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Original Article

Heat-rekindling in UVB-irradiated skin above NGF-sensitized muscle: experimental models of prolonged mechanical hypersensitivity

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Abstract: Experimental models of prolonged pain hypersensitivity in humans are desirable for screening novel analgesic compounds. In this study, heat stimuli were applied in ultraviolet-B (UVB)-irradiated skin and in the UVB-irradiated skin combined with nerve growth factor (NGF)-injected muscle to investigate 1) whether the evoked mechanical hypersensitivity by UVB irradiation would be prolonged or enhanced following heat rekindling, and 2) whether the combination between cutaneous and muscle hypersensitivity may influence the rekindling effects. Skin sensitization was induced in 25 volunteers by UVB irradiation in areas above the upper-trapezius muscle, low-back or forearm. Muscle sensitization was induced in the low back by bilateral injections of NGF. The area of cutaneous hyperalgesia was evaluated 3 days after the irradiation by mechanical pin-prick stimulation whereas the areas of allodynia were evaluated 1, 2 and 3 days after irradiation by von Frey hair assessments. Cutaneous heat stimulation (40°C for 5 min) was performed on the 3rd day to investigate its effect on the areas of cutaneous allodynia and hyperalgesia. Findings revealed that 1) allodynia and hyperalgesia developed following UVB irradiation, 2) heat stimulation of the UVB-irradiated skin enlarged both hyperalgesic and allodynic areas ($P < 0.01$), and 3) muscle sensitization did not influence the effect of UVB on allodynia or the response to heat rekindling. These data suggest that heat rekindling applied to an UVB-sensitized skin can maintain or facilitate allodynia and hyperalgesia for a longer period offering a suitable model for testing analgesic compounds when sufficient duration of time is needed for investigation of drug efficacy.

Keywords: Ultraviolet-B irradiation (UVB), heat rekindling, nerve growth factor (NGF), hyperalgesia, allodynia, muscle, skin

Introduction

The ultraviolet-B (UVB) pain model is one of the cutaneous inflammatory pain models that have been studied both in animals and humans [1, 2] and accepted as a translational model in pharmacological studies and to screen novel analgesic compounds in early phases of drug development [3, 4]. Robust UVB dose-dependent primary mechanical and thermal hyperalgesia and allodynia develop and peak at 24-48 h following the skin irradiation [1, 2, 5, 6]. However, the occurrence of secondary mechanical hyperalgesia and allodynia in the UVB model is still controversial [2, 7].

Rekindling stimulation by e.g. heat can be used to maintain peripheral input from the nociceptors at the original stimulated site and subsequently provide or sustain sensitization of central mechanisms. Heat rekindling was previously used in combination with the capsaicin pain model where a brief heat stimuli of 40°C for 5 minutes was applied resulting in maintenance of the secondary hyperalgesic area for up to 4 h [8]. This model has successfully been used for drug testing [9]. Preliminary studies in both animals [1] and humans [10] also suggest that heat rekindling can be combined with the UVB model to investigate the central mechanisms in this model manifested as secondary areas of

sensitivity to thermal or mechanical stimuli. A recent animal study showed that the combination of UVB and heat stimulation involves central sensitization featured by development of a secondary hyperalgesia that was blocked following the intrathecal administration of MK-801 (uncompetitive antagonist of the N-Methyl-D-aspartate (NMDA) receptor) [11]. Other animal studies also showed that the secondary hyperalgesia and allodynia in rats, following the UVB model, could be enhanced and prolonged by heat rekindling of the irradiated area [1, 11]. A human study performed by Eisenach and his co-workers explored the combination of UVB and heat and found that a mild heat stimulus at the UVB-irradiated site can increase the areas of secondary hypersensitivity [12]. This group also showed that intrathecal administration of ketorolac reverse allodynic but not hyperalgesic areas induced by the combination of UVB and heat. The present study aimed at further investigating the differential effects of heat rekindling on UVB-induced allodynia (tested by von-Frey) and hyperalgesia (tested by pin-prick) in two different locations (arm and back). In addition, since the UVB model is generally considered as a cutaneous pain model, it was aimed to investigate the cutaneous rekindling effect when combined with an experimental muscle pain and hypersensitivity model, which has not been investigated before. A possible convergence of nociceptive input between skin and deep tissues was hypothesised to influence the response properties of the UVB model to heat. The NGF-induced muscle pain model was selected due to the fact that this model is one of the few models that can provide a long lasting muscle hyperalgesia [13, 14].

