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Comparison of mechanisms of allodynia induced by acromelic acid A between early and late phases

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ABSTRACT

We previously showed that intrathecal administration of acromelic acid A (ACRO-A) provoked tactile allodynia in mice. As well, recent studies have demonstrated that the activation of NMDA glutamate receptor-neuronal nitric oxide synthase (nNOS) pathway and glia play crucial roles in the development and maintenance of neuropathic pain. In order to clarify their involvement in ACRO-A-induced allodynia, we investigated the effects of various agents on two mouse models at early and late-phase allodynia. The agents employed were Ca^{2+} channel α2δ ligands, NMDA and AMPA receptor antagonists, nNOS, and Ca^{2+}/calmodulin kinase II inhibitors. When injected simultaneously with ACRO-A, all of these agents blocked allodynia in the early-phase group; however, they did not block allodynia when injected 7 days after the administration of ACRO-A in the late-phase group. In order to block glial activation, astrocytic inhibitor L-α-aminoadipate (LAA) or microglial inhibitor minocycline was administrated, and allodynia was examined on day 7. Activations of nNOS and glia in the spinal cord were histochemically examined at 1 h or 1 week after injection of ACRO-A. We found that nNOS activity increased 1 h after ACRO-A injection; however, it did not increase 1 week after ACRO-A injection. Conversely, microglial activation was observed 1 week after ACRO-A injection and was significantly inhibited with minocycline treatment. Moreover, only LAA was found to inhibit late-phase allodynia.

In this study, we demonstrate that NMDA receptor activation is involved only in ACRO-A-induced tactile allodynia in the early phase, and that spinal astrocytic activation contributes to allodynia in the late phase.

1. Introduction

Neuropathic pain resulting from peripheral nerve injury is characterized by spontaneous and long-lasting pain, hyperalgesia, and allodynia (Woolf and Mannion, 1999). Peripheral nerve injury triggers long-term plastic changes along sensory pathways, from the peripheral sensory terminals to the spinal dorsal horn (Costigan et al., 2009; Latremoliere and Woolf, 2009), and synaptic plasticity is a key mechanism for neuropathic pain. Central sensitization is a glutamate receptor-dependent increase in the excitability of neurons within the central nervous system and is thought to contribute to enhanced responsiveness to sensory input following nerve injury (Latremoliere and Woolf, 2009). Glutamate is the main excitatory neurotransmitter at the vast majority of excitatory synapses, acting on three ionotropic receptor subtypes for α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA). Nociception occurs primarily through the activation of postsynaptic AMPA receptor. Under these conditions, the NMDA subtype of the glutamate channel is silent, but in the setting of inflammation and nerve injury, the increased release of neurotransmitters from nociceptors will sufficiently depolarize postsynaptic neurons to activate quiescent NMDA receptor (Basbaum et al., 2009). The consequent increase in intracellular calcium leads to the activation of several calcium-dependent enzymes, such as Ca^{2+}/calmodulin kinase II (CaMKII), phospholipase A_2 (PLA_2), and neuronal nitric oxide synthase (nNOS). Prostaglandin E_2 (PGE_2) and NO have been proposed to function as retrograde messengers and to facilitate neurotransmitter release from primary afferent terminals in the spinal dorsal horn (Kuner, 2010).

Recent studies have strongly identified spinal glia as key factors in the induction and maintenance of neuropathic pain (Watkins et al., 2001; Watkins and Maier, 2005; Mika et al., 2013). After inflammation

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or nerve injury, glia are activated and release cytokines that modulate neuronal activity and synaptic plasticity. Minocycline, a microglial specific inhibitor, exerted anti-allodynic and anti-hyperalgesic effects on neuropathic pain models (Raghavendra et al., 2003; Padi and Kulkarni, 2008; Guasti et al., 2009; Mika et al., 2009; Mei et al., 2011), and L-α-aminoadipate (LAA), a specific astrocytic toxin, inhibited neuropathic pain after spinal nerve ligation (Wang et al., 2009).

The ingestion of *Clitocybe acromelalga*, a poisonous mushroom found in Japan, causes severe tactile pain (allodynia) which can continue for over a month. By monitoring the lethal effect of *Clitocybe acromelalga* toxins in mice, 2 isomers of acromelic acid (ACRO), ACRO-A and ACRO-B, were isolated from the mushroom (Konno et al., 1983). ACRO belongs to a class of so-called kainoids bearing a pyrrolidine dicarboxylic acid represented by kainic acid. We previously showed that intrathecal (*i.t.*) administration of ACRO-A provoked prominent tactile allodynia at an extremely low dose of 1 fg/mouse (Minami et al., 2004), and that the allodynic effect of ACRO-A by a single *i.t.* administration in conscious mice was long-lasting and observed up to 4 weeks after adminsitration (Soen et al., 2007).

Recent studies have demonstrated that the activation of NMDA receptor-nNOS pathway and glia, *i.e.* astrocytes and microglia, in the spinal cord play crucial roles in the development and maintenance of neuropathic pain (Basbaum et al., 2009; Mika et al., 2013; Ji et al., 2014). In order to clarify their involvement in poisoning, we investigated the effects of various agents on ACRO-A-induced allodynia at early and late phases.

