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Formalin injection produces long-lasting hypersensitivity with characteristics of neuropathic pain

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ABSTRACT

The purpose of this study was to investigate whether 1%, 2% or 5% formalin injection produce hypersensitivity with characteristics of the neuropathic pain induced by spinal nerve injury. Formalin injection (1%, 2% and 5%) produced concentration-dependent long-lasting (at least 14 days) mechanical allodynia and hyperalgesia in both paws. Likewise, L5/L6 spinal nerve ligation induced allodynia and hyperalgesia in both paws. The intensity of hypersensitivity was greater in the ipsilateral than in the contralateral paw in all models. Systemic gabapentin or morphine completely reduced 1% formalin-induced hypersensitivity. In contrast, both drugs were not able to fully diminish 2–5% formalin- and nerve injury-induced hypersensitivity. Indomethacin produced a significant effect in the chronic 1% formalin test. Conversely, this drug did not modify 2 or 5% formalin- and nerve injury-induced hypersensitivity. Spinal nerve injury and 2–5%, but not 1%, formalin injection enhanced ATF3 protein expression and immunofluorescence in dorsal root ganglia (DRG) in a time-dependent manner. Furthermore, 2–5%, but not 1%, formalin injection or spinal nerve injury also enhanced $\alpha_2\delta$–1 subunit protein levels in DRG. Our results suggest that 5% and, at lesser extent, 2% formalin injection produces long-lasting hypersensitivity with a pharmacological and molecular pattern that resembles neuropathic pain induced by spinal nerve ligation.

1. Introduction

The formalin test is a widely used model of inflammatory pain. This model has been used in mice, rats and dogs (Rodríguez and Pardo, 1968; Dubuisson and Dennis, 1977; Hunskaar et al., 1986; Murray et al., 1988; Tjølsen et al., 1992; Abbott et al., 1995). In rodents, nociceptive responses typically consist of lifting, biting/licking and flinching of the hind paw after injection of formalin into the dorsal or plantar surface of the paw (Dubuisson and Dennis, 1977; Tjølsen et al., 1992; Abbott et al., 1995). Formalin injection produces a biphasic nociceptive behavior; a phase 1 (about 5–10 min) followed by a quiescent period (about 5 min) and then phase 2 (about 30–40 min). Interestingly, it has been shown that formalin produces a comparable pattern of activity in dorsal horn neurons (Dickenson and Sullivan, 1987a, 1987b). It is widely accepted that initial barrage of C-fiber activity in phase 1 leads to a phase 2 which depends on inflammatory response and central sensitization (Coderre et al., 1990; Yamamoto and Yaksh, 1992). In addition, there is evidence for ongoing peripheral nerve activity during phase 2 (Coderre et al., 1990; Puig and Sorkin, 1996).

Besides the acute effects of formalin (about 1 h), this irritant produces long-lasting secondary thermal and mechanical hyperalgesia (Fu et al., 2000, 2001; Vierck et al., 2008) as well as mechanical secondary allodynia and hyperalgesia in both paws (Ambriz-Tututi et al., 2009, 2011). Formalin (5%) injection into the dorsum of the paw produces hyperalgesia 1–3 days after injection, which lasts for 3–6 weeks (Fu et al., 2000, 2001). Lower concentrations of formalin (1–2%) still produce long-lasting secondary allodynia and hyperalgesia (Ambriz-Tututi et al., 2009, 2011). The mechanisms leading to the long-lasting hypersensitivity induced by formalin are unknown. However, since formalin (2–5%) enhances expression of activating transcription factor 3 (ATF3) in DRG 3 days after injection (Tsujino et al., 2000; Braz and Basbaum, 2010), we hypothesized that formalin injection could produce a profile of hypersensitivity similar to that observed in rats subjected to spinal nerve injury. Furthermore, we hypothesized that formalin injection could produce changes in the expression of ATF3 and other markers (calcium channel $\alpha_2\delta$–1 subunit [$\alpha_2\delta$–1 subnit]) in DRG similar to those produced by nerve injury. Thus, in this study we assessed the pharmacological and molecular profiles of the long-lasting hypersensitivity induced by formalin in

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comparison to a spinal nerve ligation model.

2. Materials and methods

2.1. Animals

These experiments were carried out in female Wistar rats. Sex of rats was chosen based on previous observations in our laboratory in which we did not find significant differences in formalin- or spinal nerve injury-induced nociceptive responses between male and female rats (Caram-Salas et al., 2007; Cervantes-Durán et al., 2016). For the evaluation of formalin-induced long-lasting hypersensitivity, we used rats at 8–10 weeks of age (body weight 180–200 g). In the case of the L5/L6 spinal nerve ligation model, we used animals of 6 weeks (140–160 g). Animals had free access to food and drinking water before the experiments. All experiments followed the Guidelines of Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and were approved by our local Ethics Committee (Protocol 042-13, Cinvestav, Mexico City). Efforts were made to minimize the number of animals used.

2.2. Induction and assessment of long-lasting allodynia and hyperalgesia

Rats were restrained gently to inject formalin (1%, 2% or 5%) subcutaneously (s.c.) into the dorsal surface on the right hind paw. Other rats were prepared according to the method of Kim and Chung (1992). Briefly, animals were anesthetized with a mixture of ketamine (45 mg/kg, i.p.) and xylazine (12 mg/kg, i.p.). After surgical preparation and exposure of the dorsal vertebral column, the left L5 and L6 spinal nerves were exposed and tightly ligated with 6-0 silk suture distal to the dorsal root ganglion. In the sham group, the surgical procedure was identical to that described above, except that the spinal nerves were not ligated. Rats were allowed to recover from surgery for 14 days before testing pain-related behavior.

