

Monocyte chemoattractant protein-1-induced excitation and sensitization to mechanical stimulation of mechanosensitive C-fiber afferents in rat skin

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ABSTRACT

It has been previously demonstrated that chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) increases the excitability of nociceptive neurons after peripheral nerve injury or inflammation. Moreover, decreased nocifensive mechanical threshold in behavioral tests and increased calcium influx in cultured dorsal root ganglion neurons by MCP-1 application have been reported. However, the effects of MCP-1 on peripheral afferent terminals have not been studied yet. The present study aimed to examine the effect of MCP-1 on the response of cutaneous unmyelinated afferents. For this purpose, single fiber recordings of mechanosensitive C-afferents were made in vitro from skin-saphenous nerve preparations excised from rats euthanized by CO₂. Since IB4-positive neurons were previously implicated in MCP-1 induced mechanical hyperalgesia, sensitivity to α , β -methylene ATP (metATP), an indicator of IB4-positive neurons, was also studied. Application of MCP-1 100 ng/ml to the receptive field elicited excitation in one half of mechanosensitive unmyelinated afferents in the skin. MCP-1 also sensitized metATP insensitive fibers to mechanical stimulation, but not metATP sensitive fibers. The incidence of heat sensitive fibers was decreased in the MCP-1 treated group with a decrease in the response threshold. These results demonstrate MCP-1 is an effective stimulant of mechanosensitive unmyelinated peripheral afferents in the rat skin.

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1. Introduction

Monocyte chemoattractant protein-1 (MCP-1; CCL2 according to the systematic nomenclature) is a member of the chemokine family of small proteins, and plays a prominent role in the trafficking of monocytes/macrophages. MCP-1 is expressed in a subset of sensory neurons and that its expression and its receptor (CC chemokine family receptor 2, CCR2) are strongly upregulated by peripheral nerve injury or inflammation (Tanaka et al., 2004; White et al., 2005; Jeon et al., 2008; Zhang et al., 2013). The importance

of MCP-1/CCR2 system in the spinal cord has also been reported: nerve injury induced the expression of CCR2 in microglia in the spinal cord, and hyperalgesia/allodynia was less in CCR2 null mice (Abbadie et al., 2003). More recently, Toyomitsu et al. reported that MCP-1 promotes P2X₄ receptor trafficking to the cell surface of microglia (Toyomitsu et al., 2012). Different from these results, increased expression of MCP-1 was reported in astrocytes after nerve injury (Gao et al., 2009). It was also shown that TRPV1 was implicated in CCL2-induced excitatory postsynaptic currents (Spicarova et al., 2014).

In peripheral neurons, the following functions of MCP-1 have been reported. MCP-1 evoked intracellular calcium elevation and CGRP release in cultured dorsal root ganglion (DRG) neurons from neonatal rats (Qin et al., 2005). In cultured DRG neurons infected with adenoviruses expressing CCR2, activation of this CCR2 by MCP-1 also sensitized these DRG neurons to capsaicin (Jung et al., 2008). MCP-1 has also been shown to increase sodium channel Na_v1.8 activity in DRG neurons via intracellular mechanism through CCR2 (Belkouch et al., 2011). Meanwhile, Bogen et al. (2009) reported acute peripheral administration of MCP-1

Abbreviations: MCP-1, monocyte chemoattractant protein-1; metATP, α , β -methylene ATP; CCR2, CC chemokine family receptor 2; DRG, dorsal root ganglion; IB4, isolectin B4; BK, bradykinin; TRPV1, transient receptor potential vanilloid 1.

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induced mechanical hyperalgesia, and suggested the possibility that isolectin B4 (IB4)-positive sensory neurons contribute to hyperalgesia without activating CCR2.

Although the excitatory effect of MCP-1 on cultured DRG neurons or mechanical sensitization in behavioral experiments has been demonstrated as described above, there have been no studies on the excitatory or mechanical sensitizing effects of MCP-1 upon peripheral afferent terminals. We therefore studied whether MCP-1 affected the activities of unmyelinated afferents, using single fiber recording from rat skin-nerve preparations *in vitro* (Reeh, 1986) with special attention to sensitivity to α,β -methylene ATP (metATP), which is considered to be an indicator of IB4-positive neurons (Vulchanova et al., 1998; Bradbury et al., 1998).