The current study was designed to test the effect of repeated heat stimuli on mechanical sensitivity in a human model of UVB-irradiated skin alone and in combination with NGF-sensitized muscle. It was hypothesised that 1) UVB induced allodynic and hyperalgesic areas and application of heat enlarged these areas, 2) the heat rekindling has different effects on allodynia or hyperalgesia areas when NGF-induced muscle sensitization is combined with UVB model. This study would not only shed light on potential mechanisms underlying development or maintenance of allodynia or hyperalgesia but also provide a basis for a human experimental model that can be suitable for potential analgesic drug screening.

Material and methods

Subjects

Twenty-five healthy subjects of Caucasian descent participated in the study (11 females and 14 males, mean age ± SD: 25.6 ± 3.8 years) which consisted of two experiments. In Experiment I, the effect of heat rekindling on a UVB-irradiated area was tested (N = 9, 3 females, 30 ± 2 years, and 6 males, 26.3 ± 2.7 years) and the area of hyperalgesia was mapped. In Experiment II, the effect of heat rekindling was tested in a model of UVB alone and UVB+NGF. Sixteen new subjects (8 female, 25.4 ± 4.5 years, and 8 males, 23.6 ± 3.2 years) participated in this experiment and the area of allodynia was mapped. Exclusion criteria were any current acute or chronic pain condition, a history of drug abuse, use of analgesics within one week, any tattoos or skin diseases on the relevant areas, participation in more than two back training sessions per week, participation in other clinical trials the preceding 4 weeks or during the study, pregnancy or lactation. All subjects were instructed to avoid UV exposure during the study period. The study was performed in accordance with the Declaration of Helsinki and approved by the regional Ethical Committee (N-20120067, N-20110066). All subjects received written and oral information about the study and provided written informed consent prior to the experiments.

UVB irradiation protocol

One week before the study, the individual minimal erythema dose (MED) for UVB irradiation was determined using a calibrated UVB source (wavelength 290-320 nm; Saalmann Multitester, Saalmann, SBC LT 400 Herford, Germany). The MED is the minimum amount of UVB energy (J/cm^2) that produces an erythema with distinct borders 24 h after exposure. Five circular skin spots with a diameter of 1.5 cm at the anterior surface of the right forearm were irradiated with a geometric series of UVB doses ranging from 40 to 100 mJ/cm^2. In this experiment, the skin areas were then irradiated by UVB at 3 times of the individual MED as previously described [5]. This protocol was followed similarly for both experiments I and II.

Heat rekindling protocol

A thermode stimulator of 3 x 3 cm (PATHWAY ATS, Medoc Ltd, Israel) was placed on the UVB-

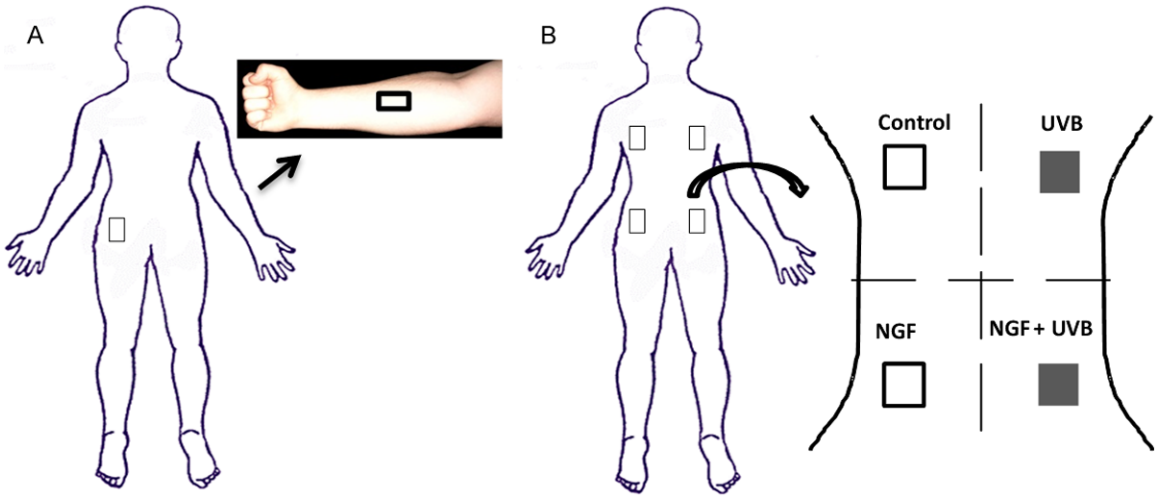

Figure 1. Representation of experimental sites for experiment I (arm and low back, A) and experiment II (low back, B).

