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Research report

The up-regulation of spinal Toll-like receptor 4 in rats with inflammatory pain induced by complete Freund's adjuvant

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ABSTRACT

Peripheral inflammation induces central sensitization that displays the features by the development of pain hypersensitivity to the stimuli. It has been shown that activation of glia contributes to the development of behavioral hypersensitivity after peripheral inflammation. It has been suggested that Toll-like receptor 4 (TLR4) primarily expressed on microglia affects central pain response. The present study was designed to examine the expressions of TLR4 and microglia in the spinal cord in different time points of inflammatory pain induced by complete Freund's adjuvant (CFA). The results show that CFA induces significant pain hypersensitivity and paw edema as well as spinal dorsal horn (SDH) microglia activation with the increased expressions of OX-42 and TLR4 during the inflammatory pain, respectively. The quantification of TLR4 with Western Blot analysis also suggests the same patter with the morphological results during the progress of inflammatory pain. In addition, chronic minocycline hydrochloride intrathecal injection reverses pain hypersensitivity and suppresses activation of microglia and TLR4 induced by CFA, but has hardly any effects on paw edema. Taken together, our data demonstrate the importance of TLR4 and microglia in rats in CFA inflammatory pain states, and suggest that blockade of microglia should likely be considered as a therapeutic opportunity.

1. Introduction

Inflammation causes peripheral and central sensitization. The processes of sensitization are thought to be related with spontaneous pain, hyperalgesia (exaggerated pain response on noxious stimulation) and allodynia (pain response on normally non-painful stimulation). It is reported that central neuroinflammation and neuroimmune activation may involved in central sensitization, allodynia and hyperalgesia (Watkins and Maier, 2001). In the spinal cord, immunelike glia (microglia and astrocytes) are important mediators of central sensitization (Miller, 2005). The glial activation in the spinal cord has been reported in inflammatory pain rat model following intra-plantar complete Freund's adjuvant (CFA) injection (Raghavendra et al., 2004). The activated glia and subsequent release of pro-inflammatory mediators have been implicated in initiating and maintaining pain response (Watkins and Maier, 2003; Watkins et al., 2001). Both microglia and astrocyte are considered to be important contributors to the early and the late stages of chronic pain pathologies, respectively (Raghavendra et al., 2004; Milligan, 2009). It is believed that receptors on the surfaces of microglia recognize invariant molecular structures of pathogens. Toll-like receptors (TLRs) are transmembrane signaling proteins that are expressed in the cells of innate immune system (Csullog et al., 2001). It has been suggested that Toll-like receptor 4 (TLR4) primarily expressed on microglia affects central pain response by ultimately inducing the production of reactive oxygen species and cytokines related to the pain response (DeLeo et al., 2000; Lehnardt et al., 2003; Tanga et al., 2005). Thus, TLR4 plays a role in glial dysregulation and leads to the development of pain.

Although some literatures are available regarding TLR4 and microglia expressions in inflammatory pain induced by CFA, the conclusion attained varies extensively. Some studies have shown microglia activation following the inflammatory insult in the CFA model (Raghavendra et al., 2004; Lehnardt et al., 2003), but other has suggested that there are no obvious activation of microglia following intra-plantar CFA (Clark et al., 2007). The aim of the present study was to test the hypothesis that (1) CFA injection causes peripheral and central sensitization relating with pain

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hypersensitivity; (2) CFA injection activates spinal microglia and TLR4 in spinal cord; (3) inhibitory effects on spinal microglia activation can partly inhibit pain hypersensitivity; (4) TLR4 is primarily expressed on microglia.

2. Materials and methods

2.1. Animal

Male *Sprague-Dawley* rats (200–230 g) were provided by Laboratory Animal Center of the Fourth Military Medical University (FMMU, Xian, PR China). All experimental protocols were in accordance with the Guideline for the Animal Care and Use Committee for Research and Education of the FMMU. All efforts were made to minimize the number of animals used and their suffering.

2.2. Intrathecal cannulation and drugs administration

Under sodium pentobarbital anesthesia (40 mg/kg *i.p.*), rats were surgically prepared for intrathecal cannulation using a method from a previous study (Storkson et al., 1996). Briefly, a dorsal midline incision (3 cm) was made at the level of T3-T4 vertebrals and a polyethylene catheter (PE-10 catheter, BD Diagnostics, Sparks, MD, USA) filled with 0.9% NS was inserted into the subarachnoid space of the lumbar and sacral enlargement. The rats were allowed to recover for a week before being used. According to modified neurologic severity scores, the rats were tested by motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive) and reflex tests. The rats showing neurological deficits postoperatively were excluded. At the end of the experiments, the location of the distal end of the catheter was verified when the spinal cord were removed. Minocycline hydrochloride (100 μg) (Sigma, St. Louis, MO, USA) was delivered via the intrathecal cannula. Minocycline was a potent inhibitor of microglial activation without direct effects on astrocytes, oligodendroglia, and neurons (Amin et al., 1996; Tikka et al., 2001). The drug was dissolved in normal saline (NS) to a volume of 10 μl and injected followed by a flush of 10 μl NS.

