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Mechanisms and time course of menthol-induced cutaneous vasodilation

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ABSTRACT

Menthol is a vasoactive compound that is widely used in topical analgesic agents. Menthol induces cutaneous vasodilation, however the underlying mechanisms are unknown. Determining the rates of appearance and clearance of menthol in the skin is important for optimizing topical treatment formulation and dosing. The purpose of this study was to determine the mechanisms contributing to menthol-mediated cutaneous vasodilation and to establish a time course for menthol appearance/clearance in the skin. Ten young (23 ± 1 years, 5 males 5 females) subjects participated in two protocols. In study 1, four intradermal microdialysis fibers were perfused with increasing doses of menthol (0.1–500 mM) and inhibitors for nitric oxide (NO), endothelium derived hyperpolarizing factors (EDHFs), and sensory nerves. Skin blood flow was measured with laser Doppler flowmetry and normalized to $\%CVC_{max}$. In study 2, two intradermal microdialysis fibers were perfused with lactated Ringer's solution. 0.017 $mL \cdot cm^{-2}$ of a 4% menthol gel was placed over each fiber. 5 μL samples of dialysate from the microdialysis fibers were collected every 30 min and analyzed for the presence of menthol with high performance gas chromatography/mass spectrometry. Skin blood flow (laser speckle contrast imaging) and subjective ratings of menthol sensation were simultaneously obtained with dialysate samples. In study 1, menthol induced cutaneous vasodilation at all doses ≥ 100 mM (all $p < 0.05$). However, inhibition of either NO, EDHFs, or sensory nerves fully inhibited menthol-mediated vasodilation (all $p > 0.05$). In study 2, significant menthol was detected in dialysate 30 min post menthol application (0.89 ng, $p = 0.0002$). Relative to baseline, cutaneous vasodilation was elevated from minutes 15–45 and ratings of menthol sensation were elevated from minute 5–60 post menthol application (all $p < 0.05$). Menthol induces cutaneous vasodilation in the skin through multiple vasodilator pathways, including NO, EDHF, and sensory nerves. Topical menthol is detectable in the skin within 30 min and is cleared by 60 min. Skin blood flow and perceptual measures follow a similar time course as menthol appearance/clearance.

1. Introduction

Menthol is the active ingredient in many topical analgesic agents that elicits a cold sensation by acting on transient receptor potential melastatin 8 (TRPM8) channels (Peier et al., 2002). Because of its wide ranging use in topical products, it is important to understand how menthol is penetrating the skin and affecting blood flow and sensation. Utilizing laser speckle contrast imaging, we recently demonstrated the topically applied menthol gel induces vasodilation of the cutaneous microvasculature (Craighead and Alexander, 2016).

TRPM8 channels are expressed in vascular endothelium (Johnson et al., 2009) and smooth muscle (Yang et al., 2006). The literature on the vasoactive effects of menthol is varied, with most (Cheang et al., 2013; Craighead and Alexander, 2016; Johnson et al., 2009; Sun et al., 2014) but not all (Olive et al., 2010; Topp et al., 2013; Topp et al., 2011) studies finding that menthol possess vasorelaxent properties. However, there is not a consensus on the mechanism(s) through which menthol mediates vasodilation. There is evidence that menthol acts through nitric oxide (NO)(Johnson et al., 2009), RhoA/Rho kinase (Sun et al., 2014), and by altering smooth muscle calcium concentration (Cheang et al., 2013; Ito et al., 2008). The disparate findings may be due to the use of different animal models and vascular beds. Because of menthol's presence in many topical agents, the human cutaneous circulation is a clinically relevant vascular bed in which to examine the vasoactive effects of menthol.

