Pioglitazone Attenuates Tactile Allodynia and Thermal Hyperalgesia in Mice Subjected to Peripheral Nerve Injury

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Abstract. To clarify the role of peroxisome proliferator activated receptor $\gamma$ (PPAR$\gamma$) in neuropathic pain, we examined the effect of pioglitazone, a PPAR$\gamma$ agonist, on tactile allodynia and thermal hyperalgesia in a neuropathic pain model. Mice were subjected to partial sciatic nerve ligation (PSL) and given pioglitazone (1–25 mg/kg, p.o.) once daily. PPAR$\gamma$ was distributed in the neurons of the dorsal root ganglion and the dorsal horn of the spinal cord and in the adipocytes at the epineurium of the sciatic nerve in naive mice. PSL elicited tactile allodynia and thermal hyperalgesia for two weeks. Administration of pioglitazone for the first week after PSL attenuated thermal hyperalgesia and tactile allodynia, which was dose-dependent and blocked by GW9662 (2 mg/kg, i.p.), a PPAR$\gamma$ antagonist. Administration of pioglitazone for the second week also relieved tactile allodynia, but administration one week before PSL had no effect. A single administration of pioglitazone to mice on day 7 of PSL did not alter tactile allodynia and thermal hyperalgesia. PSL-induced upregulation of tumor necrosis factor-$\alpha$ and interleukin-6, which are essential for neuropathic pain, was suppressed by pioglitazone for the first week. This suggests that pioglitazone alleviates neuropathic pain through attenuation of proinflammatory cytokine upregulation by PPAR$\gamma$ stimulation.

Keywords: interleukin-6 (IL-6), neuropathic pain, partial sciatic nerve ligation, peroxisome proliferator activated receptor $\gamma$ (PPAR$\gamma$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$)

Introduction

Neuropathic pain is characterized by pain in the absence of a stimulus and 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. However, currently, there are no good pharmacotherapies for neuropathic pain (1).

Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor belonging to a nuclear hormone receptor superfamily, containing three isoforms ($\alpha$, $\beta/\delta$, and $\gamma$). PPAR$\gamma$ 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 (2). Two reports indicate that PPAR$\gamma$ ligands can reduce neuropathic pain in animal models (3, 4). Nonetheless, further information on distribution of PPAR$\gamma$ in pain pathways is not available and comparison of various dose regimens of PPAR$\gamma$ agonists has not been provided. In addition, those studies have not revealed the effects of PPAR$\gamma$ agonists on up-regulated proinflammatory cytokines in the pain pathway, which is the molecular substrate for neuropathic pain.

In the present study, we identified the types of cells expressing PPAR$\gamma$ in pain pathways in naive mice. Furthermore, we show that pioglitazone, a PPAR$\gamma$ agonist, alleviated tactile allodynia and thermal hyperalgesia elicited by peripheral nerve injury associated with inhibition of up-regulation of proinflammatory cytokines in the pain pathway.
Materials and Methods

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). The sciatic nerve (SCN) of right lateral hindlimb 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. (5). As control treatment, called sham, the SCN of right lateral hindlimb in mice was exposed, but was not subjected to ligation. 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).

Immunohistochemistry
The naive mice, ones without drug administration and surgery, were fixed with 4% paraformaldehyde under anesthesia, and the tissues were dissected. The spinal cord and the dorsal root ganglion (DRG) were cut transversely (20-μm-thick) with a cryostat, while the SCN was cut longitudinally. The sections were incubated with an antibody against PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and NeuN, a specific marker for neurons (Chemicon, Temecula, CA, USA). Then, the sections were incubated with secondary antibody solution (Alexa Fluor 488- or 596-conjugated antibody to the appropriate IgG; Molecular Probes, Eugene, OR, USA). Bodipy 493/503 (Molecular Probes) was used to stain neutral lipids in the adipocytes. Fluorescent images were captured with a microscope.

