Application of menthol to the skin of whole trunk in mice induces autonomic and behavioral heat-gain responses

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WHEN AMBIENT TEMPERATURE IS decreased in mammals, autonomic and behavioral heat-gain responses appear and stabilize their core temperature. Heat-gain responses of autonomic means are shivering and nonshivering thermogenesis and skin vasoconstriction. Heat-gain responses of behavioral means are controlled by locomotion or other behaviors. Different heat-gain responses are likely to be triggered by different combinations of skin and core temperatures (24). However, heat-gain responses to decreased ambient temperature take place before core temperature drops (14, 33). Thus, skin temperature plays a primary role in triggering heat-gain responses to decreased ambient temperature. Nevertheless, it has not been clarified what molecules in cutaneous sensory nerve endings mediate cooling-induced heat-gain responses.

Recent studies have identified temperature-sensitive ion channels in cell bodies of sensory neurons. In transient receptor potential (TRP) nonselective cation channel family, there are some thermo-TRP channels (6, 12). TRPM8 and TRPA1 are activated when temperature is lower than thresholds (20, 21, 28, 30), while TRPV1–4 are activated when temperature is higher than thresholds (7, 8, 16, 22, 29, 34, 35). Stimulation of skin TRPM8 with menthol, a specific agonist for TRPM8, evokes cold sensation in humans (15, 37), which suggests that TRPM8 is involved in behavioral heat-gain responses. Furthermore, recent studies in TRPM8-deficient mice have shown that TRPM8 is required for cold sensation in mice (5, 10, 11). These results led us to hypothesize in the present study that TRPM8 mediates cooling-induced autonomic and behavioral heat-gain responses.

MATERIALS AND METHODS

Animals and Housing Conditions

Male C57BL/6 mice (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). They were housed in plastic cages under standard environmental conditions (27°C; 12:12-h light-dark cycle; lights on at 0600) with free access to rodent laboratory chow and drinking water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University.

Menthol Application

Mice were lightly anesthetized with 5% isoflurane. We applied menthol [L-menthol 1–10% wt/vol (64–640 mM), Nacalai Tesque, Kyoto, Japan] or its vehicle (100% ethanol) to the skin of whole trunk instead of cooling. Use of menthol is the only practical way to specifically stimulate skin TRPM8 because cooling should stimulate multiple types of temperature-sensitive channels. If menthol application induces heat-gain responses, it implies that TRPM8 is a molecule in cutaneous sensory endings that mediates cooling-induced heat-gain responses. Since each heat-gain response is independently controlled, we examined different types of autonomic and behavioral heat-gain responses.
The same mice were repeatedly treated with menthol only in experiments of repeated menthol application and dose dependency analysis. In other experiments, each mouse was treated with menthol only once. Diclofenac (10 mg/kg) and LPS (100 mg/kg) were intraperitoneally injected.

Body Temperature and Heat Loss Index

To measure core temperature ($T_{core}$) in a freely moving mouse, a SubCue mini Datalogger (SubCue, Calgary, Canada) was implanted into peritoneal cavity under pentobarbital sodium anesthesia (70 mg/kg). The data logger started to record abdominal temperature every 5 min 7 days after the implantation. After experiments, each mouse was anesthetized with pentobarbital sodium and killed by decapitation. Temperature records were read out from data loggers.

To assess the changes in the peripheral vasomotor tone, skin temperature at the base of tail ($T_{tail}$) was detected with thermography, Thermotracer TH3100MR (NEC San-ei Instrum, Kyoto, Japan). Heat loss index (HLI) was calculated by the following equation (26, 32):

