TRPM8 is the principal mediator of menthol-induced analgesia of acute and inflammatory pain

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ABSTRACT

Menthol, the cooling natural product of peppermint, is widely used in medicinal preparations for the relief of acute and inflammatory pain in sports injuries, arthritics, and other painful conditions. Menthol induces the sensation of cooling by activating TRPM8, an ion channel in cold-sensitive peripheral sensory neurons. Recent studies identified additional targets of menthol, including the irritant receptor, TRPA1, voltage-gated ion channels and neurotransmitter receptors. It remains unclear which of these targets contribute to menthol-induced analgesia, or to the irritating side effects associated with menthol therapy. Here, we use genetic and pharmacological approaches in mice to probe the role of TRPM8 in analgesia induced by L-menthol, the predominant analgesic menthol isomer in medicinal preparations. L-menthol effectively diminished pain behavior elicited by chemical stimuli (capsaicin, acrolein, acetic acid), noxious heat, and inflammation (complete Freund’s adjuvant). Genetic deletion of TRPM8 completely abolished analgesia by L-menthol in all these models, although other analgesics (acetaminophen) remained effective. Loss of L-menthol–induced analgesia was recapitulated in mice treated with a selective TRPM8 inhibitor, AMG2850. Selective activation of TRPM8 with WS-12, a menthol derivative that we characterized as a specific TRPM8 agonist in cultured sensory neurons and in vivo, also induced TRPM8-dependent analgesia of acute and inflammatory pain. L-menthol– and WS-12–induced analgesia was blocked by naloxone, suggesting activation of endogenous opioid-dependent analgesic pathways. Our data show that TRPM8 is the principal mediator of menthol-induced analgesia of acute and inflammatory pain. In contrast to menthol, selective TRPM8 agonists may produce analgesia more effectively, with diminished side effects.

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1. Introduction

Menthol, the cooling natural product of peppermint, is widely used in preparations for pain relief in sports injuries, arthritics, and other painful conditions [14,25]. In animal models, topical, oral or systemic administration of menthol elicits analgesia of acute, inflammatory, and neuropathic pain [17,29,40]. Menthol, and other cooling natural products such as eucalyptol, activate TRPM8, a Transient Receptor Potential (TRP) ion channel, an essential detector of cold stimuli in sensory neurons [2,13,37,38,41]. Menthol, through activation of TRPM8, may have effects similar to tissue cooling, which reduces pain in some injury states. In TRPM8-deficient mice, cooling-induced analgesia was less effective toward formalin-induced and neuropathic pain [13,31]. In rats, intrathecal injection of TRPM8 antisense DNA reduced the analgesic effects of menthol toward neuropathic pain [43,51].

Studies using TRPM8 gene–deficient mice to probe the role of TRPM8 in menthol-induced analgesia have not been reported, and some recent findings suggest that TRPM8-independent mechanisms are responsible for menthol analgesia. Menthol and eucalyptol have poor specificity for TRPM8 and interact with other TRP channels, including TRPA1 and TRPV3 [34,60]. These ion channels may elicit pain and inflammatory symptoms in some patients treated with menthol preparations, but may also contribute to analgesia [21,25,61]. For example, eucalyptol was found to inhibit human TRPA1 channels, exerting a mild analgesic effect on pain elicited by TRPA1 agonists in human subjects [54]. Menthol has similar inhibitory effects on murine TRPA1 channels at higher concentrations, but not on human TRPA1 [28,65]. Other studies suggest that menthol analgesia is mediated by TRP channel-independent mechanisms. Menthol was found to inhibit neuronal
voltage-dependent Ca\(^{2+}\) channels [49,52]. Menthol was shown to activate GABA\(_{A}\)-receptors, which may induce central inhibition of nociception [11,63,67]. Menthol may also elicit analgesia by inactivating voltage-gated sodium channels mediating action potentials in sensory neurons [19]. Menthol was also found to inhibit nicotinic acetylcholine receptors, and serotonin-gated ion channels, known to contribute to pain signaling [22,24].

In addition to the lack of selectivity, the contradictory outcomes of the above studies may have resulted from the use of different stereoisomers of menthol [27]. The predominant isomer (-98%) in natural mint oils is L-menthol, with small amounts of D-menthol and other isomers present [14]. L-menthol has a lower sensory detection threshold for cooling and more potent analgesic activity than D-menthol, and carries the "minty" smell associated with peppermint, whereas D-menthol has a phenolic smell [14,16-18]. Most mentholated analgesic products exclusively contain L-menthol [9].

Here, we compare the analgesic effects of L-menthol in wild-type and TRPM8-deficient mice in models of acute cutaneous, visceral, and inflammatory pain. L-menthol efficiently inhibited pain behavior induced by a diverse set of chemical and physical noxious stimuli. Menthol analgesia was completely abolished in Trpm8\(^{-/-}\)mice, and in wild-type mice treated with a selective TRPM8 inhibitor. Moreover, a recently identified specific TRPM8 agonist recapitulated analgesic effects of menthol. These results suggest that TRPM8 is the principal mediator of menthol's analgesic effects in vivo.

