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Full Paper

Local Administration of a Synthetic Cell-Penetrating Peptide Antagonizing TrkA Function Suppresses Inflammatory Pain in Rats

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Abstract. Novel agents that inhibit nerve growth factor signaling are required for the treatment of inflammatory pain. The present study investigated the effect of local administration of inhibitory peptide of TrkA (IPTRK3), a synthetic cell-penetrating peptide that antagonizes TrkA function, in complete Freund's adjuvant (CFA)–induced hyperalgesia in rats. Three hours after subcutaneous injection of CFA into the plantar surface of the rat's left hind paw, 10 mM IPTRK3 was injected at the same site. Thermal and mechanical hyperalgesia were tested in the ipsilateral hind paw until 7 days after CFA injection. The ipsilateral dorsal root ganglion (DRG) was dissected out for immunohistochemical analysis of transient receptor potential vanilloid subfamily member 1 (TRPV1) channels and TrkA. Local injection of this peptide significantly suppressed both thermal and mechanical hyperalgesia produced by CFA and also significantly reduced TRPV1 expression at the DRG. These results suggest that local administration of IPTRK3 is likely effective in the treatment of inflammatory pain in rats.

Keywords: cell-penetrating peptide, inflammatory pain, Tat, TrkA, transient receptor potential vanilloid subfamily member 1 (TRPV1)

Introduction

As there are limited varieties of conventional therapeutics (e.g., non-steroidal anti-inflammatory drugs, opioids, local anesthetics) for the management of severe inflammatory pain, novel drugs to inhibit nerve growth factor (NGF) signaling are needed (1, 2). Increased expression of either NGF or its high-affinity receptor TrkA, which exacerbates pain states, is reported in inflammatory tissues, including burn-injured tissue (3, 4), postoperative surgical sites (5, 6), and human degenerative lumbar facet joints (7). Local injection of complete Freund's adjuvant (CFA), which is utilized for the inflammatory pain model, also increases NGF expression in the skin (8).

Local injection of NGF induces either thermal or mechanical hyperalgesia in rats (9). Several studies have revealed the mechanisms of NGF-induced hyperalgesia (1, 9, 10). NGF binds to TrkA in the cell membrane of peripheral nerves, and the activated TrkA in turn sensitizes transient receptor potential vanilloid subfamily member 1 (TRPV1) channels (1). Both NGF and TrkA are transported retrogradely to the cell bodies of the dorsal root ganglion (DRG) (10), where they enhance expression of TRPV1 with other pronociceptive proteins in the DRG (1, 2).

Previously, we developed a new inhibitor of NGF signaling, an inhibitory peptide of TrkA (IPTRK) activity with both the cell-penetrating peptide and the amino acid sequence corresponding to the activation loop of TrkA (YGRKKRRQRRR-acp-SRDIYSTDYYR-NH_2, acp = epsilon-aminocaproic acid) (11). We named this peptide IPTRK3, since it was the third peptide we tested. When IPTRK3 is injected locally, it is expected to sup-

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press intracellular TrkA activity after penetrating the cell membrane of peripheral nerves, from which it would be transported retrogradely to the DRG, where it may cause suppression of the expression of TRPV1 in the DRG in vivo (11). In the present study, we investigated the effect of local administration of IPTRK3 at the site of inflammation in a CFA-induced hyperalgesia model and examined its effect on TRPV1 expression in rats.

Materials and Methods

Experimental animals

The study protocol was approved by the Institutional Animal Research Committee and was performed in accordance with the Ethical Guidelines of the International Association for the Study of Pain (12). Adult male Sprague-Dawley rats (weighing 200 – 250 g) were used for this study. Rats were housed in a room maintained at 25°C and illuminated in a 12:12 h cycle. Rats were provided with ad libitum access to standard rodent chow and water.

Synthetic cell-penetrating peptide antagonizing TrkA function

IPTRK3 has two components: one is the cell-penetrating peptide sequence based on the human immunodeficiency virus type1 Tat (transactivator of transcription)–derived peptide (47-YGRKKRRQRRR-57), and the other is the amino-acid sequence of the activation loop of TrkA (666 -SRDIYSTDYYR- 676), which inhibits TrkA activity (11). Acp was inserted as a highly flexible spacer between these two amino acid sequences. IPTRK3, designed as YGRKKRRQRRR-acp-SRDIYSTDYYR, was synthesized and purified by HPLC (Peptide Institute, Osaka).

