Dose–response study of topical allyl isothiocyanate (mustard oil) as a human surrogate model of pain, hyperalgesia, and neurogenic inflammation

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Published in:
Pain

DOI (link to publication from Publisher):
10.1097/j.pain.0000000000000979

Publication date:
2017

Document Version
Accepted author manuscript, peer reviewed version

PAIN Publish Ahead of Print
DOI: 10.1097/j.pain.0000000000000979

A dose-response study of topical allyl-isothiocyanate (mustard oil) as human surrogate model of pain, hyperalgesia, and neurogenic inflammation

Running head: AITC as a human model of TRPA1-induced pain

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Article category: Original manuscript

Disclosures: No conflicts of interests to declare

Conflict of interest: None to declare
Number of pages: 20 (all inclusive)
Number of figures/tables: 5 (2 in color)

Key words: Human surrogate pain model; hyperalgesia; allodynia; allyl-isothiocyanate; mustard oil; TRPA1; TRPV1

Abstract

Despite being a ubiquitous animal pain model, the natural TRPA1-agonist allyl-isothiocyanate (AITC, also known as "mustard oil") has only been sparsely investigated as a potential human surrogate model of pain, sensitization, and neurogenic inflammation. Its dose-response as an algogenic, sensitizing irritant remains to be elucidated in human skin.

Three concentrations of AITC (10%, 50%, 90%) and vehicle (paraffin) were applied for 5 min to 3x3 cm areas on the volar forearms in 14 healthy volunteers, and evoked pain intensity (visual analog scale 0-100 mm) and pain quality were assessed. In addition, a comprehensive battery of quantitative sensory tests was conducted including assessment of mechanical and thermal sensitivity. Neurogenic inflammation was quantified using Laser Imaging perfusion (FLPI). Erythema and hyperpigmentation were assessed before, immediately after, and ≈64 hours after AITC exposure.

AITC induced significant dose-dependent, moderate-to-severe spontaneous burning pain, mechanical and heat hyperalgesia as well as dynamic mechanical allodynia ($p < 0.05$). No significant differences in induced pain hypersensitivity were observed between the 50% and 90% AITC concentrations. Acute and prolonged inflammation was evoked by all concentrations and assessments by FLPI demonstrated a significant dose-dependent increase with a ceiling effect from 50% to 90%.

Topical AITC application produces pain and somatosensory sensitization in a dose-dependent manner with optimal concentrations recommended to be >10% and ≤50%. The model is translatable to humans and could be useful in pharmacological proof-of-concept studies of TRPA1-antagonists, analgesics and anti-inflammatory compounds or for exploratory clinical purposes, e.g. loss- or gain-of-function in peripheral neuropathies.

1. Introduction

The natural transient receptor potential cation channel A1 **(**TRPA1)-agonist allyl-isothiocyanate (AITC), also known as "mustard oil", has been used ubiquitously as an *in vivo* pain model in hundreds of preclinical studies [8,16,17,45,54]. It generates inflammation and spontaneous pain behavior as well as robust thermal and mechanical hyperalgesia [31,35,66]. Following cutaneous application, this symptomatology is evoked through activation of TRPA1-positive epidermal C-nociceptors. Notably, several additional animal models beyond AITC application, rely partly or entirely on TRPA1 as their substrate, e.g. models using formalin and trans-cinnamaldehyde [21,54,82].

The physiological role of TRPA1 in the sensory nervous system is not only restricted to chemonociception [39,47,53,60]; the channel has also been suggested to play important roles in mediating cold pain sensation [6,23,80], pruriception [28,46,84], the cough reflex [24,64] and to act as an immunological co-activator [15,69]. The TRPA1-channel is substantially co-expressed with transient receptor potential cation channel V1 (TRPV1) on peptidergic nociceptors [34] and these frequently act in conjunction to detect numerous environmental chemical irritants although

it is currently unclear to which extend they are functionally dependent on each other [22,70,79]. Recently, TRPA1 has been widely implicated in a number of painful and inflammatory conditions and is considered a promising potential target for the development of, amongst others, analgesic, antipruritic and anti-inflammatory pharmacotherapeutics [4,21,36,64]. For instance, the channel has been proposed to play a role in the pathoetiology of numerous disorders based on *in vivo* studies including neuropathic pain [9], migraine [11] and arthritis [29] and expression of the channel has been found upregulated in human tissues in conditions such as oral lichen planus [40], post-burn pruritus [85] and inflammatory bowel disorder [41] – conditions that are often associated with neuro-inflammation and pain. In line with these observations, a number of TRPA1-antagonists are currently being developed to target conditions such as neuropathic pain, irritable bowel diseases, inflammatory skin disorders and itch [4,12,21,36,64].