irradiated area and kept by means of a Velcro tape. The rekindling was performed by applying 40°C for 5 min [12]. During the first 8 seconds, the temperature raises 1°C per second from a starting temperature of 32°C to a steady state temperature of 40°C. Two cycles of rekindling were performed, with 40 min interval similar to the paradigm for the combined heat/capsaicin model [12]. This protocol was followed similarly for both experiments I and II.

NGF injection

Muscle soreness in the lower back was induced in experiment II by two injections (one at each side) of NGF (0.2 ml, 5 μg/ml, sterile solutions of recombinant human NGF were prepared by the pharmacy at Aalborg hospital) performed on the erector spinae at the L4 level approximately 3.5 cm lateral to the spinous process. Dose and protocol for the NGF injections have already been used before by Hayashi and collaborators, whereas the same location has been used by Deising and co-workers [31, 32].

The day after the NGF injections, the degree of muscle soreness was evaluated in the beginning of each session, using a 7-points Likert scale anchored with 0: "a complete absence of soreness", 1: "a light soreness in the muscle felt only when touched/a vague ache", 2: "a moderate soreness felt only when touched/a slight persistent ache", 3: "light muscle soreness when lifting objects or carrying objects", 4: "a light muscle soreness, stiffness or weakness when flexing the back", 5: "a moderate muscle soreness, stiffness or weakness when flexing the back" and 6: "a severe muscle soreness, stiffness or weakness that limits my ability to move" [33].

Mapping the area of hyperalgesia

In experiment I, weight calibrated pin pricks were used to map the area of hyperalgesia. One pin was selected based on the mechanical pain threshold of each subject at baseline (one for the arm and one for the back). The pain threshold was evaluated by using a set of pin-prick with diameter tip of 0.6 mm and weights of 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 50.1 and 60.0 g. Starting from the lightest weight, each pin was applied for 2 s in the area until the subject felt that the sensation changed from "pricking" to "sharp painful pricking". Two repeated stimulations were performed with each pin-prick. The weight of the pin-prick, which induced the "sharp pricking" for both stimuli, was defined as the mechanical threshold and used for mapping of the hyperalgesic area. To map the area a custom-made template was created from a soft plastic punched map. The template included small holes, located in 8 linear sequences arranged vertically, horizontally and diagonally, which met in the center. There was 0.5 cm between each hole. The template was placed on the skin so that the place where the 8 lines meet was located in correspondence of the midpoint of the irradiation. Starting approx. 8 cm from this point, the pin stimulator was placed gently on the skin through each hole. The pin was applied for 1 second at an interval