2.3. Inflammatory pain model

Inflammation was induced by subcutaneous injection of 100 μl CFA (50 μg of *Mycobacterium butyricum*; Sigma) into the center of the plantar surface of left hind paw in conscious rats. A volume of 100 μl NS was injected into the contralateral equivalent area to serve as a sham.

2.4. Behavioral tests

Animals were habituated to the testing environment for 3 days (d) before testing. Briefly, the paw withdrawal threshold to mechanical stimulus was determined using von Frey filaments (Stoelting, Kiel, WI, USA). The hind paw was pressed with one of a series of von Frey filaments with gradually increasing pressure applied to the plantar surface for 5–6 seconds (s) for each filament. Each filament was applied 10 times and the minimal value which caused at least 6 responses was recorded as the paw withdrawal thresholds (PWTs). Acute withdrawal, biting, licking or shaking of the ipsilateral hind limb and vocalization were considered to be positive signs of withdrawal (Chaplan et al., 1994). Thermal hyperalgesia was tested using a method that was modified from Hargreaves (Hargreaves et al., 1988). The rats were habituated to an environment that included an individual Perspex box on an elevated glass table and a portable radiant heat source under the glass table. The heat source was focused on the intra-plantar surface during testing. The withdrawal latency was defined as the time to withdraw the hind paw from the heat source, and 40 s was regarded as the cut-off point to avoid tissue damage. The behavioral tests were performed in a double-blind manner.

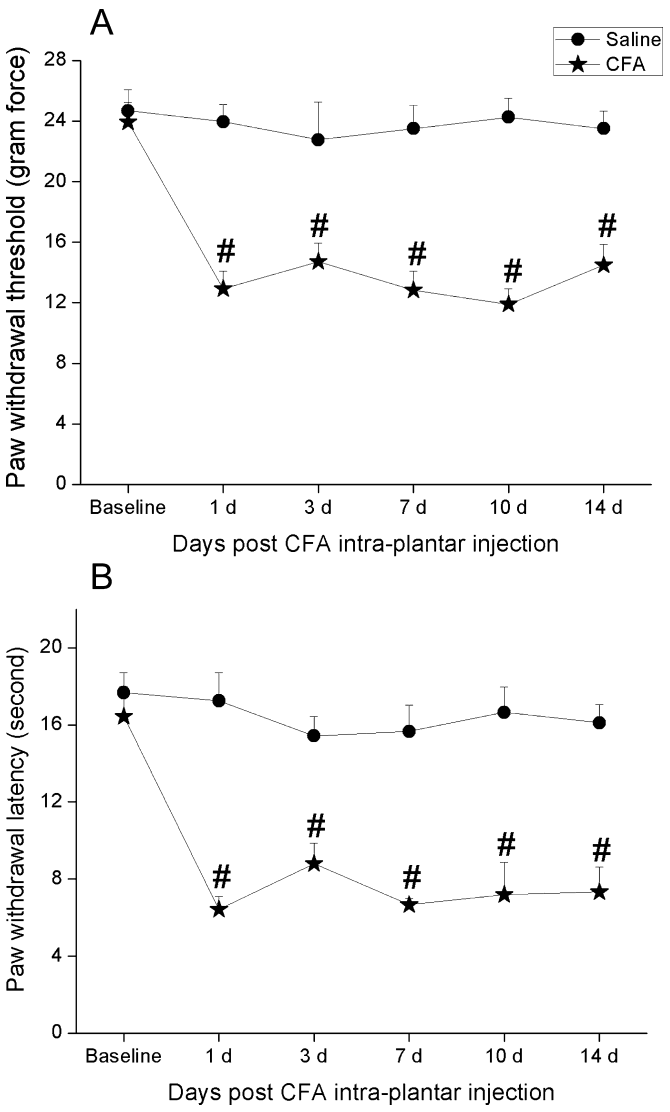

Fig. 1. Graphs showing time course of PWT and PWL in the mechanical allodynia (A) and thermal hyperalgesia (B) tests, respectively. The filled circles and stars represented the saline sham and the CFA groups, respectively. #, $P < 0.05$ compared to the saline sham groups.

2.5. Paw edema measurement

Edema formation was measured by changes in paw volume. The quantitative detection of paw volume was made by the method before (Daher et al., 2005). In brief, the paw was immersed into a cuvette filled with a solution of 2.5% Extran in water (v/v) without touching the cuvette. The cuvette was fixed on the plate of an electronic scale (precision of 0.01 g), and the immersion was accompanied by the increasing of the weight and of the liquid scale in the cuvette. The density of cuvette solution was 1 mg/ml, and the value displayed by the scale was assumed to represent the paw volume.

2.6. Immunofluorescence

Immunohistochemistry staining was performed using the open book method (Wang et al., 2009). Rats were perfused and the lumbosacral enlargements were dissected and cryoprotected in