Peripheral-Type Benzodiazepine Receptor Antagonist Is Effective in Relieving Neuropathic Pain in Mice

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Abstract. cDNA microarray analysis showed the expression of peripheral-type benzodiazepine receptor (PBR) mRNA is slightly enhanced in the spinal cord of mice with spinal nerve injury (SNL) as compared with sham-operated mice. PBR transports cholesterol to the mitochondria, where cholesterol is converted to pregnenolone. Pregnenolone is then metabolized to progesterone, an activator of progesterone receptor, and further metabolized to produce allopregnanolone and 3α,21-dihydroxy-5α-pregn-20-one (3α,5α-THDOC), positive allosteric modulators and activators of the GABA_A receptor. In the present study, we first tested whether the enhanced PBR expression is causally related to neuropathic pain, and we found that the PBR antagonist PK11195 is effective in reducing SNL-induced mechanical allodynia and thermal hyperalgesia. Next we tested whether the PK11195-induced antinociception is attributable to reduced neurosteroid synthesis, which may possibly lead to reduced activation of the progesterone receptor and/or GABA_A receptor. We found that allopregnanolone and 3α,5α-THDOC are effective in reducing the anti-hyperalgesic effect of PK11195, suggesting a partial contribution of reduced GABA_A-receptor activation to PK11195-induced antinociception.

Keywords: peripheral-type benzodiazepine receptor, spinal nerve injury, neuropathic pain, PK11195, GABA_A receptor

Introduction

The peripheral-type benzodiazepine receptor [PBR, also termed as translocator protein (18 kDa) TSPO] is a component of a multimeric protein complex that is located in the mitochondrial membrane (1–3). In the central nervous system (CNS), PBR is known to be expressed mainly in glial cells and at low levels in neurons and plays a major role in cholesterol transport. A mitochondrial cytochrome P450 cholesterol side-chain cleavage enzyme (P450SCC) then converts the transported cholesterol to pregnenolone, which is the first and rate-limiting step in neurosteroid synthesis (4). Alterations of PBR expression are known to be associated with several CNS disorders such as neurodegenerative diseases, neuropsychiatric disorders, and gliomas (3). However, how altered expression of PBR resulted in these illnesses is not fully clarified. In the neuropathic pain model, up-regulation of PBR mRNA in dorsal root ganglion (DRG) and spinal cord after peripheral nerve injury in rat has been reported (5–7), and the involvement of PBR in sensory axon regeneration has been indicated (8). These data may also imply that the PBR is involved in the induction and/or maintenance of neuropathic pain. We have shown previously that intrathecal injection of the progesterone-receptor antagonist ICI 182,780 is effective in blocking neuropathic pain symptoms (9). Progesterone is a neurosteroid and synthesized through the pathway involving PBR (10). Thus possible up-regulation of progesterone might be responsible for the induction of neuropathic pain. It was shown previously that the peripheral nerve injury activates spinal microglia and this microglial activation is the key factor inducing neuropathic pain (11). A previous study also showed that activated microglia exhibit higher expression of PBR and that the PBR...
antagonist PK11195 reduces microglial activation (12). Furthermore, the expression of the potassium-chloride co-transporter KCC2 in the lamina I neuron of the spinal cord was shown to be reduced by spinal nerve injury and the chloride concentration in the neuronal cell interior is increased and shifts $E_{\text{ion}}$ (13). Thus activation of $\text{GABA}_A$ receptors will result in excitation of lamina I neurons instead of suppression of excitation observed in the non-injured neurons. Involvement of microglial activation in this pathway has been documented (14). The neurosteroids allopregnanolone and tetrahydrodeoxy corticosterone ($3\alpha,5\alpha$-THDOC) are synthesized through a pathway involving PBR in microglia and these neurosteroids are known to function as positive allosteric modulators of $\text{GABA}_A$-receptor expressed in neurons (15, 16). These neurosteroids at higher concentrations ($\geq 100$ nM) are also known to directly activate $\text{GABA}_A$ receptors (15). Thus enhanced production of these neurosteroids through the enhanced expression of PBR might be responsible for inducing neuropathic pain.

In the present study, we have first tested whether PBR expression would be changed in the spinal cord ipsilateral to the spinal nerve injury in mice. Then we have investigated the possible involvement of PBR activation in neuropathic pain.

**Materials and Methods**

**Animals**

Male C57BL/6J mice (Clea Japan, Inc., Tokyo) weighing 22 – 26 g were used. They were housed three per cage under controlled temperature ($22 \pm 1^\circ\text{C}$) and humidity ($55 \pm 10\%$) with a 12-h light/dark cycle with food and water freely available. All behavioral experiments were performed in a sound-proof room during the light cycle (7:00 AM – 7:00 PM). Experiments were conducted with the approval of the Animal Care Committee of Tokyo Medical and Dental University and according to the guidelines for investigations of experimental pain in animals published by the International Association for the Study of Pain (17).