2. Material and methods

2.1. Experimental animals

Twenty-four male Sprague-Dawley rats weighing 300–480 g (9–14 weeks) were used in this study. The animals were purchased from a breeding company (SLC Inc., Shizuoka, Japan) and kept 1–3 per cage under a 12 h light/dark cycle (light between 07.30 and 19.30 h). Room temperature was kept at 22–24 °C. The animals had free access to food and water. All parts of the study were conducted in accordance with the Regulations for Animal Experiments in Chubu University and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institution in Japan.

2.2. Electrophysiological recordings of single afferent fibers

Rat hindpaw skin-saphenous nerve preparation was used, and the methods of recording single fiber activities, stimulation of the fibers and data analysis were basically the same as previously reported (Banik et al., 2001; Taguchi et al., 2010). Briefly, the skin from one or both hindlimbs was carefully removed with the saphenous nerve attached after CO₂ euthanasia. The isolated skin was then pinned, outside down, in the perfusion chamber and maintained at 32 ± 0.5 °C (pH 7.4) under superfusion with a modified Krebs–Henseleit solution (Krebs solution), which contained (in mM) 110.9 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 20.0 glucose. The superfusate was continuously bubbled and equilibrated with a gas mixture of 95% O₂ and 5% CO₂. The saphenous nerve was drawn through a hole into a recording chamber, placed on a small mirror and covered with a layer of liquid paraffin. Small filaments of the nerve were repeatedly split with sharpened watchmaker forceps until a single unit activity could be recorded. Action potentials (APs) were recorded and analyzed with Labchart Software (AD Instruments, Bella Vista, Australia) and a DAPSYS data acquisition processor system (Brian Turnquist, <http://www.dapsys.net>). A single cutaneous C-fiber was identified by manual probing of the receptive fields (RFs) with a blunt glass rod. The conduction velocity (CV) of the fibers was then calculated from the distance and conduction latency of a spike induced by electrical stimulation of the RF. Fibers with CV of <2.0 m/s were classified as C-fibers.

The distribution (size and location) of the RFs of the fibers was mechanically investigated with a blunt glass rod as well as with von Frey hairs (0.5 mm in diameter, 3.3–101.3 mN) for a detailed search, and then ramp mechanical stimulation was performed (as described below). A metal ring (6 mm i.d.; height, 7 mm; volume, 0.5 ml) was placed over the identified RF for spot stimulation on the RF with chemical solutions. To investigate the effect of MCP-1 on the mechanical response, ramp mechanical stimulation with a servo-controlled mechanical stimulator was repetitively applied

every 10 min during the 30 min MCP-1 application period. After that, three kinds of chemicals (α,β -methylene ATP, pH 6.2 Krebs-phosphate buffer solution and bradykinin) were applied in this order for 1 or 2 min, and finally heat stimulation (32–50 °C, 30 s) was applied for fiber classification.

2.3. Mechanical stimulation (pressing the receptive field)

To quantitatively evaluate the mechanical sensitivity of a fiber, its receptive field of each fiber was given a ramp mechanical stimulus by a stimulator with feedback regulation of the force (manufactured by Aizawa S, Goto College of Medical Arts and Science, Tokyo, Japan). The stimulator had a plastic, cylindrical probe with a flat circular tip (tip size 2.28 mm²). In the ramp mechanical stimulus, the force was linearly increased from 0 to 245 mN in 12.5 s. Mechanical stimulation was repeated several minutes before and at 10, 20 and 30 min during the MCP-1 application.

We used the same response criteria as in our previous study with cutaneous afferents to determine whether a fiber was responsive to a stimulus (Taguchi et al., 2010). Specifically, a fiber was defined to be sensitive to mechanical stimulation when it fulfilled the following criteria: (1) the net increase in the discharge rate during the stimulus period was more than 0.1 imp/s over the background discharge rate (BDR) during the control period of 60 s immediately before the onset of the mechanical stimulus, and (2) the instantaneous discharge rate of two consecutive discharges exceeded the mean + 2 SD of the BDR. The mechanical threshold was defined as the intensity that induced a discharge exceeding the mean + 2 SD of BDR when there were two or more consecutive discharges exceeding this level. If a fiber that exhibited a response at the first mechanical stimulation thereafter showed no response up to 245 mN, then the mechanical threshold was defined to be 245 mN.

