Original Article

Pioglitazone attenuates tactile allodynia and microglial activation in mice with peripheral nerve injury

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ABSTRACT: To test the possibility of a peroxisome proliferator activated receptor (PPAR)γ agonist to treat neuropathic pain, we examined the effects of pioglitazone, a PPARγ agonist, on tactile allodynia and expression of activated microglia in the dorsal horn of spinal cord using neuropathic pain model. The unilateral sciatic nerve was partially ligated (PSL) in male ICR mice. Pioglitazone (1-25 mg/kg p.o.) was administrated to mice once daily for five days immediately after PSL. We stimulated the footpad of the hind paw of mice using a von Frey filament to estimate tactile allodynia on day 5 of PSL. The activated microglia in the lumbar spinal cord was observed by immunohistochemistry with anti-Iba1 antibody, a marker for activated microglia. The number of Iba1-immunoreactive cells was counted in the dorsal horn spinal cord. On day 5, significant allodynia was developed in PSL mice. Pioglitazone significantly attenuated the tactile allodynia in a dose of 1-25 mg/kg. However, these doses of pioglitazone did not affect nociceptive responses in sham mice. Moreover, on day 6, the number of activated microglia was significantly increased in the ipsilateral dorsal horn of mice. The increase in the number of activated microglia induced by PSL was significantly suppressed by pioglitazone (1-25 mg/kg p.o.). Pioglitazone did not affect the number of activated microglia in sham mice. These results suggest that PPARγ activation inhibits the development of tactile allodynia and the expression of activated microglia in the dorsal horn of spinal cord in mice with PSL-induced peripheral nerve injury.

Keywords: Ligation, Neuropathic pain, Sciatic nerve, Spinal cord, Thiazolidinedione

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1. Introduction

Neuropathic pain is characterized by pain in the absence of a stimulus and/or by reduced nociceptive thresholds so that normally innocuous stimuli produce pain. This is a burdensome and potentially debilitating pain state. Numerous studies using animal models have proposed candidates for therapeutic targets to reduce neuropathic pain. The therapeutic strategies for neuropathic pain aim to reduce the excitability of neurons in the peripheral nervous system and/or the CNS by modulating the activity of ion channels or by mimicking and enhancing endogenous inhibitory mechanism. However, currently, there are no effective pharmacotherapies for neuropathic pain (*1*).

Microglial cells have a key role in the response to direct injuries of the central nervous system elicited by trauma or ischemia, in autoimmune diseases, and in neurodegenerative disorders (*2*). Recent evidence indicates that activated microglia are key cellular intermediates in the pathogenesis of nerve injury-induced pain hypersensitivity. Microglial activation leads to increased synthesis of the protease (*3*) and the cytokines (*4*). Direct modulation of dorsal horn neuron activity by these cytokines may be involved in the development of neuropathic pain. Therefore, targeting glia could provide opportunities for disease modification by aborting neurological alterations that support the development of persistent pain.

Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor belonging to a nuclear hormone receptor superfamily, containing three isoforms (α, β/δ and γ). PPARγ plays a critical physiological role as a primary lipid sensor and regulator of lipid metabolism. Thus, its ligands are clinically used for treatment of some diseases, including type 2 diabetes (*5*). However, PPARγ has additional effects on cellular physiology. Activation of PPAR has been shown to suppress inflammation in peripheral macrophages and in models of human autoimmune disease (*6*). Recently, it has been found

that PPARγ ligands have promising therapeutic use in neurological diseases involving neuroinflammation, such as Alzheimer's disease and multiple sclerosis (*7*). There are two reports indicating that PPARγ ligands can reduce neuropathic pain in animal models (*8,9*). Nonetheless, further information on activation of microglia and neuropathic pain induced by peripheral nerve injury is not available. In the present study, we examined the correlation of effect of PPARγ agonist pioglitazone on tactile allodynia and on microglia activation in the dorsal horn of spinal cord elicited by partial sciatic nerve ligation (PSL).

2. Materials and Methods

2.1. *Subjects and surgery*

Male ICR mice (5-week-old: Japan SLC, Hamamatsu) were anesthetized with pentobarbital (80 mg/kg, i.p., Dainippon Pharmaceuticals Co., Osaka, Japan). The sciatic nerve (SCN) was exposed just below the hip bone, and half of the sciatic nerve was tightly ligated with silk suture thread (PSL), according to the modified method of Seltzer *et al.* (*10*). The procedures used in these studies were approved by the Animal Research Committee of Wakayama Medical University in accordance with Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and The Guidelines for Animal Experiments in Wakayama Medical University (approval number 271).

