Microvascular Research 107 (2016) 39–45

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Topical menthol increases cutaneous blood flow

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ARTICLE INFO

Article history:
Received 7 March 2016
Revised 25 April 2016
Accepted 26 April 2016
Available online 27 April 2016

Keywords:
Cutaneous
Menthol
TRPM8
Microvascular
Ilex paraguariensis

ABSTRACT

Menthol, the active ingredient in several topically applied analgesics, activates transient receptor potential melastatin 8 (TRPM8) receptors on sensory nerves and on the vasculature inducing a cooling sensation on the skin. *Ilex paraguariensis* is also a common ingredient in topical analgesics that has potential vasoactive properties and may alter the mechanisms of action of menthol. We sought to characterize the microvascular effects of topical menthol and ilex application and to determine the mechanism(s) through which these compounds may independently and combined alter cutaneous blood flow. We hypothesized that menthol would induce vasoconstriction and that ilex would not alter skin blood flow (SkBF). Three separate protocols were conducted to examine menthol and ilex-mediated changes in SkBF. In protocol 1, placebo, 4% menthol, 0.7% ilex, and combination menthol + ilex gels were applied separately to the skin and red cell flux was continuously measured utilizing laser speckle contrast imaging (LSCI). In protocol 2, seven concentrations of menthol gel (0.04%, 0.4%, 1%, 2%, 4%, 7%, 8%) were applied to the skin to model the dose–response curve. In protocol 3, placebo, menthol, ilex, and menthol + ilex gels were applied to skin under local thermal control (34 °C) both with and without sensory nerve blockage (topical lidocaine 4%). Post-occlusive reactive hyperemia (PORH) and local heating (42 °C) protocols were conducted to determine the relative contribution of endothelium derived hyperpolarizing factors (EDHFs)/sensory nerves and nitric oxide (NO), respectively. Red cell flux was normalized to mean arterial pressure expressed as cutaneous vascular conductance (CVC: flux·mm Hg^{-1}) in all protocols. Topical menthol application increased SkBF compared to placebo (3.41 ± 0.33 vs 1.1 ± 0.19 CVC: $p < 0.001$). During the dose–response, SkBF increased with increasing doses of menthol (main effect, $p < 0.05$) with an ED_{50} of 1.0%. Similarly, SkBF was increased after menthol application during PORH (3.62 ± 0.29 vs. 2.50 ± 0.21 flux·mm Hg^{-1}; $p < 0.001$), but not local heating (2.98 ± 0.24 vs 2.86 ± 0.32 flux·mm Hg^{-1}; $p = 0.44$). Concurrent sensory nerve inhibition attenuated menthol-mediated vasodilation at thermoneutral baseline (1.29 ± 0.19 flux·mm Hg^{-1}; $p < 0.001$) and during PORH (2.79 ± 0.28 flux·mm Hg^{-1}; $p < 0.001$), but not during local heating (3.45 ± 0.21 flux·mm Hg^{-1}; $p = 0.1$). Topically applied menthol, but not ilex, dose-dependently increases blood flow in the cutaneous microvasculature. This increase in blood flow is mediated, in-part by sensory nerves and EDHFs.

Introduction

Topically applied analgesic gels are commonly used in clinical practice to relieve muscle and joint pain. The purported analgesic mechanism of action for these agents is gate control theory (Melzack, 1996). Menthol, the active ingredient in many topically applied analgesics, activates transient receptor potential melastatin 8 (TRPM8) channels which are part of a family of non-selective cation channels which also open in response to cool temperatures (8–28 °C) (McKemy et al., 2002). According to gate control theory, menthol elicits an analgesic effect by activating TRMP8 channels located on sensory nerves and on C and A_δ nociceptors to reduce pain transmission (Liu et al., 2013; Premkumar and Abooj, 2013). While these topically applied agents are effective at reducing perceived pain from various musculoskeletal injuries (Airaksinen et al., 2004; Hill and Sumida, 2002) they may also have other non-specific effects on neural and vascular tissues containing TRMP8 receptors.

