Muscular Heat and Mechanical Pain Sensitivity After Lengthening Contractions in Humans and Animals

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Abstract

Mechanical sensitivity of muscle nociceptors was previously shown to increase 2 days after lengthening contractions (LC), but heat sensitivity was not different despite nerve growth factor (NGF) being upregulated in the muscle during delayed onset muscle soreness (DOMS). The discrepancy of these results and lack of other reports drove us to assess the heat sensitivity during DOMS in humans and to evaluate the effect of NGF on the heat response of muscle C-fibers. Pressure pain thresholds (PPTs) and pain intensity scores to intramuscular injection of isotonic saline at 48°C and capsaicin were recorded in humans after inducing DOMS. The response of single unmyelinated afferents to mechanical and heat stimulations applied to their receptive field (RF) was recorded from muscle-nerve preparations in vitro. In humans, PPTs were reduced but heat and capsaicin pain responses were not increased during DOMS. In rats, the mechanical but not the heat sensitivity of muscle C-fibers was increased in the LC group. NGF applied to RF facilitated the heat sensitivity relative to the control. The absence of facilitated heat sensitivity after LC, despite the NGF sensitization may be explained if the NGF concentration produced after LC is not sufficient to sensitize nociceptor response to heat.

Perspective: This article presents new findings on the basic mechanisms underlying hyperalgesia during DOMS, which is a useful model to study myofascial pain syndrome, and the role of NGF on muscular nociception. This might be useful in the search for new pharmacological targets and therapeutic approaches.

Keywords: *Muscle heat sensitivity; capsaicin; Delayed-onset muscle soreness; Lengthening contraction; Nerve growth factor (NGF).*

Introduction

Muscle pain is a common condition with multiple etiologies. As an example, delayed-onset muscle soreness (DOMS) is a frequently occurring condition that usually, but not necessarily, appears after intense exercise and lengthening contractions (LC) of the muscle. It is characterized by muscle soreness on movement, but not during rest, and tenderness that appear after some delay, peak 24 - 48 hours after the exercise, and usually disappear within a week[7, 8]. DOMS in humans and its corresponding animal models have been used to study the mechanisms of muscle mechanical hyperalgesia[17, 23, 41].

Nerve growth factor (NGF) has been proposed as an important substance involved in the muscle hyperalgesia following LC. NGF is an essential neurotrophic protein involved in maintenance and differentiation of sensory and sympathetic neurons and later in adulthood in nociception and pathological pain condition[9, 28]. NGF is released from various structures including skeletal muscle tissue[2, 32]. Moreover, NGF sensitizes muscle nociceptors[29, 32, 40] and induces mechanical hyperalgesia, which lasts for up to 7 days in humans[39] and up to 2 days in rats[32]. NGF plays an important role in hyperalgesia during inflammation[19, 27, 37] . In addition to mechanical sensitization, heat sensitization has been observed in models where NGF has been injected intradermally[14, 36], and neutralization of NGF has been reported to block both heat and mechanical hyperalgesia during skin inflammation[25]. In a DOMS model, the NGF protein has been reported to be up-regulated after LC and up-regulated NGF is considered to induce mechanical sensitization since administration of an anti-NGF antibody reversed the mechanical hyperalgesia after LC[32]. There are, however no reports on the effect of NGF on muscle nociceptor heat sensitivity. Interestingly, the mechanically sensitized C-fiber afferents in the DOMS model are probably due to NGF[32] but the nociceptor sensitivity to heat is reported to remain unchanged[42]. The discrepancy of these results and the lack of other reports drove us to assess the heat

sensitivity during DOMS in humans, to reassess the heat sensitivity of primary muscle afferents after LC and to evaluate the effect of NGF on their heat sensitivity.

The mechanical hyperalgesia after LC seems to be mediated, at least partially, by the Transient receptor potential V1 (TRPV1) ion channel as TRPV1 antagonists reduce the increased mechanical sensitivity[15]. TRPV1 is known to respond to noxious heat, capsaicin, and low pH[11]. TRPV1 itself is not sensitive to mechanical stimulation, but a role for TRPV1 not only in heat but also mechanical hyperalgesia induced by muscle inflammation has been reported[43]. In humans thermally-induced muscle pain has been demonstrated by injections of warm and cold isotonic saline[18] but it is not known if this type of muscle pain is facilitated in the DOMS model.

In this study it was hypothesized that heat and capsaicin nociception was facilitated during DOMS, possibly mediated by NGF, in humans. It was also examined if muscle heat and capsaicin-induced pain and nociceptor activity are facilitated after LC in animals and if NGF can sensitize rat muscle nociceptors to heat in vitro.

Materials and methods

Human study

Subjects

Eleven healthy, adult subjects participated (6 male and 5 females); subjects aged 20 - 37 years (mean 25.8). We used a number similar to previous studies on the subject.[17] The subjects had no pain complaints or history of injuries to the lower leg and were not taking any medication. Subjects were given a detailed verbal explanation of the experimental procedure and signed an informed consent form prior to inclusion in the study. The study was conducted in Aalborg, Denmark after approval by the ethics committees of Aalborg University (N-20070042) and Nagoya University (No. 298) and in accordance with the Declaration of Helsinki.

