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Abstract

The TRPM8-agonist L-menthol has been used traditionally for its topical counter-irritant properties. While the use of topical L-menthol for pain is casuistically established, evidence regarding its efficacy is negligible. This study aimed to characterize the effect of L-menthol as a counter-irritant on cutaneous pain and hyperalgesia provoked by topical application of the TRPA1-agonist trans-cinnamaldehyde (CA). In a randomized, double-blinded study CA was applied to a 3x3cm area of the volar forearm evoking neurogenic inflammation, pain, mechanical and thermal hyperalgesia in 14 healthy volunteers. In one of two sessions, 1) 10% CA alone, 2) 40% L-menthol + 10% CA applied simultaneously were administered for 20min throughout which the subjects rated the pain intensity on a VAS_{0-10}. Extensive quantitative sensory testing was conducted and superficial blood flow (neurogenic inflammation) was recorded. Administration of CA evoked spontaneous pain, neurogenic inflammation, thermal hyperalgesia, and primary and secondary mechanical hyperalgesia. Co-administration of topical L-menthol reduced spontaneous pain intensity ($P<0.01$), neurogenic inflammation ($P<0.01$), primary mechanical hyperalgesia ($P<0.05$), secondary mechanical hyperalgesia ($P<0.05$), and heat hyperalgesia ($P<0.05$), but not cold hyperalgesia. L-menthol exhibited inhibitory effects on simultaneously established pain, hypersensitivity, and neurogenic inflammation in a human TRPA1-induced pain model. Potent TRPM8-agonists could be useful as topical anti-hyperalgesics.

The study and the trial protocol is registered and approved by the local research ethics committee under the jurisdiction of the Danish Medicines Agency no.: N-20130005. The protocol also is registered at Clinicaltrials.gov under the no.: NCT02653703.

Perspective: Drugs interacting with TRP-channels are of great therapeutic potential. In the present study we established cutaneous pain and hyperalgesia using the TRPA1-agonist trans-cinnamaldehyde. Subsequently, we showed that the frequently used topical counter-irritant and TRPM8-agonist; L-menthol, decreased evoked pain, hyperalgesia and inflammation, indicating both direct and indirect anti-nociceptive mechanisms.

1. Introduction

The naturally occurring substance L-menthol is well known to produce a noticeable cooling sensation and modest analgesia under certain conditions. The cooling sensation is well-established to be a consequence of L-menthol's sensitization of the cold receptor TRPM8, located primarily on cutaneous Aδ-fibers and to a lesser extent C-fibers, while the analgesic properties are far less clear, with multiple proposed mechanisms in play.[6,46,63] In western medicine, Dr. A. Wright was the first to suggest pain-relieving properties of menthol in 1870 in a paper published in *The Lancet*. He observed that topical ointment with peppermint oil (containing L-menthol) reduced pain in patients suffering from post-herpetic neuralgia and several studies have since substantiated the claim.[15,19,39,44,45,65] Today, L-menthol is widely used in ointments to counteract local inflammation, eczema and joint aches, in lozenges to relieve throat soreness and also in nasal sprays to decongest and de-inflame the upper airways in case of common colds and fl. Moreover, FDA has listed L-menthol as a local anesthetic, antipruritic and antitussive compound.[18] Most usage is subclinical and it is unclear whether the counter-irritant effects of L-menthol share a similar mechanism between various target tissues to which it is routinely applied.

Paradoxically, numerous recent studies (reviewed in [3]) describing the somatosensory alterations induced by high concentrations of topical L-menthol have found that the substance is capable of inducing prolonged cold hyperalgesia, spontaneous pain and primary and secondary hyperalgesia. As such, labeling L-menthol as a counter-irritant is seemingly more accurate than labeling it as a local anesthetic. The relative promiscuity among the natural transient receptor potential (TRP) channels agonists is also applicable for L-menthol, which interacts with several TRP-channels besides TRPM8 as well as other receptors often in a concentration-dependent manner.[20,33,44,45]. However, a recent pre-clinical study convincingly ascertained TRPM8 to be the principal mediator of L-menthol-induced analgesia.[45] Despite the widespread recognition of L-menthol's moderate pain-relieving properties to pre-existing painful or pruritic conditions, very little is known with regards to the characteristics of these anti-irritant effects and potential underlying mechanisms in humans.

This study aimed at assessing the counter-irritant properties of topical L-menthol on neurogenic inflammation, primary and secondary mechanical hyperalgesia and thermal hyperalgesia established by

the local irritant trans-cinnamaldehyde (CA). CA works by sensitizing the thermo-receptive and inflammation-facilitatory ion-channel TRPA1.[57] Topical application of 10% CA has previously been shown to cause mild to moderate pain, mechanical and thermal hyperalgesia and neurogenic inflammation in human skin and rodents.[23,40,43,66] At lower concentrations, topical CA has been found to evoke itch in rodents and also in humans.[25,66] The primary outcome of this study was CA-evoked peak pain intensity monitored for 20 min post application, with versus without L-menthol co-application, while secondary outcome measures included quantitative sensory testing (QST) parameters and neurogenic inflammation.

This study is the first to present a comprehensive quantitative somatosensory assessment of the counter-irritant and anti-inflammatory properties of topically applied high-concentration of TRPM8-agonist L-menthol on TRPA1-sensitized human skin, including measurement of secondary hyperalgesia and vasomotor reactivity.

2. Methods

2.1 Study design and subjects

A total of 14 healthy subjects including 5 males and 9 females, aged 20–33 years (mean: 23.2 ± 0.92) were recruited. All subjects were pain-free, without previous known neurological, dermatological or musculoskeletal disorders, and were instructed not to consume alcohol, nicotine, caffeine and medication of any kind 24 h prior to the experiment. All subjects signed a statement of informed consent in accordance with the Helsinki Declaration, before participation. The regional ethics committee approved the experiment (study no.: N-2013-0005). The same investigator (HHA) conducted all experimental sessions. The study was carried out in a double-blinded manner with balanced randomization of both the order of substances applied and dominant versus non-dominant arm (See fig. 1 for an overview of the study design and notice simultaneous application of L-menthol + CA). Hence, both the investigator and the subjects were blinded to the substance being applied. Two cotton pads soaked in a solution of 40%(w/v) L-menthol + 10%(v/v) CA were placed in the laboratory 20 min before the onset of testing. This was done to create an overwhelming ambient smell of both of the applied substances, in order to inhibit the investigator and participants ability to identify the order of solutions investigated by olfaction. Such a procedure has previously been utilized in previous studies using highly odorous compounds to avoid unmasking the investigator and subjects.[24,40,43] The ambient temperature was kept at ≈21 °C in all experimental sessions.