Despite its very widespread usage as a model of TRPA1-induced pain in animals, the AITC-model has not been extensively studied in humans [25,37,38,83]. The few previous studies applying topical AITC in humans predate the discovery of TRPA1 and mainly assessed certain aspects of the somatosensory aberrations or neuronal events induced by AITC [25,37,38,75,83]. Does-response features of this model have not previously been investigated. Given the difficulty and high failure-rate associated with turning preclinical findings on promising anti-nociceptive and anti-hyperalgesive compounds into feasible analgesics for human use [54], translatable mechanism-specific human surrogate models of pain, such as the AITC model, are warranted [5,63] and it is thus necessary to have dose-response information for the sensitization features of interest. Moreover, AITC might constitute a relevant receptor-specific pain probing modality, which could be applied to gain insights into pain mechanisms e.g. loss- or gain-of-function in patients with neuropathic pain [33,57].

The aim of the present study was to assess the dose-response features of topical AITC provocations on evoked pain and pain-associated sensory aberrations. Beyond evaluating evoked pain intensity and quality, a comprehensive battery of quantitative sensory tests was conducted together with evaluation of neurogenic inflammatory responses.

2. Methods

2.1 Study design and subjects

Fourteen healthy subjects including 10 males and 4 females, aged 19–28 years (mean (±SD): 23.5 ± 3.1 years) were recruited. The subjects were pain-free, without previous known neurological, dermatological, allergic or musculoskeletal disorders. 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-20160066). Prior to the onset of the experiment all subjects were instructed verbally and in writing that all experimental procedures could be stopped immediately if the pain or discomfort became intolerable, as well as that they are free to withdraw from the study at any point without incurring consequences. The study was carried out in a double-blinded manner with balanced randomization of both the order of AITC applications, the application sites within each volar forearm (proximal vs. distal locations) and dominant versus non-dominant arm. Randomization of right vs. left was done under the condition that arms always

shifted between the applied solutions. Both the investigator and the subjects were blinded to the AITC concentration/vehicle being applied. Every time a substance was administered, a bottle of pure 99% allyl-isothiocyanate was opened in the laboratory. This was done to create a powerful ambient smell of mustard oil, in order to inhibit the investigator and participants' ability to identify the order of solutions investigated, by olfaction. A similar procedure has previously been utilized in studies using highly odorous compounds to avoid unmasking of subjects and investigator [26,58,61]. The study was conducted as a single session lasting approximately 2.25-2.75 hours, wherein the participants were exposed to all four solutions, interspersed with short breaks following each panel of sensory testing (Fig. 1). The ambient temperature was kept at ≈21-22 °C in all sessions.

2.2. Application of allyl-isothiocyanate

AITC (≥99%) was obtained from Sigma Aldrich (Broendby, Denmark) and dissolved in pure paraffin (European Pharmacopoeia, Løve-Apoteket, Aalborg, Denmark) at concentrations of 10%, 50% and 90% (all v/v concentrations). These concentrations were determined by precedence in the literature showing that 100% AITC evokes a considerable pain and allodynia [38] and by initial pilot studies showing that much lower concentrations were likely sufficient to induce similar pain, hyperalgesia, and prominent neurogenic flare. Paraffin was chosen as the vehicle because of 1) its previous application for the purpose of applying AITC, 2) its lack of toxicity, 3) its lack of somatosensory effects. A one mL aliquot of each concentration was dispensed into a 3 × 3 cm cotton pad and placed in a custom-made polypropylene chamber (3 x 3 x 0.18 cm) (Aalborg University, Aalborg, Denmark). The chamber containing the cotton pad was then fixed to the pre-marked skin areas on the volar forearms using medical tape (BSN, Hamburg, Germany). With two pre-marked 3x3 cm areas on each volar forearm, the same skin areas were never re-used. The two areas were 6 cm apart (each 3 cm away form the midpoint between the elbow and wrist creases) to assure non-overlapping flare quantification. The application configuration was applied for 5 min before being carefully removed after which residual AITC/vehicle solution was gently washed of the skin. The AITC was deliberately removed before proceeding, because initial pilot experiments demonstrated that if the skin area is not cleaned thoroughly, the induced pain would only subside slowly and increased hyperpigmentation would occur.

2.3. Pain assessment

For each concentration applied, the participants were instructed to rate the pain intensity for 5 min on a 100 mm visual analog scale (VAS) ranging from "*no pain*" = 0, to "*worst imaginable pain*" = 100. To this end, a digital VAS using eVAS software (Aalborg University, Denmark) installed on a 10.1" Samsung tablet computer (Samsung Electronics, Seoul, Korea) was used and the pain intensity was sampled at 0.2 Hz. Subjects expressed their pain quality by choosing word descriptors from a validated brief descriptive Danish version of the McGill Pain Questionnaire (MPQ). A list of the applied sensory descriptors can be found in [62]. Pain quality was determined at 4 minutes post AITC application corresponding to a time point where moderate to intense pain was perceived in the majority of subjects. After the pain assessment, Full-Field Laser Perfusion Imaging (FLPI) was conducted and the subjects were asked to report when the pain subsided before proceeding with sensory testing.

