For measurement of relative cerebral blood flow, mice were ventilated with a small animal ventilator (Physio Suite, Kent Scientific, Torrington, CT), and cerebral cortical perfusion (CCP) was measured by laser Doppler flowmetry (ADInstruments, Colorado Springs, CO). As a method to demonstrate cerebral blood flow, mice were ventilated with a 10% CO\(_2\) gas mixture for 5 min to elicit an increase in CCP. At the end of the experimental protocol, arterial pH and PCO\(_2\) were measured by Truepoint blood gas analyzer (Diamond Diagnostics, Holliston, MA). Three groups were set up for each index: vehicle, WT+DHC, and TRPV1 KO+DHC. In each group, mice were injected subcutaneously under the back skin with either 1.25 mg/kg DHC or 20% DMSO in saline as a vehicle. The total volume of the injection was 1 µl/g body wt. Data were continuously collected by Power Lab and analyzed by Lab Chart 7 (ADInstruments).

TRPV1 desensitization test. Mice were divided into four groups: WT with high dose of DHC, WT with therapeutic dose of DHC, WT with vehicle (20% DMSO in saline), and TRPV1 KO with therapeutic dose of DHC. The high dose was based on established protocols for producing DHC-mediated TRPV1 desensitization (40). In the high-dose group, mice were treated with 30 and 15 mg/kg DHC on 2 consecutive days. The therapeutic dose of DHC was based on the dose required to produce mild hyperthermia. In the therapeutic dose groups, 1.25 mg/kg DHC was administered twice on two consecutive days. After 1 mo recovery, all of the mice were treated with 1.25 mg/kg DHC and core temperature was subsequently recorded for 90 min in 5-min intervals (IPTT-300).

Focal cerebral I/R mouse model. Focal cerebral I/R was induced by left middle cerebral artery (MCA) and left common carotid artery (CCA) occlusion for 60 min followed by 24 h reperfusion, as described previously (54). In anesthetized mice, the distal left MCA was exposed by making a 1-mm burr hole rostral to the fusion of the zygomatic arch with the squamosal bone. The left MCA was lifted slightly above the surface of the brain and fixed by a 127-µm diameter stainless steel wire while the left CCA was occluded by a small vascular clamp. Cessation of blood flow distal to the steel wire was visually confirmed. After ischemia for 60 min, the wire and the clip were gently removed to initiate the 24-h reperfusion period. To detect the effect of DHC on cerebral I/R, mice were randomly divided into three groups: I/R plus implanted osmotic pump containing saline (vehicle), I/R plus implanted osmotic pump with DHC (DHC hyperthermia), and I/R plus implanted osmotic pump with DHC but maintained normothermic with heat support (DHC normothermia). In another set, TRPV1 KO mice were used to determine whether the DHC-induced effect on cerebral I/R is specific to TRPV1 channel activation. Three groups were set up: I/R with no treatment (WT), TRPV1 KO mice with I/R (TRPV1 KO), and TRPV1 KO mice with I/R plus implanted osmotic pump containing DHC (TRPV1 KO+DHC). Either saline or DHC was infused by using the osmotic pump method. The osmotic pump was implanted at the time when reperfusion was initiated. Pump implantation was timed so that the effective dose of DHC required to produce mild hypothermia (32–34°C) would be achieved shortly after the first 90 min of recovery/reperfusion (based on pilot studies). After the surgery, anesthesia was discontinued and all mice were kept in a recovery cage with heat support to maintain a normothermic Tcore. After 90 min, all mice except the DHC (normothermia) group, were transferred to a room temperature environment (22°C). DHC (normothermia) group mice were maintained with heat support for 10 h, at which point mice were able to maintain Tcore above 36°C without support. Mice Tcore was measured every 15 min during the first 4 h of the reperfusion period in all groups. In the DHC (hypothermia) group, three mice with a Tcore lower than 30°C were excluded in the analysis. One mouse died as a result of complications with the anesthesia. In the DHC (normothermia) group, four mice with a Tcore lower than 35°C due to insufficient heat support were excluded in the analysis.

Behavioral tests. Neurofunction in mice was tested by spontaneous activity, symmetry in the movement of four limbs, outstretched forepaw, climbing, body proprioception, and response to vibrissae touch as previously described (16). Individual mice were evaluated for each of these tests and scored from 0 (minimum response) to 3 (maximum response). The maximum possible score for all tests combined was 18. Each mouse was evaluated before the surgery (to exclude any potential poor baseline responders) and after 24 h of reperfusion. The summary score represents the neurofunction of the mouse at 24 h reperfusion.

