Attenuating phosphorylation of p38 MAPK in the activated microglia: A new mechanism for intrathecal lidocaine reversing tactile allodynia following chronic constriction injury in rats

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Abstract

Increasing evidences approve the long-term analgesia effects of intrathecal lidocaine in patients with chronic pain and in animal peripheral nerve injury models, but the underlying mechanism remains elusive. Previous evidences suggest that the activation of the p38 MAPK signaling pathway in hyperactive microglia in the dorsal horn of spinal cord involves in nerve injury-induced neuropathic pain. In this study, we demonstrate that attenuating phosphorylation of p38 MAPK in the activated microglia of spinal cord, at least partly, is the mechanism of intrathecal lidocaine reversing established tactile allodynia in chronic constriction injury model of rats. This finding not only provides a new insight into the mechanisms underlying long-term therapeutic effects of lidocaine on neuropathic pain, but also reveals one more specific drug target for analgesia.

Keywords: p38 MAPK; Microglia; Neuropathic pain; Rats; Chronic constriction injury

Neuropathic pain afflicting millions of people worldwide has become one of the most significant health problems [5,15]. Microglia play a very important role in establishing and maintaining common pain states. The activation of microglia in spinal cord was discovered in various types of neuropathic pain animal models, which indicate that activated microglia are key cellular intermediaries in the development of nerve injury-induced pain hypersensitivity [28,35,36]. Furthermore, phosphorylation of p38 mitogen-activated protein kinase (MAPK), which is selectively expressed in hyperactive microglia of spinal dorsal horn, is essential for activation of microglia. Intrathecal administration of p38 MAPK inhibitor at the spinal level transiently suppresses nerve injury-induced tactile allodynia, implying that ongoing activation of the kinase is necessary for sustaining allodynia [8]. Intrathecal administration of the local anesthetic lidocaine brings significant relief to those who suffer from neuropathic pain syndromes [29,32,33]. The mechanism underlying the long-lasting anti-allodynic effect of intrathecal lidocaine remains unclear. Some researchers suggest that the inhibition of lidocaine on immune cells could contribute to some of the potent analgesic effects of lidocaine reported in burn patients and in patients having undergone surgery [19,34].

Lidocaine can inhibit cellular functions of neutrophils and macrophages in vitro, including priming, adhesion, phagocytosis. As resident immune cells in central nervous system (CNS), microglia share numerous similarities with macrophages, in their immunological and functional properties [23]. Thus, it is reasonable to assume that intrathecal lidocaine produces the anti-allodynic effect by attenuating phosphorylation of p38 MAPK in the activated microglia in spinal cord. Here, we demonstrate for the first time that intrathecal injection of lidocaine in the rats with chronic constriction injury indeed remarkably suppressed the p38 MAPK activation in the hyperactive microglia in the L4–L5 dorsal horn. Our results suggest that lidocaine may relieve neuropathic pain at least partly by suppressing p38 MAPK activation in microglia of spinal cord.

Male Sprague–Dawley rats weighing between 230 and 250 g were housed at 26 °C temperature with a 12 h light/dark cycle and were fed food and water ad libitum. In the present study, all the animals have been treated in accordance with the guidelines of National Institute by the Institutional Animal Care Committee.

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CCI surgery which produces disorders of pain sensation like those seen in human was carried out as described previously [2]. In brief, the rats were anesthetized with 10% chloral hydrate (3 ml/kg, i.p.), and a 7-mm segment of the left common sciatic nerve was exposed at the mid-thigh level, proximal to the sciatic trifurcation. Four ligatures (4–0, silk thread) were tied around the nerve at intervals of 1 mm, and ligatures were tied loosely enough.

Seven days after CCI surgery, under ether anesthesia, a polyethylene catheter (PE-10) was introduced into the subarachnoid space by a small puncture through a slit in the L5–L6 interspace, and advanced 3–4 cm into the intrathecal space to reach the lumbar enlargement of the spinal cord [26]. The right interspace, and advanced 3–4 cm into the intrathecal space to polyethylene catheter (PE-10) was introduced into the subarachnoid space. The right jugular vein and left carotid artery of the rats were cannulated with 22-gauge intravenous catheters (BD Angiocath™; Brazil), for administration of vasoconstrictors and measurements of mean arterial blood pressure (MAP), respectively. Refitted 14-gauge intravenous catheters (BD Angiocath™; Brazil) were advanced endotracheally for preparation of mechanical ventilation.