2.4. Quantitative sensory testing (QST)

The QST battery of the present study was partly derived from the guidelines of the German Research Network on Neuropathic Pain (DFNS) [68]. The verbal instructions (in Danish) for participants from the DFNS protocol were derived from the supplementary materials of Olsen *et al.* (2014) [61]. The terminology used to describe the induced sensory derangement are defined by Sandkühler (2009) [71].

2.4.1 Thermal detection and pain thresholds: Tests for cold detection threshold (CDT), warmth detection threshold (WDT), cold pain threshold (CPT), and heat pain threshold (HPT) were performed using a Medoc Pathway (Medoc Ltd, Ramat Yishay, Israel) equipped with a 3 × 3 cm advanced thermal stimulator probe with a baseline temperature of 32°C. Ramping stimuli of 1 °C/s were delivered in each application area following AITC and when the subjects identified the associated threshold (first perception of cold or warmth and first perception of cold or heat pain) they pressed a button that returned the temperature of the probe to the baseline at a rate of 5 °C/s. The results were calculated as the arithmetic mean of the thresholds from three repeated ramps.

2.4.2 Mechanical pain thresholds and sensitivity: To evaluate the mechanical pain threshold (MPT), a set of 7 weight-calibrated pinprick stimulators (MRC Systems, Germany) with weights of 8, 16, 32, 64, 128, 256, and 512 mN was applied. During five ascending/descending series of stimuli, the subjects were instructed to report when a perception of 'sharpness' or 'pricking pain' was first sensed. The final MPT was calculated as the geometric mean of the values obtained in the five series of stimuli. The mechanical pain sensitivity (MPS) was assessed to detect pinprick hyperalgesia to suprathreshold stimulation. The seven pinprick stimuli were applied in ascending order three times and for each stimulus the subjects were instructed to rate the pain intensity on a numerical rating scale (NRS) from 0–10 (0 = *no pain*, 10 = *worst imaginable pain*). Subjects were explicitly instructed to use decimals as suitable. The final MPS was calculated as the arithmetic mean of three consecutive series of the seven stimuli. The wind-up ratio (WUR) was assessed using the pinprick stimulator one intensity above the average MPT. The subjects were asked to rate their pain intensity following a single stimulus and thereafter of a subsequent series of 10 consecutive stimuli (1 stimuli/s) applied within the area of substance application. This procedure was repeated three times.

2.4.3 Allodynia: Dynamic mechanical allodynia (ALL) was assessed using a normally innocuous stimuli moving, i.e. a standardized sensory brush (SENSElab 05-brush, Somedic, Hörby, Sweden) exerting a force of 200–400 mN. It was always confirmed that subject perceived the stimulation as non-painful in an area at least 10 cm away from the relevant AITC-pretreated skin. Subsequently, the tactile stimulus was applied three times with a single stroke of approximately 3 cm in length within the AITC-pretreated skin. The subjects were instructed to rate whether pain occurred and if so to rate the pain intensity on the same numerical rating scale used for MPS.

2.5 Neurogenic inflammatory response and pigmentation

FLPI was used to assess the superficial blood perfusion immediately after removing the AITC/paraffin chamber. The measurements were conducted using a MoorFLPI-1 (Moor Instruments Ltd, Axminster, UK). The perfusion assessment was conducted with a 35-cm distance between the camera and the application area with an exposure time of 8.3 ms and 160

units of gain. The FLPI data were analyzed using MoorFLPI Review V4.0 proprietary software. The induced increases in average and peak superficial blood perfusion within the 3 x 3 cm application areas were used as proxies for primary neurogenic inflammation intensity. Moreover, the axon-reflex flare size was calculated as the area associated with the AITC provocation exhibiting a ≥30% increase compared to the surrounding perfusion background. The size was quantified in cm^2 by relative comparison to the known size of the 3 x 3 cm pre-marked application area. The 9 cm^2 in which AITC was applied was then subtracted from the score thus giving rise to an estimate of the size of the secondary neurogenic inflammation or the "reflex response". This method has previously been applied to quantify the neurogenic inflammatory response to various chemical irritants [1,2,61]. The erythema (redness) and skin pigmentation were measured with a spectrometer designed for cutaneous use (ColorMeter, DSM II, Cortex Technology, Hadsund, Denmark) as previously utilized [56] . The device provides a read-out of erythema and pigmentation based on the light absorption characteristics of skin for wavelength corresponding to hemoglobin (erythema) and melanin (pigmentation). Erythema and pigmentation data was recorded before, immediately after AITC removal and ≈64 hours (48-96 hours range) after the experimental session.