2. Methods

2.1. Animals

Experimental procedures were approved by the Institutional Animal Care and Use Committees of Yale University. Mice were housed at facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in standard environmental conditions (12-hour light-dark cycle and 23°C). Food and water were provided ad libitum. Trpa1\(^{-/-}\) and Trpm8\(^{-/-}\) mice were a gift from David Julius (University of California-San Francisco) and backcrossed into C57BL/6 background using marker assisted backcrossing (Charles River Laboratories, Wilmington, MA). Trpv1\(^{-/-}\) mice were purchased from Jackson Laboratories (Stock# 003770), Bar Harbor, ME, and crossed with Trpa1\(^{-/-}\) mice to produce Trpv1\(^{-/-}\)/Trpa1\(^{-/-}\) mice. C57BL/6 wild-type mice were purchased from Charles River Laboratories. Male mice 8 to 12 weeks of age were used in all experiments.

2.2. Chemicals

L-menthol and eucalyptol were purchased from Fisher Scientific (Pittsburgh, PA); WS-12 from obtained from Tocris Bioscience (Bristol, UK); AMG258 was a gift from Amgen Inc. (Thousand Oaks, CA); and Complete Freund's Adjuvant (CFA) was purchased from Rockland Immunochemicals (Gilbertsville, PA). Acetic acid, mustard acid, acetaminophen, capsaicin, acrolein, and naloxone were obtained from Sigma-Aldrich (St. Louis, MO).

2.3. Behavioral tests

2.3.1. Hot plate test

L-menthol at a dose of 10 mg/kg was prepared in dimethyl sulfoxide (DMSO), further diluted in phosphate-buffered saline (PBS), and applied through oral gavage to wild-type male C57BL/6 or Trpm8\(^{-/-}\) mice (8-12 weeks old) in a volume of 50 mL/kg. WS-12 (10 mg/kg) was prepared in ethanol, further diluted in corn oil, and injected (i.p.) into mice in a volume of 5 mL/kg. WS-12 showed poor solubility in aqueous media, and needed to be used at dosages equivalent to menthol in in vivo tests. After 30 minutes, mice were placed into a transparent chamber with a stainless steel hot plate set to a temperature of either 52°C or 55°C (ITTC, Woodland Hills, CA). A baseline test of paw withdrawal latency was done 1 week before to ensure that there were no differences among the groups. Behavior was then videorecorded, and the latency to exhibit nociceptive responses (such as lifting and licking of the hind paw or jumping) was calculated using a stop watch.

2.3.2. Acetic acid writhing test

Mice were first habituated in transparent observation chambers for 30 minutes before the test. Mice were given L-menthol (10 or 20 mg/kg), eucalyptol (200 mg/kg), or acetaminophen (50 mg/kg) by i.p. injection 30 minutes before testing, prepared in corn oil and administered i.p. to mice in a volume of 5 mL/kg. Thirty minutes later, mice were injected i.p. with 0.6% acetic acid (10 mL/kg). Numbers of stretching (writhing) movements were recorded for 20 minutes by a camera located underneath the chamber and counted thereafter.

2.3.3. Tail flick test

Mice were restrained in a small restraining chamber allowing the free movement of the tails. Mice were trained in the chamber for 40 minutes daily for 1 week before the test. Tails of the mice were exposed to radiant heat, and the tail flick latency was automatically recorded by the testing device (ITTC, Woodland Hills, CA). Tail-flick latencies were averaged from 3 separate tests conducted within a 1-minute interval. L-Menthol (10 or 20 mg/kg) was prepared in corn oil and then i.p. injected into mice at a volume of 2.5 mL/kg 30 minutes before testing.

2.3.4. Chemically induced nociceptive behavior

Mice were placed into transparent chambers and habituated for 30 minutes before testing. Compounds were injected into the hind paw of mice using a 1-ml syringe and 30-gauge needle as follows: L-menthol (50 nmol) was co-injected with capsaicin (5 nmol) or acrolein (25 nmol) in a total volume of 50 μL dissolved in saline. WS-12 (6 nmol) was injected with capsaicin (2 nmol) or acrolein (30 nmol) in a total volume of 20 μL dissolved in corn oil. Nociceptive behavior (licking, flinching, or biting of injected paw) was recorded for 10 minutes and quantified thereafter.

2.3.5. Capsaicin-induced mechanical hyperalgesia

Mice were habituated for 1 hour to wire mesh screen surface before testing. Paw withdrawal thresholds were determined using a series of von Frey filaments (0.008-6.00 g) pressed against the plantar surface of the hind paw in ascending order beginning with the finest fiber following standard procedures [10,42]. The minimum force (g) that caused the mouse to withdraw its hind paw away from the filament was considered as the withdrawal threshold. For each paw, a von Frey hair was applied 5 times at 10-second intervals. The threshold was determined when paw withdrawal was observed in more than 3 of 5 applications. A withdrawal response was considered valid only if the hind paw was removed completely from the platform. If the paw withdrawal response was ambiguous, the application was repeated. Thresholds were measured at 0, 15, 30, 45, and 60 minutes after application of capsaicin (400 pmol) or capsaicin + L-menthol (20 nmol) in a 20-μL volume.