According to our data from preliminary research, during which CCI rats received various concentrations of lidocaine (from 0% to 1.8%), we found 1.2% intrathecal lidocaine exerted the best analgesic effect on the CCI rats (data not shown). So in this research, the rats were randomly divided into four groups as follows: normal rats without any treatment (NORM), CCI rats without any treatment (CCI), CCI rats + intrathecal injection with 0.5 ml of 0.9% saline as sham group (CCI + SALI), CCI rats + 0.5 ml intrathecal injection with 1.2% lidocaine (CCI + LIDO). Lidocaine was diluted in 0.9% saline to 0.5 ml equally in all the groups, to avoid the influence of different intracranial pressures on the development of pain [30,31], and were injected into the intrathecal catheters at a constant rate of 0.05 ml/min. The rats were mechanically ventilated (Rat Ventilator, RSP1002, Kent Scientific Inc., USA) with air immediately at the sight of irregular respiration. Meanwhile, MAP was continuously monitored until the rat recovered completely from intrathecal lidocaine therapy. When MAP dropped about 30% from pre-therapy value, the rat was treated by single intravenous administration of dopamine 2 mg·kg⁻¹. There were no significant difference between group CCI + SALI and group CCI + lidocaine in operation time, the consumption of antibiotic and dopamine, the recovery rate, and the weight of rats after operation (data not shown). These rats were tested behaviorally each day after intrathecal lidocaine, until tenth day.

The rats were placed on a metal mesh floor. To assess the mechanical allodynia, a set of von Fray filaments ranging from 1.3 to 26 g was applied (Stoelting CO) in ascending order. The 60% paw withdrawal threshold (PWT) was determined by the up-down method. Each rat was measured three times, and the average values were obtained.

Four days after intrathecal injection of lidocaine, CCI rats showed the highest mechanical threshold according to behavior test. On the day, the rats were deeply anesthetized with 10% chloral hydrate and perfused transarteriae aorta with 0.9% saline (37 °C) 60 ml, followed by 4% paraformaldehyde containing 0.1% picric acid in 0.1 M PB (4 °C). The L4–L5 lumbar spinal cord was excised, postfixed for 3 h at 4 °C in 4% paraformaldehyde, and placed in 20% sucrose solution for 72 h at 4 °C. Spinal cord were embedded in OCT, and then cut with a microtome (Leica CM1900, Germany) in 14-μm section slices.

The spinal cord sections were incubated for 24 h at 4 °C in the primary antibody. Anti-phospho-p38 MAPK (anti-rabbit, 1:100; Signalway Antibody); marker of microglia, OX42 (anti-mouse, 1:50; Chemicon); marker of neurons, NeuN (anti-mouse, 1:500; Serotec), marker of astrocytes, GFAP (anti-mouse, 1:200; Chemicon) were used to identify the type of phospho-p38 MAPK positive cells. Following incubation, tissue sections were washed three times by PBS and incubated for 45 min at 37 °C in the secondary antibody solution (anti-rabbit IgG-conjugated or anti-mouse IgG-conjugated 1:100; Jackson Immunoresearch Laboratories Inc.).

The images of immunostaining p-p38 in spinal cord sections were captured by Neurolucida upright microscope (Nikon Eclipse E600). For quantitative assessment of the changes in immunofluorescence intensity of the p-p38 expression in the spinal cord section, we measured the average pixel intensity per 0.5 mm² area within medial pial layer in the superficial layer of three sections [36], analyzed by image analysis software (Image-Pro® Plus 5.0).

Four days after intrathecal injection lidocaine, the rats were killed quickly under ether. The dorsal horn of L4–L5 lumbar spinal cord was quickly removed, washed three times with PBS, and homogenized in a SDS sample buffer containing a mixture of phosphatase inhibitors and protease inhibitors. Protein samples were dissolved in 4× sample loading buffer, denatured at 50 °C for 20 min, separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 1% milk in TBST for 1 h at room temperature and incubated 24 h at 4 °C with anti-p38 antibody (anti-rabbit, 1:500; Signalway Antibody) or anti-actin antibody (anti-mouse, 1:50,000; Cell Signaling) in TBST + 1% BSA [18]. The blots were incubated for 1 h at room temperature with HRP-conjugated secondary antibody (Chemicon; 1:5000), developed in ECL solution for 1.5 min, exposed to X-ray film.