Drug administration
Pioglitazone (1 – 25 mg/kg, p.o.) or its vehicle [0.5% carboxymethyl cellulose (CMC)] was given once daily for the indicated periods (for all but experiments in Fig. 4) or once on day 7 (for experiments in Fig. 4). To evaluate whether the effect of pioglitazone was mediated by PPARγ, GW9662 (Sigma, St. Louis, MO, USA), a PPARγ antagonist, or its vehicle [3% dimethyl sulfoxide (DMSO)] was administered i.p. at 2 mg/kg 1 h before pioglitazone. Pioglitazone hydrochloride was kindly donated by Takeda Pharmaceutical Company (Osaka).

Behavioral test
We observed withdrawal responses of a hind paw when the plantar surface was contacted with calibrated von Frey filaments (0.4 or 0.6 g; Stolting, Wood Dale, IL, USA). Tactile allodynia was calculated as the number of mice showing hind paw withdrawal in response to single stimulations. To make sure that the method employed in the present study was appropriate for evaluation of tactile allodynia, we also estimated tactile allodynia in another way: stimulation with von Frey filament was applied to the hind paw plantar five times, and the number of evoked withdrawal responses was transformed to the ratio of withdrawal responses to the number of times of stimulation, five times. When tactile allodynia and the effects of pioglitazone (25 mg/kg, p.o., once daily on day 0 to 6) on day 7 and day 14 of PSL were evaluated with that ratio (data not shown), we obtained the same tendency when evaluated with the number of animals employed in the present study (Fig. 2A). Thermal sensitivity was determined by using hind-paw withdrawal latencies to a radiant heat stimulus according to Hargreaves’ method (6). Five latencies were obtained per animal on each day following PSL and were averaged.

Western blotting
We determined the change in expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the SCN (1 cm in length, including the ligation region), the DRG (L4-6), and the dorsal horn of the lumbar spinal cord. The immunoblot analysis was performed on freshly dissected tissue. SDS-PAGE-resolved samples were electrophoresed and transferred to nitrocellulose membranes and then probed with primary antibodies: rabbit anti-TNF-α antibody (Hycult Biotechnology, Uden, Netherlands), rabbit anti-IL-6 antibody (Chemicon, Temecula, CA, USA), and β-tubulin (Santa Cruz Biotechnology). Then, the probed membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence detection. The chemiluminescence was detected by Chemiluminiator (ATTO, Tokyo). The intensity of chemiluminescence was analyzed by ImageJ (NIH, Bethesda, MD, USA) and normalized to that of β-tubulin.

Statistical analyses
Statistical significance was assessed by using Fisher’s exact probability test (Figs. 2A – C, 3A and B, and 4A) or a one-way ANOVA followed by the Tukey-Kramer post hoc test (Fig. 2D, 4B, and 5A – F). Significance was set at the P<0.05 level.

Results
Immunohistochemistry revealed that PPARγ was distributed in the pain pathway from the sciatic nerve.
to spinal cord in the naive mice (Fig. 1). Immunoreactivity for PPARγ was observed in the dorsal horn of the spinal cord (Fig. 1B) and the DRG at the L5 level (Fig. 1E). They were co-localized with immunoreactivity for NeuN (Fig. 1: A and D), indicating that PPARγ was expressed in the neurons. In the SCN, immunoreactivity for PPARγ was distributed exclusively in the epineurium (Fig. 1H) and was localized eccentrically in the BODIPY-positive cells, adipocytes (Fig. 1G).

The distribution of PPARγ in the nociceptive pathway, as mentioned above, gives an indication of the possible role of PPARγ in pain development and/or modulation. We tested the effects of pioglitazone, the PPARγ agonist, on tactile allodynia elicited by peripheral nerve injury. Pioglitazone was administered once daily from immediately after PSL to day 6 following PSL. PSL increased the number of mice showing hindpaw withdrawal in response to innocuous mechanical stimulation. The number of mice with nociceptive responses was significantly greater in the PSL group with vehicle administration after day 3 following PSL than in the sham group with vehicle administration (Fig. 2A). PSL-induced tactile allodynia was significantly attenuated by daily administration of 25 mg/kg pioglitazone: after day 5 of PSL, PSL-induced tactile alldynia was inhibited by pioglitazone, and the inhibitory effect was maintained by day 14, even when pioglitazone administration was ceased on day 7. There was no significant difference between the number of mice with nociceptive responses in the sham group with vehicle and with pioglitazone. The relief of tactile allodynia by pioglitazone was dose-dependent (1 – 25 mg/kg) (Fig. 2B). The inhibitory effect of pioglitazone on tactile alldynia was attenuated by pretreatment with the PPARγ antagonist GW9662 (2 mg/kg, i.p.), which alone had no significant influence on nociceptive responses in sham and PSL (Fig. 2C). In addition, the
effect of pioglitazone on PSL-induced thermal hyperalgesia was examined. Figure 2D shows that the PSL group given vehicle had significantly shorter withdrawal latencies, and this was reversed by pioglitazone when tested on day 7 after PSL.