2.3.6. CFA test

CFA was injected into the plantar surface of the right hind paw of each mouse at a volume of 20 μL on day 0. For control mice, saline was injected instead. On days 1, 2, and 3, the paw withdrawal
2.3.3. Rotarod test

Mice were placed on a rotating cylinder with the speed increasing from 5 to 40 rpm in 5 minutes (IITC, Woodland Hills, CA) on 4 consecutive days for habituation. Tests were repeated 3 times, with 5-minute breaks. Fall latency times were determined by stopwatch and averaged for the 3 tests. All behavioral tests were performed by an experimenter blinded to experimental conditions.

2.4. Cell culture and Ca^{2+} imaging

Adult mouse dorsal root ganglia were dissected and dissociated for 1-hour incubation in 0.28 WJ/ml Liberase DH Research Grade high Disperse concentration (Roche, Germany), followed by washes with PBS, trituration, and straining of debris (70-μm-pore cell strainer, Falcon, MA). Neurons were cultured in neurobasal-A medium (Invitrogen, Grand Island, NY) with B-27 supplement, 0.5 mmol/L glutamine, and 50 ng/ml nerve growth factor (Calbiochem, La Jolla, CA) on an 8-well chambered coverglass coated with polysine (Sigma, St. Louis, MO) and laminin (Invitrogen). HEK-293T cells were cultured and transfected as described elsewhere [26]. Ca^{2+} imaging was performed 24 hours after dorsal root ganglia (DRG) dissociation or cell transfection. Medium was replaced by modified standard Ringer’s bath solution (in mmol/L): 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, and 10 HEPES, pH 7.4. Cells were loaded with Fura-2 AM (10 μmol/L, Calbiochem, CA) for 45 minutes and subsequently washed and imaged in standard bath solution. Ratio-metric Ca^{2+} Imaging was performed on an Olympus IX51 microscope with a Polychrome V monochromator (Till Photonics, Gräfelfing Germany) and a PCO Cooke Sensicam QE CCD camera and Imaging Workbench 6 imaging software (Indec, Santa Clara, CA). Fura-2 emission images were obtained with exposures of 0.1 millisecond at 340 nm and 0.1 millisecond at 380-nm excitation wavelengths. The ratio of the fluorescence intensity obtained at 340 and 380 nm was used to determine the Ca^{2+} signal.

2.5. Statistical analysis

Statistical comparisons were made between groups using the Student t test, or 1-way analysis of variance (ANOVA), followed by the Tukey post hoc test, with values of P < .05 considered significant. Data in bar graphs in the figures are the mean and standard error of the mean (SEM).

3. Results

3.1. TRPM8 is essential for L-menthol analgesia of acute heat- and capsaicin-induced pain in mice

Systemic or topical administration of L-menthol is known to increase pain thresholds in mice and rats in the hot plate test [17,29,57]. We compared the analgesic action of systemic L-menthol in wild-type C57BL/6 and Trpm8−/− mice. L-menthol (10 mg/kg, p.o.) was administered 30 minutes before testing at plate temperatures of 52°C or 55°C. In wild-type mice, L-menthol approximately doubled paw withdrawal latencies at both temperatures (Fig. 1A). This analgesic effect was completely abolished in Trpm8−/− mice (Fig. 1A). Topical application of menthol to the mouse paws (30% in ethanol, 5 minutes before testing [52°C plate temperature]) also produced a strong analgesic effect that was again abolished in Trpm8−/− mice (Fig. 1B and C). In control experiments, this concentration of L-menthol did not lead to acute nociceptive behavior when applied topically, compared to ethanol in control animals (observed over 10 minutes; n = 7 animals each). We also observed that L-menthol has analgesic effects in the mouse tail-flick test, another model of thermally induced pain. L-menthol (20 mg/kg, i.p., 30 minutes before testing) produced a significant increase in tail-flick latencies in wild-type mice (Fig. 1D). This effect was again abolished in Trpm8−/− mice (Fig. 1D). The same dose of L-menthol (20 mg/kg, i.p.) did not change tail latencies in control rotarod experiments, suggesting a specific analgesic effect (Supplementary Fig. 1).