2.4. MCP-1 application

MCP-1 (Invitrogen, Carlsbad, CA, USA) and PBS were preheated to 32 °C and applied manually into the ring. Recorded C-fiber afferents were randomly divided into three groups. One group received a PBS application (0.01 M PBS, pH 7.4, PBS group) on the RF as a control, another received an MCP-1 application (100 ng/ml in 0.01 M PBS if not otherwise noted, MCP-1 group). The remainder received 10 ng/ml of MCP-1. MCP-1 was washed out soon after the mechanical stimulation sequence.

Positive sensitivity to MCP-1 was defined based on the same criteria as for the other sensitivities except that the net increase in the discharge rate was calculated as follows: the BDR during 60 s immediately before MCP-1 application was subtracted from the discharge rate which was calculated over 60 s immediately before the onset of each mechanical stimulus during MCP-1 application.

2.5. Chemical and heat stimulation

pH 6.2 Krebs-phosphate buffer solution (60 s), bradykinin (BK, 10 μ M, 60 s, Peptide Institute, Inc., Osaka, Japan) and thermal solutions were applied to the ring positioned on the RF. pH 6.2 Krebs-phosphate buffer solution, which contained (in mM) 11.5 NaH₂PO₄, 5.0 Na₂HP₄, 120 NaCl, 5.9 KCl, 1.0 CaCl₂, 1.2 MgSO₄, and 20.0 glucose, was continuously bubbled with O₂. The solutions were applied at a speed of approximately 7.5 ml/min through the hand-made tubing system with heat exchanger to control the temperature of the stimulants. A thermocouple was positioned at the RF to monitor the temperature. The tip of a suction tube was also set in the ring to avoid spilling and spreading of the solution outside the ring. The same as MCP-1 application, metATP (1 mM in 0.01 M PBS, 120 s, Sigma, St. Louis, MO, USA), P2X receptor agonist, was preheated to 32 °C and applied manually into the ring. The

Table 1
Summary of basic properties and sensitivities of the recorded fibers.

Treatment	Conduction velocity (m/s)	No. of responding fibers (%)			Background activity before MCP-1 (imp/s)	Basal response to mechanical stimulation	
		metATP	pH 6.2	Bradykinin		Response magnitude	Threshold (mN)
PBS (<i>n</i> = 19)	0.57 ± 0.03	8 (42)	1 (5)	6 (32)	0.04 ± 0.02	18.7 ± 4.8	98.0 ± 10.8
MCP-1 (<i>n</i> = 18)	0.64 ± 0.04	7 (39)	3 (17)	6 (33)	0.05 ± 0.03	25.4 ± 5.3	99.0 ± 12.4

RF was continuously superfused with Krebs solution (32 °C) before and after application of stimulants. MetATP, pH 6.2, BK, and heat (32–50 °C, 30 s) were applied, in that order. The application interval was more than 5 min after the firing ceased. The same response criteria as for the mechanical response were employed except that the discharge rate was calculated only during the stimulus period for metATP and heat, and both during the stimulus period and after 60 s for pH 6.2 and BK. The heat threshold was defined the same as for the mechanical threshold.

2.6. Statistical analysis

Results for the distribution of heat thresholds and maximum discharge rate after MCP-1 application are expressed as median with interquartile range (IQR). The other results are shown with mean ± SE. The differences in the average number of APs, response magnitude to mechanical stimulation and the mechanical threshold between the MCP-1 and PBS group were statistically analyzed with repeated measures ANOVA followed by Bonferroni's multiple-comparison test, compared with the control (PBS) group, when appropriate. Comparison of heat threshold and maximum discharge rates was examined with the Mann–Whitney *U*-test. The incidence of fibers responding to a stimulus was compared with Fisher's exact probability test. *p* < 0.05 was considered significant.

3. Result

3.1. General

A total of 53 cutaneous C-fiber afferents were recorded from both hindlimbs (47 from the left, 6 from the right) and randomly divided into three groups: 19 fibers to PBS group, 18 fibers MCP-1 group, 16 fibers to 10 ng/ml MCP-1 group. The basic properties and sensitivities to stimuli of the fibers in the PBS and MCP-1 groups are summarized in Table 1. There were no significant differences between groups in any term (Mann–Whitney *U*-test).

