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Topical thermal therapy with hot packs suppresses physical inactivity-induced mechanical hyperalgesia and up-regulation of NGF

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Abstract We focused on the analgesic effect of hot packs for mechanical hyperalgesia in physically inactive rats. Male Wistar rats were randomly divided into four groups: control, physical inactivity (PI), PI + sham treatment (PI + sham), and PI + hot pack treatment (PI + hot pack) groups. Physical inactivity rats wore casts on both hind limbs in full plantar flexed position for 4 weeks. Hot pack treatment was performed for 20 min a day, 5 days a week. Although mechanical hyperalgesia and the up-regulation of NGF in the plantar skin and gastrocnemius muscle were observed in the PI and the PI + sham groups, these changes were significantly suppressed in the PI + hot pack group. The present results clearly demonstrated that hot pack treatment was effective in reducing physical inactivity-induced mechanical hyperalgesia and up-regulation of NGF in plantar skin and gastrocnemius muscle.

Keywords Physical inactivity · Mechanical hyperalgesia · Nerve growth factor · Thermal therapy · Analgesia

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Introduction

Persons who have health problems treated by casting or bed rest often become physically inactive. However, this may cause undesirable side effects. A recent study showed that long-term bed rest induced disuse atrophy, muscle damage, and pain [1]. In other physically inactive conditions, casting also induced disuse atrophy, joint contracture and cutaneous mechanical and/or cold hyperalgesia in human and animal studies [2–5]. These studies suggest that some physically inactive conditions induce some pain conditions. Muscular atrophy caused by cast immobilization induces the infiltration of macrophages in the skeletal muscle [6]. Macrophages release nerve growth factor (NGF) [7]. NGF is known to be involved in cutaneous tactile allodynia and muscular mechanical hyperalgesia in some disease states [8–13]. An increase in NGF protein in the plantar skin and the up-regulation of NGF positive neurons in the dorsal root ganglions (DRGs) have also been shown to be induced by cast immobilization [14–16]. Furthermore, we have reported that physical inactivity increases the amount of NGF protein in the gastrocnemius muscle [17]. These results suggest that NGF may play a pivotal role in the skin and muscular mechanical hyperalgesia induced by physical inactivity.

Exercise and manual therapy have been reported to be highly effective analgesic therapies in clinical studies [18–21]. However, pain induced by joint movement in physically inactive patients may limit their exercise or the manual therapy they can receive. Thermal therapy is also well known to be useful for treating pain in humans [22–25]. Innocuous heat stimulus increases the tissue temperature, resulting in increased blood flow and tissue metabolism [26]. Increasing blood flow facilitates the supply of nutrients and oxygen to the site of wounds, and

results in tissue healing [27, 28]. Tissue healing is believed to accelerate pain reduction. However, the histological and biochemical/molecular changes induced by innocuous heat treatment have not been well studied, and the mechanism of this analgesic action remains largely unknown. In the present experiment, we investigated the effects of thermal treatment with hot packs on mechanical hyperalgesia and the expression of nerve growth factor in the skin and muscle in an animal model of physical inactivity. A preliminary result has been presented in abstract form [17].

Materials and methods

Animals

All experiments were conducted with the approval of the animal experiment committees of Nagoya Gakuin University (approval number: 2007-004) and Kanazawa University (approval number: AP-153588), and in accordance with the Ethical Guidelines of the International Association for the Study of Pain.

Male Wistar rats ($n = 38$, aged 8 weeks) weighing 260 g at the beginning of the experiments were used in this study. These rats were randomly divided into four groups: naive control (CON, $n = 10$); physical inactivity (PI, $n = 10$); physical inactivity and sham treatment (PI + sham, $n = 8$); and physical inactivity and hot pack treatment (PI + hot pack, $n = 10$). The rats were housed in plastic cages with an ambient temperature of 23 ± 1 °C and maintained on a 12-h light/dark cycle. They had free access to food and water.