TRPV1, the capsaicin receptor, is a crucial mediator of heat-evoked pain in human beings and in rodents [8,46,47]. Because we observed that L-menthol inhibited heat-evoked pain, we investigated whether L-menthol would also reduce nociceptive behavior caused by specific pharmacological activation of TRPV1 after injection of capsaicin into the plantar surface of the mouse hindpaw (5 nmol/50 μl) (Fig. 1E). Coinjection of L-menthol (intraplantar, 50 nmol/50 μL = 1 nmol/ml) strongly diminished capsaicin-induced nociceptive responses in wild-type mice. In Trpm8−/− mice, capsaicin-induced nociceptive behavior was as robust as in wild-type mice (Fig. 1E). However, the analgesic effect of L-menthol was completely absent in Trpm8−/− mice (Fig. 1E). As TRPA1 may functionally interact with TRPV1 in capsaicin-sensitive sensory neurons expressing both channels, and as murine TRPA1 channels were found to be inhibited by high L-menthol concentration, we examined the actions of L-menthol in Trpa1−/− mice. The Trpa1−/− mice showed normal nociceptive responses to capsaicin, and we found that the analgesic activity of L-menthol was not different from its activity in wild-type mice (Fig. 1E, tested by analysis of variance, followed by Tukey post hoc test, P > .05). Mechanical hyperalgesia caused by intraplantar injection of capsaicin (400 nmol/20 μL) was also inhibited by L-menthol when co-injected (20 nmol/20 μL = 1 nmol/ml) in wild-type mice (Fig. 1F). Trpm8−/− mice showed normal mechanical hyperalgesia after injection of capsaicin, but L-menthol’s analgesic effects were absent (Fig. 1G).

3.2. L-menthol requires TRPM8 for inhibition of TRPA1-induced acute and inflammatory pain

Acrolein, the major reactive irritant in cigarette smoke, stimulates sensory nerves through activation of TRPA1 [1,14]. We recently reported that L-menthol inhibited acrolein-induced respiratory irritation responses in mice [64]. Acrolein is also generated endogenously as a product of lipid peroxidation, similar to other endogenous lipid peroxidation products thought to cause inflammatory pain through activation of TRPA1 after tissue injury [35,39,59]. To examine the effects of L-menthol on TRPA1-induced pain behavior, we compared acute nociceptive responses of mice injected with acrolein (25 nmol/50 μL) into the plantar surface of the hindpaw, with or without coinjection of L-menthol (50 nmol/50 μL = 1 nmol/ml). Wild-type mice showed obvious acrolein-induced TRPA1-dependent nociceptive behavior that was strongly inhibited by L-menthol (Fig. 2A, Supplementary Fig. 2A). Trpm8−/− mice showed equally strong responses to acrolein, but the analgesic effects of L-menthol were completely absent (Fig. 2A).

Systemic treatment of mice with a mixture of D- and L-menthol was recently reported to diminish mechanical pain hypersensitivity in the CFA model of inflammatory pain [40]. Several pharmacological and gene knockout studies have implicated TRPA1 as a major mediator of hyperalgesia and allodynia in this model [12,15,32,36]. We examined the effect of locally administered L-menthol (60 nmol/20 μl, or vehicle, intraplantar injection. 30 minutes before testing) by von Frey hair analysis of wild-type mice on the 3 days following CFA (20 μl) injection into the hindpaw (Fig. 2B). Mice injected with L-menthol showed significantly diminished mechanical hyperalgesia (Fig. 2B). The role of TRPM8 in the analgesic effect of L-menthol in the CFA model was probed...
**Fig. 1.** Effects of L-menthol on thermal- and capsaicin-induced pain behavior in wild-type and Trpm8−/− mice. (A) Paw withdrawal latencies in the hot plate test (52° or 55°C) in wild-type mice (black) and Trpm8−/− mice (blue) treated with L-menthol (Ment, 10 mg/kg) or vehicle (PBS, Veh) through oral gavage (p.o.) 30 minutes before testing. n = 7–10 mice per group. (B) Paw withdrawal latencies of wild-type mice in the hot plate test (52°C) after topical application of 30% L-menthol and ethanol (Veh). The 2 hind paws were immersed into vehicle or L-menthol solution for 5 seconds and allowed to dry for 1 minute. n = 7–9 mice per group. (C) Paw withdrawal latencies of Trpm8−/− mice in the hot plate test after topical application of 30% L-menthol and ethanol. Experiment as in panel B. n = 8 mice per group. (D) Tail-flick latencies of wild-type and Trpm8−/− mice treated with vehicle or L-menthol (10 or 20 mg/kg, i.p.) 30 minutes before testing. n = 6–7 mice per group. (E) Effects of L-menthol on capsaicin-induced nocifensive behavior (flinching, lifting, and biting) in wild-type mice. Trpm8−/− or Trpa1−/− mice. Vehicle (PBS), L-menthol (50 nmol) or capsaicin (5 nmol) or capsaicin in combination with L-menthol (5 nmol+50 nmol) were injected (in 50-μL volume) into the plantar surface of the hind paw and nocifensive behavior was recorded for 10 min. n = 6–12 mice per group. (F) Effects of L-menthol on capsaicin-induced mechanical hyperalgesia in wild-type mice. Capsaicin (400 pmol) or capsaicin plus L-menthol (20 nmol) were injected into the plantar surface of the hind paw in 20-μL volume. Paw withdrawal thresholds were measured with von Frey hair filaments at time points indicated. n = 6 mice per group. **P < .01, *P < .05 vs Veh, "P < .01, *P < .05 vs Cap + Veh (G) Effects of L-menthol on capsaicin-induced mechanical hyperalgesia in Trpm8−/− mice. Experiment performed as in panel E. n = 6 mice per group. **P < .01, *P < .05. NS = not significant (P > .05).