3.2. Excitation by MCP-1

The background activity of the MCP-1 group before application was not significantly different from that of the PBS group (*p* > 0.05; Mann–Whitney *U*-test, Table 1). MCP-1 application induced excitation in nine fibers. An example of this excitation is shown in Fig. 1A. Intermittent and occasionally vigorous discharge started around 15 min of the 30 min MCP-1 application period, and the excitation

of fibers disappeared immediately after MCP-1 was washed out with Krebs solution. The responses of this fiber to other stimuli are shown in Fig. 1C–E. The mechanical threshold before MCP-1 application was 126.6 mN, shown in Fig. 1C. This fiber did not respond to heat or chemical stimulation except for BK (Fig. 1D, E).

The incidence of fibers excited by MCP-1 was significantly higher than that excited by PBS at each time point (*p* < 0.05, at 10 and 20 min; *p* < 0.01, at 30 min; Fisher's exact probability test, Table 2). The average number of evoked APs of all fibers challenged with MCP-1 100 ng/ml was significantly larger than in the PBS group (*p* < 0.01 in treatment, n.s. in time, repeated measures ANOVA; *p* < 0.05 MCP-1 vs PBS at 30 min, Bonferroni's multiple-comparison test, Fig. 1B). Evoked APs in the MCP-1 sensitive afferents (*n* = 9) increased ten times 30 min after application was started (4.0 ± 1.8 imp before, 43.1 ± 16.1 imp; 30 min after, 60 s). There was no significant difference in the incidence of MCP-1 excited fibers between metATP sensitive and insensitive fibers (number of excited fibers: 3 of 7 metATP sensitive fibers; 6 of 11 metATP insensitive fibers, Fisher's exact probability test).

The incidence of excited fibers at a lower concentration (10 ng/ml) of MCP-1 was 25% (4 of 16).

3.3. Mechanical sensitization

The sensitizing effects of MCP-1 on the mechanical response were compared with regard to metATP sensitivity. MetATP excited 8 of 19 fibers in the PBS group and 7 of 18 fibers in the MCP-1 group (Table 1). The mechanical response of metATP sensitive fibers did not show any change after MCP-1 application (repeated measures ANOVA, Fig. 2A), whereas metATP insensitive fibers showed increased mechanical response with time after MCP-1 application, which was significantly different from the PBS group, and a significant increase was detected at 20 min after MCP-1 application compared with the PBS group (*p* < 0.01 in the main effect of treatment, repeated measures ANOVA; *p* < 0.05 MCP-1 vs PBS at 20 min, Bonferroni's multiple-comparison test, Fig. 2B). The response threshold was not significantly changed in either metATP sensitive or insensitive fibers (metATP sensitive fiber: 102.4 ± 13.5 mN (*n* = 15), metATP insensitive fiber: 95.8 ± 10.2 mN (*n* = 22), *p* > 0.05, Mann–Whitney *U*-test).

We also checked whether there was any relationship between MCP-1 induced excitation and sensitization to mechanical stimulation. There was a slight tendency for the mechanical response to increase with time after MCP-1 application in both the MCP-1 sensitive and insensitive groups, without a significant difference between the groups (repeated measures ANOVA, Fig. 2C).

Table 2
Summary of the excitation by MCP-1 and heat sensitivity.

Treatment	Excitation No. of responding fibers (%)			Heat sensitivity No. of responding fibers to heat (%)
	10 min	20 min	30 min	
PBS (<i>n</i> = 19)	0 (0)	2 (11)	2 (11)	17 (89)
MCP-1 (<i>n</i> = 18)	4 (22)*	7 (39)*	90 (50)**	8 (44)**

* *p* < 0.05 significant difference when compared with PBS group data, Fisher's exact probability test.