All data were presented as mean ± S.D. In the part of behavioral tests, the mean values comparisons were analyzed using two-way ANOVA test. Student’s t-test was used to verify the change in the intensity of immunoreactive cells and of protein bands. The criterion for statistical significance was p < 0.05.

Six days after CCI surgery, the withdrawal threshold (PWT) of ipsilateral side hind paws of rats was significantly decreasing. Intrathecal administration of 1.2% lidocaine significantly reversed the established tactile allodynia in CCI rat (p < 0.05, Fig. 1), and the anti-allodynic effect lasted until 8 days after injection. In contrast, in the rats which were infused with saline,
Fig. 1. Intrathecal injection of lidocaine suppressed the established mechanical allodynia caused by CCI. The mechanical paw withdrawal threshold (PWT) by von Frey stimulation to the hind paws was assessed on day before CCI, 3, 6 days after CCI (post-CCI), and 2, 4, 6, 8, 10 post-intrathecal lidocaine (post-drug). The PWT between contralateral side and ipsilateral side was significantly different after CCI surgery, and it was obviously reversed after intrathecal injection of lidocaine in CCI rat, but not changed by intrathecal saline. The anti-allodynic effect of CCI + lidocaine group maintained 8 days after injection. Error bars represent mean ± S.D. (n = 6) *p < 0.05, **p < 0.01 vs. CCI + saline group.

no attenuation or reversal of tactile allodynia was observed (Fig. 1).

Compared to normal rats, immunostaining showed the phospho-p38 MAPK on the ipsilateral side of saline-treated or untreated CCI rats was obviously increased (Fig. 2), and almost all phospho-p38 MAP positive cells were double-labeled with OX42 12 days after nerve injury, indicating that activation of p38 MAPK in the dorsal horn is highly restricted in individual hyperactive microglia (C), neither in neurons (D) nor in astrocytes (E) following CCI surgery. Intrathecal lidocaine leaded to a reduction in p-p38 positive cells (p < 0.05, Fig. 3).

In Western blot analysis, we found that the change of band intensity of phospho-p38 MAPK protein in spinal cord had the same result as immunofluorescence analysis (Fig. 3R and S).

These results indicate that intrathecal lidocaine can significantly reverse the established tactile allodynia, at least partly by suppressing the p38 MAPK activation in microglia of spinal cord.

In the present study, we found single intrathecal lidocaine alleviated established tactile allodynia for about 8 days in CCI model, and firstly showed that intrathecal lidocaine significantly suppressed p38 MAPK activation in microglia of spinal cord, which occurred exclusively in hyperactive microglia in the dorsal horn of CCI rats.

As a widely used clinical drug, lidocaine was introduced to manage chronic and refractory pain for many years [29,32,33]. Accumulating studies suggest that intrathecal lidocaine has a long-term anti-allodynic effect on patients and animals with neuropathic pain at the dorsal horn level [21,22,25], but the precise mechanism underlying the anti-allodynic effects of lidocaine remains elusive. Some studies concerned with the ability of lidocaine in reducing the ectopic discharge after nerve injury by blocking the voltage-gated sodium channel [17]. However, since the elimination half-life of lidocaine is about 2 h [3], it seems impossible that lidocaine still functions in spinal cord several days after single intrathecal injection. Therefore, it was unreasonable to explain simply that the long lasting anti-allodynic effect of single intrathecal lidocaine was mediated by transiently suppressing the ectopic discharge or blocking the nerve conductance. Recent literature also pointed out that not all spontaneous ectopic discharges were induced by abnormal sodium channel activities, and not all neuropathic pain symptoms were underlain by spontaneous ectopic discharges [17]. So there should be another mechanism for the long-lasting anti-allodynic effects of lidocaine.

It is increasingly appreciated that neurons are not the only players that drive the establishment and maintenance of common clinical pain states. Glia and immune cells also have important roles. Neuropathic pain shares many features with a neuroimmune disorder [24]. Microglia cells are the major immunocompetent cells in the CNS. They share many similarities with macrophages of peripheral tissues. For example, MHC class II, complement receptors, cytokine/chemokine receptors, can be identified on microglia in CNS, like those on lymphocytes and macrophages in peripheral immunization system. Microglia