TRMP8 receptors are expressed in both neural (Babes et al., 2011) and vascular cells (Johnson et al., 2009; Yang et al., 2006). The therapeutic effects of topical analgesics containing menthol may be mediated in-part through its action in the skin itself. Several skin-specific techniques have been developed to examine neurovascular signaling in the cutaneous circulation including the relative contributions of sensory nerves, endothelium derived hyperpolarizing factor(s) (EDHFs) (Lorenzo and Minson, 2007) and nitric oxide (NO)-dependent (Kellogg et al., 1999) signaling pathway. Specifically, after a brief period of arterial occlusion (5 min) the following increase in skin blood flow (SkBF) is mediated through sensory nerves and EDHF -dependent mechanisms (Lorenzo and Minson, 2007). Locally heating a small area of skin causes a brief

Abbreviations: TRPM8, transient receptor potential melastatin 8; NO, nitric oxide; SkBF, skin blood flow; EDHF, endothelium derived hyperpolarizing factor; PORH, post occlusive reactive hyperemia; LSCI, laser speckle contrast imaging; MAP, mean arterial pressure; CVC, cutaneous vascular conductance; THR, total hyperemic response; ED_{50}, effective dilation 50%.

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http://dx.doi.org/10.1016/j.mvr.2016.04.010
0026-2862/

axon reflex that is in-part mediated by sensory nerves. The axon reflex is followed by a nadir and then a prolonged plateau in vasodilation that is predominantly dependent on the production of NO from endothelial NO synthase (Minson et al., 2001). Sensory nerves do not play a role in the NO-mediated plateau in SkBF (Hodges et al., 2009). Menthol is an agonist for TRPM8 channels and induces either vasodilation or vasoconstriction dependent upon the initial level of vascular tone (Johnson et al., 2009) through alterations in sensory nerve (Johnson et al., 2009), NO (Johnson et al., 2009), and RhoA/ROCK vasoconstrictor pathways (Sun et al., 2014). Work on human arterial blood flow suggests that menthol acts as a constrictor (Olive et al., 2010; Topp et al., 2013). However, the effect of menthol at clinically relevant concentrations on cutaneous neurovascular control in humans is presently unknown.

In addition to menthol, commonly used topical analgesic gels (BioFreeze®) also contain *Ilex paraguariensis*, a plant-derived additive that contributes to the texture of BioFreeze®. Ilex has documented antioxidant properties (Boaventura et al., 2012; Gao et al., 2013b; Leonard et al., 2010) and increases NO bioavailability, while decreasing vasoconstrictors including endothelin and thromboxane B2 (Gao et al., 2013a), in both human and rat models (Muccillo Baisch et al., 1998; Schinella et al., 2005). However, the effects of topically applied ilex and possible interactions with menthol on cutaneous blood flow are unclear.

The aim of this study was to determine the separate and combined effects of menthol and ilex on SkBF. We also sought to elucidate mechanisms by which ilex and menthol may alter SkBF by utilizing non-invasive skin specific stimuli (PORH, and local heating). We hypothesized that topical menthol would induce cutaneous vasoconstriction through sensory nerve mediated mechanisms, while topical ilex would have no effect on SkBF.

Material and methods

Subjects

Experimental protocols were approved by the institutional review board of The Pennsylvania State University and conformed to the Declaration of Helsinki. Voluntary written and verbal consent were obtained from all subjects prior to participation in the study. Each protocol was performed on different groups of 10 young, healthy subjects. All subjects were apparently healthy, normally active, nonsmokers, not on prescription medication, and did not present with any chronic disease.

Instrumentation

All experiments took place in a thermoneutral laboratory with subjects in a semi-supine position. Subjects refrained from consuming alcohol for at least 12 h before the experiment and did not consume caffeine or exercise on the day of the experiment. The subject's left arm was placed in a supinated position on a vacuum cushion to limit movement artifact during data collection.