Infarct size measurement. After the behavioral test, mice were euthanized and perfused with 10 ml ice-cold saline via the aorta. Brains were isolated and sectioned into four 2-mm slices in a mouse brain matrix (Kent Scientific). Brain slices were stained by 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich T 8877, St. Louis, MO) and analyzed as previously described (32). All of the slices were photographed the day after staining. The infarct area was analyzed in Photoshop CS3 and defined by the unstained area (white color). Infarct volume was quantified by dividing the total volume of the infarct area by the edema index, which was calculated by dividing the total volume of left hemisphere by the total volume of right hemisphere from the four slices. Infarct volume is presented as the percentage of infarct volume compared with the right hemisphere volume.

Statistical analysis. The behavioral test score data is expressed as median; a one-way analysis of variance on Ranks was used to compare the difference within the three groups with a Kruskal-Wallis test for individual comparisons. All the other data are expressed as means ± SE. One-way ANOVA was used to evaluate differences between three groups. Two-way RM ANOVA was used for comparing groups with repeated measures, using the Holm-Sidak test for individual comparisons.

RESULTS

DHC produces a stable and reversible hypothermic response specifically via TRPV1 activation. Figure 1A summarizes mouse Tcore in response to 4 h of continuous DHC infusion (1.25 mg·kg\(^{-1}\)·h\(^{-1}\)·sc). DHC was delivered by infusion pump to conscious and freely moving mice loosely tethered by a segment of PE-10 tubing and a 40-gauge thermocouple wire. Since we were unable to find prior studies demonstrating specificity of the DHC-mediated hypothermic response to TRPV1 channels, we additionally examined the Tcore response in TRPV1 KO mice. Before the infusion, there was no significant difference in Tcore.
within WT, vehicle control, and TRPV1 KO groups. After 70 min of DHC infusion, the WT group temperature began to drop. By 100 min of infusion, the mean Tcore stabilized at 33.0 ± 0.2°C in the WT group compared with 36.7 ± 0.4°C in the TRPV1 KO group or 36.9 ± 0.4°C in the vehicle control group (n = 4/group, P < 0.001, power = 1.0). After the DHC infusion was stopped, the temperature in the WT group spontaneously recovered to 36.3 ± 0.2°C. Infusion of vehicle in WT mice did not produce any drop in Tcore. DHC infusion in TRPV1 KO mice did not induce any drop in Tcore, demonstrating the specificity of DHC-mediated hypothermia to TRPV1 receptors.

Subcutaneous infusion of DHC (1.25 mg·kg⁻¹·h⁻¹) by osmotic pump produced lasting hypothermia within the therapeutic range (32–34°C) beginning within 2 h after pump implantation (Fig. 1B). The osmotic pumps were loaded with sufficient DHC to produce very consistent and stable hypothermic responses. However, in 25% of the mice (1 in 4 animals), osmotic delivery of DHC produced undesirable variability in the resulting Tcore, which is evident in the greater SEM values beginning at 8 h infusion (Fig. 1B). The mean temperature reached after termination of DHC infusion was greater than the preinfusion Tcore but did not exceed the upper limit of the typical circadian variance in these mice. Note that the infusion was started in the sleep cycle (when Tcore is lowest) and terminated in the wake cycle (when Tcore is highest).

The dose of DHC that produces mild hypothermia transiently decreases blood pressure but has no effect on heart rate (HR) or cerebral blood flow. An ideal hypothermic agent should not produce significant perturbations of cardiovascular parameters. Thus, as an initial step in evaluating the potential suitability of DHC-induced hypothermia for clinical application, HR, mean femoral artery blood pressure (BP), and CCP were measured before and after acute DHC treatment. Figure 2, A and B, shows that within 10 s after DHC treatment (1.25 mg/kg sc), BP in the WT group slightly decreased 5.8 ± 0.8 mmHg within the first minute after the injection (n = 3/group, P = 0.01, power = 0.840). The drop in BP was transient and recovered to pre-DHC value within 4 min. There was no effect on BP in the TRPV1 KO or vehicle-treated WT mice (n = 3/group, P > 0.05). To evaluate the contribution of parasympathetic activation in the transient drop of BP, atropine (5 mg/kg sc) was administered 10 min before DHC injection. The DHC-mediated dip in BP was completely blocked by atropine pretreatment (Fig. 2, A and B).