Allodynia and hyperalgesia in animal models is temporally differentiated into two phases: development and maintenance (1). To test whether pioglitazone had a phase-specific effect on neuropathic pain, we administered 25 mg/kg pioglitazone at the different phases of tactile allodynia. When pioglitazone was given on day 7 to day 1 before PSL, PSL-induced tactile allodynia was not significantly affected on day 7 following PSL (Fig. 3A). On the other hand, administration of pioglitazone on day 7 to day 13 following PSL reduced the magnitude of tactile allodynia on day 14 (Fig. 3B).

We also examined the acute effect of pioglitazone. Mice without exposure to drug received a single administration of pioglitazone (25 mg/kg, p.o.) on day 7 of PSL, followed by evaluation of nociceptive responses for 4 h after drug administration. Acute administration of pioglitazone did not alter either tactile allodynia (Fig. 4A) or thermal hyperalgesia (Fig. 4B) in mice subjected to PSL, and it was without significant effect on sham mice.

Some inflammatory cytokines, such as TNF-α and IL-6, are reportedly essential for establishment of neuropathic pain (7). The effect of 25 mg/kg pioglitazone was tested on the expression of TNF-α and IL-6 in the pain pathway: SCN, L4-6 DRGs, and the dorsal horn of lumbar spinal cord (Fig. 5). PSL induced significant increases in the expression of both cytokines in all examined tissues on day 7 following PSL. Pioglitazone, when administered for seven consecutive days after PSL, had no significant effect on the expression of TNF-α and IL-6.
immediately after PSL, abrogated the up-regulation of both cytokines in all examined tissues without significant change in the sham group.

Discussion

PPARγ is present predominantly in adipose tissues and has a role as a master regulator in the formation of fat cells (2). The expression of PPARγ has been investigated in several rodent tissues. There were only two reports on the presence of PPARγ in the dorsal horn of spinal cord (3, 8) and it was unclear which cells in this area expressed PPARγ. Our study not only confirmed these findings but also found that PPARγ was localized in the neurons of the dorsal horn of the spinal cord (Fig. 1A). Furthermore, we demonstrated for the first time the expression of PPARγ in the DRG neurons (Fig. 1D) and in the adipocytes in the perineurium of the SCN (Fig. 1G). Our findings provide the precedence for further studies to determine the relationship between neuropathic pain and PPARγ expression in the pain pathway.

Using a spinal cord injury (SCI) model, Park et al. revealed that PPARγ agonists prevented thermal
hyperalgesia (4). This model is clinically useful for studying serious motor dysfunction after SCI. The motor dysfunction, however, makes it difficult to evaluate withdrawal responses of an injured paw to nociceptive stimuli. In our study, mice subjected to PSL showed motor paralysis immediately after PSL, but recovered in a few days (5). Additionally, the finding by Park et al. that pioglitazone improved motor paralysis in the SCI model (4) 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 in studies of neuropathic pain.