$$HLI = \frac{T_{core} - T_{tail}}{T_{core}} \times 100$$

Fig. 1. A: core temperatures of 10% menthol-treated mice (solid circles) and vehicle-treated mice (open circles) (each $n = 8$). Both reagents were applied at 0 min. Core temperatures of both groups transiently decreased at 5 min ($t$-test; $P < 0.05$ between 0 and 5 min in each group). Core temperature of menthol-treated mice was significantly higher than that of vehicle-treated mice during 20–120 min (two-way ANOVA; $P < 0.05$ between menthol treatment and vehicle treatment, $t$-test; $P < 0.05$ between 20 and 120 min). B: effects of repeated application of 10% menthol on core temperature elevation. Menthol was applied 3 times every other day. The increases at 60 min from menthol application were shown (each $n = 5$). The increases were not significantly different among the three trials (one-way ANOVA; $P > 0.05$). C: effects of menthol concentration on core temperature elevation. Mice were treated with 1% (open triangle), 5% (solid triangle), 10% menthol (solid circles), and vehicle (open circles) (each $n = 5$). Each concentration of menthol significantly elevated core temperature compared with vehicle (two-way ANOVA; $P < 0.05$ between each dose of menthol and vehicle). In addition, menthol-induced elevation of core temperature was dose-dependent (two-way ANOVA; $P < 0.05$ between each dose). D: menthol-induced hyperthermia in diclofenac-pretreated mice (solid circles) and saline-pretreated mice (open circles). Core temperatures were not significantly different between the two groups (each $n = 8$) (two-way ANOVA; $P > 0.05$ between diclofenac pretreatment and saline pretreatment). E: LPS-induced fever in diclofenac-pretreated mice (solid squares) was significantly suppressed compared with that in saline-pretreated mice (open squares) (each $n = 8$) (two-way ANOVA; $P < 0.05$ between diclofenac pretreatment and saline pretreatment). F: $O_2$ consumption of menthol-treated mice (solid circles) was significantly higher than that of vehicle-treated mice (open circles) (each $n = 9$) (two-way ANOVA; $P < 0.05$ between menthol treatment and vehicle treatment $t$-test; $P < 0.05$ during 26–117 min).
H LI = (T tail − T a)/(T core − T a), where T a is ambient temperature of 27°C.
H LI eliminates direct influences of both T a and T core on skin temperature. The value of H LI varies from 0 (full vasoconstriction) to 1 (full vasodilation). The H LI has been successfully used to evaluate the thermoregulatory vasomotor response of the guinea pig ear (25) and rat tail (27).

Oxygen Consumption

Oxygen consumption was measured in rectangular chambers (125.4 cm² floor and 6.5 cm in height). Each mouse was habituated to the chamber for half a day before trials with free access to laboratory chow and drinking water. Menthol or vehicle was applied as described above. Expired gas and room air were analyzed with O₂ analyzer (model RL-600, AlcoSystem, Tokyo, Japan). Room air was pumped through the chambers at a rate of 0.5 ± 0.05 l/min. Expired air was dried and directed to the O₂ analyzer. The data for each chamber were obtained every 13 min. Oxygen consumption (V˙O₂) was calculated using the following equation: V˙O₂ = (FEN₂/FIN₂)·FIO₂ − FEO₂·VT·10 (ml/min), where FEN₂ is the concentration of nitrogen in the expired air, FIN₂ is the concentration of nitrogen in the room air, FIO₂ is the concentration of oxygen in the expired air, FEO₂ is the concentration of oxygen in the room air, and VT is the air flow through the chamber corrected to standard temperature and pressure, dry.

Shivering Recorded by Electromyogram

Electrodes were made from a pair of Teflon-coated silver wire (0.18-mm diameter) with 3-mm exposed tips. The electrode tips were separated by 5 mm. Under isoflurane anesthesia, electrodes were inserted under the back skin and placed on the back muscles. Electrode leads were attached to a connector, which was fixed on the neck skin using glue. After recovering from anesthesia, each mouse was placed in a plastic cage with a lid (480 cm² floor and 17 cm in height). Experiments started 3 h after electrode placement. First, we measured baseline electromyogram (EMG) for 3 min. Then mice were treated with menthol or vehicle as described above. After 1 h, EMG was measured for 15 min. On the next day, the experiment was repeated in the same mice with the other reagent. EMG signals were noise-filtered (high-pass filter 10 Hz, low-pass filter 5,000 Hz, 60 Hz notch), amplified with BioAmp (ADInstruments, Nagoya, Japan) and recorded at a rate of 4,000 points/s with PowerLab (ADInstruments). Amplitude of EMG, root mean square (RMS), was analyzed with IGOR Pro (WaveMetrics, Lake Oswego, OR).

