Change of vanilloid receptor 1 expression in dorsal root ganglion and spinal dorsal horn during inflammatory nociception induced by complete Freund's adjuvant in rats

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Received 11 December 2003; accepted 8 January 2004

DOI: 10.1097/01.wnr.0000118721.38067.44

The present study aimed to systematically observe the change of vanilloid receptor 1 (VR1) during inflammatory nociception induced by intraplantar injection of complete Freund's adjuvant (CFA) into the left hind paw in rats. Hot plate latency (HPL) was used to evaluate resulting thermal hyperalgesia and immunohistochemistry to observe VR1 expression in dorsal root ganglion and spinal cord dorsal horn. Results showed that HPL decreased from day 1 to day 28 after CFA injection, with shortest at day 14. VR1 expression correspondingly increased from day 1 to day 21 with peak at day 14, and returning to the control level at day 28. A shift of VR1 expression from small to medium DRG neurons over the observation period was seen. These results suggest that VR1 could play an important role in the early stage, but not the late stage, of CFA inflammatory nociception. *NeuroReport* 15:655–658 © 2004 Lippincott Williams & Wilkins.

Key words: Complete Freund's adjuvant; Dorsal root ganglion; Inflammatory pain; Spinal cord; VR1

INTRODUCTION

Vanilloid receptor 1 (VR1) is a ligand-gated selective cation channel expressed in nociceptors [1]. It can be activated by capsaicin, the main pungent ingredient in hot chilli peppers, other vanilloids, heat ($>43^{\circ}C$) and low pH. Dorsal root ganglion (DRG) and the dorsal horn of the spinal cord are very important processing stations of the senses. VR1 is highly expressed in DRG although not in the CNS [1]. Many reports suggest that VR1 participates in acute pain [1–3]. Other studies have also demonstrated changes in VR1 protein expression in hind paw skin, sciatic nerve and DRG 2 and 7 days after complete Freund's adjuvant (CFA) injection [4,5]. Application of the VR1 antagonist, capsazepine (CPZ), and other antagonists were shown to produce marked antinociception in the formalin model of pain in mice, and to reduce inflammation-induced thermal hyperalgesic responses in the carrageenan model of pain in rats [6,7]. Two recent experiments performed in mutant mice that lack VR1 demonstrated that VR1 was essential for hyperalgesia induced by either acid or heat [8,9]. Previous reports have concentrated on the role of VR1 during a relatively short period (≤ 1 week) following inflammatory nociception. Therefore, we attempted to determine how VR1 changes during the full time course of inflammatory nociception. In the present study, we observed the change of VR1 expression in DRG and spinal dorsal horn at different time points over a relatively long period, 28 days, after CFA injection.

MATERIALS AND METHODS

Experimental animals: Male Sprague–Dawley rats (150–200 g) were housed under natural light:dark cycles and provided water and food *ad lib*. They were habituated to the testing paradigms for 3–5 days before data collection. All protocols were approved by the Animal Care and Use Committee of our university.

Hot plate test (HPL): Rats were habituated to the experimental environment for 30 min in their cage and then placed on a hot plate ($52\pm0.5^{\circ}C$). Time until the rat jumped or licked either of its hind paws was recorded after it was placed on the plate. The average of three measures was used as the control value.

Establishment of CFA inflammatory pain model: Two days after HPL test, the same rats received an injection of 100 µl CFA (Sigma-Aldrich, St. Louis, USA) into the plantar surface of the left hind paw to induce inflammatory nociception [10]. Classical signs of acute inflammation including edema, redness and heat were most intense on days 1–3 after injection, and lasted >4 weeks.

Immunohistochemical staining of VR1: Rats were over-anesthetized and then transcardially perfused with normal saline and 4% paraformaldehyde (pH 7.4). The left L5 DRG and the lumbar enlargement of the spinal cord were quickly removed, post-fixed with paraformaldehyde, and replaced with sucrose. The 10 μm sections of DRG and the spinal cord were immunostained according to the ABC method [11]. Briefly, sections were incubated sequentially with: (1) rabbit anti-rat IgG primary antibody (Calbiochem, Oncogene, USA) against VR1 protein (for DRG, 1:400; for spinal cord, 1:200) for 24 h at 4°C, (2) biotinylated goat anti-rabbit secondary antibody (1:200, 1 h; Vector, USA) at 37°C, and (3) avidin-biotin peroxidase complex-standard (1:100, 2 h; Vector) at room temperature. Bound peroxidase was visualized by incubation with 0.05% diaminobenzidine (Sigma-Aldrich) and 0.003% H_2O_2 in PB.