2.2. Application of CA and L-menthol

CA (≥99%) and L-menthol (≥99.9%) were obtained from Sigma Aldrich (Broendby, Denmark) and dissolved in 90% ethanol at concentrations of 10%(v/v) CA and 40%(w/v) L-menthol + 10%(v/v) CA. The chosen concentrations were determined by precedence in the literature showing that 10% CA evokes a considerable neurogenic flare and prolonged mechanical hyperalgesia [39,40,43] and that the maximal solubility of pure L-menthol in ethanol is approximately 40%. Ethanol was chosen as a vehicle because of 1) its ability to dissolve a high concentration of L-menthol, 2) its low toxicity for an organic solvent, 3) its marginal somatosensory sensory effects and 4) the precedence of using it in literature.[3,4,39,40,43]

One mL aliquot of each test substance was dispensed onto a 3 × 3 cm cotton pad and placed on a 5 × 5 cm sheet of medical tape. Between the cotton pad and the tape, a layer of plastic-film was added to inhibit the evaporation of the solutions. This configuration was applied for 20 min while the subjects were in a ≈30° incline position with their arms supinated. To assure that the applied substances did not interact or showed instability in the vehicle nuclear magnetic resonance spectroscopy (NMR) was performed on the vehicle, the individual solutions of 40% L-menthol and 10% CA and the combined solution containing both 40% L-menthol and 10% CA. The NMR spectra were recorded in CDCl3 on a Bruker AVIII-600 spectrometer with a 5 mm TCI (H–C/N–D) probe at 298 K. The NMR confirmed that the utilized substances are stable in the applied solutions and do not partake in any chemical reactions with the vehicle or each other, demonstrated by the lack of new peak formation in the mixture solution, see supplementary material 1, fig. 1-4.

2.3. Pain assessment during application

The primary outcome of the study was evoked peak pain intensity during the 20 min of application of the two substances. The participants were required to rate the overall pain intensity on a visual analog scale (VAS) every 1 min (ranging from “no pain” = 0, to “worst imaginable pain” = 10) and to apply qualitative pain descriptors based on the validated brief descriptive Danish version of the McGill Pain Questionnaire (MPQ) at time points of 5, 10, 15 and 20 min.[47] ‘Hot/burning’ and ‘cold/freezing’ were re-added to this modified edition of the questionnaire.

2.4. Perceived area of pain

At the first time point of a decrease in spontaneous pain intensity (i.e. corresponding to the individual peak pain level), the subjects marked the area of perceived pain on a digital generalized arm-chart using a 10.1" Samsung Note tablet (Samsung Electronics, South Korea) equipped with *Navigate Pain* software version 1.9.1 (Aalborg University, Aalborg, Denmark) for further analysis of the spatial pain profile.[9] The subjects were instructed to draw the area from which pain was sensed irrespective of area of redness, innocuous sensations such as warmth and the knowledge of the location of the application pad. The area of pain was later calculated with *Navigate Pain* as a percentage of the total chart area.

2.5. Quantitative sensory testing (QST)

The QST protocol was partly derived from the QST guidelines of the German Research Network on Neuropathic Pain (DFNS).[51] The verbal instructions (in Danish) for participants from the DFNS protocol were derived from the supplementary materials of Olsen *et al.* (2014). [43] The terminology used to describe the induced sensory derangement are defined in Sandkühler (2009).[53]

2.5.1. Thermal thresholds: Tests for cold detection threshold (CDT), warmth detection threshold (WDT), cold pain threshold (CPT), and heat pain threshold were performed with a Medoc Pathway (Medoc Ltd, Ramat Yishay, Israel) equipped with a 3 × 3 cm advanced thermal stimulator probe where the baseline temperature was set to 32 °C. Ramp stimuli of 1 °C/s were delivered and when the subjects identified the associated threshold he/she pressed a button that returned the temperature to the baseline at a rate of 5 °C/s. The test result was calculated as the arithmetic mean of the outcome from three repeated stimuli.

2.5.2. Mechanical sensitivity: The mechanical detection threshold (MDT) was determined using a set of 20 calibrated Von Frey filaments (North Coast Medical, Gilroy, CA, USA) with exerted forces ranging from 0.008 g to 300 g (0.078 mN to 2.9 N) applied over three series of stimuli with ascending/descending weight. Each stimulus lasted 1-2 s and intervals between stimuli were approximately 2 s. The subjects were asked to report upon any sensation from the area. The resulting MDT was calculated as the geometric mean of the values obtained in each of the five series of stimuli. To evaluate the mechanical pain threshold (MPT) and mechanical pain sensitivity, an electronic von Frey transducer and an electronic coVAS connected to a SENSE-Box setup were used (both items: Somedic, Hörby, Sweden). Five ramp stimuli from 0-110 g at a rate of 5g/1 sec with the default stimulus probe were conducted at different locations within the application area. The subjects were instructed to continuously rate the perceived pain on the coVAS ranging from “No pain” (0) to “worst imaginable pain” (10). The arithmetic mean of the VAS scores yielded three MPTs (fixed to VAS = 0.5, 1 and 2) and an area-under-the-curve (AUC). To map the area of secondary mechanical hyperalgesia (SH) in response to pin prick stimulation, a set of 9 pinprick stimulators (Aalborg University, Aalborg, Denmark) were used. A pin was selected based on the MPT of each individual subject. Stimuli were applied from eight different paths arranged octagonally around the application area in steps of 1 cm at intervals of 2 s. Stimulation started approximately 6 cm away from the

application area and continued towards the application site until the volunteer reported an increase in sharp/pricking pain.