** *p* < 0.01 significant difference when compared with PBS group data, Fisher's exact probability test.

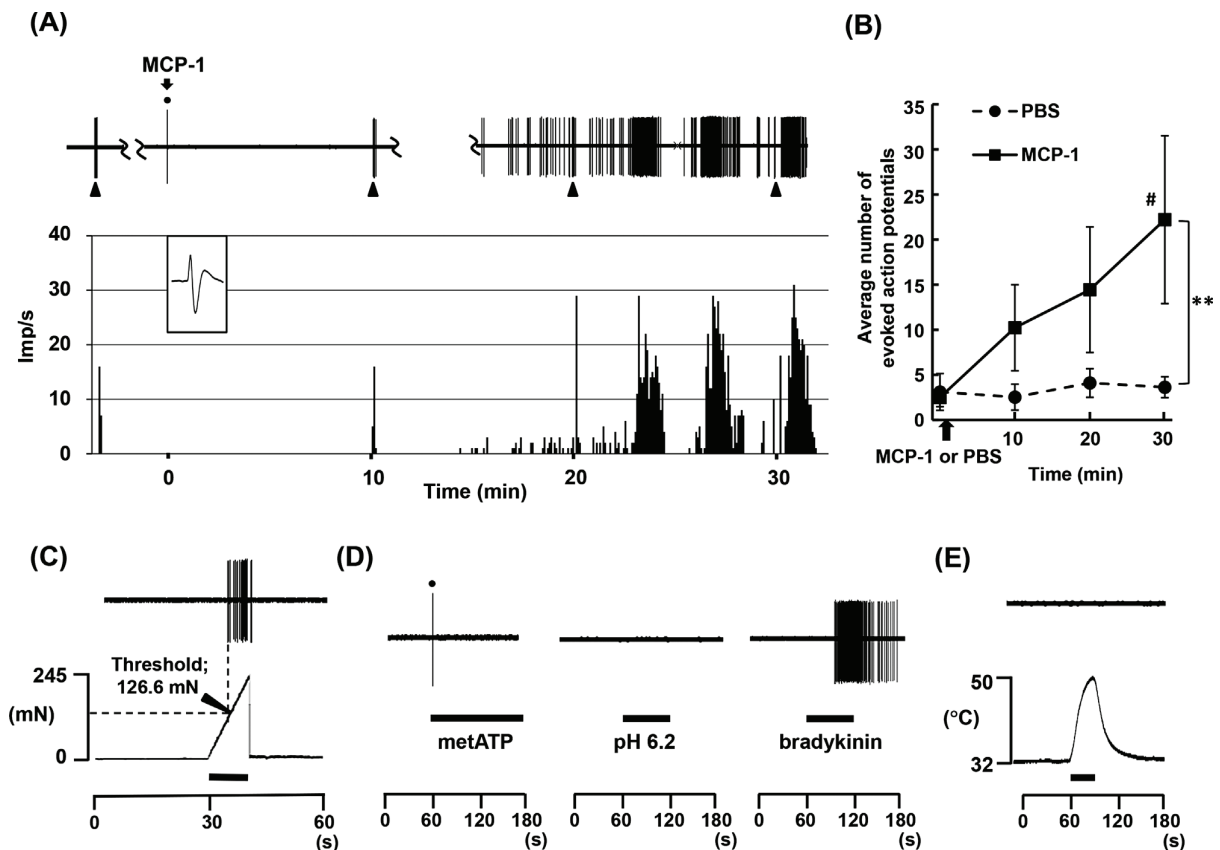


Fig. 1. Responses of an afferent fiber to MCP-1, mechanical, chemical and heat stimulation.

(A) Response to MCP-1 100 ng/ml. Original registration of the nerve activity (upper graph, same in C–E), markers of mechanical stimuli (black arrow heads) at every 10 min and corresponding time histogram of the discharges (lower trace). Inset in the time histogram represents the shape of the AP of this C-fiber in the expanded time scale. Conduction velocity of this fiber was 1.16 m/s. The time point when MCP-1 application was started is indicated by the arrow (time 0). A black dot above the nerve recording shows an artifact contaminated at the moment when MCP-1 was applied (same as in D when metATP was applied). (B) Average number of APs evoked by MCP-1. APs were counted over 60 s every 10 min during 30 min MCP-1 application period. ** $p < 0.01$ between treatment groups (repeated measures ANOVA), and # $p < 0.05$ compared with PBS group at 30 min (Bonferroni's post hoc tests, $n = 19$ in PBS group, $n = 18$ in MCP-1 group). (C) Response to mechanical stimulation before MCP-1 application. Original registration of the nerve activity, recording of the force applied by a servo-controlled mechanical stimulator to the receptive field of the fiber. Stimulation period is marked with a black bar (12.5 s). Mechanical threshold of this fiber was 126.6 mN. (D) Responses to metATP 1 mM, pH 6.2 and BK 10 μ M. Application period is marked with a black bar (120 s for metATP and 60 s for pH 6.2 and BK). This fiber responded to neither metATP nor pH 6.2. (E) Response to heat. Temperature recording is given under the nerve activity. Stimulation period is marked with a black bar (30 s). This fiber did not respond to heat stimulation.