2.6 Statistics

Sample size calculation was conducted based on previously obtained data applying similar pain models [61] and using the approached outlined for crossover designs [56]. 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 visual inspection of Q-Q plots and the Shapiro-Wilk normality test when necessary. Average and peak pain intensity induced by the AITC were calculated and compared from VAS-recordings while categorical variables are shown as % of distribution without further hypothesis testing. The primary analyses were conducted using repeated measures analyses of variations (RM-ANOVAs) with the factor: *concentration* (four levels) using Fisher's least significant difference (LSD) test *post hoc* test in a step-wise manner. For erythema and pigmentation data, an additional level of *time* was added to the RM-ANOVA and values were always normalized to the values obtained from the vehicle area to account for day-to-day variability. While all statistical tests were conducted using raw data, the temporal profiles of evoked pain (Fig. 2A) were downscaled to one data point per 0.5 min by calculating the average of six 0.2 Hz ratings. Z-scores were calculated to provide a relativized overview of induced sensory sensitization using the formula [$Z = (\chi-\mu)/\sigma$] where "χ" is the average value following AITC, "μ" is the average value in the vehicle condition and "σ" is the combined standard deviation for the parameter across the four solutions. 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) and graph plotting was conducted in GraphPad Prism. A p-value of ≤ 0.05 was considered significant.

3. Results

All enrolled participants finished the study and topical administration of AITC was well tolerated and did not produce any unexpected side effects (e.g. local edema, blistering, desquamation or systemic reactions). No subjects withdrew from the study nor terminated any of the experimental procedures. The spontaneous pain evoked by AITC generally resolved quickly after removing residual solution. AITC-evoked pain subsided quickly after removing residual solution and all subjects confirmed that no pain remained before the sensory testing was started. Due to technical issues, the erythema and pigmentation data is missing for one subject (excluded from relevant analyses, n = 13). The study was designed to assess the dose-response function of AITC, not to detect any potential sex differences in AITC-induced responses and hence no sub-analysis on data was conducted for this purpose. No significant differences were observed related to order of AITC administration or arm dominancy.

3.1. Evoked pain intensity and quality

The most frequently reported descriptors of the evoked pain quality were: "warm/burning" (10% n = 5/14, 50% = 10/14, 90% = 10/14), "searing" (10% = 8/14, 50%= 9/14, 90% = 7/14) and pricking/stinging (10% = 7/14, 50% = 3/14, 90% = 5/14). Four subjects described the vehicle as inducing a mild "cold/freezing" sensation. The mean pain intensity was a dose-response-dependent for both mean ratings and peak pain ($F(3,39) = 29.9$, $p < 0.001$), see Fig. 2A and 2B. Post hoc testing revealed that all AITC conditions induced more intense pain than the vehicle ($p < 0.01$) and that both 50% and 90% AITC induced higher pain intensity than 10% AITC ($p < 0.01$). This was true for both mean and peak pain ratings. No significant difference was found between 50% and 90% AITC. Peak pain intensity reached to 41.43 ± 6.17 for 10% AITC, 56.9 ± 7.77 for 50% AITC, and 59.9 ± 7.46 for 90% AITC. Negligible pain was reported following the vehicle application in two subjects who rated it on the VAS (both < 5 out of 100).

3.2. Thermal sensory sensitivity

Thermal cold as well as warmth detection thresholds remained unchanged following application of AITC regardless of the concentration applied ($F(3,39) = 0.35$, $p > 0.7$ and ($F(3,39) = 2.83$, $p > 0.05$) indicating that neither thermal hypo- nor hyperesthesia was induced by AITC (Fig. 2C). The cold pain threshold was, however, altered by AITC ($F(3,39) = 3.93$, $p < 0.05$), with post hoc tests revealing that cold hypoalgesia was only significantly elicited in the 90% AITC condition ($p < 0.05$) compared with vehicle. Conversely, HPT decreased significantly and in a dose-dependent manner ($F(3,39) = 86.0$, $p < 0.001$). While 10% was insufficient to produce heat hyperalgesia, both 50% and 90% evoked substantial decrease in HPT from 40.9 ± 0.92 °C in the vehicle condition to 37.1 ± 0.47 °C following 50% AITC and 36.8 ± 0.50 °C following 90% AITC (Fig. 2D). Heat pain thresholds were also significantly lower in the 50% and 90% AITC conditions compared to the 10% condition, but no difference was found between 50 and 90% AITC concentrations.