Human experimental protocol

The experimental protocol (Fig. 1A) consisted of 4 sessions (day 0, day 3, day 4 and day 7) in which pressure pain thresholds (PPTs) were measured on the tibialis anterior (TA) muscle of both legs. DOMS was induced during the second session (day 3) and hyperalgesia development was assessed on the following sessions. On days 0 and 4 (baseline measurement and 24 h after DOMS induction, respectively) PPTs were measured before and after i.m. injections of isotonic saline at room temperature (control saline), isotonic saline heated to 48 ºC (heated saline), and capsaicin (Fig. 1A). Injections were given to the 3 most mechanically sensitive sites of the TA muscle and the same injection procedure was repeated at the anatomically corresponding site on the control leg. Selection of injection sites was done on days 0 and again in day 3 before inducing DOMS. Selection of control/DOMS (right or left leg) was randomized.

DOMS induction and assessment

DOMS was induced by LC of the TA muscle as previously reported[16]. Briefly, the subject stood on a 13 cm high metal platform, placed approximately 45 cm from a wall. Subjects were instructed to stand with the heel of the experimental leg on the edge of the platform with the mid- and forefoot extending over the edge. The palms of the subject's hands were placed on the wall for support only. After that, the subject raised the non-experimental leg over the platform to the hip and knee level and while standing on a single leg, the subject performed a slow plantar flexion of the foot and ankle allowing the forefoot to descend until the toes touched a cushion (approximately 2 cm thick) placed below the platform. At this point, the non-experimental leg was extended until weight bearing and used to assist in returning the subject to the initial starting position. This process was repeated 10 times per set for 3 sets separated by a 20 s rest period. To corroborate that the exercise was intense enough to induce DOMS, subjects were asked to heel-walk and poor performance of this task on the experimental side was used as a clinical sign of muscular fatigue, this strongly suggested the subject would develop DOMS 24-48 hours later[16], as none of the subjects was a trained athlete accustomed to intense exercise. No subject was actually successful at heel-walking without any obvious impairment. Subjects were given a modified Likert scale diary[16] to assess the self-perceived soreness at nights during all the days they were enrolled in the experiment.

Pressure algometry

The effects of DOMS on the injection-induced mechanical hyperalgesia were assessed as follows. The pressure pain thresholds were measured on ten sites bilaterally (5 on each leg), 2 cm apart, on the muscle belly of the tibialis anterior (TA) muscle. The points were located between 6 - 10 cm and 16 - 20 (depending on the subject height) cm distal to the apex patellae, to ensure that they were on the belly of the TA muscle. PPTs were measured in a random order using a computer-controlled pressure algometer (Aalborg University, Denmark)[5]. Pressure stimulation was applied perpendicularly to the

skin surface. A kapok-filled vacuum cushion stabilized the shin during the stimulation and the angle of pressure application. The probe was circular (1 cm^2), covered with firm rubber and a disposable latex sheath. The pressure stimulation was computer-controlled and increased at a constant rate (59 kPa/s). The subject stopped the pressure stimulation at PPT via a push button. The PPT was defined as "the point at which the pressure sensation just becomes painful". Measurements were repeated 3 times and the average of these values was used in further analysis. The PPTs related with the five assessment sites on each leg before the injections on days 0 and 4 were used to define the three sites on each leg with the lowest PPTs to be reassessed after the injection procedure. To ensure consistency of assessment, the measuring sites were marked with a felt tip marker on the first session and re-marked as needed, and subjects were asked to be careful of not removing these marks through the duration of the experimental protocol. The PPTs for the three sites were recorded before the first injection and 2 minutes after the end of the induced pain of the last of the 3 injections. Site selection was done on day 0 and on day 3. PPTs of the sites selected on day 3 were compared from day 3 to day 7 to assess the changes induced by the exercise.

Warm saline and capsaicin-induced muscle pain

The first of the 3 PPT sites, randomly selected, was injected over 20 seconds with 1.5 mL of sterile control saline using a 10 mL syringe with a 27 G needle. Movement of the needle in conjunction with a very slight dorsiflexion/inversion movement performed by the subject was used to determine the depth of the injection, to ensure that the saline is delivered just below the crural fascia. The second site was injected with 1.5 mL of 48 °C warm sterile isotonic saline (heated saline) according to the procedure previously described[18], using the same size of syringe, depth and injection time as before. The temperature of the remaining saline was measured immediately after the injection and confirmed to be above 47 °C. The third site was injected with 0.5 mL, 20 μg of capsaicin. The order of the injections

was the same every time, from weaker to stronger stimulus to reduce the influence of previous injections. All sterile solutions were prepared by Aalborg Hospital Pharmacy, Denmark. Injections were separated by a 2 min window after the resolution of pain induced by the previous injection. Time schedule of injections and PPT measurements is shown Fig. 1A. Subjects rated their pain intensity after each injection using an electronic visual analogue scale (VAS) in which the bottom of the scale represented "no pain" while the top was marked as "maximum pain" imaginable. The VAS output was measured from onset to resolution of pain. The VAS peak was extracted.