2.6. Neurogenic inflammation (vasomotor responses)

Before (baseline) and immediately following substance application, infrared thermography (A40 Thermocamera, FLIR Systems, Wilsonville, OR, USA) and speckle contrast flowmetry (MoorFLPI, Moor Instruments, Devon, UK) were performed. Infrared thermography was conducted at a 40-cm distance between the camera lenses and the application area with an exposure time of 18 ms. Pictures were analyzed on the proprietary software using the arithmetic mean of the temperature in the 3 x 3 cm application area as region of interest (ThermaCAM Researcher Pro 2.10, FLIR Systems, Wilsonville, OR, USA). The perfusion assessment was conducted with a 30-cm distance between the head of the laser and the application area with an exposure time of 8.3ms. Single frame images were analyzed on appertaining software (MoorFLPI Review V 4.0, Moor Instruments), upon which the arithmetic mean flux (arbitrary units) was calculated. The longitudinal analysis of the spatial dispersion of neurogenic inflammation was performed using the line histogram tool. A 7-cm line centered in the area of application was placed longitudinally along the volar forearm and the perfusion intensity was recorded every 2.5 mm, an approach described in details previously.[43] This allows for quantification of increased superficial blood perfusion both within the area of application (primary flare) and beyond the application area (secondary flare).

2.7. Statistics

Sample size calculations were conducted based on previously obtained data applying similar pain models [43] and using the approached outlined in [37] for crossover designs. The obtained data are presented as arithmetic or geometric means (non-linear stimulus modes) ± the standard error of the mean (SEM), unless otherwise stated. Data from all assessments were tested for normality using the Shapiro-Wilk normality test with and without log-transformation (MDT and pain area data remained non-normal). Average and peak pain intensity (primary outcome) were calculated and compared from VAS-recordings while categorical variables are shown as % -distribution without further hypothesis testing. Secondary outcome variables with 2 measurements were compared using the Student *t*-test or the Wilcoxon rank-sum test (non-parametric data), i.e. data from VAS-recording, area assessment

(non-parametric) and quantification of SH. Variables with 3 measurements or more were analyzed with RM-ANOVA using the Bonferroni post-hoc test, i.e. measures of mechanical sensitivity (with the exception of SH), thermal sensitivity, superficial blood perfusion and skin temperature. In addition to the pairwise post-hoc tests of the FLPI data the AUC was calculated and statistically evaluated for each individual data point. Correlational analyses where performed between parameters of spontaneous pain intensity and thermal, mechanical and blood flow aberrations using Pearson product-moment correlation coefficient (parametric data) or Spearman's rank correlation coefficient (non-parametric data). Both absolute and relative QST values were assessed for associations. Data handling and calculation of descriptive statistics were carried out in Excel, while statistical comparisons were performed in SPSS (Both software packages: Windows, Redmond, WA, USA). A p-value (P) ≤ 0.05 was considered significant.

3. Results

All enrolled participants finished the study and topical application of CA and L-menthol was well tolerated and did not produce unexpected or unwanted side effects (such as local edema or systemic reactions). The mechanical threshold data obtained from one outlier (participant no. 9) was excluded because MPT levels (see section 2.5.2) at baseline were not reached during the devised stimulus paradigm. No significant differences were observed related to randomization or arm dominancy. The study was not designed to detect potential gender differences. While the average peak pain score (primary outcome) for 10% CA was < 3, a total 9/14 subjects had peak pain scores ≥ 3. However, 3 individuals reported pain peak values ≤ VAS 1. Unless specifically stated, these low responding individuals are included in the results below.

3.1. Spontaneous pain intensity and quality

CA produced mild pain (see fig. 2A and C), peaking after approximately 16 min of application, as 2.84 ± 0.42 for CA and 2.10 ± 0.32 (on VAS 0-10) for CA + L-menthol. CA-evoked cutaneous pain was partially relieved by co-application of L-menthol ($P \leq 0.01$, paired student t-test). If excluding the three subjects with peak pain intensity scores of ≤ 1, the peak pain intensity for the remaining group was 3.87 ± 0.46 for the CA and 3.05 ± 0.57 for the CA + L-menthol condition ($P \leq 0.05$). The spatial and temporal pattern of the pain was not significantly altered by concomitant administration of L-menthol (fig. 2D and F). However, observationally it appears that CA alone gave rise to a more homogeneous spatial profile (see fig. 2F), but this tendency was not quantified. A noteworthy positive correlation was observed between pain intensity after CA application and pain area size ($\rho = 0.592$, $P = 0.026$), however, this association was not significant in the CA + L-menthol group.

During application of CA the most common qualitative descriptors from the MPQ were “pricking/stinging” (54%), “warm/burning” (43%), “cold/freezing” (40%), “searing” (31%) and “itching” (26%). During co-application of CA and L-menthol the most frequent descriptors applied were “cold/freezing” (69%), and “searing” (38%), see fig. 2C. Paradoxically, “warm/burning” and “cold/freezing” were occasionally reported simultaneously during both CA and CA + L-menthol application.

3.2. Thermal thresholds

CDT was significantly increased by the solution containing both CA and L-menthol, but not CA alone, in accordance with previous findings, ($P \leq 0.01$, fig. 3A). WDT was increased after application of CA (fig. 3B). Concomitant application of L-menthol re-established WDT to the baseline levels ($P \leq 0.05$). As expected, CA + L-menthol drastically reduced CPT from 12.87 ± 2.49 °C at baseline to 20.43 ± 2.01 °C. ($P \leq 0.01$). Administration of CA alone also induced cold hyperalgesia ($P \leq 0.05$), although not to the same extent as the solution containing L-menthol (fig 3C). HPT was significantly decreased following CA administration, but was unchanged under L-menthol co-administration; however, an insignificant trend towards an HPT decrease was observed ($P = 0.077$). In summary, L-menthol had a counteractive effect on increased warmth and heat pain sensitivity while establishing or aggravating aberrations pertaining to innocuous and non-noxious cold sensation (see fig. 3 for an overview).