IPTRK3 was dissolved in phosphate-buffered saline (PBS) to the concentration of 10 mM/L and then stored at −30°C. We selected this concentration of 10 mM/L of IPTRK3, which is over a hundred times the concentration required for the inhibitory effects of TrkA activity both in vitro and in cell cultures (30 – 60 μM), as shown in our previous study (11), because local anesthetics are reported to gradually diffuse into surrounding tissues after local injection and get diluted to approximately a hundredth or less of the original concentration in the peripheral nerve (13).

Induction of hyperalgesia

Hyperalgesia was induced by subcutaneously injecting 50 μL of CFA (Sigma-Aldrich, St. Louis, MO, USA) into the plantar surface of the left hind paw of the rats using a 30-gauge hypodermic needle under sevoflurane anesthesia. Classical signs of inflammation, including edema and redness, were observed for at least 7 days.

Behavioral assessments

The mid-plantar area of the left hind paw was tested for thermal hyperalgesia (14). The light heat source, applied for a maximum of 20 s, was adjusted to produce withdrawal latencies of approximately 10 s (Model 7370 Plantar Test; Ugo Basile, Milan, Italy). Rats were acclimatized to the testing environment, and paw withdrawal latencies of the left hind paws, as the noxious heat threshold, were measured.

Mechanical hyperalgesia was also tested at the mid-plantar area of the left hind paw, using an automated version of the von Frey hair assessment (Model 37400 Dynamic Plantar Aesthesiometer, Ugo Basile). After rats were acclimatized to the testing environment, a mechanical stimulus, using a pointed metallic filament, was applied to the plantar surface of the hind paw through a wire mesh–bottomed cage until the paw was withdrawn or the preset cut-off was reached (50 g). The mechanical threshold was defined as the force in grams at which the rat withdrew its paw.

The noxious heat and mechanical thresholds were separately measured in each group of rats. The threshold was measured five times in each rat and then averaged. Stimulus interval was 5 min. All measurements were performed in a blinded fashion, with the investigator unaware of the injected agent, whether IPTRK3 or PBS.

The effect of IPTRK3 on CFA-induced inflammatory pain

To assess the effect of IPTRK3 on CFA-induced inflammatory pain, we once again anesthetized the rats with sevoflurane 3 h after CFA injection and then injected 50 μL of either 10 mM IPTRK3 (n = 5) or PBS (n = 6) subcutaneously into the same site as the CFA injection, using a 30-gauge hypodermic needle under sevoflurane anesthesia. Paw withdrawal latencies were measured before and at 2, 4, and 6 h and 2, 4, and 7 days after CFA injection. Mechanical thresholds were also measured in the same way in other rats injected with either IPTRK3 (n = 5) or PBS (n = 5).

We also evaluated whether IPTRK3 itself affects the noxious heat threshold or not. Either 50 μL of 10 mM IPTRK3 alone (n = 6) or PBS (n = 6) alone, without prior CFA administration, was injected subcutaneously into the plantar surface of the left hind paw of the rats, using a 30-gauge hypodermic needle under sevoflurane anesthesia. Paw withdrawal latencies were measured before injection as control values and at 2 h and 2 and 7 days after injection.

Immunohistochemistry

Three hours after CFA injection, rats were injected locally with either IPTRK3 (n = 4) or PBS (n = 4). Then, 1 h after local administration of peptide/PBS, all rats were over-anesthetized with sevoflurane and perfused transcardially with saline, followed by 4% paraformaldehyde. The left L4/5 DRGs were dissected out, post-fixed in 4% paraformaldehyde, and transferred to 20% sucrose overnight for cryoprotection. Ten-micron-thick sections of the DRG were cut on a cryostat and processed for TRPV1 and TrkA immunohistochemistry. Sections were incubated overnight in rabbit anti-TRPV1 antibody (1:3000; Neuromics, Edina, MN, USA) or rabbit anti-TrkA antibody (1:200; Millipore, Billerica, MA, USA) in 10 mM PBS at 4°C. This was followed by incubation in biotinylated anti-rabbit IgG (1:50; Vector Labs, Burlingame, CA, USA) for 1 h and in avidin-biotin complex (Vector Labs) for 1 h at room temperature. Sections were exposed to 3,3′-diamino-benzidine-4HCl (Wako, Osaka) in 50 mM Tris buffer, pH 7.4, containing 0.2% H_2O_2.