3.3. Mechanical hyperalgesia and allodynia

AITC induced dose-dependent mechanical hyperalgesia as evident by increased MPS, including mechanical stimuli in the suprathreshold range, was significantly increased for all AITC concentrations but no clear dose-dependency was evident in the response (Fig. 3A). However, trends toward higher MPS following 50% and 90% AITC compared to the 10% AITC were evident ($p = 0.051$ and 0.099, respectively). MPT ($F(3,39) = 8.41$, $p < 0.01$) decreased following 50% and 90% AITC administration with approximately ⅔ (Fig. 3B). While all AITC concentrations evoked significant decreases in MPT with respect to vehicle, 50% and 90% AITC yielded significantly further decreases compared with 10% ($p < 0.05$). The wind-up-ratio (WUR) to pinprick pain stimuli was unaffected by AITC ($F(3,39) = 1.34$, $p > 0.05$, data not shown). AITC induced significant dose-dependent allodynia to brush ($F(3,39) = 15.8$, $p < 0.001$). For 10% AITC 9/14 subject reported allodynia, for 50% 11/14 reported it and for 90% 13/14 reported it. Again, all AITC concentrations induced significant allodynia compared to vehicle, but 50% and 90% AITC induced more pronounced allodynia than 10%, while no differences was present between 50% and 90% AITC. The brush-evoked allodynia was always perceived as non-painful in normal intact skin on the volar forearm treated with vehicle. Notably, most subjects spontaneously reported pain associated with gentle wiping of the skin immediately next to the application areas treated with 50% and 90% AITC, when residual solution was removed from the application site. This indicates that while not quantified in the present study, secondary allodynia to innocuous touch was evoked. For a comparative overview of the somatosensory aberrations induced by the AITC (see Fig. 4).

3.4. Neurogenic inflammatory response

All concentrations of AITC induced robust neurogenic inflammation for mean ($F(3,39) = 38.6$, $p < 0.001$) and peak values ($F(3,39) = 34.1$, $p < 0.001$). Clear dose-response relationships were evident for these parameters although no significant differences were detected between 50% and 90% AITC for both mean and peak perfusion (Fig. 5A and B). Notably, all AITC concentrations also induced a secondary inflammatory response known as “axon-reflex-flare” ($F(3,39) = 56.8$, $p < 0.001$), The size of this response was also dose-dependent with the 90% AITC concentration inducing a larger axon-reflex-flare than the 10% concentration ($p > 0.05$), with 17.0 ± 1.29 cm vs. 13.0 ± 1.87 cm, respectively (Fig. 5C). For primary erythema measured by spectroscopy there was only an effect of *time* ($F(2,24) = 131.3$, $p < 0.001$) but not for *concentration* ($F(2,24) = 2.56$, $p = 0.099$) nor was there an effect of *concentration* x *time* interaction. Post hoc testing showed that erythema increased following all AITC concentrations and then decreased considerably when measured approximately 64 hours after the initial AITC exposures, although the erythema still remained detectable ($p < 0.01$). At 64 hours, there was generally no visually perceptible inflammation in the AITC-treated area (Fig. 5D). For pigmentation no significant changes occurred at group level for neither *time* ($F(2,24) = 0.86$, $p = 0.44$) nor *concentration* ($F(2,24) = 0.19$, $p = 0.44$). However, slight but visually discernable hyperpigmentation occurred in 6/14 subjects following 50% AITC and 8/14 following 90%, indicating poor resolution for colorimetry of pigmentation (Fig. 5E). This hyperpigmentation generally resolved within 1-2 weeks after the study start.

4. Discussion

The present study showed that 5 min topical application of AITC (mustard oil) produces pain and somatosensory sensitization in a dose-dependent manner in healthy human skin. The model evoked robust mechanical hyperalgesia, allodynia, heat hyperalgesia, and neurogenic inflammation. Notably, no significant additional sensitization and pain was achieved when using 90% compared with 50% AITC, indicating a ceiling effect. Based on the present study, AITC concentrations of >10% and ≤50% are likely ideal for elicitation of robust sensory pain symptomatology, while minimizing unnecessary exposure.

4.1 Evoked pain intensity and quality

AITC induced dose-dependent, predominantly burning pain, conceivably corresponding to its activation of TRPA1-expressing C-nociceptors in the epidermis. This is well aligned with a previous paper by Koltzenburg *et al.* (1992) reporting very similar finding related to chemogenically-induced pain and pain quality for 100% (pure) AITC applied to a smaller area (2x2 cm) for 5 minutes. The predominant burning sensation is also in line with additional previous evidence [37,49]. Notably, the relatively rapid onset (1-3 min) of pain following topical administration has previously been observed [38] corresponding to the temporal profile observed in the present study. The AITC model works noticeably faster than topical capsaicin (a TRPV1-agonist), a feature likely related to the fact that it is a smaller and more soluble molecule. Notably, the topical capsaicin-model has been extensively used as a TRPV1-pain and inflammation specific model in early pharmaceutical proof-of-concept studies for drugs developed for various clinical conditions involving TRPA1 [64].