Formalin- and spinal nerve ligation-induced hypersensitivity was assessed at baseline and 1, 3, 7 and 14 days after injury. However, drugs were administered only 14 days after formalin injection or spinal nerve injury. Rats were placed into testing cages on a wire mesh bottom and allowed to acclimate for about 40 min. Measurements were determined by applying two von Frey filaments (Stoelting Co, Wood Dale, IL) to the base of the third toe on the plantar surface of both paws 10 times during each testing period to determine the response frequency for each filament. Three trials were completed to determine the paw response frequency. A force of 10 mN (1 g) does not activate cutaneous nociceptors in *naïve* rats as it does not lead to a paw withdrawal. Thus, responses to this filament are an indication of the presence of allodynia. Likewise, a force of 250 mN (26 g) or more is considered a noxious stimulus (Leem et al., 1993) and then hyperalgesia occurs when there is an increased response to these stimuli. Rats were killed in a CO_2 chamber at the end of the experiment.

2.3. Western blot analysis

Western blot analysis was used to determine the expression of ATF3 and $\alpha_2\delta$–1 subunit in the ipsilateral DRG. *Naïve, sham*, ligated and formalin-treated rats were killed by decapitation. The L4-L6 DRGs were dissected. Immediately, tissues were frozen and stored at −70 °C. Tissues from individual animals were homogenized in ice-cold lysis buffer (in mM: 150 NaCl, 50 Tris–HCl, 1 EDTA), pH 7.4 for 2 min at 4 °C. The protease inhibitors PMSF (2 mM), aprotinin (6.8 μg/ml), leupeptin (4 μg/ml), pepstatin A (4 μg/ml) and the surfactant 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) were added to the lysis buffer immediately prior to use. The homogenate was then centrifuged (Eppendorf, Hamburg) at 20,800×*g* for 10 min to remove cellular debris. The resultant supernatant was used to measure protein concentration by the Bradford's method (Bio-Rad, Hercules, CA).

One hundred and fifty μg of total protein were resolved by denaturing in 8% and 12% SDS–polyacrylamide gel electrophoresis for $\alpha_2\delta$–1 subunit and ATF3, respectively and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk or 2% casein in PBS at pH 7.4 (in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4 and 2 KH_2PO_4) with 0.05% Tween-20 for 1 h. After that, they were washed and incubated overnight at 4 °C in 1% non-fat dry milk/PBS containing rabbit anti-ATF3 (1:500, Santa Cruz Biotechnology, Cat # C-19: sc-188, Dallas, Texas) or rabbit anti-$\alpha_2\delta$–1 subunit (1:1000, Alomone Labs, Cat # CC-015, Jerusalem) antibodies. Membranes were incubated for 1 h at room temperature in 1% non-fat milk/PBS containing the horseradish peroxidase-conjugated secondary antibody (anti-rabbit, Cat # 111-035-003, 1:6000, Jackson Immunoresearch, West Grove, PA). Protein signal detection was achieved with the ECL chemiluminescence system (ECL plus, Amersham, UK). The next day, blots were stripped and incubated with a monoclonal antibody directed against β-actin (1:10000, Cat # GT5512, GeneTex, Irvine, CA), which was used as internal control to normalize ATF3 or $\alpha_2\delta$–1 subunit protein expression level. Scanning of the immunoblots was performed and the bands were quantified by densitometry using an image analysis program (Image Lab Software Version 5.2.1, Bio-Rad, Hercules, CA).

2.4. Immunohistochemistry

Rats were anesthetized under isoflurane flow and perfused intra-aortically with 250 ml of ice-cold, 0.1 M buffer phosphate [containing (mM): 137 NaCl, 2.7 KCl, 10 Na_2HPO_4 and 2 KH_2PO_4] with heparin (10000 U/L), pH 7.4, at a flow rate of 18–25 ml/min, followed by 4% paraformaldehyde +12% picric acid solution in PBS. After perfusion, ipsilateral L4-L6 DRGs were collected and then cryoprotected by 72 h in 30% sucrose in PBS, embedded in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA), frozen with dry ice and stored at −70 °C.

Sections were cut from frozen blocks with a cryostat (Leica CM1950, Nussloch, Germany) at a thickness of 14 μm. The sections were mounted onto gelatin-coated slides. Pap-pen (Super Pap-Pen, Cat. # h2802, EB Sciences, East Granby, CT) was used to draw a hydrophobic ring around the sections and the slides were allowed to air-dry for 1 h at room temperature. Sections were first washed 3 times with 0.1 M PBS pH 7.4., followed by a blocking step with 3% normal donkey serum in PBS +0.3% TX-100 for 2 h. Then, the sections were incubated overnight at 4 °C in 1% blocking solution containing the primary antibodies. Immunodetection of ATF3 in DRG was performed by using a rabbit anti-ATF3 antibody (1:500, Cat. # C-19: sc-188, Santa Cruz Biotechnology, Inc, Dallas, Texas). Sections were washed in 0.1 M PBS and incubated for 3 h at room temperature with the corresponding secondary antibody conjugated to Cy3 (Cy™3 AffiniPure Donkey Anti-rabbit IgG [H+L], 1:600, Cat. #711-166-152, Jackson ImmunoResearch, West Grove, PA).

2.5. Immunofluorescence microscopy

Fluorescence images were captured using an immunofluorescence microscope (Nikon™ ECLIPSE Ni, Melville, NY) at the same exposure and fluorescent lamp intensity settings using a 10× objective. Images were analyzed using the Image-Pro Premier 9.1 software (Media Cybernetics Inc, Rockville, MD).

To determine the percentage of ATF3+ neurons, 6–8 sections corresponding to 600–1200 neurons for each DRG per animal were counted. The percentage of ATF3-labeled neurons was calculated by dividing the number of ATF3+ neurons by the total number of neurons counted ×100.