Animal study

Experimental animals

Seventy four male Sprague–Dawley rats (SLC, Shizuoka Prefecture, Japan) weighing 340 – 440 g (10 – 13 weeks) were used in this study. Twenty-five of them received LC 2 days before single fiber recording and provided the same number of preparations (LC group), twenty rats provided 32 preparations that served as the control (CTR group); the remaining 29 animals provided 43 preparations (from both left and right legs), divided in 21 preparations that received NGF injections and 22 that received PBS injections as control. Differences in preparation yield are due to lost preparations in failed experiments. From these animals, 100 muscle-nerve preparations were obtained. The animals were kept one to three per cage under a 12-hour light/dark cycle (light between 0700 and 1900 h or 0800 and 2000 h) in an air-conditioned room (22 – 24 °C). Food and water were available without restriction. All experimental procedures were conducted according to the Regulations for Animal Experiments in Nagoya University and Chubu University, and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions in Japan, and were approved by the Animal Care Committee, Nagoya University and Chubu University.

LC protocol

The animals received LC as described in previously[41]. Rats were anesthetized with pentobarbital sodium 50 mg/kg, administered intraperitoneally (i.p.). Rectal temperature was kept in the physiological range (37 – 38 °C) with a heating pad during the exercise period. A pair of needle electrodes were transcutaneously inserted near the right common peroneal nerve, and EDL muscle was contracted by electrically stimulating it with a current magnitude of three times the twitch threshold, frequency of 50 Hz with a pulse width of 1 ms, and stimulus period of 1 s. The EDL muscle was simultaneously stretched by a servomotor over a 1 s period and then returned to the starting position over a 3 s period. This pattern was repeated every 4 s for a total of 500 repetitions.

Electrophysiological recordings of single afferent fibers

Single fiber recordings were performed as previously described[42]. The EDL muscle was excised with the common peroneal nerve attached under pentobarbital anesthesia (50 mg/Kg, i.p.). The LC group included preparations only from the right (exercised) side (n = 25) while the CTR group (n = 32) contained preparations from both right (n = 14) and left (n = 18), the rest of the preparations were used in the NGF experiment (see below). One single nerve fiber activity was recorded from each preparation. Animals were killed in a CO_2 chamber after dissection of the preparation. The preparation was then placed in an organ bath with the tendinous ends of the EDL muscle pinned to the test chamber. The preparation temperature was maintained at 34.0 ± 0.5 °C (pH 7.4) and continuously superfused with modified Krebs–Henseleit solution (Krebs solution) containing (in mM) 110.9 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 20.0 glucose. The superfusate was continuously bubbled and equilibrated with a gas mixture of 95% O_2 and 5% CO_2. Small segments of the nerve were split repeatedly with sharpened forceps until single-unit activity could be recorded. All the action potentials were recorded with conventional method and analyzed with DAPSYS (Brian

Turnquist, http://www.dapsys.net, USA) data acquisition processor system and Labchart Software (AD Instruments, New Zeland).

Inclusion criteria for muscle thin afferents were: 1) sensitive to mechanical stimulation from probing with a glass rod, 2) no intensity-dependent increase in the discharge rate while the muscle was stretched by a length of a few millimeters, 3) conduction velocity (CV) slower than 2.0 m/s.
The size and location of the receptive fields of a fiber was mapped with a von Frey hair (0.5 mm in diameter) two grades stronger than the threshold and that consistently induced a response. Once a fiber was identified, mechanical, chemical, and thermal stimulations were applied to the receptive field in most instances following this order: 1) a ramp mechanical stimulation with a servo-controlled mechanical stimulator, 2) Krebs solution (pH 7.4), 3) heat (slow heating ramp from 34 to 50 °C), and 5) capsaicin (1 μM). Capsaicin was applied at least 10 minutes after the stimulation with heat. Other preparations used for NGF injection series were stimulated in the following order: same as 1)~3) described above, 4) NGF or phosphate buffered saline (PBS) injection into the muscle near the receptive field, and 5) heat, every 10 minutes for up to 1 hour.

Intervals between stimuli varied: When a stimulus induced no excitation, the interval before the next stimulus was set to about 5 min, and when the previous stimulus induced an excitation, more than 10 min elapsed after the end of the response before a new stimulus was started. Before each stimulation, the spontaneous activity of the fiber was calculated during a control period for 60 s immediately before the stimulation.

Mechanical stimulation

Mechanical stimulation was performed as previously described[42] using a stimulator probe with a flat round tip (size 2.28 mm^2). The stimulation ramp increased linearly during 10 seconds from 0 to 86 kPa (Fig. 1B) and was applied to the most sensitive part of the receptive field. The mechanical threshold was defined as the intensity that induced a discharge that exceeded the mean frequency plus 2 standard deviations of spontaneous discharges during the control period, when there were at least two or more consecutive discharges exceeding this level. The response magnitude was evaluated as elicited impulses per second, including all impulses from the first impulse after crossing the mechanical threshold until the last impulse before the end of the stimulation; only impulses above the mechanical threshold were considered to be a response, while all the rest were considered spontaneous activity.

Stimulation with capsaicin

After the mechanical stimulation, chemical solutions were superfused on top of the mechanically sensitive receptive field through a metal tube (2.2 mm in diameter) that went through a heat exchanging system to keep the solutions at the same temperature as the stimulation chamber. The opening of the tube was placed as close as possible to the receptive field but separated enough for the solution to flow out and that the outflow would not induce any mechanically induced response. The speed of flow was set at 5 mL/30 s. A thermocouple on the tip of the metal tube monitored the temperature on top of the receptive field. Capsaicin was prepared as stock solution at 1 mM in 99% ethanol and diluted in Krebs solution to the final 1 μM concentration.