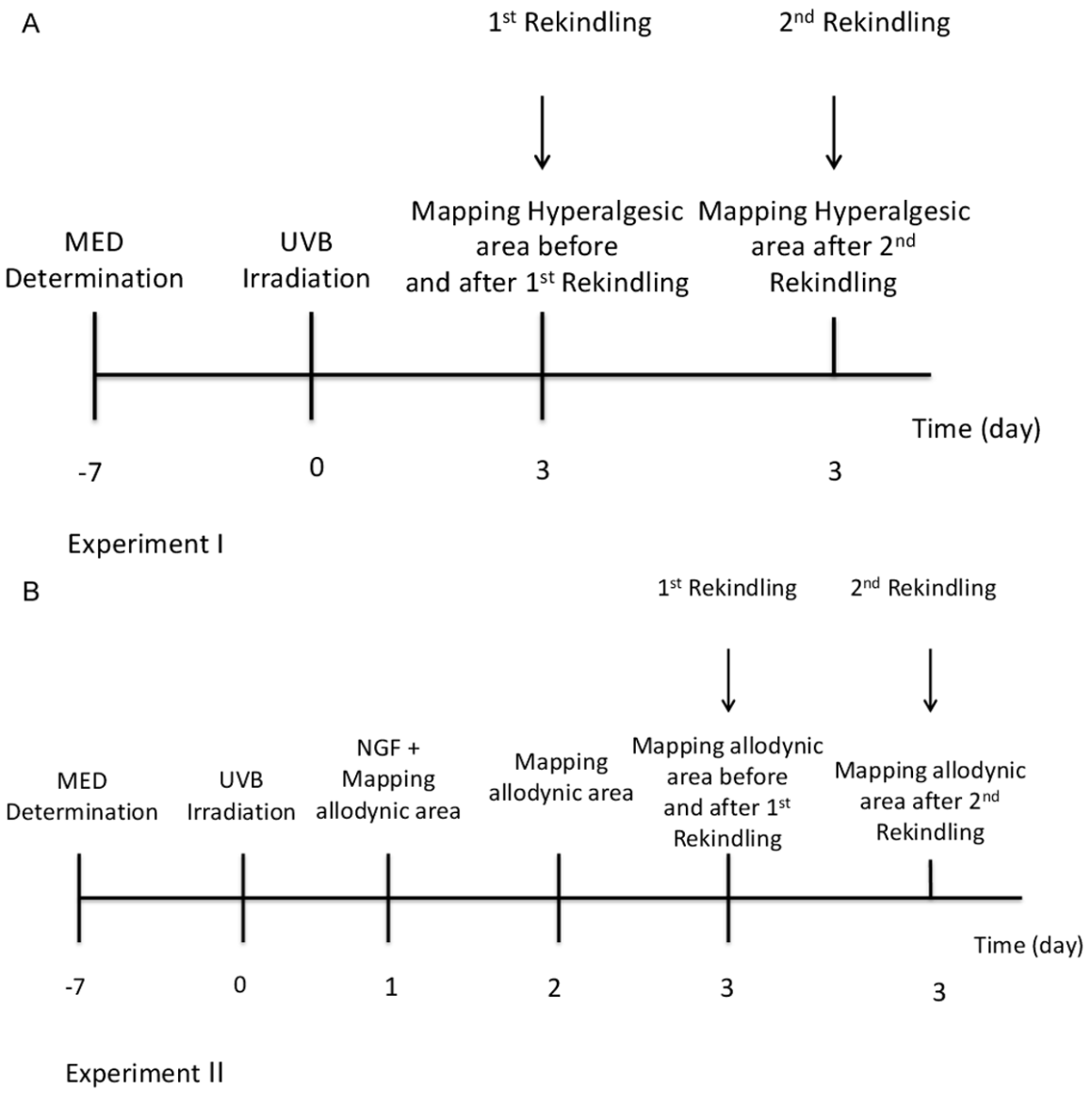

Figure 2. Schematic overview of experiment I (A) and experiment II (B).

of 2 seconds between each stimulus. The subject was asked to report when the "sharp pricking" changes into "a sharp painful pricking", or "pain", and these points were marked on the template. The hyperalgesic mapping assessment typically took approximately 10 min. The defined area was then transferred to a transparent film and the area of hyperalgesia was calculated by using the software Vista Metrix (version 1.38.0m, Skill Crest LLC).

Mapping the area of allodynia

In experiment II, a single von Frey hair stimulator (26 g) was used on a similar template as described above. The von Frey hair was placed gently on the skin through each hole and the force load applied for 1 second at an interval of 2 seconds between each stimulus. The subject was asked to report when the "tingling" changes into "a different feeling", "unpleasant", and these points were marked on the template. The allodynic mapping assessment typically took few min. The defined area was then transferred to a transparent film and the area of allodynia was calculated following the similar procedure described above.