Changes in the mechanical responses of metATP insensitive fiber with MCP-1 10 ng/ml application were 112.3–86.4%, which was not significant (repeated measures ANOVA).

3.4. Sensitivities to chemical and heat stimulations

We did not observe any significant differences between PBS and MCP-1 groups in the incidence of fibers responding to metATP, pH 6.2, and BK (Table 1, Fisher's exact probability test). In contrast, the incidence of heat sensitive fibers was significantly less in the MCP-1 group compared with the PBS group (PBS group, 89% (17 of 19); MCP-1 group, 44% (8 of 18); $p < 0.01$, Fisher's exact probability test; Table 2). Six of 9 fibers (66%) in the MCP-1 100 ng/ml group that were excited by MCP-1 were not heat sensitive. The distribution of the heat threshold of each group is shown in Fig. 3A. The heat threshold was 41.0 °C (IQR: 36.4–44.6 °C) in the PBS and 35.8 °C (IQR: 34.4–37.0 °C) in the MCP-1 group. There were no afferents in MCP-1 group with a heat threshold over 41 °C, and thus the distribution of the threshold temperature tended to be lower in the MCP-1 group ($p = 0.0583$, Mann–Whitney U -test). The number of heat responding fibers was 4 each in both metATP sensitive

and insensitive fibers of the MCP-1 group (no significant difference, Fisher's exact probability test).

With MCP-1 10 ng/ml, 10 of 16 fibers (63%) were heat sensitive, and the threshold was 37.2 °C (IQR: 35.6–38.5 °C). There was only one fiber whose threshold was over 41 °C in this group. It must be noted that three of the four fibers excited by MCP-1 10 ng/ml were not heat sensitive.

The maximum average discharge rate, which was determined by comparing the average discharge rate for 60 s at 10, 20 and 30 min during MCP-1 application, and the heat sensitivity of the MCP-1 group are summarized in Fig. 3B. All 5 fibers with maximum discharge rates greater than 0.5 imp/s were not heat sensitive. The mean maximum discharge rate was 0.41 imp/s (IQR: 0.04–0.92 imp/s) in heat insensitive fibers ($n = 10$) and 0.04 imp/s (IQR: 0.00–0.18 imp/s) in heat sensitive fibers ($n = 8$) in the MCP-1 group (no significant difference, Mann–Whitney U -test). When we focus on the MCP-1 sensitive fibers, the median for the maximum discharge rate was 0.79 imp/s (IQR: 0.52–1.51 imp/s) in heat insensitive ($n = 6$) fibers and 0.23 imp/s (IQR: 0.20–0.28 imp/s) in heat sensitive ($n = 3$) fibers.

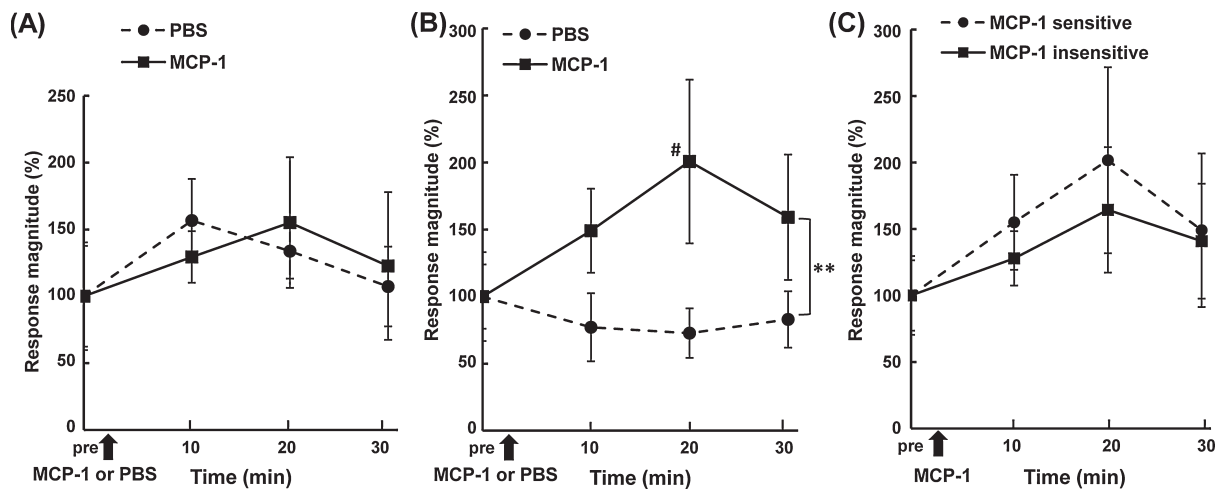


Fig. 2. Changes in the response magnitude to mechanical stimulation after MCP-1 application.