Heat stimulation and NGF injection

For thermal stimulation, heated Krebs solution was superfused on the receptive field through the same tube that was used for capsaicin application. The solution was pre-warmed slowly though the heat-exchange system with the purpose to create a slowly increasing ramp stimulation from 34 ± 0.5 ºC to

50 ± 0.5 ºC in 30 seconds (Fig. 1C). Temperature was measured with the thermocouple placed on the tip of the superfusion tube. To avoid extreme heat stimulation-induced desensitization due to repeated stimulation an active cooling system was implemented, reducing the period of exposure to heated solution during the cooling down period to less than 30 seconds (usual time without active cooling was ~1 min).

To evaluate the effect of NGF on the heat sensitivity (NGF series) of muscle nociceptors, a series of thermal stimulations were done. Five minutes after the first thermal stimulation, NGF (0.8 μM in 0.01 M PBS, 20 μL) or PBS (0.01 M, 20 μL, as a control) was injected into the belly of the EDL muscle, approximately 2 mm away from the receptive field of the recorded afferents to avoid inducing any mechanically-dependent response during injection. Injection was done slowly during approximately 10 s. Ten minutes after the injection, thermal stimulation was repeated every ten minutes for up to one hour. One min before each heat stimulation the spontaneous activity was recorded and later this was analyzed to search for NGF induced sensitization on the fiber baseline activity.

The criteria for the temperature response threshold were the same as in the mechanical stimulation, but the response intensity was evaluated as total elicited impulses. In case the fiber did not show any discharge during the stimulation period, the fiber was considered to be non-responsive to heat, but the injection and heat stimulation were continued similar to heat-sensitive fibers. For the purpose of analysis, in these situations the heat response threshold of the fiber was considered to be above the cutoff value and the cutoff value was used as the heat response threshold.

Statistical analysis

Collected data from all the experiments was analyzed using GraphPad Prism 5 Software, Sigma Plot 11 (Systat Software, Inc) software or Statistica (Statsoft). A majority of PPT and VAS parameters passed the Shapiro-Wilk normality and Kolmogorov-Smirnov tests. These data were analysed by three-way repeated measures analysis of variance (RM-ANOVA) with the factors: *Legs (LC, control), Injection site (control saline, heated saline, capsaicin)* and *time (pre and post injections or pre and post exercise and injection)*. Pre and post injection on day 0 were analyzed separated from the rest of PPTs since the type of injection and the injected site was not the same as the one used on day 4. The modified Likert scale scores were analyzed by two-way RM-ANOVA with factors *Legs* and *Days (days 0 to 6)*. The Newman-Keul's (NK) test was used for post-hoc comparisons incorporating correction for the multiple comparisons in case of significant factors or interactions.

In animal experiment statistical analyses were performed by Mann-Whitney test on the mechanical and heat thresholds and responses after LC since most of the studied groups did not pass a Shapiro-Wilk normality test. Response percentages to heat and capsaicin were compared using a Fisher's exact probability test. Two-way RM-ANOVA on factors time and treatment (PBS or NGF) after transformation of the data to ranks, followed by a Holm–Sidak comparison if warranted was used for the heat thresholds and responses after NGF injections. A p value of <0.05 was considered significant. All data were expressed as mean ± standard error (SEM), or median and interquartile range (IQR) when appropriate.

Results

Mechanical and heat sensitivity during DOMS in humans

The ANOVA on Likert scores showed an interaction between legs and days (Fig. 2A; RM-ANOVA: $F(6) = 4.1$, $P < 0.001$). The two injection days (0 and 4) resulted in higher scores in both legs compared with Days 1, 2, 5, and 6 (NK test: $p < 0.001$). At the nights immediately after exercise (day 3) and 24 h post exercise (day 4) Likert scores of soreness were significantly higher in the LC leg when compared with the contralateral side (NK test: $p < 0.0006$), showing the presence of DOMS after LC.

Pressure Pain Thresholds after LC and injections

The analysis of PPTs at days 3 to 7 resulted in a significant interaction between leg and time (Fig. 2B; RM-ANOVA: $F(3) = 3.6$, $p < 0.02$) illustrating that on day 4-pre the PPTs in the LC leg were significantly reduced compared with the control leg and pre-exercise (day 3) and day 7 levels (NK test: $p < 0.03$), confirming the presence of mechanical hyperalgesia on the exercised leg before the 2nd injections.

Independent of injection type the PPT levels after injections were significantly reduced at day 4 compared with pre injection values at day 4 and values at days 3 and 7 (average of three injection sites are shown in Fig. 2B-right, NK test: $p < 0.02$). Likewise, the post-injection PPT values in both legs at day 0 were reduced compared with the pre-injection levels (average of three sites are shown in Fig. 2B-left, RM-ANOVA: $F(1) = 6.7$, $p < 0.03$; NK test: $p < 0.03$). There was no significant difference between CTR and LC group ($p = 0.2$)

Heat and capsaicin-induced muscle pain

The VAS scores following injections on day 0 and 4 (after LC) showed that the VAS peak after heated saline (average: 4.3 ± 0.1 cm) and capsaicin (average: 6.7 ± 0.1 cm) were higher than control saline (average: 2.4 ± 0.1 cm; Table 1), and capsaicin gave higher scores than heated saline (RM-ANOVA: $F(2) = 48.2$, $p < 0.000001$; NK test: $p < 0.0006$). No significant interactions between legs or the effect of the injections were found.