Physical inactivity model

Rats in the PI, PI + sham, and PI + hot pack groups were cast immobilized for 4 weeks. They were anesthetized with isoflurane (2–4% in the air, delivered through a mask, Univentor, Zejtun, Malta), and their two hind limbs were encased by plaster casts (ALCARE Co, Ltd, Tokyo) in full plantar flexed position for 4 weeks (Fig. 1a, b). These casts were removed for about 2 h every day for 5 days per week under anesthesia for hot pack/sham application and withdrawal threshold measurements, after which the casts were put back on (Fig. 1d).

Treatment

After the temporary cast removal, the rats in the PI + sham and the PI + hot pack group were treated with a hot pack (Hildpack-L, Maruho Corporation, Osaka, Japan). The rats

in the PI + hot pack group were anesthetized with isoflurane and laid on a hot pack that had been heated in hot water (50 °C) with their right side down (Fig. 1a, c). The rats in the PI + sham group were treated with a hot pack of the room temperature as a control. These treatments were performed for 20 min 5 times per week.

Skin temperature measurement

We measured skin temperatures using infrared thermometers (HORIBA, Ltd, Kyoto, Japan) during the casting period. The skin surface temperature was measured shortly after cast removal (within 60 s) and then once a week under anesthesia at the heel and the head of fibula in the right hind limb in each group. Apart from this, to get a preliminary rough estimate of the temperature change induced by hot pack treatment, the skin temperature was measured once before and after the hot pack ($n = 10$) or sham treatment ($n = 8$) in the naïve rats.

Mechanical withdrawal threshold measurements

After cast removal and treatment, we measured the mechanical withdrawal threshold (Fig. 1d). Rats were placed in a modular animal enclosure with a wire mesh bottom ($20 \times 20 \times 13.5$ cm), and acclimated for 20–30 min before being subjected to the von Frey test. After acclimation, mechanical stimulus was delivered to the plantar surface of the right hind paw by pressing a 0.5-mm-diameter filament using a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) positioned beneath the bottom of the enclosure. The filament delivered an increasing upward force from 0 to 490 mN over 10 s. The force at which a paw withdrawal response occurred was recorded. Measurements were repeated 5 times at 2-min intervals. The minimum and maximum values were excluded, and the average of the remaining three values was calculated and taken as the withdrawal threshold of the skin.