Nonshivering Thermogenesis

Phosphorylated and nuclear-translocated NF-κB p65 (pNF-κB) was used as a marker of activities of brown adipose tissue (BAT) (23). Mice were treated with menthol or vehicle as above. One hour after the treatment, they were anesthetized with pentobarbital sodium and perfused with 15 ml of 4% lidocaine solution from the left cardiac ventricle. Lidocaine was used to suppress nerve activity during dissection of the mice. Interscapular BAT was collected and immediately frozen in dry ice powder. Tissue samples were cut into 30-μm-thick slices by a cryostat and thaw-mounted on silane-coated slide glasses. The sections were fixed in 10% (vol/vol) formalin in 0.1 M PBS for 10 min. After washing with 0.1 M PBS, the sections were blocked for 30 min with 10% (vol/vol) normal donkey serum in 0.1 M PBS containing 0.2% Triton X-100 and 0.03% sodium azide. The sections were incubated with rabbit anti-pNF-κB p65 (Ser 276) (1:100, Cell Signaling Technology, Beverly, MA) at room temperature overnight. After washing in 0.1 M PBS, the sections were incubated with AlexaFluor 488-conjugated anti-rabbit IgG (H & L) (1:500, Molecular Probes, Carlsbad, CA) for 2 h at room temperature. Cellular nuclei were stained with TOPRO3 (1:1,000, Molecular Probes). Fluorescent signals were captured by a laser confocal microscope (Radiance 2000, Bio-Rad, Foster City, CA). The fluorescent images were processed using Adobe Photoshop (Adobe). The percentage of pNF-κB-positive nuclei was calculated from at least 200 BAT cells in each mouse.

Behavioral Selection of Floor Temperature

To examine whether menthol application changes thermal preference of mice, a menthol- or vehicle-treated mouse was placed in a longitudinal plastic cage (16 cm × 27 cm in size), the floor of which was divided into two temperature zones. One half of the floor (16 cm × 13.5 cm in size) was kept at 35°C using a heating pad under the

Fig. 2. Representative electromyograms (EMGs) of menthol-treated mouse (A), vehicle-treated mouse (B), mouse exposed to 4°C (C), and mouse exposed to 27°C (D). E: changes in EMG amplitude (RMS) from the baseline values, that is, EMG amplitude prior to menthol or vehicle treatment. Change in EMG amplitude of menthol-treated mice was significantly larger than that of vehicle-treated mice (t-test; P < 0.05).
cage, and the other half was left unheated (27°C). Before the experiment, a mouse was habituated to the same type of plastic cage with 27°C floor temperature for more than 20 min. Thereafter, the mouse was transferred to the center of the cage with two temperature zones. Mouse movement was recorded by night-vision system of a digital video camera for 20 min (1,800 frames/min). We measured how long a mouse stayed on 35°C floor every 1 min using Image J software with a macro program of our own making.

**Menthol-Induced Activation of Sensory Nerve Endings**

To examine whether menthol application to skin stimulates local nerve endings, we applied menthol to the right half of the trunk in pentobarbital sodium-anesthetized mice. They were placed on a heating pad (35°C) to prevent drop of core temperature. Ten minutes after the menthol application, mice were perfused with 4% lidocaine. Their vertebral bones containing spinal cord and dorsal root ganglia were collected and frozen. Tissue samples were cut into 20-μm-thick sections at the L2 DRG level. Immunohistochemistry for pNF-κB was conducted as above. After immunohistochemistry, following the staining of cells with methyl green and nuclei with DAPI, the images were captured by a CCD camera. The sizes and number of cells were measured using Adobe Photoshop and Image J software.