**Fig. 2.** Effects of L-menthol on acute acrolein-induced pain and CFA-induced mechanical hyperalgesia in wild-type, Trpm8−/−, or TRPM8 antagonist–treated mice. (A) Nocifensive behavior in wild-type mice (black) or Trpm8−/− (blue) mice, quantified over 10 minutes after hindpaw injection of vehicle (Veh, PBS), acrolein alone (Acrol, 25 nmol in 50 μL PBS) or acrolein plus L-menthol (50 nmol, co-injected), n = 9–10 mice per group. **P < .01, NS: not significant (P > .05). (B) Effects of L-menthol on CFA-induced mechanical hyperalgesia in wild-type mice, measured by von Frey hair analysis. Baseline thresholds were measured before CFA injection (day 0). On days 1, 2, and 3, mice were injected into the plantar surface of the paw with L-menthol (Ment, 60 nmol in 20 μL green) or vehicle (PBS, red) 30 minutes before testing. Non-CFA-treated mice are shown for comparison (black). n = 7–8 mice per group. **P < .05 vs CFA+Veh, **P < .01 vs Control, NS = not significantly different from CFA+Veh. (C) Effects of L-menthol on CFA-induced mechanical hyperalgesia in Trpm8−/− mice. Experiment performed as in panel B. AMG2850 was administered (30 mg/kg, L.p.) 45 minutes before von Frey test. L-menthol was injected into hindpaw 15 minutes later. n = 7–8 mice per group. (D) Effects of L-menthol on CFA-induced mechanical hyperalgesia in Trpm8−/− mice. Experiment performed as in panel B except that Trpm8−/− mice were used. n = 7–8 mice per group.
including the stomach, intestine, liver, and bladder [23]. Many folk medicine traditions recommend menthol- and eucalyptol (1,8-cineol)-containing essential oils for the treatment of visceral discomfort and pain. We examined the role of TRPM8 in L-menthol and eucalyptol-induced analgesia in the acetic acid model of visceral pain [17,48]. In wild-type mice, writhing movements in response to acetic acid (0.6%, 10 mL/kg, i.p.) were strongly reduced by pretreatment with either L-menthol (10 or 20 mg/kg, i.p.) or eucalyptol (200 mg/kg, i.p.) (Fig. 3A). Eucalyptol had no effects on locomotion in the rotarod assay at this dose (Supplementary Fig. 1). Trpm8−/− mice responded normally to acetic acid. However, the analgesic effects of L-menthol and eucalyptol were completely abolished in these mice (Fig. 3A). In control experiments, wild-type and Trpm8−/− mice were equally sensitive to analgesia by acetaminophen (50 mg/kg, i.p.). Acetic acid–induced writhing behavior was normal in Trpa1+/− mice (Fig. 3B). In contrast, this behavior was completely absent in Trpv1−/− mice, suggesting that TRPV1 is the major mediator of the pro-algesic effects of weak acids such as acetic acid (Fig. 3B).

3.5. WS-12 is a specific agonist of TRPM8 channels in sensory neurons

TRPM8 agonists such as L-menthol, eucalyptol, or icilin were recently found to interact with other TRP ion channels, including TRPA1 and TRPV3, and other neuronal targets, including sodium channels, calcium channels, and GABA receptors [28,34,40,50,53,60,63]. These interactions may contribute to analgesia, but may also underlie the irritating and pro-inflammatory side effects observed in many patients treated with these agents. The development of specific TRPM8 agonists would allow more focused studies of the analgesic effects of this ion channel, and may provide improved analgesics with diminished side effects. In tests on heterologously expressed TRP ion channels, the menthol derivative WS-12 was identified as a potentially selective agonist of TRPM8 [5,33,62]. In control Ca2+-imaging experiments in HEK293t cells, WS-12 (10 μmol/L) activated mouse TRPM8 (mTRPM8), but neither activated nor inhibited mTRPA1 (pre-activated by mustard oil), and minimally inhibited human TRPA1 (Supplementary Fig. 3). In contrast, menthol and eucalyptol both strongly inhibited mTRPA1 and hTRPA1 channels pre-activated by mustard oil (Supplementary Fig. 3). The specificity of WS-12 for TRPM8 has not been systematically corroborated in cultured primary neurons, or in vivo. In Ca2+ imaging experiments, we challenged cultured mouse DRG neurons with a TRPM8-saturating dose of WS-12 (1 μmol/L or 10 μmol/L), and subsequently with mustard oil (70 μmol/L), capsaicin (1 μmol/L), and KCl (40 mmol/L). Approximately 10% to 14% of cultured wild-type neurons were responsive to WS-12 (Fig. 4A and C). These responses were almost completely abolished in DRG neurons cultured from Trpm8−/− mice (Fig. 4B and C). The absence of TRPM8 did not affect responsiveness to capsaicin or mustard oil (Fig. 4B and C). L-menthol (250 μmol/L) activated a larger population of DRG neurons (approximately 34%) than WS-12. In neurons dissociated from Trpm8−/− mice, a significant number of neurons retained sensitive to L-menthol (~13%). Recent studies reported that TRPA1 is activated by menthol, a result that we confirmed in the present study (Supplementary Fig. 3). To examine the contribution of TRPA1 to the residual TRPM8-independent neuronal responses to L-menthol, we analyzed the effect of L-menthol in neurons cultured from Trpa1+/−/Trpa1−/− double-deficient mice. L-menthol responsiveness was almost completely abolished in these neurons, suggesting that TRPA1 mediates the residual L-menthol–induced Ca2+-influx (Fig. 4C). At a very high concentrations (1 mmol/L), the effects of L-menthol were diminished, likely due to inhibition of murine TRPA1 channels, with L-menthol activating some neurons in the absence of TRPA1 and TRPM8 (Fig. 4C). Population analysis revealed that a subset of WS-12 responsive neurons (13 of 23) also responded to capsaicin, suggesting that TRPM8 and TRPV1 are co-expressed in a subpopulation of DRG neurons. Taken together, the above data suggest that WS-12 is a highly potent and specific TRPM8 channel agonist in DRG neurons.