4.2. Thermal sensory sensitivity

AITC did not induce changes in the detection of innocuous cold and warmth, indicating that afferent units conveying these sensations, within the detection threshold range, are likely TRPA1-negative. This corresponds to the prevailing notion of these fibers being myelinated cold Aδ-fibers and warmth-selective C-fibers, respectively [14]. While the effect of AITC-induced TRPA1-activation on thermal detection thresholds have not previously been assessed, the present results do correspond to findings stemming from the trans-cinnamaldehyde model, which similarly have no, or very limited effects on thermal detection thresholds [2,58,61]. Oppositely, CPT was decreased by the 90% AITC concentration signifying cold hypoalgesia and a similar, but insignificant trend was observed for 50%. This observation has previously been made for irritants inducing burning pain, for instance capsaicin and trans-cinnamaldehyde, and it has been suggested that this could be a consequence of the pleasantness or relieve associated with cold counter-stimuli on the affected skin. It is paradoxical, that both TRPA1-agonists; trans-cinnamaldehyde and here AITC induce cold hypoalgesia and burning pain [58,67], given that TRPA1 has previously been implicated in cold pain sensation [19,32,51]. Cold pain is hypothesized to rely on a common nociceptive pathway for both nociceptive heat and cold along with simultaneous activation of innocuous cold Aδ-fibers. It has been suggested that $TRPA1^{+}$-afferents may mediate the burning component of noxious cold [18,58]. However, recent evidence indicates that TRPA1 is - by no means - selective to cold pain sensation [24,28,46,64,84] and that

the channel acts more as a general transducer and amplifier to certain noxious and pruritic stimuli [35]. Lastly, the evidence implicating TRPA1 in cold pain sensation is almost entirely derived from rodent studies and since peptidergic nociceptors in humans and rodents do exhibit notable dissimilarities, preclinical findings are not necessarily fully transferable [74]. Dose-dependent heat hyperalgesia, as evident from increased HPT, was provoked by AITC. Notably, both 50% and 90% AITC reduced the HPT to ≈ 37°C, i.e. approximately 2.5°C higher than HDT in the vehicle-treated skin. Heat hyperalgesia is conceivably evoked by primary sensitization of fibers responsible for conveying heat pain around the pain threshold territory, i.e. PmC- and Aδ-fibers [44,52]. Following TRPA1-activation by AITC these units respond at temperatures normally insufficient in evoking action potential and perhaps even at temperatures below that of the skin *in situ*. Given the estimated 65% co-expression of TRPA1/TRPV1 on peptidergic nociceptive fibers in rats [34], TRPA1-induced heat hyperalgesia, and perhaps cold hypoalgesia, is likely produced through same subpopulation of fibers that mediate these features in the capsaicin-model [20].

4.3. Mechanical hyperalgesia and dynamic allodynia

AITC induced dose-dependent decreases in MPT and corresponding increases in pain in response to mechanical supra-threshold pain stimuli (increased MPS), although no differences were present between the three AITC concentrations for the latter outcome parameter, even when comparisons were conducted with the LSD-test. Prolonged mechanical hyperalgesia has previously been described following a 5-min application of 100% AITC; however, in that study the secondary hyperalgesic area was mapped with a single 23g von Frey filament [38], contrasting the present quantification of primary hyperalgesic severity. Normal perception of pinprick pain is thought to primarily reflect type-1 Aδ-, and to a lesser extent, PmC-fiber input [48,78]. The primary hyperalgesia to pinprick stimuli observed in the present study likely reflects either primary sensitization of the aforementioned mechanoreceptive units, and/or, perhaps more likely, sensitization occurring at the level of the spinal dorsal horn (central sensitization) [43]. Strikingly, a previous study has found that primary mechanical hyperalgesia induced by AITC persists during an A-fiber block, while a microneurography study has found that PmC-fibers were not sensitized following AITC application [38,75]. Mechanistically, the aforementioned sensitization would likely be evoked by activation of TRPA1-positive nociceptive peripheral afferent C-fibers [81] and subsequent effects on the second order nociceptive neurons in the dorsal horn [77]. These central neurons, in turn, receive convergent input from Aδ-nociceptors thus mediating an increased sense of mechanically evoked pricking pain (pinprick hyperalgesia) [42,77].This explanatory model of centrally mediated sensitization could also explain the pronounced, dose-dependent AITC-induced elicitation of allodynia, which has also previously been observed lasting for at least 30 min post 5-min application of 100% AITC [38]. For allodynia, thickly myelinated low-threshold primary Aβ-afferents, rather than Aδ-fibers, normally mediating the sense of light touch, would converge onto the nociceptive dorsal horn neurons following the preceding AITC-induced nociceptor barrage and produce the sensations of brush-evoked pain [42,43,81]. This explanation is well aligned with the fact that a previous paper on AITC-induced hyperalgesia in humans reported significant secondary hyperalgesia (an aspect of sensitization not quantified in the present study), which is recognized to be a centrally mediated phenomenon affecting Aδ- (pinprick hyperalgesia) and Aβ-fibers (stroke-evoked allodynia). The wind-up-ratio for pinprick stimuli was unaffected by the AITC-provocations, which is in line with findings in capsaicin-