Figure 2C summarizes mean BP responses to DHC at 1.25 and 2.5 mg/kg. Both doses showed a modest but statistically significant drop in the first 2 min after DHC injection (n = 3/group, P < 0.001, power = 0.98). There was no difference in the drop between the two groups (5.8 ± 0.8 vs. 5.1 ± 0.4 mmHg, respectively, within the first minute, n = 3/group, P = 0.709).

Despite the slight drop in BP in the DHC-treated WT mice, there was no apparent drop in HR due to specific TRPV1 activation in WT mice. Although there was a small dip in HR within the first minute of drug infusion, it was evident in all three groups studied (DHC, vehicle, and TRPV1 KO + DHC), suggesting that the effect is not mediated by DHC treatment or TRPV1 activation (Fig. 3A, n = 6/group, P = 0.281). Furthermore, injection of twice the treatment dose of DHC (2.5 mg/kg) also failed to demonstrate a significant difference in HR between groups (Fig. 3B, n = 3/group, P = 0.744).

We next sought to determine whether DHC infusion produced acute or delayed changes in cerebral blood flow. As noted above, we found a mild decrease in BP immediately after DHC infusion. In addition, other labs have found acute effects of TRPV1 agonist infusion in other species when delivered intravenously (7, 14). TRPV1 agonism has also been demonstrated to produce vasodilation in certain vascular beds (8, 18, 55). Thus it was important to determine whether DHC infusion produced changes in cerebral blood flow either by acute drop in BP or direct vasodilation of cerebral resistance vessels.

Experiments were designed to measure CCP by laser Doppler flowmetry. Administration of 1.25 mg/kg DHC to WT mice

![Diagram](http://ajpregu.physiology.org/)

Fig. 2. DHC transiently decreased mean arterial blood pressure (MAP) in anesthetized mice through parasympathetic activation. A: representative recordings of MAP before and after DHC or vehicle injection in WT or TRPV1 KO mice. B: summary of the blood pressure changes during the first 4 min after DHC or vehicle injection (P < 0.05, n = 3/group). C: summary of the dose dependence of DHC on MAP (n = 3/group). Arrow indicates the injection time point.
CO2 inhalation (10%) produced a robust increase in CCP in all groups (Fig. 3D). As a positive control for viable cerebrovascular reactivity (36), 10% CO2 inhalation did not produce measurable changes in CCP over a 20-min period compared with either vehicle control or with DHC-treated TRPV1 KO mice (Fig. 3D). Blood gas and pH values were within normal limits for all groups measured at the conclusion of the DHC (or vehicle) response (Table 1).

The dose of DHC that produces mild hypothermia does not produce TRPV1 desensitization after consecutive injections. It has been shown that high doses of DHC (30 and 15 mg/kg consecutive treatments over 2 days) causes long-term damage or desensitization of TRPV1-containing sensory fibers in mice (40). To test whether our therapeutic hypothermic dose of DHC (1.25 mg/kg) alters subsequent TRPV1-dependent hypothermic responses, anesthetized mice were treated with either high dose or therapeutic dose DHC on 2 consecutive days. A third group was treated with vehicle only. After 1 mo of recovery, all mice were treated again with the therapeutic dose of DHC (1.25 mg/kg) to evaluate the hypothermic capacity. Mice that received high-dose DHC treatment demonstrated nearly complete elimination of the hypothermic response, similar to the DHC response in TRPV1 KO mice (Fig. 4A, n = 3/group). In contrast, mice previously treated with therapeutic doses of DHC (1.25 mg/kg × 2 days) demonstrated an undiminished hypothermic response compared with the vehicle-treated group. In a separate experiment, a single mouse was injected with double the therapeutic dose of DHC (2.5 mg/kg) on 4 separate days over a 6-day period (Fig. 4B). Note that the hypothermic response was reproducible despite receiving multiple injections and a cumulative dose of 10 mg/kg DHC.