**Statistical Analysis**

The data are reported as means ± SE. Core temperature, oxygen consumption, HLI, and thermal preference were compared across treatment and time points by a two-way ANOVA for repeated measurements followed by a post hoc test. Effects of repeated menthol
application were assessed by a one-way ANOVA. Dose dependency of menthol effect was analyzed across treatment and time points by two-way ANOVA for repeated measurements. Comparisons of two values were made by a t-test. A P value of 0.05 was set for statistical significance.

RESULTS AND DISCUSSION

Autonomic Heat-Gain Responses

Menthol-induced increase in core temperature. When 10% menthol or vehicle was applied to the skin of whole trunk in mice, core temperature decreased transiently (t-test; \( P < 0.05 \) between 0 and 5 min in each groups) (Fig. 1A). This transient decrease seems to be due to increased heat loss caused by ethanol evaporation. Core temperature recovered to the baseline within 15 min. Thereafter, core temperature of vehicle-treated mice stayed around the baseline level, whereas that of menthol-treated mice continued to rise until 30 min. This resulted in 1.3°C difference in core temperature between the two groups at 30 min (Fig. 1A). The hyperthermic effect of menthol continued for 100 min (two-way ANOVA; \( P < 0.05 \) between menthol treatment and vehicle treatment, t-test; \( P < 0.05 \) during 20–120 min). To examine whether hyperthermic response to menthol is changed by repeated applications of it, we treated mice with 10% menthol 3 times every other day. Elevations in core temperature 60 min after menthol application were almost the same (one-way ANOVA; \( P > 0.05 \)), indicating that neither desensitization nor sensitization occurred under these experimental conditions (Fig. 1B). Dose dependency of the menthol action was examined in mice treated with 1%, 5%, 10% menthol, and vehicle (Fig. 1C). Each concentration of menthol significantly elevated core temperature compared with vehicle treatment (two-way ANOVA; \( P < 0.05 \) between each dose of menthol and vehicle). In addition, menthol-induced elevation of core temperature was dose dependent (two-way ANOVA; \( P < 0.05 \) between each dose). Thus, 10% menthol was used in all experiments below. This concentration of menthol has been reported to evoke cold sensation in humans when applied to the skin (15, 37).

To examine whether menthol-induced hyperthermia is fever, mice were pretreated with diclofenac (a prostaglandin synthesis inhibitor) intraperitoneally 1 h before 10% menthol application. Diclofenac did not influence menthol-induced hyperthermia (two-way ANOVA; \( P > 0.05 \) between diclofenac pretreatment and saline pretreatment) (Fig. 1D), while the same dose of diclofenac significantly suppressed LPS-induced fever (two-way ANOVA; \( P < 0.05 \) between diclofenac pretreatment and saline pretreatment) (Fig. 1E). These results indicate that menthol-induced hyperthermia is not fever. Next, we examined what kind of autonomic heat-gain responses are involved in the elevation of core temperature.

Oxygen consumption. To examine whether menthol application raises heat production in whole body, we measured \( O_2 \) consumption (Fig. 1F). In response to 10% menthol or vehicle, \( O_2 \) consumption was almost doubled at 13 min. At this time point, \( O_2 \) consumption was not significantly different between the two groups (t-test; \( P > 0.05 \)). After this initial rise, \( O_2 \) consumption in vehicle-treated mice gradually declined and returned to the baseline level by 39 min after the application. On the other hand, \( O_2 \) consumption in menthol-treated mice declined more slowly and was maximally 31% higher than that in vehicle-treated mice from 26 min to 117 min after the application (two-way ANOVA; \( P < 0.05 \) during 26–117 min).

Because there was no visible enhancement of external work in mice after menthol or vehicle application, consumed \( O_2 \) seemed to be mostly used for heat production. The initial rises in \( O_2 \) consumption at 13 min were temporally consistent with
The recovery of core temperature from hypothermia (Fig. 1A), suggesting that $O_2$ was initially used for heat production to recover core temperature in both groups. On the other hand, the menthol-specific rise in $O_2$ consumption from 26 to 117 min seemed to be used for heat production that caused menthol-induced hyperthermia.