The present studies are the first to report that pioglitazone reduces peripheral nerve injury–induced tactile allodynia and thermal hyperalgesia in mice, when repeatedly administered p.o. after the injury (Fig. 2). This effect was dose-dependent and mediated by PPARγ. Moreover, pioglitazone inhibited tactile allodynia behavior, even when given from day 7 to day 13 following PSL (Fig. 3B), during which allodynia had already developed. These results suggest that pioglitazone inhibits allodynia at both the induction phase and the maintenance phase. What mechanisms underlie the effect of pioglitazone is still open in the present study. It is generally believed that glial cells contribute to hypersensitivity in chronic pain conditions; spinal microglia and astrocytes play a major role in the development and maintenance of allodynia, respectively (1). Both types of activated glia express PPARγ in cultures and in injured brain (9). In addition, thiazolidinedione derivatives, including pioglitazone, were reported to inhibit induction of inflammatory mediators in lipopolysaccharide-stimulated astrocyte cultures and microglia cultures (10). We therefore, propose a hypothesis that pioglitazone may control neuropathic pain through activation of PPARγ in glial cells activated at different phases. Further studies are needed to determine the expression and function of PPARγ in activated spinal glia after PSL.

On the other hand, PPARγ was distributed in the neurons in the DRG and the spinal cord and in the adipocytes in the SCN of naive mice (Fig. 1). In this context, administration of pioglitazone prior to PSL was ineffective in reducing PSL-induced tactile allodynia (Fig. 3A). Thus, pioglitazone-primed PPARγ in the neurons and the adipocytes alone may be not enough to prevent tactile allodynia induced by PSL. The pathological role of PPARγ in neurons and adipocytes in neuropathic pain is still unclear, whereas DRGs and spinal neurons can secrete some inflammatory mediators that may be the basis for neuropathic pain (11, 12). The adipocytes also can secrete inflammatory cytokines, such as IL-6 and TNF-α (13), which are reportedly essential for neuropathic pain (7). Elucidation of temporal change in expression and function of PPARγ in the neurons and the adipocytes after PSL may help clarify their role in neuropathic pain.

Churi et al. reported that mechanical and cold hypersensitivity in the spared nerve injury model was decreased by a single intrathecal administration of another thiazolidinedione, rosiglitazone, whereas a single intraperitoneal and intracerebroventricular administration of it did not alter them (3). They concluded that spinal PPARγ activation was required for the acute effect of a single injection of PPARγ agonist. To test whether pioglitazone had acute effect on neuropathic pain, we examined the effect of a single p.o. administration, another systemic administration, of pioglitazone on developed neuropathic pain. A single administration of pioglitazone did not influence tactile allodynia and thermal hyperalgesia on day 7 of PSL (Fig. 4: A and B), which is consistent with the report by Churi et al. On the other hand, it remains unclear how much p.o. administered pioglitazone was disposed in the spinal cord in the present study. The higher doses of pioglitazone, enough to stimulate spinal PPARγ, might attenuate neuropathic pain, when a single administration is given, since a part of the orally given pioglitazone crosses blood brain barrier in rats (14).

Some proinflammatory cytokines contribute to neuropathic pain. TNF-α and IL-6 are accepted mediators underlying tactile allodynia and thermal hyperalgesia induced by peripheral nerve injury (7). SCN injury increases the levels of both cytokines in the SCN, DRG, and spinal cord (15 – 19), which agrees with the present study (Fig. 5). Transcription of both cytokines is regulated by transcription factor binding sites within the promoter. It has been reported that promoter activities of both TNF-α and IL-6 are driven by binding of NF-κB, AP-1, and STAT, primary proinflammatory transcription factors (20). PPARγ may also regulate genes for proinflammatory proteins, not through binding to PPARγ response element but by interaction with all transcription factors as described above (21). We therefore, tested whether pioglitazone blocked PSL-induced up-regulation of both cytokines. As expected, administration of pioglitazone after PSL reduced increases in the expression level of both cytokines in all examined tissues (Fig. 5). These results strongly suggest that pioglitazone alleviates tactile allodynia and thermal hyperalgesia, at least in part, through inhibiting up-regulation of proinflammatory cytokines. A future study is needed to reveal whether blockade of up-regulation of both and/or each cytokine is essential for pain relief by PPARγ.
In conclusion, PPARγ stimulation may provide a novel therapeutic approach for the treatment of neuropathic pain at the induction and maintenance phases. A promising clinical use might include prevention of the progress of neuropathic pain induced by major surgery and chemotherapy and treatment of established neuropathic pain such as diabetic peripheral neuropathy.

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