A Randall–Selitto analgesiometer (Ugo Basile, Comerio, Italy) equipped with a probe of tip diameter 2.6 mm was used to measure the withdrawal threshold of the right gastrocnemius muscle. Use of a large diameter probe enabled measurement of the withdrawal threshold of the deep tissue [29]. The nociceptive threshold was defined as the force that induced a withdrawal response to an increasing pressure stimulus from 0 to 2450 mN. Measurements were repeated 5 times at 1-min intervals, and the average of three values after excluding the minimum and maximum values was taken as the withdrawal threshold of the rat in each experimental session.
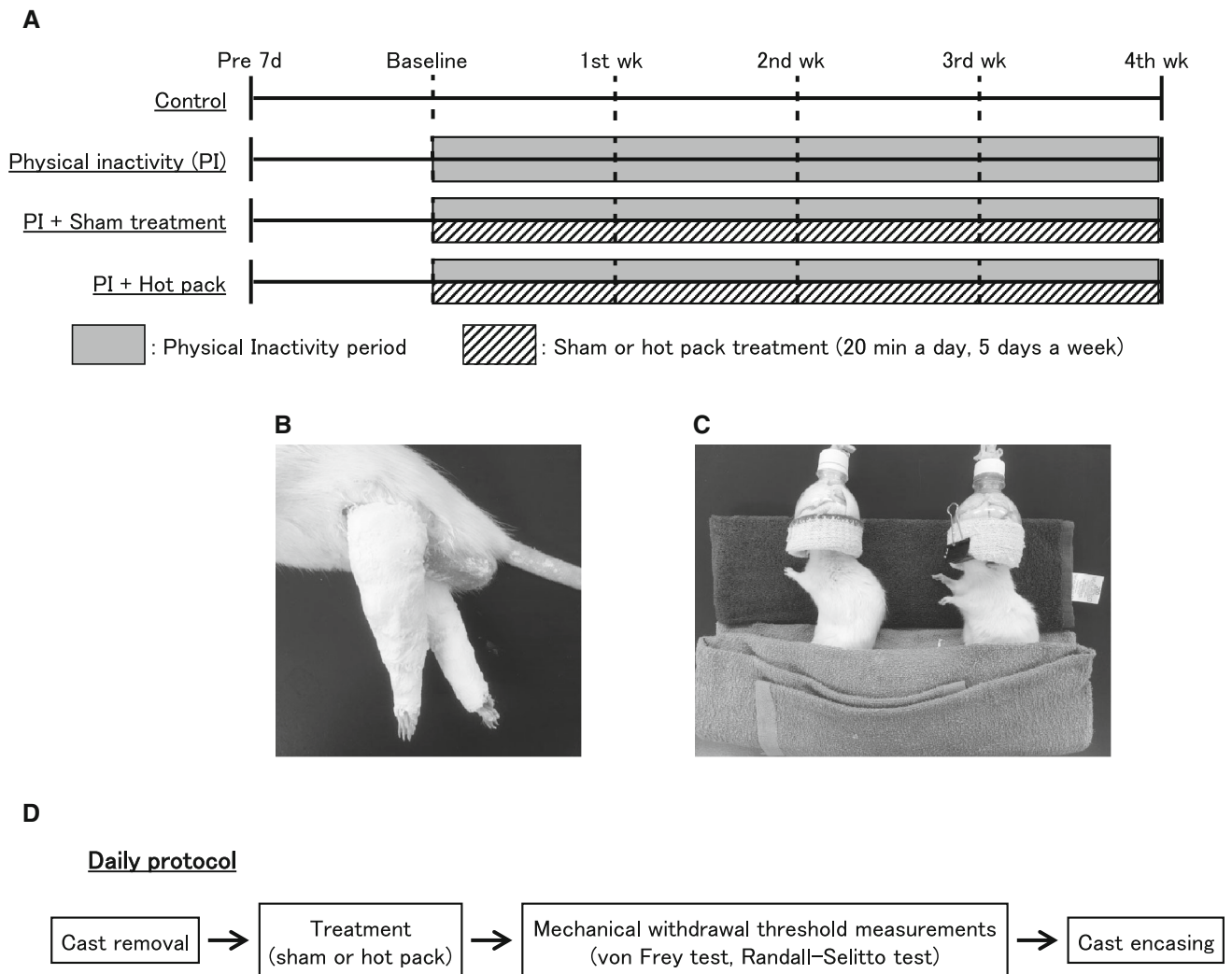
Fig. 1 Hot pack treatment protocols for the experimental groups. **a** Treatment groups and time schedule of the treatment. Rats were divided into four groups: naïve control (CON), physical inactivity (PI), PI + sham, and PI + hot pack. **b** Photograph of casted hind limbs. Both hind limbs of rats in the PI, PI + sham, and PI + hot pack group were immobilized in full plantar flexed position using plaster casts for 4 weeks. **c** Photograph showing how hot pack was applied to rats. PI + sham and PI + hot pack groups were anesthetized with isoflurane and placed in a left lateral position on the hot pack and covered for 20 min a day, 5 days per week. **d** Daily protocol. Firstly, casts were removed from rats in the PI + sham and the PI + hot pack groups. Secondly, they received the sham or hot pack treatment. Next, their mechanical withdrawal threshold was examined. Finally, casts were put back on both hind limbs. In the PI group, the withdrawal threshold was measured after cast removal

Enzyme-linked immunosorbent assay for NGF

At the end of experiment, the plantar skin and gastrocnemius muscle were excised under anesthesia (pentobarbital sodium, i.p. 50 mg/kg) and quickly frozen in liquid nitrogen. The frozen plantar skin and gastrocnemius muscle were crushed into small pieces using a pestle and mortar. The pieces of the plantar skin and gastrocnemius muscle were homogenized 1:10 (w/v) in lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitor in distilled water). Homogenates were centrifuged for 15 min at 15,000 rpm at 4 °C. Supernatants were preserved at − 80 °C until assay. NGF protein levels were measured using a rat beta-NGF DuoSet ELISA kit (DY556, R&D Systems, Inc., Minneapolis, MN, USA) and total protein levels using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturers' protocols.