The stained sections of the DRG were analyzed under a light microscope for TRPV1-positive neuron distribution. Every fourth section was picked from a series of consecutive DRG sections, ten sections being counted for each DRG. Results of microscopy were expressed as the percentage of TRPV1-positive neurons (15).

Statistical analyses

Data were analyzed using one-way analysis of variance with Bonferroni *post hoc* analysis. Statistical significance was established at the $P < 0.05$ level. All values are reported as the mean ± S.D.

Results

IPTRK3 suppresses CFA-induced hyperalgesia

Paw withdrawal latencies after CFA injection in the PBS group decreased significantly from 2 h to 7 days (Fig. 1). IPTRK3 showed significant suppression of these decreases in paw withdrawal latencies compared to the PBS-injection group from 4 h to 4 days. Mechanical thresholds after CFA injection in the PBS group decreased significantly from 4 h to 4 days (Fig. 2). The CFA-induced significant reduction of mechanical threshold, however, was only observed at 4 and 6 h in the IPTRK3 group. There was a significant difference in mechanical thresholds between IPTRK3 and PBS groups at 6 h. These results suggest that IPTRK3 likely suppresses both CFA-induced thermal and mechanical hyperalgesia.

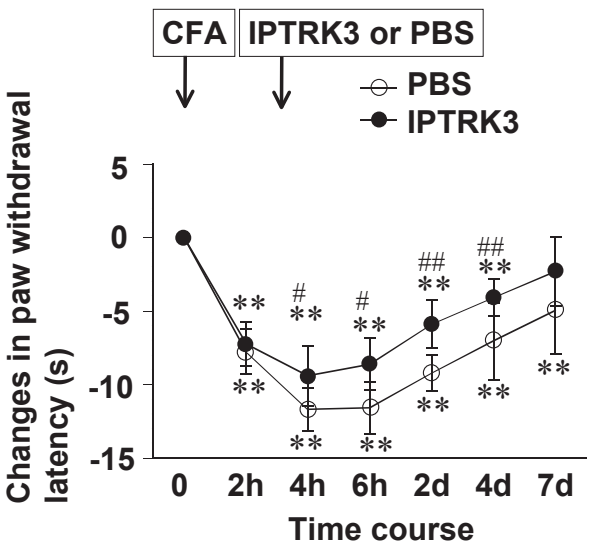

Fig. 1. CFA-induced thermal hyperalgesia in rats. Time course of changes in paw withdrawal latency were investigated starting before subcutaneous injection of CFA (base line value, time point 0 h) and at the time points of 2, 4, and 6 h and 2, 4, and 7 days after CFA injection. Either PBS (n = 6) or IPTRK3 (n = 5) was injected subcutaneously 3 h after CFA injection. $^{**}P < 0.01$, compared with the baseline value at 0 h in each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, with comparison between values in the two groups at the same time point. Results represent means ± S.D.