Experimental protocol - experiment I

Two rectangular areas (3 x 4 cm) located on the low back (approximately 3-5 cm from the spinal

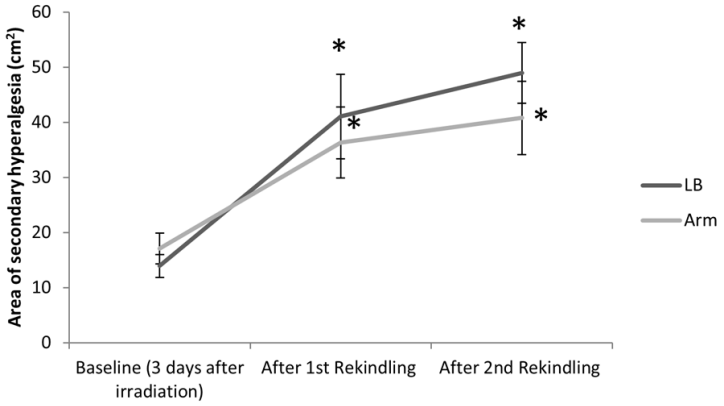

Figure 3. Mean (± SEM, N=9) area of hyperalgesia (cm^2) in the forearm and low back before and after each cycle of heat rekindling at Day 3 (*P < 0.01 compared with baselines).

cord and 8 cm from the iliac crest) and in the middle forearm were irradiated with UVB (**Figure 1A**). Three days after irradiation, two cycles of heat rekindling were performed, with 40 min interval. The area of hyperalgesia was assessed at baseline (before the first heat stimulation) and immediately after each cycle of the rekindling (**Figure 2A**). Hyperalgesic reactions inside and outside the irradiated areas until day 3 were studied with other modalities and reported elsewhere (article in preparation).

Experimental protocol - experiment II

Two rectangular areas (3 x 4 cm) on the low back (approximately 3-5 cm from the spinal cord and 2-3 cm from the iliac crest) and on the upper trapezius muscle (approximately 3-5 cm from the spinal cord and 8 cm from the clavicle) were UVB-irradiated and the contralateral mirrored area was used as control (**Figure 1B**). The side for UVB irradiation was chosen at random and balanced between subjects. The day after irradiation, the subjects received two injections of NGF (one on each side) into the erector spinae muscle at level of L4 approximately 3.5 cm lateral to the spinous process in order to induce muscle soreness in the low back. Three days after irradiation, two cycles of rekindling were performed, with 40 min interval. The area of allodynia was assessed at the day of experiment and 1, 2, and 3 days after irradiation and after each cycle of rekindling (**Figure 2B**). Hyperalgesic reactions inside and outside the irradiated areas until day 3 were studied with other modalities and reported elsewhere (article in preparation).

Statistical analysis

All values are presented as means and standard error of the mean (SEM). Statistical analysis was performed using SPSS (V19 2010, IBM SPSS ©,). The Kolmogorov-Smirnov test was used to check the normal distribution of the collected data. For the area of hyperalgesia repeated measures analysis of variance (ANOVA) was used with factors *sites* (arm and back) and *repetition* (baseline, after 1st rekindling, after 2nd rekindling). For the area of allodynia, the repeated measures ANOVA was used with factors *sites* (UVB and UVB + NGF), and *time* (1, 2 and 3 days after irradiation). Day 3 was considered as baseline for heat rekindling effect on allodynia, and repeated measures ANOVA was performed with factors of *sites,* and *repetition*. The ANOVA was followed by Bonferroni post-hoc test in case of significant factors or interactions. *P*-values less than 0.05 were considered statistically significant.

Results

All participants completed the study and no safety issues were recorded or reported.

Area of secondary hyperalgesia after heat rekindling (experiment I)

Three days following the UVB irradiation the area of secondary hyperalgesia was present in both forearm (17 ± 3 cm^2) and low back (14 ± 2 cm^2). The ANOVA of the hyperalgesia showed a time effect indicating that following the first cycle of heat rekindling, the areas were significantly enlarged both in the forearm and low back (ANOVA: $F_{2,7} = 18.9$, $P < 0.01$) compared with baseline and remained enlarged following the second cycle of the heat rekindling (**Figure 3**).

Muscle soreness and area of allodynia (experiment II)

The day after NGF injection, low muscle soreness was present in all subjects. This was based on the mean Likert scale score (4.1 ± 0.2).