Image analysis: The images of immunostained DRG and the spinal dorsal horn sections were captured with a CCD camera under a microscope (Leica Instruments, Germany). Signal intensity was calculated with the SCION Image, an NIH image-processing and analysis software system. The area of each neuron was calculated using the Leica Qwin software system [12,13]. The ratio of VR1-positive cells compared with the total neuronal profiles and the average intensity of VR1-positive neuronal profiles of DRG were calculated in a blind fashion to identify any relative changes in VR1 expression in the DRG neurons. The average intensity was defined as the difference between average gray value (mean density) within each DRG section and its background. The percentage of VR1-positive neuronal profiles per each DRG belonging to corresponding cell size was compared for cell size distribution. The average intensity of the superficial layers of the spinal dorsal horn was also defined as the difference between average gray value within left superficial layer and white matter.

Statistical analysis: Data are expressed as mean ± s.e.m. Differences among time points were analyzed with one-way ANOVA for repeated measures, followed by Dunnett's *post-hoc* test. Significance was determined as $p < 0.05$.

RESULTS

Thermal hyperalgesia of the left hind paw following CFA injection: The time course of thermal hyperalgesia reflected by HPL from day 1 to day 28 after CFA injection is shown in Fig. 1. Latencies after CFA injection decreased significantly ($p < 0.05$ or 0.01) from day 1 to day 28 with the shortest at day 14.

Change of VR1 expression in the left L5 dorsal root ganglion following CFA injection: VR1 immunoreactivity (*ir*) in DRG was measured on the pre-injection day (control) and on days 1, 3, 7, 14, 21, and 28 following CFA injection. Results from the pre-injection day and days 1, 14 and 28 are shown in Fig. 2a. The average intensity of VR1-positive neuronal profiles in the left L5 DRG increased significant from day 1 until day 21 ($p < 0.05$ or 0.01, $n = 3$), and then returned at day 28 to the control level as at the pre-injection day ($p > 0.05$; Fig. 2b). The percentages of VR1-positive neurons at the different time points mentioned in Fig. 2a are shown in Fig. 2c. The ratio of positive to total neurons in inflamed DRG at day 14 after CFA injection was increased significantly compared with that at pre-injection (Fig. 2c). Area frequency histograms (Fig. 3) show that VR1 expression on pre-injection day and on day 1 after injection is almost entirely in small neurons (cell area $< 600\,\mu m^2$). At days 14 and 28, the ratio of VR1-positive neurons significantly increased within small-to-medium ($600\,\mu m^2$–$1000\,\mu m^2$) neurons compared with that on pre-injection day and on day 1 ($p < 0.05$).

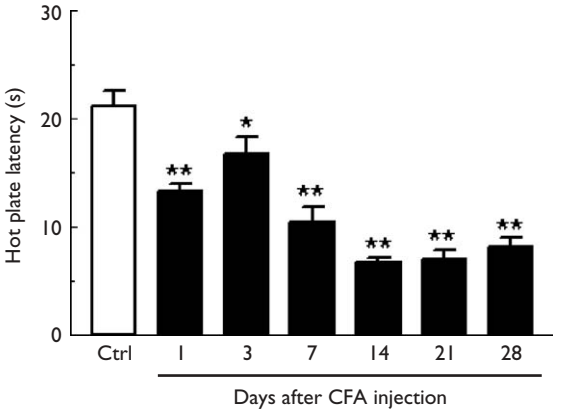

Fig. 1. Thermal hyperalgesia after CFA injection into the left hind paw of rats, as shown by changes in hot plate (52 ± 0.5°C) latency (HPL). Data were collected on the day before CFA injection as control (open box) and at indicated days after CFA injection (closed boxes). There was a significant decrease in HPL from day 1 to day 28 after CFA injection compared with the control group. * $p < 0.05$, ** $p < 0.01$ compared with the pre-injection day ($n = 10$).