3.3. Mechanical sensitivity

MPT was decreased by CA at VAS=1 and 2 ($P \leq 0.05$), but not significantly at VAS=0.5. Moreover, at VAS=1 co-administration of CA + L-menthol led to a reduction in MPT (fig. 4A). When comparing MPS AUC for the entire stimulus response function, no significant differences were found (fig. 4B), i.e. supra-threshold mechanical sensitivity was unaltered by co-application of L-menthol. An average area of 29.43 ± 2.03 cm^2 of SH was induced by topical CA administration. Co-application of L-menthol resulted in a significantly smaller area of SH ($P \leq 0.01$, paired Student t-test) measuring 15.43 ± 2.06 cm^2, indicative of typical counter-irritancy since high-concentration L-menthol alone has previously been shown to induce large areas of SH [8] (fig. 4C). No significant changes from baseline were observed in MDT following application of CA or CA + L-menthol (an insignificant trend towards a decrease in mechanical sensitivity was observed with CA alone ($P = 0.089$, fig 4D).

3.4. Skin temperature and cutaneous perfusion (neurogenic inflammation)

Strong neurogenic inflammation, as evidenced by increased cutaneous blood perfusion, was observed following the application of CA alone and CA + L-menthol. However, when comparing the results from the longitudinal dispersion of the perfusion, significant differences were observed particularly in the secondary area (see fig. 5A). While only minor differences in superficial perfusion produced by application of CA and CA + L-menthol were established within the primary area of application (grey area, fig. 5A), CA alone produced a very powerful secondary neurogenic flare, which

extended beyond 2 cm from the primary area of application ($P \leq 0.01$). On the contrary, CA and L-menthol applied together only produced a very modest secondary neurogenic flare.

Skin temperature was increased by topical application of CA and CA + L-menthol ($p < 0.01$, fig. 5B). However, there was no significant difference between the temperature increase induced by the two substances.

4. Discussion

High concentration L-menthol has been widely used as a human pain model particularly for cold hyperalgesia. In the present study we sought to evaluate the analgesic, anti-hyperalgesic and anti-inflammatory effect of L-menthol to CA-induced sensory and vasomotor symptomatology, and as such did not include a condition with L-menthol alone, which is well known to induce prolonged cold hyperalgesia, spontaneous pain and primary and secondary hyperalgesia.[3,8,34,43,50,62,63]

Topical application of 10% CA provoked mild pain, primary and secondary mechanical hyperalgesia, heat and cold hyperalgesia and substantial primary and secondary neurogenic inflammation. Notably, 3 subjects were considerably below the averagely reported pain peak values and scored ≤ 1 (0.80 ± 0.05) on the VAS versus 3.87 ± 0.46, for the remaining 11 subjects. This could be indicative of a CA non-/low-responding phenotype, but a larger study is needed to assess whether or not this is in fact a distinct subgroup or merely a consequence of natural inter-individual variability. Simultaneous administration of 40% topical L-menthol together with CA completely or partially reversed spontaneous pain, primary and secondary mechanical hyperalgesia, heat hyperalgesia and neurogenic inflammation (temperature and blood perfusion), but aggravated cold hyperalgesia. In other words, L-menthol had a significant inhibitory effect on the primary outcome (peak pain intensity induced by CA) and a number of secondary outcome related to thermal and mechanical sensitivity and neurogenic inflammation. Below, fig. 6 summarizes the two most likely potential mechanisms behind the observed antagonistic effect, i.e. either through spinal segmental inhibition or through peripheral receptor-mediated antagonism. It should be noticed that these could likely working in a complementary fashion.

4.1. Spontaneous pain intensity and quality

Application of 10% topical CA induced a mild spontaneous pain in this study, see fig. 2a and b. These findings are well in line with previous reports on topical CA in a 10% concentration as human surrogate model of pain, although the level of induced pain differ somewhat between studies, i.e. peak $VAS_{0\text{-}10}$ = 3±0.7 [40] (non-responders excluded), peak $VAS_{0\text{-}10}$ ≈3.7±0.6 [39], $VAS_{0\text{-}10}$ = 2.3±0.63 [50] and $NRS_{1\text{-}100}$ = 10.3±2.8 [43], which is likely related to small methodological differences, such as applied pain quantification, sampling frequency and participants. While 9/14 subjects did report peak pain > 3, 10% CA appear to induce mild pain on average. This mild inflammatory cutaneous pain bears resemblance

to inflammatory dermatological conditions such as atopic eczema or contact dermatitis, which beyond chronic itch is often characterized by mild pain.[10,41] Notably, the pathoetiology of these conditions have also been tightly associated with TRPA1.[14,42,52] In lieu of the significant differences in pain processing, pain sensitivity and TRP-expression between different tissues and anatomical locations [13,27,35,36], the CA pain model probably needs to be re-implemented in various tissues if to, for instance, mimic mucosal inflammation and pain associated with e.g., throat soreness.

In a subgroup of participants CA induced significant itch, a sensation likely partially masked by the presence of pain, since itching is shown to increase with lower topical CA concentration. [25] With co-administration of L-menthol, the pain quality shifted towards a "cold/freezing" character due to the sensitizing effect of L-menthol on $TRPM8^+$ cold-mediating fibers (Peier et al., 2002; Wasner et al., 2004). Mechanistically, CA-induced pain is likely mediated by activation of $TRPA1^+$ C-fibers, which partially co-localize with TRPV1 on nociceptive mechano-insensitive and polymodal C-fibers.[40,54,58] It is conceivable that the reduction in pain intensity by co-application of L-menthol observed in the present study, is a consequence of $TRPM8^+$ Aδ-fiber-mediated segmental inhibition of nociceptive C-fiber transduction, since L-menthol, being a chemical proxy of cooling, does not possess the anti-edematous or nerve conduction inhibitory effects of actual cold-induced analgesia.[17] Supporting this notion, a recent *in vivo* study using 10% and 40% L-menthol suggested that TRPM8 is the principal mediator of L-menthol-induced analgesia in neuropathic pain associated with spinal nerve ligation. [45] However, it has been proposed that analgesic effect of L-menthol may include several mechanisms, such as desensitization of TRPV1-fibers, stimulation of κ-opioid receptors and enhancement of central glutamate-facilitated gate-control mechanisms.[21,29,33,49,67]