induced hyperalgesic skin [50]. Pinprick hyperalgesia and the itch-related phenomena of hyperknesis and alloknesis, but not dynamic mechanical allodynia, have previously been reported following use of trans-cinnamaldehyde in humans [2,58,61], but in the present study only one subject reported a sensation of itch, conceivably due to the high levels of pain. Lastly, both dynamic mechanical allodynia and hyperalgesia are very well studied characteristics of the capsaicin model, which at least in part, activates a subpopulation of nociceptive fibers co-expressing TRPA1, and thus also recruited by AITC administration.

4.4. Neurogenic inflammatory response

It is suggested that the primary cause of the herein observed acute inflammation is predominantly neurogenic in origin due to three observations; 1) the secondary flare extended several centimeters away from the application area, 2) the flare developed within minutes, 3) there was no sign of exudation (lack of wheal reactions) indicating that mast cell degranulation did not occur. The neurogenic inflammatory response is thought to primarily represent an efferent function of CMi-fibers [13,73]. With their large terminal aborizations and capacity to release e.g. the vasodilatory calcitonin-gene related peptide (CGRP), this subpopulation of afferent fibers can elicit neurogenic flare significantly beyond the area where an irritant substance is applied [72,73,76]. Animal studies have shown that capsaicin-induced desensitization only inhibits acute, but not prolonged TRPA1-mediated inflammation and that anti-histamines has no anti-inflammatory effect [7,30], while a human micro-neurography study has shown that the vast majority CMi-fibers are TRPV1-positive. Taken together this may suggest that AITC produces inflammation through parallel neurogenic and immunological mechanisms; the initial inflammatory response being predominantly neurogenic through CMi-activation and the prolonged primary inflammation measured by spectrometry being primarily immune-driven. This further raises the question of to which extent TRPA1 is an independent nociceptive transducer in human skin (see section 4.4). No differences were observed on primary neurogenic flare or axon-reflex-flare between 50% and 90% AITC, despite assessment with the LSD-test, likely indicative of a ceiling effect of either CGRP-release or vascular responsiveness to said neuropeptide or other vasoactive neuropeptides. A number of previous human studies using the TRPA1-agonist trans-cinnamaldehyde have found a similar neurogenic flare pattern [58,61], suggesting that TRPA1 is expressed on a substantial proportion of CMi-fibers.

4.5 TRPA1 as independent nociceptive transducer

TRPA1 has been proposed to play a detrimental role in the pathoetiology of numerous disorders including neuropathic pain [9], chronic inflammatory skin diseases [59], irritable bowel disorders [41], migraine [11] and arthritis [29]. Notably, aberrant TRPV1-signalling, has also been suggested to be involved in many of these conditions and there is evidence of structural and functional TRPA1/TRPV1 co-localization. However, while TRPA1 is certainly a TRPV1 independent molecular nociceptive/chemoreceptive transducer, it is unclear whether there is a phenotypically significant subpopulation of afferent fibers that are TRPA1-expressing, but TRPV1-negative. A recent study showed that TRPA1-induced hyperalgesia relies entirely on TPRV1-expression [22], contradicting the conventional notion of functional independence [10,31]. As prior studies have almost exclusively been conducted in rodents, it would be relevant to assess whether TRPA1-induced pain and inflammation is inducible in human skin where TRPV1-positive nociceptors have been defunctionalized, e.g. by pre-application of high-

concentration of capsaicin [3,27]. Using such defunctionalization of TRPV1-fibers in humans, it has recently been shown that a PAR2-mediated itch provocation, principally relying on co-activation of TRPA1, is abolished when TRPV1-positive fibers have been ablated by prolong 8% capsaicin application [3]. This seems to indicate that at least the entire subpopulation of pruriceptive nociceptors characterized by PAR2/TRPA1-expression is also TRPV1-positive. In most aspects the observed pain response for AITC is similar to that known from topical capsaicin application [33,38,61]. However, likely associated with differences in skin penetration rates of these two chemical substances, AITC evokes pain and hyperalgesia more rapidly than capsaicin. Several limitations adhere to the results of the present study: 1) While steps were taken to assure blinding of subjects and the investigator, it was difficult to avoid unmasking of the vehicle condition given that no visible flare or no pain was present as opposed to the AITC conditions. 2) Although dose-response features were assessed in participants of both sexes, the study was not designed to detect potential sex differences, which are well established to exist in several experimental models and clinical pain conditions [55,65]. Future studies on TRPA1-induced pain and sensitization should address this key issue for this particular type of pain provocation. 3) While sensory testing was nominally conducted after the spontaneously evoked pain had subsided, it cannot be excluded that low-intensity discharges within the area continued and/or that pain sensitization could have been rekindled or maintained by the somatosensory testing on the treated skin. However, in order to minimize this possibility, the assessment of heat pain thresholds was conducted at the very end of each assessment panel for each applied solution.