Mechanical and heat sensitivity of muscle thin-fiber afferents after LC in animals

General Data

The mean CV for all recorded fibers was 0.7 m/s (n = 57), ranging between 0.3 m/s to 1.6 m/s with most fibers below 1 m/s, making it highly probable that these were C fibers. Mean conduction velocity was 0.7 m/s (± 0.1 m/s) in the control group and this was not different from the 0.8 m/s (± 0.1 m/s) in the LC group.

Spontaneous activity was low; in the control group out of 32 fibers 8 did not show any spontaneous activity and in the LC group, 4 out of 25 did not show any activity before any stimulation. Spontaneous activity was not affected by lengthening contraction: No detectable difference was observed between the median activity of the control group (0.02 imp/s; IQR 0.0 – 0.4 imp/s) and the LC group median (0.1 imp/s; IQR 0.0 – 0.35 imp/s).

Mechanical sensitivity of muscle thin-fiber afferents after LC in animals

A sample recording of mechanical response of a muscle C-fiber is shown with stimulation pattern in Fig 1B. The stimulation with the servo-controlled mechanical stimulator showed that afferents from the LC group had a median mechanical threshold of 3.4 kPa (IQR; 1.7 – 11.7 kPa, n = 25), which was significantly lower than the median threshold of 14.3 kPa (IQR; 9.2 – 34.6 kPa, n = 32, $p < 0.001$) from

the control group (Fig. 3A). In line with the decreased mechanical threshold of the exercised group the response intensity to mechanical stimulation was higher in the exercised group (Fig. 3B; $p < 0.01$). Median response in the LC group was 6.0 imp/s (IQR; 4.4 - 12.0 imp/s, n = 25) versus 3.6 imp/s (IQR; 1.2 - 5.9 imp/s, n = 32) in the CTR group.

Heat sensitivity of muscle thin-fiber afferents after LC in animals

Of the fibers studied in the control group, 50 % (n = 16) responded to heat stimulation while 66 % (n = 14) of the LC group fibers responded, this difference not being significant ($p > 0.05$, Fischer's exact test). The heat stimulation was done with a slowly increased ramp over 30 s, as seen in the figure 1C, the median response threshold temperature in the control was 45.6 ºC (IQR 39.8 – 48.0 ºC, n = 16) non-significantly different from the LC group threshold that was 45.5 ºC (IQR 43.3 – 48.4 ºC, n = 14, p = 0.5). The median of the response magnitude to the heat stimuli in the control group was 1.0 imp/s (IQR 0.5 – 2.2 imp/s) versus 0.6 imp/s (IQR 0.5 – 2.1 imp/s) in the LC group but not significantly different ($p = 0.6$).

Response of muscle thin-fiber afferents to capsaicin stimulation after LC

Not all fibers responded to capsaicin. Out of the 14 heat sensitive afferents recorded in the LC group only 9 responded to capsaicin (64.3 %) versus 10 out of 16 (62.5 %) in the control group, with no significant difference. The response to capsaicin was variable and there was no difference in it between groups ($p= 0.1$). The median of the total impulses elicited in the control group was 0.9 imp/s (IQR 0.5 - 1.5 imp/s) vs. 1.6 imp/s (IQR 0.9 – 3.8 imp/s in the LC group. Occasionally the heat insensitive afferents also responded to capsaicin, with a 27.3 % (3 out of 11), in the LC group versus only 18.75 % (3 out of 16) in the control group, this also being non-significant (Fisher's exact test, $p= 0.7$).

Enhanced mechanical sensitivity after LC in heat-sensitive fibers

The recorded afferents were classified into two groups according to their response to heat so that their mechanical thresholds and response could be compared. The heat sensitive fibers from the LC group (n = 14) showed a lower mechanical threshold ($p < 0.05$) than the CTR group (n = 16), while the heat insensitive fibers showed similar mechanical thresholds (Fig. 4A,B). Also the response to the mechanical stimulation was higher in the LC group in the heat sensitive afferents ($p < 0.01$) (Fig. 4C). On the contrary the discharge rate induced by a mechanical stimulus in the heat insensitive fibers after LC was not different from the control fibers (Fig. 4D).

Enhanced mechanical sensitivity after LC in capsaicin sensitive fibers

The capsaicin sensitive fibers showed a decreased mechanical threshold in the LC group (median 3.2 kPa, IQR 2.2 – 11.3 kPa, n = 12) vs the control group (median 15 kPa, IQR 10.6 – 38.1 kPa, n = 13, $p < 0.01$), while the capsaicin insensitive did not show any difference between groups (control median 13.6 kPa, IQR 8.2- 22.9 kPa, n = 19 vs LC median 6.4 kPa, IQR 1.9 – 18.2 kPa, n = 13, $p = 0.2$). The mechanical response followed the same pattern in the capsaicin sensitive group and was significantly higher in the LC group (median 10.7 imp/s, IQR 5.2 – 13.6 imp/s) vs the control group (median 3.6 imp/s, IQR 1.0 – 11.0 imp/s, $p < 0.05$). The capsaicin insensitive group did not show any difference in the response to mechanical stimulation between groups (CTR median 3.6 imp/s, IQR 1.3 – 5.6 imp/s vs LC median 4.9 imp/s, IQR 4.0 – 8.0 imp/s, $p= 0.2$).