3.6. Analgesia of capsaicin-, acrolein-, and heat-induced pain in mice by selective TRPM8 activation with WS-12

Although WS-12 has been characterized as a cooling agent in human beings, its effects on nociception have not been examined in vivo [62]. We inquired whether WS-12, through specific activation of TRPM8, produces analgesic effects similar to those of L-menthol. We first examined the effects of WS-12 on capsaicin-induced nocifensive behavior. Similar to L-menthol, the co-injection of WS-12 (6 nmol/20 μL) with capsaicin (2 nmol/...
Discussion

In the present study we have dissected the role of TRPM8 in the analgesic effects of L-menthol, the predominant menthol isomer in widely used analgesic treatments. L-menthol effectively attenuated pain behavior in all models of acute pain (capsaicin, heat, acrolein, acetic acid) and inflammatory pain (CFA) examined in this study. These analgesic effects were completely abolished in TRPM8-deficient mice, supporting a crucial role of TRPM8 in menthol-induced analgesia vs other proposed targets.

TRPM8 was essential for L-menthol to inhibit nocifensive responses triggered by both TRPV1- and TRPA1-activated pain pathways. L-menthol attenuated nocifensive behavior elicited by injection of capsaicin or by noxious heat in the hot plate and tail flick tests, responses associated with activation of TRPV1. TRPM8 was also crucial for L-menthol-induced analgesia in the acetic acid model of visceral pain. Acetic acid-induced pain was absent in TRPV1-deficient mice, suggesting that activation of acid-sensitive TRPV1 channels is the key noceptive mechanism causing writhing behavior. This observation is in line with prior studies demonstrating analgesic effects of selective TRPV1 antagonists in this model, and suggests that the roles of other proposed targets, such as acid-sensitive ion channels or TRPA1 in nociception caused by weak acids, need to be re-examined [44,56].

TRPM8 was also necessary for L-menthol to inhibit nocifensive behavior elicited by direct chemical stimulation of TRPA1 with acrolein. Acrolein, a reactive electrophile, is the major noxious irritant in tobacco smoke, and we recently reported that L-menthol effectively blocked acrolein-induced respiratory irritation responses mediated by airway-innervating chemosensory neurons in mice [1,64]. The physiological effects of menthol in cigarettes are currently the focus of controversial discussions and regulatory efforts, as menthol may facilitate smoke inhalation and smoking-induced diseases [64]. In the previous study, we demonstrated that a TRPM8 inhibitor, AMTB, partially abolished L-menthol’s counter-irritant effects toward acrolein and other respiratory irritants in tobacco smoke [64]. The specificity of AMTB remains poorly characterized, and its potency and pharmacokinetics in vivo

20 μL) into the mouse hindpaw significantly reduced capsaicin-induced nocifensive behavior (Fig. 5A). The analgesic effect of WS-12 was completely abolished in Trpm8+/− mice (Fig. 5A). WS-12 (6 nmol/20 μL) also diminished acrolein (30 nmol/20 μL)-induced nocifensive behavior when co-injected into the hindpaw (Fig. 5B). In the hot plate test (52°C), systemic treatment with WS-12 (10 mg/kg, i.p.) produced an obvious analgesic effect similar to that of L-menthol, and this effect was again abolished in Trpm8+/− mice (Fig. 5C). WS-12 did not change falling latencies in mice in the rotarod test at this dose (10 mg/kg, i.p.) (Supplementary Fig. 1).