Fig. 2. P-p38 MAPK dramatically increased in the dorsal horn following CCI surgery and expressed exclusively in hyperactive microglia, but not in neurons or astrocytes. (A and B) Immunofluorescence of p-p38 MAPK was detected in L4–L5 dorsal spinal cord of normal rats (A) and the rats of 14 days after CCI (B). It is clear that the p-p38 MAPK in the ipsilateral side (Ipsi) was more than that in the contralateral side (Contra). (C–E) Colocalization of p-p38 (green) with OX42 (C, red, the marker of microglia), NeuN (D, red, the mark of neuron), GFAP (E, red, the marker of astrocytes), double staining appears yellow (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
cells and macrophages originate from a monocytic cellular lineage of mesodermal. Accumulating researches reported these small glia contribute a lot to neuropathic pain [16], microglial activation is characterized by p38 MAPK phosphorylation. We also found a marked increase of the level of phospho-p38 MAPK in the microglia of dorsal horn in CCI model. This result supported that microglial regulation of tactile allodynia may require activation of p38 MAPK. But it remains unclear how activated p38 MAPK in spinal microglia contribute to the dorsal horn pain transmission network after nerve injury. The most plausible mechanism is that persistent active p38 MAP kinase in the microglia play a key role in upregulating the production of cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and prostaglandin E2 (PGE2) [7,13,14,27]. All these cytokines may act directly on the central terminals of primary afferent neurons and dorsal horn neurons [16,20,24]. On the other hand, cytokines also provide important autocrine feedback signals to microglia themselves, inducing components of the complement cascade in microglia which further activate the p38 MAPK, establishing a feed forward cycle. Therefore, microglia, neurons and cytokine/chemokines may actively participate in the process of central sensitization. We suppose that activation of p38 MAPK in microglia may result in a vicious circulation in nerve injury induced chronic pain. Alleviating the immune response of microglia may provide a target for relieving neuropathic pain by aborting neurobiological alterations. Recently, researchers pay more attention on the effects of lidocaine on non-nervous tissue, particularly immune system
[9]. They suggest that lidocaine can effectively modulate the activation, priming, migration, phagocytosis of macrophages, as well as the synthesis and release of inflammatory mediators. Lidocaine has been proved to be successful in treating some autoimmune disease, such as interstitial cystitis, ulcerative proctitis, arthritis and herpes simplex infections.

A lot of literature reported lidocaine could inhibit secretion of such cytokines, as IL-8, IL-6, IL-1β, and TNF-α in some immunocell line [1,10–12]. Microglia, as a tissue-specific mononuclear phagocyte, have many properties in common with macrophages of peripheral tissues. It is reasonable to imply that lidocaine can also affect the microglial function as it does in other immunocells, further interrupting the vicious cycle of positive feedback between cytokines and microglia in the process of neuropathic pain, so as to relieve chronic pain. Our results showed that intrathecal lidocaine could dramatically decrease the phospho-p38 MAPK in the microglia of spinal cord 4 days after injection of 1.2% lidocaine. Our behavioral result was in agreement with the clinical study by Yokoyama et al. [33], who reported sustained analgesic effects of spinal anesthetic therapy with lidocaine for the relief of neuropathic pain. However, our dose of lidocaine was larger than the routine clinical dose [30]. Lan et al. [12] reported that endothelial IL-1β, IL-6, and IL-8 concentrations were attenuated by administration of lidocaine at high concentration. This evidence may provide a reasonable explanation for why intrathecal injection of 500 μg lidocaine (equaled approximately 0.1% in 250 g rats) failed to alleviate tactile allodynia following spinal nerve ligation in rats [6]. Therefore, it implies that high concentration of lidocaine effectively block the vicious feedback between microglia and auto-secreting cytokines in nerve injury, suppress the activation of p38 MAPK in spinal cord and thus achieve the goal of long lasting analgesia.

Our study has considerable clinical prospect. It provides new insight into the mechanisms underlying the prolonged therapeutic effects of lidocaine on neuropathic pain syndromes. Furthermore, the result reasonably explains some clinical state that was previously unclear. For example, lidocaine has neuroprotective effect on rat suffering from cerebral ischemia by attenuating the activation of p38 MAPK in cortex, which has been implicated as an important inducer for ischemia/hypoxia-induced cerebral injury [4]. This research increases our current understanding of lidocaine, and provides a new, non-opioid therapeutic avenue for treating chronic pain. However, we have to point out our research cannot explain some problems. How does lidocaine as a sodium channel blockade suppress the activation of p38 MAPK? Does it act directly on microglia or influence the interaction between neuron and microglia? Does lidocaine have the same effect of the activation of p38 MAPK on different nerve injury model in rat? We believe these problems could be solved in further study.

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