Statistical analysis

All data are presented as the mean ± SEM. Statistical analyses were performed using SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). Skin temperatures and withdrawal thresholds were analyzed by two-way repeated measures analysis of variance (ANOVA) followed by

Holm–Sidak multiple comparison tests. NGF protein was analyzed by one-way ANOVA followed by Holm–Sidak multiple comparison test. Results were considered statistically significant when $p < 0.05$.

Results

Change of skin temperature after hot pack therapy

The skin temperatures at the heel and the head of the fibula in each group are chronologically shown in Table 1. There was no main effect of interaction and no significant difference in the temperatures among the four groups or five measurements at the heel ($p > 0.05$, respectively) although there was a significant difference among four groups at the head of the fibula ($p < 0.05$). However, there was no main effect of interaction ($p > 0.05$).

In a preliminary experiment using some naïve rats, the skin temperature was separately measured before and after the hot pack and sham treatments. After treatment, the temperature of the skin at the right heel remained at almost the same level (29.9 ± 1.2 °C) with the sham treatment of naïve rats ($p > 0.05$) and increased to 35.1 ± 0.6 °C with the hot pack treatment of naïve rats ($p < 0.05$, Fig. 2). The temperature of the skin at the head of the right fibula was 31.3 ± 1.3 °C with the sham treatment of naïve rats and 35.7 ± 0.5 °C with the hot pack treatment of naïve rats. The skin temperature after treatment with the hot pack treatment of naïve rats was significantly higher in both the heel and the head of the fibula compared with the sham treatment of naïve rats ($p < 0.05$).
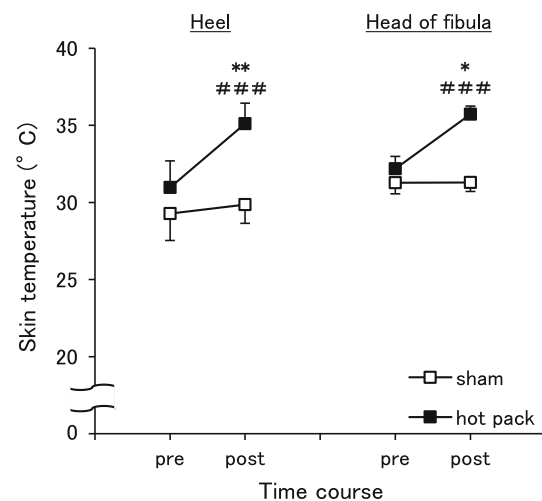
Fig. 2 Changes in skin temperature after treatment. The skin temperatures of the right heel (left graph) and the head of fibula (right graph) were significantly increased after the hot pack treatment compared to the sham treatment. *$p < 0.05$ and **$p < 0.01$ vs the sham treatment. $^{###}p < 0.001$ vs pre within the hot pack treatment