Change of VR1 expression in the superficial layers of spinal dorsal horn following CFA injection: In normal rats, VR1-*ir* could be detected in the superficial layers (lamina I and inner lamina II) of the spinal dorsal horn (Fig. 4a). The distribution pattern of VR1-*ir* did not change in the spinal cord, but the intensity increased significantly from day 1 until day 21 ($p < 0.05$ or 0.01, $n = 3$). Quantitative analysis of the optical densities is shown in Fig. 4b. Compared with pre-injection, the intensity reached peak at days 7 and 14 after CFA injection ($p < 0.01$, $n = 3$), but returned at day 28 to the control level ($p > 0.05$).

DISCUSSION

The mechanisms underlying thermal hyperalgesia are of great interest but not fully elucidated at present. VR1 is an important molecule for the development of thermal hyperalgesia under the inflammatory pain state. The present study systematically observed the time course of thermal hyperalgesia up to 28 days in rats with CFA-induced inflammatory thermal hyperalgesia (Fig. 1). We show that VR1 protein levels in the left L5 DRG and the superficial layers of the spinal dorsal horn increased significantly from day 1 to day 21 after CFA injection, with a peak at day 14 (Fig. 2, Fig. 4). To our knowledge, this is the first study of this phenomenon in rat CFA model over such a long period.

Since it was successfully cloned [1], the role of VR1 in the normal heat sensation and in pain conditions (especially thermal hyperalgesia) has attracted great interest. Many researchers have already demonstrated its role in acute pain.