Pharmacological studies using opioid receptor antagonists indicate that the analgesic effects of menthol depend on endogenous opioid pathways [17,57]. We used naloxone, a non-selective opioid receptor antagonist, to examine whether WS-12-induced analgesia in the hot plate test would also depend on opioid receptor function. Inhibiting opioid receptors (naloxone, 5 mg/kg, i.p.) significantly diminished the analgesic effect of WS-12 (52°C plate temperature), suggesting that both menthol and WS-12 activate similar opioid mechanisms downstream of TRPM8 (Fig. 5D).

**Fig. 4.** Effects of cooling agent WS-12 on mouse DRG neurons, measured by ratio metric Ca2+-imaging. (A) Representative Ca2+-signals in wild-type DRG neurons elicited by WS-12 (1 μmol/l), followed by mustard oil (MO, 70 μmol/l), capsaicin (Cap, 1 μmol/l), and KCI (40 mmol/l). (B) Representative Ca2+-signals in TrpmS+/− DRG neurons. Experiments performed as for panel A. (C) Percentage of wild-type, TrpmS+, and TrpmS−/− DRG neurons responding to Cap, MO, WS-12 (1 or 10 μmol/l) and L-menthol (Ment, 250 μmol/l, or 1 mmol/l). Each column shows average percentages from 5 to 8 separate tests, and each test contains 40 to 60 neurons. Neurons were defined as responsive when the increase in Fura-2 emission ratio (340 nm/380 nm) in a given neuron exceeded 10% of the KCl response. *P < 0.01, **P < 0.05, NS = not significant (P > 0.05). (D) Population analysis of wild-type DRG neurons responding to WS-12, MO and Cap. Agonists were applied as for panel A. n = 241 neurons, 6 fields.
Fig. 5. Effects of WS-12 on acute thermal-, capsaicin-, and acrolein-induced nocifensive behavior. (A) Nocifensive behavior of wild-type (black) or Trpm8−/− (blue) mice after local injection of vehicle (corn oil); WS-12 (6 nmol), capsaicin (2 nmol), or capsaicin (2 nmol) in combination with WS-12 (6 nmol), all administered in 20 μL injection volume. n = 6–12 mice per group. (B) Nocifensive behavior in response to paw injections of vehicle (corn oil), acrolein (30 nmol/20 μL), and acrolein and WS-12 (6 nmol/20 μL) together, 20 μL injection volume each recorded as for panel A. n = 6–7 mice per group. (C) Paw withdrawal latencies in the hot plate test (52°C) in wild-type (black) or Trpm8−/− mice, injected i.p. with vehicle (corn oil) or WS-12 (10 mg/kg) in a volume of 5 mL/kg, 30 minutes before testing. n = 8–12 mice per group. (D) Effects of naloxone on WS-12–induced analgesia in the hot plate test (52°C). WS-12 (10 mg/kg) was administered as for panel C 30 minutes before testing. Fifteen minutes later, naloxone (Nalox, 2 or 5 mg/kg, i.p.) or vehicle (Veh, PBS) in a volume of 5 mL/kg was injected. n = 8–12 mice per group. *P < .01, **P < .05. NS = not significant (P > .05).

require further study. Our present data derived from Trpm8−/− mice show complete absence of L-menthol's analgesic effects towards acrolein-induced pain. This result supports a role of TRPM8 in L-menthol–induced counterirritation in the respiratory system that is innervated by sensory neurons showing TRP channel expression patterns and connectivity very similar to nociceptors [3]. Acrolein is also a lipid peroxidation product and resembles other endogenous reactive products generated during inflammation and activating TRPA1 [35,39,59]. We demonstrated that L-menthol diminished inflammatory mechanical hyperalgesia in ORG neurons of wild-type mice, demonstrating that these mice have no general defects preventing analgesia. Although not as comprehensive as for menthol, our examination of eucalyptol's analgesic effects points to a crucial role of TRPM8 as an analgesic target of this widely used natural product. In the acetic acid model, eucalyptol (1,8-cineol) strongly reduced writhing behavior, with an efficacy very similar to that of L-menthol. This effect was abolished in TRPM8-deficient mice, supporting the idea that activation of TRPM8 by eucalyptol activates analgesic pathways. This finding differs from the outcome of a recent study observing that eucalyptol is acting as an analgesic through the inhibition of TRPA1 [54]. As explained above, our data suggest that acetic acid–induced pain behavior is triggered by activation of TRPV1, demonstrating that eucalyptol can inhibit pain responses triggered by pathways that do not involve TRPA1. Eucalyptol's analgesic effects may depend on its pharmacological actions on 1, or both, ion channels, depending on the pain-inducing stimulus. With WS-12, we identified a specific pharmacological tool enabling identification and characterization of TRPM8-expressing cultured sensory neurons. WS-12, previously characterized as a selective agonist of TRPM8 in heterologous expression systems, exhibited high specificity for TRPM8 in cultured DRG neurons [33]. WS-12 induced Ca2+-influx in DRG neurons of wild-type mice, but not in neurons cultured from TRPM8-deficient mice. In DRG cultures, L-menthol activated a larger population of sensory neurons than WS-12 and a significant number of TRPM8−/− neurons retained sensitivity to L-menthol, indicating that L-menthol activated additional TRPM8-independent Ca2+-influx pathways. This residual response was absent in DRG neurons from Trpm8−/−/Trpa1−/− double-deficient mice, suggesting that TRPM8
and TRPA1 are the major excitatory L-menthol receptors in sensory neurons [28]. WS-12–responsive cells fell into 2 categories. One cell population was responsive to WS-12 and also to mustard oil and capsaicin, the agonists of TRPA1 and TRPV1, respectively. This population may represent a nociceptor population [55,58]. The other neuronal population was responsive to WS-12, but not to capsaicin or mustard oil. It is likely that this population initiates the sensation of cool temperatures when TRPM8 is activated [55,58].