Hot pack therapy suppressed the development of mechanical hyperalgesia induced by physical inactivity
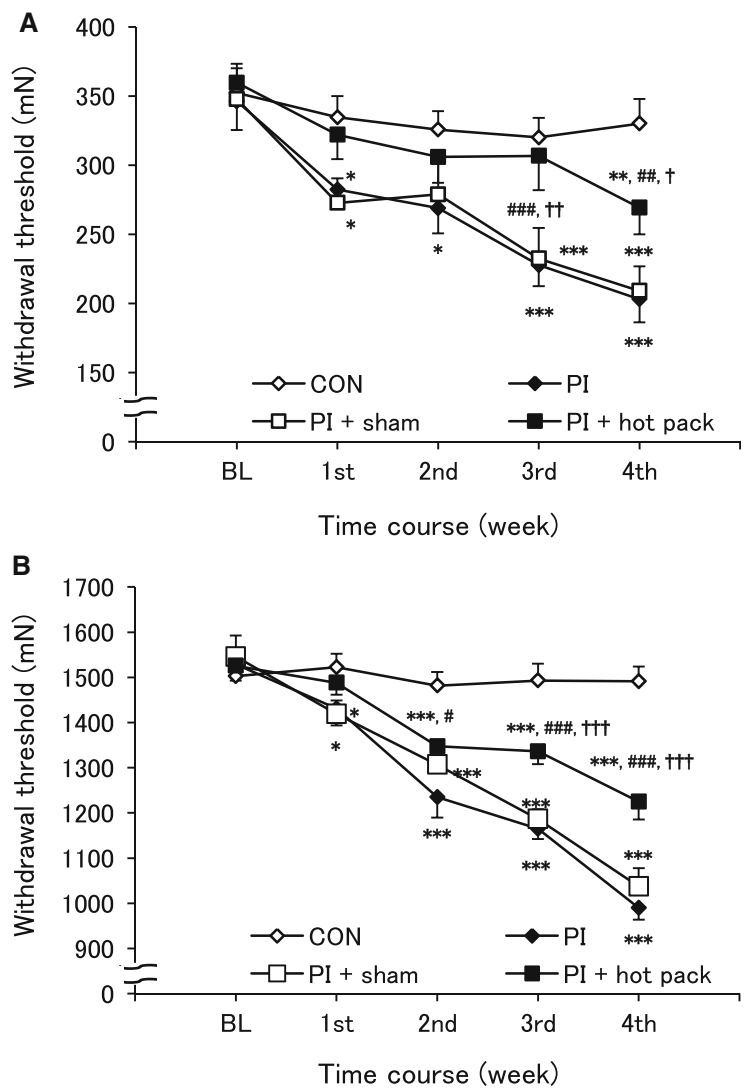
There were significant differences among the four groups or five measurements for the mechanical withdrawal thresholds of the plantar skin ($p < 0.001$, respectively). There was also a significant interaction between groups and measurements ($p < 0.01$). The mechanical withdrawal thresholds of the plantar skin in the PI group gradually decreased from 1 week after casting compared with baseline (BL), while that in the CON group remained unchanged ($p < 0.05$–0.001, Fig. 3a). The withdrawal threshold in the PI + sham group decreased similarly to that in the PI group. The withdrawal thresholds of both groups were significantly lower than that of the CON group

Table 1 Skin temperatures before treatment at the heel and head of fibula in each group

	Group	Skin temperatures				
		Baseline	1st week	2nd week	3rd week	4th week
Heel	CON	30.2 ± 1.3	28.3 ± 0.9	25.9 ± 0.6	27.9 ± 1.3	26.6 ± 1.1
	PI	29.5 ± 1.4	28.2 ± 1.1	27.3 ± 0.6	27.4 ± 1.0	25.5 ± 0.7
	PI + sham	29.2 ± 1.8	28.1 ± 1.4	28.7 ± 1.2	28.6 ± 1.9	25.3 ± 0.3
	PI + hot pack	31.1 ± 0.9	28.2 ± 1.1	25.7 ± 1.1	29.8 ± 1.5	27.4 ± 0.9
Head of fibula	CON	33.8 ± 0.2	33.6 ± 0.2	33.0 ± 1.0	33.9 ± 0.2	33.7 ± 0.4
	PI	33.5 ± 0.6	32.2 ± 0.6	31.5 ± 0.8	32.2 ± 0.3	31.7 ± 0.5
	PI + sham	31.9 ± 0.8	32.1 ± 0.6	31.5 ± 0.6	32.6 ± 0.6	31.7 ± 0.4
	PI + hot pack	33.8 ± 0.3	33.4 ± 0.6	32.6 ± 1.1	33.1 ± 0.7	32.4 ± 0.5