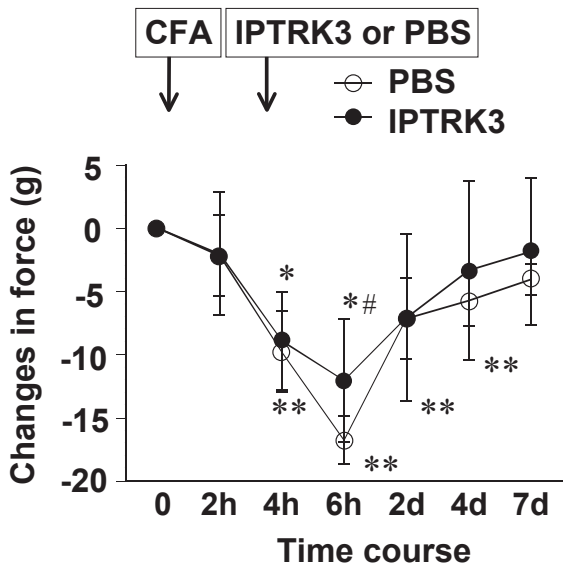

Fig. 2. CFA-induced mechanical hyperalgesia in rats. The time course of changes in the mechanical threshold was investigated starting before subcutaneous injection of CFA (baseline value, time point 0 h) and at the time points of 2, 4, and 6 h and 2, 4, and 7 days after CFA injection. Either PBS (n = 5) or IPTRK3 (n = 5) was injected subcutaneously 3 h after CFA injection. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the baseline value at 0 h in each group. $^{\#}P < 0.05$, comparison between values in the two groups at the same time point. Results represent means ± S.D.

We also examined the changes in paw withdrawal latencies after subcutaneous injection of IPTRK3 or PBS without CFA. There were no significant differences in noxious heat thresholds between and within groups of rats injected with IPTRK3 or PBS alone at 2 h and 2 and 7 days. These results suggest that IPTRK3 itself induces neither thermal hypoalgesia nor hyperalgesia.

IPTRK3 suppresses the expression of TRPV1 at the DRG

Protein expressions of TRPV1 and TrkA at the DRG are shown in Figs. 3 and 4, respectively. Expression of TRPV1 at the DRG after CFA injection was significantly suppressed by IPTRK3 (Fig. 5). On the other hand, there was no significant difference in TrkA expression in the DRG between PBS and IPTRK3 groups (Fig. 5).

Discussion

IPTRK3, a cell-penetrating peptide with TrkA inhibitory activity, suppressed CFA-induced thermal and mechanical hyperalgesia after local administration at the site of CFA injection, together with reduction of protein expression of TRPV in the ipsilateral DRG.

NGF antagonists can be categorized as NGF-capturing agents, antagonists at the NGF-binding site, and antagonists of TrkA function (1). K252a, an antagonist of TrkA function, is reportedly effective for inflammatory pain in rats (16, 17). However, K252a likely has many adverse effects because of lack of specificity for TrkA (1). IPTRK3 also belongs to the class of antagonists of TrkA function. In our previous study (11), this peptide showed no effect on tyrosine kinase activities of the insulin receptor and epidermal growth factor receptor in vitro. Further studies are needed to investigate the effect of IPTRK3 on other pronociceptive protein kinases, such as mitogen-activated protein kinase, protein kinase C, c-Jun N-terminal kinase, and Ca^{2+}/calmodulin-dependent protein kinase.

Single injection of a drug at the inflammatory area is a fairly useful regimen for the clinical management of severe inflammatory pain states, especially if it provides a prolonged analgesic effect. A single injection of local anesthetic at the site of inflammation, however, induces a transient analgesic effect (18 – 21). Therefore, continuous infusion of local anesthetics, either locally or systemically, is needed for prolonged analgesia (20, 22, 23). As single injection of IPTRK3 showed sufficient inhibitory effects on thermal hyperalgesia lasting for at least 4 days in the present study, a single local injection of IPTRK3 at the site of inflammation would be a favorable regime for severe inflammatory pain states, for which conventional therapy does not work.

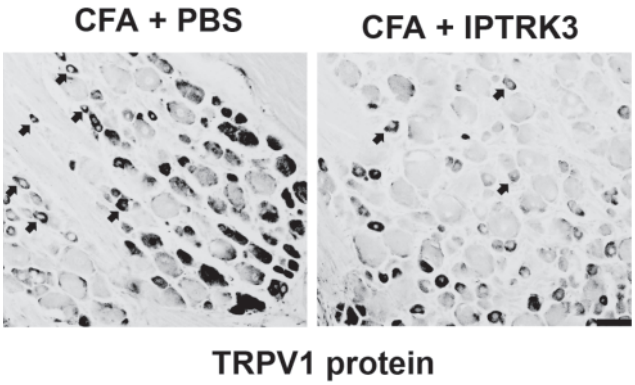

Fig. 3. Immunohistochemistry for TRPV1 channels in the dorsal root ganglion 4 h after subcutaneous injection of CFA. Either PBS or IPTRK3 was injected subcutaneously 3 h after CFA injection. Arrows indicate typical TRPV1 protein. Scale bar, 50 μm.