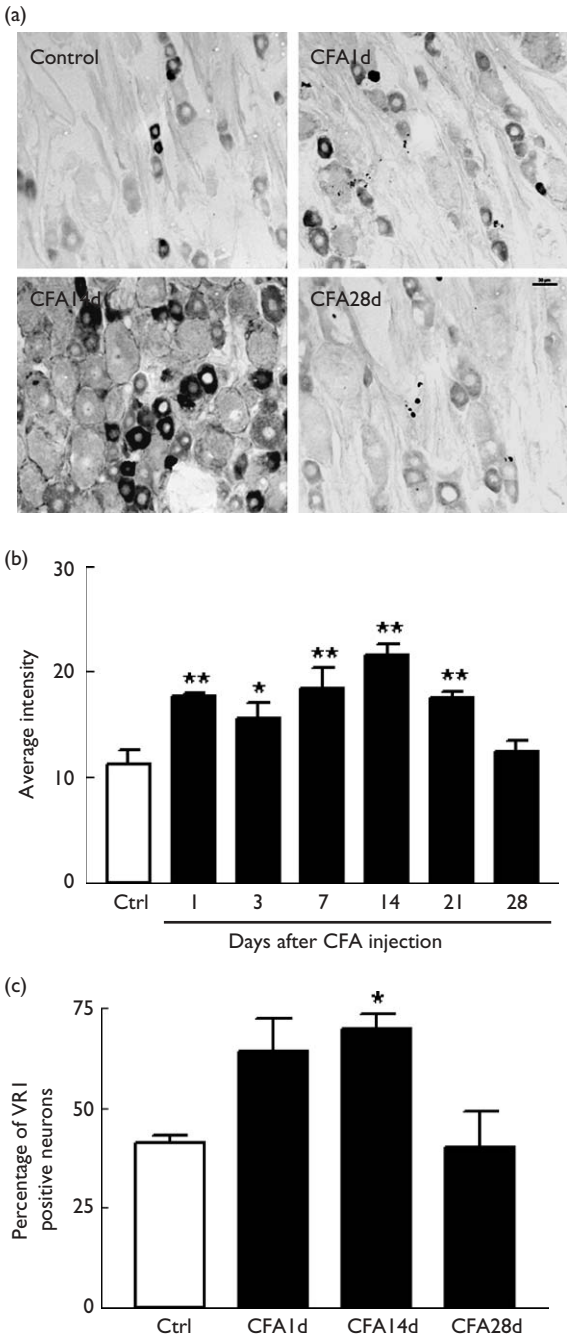

Fig. 2. The change of VR1 expression in the left L5 DRG in rats during inflammatory nociception induced by CFA injection into left hind paw. (**a**) Immunohistochemistry of VR1 expression in DRG at the pre-CFA injection day (Control), day 1, 14 and 28 after CFA injection. Bar=30μm. (**b**) Quantification of average intensity of VR1-positive neuronal profiles in the left L5 DRG as shown in (a). VR1 expression increased from day 1 to 21 following CFA injection, but fell back at day 28 to the control level as the pre-injection day. $*p<0.05$, $**p<0.01$ *vs* pre-injection ($n=3$). (**c**) Ratio of VR1-expressing neurons to the total. The ratio of neurons expressing VR1 was significantly increased at 14 days after CFA injection. $*p<0.05$ *vs* pre-injection (control) ($n=3$).

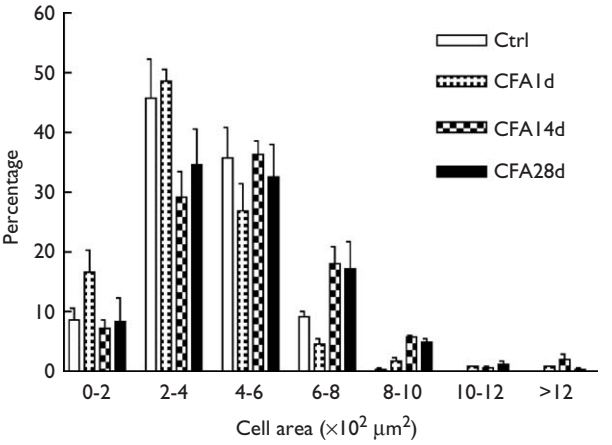

Fig. 3. Area–frequency distribution of VR1-positive neurons in the left L5 DRG in rats at the pre-injection day and days 1, 14, 28 after CFA injection. The percentage of VR1-expressing neurons from each DRG that belonged to corresponding cell size was indicated. At day 14 and day 28, VR1-positive staining appeared more frequently in medium DRG neurons (600–1000 μm^2). Among these neurons, the percentage of VR1-positive neurons (cell area 600–800 μm^2) was significantly higher, compared with pre-injection (control) and day 1 ($p < 0.05$); similar change was observed in the 800–1000 μm^2 cell area VR1-positive neurons ($p < 0.01$).

For example, the peripheral VR1 increased in the inflamed rat hind paw and DRG 2 and 7 days after CFA injection, respectively [4,5]. Selective VR1 antagonists could alleviate thermal hyperalgesia in formalin and carrageenan models of pain in rats [6,7]. Our results confirm the role of VR1 during this period of hyperalgesia. From day 1 to day 21 after CFA injection into the hind paw of rats (Fig. 1), VR1 expression in DRG and the superficial layers of the spinal dorsal horn increased, with a peak at days 7–14; the ratio of VR1-positive neurons per DRG at day 1 and day 14 after CFA injection reached 1.5-fold and 1.7-fold of the control level. There was a shift of VR1 expression from small to medium neurons (Fig. 3). Pre-injection and on day 1, the VR1-positive cells were mainly small DRG neurons (cell area $<600\,\mu m^2$), but at day 14 and day 28, VR1 began to appear in medium neurons (600–1000 μm^2). The medium DRG neurons are myelinated Aδ fibers, which are very important in the development of hyperalgesia. This shift of VR1 to the medium neurons gives a new explanation for VR1 in the inflammatory hyperalgesia. From our results, the so-called early stage or phase of CFA inflammatory nociception is during the first 21 days following CFA injection.

Our results also show that at day 28 after CFA injection (Fig. 1), VR1 expression in DRG neurons and the superficial layers of the dorsal horn was no longer elevated, although the thermal hyperalgesia was still present. This is a very interesting phenomenon, suggesting that VR1 in DRG and the spinal dorsal horn might play different roles in the early and late stage of the CFA inflammatory nociception. It raises interesting questions, such as: Why is VR1 expression increased in the early stage? Under what kind of regulatory mechanism(s) does the increased VR1 return in the late stage? What is the physiological significance of this change of VR1 expression? These questions need further investigation, especially through functional experiments.

In summary, VR1 expression in DRG and the superficial layers of the spinal dorsal horn increased in the early stage and decreased in the late stage of CFA-induced inflammatory nociception. Thus, the present study provides evidence that VR1 might play different roles in the development of thermal hyperalgesia at different stages in CFA inflammatory nociception in rats.