Muscle shivering. Muscle shivering is a major source of heat production. To explore whether menthol treatment induces muscle shivering, we performed EMG recordings of back muscle 60 min after 10% menthol application. This time point was chosen because core temperature and $O_2$ consumption in menthol-treated mice were higher than those in vehicle-treated mice. Large EMG activity was intermittently observed in menthol-treated mice (Fig. 2A) but not in vehicle-treated ones (Fig. 2B). For comparison, EMG was recorded when mice were exposed to 4°C (Fig. 2C) for 60 min. The EMG signals were similar between menthol-treated mice and 4°C-exposed mice. Amplitude (RMS) of EMG in menthol-treated mice was significantly higher than that in vehicle-treated ones ($t$-test; $P < 0.05$) (Fig. 2D), indicating that menthol treatment induced muscle shivering.

Nonshivering thermogenesis. Another major source of heat production in rodents is BAT. To examine whether menthol application induces BAT activity, we carried out immunohistochemistry of pNF-κB, which can be a marker of activated brown adipocytes (23). The number of pNF-κB-positive nuclei in BAT was dramatically increased in mice when exposed to 4°C for 60 min ($t$-test; $P < 0.05$) (Fig. 3A, B, and E). Similar results were observed when mice were treated with 10% menthol for 60 min ($t$-test; $P < 0.05$) (Fig. 3C, D, and F), indicating that menthol treatment induced BAT activity.

Heat loss index. To examine whether menthol application induces vasoconstriction, we measured tail surface temperature in addition to core temperature. HLI, inversely reflecting the degree of tail skin vasoconstriction, was calculated at four time points (a, just before treatment; b, core temperature recovered to the level of a; c, core temperature reached the highest level; and d, 10 min after c) (Fig. 4A). HLI of 10% menthol-treated mice was significantly lower than that of vehicle-treated mice at time point “b” ($t$-test; $P < 0.05$ between menthol treatment and vehicle treatment) (Fig. 4B and C), indicating that menthol induces vasoconstriction when core body temperature was rising most quickly.

Thus, all typical autonomic heat-gain responses were induced by application of 10% menthol to the skin of whole trunk in mice. These autonomic heat-gain responses should increase heat content of the body in a thermally neutral environment, and, consequently, result in hyperthermia.

Behavioral selection of floor temperature. We examined whether menthol application changes thermal preferences of mice. This experiment was done during the dark period when mice were awake. After application of 10% menthol or vehicle, mice were put on the border between a 35°C floor and a 27°C floor and were allowed to move freely. Menthol-treated mice spent significantly longer time on 35°C floor than vehicle-treated mice from 6 min to 20 min after the application (two-way ANOVA; $P < 0.05$ between menthol treatment and vehicle treatment, $t$-test; $P < 0.05$ during 6–20 min) (Fig. 5). This result is in line with a study by Almeida et al. (1), in which intravenous injection of menthol evoked heat-seeking behavior in rats.

When we performed the experiment during the light period, time spent on 35°C floor was not significantly different between menthol-treated mice and vehicle-treated ones. Although the reason for this day-night difference of behavior is unclear, a similar day-night difference was reported in LPS-
injected rats that were allowed to move freely in a thermal gradient (31).

Recently, papers from three groups (5, 10, 11) showed that TRPM8-deficient mice spent more time on the low-temperature floor than wild-type mice, indicating that deletion of TRPM8 reduced behavioral avoidance of low temperature. On the other hand, the present study and those by Almeida et al. (1, 2) indicated that stimulation of TRPM8 facilitated heat-seeking behavior. Thus, the TRPM8-deletion studies and TRPM8-stimulation studies are complementary to each other, providing a strong evidence for the role of TRPM8 in behavioral response to low temperature.