Protocol 1

Each subject completed four trials where forearm blood flow over a 60 cm^2 area of the ventral forearm was measured with a full-field laser perfusion imager (Moor instruments) that utilizes laser speckle contrast imaging (LSCI) to obtain relative measures of SkBF, expressed as flux. LSCI works through measuring fluctuations in the speckle pattern of laser light as blood flows through the skin (Briers, 2007). The full-field laser perfusion imager was set with a time constant of 1.0 s, display rate of 25 Hz, and exposure time of 4 milliseconds. During each trial, one of four gels was applied to the skin in a double blind randomized fashion: (1) placebo (2) menthol (4%) (3) ilex (0. 07%) and (4) menthol + ilex. The menthol + ilex gel was commercially available BioFreeze® gel. The other gels were formulated by the manufacturer, de-identified and coded. The formulations were exactly as the commercially available gel except for the removal of menthol and/or ilex. All investigators were blinded as to the identity of the gels until the completion of data collection and analysis.

The measurement area was marked on each subject and clear cellophane wrap was placed over the arm to limit evaporation. LCSI was conducted with and without cellophane wrap prior to gel application in nine experiments and was found not to alter baseline blood flow ($p = 0.185$). Baseline blood flow was measured on the forearm for 15 min, after which the cellophane wrap was temporarily removed and 1ml of test gel was applied evenly over the marked area. The clear cellophane wrap was placed back into position and blood flow was measured until a minimum of 15 min of a steady plateau in SkBF was obtained. Subjects completed one trial for each treatment condition (placebo, menthol, ilex, menthol + ilex). Pilot work indicated that a 24 h washout was sufficient to allow abatement of the effects of previous gel exposure, so a minimum 24 h washout between trials was employed.

Arterial blood pressure was measured via brachial auscultation every five minutes and heart rate was continuously monitored throughout the experiment (Datex-Ohmeda Cardiocap/5). Mean arterial pressure (MAP) was calculated as diastolic blood pressure plus one third pulse pressure. All data were normalized to cutaneous vascular conductance (CVC: flux/MAP).

Data analysis

Data were analyzed, recorded, and stored offline for analysis (moorFLPI Review V3.0). Baseline values were determined as the average of a five minute period of stable blood flow prior to gel application. Peak CVC was determined as the average of a five minute plateau in SkBF post gel application. A two-way mixed model repeated-measures ANOVA was conducted to detect gel and condition (i.e., post-gel) differences in SkBF. Alpha was set at $p < 0.05$.

Protocol 2

After establishing 4% menthol gel as a vasodilator in protocol 1, we sought to characterize the dose–response curve between menthol concentration and changes in CVC. Seven different gels containing 0.04%, 0.4%, 1%, 2%, 4%, 7%, and 8% menthol were utilized for this experiment. These concentrations were chosen to test menthol concentrations above and below those contained in commercially available analgesic gels, with 7–8% menthol representing the highest concentrations available that would emulsify in the gel.

In a group of 10 subjects, four 15 cm^2 areas for gel application were identified on the ventral forearm. Volumes of 0.25 mL of each gel were applied to the marked sites in a randomized manner. This volume of gel was chosen to match the same amount of gel/cm^2 as utilized in protocol 1. Both researchers and subjects were blind to the identity of each gel. Immediately after gel application, all four sites were covered with clear cellophane wrap as in protocol 1. SkBF was measured with LSCI. Once flux at any individual site reached a plateau for five minutes the cellophane wrap was removed from that location only and any excess gel was removed. SkBF was then continuously measured until flux returned to basal values for a minimum of five minutes. Blood pressure and heart rate were monitored as in protocol 1.

Peak SkBF was measured as the highest CVC recorded during menthol administration for each menthol dose. Total hyperemic response (THR) was determined for each menthol dose as the integrated increase in SkBF with baseline subtracted, expressed as CVC multiplied by time in seconds (CVC·s), as previously described (Wong et al., 2003). One-way ANOVAs were run to detect between gel differences. Alpha was set at $p < 0.05$. Menthol concentrations were log transformed and data were curve modeled as a four parameter logistic equation without minimum or maximum constraints (GraphPad Prism 6). Effective dose 50% (ED_{50}),