4.2. Thermal thresholds

Cold hyperalgesia was established by both CA and more significantly with simultaneous CA + L-menthol administration due to its established interactions with cold receptor $TRPM8^+$, indicating that L-menthol acts to increase pain sensitivity on this particular parameter. As for the L-menthol containing solution this is well in line with previous results.[4,8,63] However, CA, herein found to induce cold hyperalgesia has previously been found to produce contradictory CPT results including cold hypoalgesia, cold hyperalgesia or simply no significant alterations.[3,39,40,43,50] Cold pain sensation is hypothesized to rely on a mutual nociceptive pathway for both nociceptive heat and cold producing a

sensation of burning pain, with simultaneous activation of innocuous cold Aδ-fibers acting as a modality switch.[5,12] Upon application of CA alone, but not combinatorial application of CA + L-menthol, the HPT was significantly decreased, suggesting that L-menthol partly reverses CA-induced heat hyperalgesia. This is well in line with previous results from studies on the somatosensory effects of high-concentration L-menthol suggesting that it decreases HPT without pre-established heat hyperalgesia, potentially through sensitization of TRPV3 [8,39,40], as is the case in this study. CDT was found to increase the response to the CA + L-menthol, but not to CA alone, while WDT was decreased by CA alone, indicative of sensitization of non-noxious primary afferent conveying warmth, but not by L-menthol + CA, in lines with L-menthol having cooling properties and CA inducing warm/burning sensations. Although a convenient organic solvent, ethanol has been shown to induce subtle somatosensory aberrations in itself particularly with regards to thermal detection thresholds [39,40,43,63], which may also be the case in this study. As evidenced by *in vitro* data, the modulatory properties of ethanol on somatosensation is proposed to be either directly through TRPV1 sensitization [59] or indirectly through dissolution of the outer epidermal layers.[32]

4.3. Mechanical thresholds

MPTs at VAS 1 was decreased by CA and CA + L-menthol, which is overall in accordance with the literature.[8,40,63] At VAS 2, only CA alone produced a significantly decreased MPT highlighting a more pronounced effect on mechanoception than combinatorial application of CA and L-menthol and implying an antagonistic interaction between the two substances. A similar, but insignificant, trend ($P = 0.089$) was observed in MPS, indicating a minimal anti-hyperalgesic effect of L-menthol at suprathreshold stimulation-intensities. Mechanistically, the reduced pinprick hyperalgesia associated with combinatorial administration of L-menthol and CA is suggested to rely on L-menthol-induced local counter-irritancy through TRPV1 nociceptor desensitization or segmental Aδ-fiber-facilitated inhibition of mechanical pain perception.[21,33,45,63] Not previously shown, co-administration of L-menthol decreased the SH established by CA. Since SH is generally recognized to be a centrally mediated mechanism[2,30,48], this is likely a consequence of an overall diminished nociceptive drive, i.e. through decreased spontaneous pain and neurogenic inflammation. MDT remained unaffected in this study plausibly because neither CA nor L-menthol sufficiently affected tactile C- or Aβ-fiber activity.[8]

4.4. Neurogenic inflammation

In line with previous studies [25,39,40,43], topical application of CA induced neurogenic inflammation both within the area of application (primary flare) and substantially beyond the application area (secondary flare). This increased was also reflected on increased skin temperatures. Neurogenic inflammation is produced upon stimulation of peptidergic nociceptive or pruriceptive fibers, which release locally acting vasodilatory peptides, most prominently calcitonin gene-related peptide and substance P.[31,54] Both polymodal fibers and mechano-insensitive-fibers are capable of producing neurogenic inflammation, but only mechano-insensitive-fibers have the ability to produce significant secondary flare since their terminal branching makes up a much larger receptive field than that of polymodal fibers. It has previously been shown that L-menthol alone produces a small but significant increase in superficial blood flow [43,63], likely because approximately 15% of $TRPM8^+$ cold-responsive mouse dorsal root ganglion neurons co-express TRPV1, or due to direct modulation of vascular tone.[8,61] The strong neurogenic flare and pain induced by CA are likely consequences of a 50% C-fiber co-expression between $TRPA1^+$ and peptidergic $TRPV1^+$ fibers, since the pruriceptive $TRPV1^-$ population of $TRPA^+$ fibers are normally non-peptidergic Mas-related G-protein coupled receptor-positive X1, incapable of producing neurogenic inflammation as shown by [7,56,64,68]. Interestingly, co-administration of CA and L-menthol caused a primary flare reaction very similar to that of CA alone, but significantly decreased the secondary neurogenic flare suggestive of a specific local inhibitory effect of L-menthol on axon-reflex-flare producing mechano-insensitive nociceptors. Since TRPA1 is also a key player in pulmonary detection and reaction to irritants and in the non-histaminergic itch, this could also explain the known antitussive, decongestant and anti-pruritic effect of L-menthol.[1,11,16] Despite the fact that L-menthol is being widely used as an OTC analgesic, antipruritic, decongestant and antitussive, little is known regarding its mechanism of action in these contexts on a cellular level. [3] The anti-hyperalgesic and anti-inflammatory effect observed in this study on is likely mediated by reduction of activity in the primary afferent neurons with potential explanations including interaction on the level of penetration, keratinocyte responses or receptor-desensitization. A human study using capsaicin found that L-menthol significantly cross-desensitized capsaicin-responsive C-fibers suggesting this response as a peripherally mediated counter-irritancy.[21] TRPA1-stimulation with topical CA in humans has been shown to cause 25- to 42-fold increases in local secretion of