Utilizing reactive hyperemia and local heating to assess endothelium derived hyperpolarizing factor (EDHF)/sensory nerve and NO-dependent vasodilation respectively, we recently found that menthol likely caused vasodilation through EDHFs and sensory nerves (Craighead and Alexander, 2016). However, a ceiling effect of cutaneous vasodilation with local heating may have masked any contribution from NO to menthol-mediated vasodilation. Furthermore, while reactive hyperemia and local heating in the skin are in large part pathway specific, there is a certain degree of cross talk from multiple vasodilator pathways (Brunt and Minson, 2012; Engelke et al., 1996; Medow et al.,

Abbreviations: CVC, cutaneous vascular conductance; $\%CVC_{max}$, percentage of maximum cutaneous vascular conductance; EDHF, endothelium derived hyperpolarizing factor; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; SNP, sodium nitroprusside; TRPV3, transient receptor potential vanilloid 3; TRPM8, transient receptor potential melastatin 8; TEA, tetraethylammonium.

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2007). Consequently, the mechanism(s) contributing to menthol-mediated vasodilation need to be more fully elucidated.

From a therapeutic standpoint, topical menthol containing products are used in clinical populations (i.e. arthritis, muscle strain, back pain) for their effects of sensation and pain relief. Along with elucidating menthol's mechanism(s) of action, determining the amount of menthol delivered to the cutaneous tissue and the rate of menthol appearance/clearance is important for optimizing formulation of topical menthol containing products for improving its analgesic effects.

The goals of this study were to use intradermal microdialysis, to (1) pharmacodissect the mechanism(s) through which menthol induces cutaneous vasodilation, and (2) determine a time course for menthol appearance and clearance in the cutaneous tissue following topical menthol application. We hypothesized that NO, EDHFs, and sensory nerves would all contribute to menthol-mediated vasodilation. We also hypothesized that menthol would be detectable in dialysate samples from the cutaneous tissue within 30 min on topical exposure and remain present throughout the duration of our protocol.

2. Methods

Experimental protocols were approved by the institutional review board of The Pennsylvania State University and conformed to the Declaration of Helsinki. Voluntary verbal and written consent were obtained from all subjects prior to participation in the study. Protocols were carried out in a group of 10 young, healthy, participants who did not smoke, were not pregnant or breastfeeding, and were free from any apparent cardiovascular disease. Women not taking birth control were tested during the early follicular phase, while women on oral birth control were tested during the placebo phase of their medication. Participants were free of any other medications that are known to alter vascular function.

2.1. Study 1: menthol dose-response

All experiments took place in a thermoneutral laboratory with subjects in a semi-supine position. Subjects did not exercise, or consume caffeine or alcohol, for 12 h before the experiment. Four intradermal microdialysis fibers (CMA Microdialysis; 55 kDa cutoff) were placed in the skin of the ventral forearm as previously described (Smith et al., 2011; Stanhewicz et al., 2012). After placement of microdialysis fibers, 60–90 min was given for full resolution of hyperemia due to insertion trauma.

After resolution of fiber placement hyperemia, each microdialysis site was randomly assigned either 1) control (lactated Ringer's solution), 2) 20 mM N^G-Nitro-L-arginine methyl ester (L-NAME), a non-selective NO synthase (NOS) antagonist to assess NO-dependent vasodilation, 3) 50 mM tetraethylammonium (TEA), a potassium channel blocker to determine the contribution of EDHFs or 4) Lidocaine (4% LMX4 cream applied for 60 min) over a microdialysis site perfused with lactated Ringer's to inhibit sensory nerves. L-NAME and TEA were mixed with lactated Ringer's right before use and filtered through a syringe microfilter (Acrodisc; 0.2 μm filter). Drugs were perfused with microperfusion pumps (BASi Bee Hive controller and Baby Bee syringe drive) set at a rate of 2 $\mu L \cdot min^{-1}$. Sensory nerve blockade was confirmed through lack of sensation to a needle prick (Minson et al., 2001; Wong, 2013).