5. Conclusion

The present study shows that topical AITC (mustard oil) is an effective, safe and easily applicable, human surrogate model of TRPA1-evoked pain, hyperalgesia, and neurogenic inflammation. For both evoked pain and most somatosensory sensitization parameters the model produces dose-dependent responses, with no significant additional effects beyond a concentration of 50%. To minimize risks and irritant exposure, future studies should therefore use AITC concentrations >10% to ≤50%. The model is suitable for experimental pain studies in healthy controls or patients with pain profiling and could be particularly useful in pharmacological proof-of-concept studies of TRPA1-antagonists, analgesics and anti-inflammatory drugs.

6. Acknowledgement

HHA received support from the *EliteForsk 2016 Rejse-Stipendiat* granted by *the Danish Ministry of Higher Education and Science.* The authors declare that the sources of financial support had no influence on design, data assessment, analysis and interpretation. All authors declare no conflicts of interest. Z. Rahmani is acknowledged for her work in relation to data collection.

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Figure legends

Figure 1. Flowchart of study procedures. For each of the four substance applications the assessments depicted above were conducted. AITC = allyl-isothiocyanate, ALL = allodynia; CDT = cold detection threshold; CPT = cold pain threshold; FLPI = full-field laser perfusion imaging; MPT = mechanical pain threshold; MPS = mechanical pain sensitivity; NRS = numerical rating scale; HPT = heat pain threshold; VAS = visual analog scale; WDT = warmth detection threshold; WUR = wind-up ratio. Notice that the order of AITC/vehicle application was randomized.

Figure 2. Evoked pain and thermal sensitivity following application of AITC and vehicle. A) Evoked pain. **B**) Individual peak pain intensity. **C**) Thermal cold and warmth detection thresholds. **D**) Thermal cold and heat pain thresholds. Notice that **B, C** and **D** share the legend of subfigure **D**. Significance indicators: (*/$^{\#}$) = $p < 0.05$, (**/$^{\#\#}$) = $p < 0.01$, (***/$^{\#\#\#}$) = $p < 0.001$ $^{\#}$ denotes that relevant AITC-applications were significantly different from the vehicle at the lowest observed p-value. AITC = allyl-isothiocyanate; CDT = cold detection threshold; CPT = cold pain threshold; HPT = heat pain threshold; VAS = visual analog scale; WDT = warmth detection threshold.

Figure 3. Mechanical hyperalgesia and allodynia following AITC and vehicle. **A**) Mechanical pain sensitivity. **B**) Mechanical pain threshold. **C**) Dynamic mechanical allodynia. Notice that **B** and **C** share legends. Significance indicators: (*/$^{\#}$) = $p < 0.05$, (**/$^{\#\#}$) = $p < 0.01$, $^{\#}$ denotes that

relevant AITC-applications were significantly different from the vehicle at the lowest observed p-value. AITC = allyl-isothiocyanate; mN = miliNewton; NRS = numeric rating scale.

Figure 4. Z-score plot of AITC-evoked sensitization. Z-transformed sensory changes evoked by AITC relative to the vehicle condition for thermal and mechanical assessments. AITC = allyl-isothiocyanate, ALL = allodynia; CDT = cold detection threshold; CPT = cold pain threshold; FLPI = full-field laser perfusion imaging; MPT = mechanical pain threshold; MPS = mechanical pain sensitivity; NRS = numerical rating scale; HPT = heat pain threshold; VAS = visual analog scale; WDT = warmth detection threshold.

Figure 5. Neurogenic inflammation, axon-reflex-flare, erythema and pigmentation following AITC and vehicle. A) Representative FLPI images of AITC-evoked neurogenic inflammation. **B**) Neurogenic inflammatory response as mean and peak values within the application area. **C**) Area of axon-reflex-flare. **D**) Erythema before, immediately after and 64 hours after AITC. **E**) Pigmentation before, immediately after and 64 hours after AITC. Notice that **B** and **C** as well as **D** and **E** share legends. Significance indicators: (*/$^{\#}$) = $p < 0.05$, (**/$^{\#\#}$) = $p < 0.01$, (***/$^{\#\#\#}$) = $p < 0.001$, $^{\#}$ denotes that relevant AITC-applications were significantly different from the vehicle at the lowest observed p-value. AITC = allyl-isothiocyanate; arb. = arbitrary; cm = centimeter.

Figure 1