eicosanoids and cytokines such as prostaglandin D2 and interleukin 8, respectively (VanderEnde and Morrow, 2001). Cellular interactions between $TRPM8^{+}$ and $TRPA1^{+}$ nerves and keratinocytes could alter secretion of pro-inflammatory substances, hence accounting for the herein observed effect of topical L-menthol.[33,67] It is important to infer that higher concentrations of L-menthol, as applied in the present study, have been suggested also to act on TRPA1-receptors. Evidence from patch clamp recordings indicates that L-menthol interacts with TRPA1 in a bimodal concentration-dependent manner in which high concentrations reversibly block TRPA1-channels.[28,33] This is a likely explanation for the decrease in neurogenic inflammation, which is known to be an entirely local neurogenic reflex [22], but could also explain decreases in spontaneous pain, thermal and mechanical sensitivity. This is well in line with the fact that several ongoing clinical studies are currently focusing on TRPA1-antagonists for conditions such as pain and pruritus. The fact that L-menthol might not be an entirely TRPM8-specific compound [28,33,55] means that it could interact independently of TRPM8 with pro-inflammatory mediators to, at least in part, create the observed effects. This might be aggravated by higher concentrations, which makes it desirable to test novel and more potent TRPM8-agonists, such as icilin, when these become feasible to apply in humans.

5. Conclusion

Here it is shown, for the first time, that high concentration L-menthol has significant alleviatory effects on inflammatory pain and associated sensory aberrations in a topical 10% CA-induced surrogate pain model assessed systematically by comprehensive quantitative sensory testing. Further studies are warranted to ascertain the mechanistic basis of the observed L-menthol-induced inhibition of CA-evoked neurogenic flare, pain and sensory hypersensitivity. These results suggest that novel and more potent TRPM8-agonists or dual action TRPM8-agonists/TRPA1-antagonists could be of benefit for anti-hyperalgesic and anti-inflammatory purposes.

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Author contributions

All authors were involved in the conception of the study. HHA performed the experimental sessions of the study and the primary data analysis. All listed authors were involved in drafting, discussing, revising, and approving the manuscript.

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Figure legends

Fig. 1. A flowchart of the experimental session showing test arms, application of solutions for 20 min and the assessments conducted at different time points. Test arm and solutions are listed only as an exemplification - the actual order of substance application was randomized.

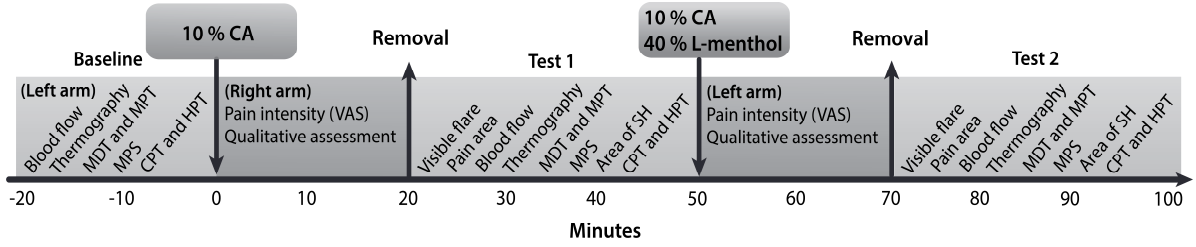
Fig. 2. Changes in pain intensity, temporality, quality and spatiality in response to application of CA and CA + L-menthol: A) Temporal pain intensity, B) Average and peak pain intensity, C) Descriptive pain quality, D) Spatial distribution of pain at individualized pain peaks, E) Duration until peak pain intensity, F) Overlays of the spatial distribution of pain. Asterisks: * = $P \leq 0.05$, ** = $P \leq 0.01$.

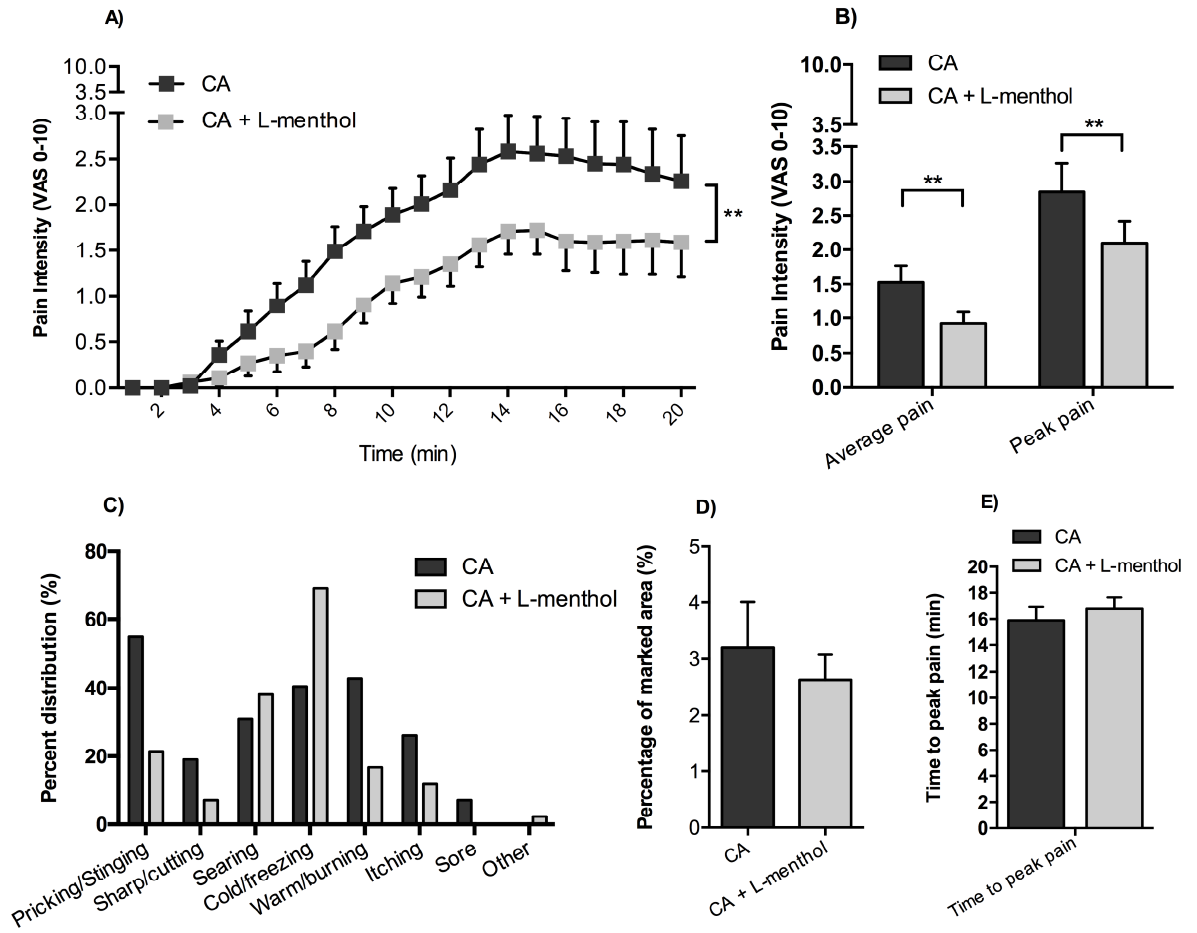
Fig. 3. Baseline averages and changes in thermal thresholds in response to application of CA and CA + L-menthol: A) Cold detection threshold, B) Warmth detection threshold, C) Cold pain threshold and D) Heat pain threshold. Asterisks: * = $P \leq 0.05$, ** = $P \leq 0.01$.