Localization of menthol-activated sensory neurons. To confirm that menthol-activated sensory neurons locally in the area of application, we applied 10% menthol to the right half of the trunk, while the left half was untreated. Ten minutes after experiments, we compared the number of pNF-κB-positive neuronal nuclei between the right and left DRG of the L2 level. Neurons with pNF-κB-positive nuclei were present only in the right DRG (Fig. 6, A and B). In three independent experiments, we obtained the same results. The proportion of pNF-κB-positive neurons to DRG neurons was 1.2% (55/4,454). The average diameter of pNF-κB-positive neurons was 18.3 ± 0.7 μm (means ± SE, n = 55). These cells are classified as small-sized neurons, and most of TRPM8-positive neurons are reported to belong to this cell population (21).

Several studies have implicated that nuclear pNF-κB is a marker of activated neurons. In neuronal cells, phosphorylation and nuclear translocation of NF-κB were brought about by elevation of intracellular Ca²⁺, which accompanies neuronal excitation (17). Furthermore, high-frequency electrical stimulation of perforant path in the hippocampus activated neuronal NF-κB (13). Thus, expression of nuclear pNF-κB in the DRG indicates that menthol indeed activated sensory neurons in this study. Furthermore, the ipsilateral expression of pNF-κB indicates that the menthol action was restricted to the area of application.

It has been a question whether cold sensation and heat-gain responses are triggered by the same receptor in the skin. The present study for the first time showed that skin menthol receptor, probably TRPM8, triggers heat-gain responses. Thus, cold sensation and heat-gain responses are evoked by the same receptor. There are two issues to be considered when we interpret the present findings. First, the present study is based on an assumption that menthol is a specific agonist of TRPM8. This assumption is, to a certain degree, reasonable because the number of menthol-sensitive DRG and TG neurons was greatly reduced in TRPM8-deficient mice (5, 10, 11). However, a small fraction of DRG and TG neurons isolated from TRPM8-deficient mice still responded to menthol with elevated intracellular Ca²⁺ (10, 11). It is not elucidated yet whether this TRPM8-independent Ca²⁺ elevation is due to the opening of voltage-sensitive calcium channels following neuronal excitation or due to the release of Ca²⁺ from intracellular stores. Indeed, in some cell lines, menthol induced Ca²⁺ release from endoplasmic reticulum and Golgi in a TRPM8-independent manner (18). Thus, although menthol can influence DRG neuron function in a TRPM8-independent mechanism, its physiological significance in electrical activity of neurons, and the subsequent heat-gain responses remains to be studied. Second, it is unclear whether TRPM8 is solely responsible for cooling-induced heat-gain responses. DRG neurons express several non-TRPM8 ion channels influenced by low temperature (3, 4, 19, 30). These two issues should be solved by studying heat-gain responses of TRPM8-deficient mice to menthol and low ambient temperature, respectively.

Perspectives and Significance

The present study provided two interesting implications for future studies. First, core temperature of homeotherms may depend on the activity of TRPM8. It is well known that birds have higher normal core temperature (40 – 43°C) than mammals (37°C). Interestingly, thermal threshold of chicken TRPM8 (34°C) is also higher than that of mouse TRPM8 (~26°C) (9), leading us to speculate that higher activity of chicken TRPM8 may cause their higher core temperature. The present finding that activating TRPM8 with menthol raises core temperature of mice supports this speculation. Thus, we can hypothesize that core temperatures of homeotherms are determined by their thermal threshold of TRPM8. To verify this hypothesis, the development of transgenic mice expressing chicken TRPM8 and studies of their thermoregulatory responses will be needed. Second, neural circuits of heat-gain responses triggered by TRPM8 may be elucidated by generating and analyzing transgenic mice expressing wheat germ agglutinin, a transsynaptic neural tracer (36), in TRPM8-expressing sensory neurons. Success in these future studies will provide great progress in our understanding of thermal information processing and body temperature regulation.

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REFERENCES