(A) Fingers sensitive to metATP. Response magnitude is presented as the percentage ratio to the averaged response before application. No significant differences between PBS ($n=8$) and MCP-1 groups ($n=7$) were detected after MCP-1 application (repeated measured ANOVA). (B) Fingers insensitive to metATP. There was a significant difference between the PBS and MCP-1 groups (** $p < 0.01$ in treatment, repeated measured ANOVA), and # $p < 0.05$ compared with the PBS group by Bonferroni's post hoc tests, $n=11$ in each group. (C) Change in the mechanical responses of fibers excited or not excited by MCP-1, $n=9$ in each group. There was no significant difference between groups (repeated measured ANOVA).

4. Discussion

The present results show that MCP-1 induced long lasting excitation in unmyelinated afferents and sensitized metATP insensitive fibers to mechanical stimulation. Attenuated heat sensitivity was also induced by MCP-1 application.

The incidence of MCP-1 induced excitation in the mechanosensitive C-fiber afferents innervating hindlimb skin of rat was higher along with the concentration of MCP-1 and the increasing application duration, but a half of fibers did not change their

excitability even with MCP-1 100 ng/ml application for 30 min. It is unclear whether MCP-1 directly acted on peripheral terminals of afferents in the present study. Several reports have shown that CCR2 are scarcely expressed in the DRG neurons (Jung et al., 2008; Richards et al., 2011). Meanwhile, Belkouch et al. (2011) reported colocalization of CCR2 and $Na_v1.8$ expression in the DRG neurons from naïve rats. According to their study, MCP-1 enhanced $Na_v1.8$ currents in almost all small primary sensory neurons, but this high incidence of the effects is different from our present results. Despite of the difference of incidence of MCP-1 responding

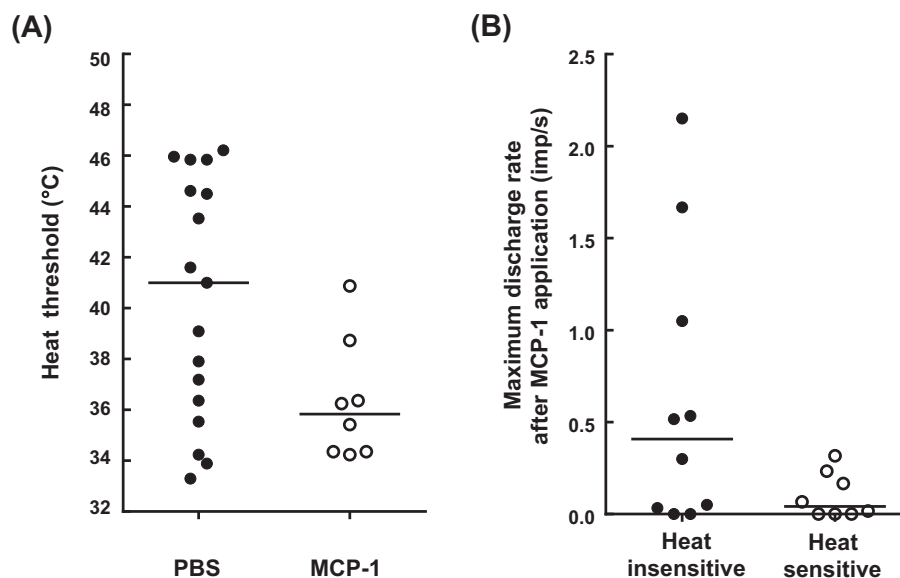


Fig. 3. Distribution of heat thresholds and maximum discharge rate after MCP-1 application.