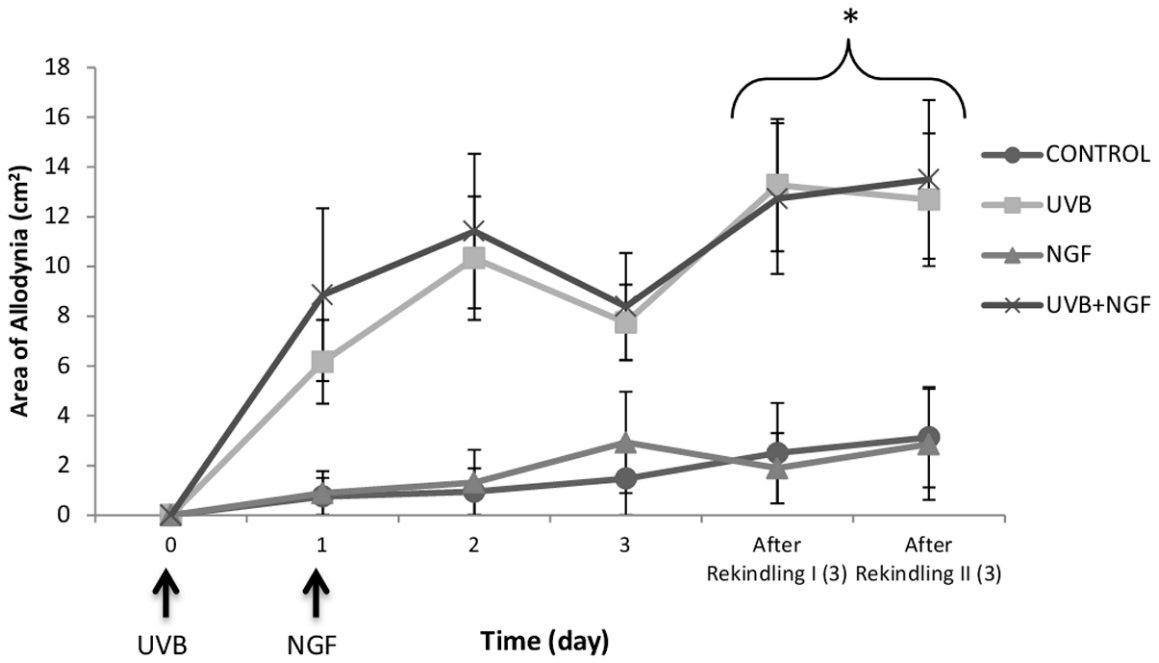

Figure 4. Mean (± SEM, N = 16) area of allodynia (cm^2) before and 1, 2 and 3 days after UVB irradiation and after each cycle of rekindling on the 4 locations. The two arrows indicate the day of UVB irradiation and NGF injection, respectively. An area of allodynia was developed 1 day after UVB irradiation and it remained present for up to 3 days. No difference was reported between days for both UVB and UVB+NGF sites. A main effect of time showed that, in the UVB and UVB + NGF sites, following the two cycles of rekindling, the allodynic area was significantly enlarged compared with baseline (3 days after irradiation) in both sites ($P < 0.01$). No difference in increase was reported between the two cycles of rekindling.

In both UVB and UVB+NGF sites, an area of allodynia was consistently developed 1 day after UVB irradiation (6.2 ± 1.7 cm^2 and 8.9 ± 3.5 cm^2 for UVB and UVB + NGF, respectively) and it remained present for up to 3 days. No difference was reported between days for both sites. Only one subject presented an area of allodynia in both the control and the NGF sites 24, 48 and 72 h after irradiation. As a consequence no statistical analysis was performed in these two locations.

Area of allodynia after heat rekindling (experiment II)

A time effect on the ANOVA of the area of allodynia after rekindling in the UVB and UVB + NGF sites showed that following the two cycles of rekindling, the allodynic area was significantly enlarged compared with baseline (3 days after irradiation) in both sites (ANOVA: $F_{2,14} = 4.1$, $P < 0.01$, **Figure 4**). No difference was reported between the two cycles of rekindling. Only three subjects presented an area of allodynia after rekindling in the control and NFG sites. As a consequence, no statistical analysis was performed in these two sites.

Discussion

The present study showed that UVB irradiation induced both areas of allodynia and hyperalgesia, which were present on the human skin independent of the site of application (low back or forearm) and that these areas were detectable at least up to 3 days following a single UVB stimulation. In addition, findings from this study demonstrated that heat rekindling could affect the areas of allodynia and hyperalgesia, regardless of the tested body region (low back or forearm). Data showed that both areas of allodynia and hyperalgesia were enlarged and prolonged by the heat rekindling. This response is valuable for testing potential analgesics that need sufficient time to modulate the hypersensitivity and pain. Another novel finding of this study was that the muscle sensitization induced by NGF did not override or potentiate the effect of UVB neither with regard to the induction of areas of allodynia and hyperalgesia; nor in response to heat rekindling.