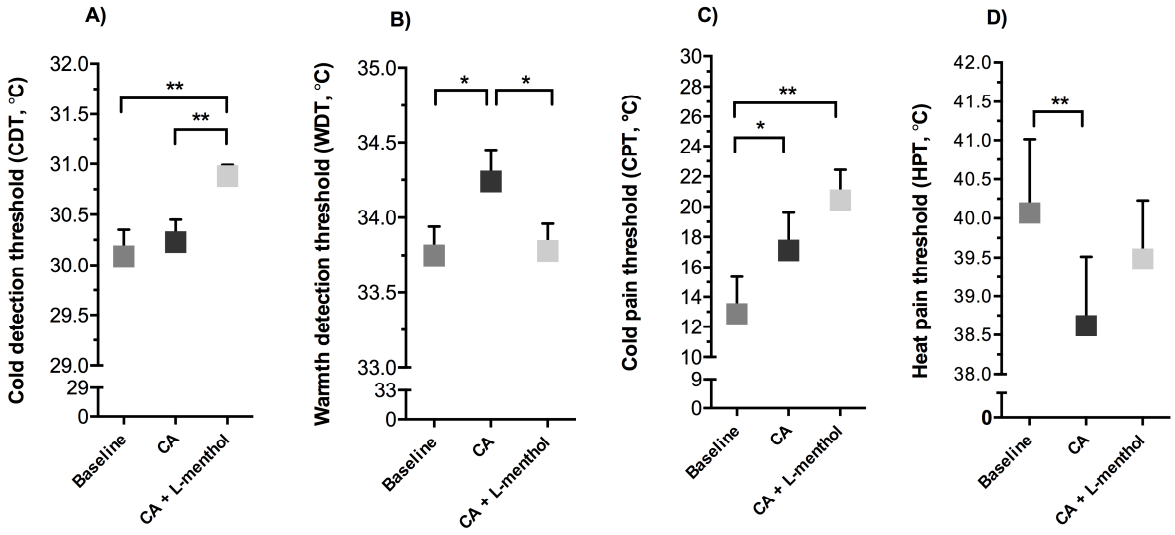
Fig. 4. Baseline averages and changes in mechanical thresholds, sensitivity and secondary hyperalgesia in response to application of CA and CA + L-menthol: A) Mechanical pain threshold at VAS = 0.5, VAS = 1 and VAS = 2. B) Mechanical pain sensitivity, C) Area of secondary hyperalgesia, D) Mechanical detection threshold. Asterisks: * = $P \leq 0.05$, ** = $P \leq 0.01$.

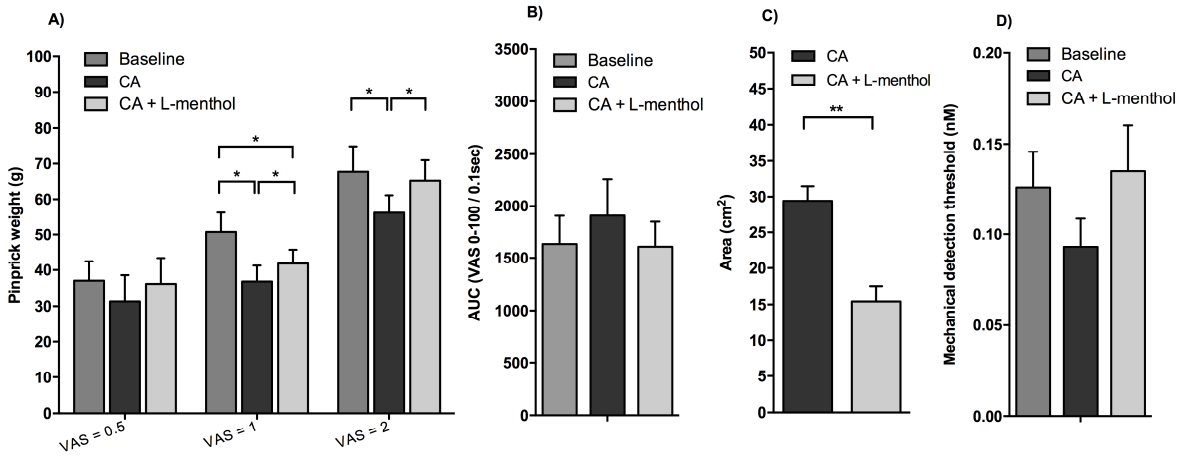
Fig. 5. Changes in the superficial blood flow and temperature following application of CA and L-menthol + CA. A) Longitudinal dispersion of neurogenic inflammation. The centered stippled line represents the center of the application area while the grey zone represents the primary (application) area. The significance indication bars in the lower right corner of fig. A show significance between entire data series whereas individual asterisks denote comparison between each location. Both with Bonferroni-correction. B) Superficial skin temperature measured by thermography in the application area. Asterisks: * = $P \leq 0.05$, ** = $P \leq 0.01$.