The effect of NGF on the heat sensitivity of muscle thin-fiber afferents

General data

Conduction velocities of the studied afferents were between 0.3 - 1.1 m/s with a mean CV for all recorded fibers of 0.5 m/s (± 0.2 m/s, n = 43), all of them were lower than 1 m/s and thus considered to

be C-fibers. Of all 43 recorded fibers 24 fibers responded to heat stimulation, these preparations randomly received either an NGF (n = 10) or PBS (n = 13) injection. Mechanical response was tested before any other stimulation and analysis did not show any difference in mechanical threshold (control median = 10.8 kPa, IQR 2.8 – 44.1 kPa vs NGF = 11.3 kPa, IQR 1.8 – 31 kPa; p = 0.5), or response intensity (control median = 5.1 imp/s, IQR 1.9- 8.7 imp/s vs NGF median 2.5 imp/s, IQR 1.4 – 3.4 imp/s; p = 0.2), between the groups.

Baseline spontaneous activity was low, for the control group the median was 0.07 imp/s (IQR 0.0 – 0.6 imp/s, n = 22) vs NGF median of 0.07 imp/s (IQR 0.0 – 0.33 imp/s, n = 21), not showing any significant difference before the injections (p = 0.5).

Heat sensitivity

Sample recordings of heat response in the control and NGF groups before and 30 min after NGF/PBS injection are shown in Fig. 5A, B. Baseline threshold to heat stimulation did not show any difference between the groups (Fig. 6), the control group threshold median was 39.5 °C (IQR 38.4 – 43.0 °C) vs. the NGF group threshold of 39.3 °C (IQR 36.4 – 43.7 °C, p = 0.7). The response median was 2.4 imp/s (IQR 0.4 – 3.3 imp/s) for the control vs. NGF 1.7 imp/s (IQR 0.5 – 5.0 imp/s); also not showing any significant difference (p = 0.7). Preliminary experiments suggested that repeated stimulations with heat, induced a slow desensitization of the fibers, presented as a diminished response to the stimuli and an increase of the firing threshold temperature. The afferents in the PBS-injected group followed the same pattern of gradual desensitization after repeated stimulations with heat (Fig. 6A). The NGF-injected group did not show any increase in the heat response threshold through the experiment and while the median threshold had small variations through time it never increased as drastically as the threshold in the control group. From 20 minutes after the injection, heat response thresholds were significantly higher in the control group and remained like that until the end of the experiment 60 minutes after the

injection ($F(1) = 14.1$, $p < 0.005$, Two way repeated measures ANOVA after transformation to ranks, analyzing for factors treatment and time).

Response magnitude to heat stimulation tended to decrease in both groups, but the response of the NGF group remained significantly higher than in the PBS group 20 minutes after injection and later (Fig. 6B; $F(1) = 8.3$, $p < 0.01$, two-way RM-ANOVA after transformation to ranks , analyzing for factors treatment and time).

On the heat insensitive fibers another phenomena was observed. Some fibers responded to heat after the injection of either PBS or NGF, specifically of the 10 heat insensitive fibers injected with NGF, 6 (60%) responded to heat after the injection, in a variable time frame, versus 18.2% (2 out of 9), of response after the injection of PBS. The percentage of response in the groups looks very different, but this was not considered to be statistically significant ($p = 0.17$, Fischer's exact test) probably because the sample number was too small.

Following the NGF administration no sensitization was observed in the form of a significant increase in spontaneous activity (Fig. 6C, two-way RM-ANOVA, $p > 0.05$).

Discussion

The novel findings of the present study were that heat sensitization was not observed during DOMS in humans and an animal model of DOMS, where NGF is assumed to be upregulated. Nonetheless, it was shown in rats that NGF could induce heat sensitization in muscle thin afferents.

Muscle heat sensitivity in DOMS

Mechanical hyperalgesia after LC has been reported in multiple studies in different muscles[15, 16, 41]. There is evidence that the TRPV1 channel is involved in mechanical hyperalgesia induced by LC[15]. In the DRG, this channel is expressed mainly in a small-sized neuron subpopulation that has unmyelinated C-fibers[11]. TRPV1 is sensitive to a variety of stimuli e.g. heat above 43 ºC, capsaicin, and protons[11] and is also thought to play an important role in the modulation of painful responses in neuropathic[21] and inflammatory pain conditions[3, 4]. In an effort to evaluate if sensitization of TRPV1 is involved in hyperalgesia in humans in DOMS induced after LC, the muscle afferents of the tibialis anterior muscle were stimulated with both heated saline and capsaicin, with the expectation to see an increased response to these stimuli during DOMS. As previously reported[17], DOMS was successfully induced by LC but the heat and capsaicin induced muscle pain was not significantly increased in the DOMS condition.