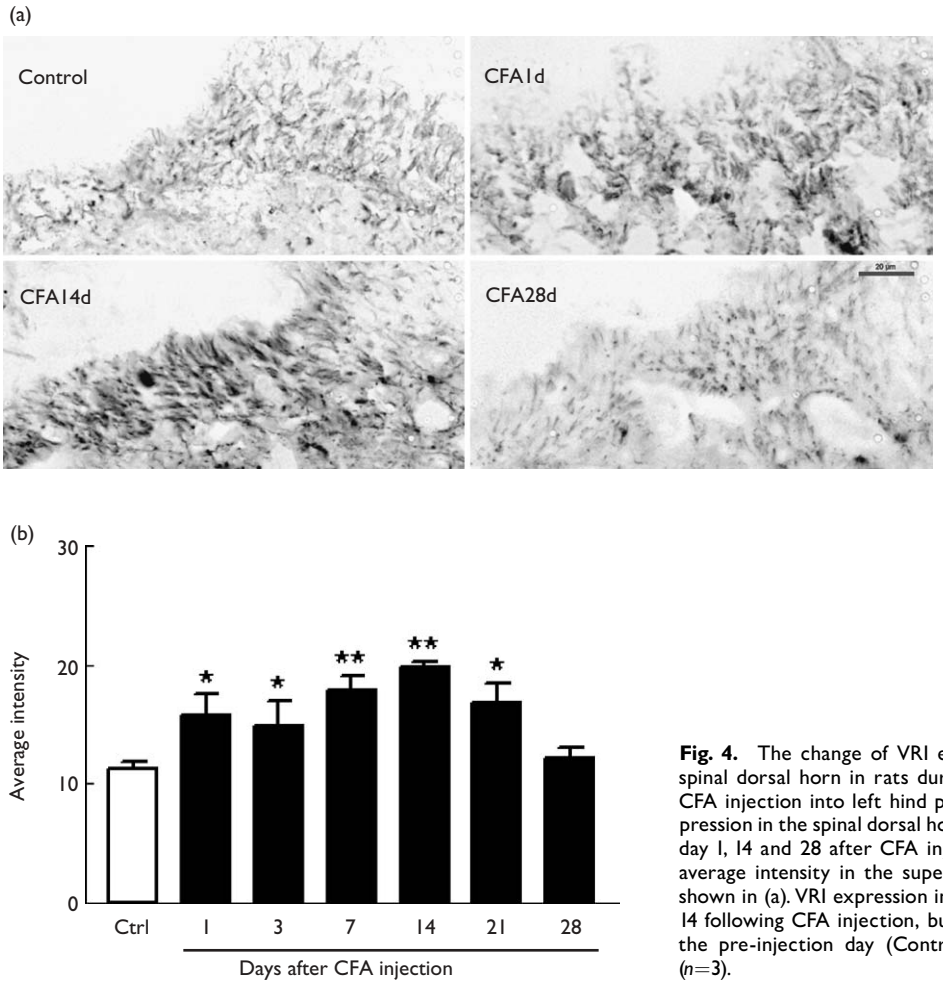

Fig. 4. The change of VR1 expression in the superficial layers of the spinal dorsal horn in rats during inflammatory nociception induced by CFA injection into left hind paw. (**a**) Immunohistochemistry of VR1 expression in the spinal dorsal horn at the pre-CFA injection day (Control), day 1, 14 and 28 after CFA injection. Bar=20 μm. (**b**) Quantification of average intensity in the superficial layers of the spinal dorsal horn as shown in (a). VR1 expression increased from day 1 to 21 with peak at day 14 following CFA injection, but returned at day 28 to the basal level as the pre-injection day (Control). $*p<0.05$, $**p<0.01$ *vs* pre-injection ($n=3$).

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Acknowledgements: This project was supported by the grants from the National Basic Research Program of China (G1999054000) and the National Natural Science Foundation of China (30330026, 30240059, 30170319). The authors thank Dr Sonya G. Wilson (Department of Neuroscience and Integrative Pharmacology, Abbott Laboratories, IL) for help in preparing this manuscript.