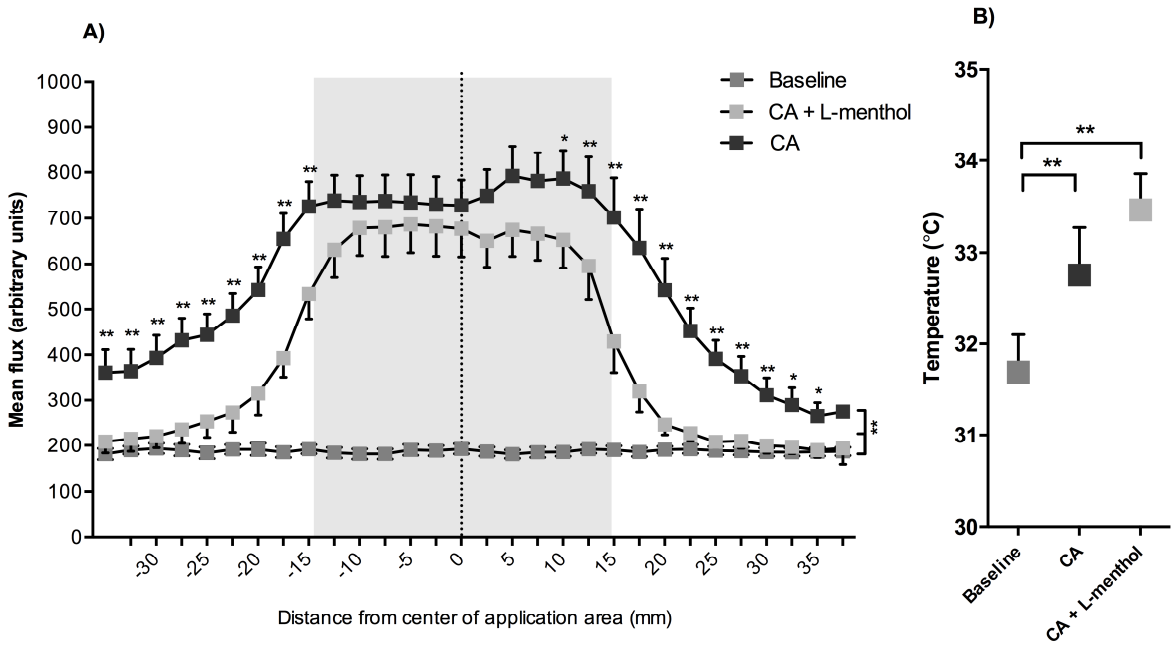
Fig. 6. Summary of suggested mechanisms (marked with "?") behind the observed antagonism between TRPA1 and TRPM8-activation on sensory and vasomotor parameters. Since the axon-reflex is a peripheral response not affected by proximal anesthetic blockade [22], it is likely that different mechanisms could be involved in reducing the neurogenic inflammation, which is likely related to high-concentration L-menthol-associated TRPA1-antagonism, and in reducing sensory symptoms perhaps via segmental inhibition.

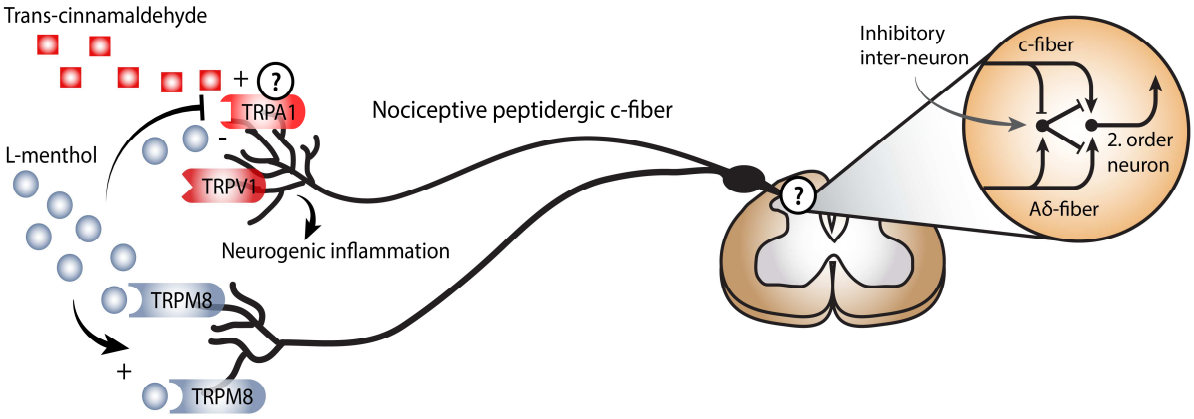



A)
Pain Intensity (VAS 0-10)
CA
CA + L-menthol
Time (min)
**
B)
Pain Intensity (VAS 0-10)
CA
CA + L-menthol
**
**
Average pain
Peak pain
C)
Percent distribution (%)
CA
CA + L-menthol
Pricking/Stinging
Sharp/cutting
Searing
Cold/freezing
Warm/burning
Itching
Sore
Other
D)
Percentage of marked area (%)
CA
CA + L-menthol
E)
Time to peak pain (min)
CA
CA + L-menthol
Time to peak pain
F)
CA
L-menthol + CA









Highlights

- Topical administration of 10% trans-cinnamaldehyde was applied to evoke pain, neurogenic inflammation, thermal hyperalgesia, and primary and secondary mechanical hyperalgesia
- Simultaneous administration of 40% topical L-menthol completely or partially reversed pain, mechanical hyperalgesia, heat hyperalgesia and neurogenic inflammation, but aggravated cold hyperalgesia
- We suggest that L-menthol's well-known counter-irritant effect is mediated in in part through spinal segmental inhibition and through peripheral receptor-mediated antagonism
- Potent TRPM8-agonists could be useful as topical anti-hyperalgesics and anti-inflammatories