The muscle pain sensitivity was increased by all the injection paradigms. PPT measurements were done before and after all injections; this might difficult the evaluation of the effect of individual substance. Still, injection volume was small and the spots distanced from each other as to allow comparison. The PPTs decreased in both the control and exercise legs, regardless of the injected substance on day 0, but no further decrease after DOMS induction compared with the control leg was observed. It is possible

that the mechanical hyperalgesia induced by the injections causes a decrease in the PPTs so drastic that there is not enough room to observe a summation effect between the DOMS and the injection induced mechanical hyperalgesia.

The subjects reported similar pain intensities to heat and capsaicin in both the exercised leg and the control and in both pre- and post-exercise conditions. Previous report confirms that at this temperature (48°C), isotonic saline induces a painful response[18] and while the injection of warm isotonic saline indeed induced a painful sensation greater than the one induced by the isotonic saline at room temperature in the present experiment, it was not potentiated in DOMS. It is possible that the heated saline used in this experiment was not warm enough to induce any response from the TRPV1 channel, but testing higher temperature stimulation was impossible due to the risk of tissue damage.

The injection of capsaicin, even in very small volumes and concentrations, causes an almost immediate painful response[6, 44]. The maximum VAS scores elicited by the capsaicin injection were similar in both control and exercised group and before and after the exercise. This could be because the pain induced by capsaicin was so high even in the control leg, it did not allow any room in the scale to show any significant difference with the exercised leg.

Muscle heat sensitivity of thin fiber afferents in animals following LC

Mechanical sensitization of muscle C-fibers after LC developed in the present experiment in concordance with previous reports[42], although the average threshold was lower than the previously reported. This result strongly suggests that the muscle preparations from the exercised group were under a DOMS-like condition, thus making this setup adequate to evaluate if individual afferents were sensitized to heat by the previous exercise. However, we were unable to detect any difference in heat

sensitivity between the exercised and the control preparations. This was in line with the present human experiment and the previous report[42]. The response to capsaicin stimulation was also similar in both groups. Although TRPV1 has been linked directly to heat hyperalgesia[38, 43], this was done in inflammatory pain models and no current studies evaluate the presence of heat hyperalgesia under DOMS-like conditions. The specific model used in this study does not show histological evidence of inflammation[15].

C-polymodal afferent fibers are the main players in mechanical hyperalgesia after LC

Interestingly it was the heat sensitive afferents, 52% of the recorded afferent fibers, which were sensitized to mechanical stimuli; while the heat insensitive fibers did not show differences in their mechanical thresholds or responses. This is in line with previous reports suggesting that C-polymodal fibers are the ones mainly involved in the mechanical hyperalgesia in DOMS[22, 42]. To add support the capsaicin sensitive fibers also showed decreased mechanical thresholds after LC and their mechanical response was also increased when compared with the controls. These capsaicin sensitive fibers must express TRPV1 and their role in DOMS mechanical hyperalgesia is accentuated when it is taken into consideration that an injection of a selective antagonist of TRPV1 completely reversed the mechanical hyperalgesia after LC[15], and when TRPV1 fibers are selectively killed by neonatal capsaicin application[24, 33], the animals do not develop mechanical hyperalgesia after exercise[26]. Together, these findings show that the polymodal C-fibers are the main target of the hyperalgesic effects of LC. How TRPV1 is related with mechanical sensitization and thus mechanical hyperalgesia is to be studied.

NGF increases heat sensitivity of C-polymodal afferents

The previous results suggest that C-polymodal afferents are mainly responsible for increased mechanical sensitivity after eccentric exercise[42]. NGF does not alter the mechanical response in muscle

thin-fiber afferents immediately after intramuscular injection[20], but after 20 minutes it causes increased mechanical sensitivity for at least 2 hours[32]. NGF content in exercised muscle is elevated after LC seen as a rise in NGF mRNA and protein after LC[32]. TRPV1 is thought to be part of this sensitization pathway[15, 34] and the effect of NGF on the heat sensitivity of muscular thin fiber afferents was investigated. The results showed for the first time that NGF can sensitize muscle afferents to heat. The mechanism of heat sensitization is still unclear. Possible mechanisms would include an NGF-induced phosphorylation of TRPV1 through phosphatidylinositol-3-kinase and protein kinase C[10], translocation of TRPV1 channels to the cell membrane[46] or an increase of the expression of TRPV1[38, 45].

The observation of activity in afferents that were originally insensitive to heat stimulation may also indicate clues to the mechanisms of heat hyperalgesia. Although it was not significant, a large percentage of the heat insensitive afferents exposed to NGF responded to heat at some time point. This suggests that either the fiber has the ability to convert to a polymodal type or that these fibers were part of a TRPV1-expressing fiber population and the TRPV1 channel indeed experienced a fast translocation to the membrane, giving the afferent the ability to detect heat at almost noxious temperatures. The reason for the lack of initial response of these fibers might be that the stimulus temperature was not high enough, or it could suggest that in these fibers TRPV1 gets recruited only under the influence of neurotrophic factors.

Different effects of DOMS and NGF on heat sensitivity of muscle nociceptors

Although the effect of NGF on heat sensitivity of muscle thin fibers was clear when injected directly into the muscle, we failed to observe changes in the sensitivity in both DOMS models, human and animal. When injected intramuscularly, NGF decreases the mechanical withdrawal threshold of rats in a dose dependent manner[32]; if the mechanisms and pathways for the effect of NGF on heat sensitivity

are similar, heat sensitization could also be induced in a dose dependent way. If this is the case, NGF released by LC might be too little and insufficient to cause a detectable heat sensitization. This is one possible explanation for this discrepant observation.