UVB-induced areas of allodynia and hyperalgesia

The UVB model is a well-established model of peripheral sensitization, where primary allodynic and hyperalgesic responses peak between 24 and 48 h in rats and in humans [3, 5, 11]. In line with those findings, the present study showed that UVB irradiation of the skin induces both allodynia and hyperalgesia lasting up to 3 days, caused by the sensitization of peripheral nociceptors in the skin. The existence of secondary hyperalgesia in the area surrounding the UVB-irradiated site is controversial. Nonetheless, secondary allodynia and hyperalgesia were present in the current study, suggesting a potential contribution of central components in the observed hypersensitivity. This finding has been confirmed by several studies showing that the UVB model also induces secondary areas of allodynia and hyperalgesia remote from the irradiated site through induction of central sensitization both in animal and humans [1, 3, 11]. However, some studies have demonstrated that UVB induces an area of hyperalgesia restricted only to the irradiated site [5, 15]. These discrepancies among studies may be due to the different methodology used to assess secondary mechanical hyperalgesia [1]. In a recent study, Gustorff and coworkers used a 25.6 g pin-prick stimulator and were able to map, 24 h after irradiation, a large area of pin prick secondary hyperalgesia (mean ± SEM, 218 ± 32 cm^2), whereas Bishop et al. failed to show any area of secondary hyperalgesia using a 10 g von Frey filament [3, 5]. In the present study the area of secondary hyperalgesia was mapped 72 h after irradiation by the use of a pin-prick stimulator corresponding to the mechanical pain threshold before sensitization, showing an area (approximately 20 cm^2) of secondary hyperalgesia. Hence, it is not unlikely that the type of mechanical stimulator influences the findings. Changes in skin sensitivity in both primary and secondary areas may be the consequence of the inflammatory process induced by UVB irradiation. Several studies have confirmed that cutaneous irradiation may induce activation of keratinocytes, fibroblasts, and immune cells leading to release of several cytokines and inflammatory mediators, including prostaglandins (PGs) as PGE2, PGF2α, and PGD2, implicated in the sensitization of the peripheral nociceptive terminals of the sensory neurons [16, 17]. In a human study, Sycha and co-workers have shown that application of the Cyclooxygenase-2 (COX-2) selective inhibitor, Rofecoxib, could reduce the area of primary and secondary hyperalgesia induced by UVB irradiation by blocking the enzyme that is directly involved in the production of PGs [18]. The involvement of central mechanism in the UVB-pain model was also demonstrated by Gustorff and colleagues who showed a reduction of the area of secondary hyperalgesia after application of opioids [19], most likely due to block of the opioid receptors present in the central nervous system [19, 20]. In the present study, a small area of allodynia was also present in the control and NGF sites. In those areas the repetitive testing procedures may have been a source of nociception activity. Another explanation might be a contralateral site effect due the central sensitization induced by the UVB. However, there is no evidence supporting the contralateral effects of a unilateral UVB irradiation model.

The effect of heat rekindling on the area of allodynia and hyperalgesia

Several research groups tried to combine pain models in order to potentiate the effect or prolongation of the responses. Heat rekindling is one of the techniques that has been applied to enhance afferent input and promote the induction of a central sensitization. Several studies have investigated the effect of the combination of capsaicin and heat on the areas of allodynia and hyperalgesia in the skin [8, 21-23]. Those studies concluded that the combined heat/capsaicin model is a reliable and reproducible model that can be used for induction of a long-lasting state of hyperalgesia and allodynia [8, 21]. Preliminary work in both animals and humans suggests that combining heat and UVB model is also possible to enhance the maintenance or expansion of the UVB-induced hyperalgesia and allodynia. The present study was conducted in two different groups of subjects that were tested for allodynia or hyperalgesia. The results of the present study confirm that heat rekindling of a UVB-sensitized skin can enhance and stabilize both areas of allodynia and hyperalgesia-induced by the UVB irradiation. Similar responses to heat rekindling suggest that the mechanisms underlying allodynia and hyperalgesia might be similarly modulated by heat. It is known that allodynia is mediated by input coming from Aβ-fibres [1, 24, 25],