(A) Distribution of heat thresholds with the median. Filled circles: PBS group ($n=17$), open circles: MCP-1 group ($n=8$). There were no fibers that had a heat threshold over 42 °C in the MCP-1 group. The threshold tended to be lower in the MCP-1 group than in the PBS group ($p=0.0583$, Mann-Whitney U -test). (B) Maximum discharge rate determined by comparing average discharge rate for 1 min at 10, 20 and 30 min after the start of MCP-1 application. Filled circles: heat insensitive fibers ($n=10$), open circles: heat sensitive fibers ($n=8$). The discharge rates of all heat sensitive fibers were less than 0.5 imp/s. Maximum discharge rates were not significantly different between heat insensitive (0.41 imp/s (IQR: 0.04–0.92 imp/s)) and sensitive fibers (0.04 imp/s (IQR: 0.00–0.18 imp/s)) in the MCP-1 group (Mann-Whitney U -test).

neurons, the excitation of mechanosensitive C-fiber afferents may be originated from $\text{Na}_v1.8$ current enhancement. On the contrary, [Abbadie et al. \(2003\)](#) have demonstrated cells expressing CCR2 in the skin, sciatic nerve and DRG neurons, and they have shown monocytes/macrophages are CCR2 positive in the dermis and around blood vessels. The inflammatory mediators secreted from non-neural cells in the tissue expressing CCR2, following MCP-1 binding, may also have been involved in the excitation.

The sensitizing effects of MCP-1 on the mechanical response were compared with regard to metATP sensitivity, because it was reported that MCP-1-induced mechanical hyperalgesia depended on IB4-positive neurons ([Bogen et al., 2009](#)). We analyzed the mechanical sensitization of afferents by MCP-1 in terms of metATP sensitivity instead of IB4 binding, as it is reported that 94.5–98.4% of DRG neurons immunoreactive to P2X_3 receptor have the ability to bind to IB4 ([Bradbury et al., 1998](#); [Vulchanova et al., 1998](#)). Contrary to our expectation, mechanical sensitization by MCP-1 100 ng/ml was observed in metATP insensitive C-afferents in the present study. A previous report showed that mechanical hyperalgesia induced by intradermal injection of MCP-1 to the dorsum of rat hind paw was mediated by IB4-positive nociceptors ([Bogen et al., 2009](#)), based on the finding that destruction of IB4-positive DRG neurons by intrathecal injection of IB4-saporin or antisense to IB4-binding protein (versican) attenuated the MCP-1 induced mechanical hyperalgesia. They reported that these treatments reduced the numbers of IB4-binding sensory neurons in the DRGs and IB4-positive central processes terminating in the dorsal horn of the spinal cord, and downregulated expression of versican in DRG neurons. However, our present recordings of peripheral unmyelinated afferents activities showed that fibers supposed to be IB4-negative, rather than -positive, contribute to mechanical sensitization by MCP-1. [Qin et al. \(2005\)](#) reported that CGRP, mainly contained in IB4-negative neurons, was released after MCP-1 (100 ng/ml) binding to CCR2 of DRG neurons. Our findings seem to be in line with their report, although they did not refer to mechanical sensitization.

In a preliminary study, we examined mechanical sensitization by MCP-1 on cultured DRG neurons by measuring mechanically activated currents using the patch-clamp method ([Kubo et al., 2012](#)), but we could not observe any mechanical sensitization ($n = 66$, data not shown). It is possible that the culture conditions or application period might not have been appropriate for detection of mechanical sensitization, or that some cells other than neurons might be necessary for sensitization by MCP-1.

The last, we also demonstrated that the MCP-1 group had fewer heat sensitive fibers than the PBS group and lacked high heat threshold fibers. The maximum discharge rate induced by MCP-1 differed between heat-insensitive and -sensitive fibers. These results might suggest that C-fibers were desensitized to heat after a higher discharge rate induced by MCP-1. This idea is supported by observations by [Jung et al. \(2008\)](#) showing that MCP-1 sensitizes the transient receptor potential vanilloid 1 (TRPV1) channel, which is considered to be involved in heat sensitivity ([Tominaga, 2007](#)), via CCR2 activation in human embryonic kidney cells co-expressing CCR2 and TRPV1 ([Jung et al., 2008](#)). Thus, TRPV1 activation could be involved in peripheral afferent excitation by MCP-1 and might lead to desensitization to heat. To clarify this point, future experiments are needed.

5. Conclusion

MCP-1 induced excitation in one half of mechanically sensitive unmyelinated afferents in the skin. It also induced mechanical

sensitization in the afferents not expressing P2X_3 receptors. To elucidate the cellular and molecular mechanism of MCP-1-induced peripheral excitation and sensitization, further experiments are needed.

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