We observed the chronological change of skin temperatures due to physical inactivity or treatment. Skin temperatures were taken soon after cast removal. Skin temperatures are expressed as mean ± SEM. There were no significant differences between any groups during the physical inactivity period

Fig. 3 Hot pack treatment attenuated the physical inactivity-induced cutaneous and muscular mechanical hyperalgesia. **a** von Frey test and **b** Randall–Selitto test were performed to measure the cutaneous and muscular mechanical withdrawal thresholds from baseline (BL) to 4th week. The decrease in the cutaneous and muscular mechanical withdrawal thresholds in the PI and PI + sham groups was not seen to the same extent in the PI + hot pack group. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs CON group. $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ vs PI group. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, and $\dagger\dagger\dagger p < 0.001$ vs PI + sham group


at all time points except the baseline. In contrast, the cutaneous mechanical withdrawal threshold of the PI + hot pack group showed almost no decrease except in the 1st week, and there were significant differences from those in the PI and PI + sham groups. It should be noted that the threshold of the PI + hot pack group was not significantly different from that of the CON group except in the 4th week. In the 4th week, the threshold of the PI + hot pack group was lower than that of the CON group, yet it was still significantly higher than those in the PI and the PI + sham groups.

In the gastrocnemius muscle, there were also significant differences in the mechanical withdrawal threshold among the four groups or five measurements or interaction ($p < 0.001$, respectively). The mechanical withdrawal threshold in the PI group significantly decreased in a stepwise fashion from the 1st week to the 4th week ($p < 0.001$, Fig. 3b). That in the PI + sham group

decreased similarly. The muscular mechanical withdrawal threshold of the PI + hot pack group also decreased but, unlike the skin withdrawal threshold, it was significantly higher than those of the PI (except at the 1st week) and PI + sham (at the 3rd and 4th weeks) groups, and significantly lower than that of the CON group except in the 1st week.

Hot pack therapy suppressed the physical inactivity-induced up-regulation of NGF

At the end of the physical inactivity period, NGF protein in the plantar skin and gastrocnemius muscle were measured, and there were significant differences in NGF protein in the plantar skin ($p < 0.01$) and gastrocnemius muscle ($p < 0.001$) among the 4 groups. It was significantly increased in the PI and the PI + sham groups compared with the CON group ($p < 0.05$–0.001, Fig. 4a, b). No
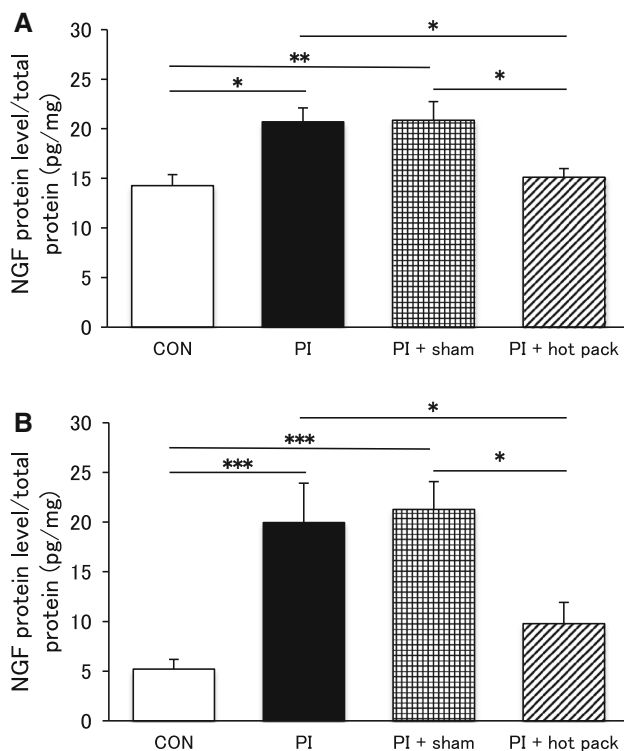
Fig. 4 Hot pack inhibited the up-regulation of NGF in the plantar skin and gastrocnemius muscle. NGF protein levels of the **a** plantar skin and **b** gastrocnemius muscle in the PI + hot pack group were significantly lower than the PI and the PI + sham group. No significant difference was observed between the PI and the PI + sham group. This elevated NGF level was suppressed by hot pack treatment. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs each other