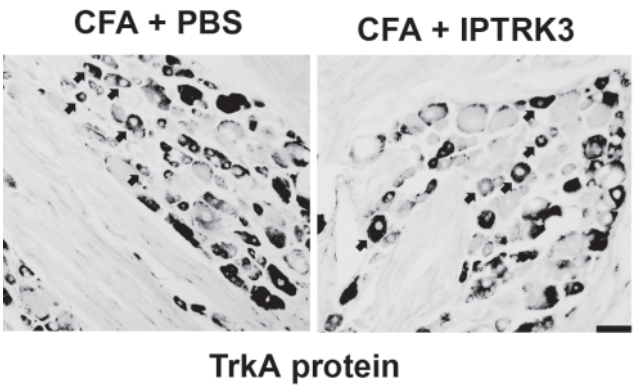

Fig. 4. Immunohistochemistry for TrkA in the dorsal root ganglion 4 h after subcutaneous injection of CFA. Either PBS or IPTRK3 was injected subcutaneously 3 h after CFA injection. Arrows indicate typical TrkA protein. Scale bar, 50 μm.

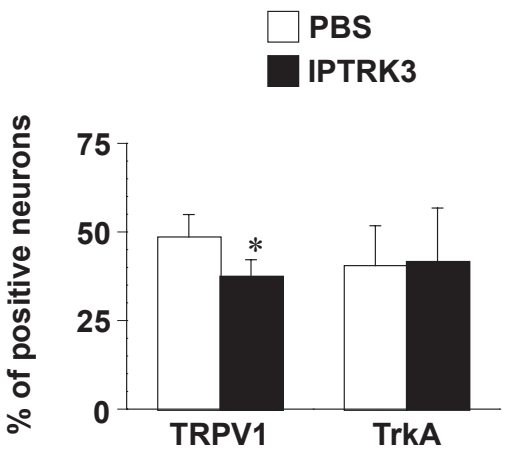

Fig. 5. Quantification of protein levels, as shown in Figs. 3 and 4, by the percentage of either TRPV1- or TrkA-positive neurons. *$P < 0.05$, comparison between PBS and IPTRK3 groups. Results represent means ± S.D. of four separate experiments in each group.

The suppressive effect of IPTRK3 on CFA-induced mechanical hyperalgesia was smaller than that on CFA-induced thermal hyperalgesia in the present study. An electrophysiological study reported that the mechanism causing mechanical sensitization of cutaneous nociceptors after CFA injection into the rat's hind paw is different from that causing thermal sensitization (24). Other investigators reported that a central mechanism causes NGF-induced mechanical hyperalgesia, whereas both peripheral and central mechanisms cause NGF-induced thermal hyperalgesia (9, 25). Although these different mechanisms inducing mechanical and thermal hyperalgesia might explain the distinction between suppressive effects of IPTRK3 on each hyperalgesia, the precise reason is unclear.

The present study showed that IPTRK3 suppressed protein expression of TRPV1 in the DRG during inflammatory pain. NGF signaling upregulates the expression of pronociceptive proteins, such as TRPV1, the brain-derived neurotrophic factor, substance P, calcitonin gene-related peptide, and the $Na_v1.8$ sodium channel in sensory neurons, that mediate nociception via peripheral and central sensitization (1, 2). TRPV1 is essential for thermal hyperalgesia induced by inflammation (15, 26). Therefore, the suppression of TRPV1 expression is one possible mechanism of the inhibitory effect of IPTRK3 on CFA-induced thermal hyperalgesia.

A limitation of this study is that the reason why a single local injection of IPTRK3 induced a prolonged inhibitory effect on CFA-induced hyperalgesia is unknown. Further studies are needed to investigate the time-course of expression of several pronociceptive proteins in peripheral and central neurons and also to examine the half-life of this peptide in vivo.

In summary, local administration of IPTRK3, a cell-penetrating peptide having direct inhibitory effects on TrkA activity, suppresses hyperalgesia induced by inflammation in rats. This peptide would be a new candidate as a viable therapeutic drug for inflammatory pain.

Acknowledgments

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