After full abatement of insertion trauma, local heating units were placed over each microdialysis site and set to 33 °C to maintain thermoneutral skin temperature. Laser Doppler flowmeter probes (Moor Instruments) were placed over each local heating unit to continually measure red cell flux. Measurement of skin blood flow with laser Doppler flowmetry is highly reproducible when the data are expressed as a percentage of maximum vasodilation (Tew et al., 2011). Data were sampled at 40 Hz with WinDaq data acquisition software (DATAQ Instruments). Blood pressure was measured every five minutes via brachial auscultation (CardioCap5, General Electric). Baseline laser Doppler flux was measured for approximately 15 min, after which the menthol dose-response commenced. Seven increasing doses of menthol (99% Menthol, Sigma-Aldrich) (0.1, 1, 10, 50, 100, 250, and 500 mM) were perfused through each microdialysis fiber in 5 min increments. Menthol was mixed with lactated Ringer's and the matching pharmacological inhibitor when appropriate. 500 mM menthol was chosen as the maximum dose as it represented the most menthol that could dissolve in lactated Ringer's when utilizing a hot plate and a stir bar. Menthol's solubility could have been increased with the addition of ethanol to the solution, however we elected not include ethanol because of its inhibitory effect on TRPM8 channels (Benedikt et al., 2007).

Following completion of the menthol dose response, 28 mM sodium nitroprusside (SNP), was perfused through the fibers at a rate of 4 $\mu L \cdot min^{-1}$ and the temperature of the local heaters was raised to 43 °C to elicit maximum vasodilation (Holowatz et al., 2005; Smith et al., 2011).

Data were stored offline for later analysis. Red cell flux was normalized to cutaneous vascular conductance (CVC: flux·mean arterial pressure^{-1}) and expressed as a percentage of site specific maximum obtained from SNP/43 °C heat (%CVC_{max}). All data were analyzed with GraphPad Prism 6 software. A 2-way ANOVA was run to detect menthol dose-site interactions. Tukey's multiple comparisons tests were conducted where appropriate. Significance was set a priori at $\alpha = 0.05$. An a priori power analysis revealed that 10 subjects was sufficient to detect a meaningful physiological difference of 10% between microdialysis treatment sites with a standard deviation of the difference of 10%, with a desired power of 0.8. Effect size (Cohen's d) was calculated for main effects of treatment in study 1. The interpretation of effect size follows the convention of Cohen: 0.2, 0.5, 0.8 correspond to "small," "medium," and "large" effect sizes respectively (Cohen, 1988).

2.2. Study 2: menthol dialysate recovery

All experiments took place under the same conditions as in study 1. Two microdialysis fibers were placed in the skin of the ventral forearm. Thirty minutes after placement of fibers and resolution of the insertion hyperemia, a 60 cm^2 (15 cm·4 cm) area on the ventral forearm was marked. This marked area included the intradermal portions of the microdialysis fibers. Skin blood flow over this area was measured for 5 min with a sample rate of 0.25 Hz utilizing a laser speckle contrast imager (Full-field Laser Perfusion Imager, Moor Instruments). Laser speckle contrast imaging measures provides a relative measure of skin blood flow that is reproducible and possesses good spatial and temporal resolution (Roustit and Cracowski, 2012; Roustit et al., 2010). Dialysate samples from outflow end of the microdialysis fibers were simultaneously collected in 300 μL glass vials with Teflon coated caps. Microperfusion pumps were set at a rate of 1 $\mu L \cdot min^{-1}$ to collect 5 μL of dialysate. After collection of baseline skin blood flow and dialysate samples was completed, the microperfusion pumps were turned off and skin blood flow measurements were paused. One mL of 4% menthol gel (Biofreeze®) was applied evenly over the 60 cm^2 area by a researcher with a gloved hand. A clear plastic covering was placed over the menthol to prevent evaporation and measurement of skin blood flow was resumed.

Topical menthol was left in place for 30 min. Subjective rating of menthol sensation was obtained from the subjects 5, 10, 15, and 30 min after menthol had been applied. The sensation scale was a 0 to 10 visual analog scale with 0 representing no sensation of menthol and 10 a "very intense" sensation.

After 30 min, the plastic covering was removed and excess menthol containing gel was wiped away using a sterile piece of gauze. Dialysate samples and menthol sensation were collected as during baseline and were repeated every 30 min for 240 more minutes (270 total experiment time). Laser speckle contrast perfusion imaging was measured