significant difference was observed between the PI and the PI + sham groups. In contrast, NGF in the PI + hot pack group was significantly lower than that in the PI and PI + sham groups ($p < 0.05$), and it was not different from that in the CON group, indicating that the increase in NGF as a result immobility was impeded by the hot pack.

Discussion

Rats with both hind limbs immobilized by cast were used as a physical inactivity model in this study. It has been previously reported that this model shows muscle atrophy and joint contracture that are similar to changes induced by bed rest [30]. Thus, our model mimics a bed rest condition. Physically inactive rats showed decreased cutaneous and muscular mechanical withdrawal thresholds, which are conditions compatible with human cutaneous and muscle hyperalgesia. We measured withdrawal threshold in the gastrocnemius muscle using a 2.6-mm-diameter probe. A previous study showed that a large probe can measure deep tissue withdrawal thresholds excluding the effect of skin [29]. In this study, our results show muscle withdrawal

threshold but not skin withdrawal threshold. They also showed up-regulation of NGF in the plantar skin and gastrocnemius muscle. It has been reported that NGF is responsible for mechanical hyperalgesia [8, 9, 11]. Murase et al. reported that the extensor digitorum longus (EDL) muscle of rats with delayed onset muscle soreness showed mechanical hyperalgesia, that NGF was up-regulated in a time course compatible with mechanical hyperalgesia, and that this mechanical hyperalgesia was reversed by the intramuscular injection of anti-NGF antibody [31]. We also previously reported that muscular mechanical hyperalgesia induced by physical inactivity was suppressed by anti-NGF antibody [17]. In addition to that result, our present finding that physical inactivity induced an increase in NGF protein and muscular mechanical hyperalgesia in the gastrocnemius muscle indicates that NGF contributes to physical inactivity-induced mechanical muscular hyperalgesia.

Furthermore, we found that hot pack treatment inhibited, though not completely, physical inactivity-induced cutaneous and muscular mechanical hyperalgesia, and that the hot pack also reduced the up-regulation of NGF protein levels. In contrast, NGF in the PI + sham group was not different from that in the PI group. We examined whether hot pack stimulation is noxious or innocuous heat stimulation in a preliminary study. The maximum temperature of the hot pack during treatment was 40.2 ± 0.4 °C. Therefore, this stimulation is presumed to be innocuous heat based on results from our preliminary study. These results suggest that the NGF suppressing effect was produced by the innocuous heat stimulation, not by the tactile or pressure stimulation from the hot pack material. It is known that heat stimuli induce an increase in blood flow to dilate vessels and increase their permeability [32]. Song et al. found that the blood flow in the skin was increased 15-fold compared with the control value while that in the muscle was also increased ninefold when rodent legs were warmed in a 45 °C water bath [33]. Noguchi et al. have also reported that moxibustion-like thermal stimulation for rats induced a 23% increase in the blood flow in the gastrocnemius muscle, which was maintained for 14 min after treatment [34]. In a preliminary study, we checked the change in muscle temperature after hot pack application in a rat by inserting a thermoprobe into the muscle. The muscle temperature of the gastrocnemius muscle was increased from 33.4 to 36.7 °C after treatment, which was smaller than the change in the skin. This might be the reason that the change in the withdrawal threshold with a hot pack was smaller in the muscle. Hot pack treatment is thought to regulate the blood flow and metabolism in the skin and the muscle, and consequently the elevated blood flow may play a role in the reduction of NGF.