2.2. *Behavioral test*

We observed the withdrawal responses of hind paw of which the plantar surface was applied with calibrated von Frey filaments (0.4 g; Stolting, Wood Dale, IL, USA) on day 5 following PSL. Tactile allodynia was calculated as the ratio of the number of hind paw withdrawals of 5 stimulations.

2.3. *Immunohistochemistry*

Six days following PSL, the mice were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 20 mL of PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbar spinal cord were dissected out and cut transversely (20-μm-thick) with a cryostat. The sections were incubated with a rabbit antibody against mouse Iba1 (Wako, Osaka, Japan). Then, the sections were incubated with secondary antibody solution (Alexa Fluor 488-conjugated antibody to the rabbit IgG, Molecular Probes, Eugene, OR, USA). Fluorescent images for a mouse were captured with a fluorescence microscope. Four to five images (400 μm × 400 μm) were taken in an area including the dorsal horn. All Iba1-positive cells were counted per the area, and the number from all the sections was averaged for each mouse.

2.4. *Drug administration*

Pioglitazone (1-25 mg/kg p.o.) or its vehicle (0.5% carboxymethyl cellulose, CMC) was given once daily from immediately after PSL to day 4 of PSL. Pioglitazone hydrochloride was kindly donated by Takeda Pharmaceutical Company (Osaka, Japan).

2.5. *Statistical test*

Statistical significance was determined by ANOVA followed by Tukey multiple comparisons' test, and set at $p < 0.05$.

3. Results

3.1. *Effect of pioglitazone on PSL-induced tactile allodynia*

We tested the effects of pioglitazone on tactile allodynia elicited by peripheral nerve injury on day 5 following PSL. Pioglitazone was administered once daily from immediately after PSL to day 4 following PSL. PSL significantly increased the ratio of withdrawal response of hindpaw to innocuous mechanical stimulation, compared to in sham group. The PSL-induced tactile allodynia were significantly attenuated by pioglitazone (1-25 mg/kg), which did not affect the ratio of nociceptive responses in sham group (Figure 1).

3.2. *Effect of pioglitazone on expression of PSL-activated microglia*

Immunohistochemistry using anti-Iba1 antibody revealed the expression of activated microglia with

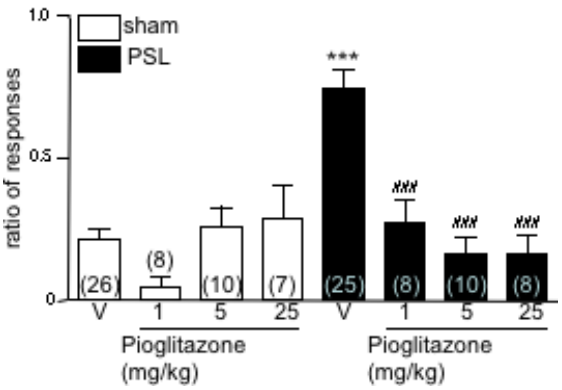

Figure 1. Effect of pioglitazone on tactile allodynia in mice subjected to PSL. Pioglitazone (1-25 mg/kg, p.o.) was administered once daily immediately after PSL to day 4 following PSL. Behavioral test was performed on day 5 of PSL. V denotes vehicle. The number in parentheses indicates the number of experiments. *** $p < 0.001$ *vs.* V/sham. ### $p < 0.001$ *vs.* V/PSL.

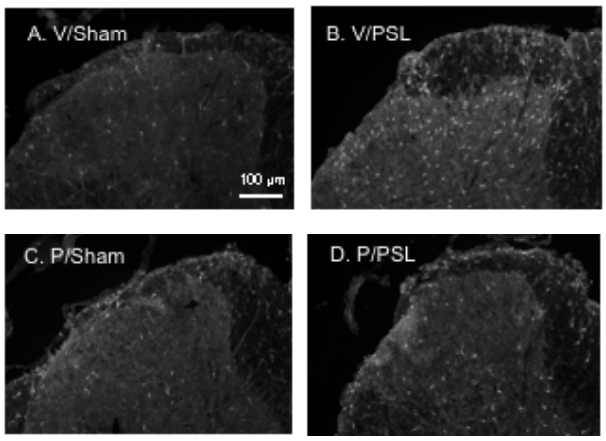

Figure 2. Pioglitazone attenuates PSL-induced up-regulation of activated microglia in the dorsal horn of spinal cord. Mice were perfused with 4% paraformaldehyde on day 6 of PSL. The sections were prepared from the dissected lumbar spinal cord and stained with anti-Iba1 antibody, a specific marker for activated microglia. Micrographs were representative of sham and PSL treated with vehicle (V) or 25 mg/kg pioglitazone (P). Dose regimen is shown at the legend in Figure 1.