DHC-induced mild hypothermia provides neuroprotection after acute cerebral I/R when initiated 90 min after reperfusion. To test the potential of DHC-induced hypothermia to provide neuroprotection following focal cerebral ischemia, DHC was infused during the reperfusion period by subcutaneous osmotic pump in mice subjected to distal MCA occlusion/reperfusion (I/R). WT mice were divided into three treatment groups: vehicle, DHC (hypothermia), and DHC (normothermia). Mice in the DHC (hypothermia) group were allowed to become hypothermic, whereas mice in the DHC (normothermia) group were provided 10 h of heat support to maintain Tcore in the normothermic range despite DHC infusion. Pumps containing DHC or vehicle were implanted just before initiating cerebral reperfusion. In all groups, mice were given heat support for the first 90 min of reperfusion to maintain normothermia during the postsurgery recovery period. There was no significant difference in Tcore within the groups during the first 90 min of reperfusion with heat support (Fig. 5A). At 90 min postreperfusion, vehicle and DHC (hypothermia) group mice were returned to ambient temperature (22°C). In the DHC (hypothermia) group, the Tcore dropped to a mean of 32.8 ± 0.5°C versus 34.7 ± 0.2°C and 35.6 ± 0.1°C in vehicle and DHC (normothermia) groups, respectively (n = 8, 9, 6 in each group, P < 0.001, power = 1.00). All mice recovered Tcore ≥ 36.0°C by 24 h reperfusion. It is noteworthy that 27% of the mice treated with DHC (3 of 11 animals) produced a greater than expected drop in Tcore during the reperfusion period. This rate of occurrence matched that of the naive mice with implanted osmotic pump from Fig. 1B. Given the reproducability and stability of the DHC-mediated hypothermia with syringe pump infusion (Fig. 1A), the variability of the hypothermic response appears to be primarily related to issues with the osmotic pumps rather than variability of the physiological response to DHC.

Table 1. Blood gas and pH values after DHC or vehicle treatment in anesthetized mice

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<thead>
<tr>
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<th>WT + DHC</th>
<th>WT + Vehicle</th>
<th>TRPV1 KO + DHC</th>
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<tbody>
<tr>
<td>pH</td>
<td>7.37 ± 0.04</td>
<td>7.41 ± 0.002</td>
<td>7.42 ± 0.02</td>
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<tr>
<td>PO2, mmHg</td>
<td>31.9 ± 2.47</td>
<td>30.2 ± 3.2</td>
<td>31.7 ± 0.7</td>
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<tr>
<td>P02, mmHg</td>
<td>371.4 ± 114.6</td>
<td>305.7 ± 88.1</td>
<td>313.3 ± 85.6</td>
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<tr>
<td>HCO3, mM</td>
<td>18.1 ± 2.2</td>
<td>19.1 ± 2.1</td>
<td>20.3 ± 0.7</td>
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Values are means ± SE, n = 3 per group. Arterial samples were collected at the end of the DHC or vehicle response. WT, wild-type mice; KO knockout mice; DHC, dihydrocapsaicin; TRPV1, transient receptor potential vanilloid 1.
Neurological function and brain injury was evaluated at 24 h reperfusion. DHC-induced hypothermia substantially decreased the infarct volume and improved neurofunctional recovery following cerebral I/R injury. Representative TTC-stained brain sections at 24 h reperfusion are pictured in Fig. 5B. The infarct volume was reduced to 1.3 ± 0.8% in the WT+DHC group versus 10.1 ± 2.0% in vehicle or 8.6 ± 2.4% in DHC (normothermia) groups (Fig. 5C, n = 8, 10, 6 in each group, P = 0.005, power = 0.831). The median behavior test score of the DHC (hypothermia) group was 16.75 compared with 15 in the vehicle group and 14.5 in the DHC (normothermia) group (Fig. 5D, n = 8, 10, 6 per group, P < 0.001, power = 0.984). The vehicle and DHC (normothermia) groups were not statistically different from each other in infarct volume or behavior test score. Comparison of individual tests demonstrated a significant difference between DHC (hypothermia) and vehicle/DHC (normothermia) specifically in “symmetry in the movement of four limbs” and “extension of forepaw” tests (Table 2A). These results demonstrated significant neuroprotection by DHC infusion and that said protection is specifically due to the hypothermic effect of DHC.