whereas the hyperalgesia is mediated by Aδ-fibres and C-fibres [1, 12, 26, 27]. These different types of fibres may respond in a similar way to the application of heat, reflecting the expansion of both allodynic and hyperalgesic areas. These results are in line with the findings of others. Davis and co-workers investigated the occurrence of secondary mechanical hyperalgesia in a rat UVB model and whether secondary hyperalgesia was enhanced by heat rekindling of the UVB-irradiated skin [1]. Using manual and automated aesthesiometer and a paintbrush, they demonstrated, for the first time in a rodent UVB model, that UVB irradiation on the plantar heel of the hind paw induced a profound secondary mechanical allodynia and hyperalgesia that peaked at 48 h following UVB irradiation and that heat rekindling of the UVB-irradiated skin further enhanced and prolonged the effect [1]. They also suggested that heat rekindling activates thermally sensitized nociceptors in the UVB-inflamed skin, resulting in an afferent barrage into the dorsal horn, which drives central sensitization [1]. In their study, no spontaneous pain was observed after rekindling, suggesting that the ongoing activity was enough to involve central mechanisms, but it was below the threshold for spontaneous pain induction [1]. The combined UVB/heat model was also validated as a translational model of inflammatory pain [11]. Only few studies have investigated the effect of the combination of UVB-pain model and heat in humans. For instance, Cookson and co-workers combined heat and UVB irradiation in human clinical trials to enhance central mechanism, concluding that the UVB model can potentially be combined with heat rekindling to increase the area of secondary hyperalgesia [10]. Based on these results, it is possible that heat rekindling may increase both Aδ-fibres and C-fibre nociceptor inputs into the spinal cord, thus, enhancing the central nervous system components' contribution and causing a more robust secondary mechanical (pin-prick) hyperalgesia and allodynia [10, 11]. Another human study has also explored the combination of UVB and heat and found that heat rekindling can induce a larger secondary mechanical hyperalgesic area compared to UVB alone [12].

This model combining the skin inflammatory sensitization induced by UVB irradiation with heat rekindling can be a suitable pain model to characterise new analgesic drugs. Wang and collaborators tested the validity of the combined UVB/heat model on detecting the efficacy of new potential antihyperalgesic drugs by Rofecoxib and the NMDA antagonist, ketamine, two drugs with different antihyperalgesic profiles [28] and found that both drugs reduced the area of secondary mechanical hyperalgesia in humans. Therefore, the UVB-heat model can be used as a valid tool to study the efficacy of both peripherally and centrally-acting analgesic drugs [28].

Effect of heat rekindling in UVB-irradiated skin and NGF-sensitized muscle

In this study the addition of muscle sensitization to the traditional UVB model seem not to change the characteristics of the UVB model, in terms of size of allodynia. It was hypothesized that the combination of deep and superficial inflammatory models might enhance the responsiveness to mechanical stimulation potentially via central hyperexcitability and convergence between superficial and deep tissue sensory input. However, data did not support this idea. The lack of this finding would not completely reject a possible interaction between the two models. Only few experimental studies have investigated the possible interaction between cutaneous and muscle hyperalgesia, reporting cutaneous hyperalgesia as a result of pain in deeper structures in e.g. osteoarthritis patients [29, 30]. So far, no studies have investigated the existence of facilitation between UVB and NGF models when applied in combination. Whether this observation is a true phenomenon due to robustness of UVB model characteristics masking the effects of NGF-induced sensitization or it is due to the NGF dose, time of application and paradigms applied in the current study is not clear and needs further investigation.

Conclusion

This study showed that UVB irradiation induced long-lasting and stable areas of allodynia and hyperalgesia. Heat stimulation of the UVB-irradiated skin enlarged the areas of allodynia and hyperalgesia. The presented combined UVB/heat model could possibly serve as a suitable experimental model to test potential analgesics that need longer duration to show the effect on pain and hypersensitivity. Both locations of low back and forearm seem to be useful test sites. Muscle sensitization induced by

NGF did not override or potentiate the effect of UVB in terms of induction of areas of allodynia and hyperalgesia nor in response to heat rekindling suggesting the robustness of the model.

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Disclosure of conflict of interest

The authors have no conflict of interest to report.

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