It is important to consider that in the DOMS setting, GDNF may also play an important part in the sensitization of nociceptors[30]. NGF and GDNF collaborate in inducing pronounced mechanical hyperalgesia[31] although they are thought to work on different sets of nociceptive neurons[35], but we do not know whether they collaborate also in thermal hyperalgesia. DOMS seems to require the involvement of both pathways to occur[31]. Interestingly, in GDNF over-expressing mice the TRPV1 expression in DRG decreases and there is no thermal hyperalgesia. At the same time there is an increase in TRPA1 channel expression[1]. TRPA1 has a role in mechanosensation[12, 13] and may also contribute to increased mechanical sensitivity in thin-fibers. This partial contribution of TRPA1 to the mechanical sensitivity could explain why mechanical sensitization was observed while heat sensitization wasn't present in DOMS in humans or after LC in rats. Possible interaction between NGF and GDNF in thermal hyperalgesia and the involvement of TRPA1 in DOMS are open for future study.

Conclusion

Heat sensitization was not observed during DOMS in humans and after LC in animals where NGF is upregulated according to previous findings. However, this study showed that in rats NGF can induce heat sensitization in muscle thin afferents. The mechanism behind this effect is still unclear, and new potential pathways need to be explored to better understand the role of NGF and TRPV1 in DOMS.

Disclosures

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Legends for figures

Figure 1. Experimental protocols and stimulation procedures.

A. Protocol timeline for the injections and assessments. B. Sample of a single fiber activity responding to mechanical stimulation and stimulus recording. C. Sample of a single fiber activity responding to thermal stimulation and stimulus recording.

Figure 2. DOMS development in human subjects.

A. Likert Scale scores over the period of the experiment. Mean ± SEM (n = 11). *** $p < 0.001$, compared to the contralateral side; # $p < 0.05$, compared to days 1, 2, 5 and 6 (NK test). Note that the scores on the day of DOMS induction (at night of day 3), and 24 hours after (day 4) were significantly higher than on the contralateral non-exercised leg. On day 0 and 4 both sides show a higher than usual score which is probably because the scale was reported at night after the experimental session in which saline and capsaicin were injected. **B.** PPT values averaged over all injected sites during the 4 days of the experiment. Mean ± SEM (n = 11). * $p < 0.03$, compared with pre-injection values day 0 or day 4-preinjection, day 3, and day 7; # $p < 0.02$ compared with day 4 pre values on the control leg and days 3 and 7 on the LC leg (NK test) . Note that the PPTs became significantly lower on day 0 in both legs after the injections. The PPTs were also decreased in the LC group 24 hours after exercise, then returned to their original values 72h after the exercise.

Figure 3. Mechanical sensitivity of single fibers after LC.

Median and IQR, N in parentheses under the groups. **A.** Mechanical thresholds of recorded afferents. The response threshold was significantly lower in the preparations taken from exercised muscles 2 days after LC (*** $p < 0.001$, Mann-Whitney test) **B.** Response intensity to mechanical stimulation. As the

thresholds decrease, the response intensity on the same afferents was higher in the LC group (** $p < 0.01$, Mann-Whitney test). Both result confirm the presence of mechanical sensitization.

Figure 4. Change in mechanical sensitivity in heat sensitive and insensitive fibers after LC. Median and IQR, N in parentheses under the groups. Mechanical Thresholds and Responses grouped by heat sensitivity. **A&B.** Mechanical thresholds; A, heat sensitive afferents; B, heat insensitive afferents. **C&D.** Response magnitude to mechanical stimuli in impulses/second; C, heat sensitive afferents; D, heat insensitive afferents. Heat sensitive afferents showed a decrease in the thresholds and an increase in the response magnitude to the mechanical stimuli after LC while the heat insensitive group did not show any change either in the threshold or the response magnitude. (* $p < 0.05$, ** $p < 0.01$; Mann-Whitney test)

Figure 5. Response to heat using a slowly increasing stimulation ramp.
A. Sample recording of responses to heat stimulations before and 30 minutes after an i.m. injection of PBS. **B.** Sample recording of responses to heat stimulations before and 30 minutes after an i.m injection of NGF. In the PBS injected preparation the response temperature threshold was increased after 30 min post-injection while in the NGF injected preparation the response threshold was actually lower than on the baseline and the response magnitude was slightly increased.

Figure 6. NGF facilitated the response to thermal stimulation.
A. Change in the heat threshold over time. Medians (CTR n = 13, NGF n = 10). **B.** Response magnitude to heat, as net increase in discharge rate (imp/s) induced by the heat stimulation. **C.** Discharge rate of spontaneous activity before and after the NGF injection. Signiticant difference between groups as a whole are shown at the right side of each graph, ** $p < 0.01$. Significant difference

at each time point is also shown, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the CTR value at the same time point. Two way repeated measures ANOVA after transformation to ranks with Holm-Sidak post-hoc test. IQRs are not included in the graphs because it oversaturated them and made them hard to read.