The present study showed that the cutaneous mechanical withdrawal threshold in the PI + hot pack group was

significantly lower than that in the CON group even though NGF protein in the PI + hot pack group was at the same level as that in the CON group at the end of experiment. Some mechanisms by which NGF induces pain have been reported [35]. In the acute phase, NGF sensitizes the transient receptor potential vanilloid receptor 1 (TRPV1) through the TrkA receptor which has the highest binding affinity for NGF, and this sensitization is related to hyperalgesia [36, 37]. NGF also activates mast cells, and activated mast cells release some pain mediators [38]. These changes also induce pain. Other mechanisms are the retrograde transport of NGF from peripheral nerve endings to DRGs [39], leading to transcriptional changes that result in the up-regulation of nociceptive proteins such as TRPV1, brain-derived neurotrophic factor (BDNF), and acid-sensing ion channel 3 (ASIC3) [40–42]. The average propagation speed of NGF within the axon is reported to be 1.3 μm/s [43], indicating that the up-regulation of some nociceptive proteins induced by NGF does not occur quickly. The difference between behavioral and biological results hints at the possibility that hot pack treatment induces temporary NGF deletion and suppresses the hyperalgesia induced by the activation of mast cells and the sensitization of TRPV1, but does not suppress the hyperalgesia induced by up-regulations of nociceptors. We also performed the behavioral pharmacology experiments investigating transient receptor potential vanilloid receptor 4 (TRPV4) and protease activated receptor 2 (PAR2), because it has recently been shown that these substances may involve mechanical hyperalgesia [44, 45]. Dose-dependent suppression of the mechanical hyperalgesia was observed after administration of TRPV4 or PAR2 antagonists, respectively, at the end of the physical inactivity period. Moreover, no significant differences were detected after injection of TRPV1 antagonist or ASIC3 antagonist [46]. PAR2 and TRPV4 may relate in physical inactivity-induced mechanical hyperalgesia.

A recent report showed that heat stimulation facilitated inflammatory reactions after muscle injury, resulting in the acceleration of muscle recovery and reduction in inflammatory reactions [47]. Some reports have investigated the effect of thermal therapy using molecular biological methods. They have shown that repeated thermal therapy up-regulated heat shock protein (HSP) [48–50]. HSPs facilitate the folding and unfolding of proteins, protein translocation, and anti-apoptotic mechanisms [51]. Anti-inflammatory roles of HSP have also been described [52]. HSP induction down-regulated the expressions of IL-8 and TNF-α mRNA and the protein levels of these cytokines [53]. In addition, heat shock factor 1, which is a major transcription activator of the heat shock gene, suppresses the transcription of prointerleukin 1-β [54]. It has been noted that TNF-α and IL-

1β induced an increase in NGF in keratinocytes [11, 55]. It is likely that thermal therapy has a suppressive effect on inflammation, and represses the up-regulation of NGF. For the moment, however, we have no data on the expression of HSP, TNF-α, and IL-1β. Further studies will be necessary to establish the mechanism of thermal therapy-induced analgesia.

Thermal therapy using hot packs is commonly used for treatment of pain. However, the analgesic effect is uncertain. Our study clearly demonstrates that hot pack treatment improves physical inactivity-induced mechanical hyperalgesia and up-regulation of NGF. NGF is known to be associated with some pain conditions, such as delayed onset muscle soreness, bone injury, diabetes, inflammation, nerve injury, osteoarthritis and low back pain [9–11, 31, 56–58]. Tanezumab is a humanized monoclonal antibody that specifically inhibits nerve growth factor and has recently become a focus as a new treatment for chronic pain [58–60]. However, the safety this drug has not yet been established because the lack of NGF induces some side effects, such as headache, upper respiratory tract infection, and paresthesia [59]. These results suggest that hot pack treatment may be an effective analgesic treatment for musculoskeletal pain, with the exception of some side effects.

Finally, we clearly demonstrated that hot pack treatment was effective in reducing physical inactivity-induced mechanical hyperalgesia and up-regulation of NGF in plantar skin and gastrocnemius muscle. The mechanisms of this hyperalgesia, NGF up-regulation and their suppression by hot pack treatment remain open for future study.

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