DHC-induced hypothermic and neuroprotective effects in cerebral ischemic stroke are specific to TRPV1 activation. TRPV1 KO mice were used to demonstrate the specific role of TRPV1 channels in DHC-mediated hypothermia and neuroprotection. Mice were divided into WT, TRPV1 KO, and TRPV1 KO+DHC groups. Heat support was provided to all three groups during the first 90 min of reperfusion. Mice were then transferred to housing cages with an ambient temperature of 22°C. There was no significant difference in Tcore among all three groups: 34.4 ± 0.4°C, 34.5 ± 0.3°C, and 34.9 ± 0.2°C (Fig. 6A; n = 5, 6, and 5 per group). TTC staining (Fig. 6, B and C) and behavioral testing (Fig. 6D and Table 2B) demonstrated no significant differences between groups. Infarct volumes were 10.6 ± 1.4%, 10.7 ± 1.0%, and 10.8 ± 2.0% for WT, TRPV1 KO, and TRPV1 KO+DHC, respectively (n = 10, 6, 6 in each group, P = 0.995). Median behavior test scores were 14.25, 14.25, and 14.75, respectively (n = 10, 6, 6 in each group, P = 0.558). These data demonstrated the critical role for TRPV1 channels in DHC-mediated hypothermia and the neuroprotective effect.

**DISCUSSION**

TH appears to have tremendous potential as a treatment for a variety of neurological injuries including ischemic stroke (4, 9, 42, 48). However, TH has yet to be evaluated for the treatment of stroke in large-scale clinical trials (1). One significant hurdle for clinical trials involves the limitations with current methods of producing hypothermia with specific regard to stroke patients. In particular, TH typically requires sedation and mechanical ventilation of an otherwise conscious patient.

<table>
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<th>Table 2. Individual behavioral tests data summary of DHC and TRPV1 groups</th>
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<tr>
<td>Vehicle (n = 10)</td>
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<td>Spontaneous activity</td>
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<td>Symmetry in the movement of four limbs</td>
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<td>Body proprioception,</td>
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Values are median (25%, 75%); *P < 0.05 compared with normothermia control.
While there have been prior studies evaluating drug-induced hypothermia through myriad mechanisms, they have typically been plagued with a number of adverse effects or limited effectiveness that have diminished the potential for translation to clinical application (3, 11, 23, 31, 52, 53). Safer and more effective methods for producing TH are needed that are compatible with conscious subjects to make TH a viable treatment option for a broader patient population. Indeed, there has been renewed interest in a variety of pharmacological methods of promoting hypothermia without the need for sedation (19, 45). In the present study, we demonstrate that application of PH through selective targeting of thermosensitive TRPV1 produces long-lasting mild hypothermia without significant adverse cardiovascular effects or desensitization upon long or repeated DHC exposures. Furthermore, we demonstrate that DHC-mediated TH is highly effective for the treatment of ischemic stroke when applied well into the reperfusion period.

The classic TRPV1 channel agonist, capsaicin, is presumed to promote hypothermia through activation of centrally located TRPV1 containing neurons and/or peripherally located sensory nerve fibers (34, 38) and has been demonstrated to effectively produce mild hypothermia in a variety of small and large mammals including nonhuman primates (15, 17, 27). The drop in Tcore results from hypothalamus-driven autonomic responses to promote heat loss, such as cutaneous vasodilation and either sweating (humans) or saliva spreading (mice) (5, 6). TRPV1 KO mice lack the hypothemeric response to capsaicin (6). DHC, another naturally occurring capsaicinoid TRPV1 channel agonist, shows slightly greater effectiveness to induce hypothermia compared with capsaicin (24, 27). In the present study, we demonstrated that, like capsaicin, DHC also acts specifically through TRPV1 channels to promote hypothermia.

One area of concern regarding the use of TRPV1 agonists is the potential for receptor desensitization or even TRPV1-containing nerve damage (22). We, therefore, examined the sustainability of TRPV1-mediated hypothermia with therapeutic DHC doses as well as doses known to produce TRPV1 ablation or desensitization (40). Our studies with DHC did not demonstrate perceivable desensitization with prolonged DHC infusion (Fig. 1) or multiple applications at double the therapeutic dose (Fig. 4). Interestingly, desensitization of the hypothemic response may be somewhat agonist dependent, as two other TRPV1 agonists [rinvanil (15, 29) and arvanil (15)] demonstrate notable attenuation of effect with either continuous infusion or repeated bolus injections.