WS-12 also showed high specificity for TRPM8 in vivo. WS-12 induced analgesia of TRPV1 (heat, capsaicin)–activated and TRPA1 (acrolein)–activated nociception. Similar to L-menthol–induced analgesia, WS-12–induced analgesia was completely absent in TRPM8-deficient mice. Both analgesia by L-menthol and WS-12 was diminished when opioid pathways were blocked by naloxone treatment, suggesting that both agents activate similar TRPM8–dependent analgesic pathways, and that TRPM8–induced analgesia relies on activation of endogenous opioid receptors. L-menthol analgesia may involve signaling by central κ-opioid receptors, as central administration of a κ-opioid was shown to diminish L-menthol–induced analgesia [17,57].

L-menthol produced TRPM8–dependent analgesia of acute and inflammatory pain over a wide range of concentrations and administration routes (oral, intraperitoneal, intraplantar, or topical), from estimated systemic levels of 60 μmol/L (at 10 mg/kg, assuming equal distribution in a 25-g mouse without metabolism) or 120 μmol/L (20 mg/kg), to a topical concentration in the molar range (at 30% in ethanol). In addition to activating TRPM8 channels, these concentrations are sufficient or by far exceed the concentrations needed to exert pharmacological effects on other targets implicated as mediators of menthol–induced analgesia, including voltage-dependent Ca²⁺ channels, GABA-A receptors, sodium channels, peripheral nicotinic acetylcholine receptors or serotonin–gated ion channels [11,19,22,44,45,52,63,67]. The role of these alternative targets in L-menthol–induced analgesia should have been unmasked in TRPM8⁻/⁻ mice. However, since we did not observe any residual analgesic effects of L-menthol in TRPM8⁻/⁻ mice, the interaction of these targets with menthol may not affect nociception, or these alternative targets may play a role in menthol analgesia only in specific situations. Some analgesic effects mediated by other targets may be specific for other menthol isomers such as D-menthol that was shown to induce analgesia by inhibiting neuronal sodium channels when these were activated by a specific membrane toxin [19]. However, D-menthol was shown to have no analgesic effects in the acetic acid–induced model and other pain models [17]. As L-menthol is by far the predominant menthol isomer in analgesic preparations, analgesia induced by other menthol isomers may not be relevant in the context of current treatment regimens.

The L-menthol concentrations used in our in vivo studies is sufficient to affect the activity of TRPA1 channels in sensory neurons. Both mouse and human TRPA1 are activated by menthol at lower concentrations, and mouse (but not human) TRPA1 is inhibited by menthol at high concentrations in vitro, findings we confirmed in our imaging analysis of heterologously expressed TRPA1 channels [28,34]. However, acrolein–induced nociceptive behavior, clearly L-menthol dependent, was not affected by co-injection of 1 mmol/L L-menthol, a concentration shown to inhibit mTRPA1 channels in vitro [28]. This apparent lack of inhibition may be due to TRPM8– and TRPA1–independent pro-algesic effects of menthol at high concentrations, as reported in earlier studies [6,28,45]. Indeed, we observed that L-menthol at 1 mmol/L activated Ca²⁺–influx into DRG-neurons from TRPM8⁻/⁻/TRPA1⁻/⁻ double-deficient mice. Metabolic mechanisms may also play a role in the in vivo effects of L-menthol, as shown by us in a previous study demonstrating that L-menthol–induced respiratory irritation depends on the activity of cytochrome P450 enzymes that may convert menthol into reactive intermediates activating TRPA1 in airway-innervating sensory fibers [64].

Although TRPM8 accounts for likely all of the analgesic activity of L-menthol, interaction of L-menthol with TRPA1 and other targets may have pro-algesic and inflammatory effects. Indeed, topical menthol treatment frequency is accompanied by skin irritation, and menthol inhalation can exacerbate asthma in some patients, both conditions in which TRPA1 has a documented role [1,7,66]. The replacement of L-menthol in analgesic and counter-irritant treatments by TRPM8–specific agents such as WS-12 may prevent these pro-inflammatory effects and allow a more focused analgesic therapy.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2013.06.043.

References