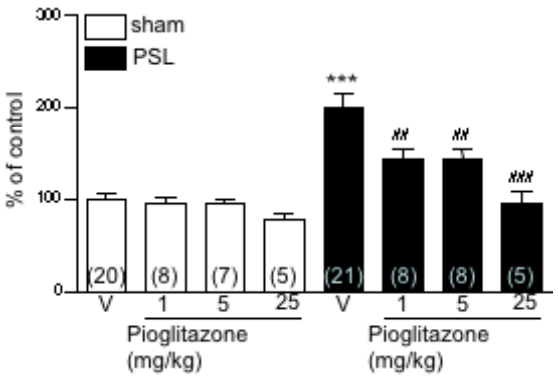

Figure 3. Effect of pioglitazone on up-regulation of activated microglia in mice subjected to PSL. The number of cells with immunoreactivity for Iba1 was counted in the dorsal horn of spinal cord. Dose regimen and immunostaining procedures are shown at the legend in Figures 1 and 2, respectively. The number of immunoreacitve cells were normalized relative to sham group treated with vehicle (control), and expressed in percentage (% of control). V denotes vehicle. The number in parentheses indicates the number of experiments. $^{***}p < 0.001$ *vs*. V/sham. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs*. V/PSL.

amoeboid morphology in the dorsal horn of spinal cord (Figure 2A). The number of cells with immunoreactivity to anti-Iba1 antibody was significantly greater in PSL group treated with vehicle than in sham group with vehicle (Figure 2B). PSL-induced increase in the number of activated microglia was significantly attenuated by administration of pioglitazone at 1-25 mg/kg (Figures 2D and 3).

4. Discussion

We examined the effect of PPARγ agonist, pioglitazone, on development of tactile allodynia and expression of activated microglia in mice subjected to peripheral nerve injury. Administration of pioglitazone for five days immediately after PSL attenuated tactile allodynia, associated with inhibition of PSL-induced expression of activated microglia in the dorsal horn of spinal cord. These results suggest that relief of tactile allodynia *via* PPARγ stimulation may be mediated by the inhibition of central sensitization through reduced activation of microglia in the spinal cord.

Pioglitazone reportedly attenuated thermal hyperalgesia and microglial activation in spinal cord injury model of rats (*9*). This model is clinically useful for study of serious motor dysfunction after spinal cord injury. The motor dysfunction, however, makes it difficult to evaluate withdrawal responses of an injured side of paw to nociceptive stimuli. In our study, mice subjected to PSL showed motor paralysis immediately after PSL, but recovered within a few days (*10*). Additionally, other finding that pioglitazone had improved motor paralysis in the spinal cord injury model (*9*) might make it even more complicated to interpret the influence of pioglitazone on thermal hyperalgesia. The PSL model, with less severe motor paralysis, is likely to be more useful studies of neuropathic pain.

The present study agrees with the well-established paradigm that peripheral nerve injury up-regulates activated microglia in the dorsal horn of spinal cord. The activated spinal microglia is required for the expression of neuropathic pain after nerve injury (*11*). Microglial activation leads to increased production of the proinflammatory cytokines, which subsequently act directly on the terminals of primary afferent neurons and on the dorsal horn neurons (*1*). The proinflammatory cytokines have another important autocrine feedback signal to microglial cells themselves, which results in fueling of the microglial inflammatory response (*12*). Proinflammatory cytokines contribute to increased spontaneous nociceptor activity and stimulus sensitivity, called central sensitization underlying neuropathic pain (*13-15*). Study on neuroinflammatory has shown that PPARγ agonist with anti-inflammatory activity suppresses production of proinflammatory mediators in the brain (*2*). We also found that pioglitazone blocked PSL-induced upregulation of proinflammatory cytokines in the dorsal horn of spinal cord, such as IL-6 and TNF-alpha, which are believed to be essential for neuropathic pain (data not shown). These facts propose a hypothesis that pioglitazone prevents development of tactile allodynia through inhibition of PSL-induced upregulation of the proinflammatory cytokines in the dorsal horn of spinal cord. On the other hand, PPARγ is expressed in the dorsal horn of spinal cord (*8,16*). These reports suggest that spinal PPARγ plays a possible role for inhibition of microglial activation. Further evidence supports the action of orally given pioglitazone on CNS: 18% of pioglitazone crosses the blood-brain barrier in rats when administered p.o. (*17*).

In conclusion, PPARγ synthetic ligands such as pioglitazone appear to be a promising drug to treat neuropathic pain involving through interfering

with microglial activation. A deep knowledge of the molecular mechanisms evoked by pioglitazone either dependent or independent of the receptor activation and of PPARγ expression in activated microglia is mandatory for the clinical use of pioglitazone with regimen for increased efficacy and safety.

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