2. Materials and Methods

2.1. Chemicals

For the purpose of the study, ACRO-A was generously donated by from Professor H. Shirahama of Hokkaido University. We procured (t)-5-methyl-10,11-dihydro-5 H-dibenzo- [A,D]cyclohepten-5,10-imine hydrogen maleate (MK-801) from Merck Research Laboratories (Rahway, NJ, U.S.A.). Pregabalin, gabapentin, L-leucine, L-isoleucine, N-nitro-L-arginine methyl ester (L-NAME) and L-α-aminoadipate (LAA) were obtained from Sigma (St. Louis, MO, U.S.A.). Joro spider toxin-3 (JSTX) and minocycline were purchased from Wako Pure Chemicals (Osaka, Japan). KN-92 and KN-93 were supplied by EMD Chemicals (San Diego, *CA*, U.S.A.). All drugs were dissolved in sterile saline on the day of the experiments and kept on ice until used.

2.2. Animals

Male ddY mice were purchased from Shizuoka Laboratory Center (Hamamatsu, Japan). The animals were housed under conditions of a 12-h light/12-h dark cycle, a constant temperature of 24 ± 2 °C, and 60 ± 10% humidity. They were allowed free access to food and water before testing. All animals conformed to the regulations of the Animal Care Committees of Osaka Medical College and Kansai Medical University and received humane care in accordance with the guideline of the Ethics Committee of the International Association for the Study of Pain (Zimmermann, 1983).

2.3. Tactile allodynia induced by i.t. injection

Studies on tactile allodynia induced by *i.t.* injection of ACRO-A were carried out as described previously (Minami et al., 2004). Briefly, a 27-gauge stainless-steel needle attached to a microsyringe was inserted between the L5 and L6 vertebrae, and the agents (each in 5 μl of saline) were injected slowly into the subarachnoid space of conscious mice. In order to evaluate the effect on ACRO-A-induced allodynia, Ca^{2+} channel $\alpha_2\delta$ ligands (pregabalin, gabapentin, L-leucine, and L-isoleucine), NMDA receptor antagonist (MK-801), Ca^{2+}-permeable AMPA receptor antagonist (JSTX), NOS inhibitor (L-NAME), and CaMKII inhibitor and its inactive enantiomer (KN-93 and KN-92) were each injected simultaneously with ACRO-A in the early-phase model and 1 week after *i.t.* injection of ACRO-A in the late-phase model. After *i.t.* injection of these agents, allodynia was assessed once every 5 min for 15 min (early allodynia) or once every 5 min for 50 min (late allodynia) by light stroking of the flank of the mice with a paintbrush in a blind fashion. The allodynic response was ranked as follows: 0 (no response), 1 (mild squeaking with attempts to move away from the stroking probe), 2 (vigorous squeaking evoked by the stroking probe, biting at the probe, or strong efforts to escape). The score for allodynia induced by ACRO-A alone was 100% and the animals were used only for a single experiment.

2.4. NADPH-diaphorase histochemistry

At 1 h or 1 week after *i.t.* injection of ACRO-A (1 fg/5 μl), the animals were anesthetized by intraperitoneal (*i.p.*) injection of pentobarbital (50 mg/kg) and perfused through the left cardiac ventricle with 50 ml of physiological saline followed by a fixative containing 4% paraformaldehyde in 0.12 M sodium phosphate (pH 7.4). Following dissection, the spinal cord was postfixed overnight in the same fixative at 4 °C and then kept for 1 day in 0.1 M sodium phosphate buffer (pH 7.4) containing 30% (w/v) sucrose. Transverse frozen sections (40-μm thick) were cut on a cryostat, and the sections were thaw-mounted on slides. For the distribution of nNOS, NADPH-diaphorase histochemistry was carried out (Mabuchi et al., 2003). The incubation was performed for 2 h at 37 °C in a reaction mixture containing 0.5 mg/ml β-NADPH, 0.2 mg/ml nitroblue tetrazolium, and 10% Triton X-100 in 0.1 M phosphate-buffered saline (pH 7.4).

2.5. Minocycline treatment and immunohistochemistry

ACRO-A (1 fg/5 μl) was *i.t.* injected into naïve mice or those treated with minocycline. Two protocols for the daily treatment of minocycline (30 mg/kg, *i.p.*) were employed according the procedures reported previously for nerve injury model (Ma et al., 2010): 1) pre-treatment, from 1 day before to day 2 after, and 2) post-treatment, from day 2 to day 5 after *i.t.* injection of ACRO-A. At 1 h or 1 week after the injection, the mice were killed and tissue preparations were carried out as described above. The sections were then blocked with 10% normal goat serum in PBST (0.1% Triton X-100 in K^+-free 0.1 M phosphate-buffered saline (pH 7.4)) for more than 30 min. For ionized calcium binding adaptor molecule 1 (Iba1) immunostaining, the sections were incubated overnight at 4 °C with rabbit anti-Iba1 antibody (1: 500; Wako, Osaka, Japan). After washing, the sections were incubated with secondary antibody, Alexa Fluor 546-goat anti-rabbit IgG antibody (1: 300; Invitrogen), for 2 h at 25 °C.

2.6. LAA treatment

We injected the astrocytic specific toxin LAA 1 week after the i.t. administration of ACRO-A in order to evaluate its effect on late allodynia. After *i.t.* LAA or saline injection, allodynia was assessed once every 5 min for 50 min.

2.7. Quantification of histochemical images

The numbers of NADPH diaphorase-positive neurons and $Iba1^+$ microglia were counted, and the intensities of NADPH-diaphorase and Iba1 staining (Romero-Sandoval et al., 2008) were analyzed by Image J software (NIH, Bethesda, MD, USA) in 4 sections of respective treatments. The experiments were carried out 4 times, and similar results were obtained.