Cardiovascular indices can contribute significantly to neuroprotective outcome following stroke and must be considered with regard to possible translational application (52, 53). In the present study, we observed a slight transient reduction in BP after 1.25 mg/kg DHC subcutaneous injection and no change in HR or cerebral cortical perfusion. This mild drop in BP was prevented by atropine, suggesting that DHC injection causes mild activation of the parasympathetic system. Interestingly, the effect of capsaicinoids on cardiovascular function may vary depending on the delivery route. In contrast to our findings with subcutaneous delivery, intravenous infusion of DHC showed a dose-dependent increase in mean BP and HR in rats (14). In the same study, however, the authors also reported that 2 of 6 rats showed transient hypotension and bradycardia (14). Intravenous infusion of capsaicin in dogs, at doses lower than 0.1 mg/kg, did not show any effect on BP and HR while doses higher than 0.3 mg/kg showed a transient rise in BP and HR (7). While it is not fully understood how intravenous DHC affects HR and BP, it is known that TRPV1 is expressed within unmyelinated C-fibers within the aortic arch and stimulation of these fibers with lumenally delivered TRPV1 agonists contributes to decreased baroreceptor sensitivity (33, 39). Thus intravenously administered TRPV1 agonists may affect BP and HR through direct inhibition of baroreceptor function. Any such effect of DHC on baroreceptor function appears to be largely absent with subcutaneous delivery of TRPV1 agonist, whereas the hypothermic response remains robust. When considered as a whole, it appears that subcutaneous administration of DHC possesses the capacity to promote prolonged hypothermia while producing less adverse cardiovascular responses.

During the completion of the present study, Muzzi et al. (29) reported that repeated application of a TRPV1 agonist, rinvanil, induces mild hypothermia and decreases the infarct volume following 24 h and 7 days reperfusion. In that study, hypothermia was initiated either during ischemia or immediately upon reperfusion (29). These authors provided evidence suggesting that manipulating Tcore with TRPV1 receptor agonists could be a promising strategy for stroke treatment. However, in clinical reality, it takes time to provide treatment for patients after stroke onset. Therefore, our treatment of DHC-induced hypothermia was initiated after 90 min of reperfusion (or 2.5 h after stroke onset), which we believe provides a more broadly achievable treatment window relevant to clinical settings. In addition, given that recovery of brain function is the ultimate determinant of success for a stroke therapy, our studies further...
incorporated neurofunctional analysis. Future studies will be needed to determine the limits of the therapeutic window for initiating successful treatment as well as whether protection afforded by this treatment is permanent.

Given that DHC may have non-TRPV1-mediated actions as well as TRPV1-mediated actions unrelated to the hypothermic effect, we evaluated the specificity and the hypothermia dependence of DHC-mediated neuroprotection. By utilizing TRPV1 KO mice and DHC-treated mice maintained normothermic, we were able to demonstrate that the neuroprotective effect of DHC infusion was specifically linked to TRPV1-mediated hypothermia. TRPV1 KO mice treated with DHC did not show hypothermia or neuroprotection after stroke. Additionally, DHC-treated mice that were maintained normothermic for the duration of reperfusion failed to demonstrate any reduction of injury or improvement in behavioral testing. Together, these findings emphasize that the major benefit of DHC infusion is through the hypothermic response and rules out significant benefit through possible temperature-independent effects of TRPV1 agonism such as TRPV1-dependent activation of eNOS and vasodilation (8, 18, 55).

In summary, we have applied a pharmacological method to induce reversible hypothermia by selective activation of TRPV1 channels. These methods produce sustained and repeatable hypothermia within the therapeutic temperature range without significant effect on HR, BP, or CBF. Furthermore, these methods are compatible with the conscious subject and thus do not require heavy sedation or muscle paralysis to achieve therapeutic Tcore. Finally, PH through TRPV1 agonism thus do not require heavy sedation or muscle paralysis to these methods are compatible with the conscious subject and without significant effect on HR, BP, or CBF. Furthermore, peatable hypothermia within the therapeutic temperature range induce reversible hypothermia by selective activation of eNOS and vasodilation (8, 18, 55).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Z.C. and S.P.M. conception and design of research; Z.C. and A.B. performed experiments; Z.C. and S.P.M. analyzed data; Z.C. and S.P.M. interpreted results of experiments; Z.C. prepared figures; S.C. and S.P.M. drafted manuscript; Z.C. and S.P.M. edited and revised manuscript; Z.C., A.B., and S.P.M. approved final version of manuscript.

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