**Animal model of spinal nerve injury**

Spinal nerve ligation (SNL) was carried out as described previously (18, 19). Briefly, under sodium pentobarbital anesthesia, the right L5 and L6 spinal nerves were exposed by removing a small piece of the paravertebral muscles and a part of the right spinous process of the L5 lumbar vertebra. The L5 and L6 spinal nerves were then carefully isolated and tightly ligated with 8-0 silk thread. After nerve ligation, the muscle, the adjacent fascia, and the skin were closed with sutures.

cDNA microarray analysis

cDNA microarray analysis was carried out in essentially the same way as described previously (20). Two weeks after spinal nerve injury or sham operation of C57BL/6J mice, L5/6 spinal cords were dissected and collected for RNA preparation. cDNA microarray analysis was performed using the CodeLink$^\text{TM}$ UniSet Mouse 10K I (Amersham Biosciences, NJ, USA) following the protocol provided by the manufacturer. Two data sets (Sham and SNL) were compared using the CodeLink$^\text{TM}$ System Software.

**Quantitative RT-PCR**

Three days and two weeks after SNL or sham operation, L5/6 spinal cords from three mice each were dissected and collected for RNA preparation. Total RNA was prepared with the miRNeasy mini kit (Qiagen, Tokyo) and cDNA was synthesized with Superscript III (Invitrogen, Tokyo). To amplify the cDNA for PBR, the following primers were used: 5’-AGTGTTCTCT TCACGGAACAAAC-3’ and 5’-AACTGTGTCTGCAGG AGACT-3’. The cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The primers used for GAPDH were 5’-AGAGACGCGCCG CATCTTCTT-3’ and 5’-CATGTAAGCCCAGTGAT CCA-3’. These PCR products were gel-purified and cloned into the pCRII vector (Invitrogen). The resultant plasmids were used as standard samples for quantification. Quantitative RT-PCR was performed by TaqMan$^\text{®}$ Gene expression assays (Mm00437828_m1 for PBR and Mm99999915_g1 for GAPDH; Applied Biosystems, Tokyo) on a 7500 real time PCR system (Applied Biosystems). The ratio of PBR/GAPDH was calculated and compared.

**Immunohistochemistry**

Three days and two weeks after SNL operation (three mice each), mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 10 ml of 1 unit/ml heparin (Mochida, Tokyo) in physiological saline, followed by 30 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cords were dissected out and postfixed in the same fixative at 4°C overnight, followed by immersion in 30% sucrose in 0.1 M phosphate buffer at 4°C overnight. The tissue was embedded in OCT compound (Sakura Finetek, Tokyo), frozen in powdered dry ice, and cut on a cryostat at a 14-μm thickness. The L5/6 spinal cord sections were processed for immunohistochemistry using the conventional method. The following antibodies were used: goat anti-PBR polyclonal antibody (several dilutions including 1:100; Santa Cruz Biotechnology, CA, USA), rabbit anti-PBR polyclonal antibody (several dilutions includ-
Intrathecal injection was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the 5th or 6th lumbar vertebra, according to a previously reported procedure (21, 22) using a 25-μl Hamilton microsyringe with a 30-gauge needle. The mouse was not anesthetized during the intrathecal injection. The success rate of accurate intrathecal injection evaluated by routine control dye injection was more than 95% in our hands. In the co-administration experiment, drugs were pre-mixed and were given in a volume of 5 μl.

**Agents**

PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide], a peripheral benzodiazepine receptor antagonist; allopregnanolone (5α-pregnan-3α-ol-20-one), a positive allosteric modulator of GABA_\_ receptor; and tetrahydrodeoxycorticosterone (3α,5α-THDOC; 3α,21-dihydroxy-5α-pregnan-20-one), a positive allosteric modulator of the GABA_\_ receptor were each first dissolved in dimethyl sulfoxide (DMSO), diluted with physiological saline, and then administered intrathecally. Progesterone (cyclodextrin-encapsulated progesterone) was dissolved in H_2O, diluted with physiological saline, and administered intrathecally. All the agents above were from Sigma. The highest final DMSO concentration used was 6%. Injection of 6% DMSO in saline did not affect threshold and latency for paw withdrawals in the mechanical and thermal tests, respectively (Fig. 3).

**Statistical analyses**

Experimental data are expressed as means ± S.E.M. The Tukey-Kramer test was used for quantitative RT-PCR analyses. In other cases, data were analyzed by the unpaired Student’s _t_-test for comparisons between two groups and one-way analysis of variance followed by post hoc Dunnett’s test for multiple comparisons. A _P_ value less than 0.05 was considered statistically significant.

**Results**

**Expression of PBR**

Using the microarray techniques, it has been shown previously that the expression of PBR mRNA is increased in rat DRG and spinal cord in the peripheral axotomy model or the SNL model of neuropathic pain (5–7). Therefore, we have confirmed whether spinal nerve injury also modifies the expression level of PBR mRNA in mouse spinal cord. Using cDNA microarray techniques, we found that the expression of PBR mRNA in mouse spinal cord is increased 1.9-fold two weeks after the SNL operation. Enhanced expression of PBR mRNA was also confirmed by quantitative RT-PCR. Total RNA was prepared from mouse spinal cord three
days and two weeks after SNL or sham operation. We used GAPDH as a control to evaluate the expression of PBR in each sample, and we found that the expression of PBR mRNA in mouse spinal cord is increased 2.4-fold on day 3 and 2.1-fold two weeks after the SNL operation (Fig. 1).

Next, the expression of PBR protein in the spinal cord was examined by immunofluorescence techniques. We tested two different commercially available antibodies against PBR, but we could not detect specific signals for PBR protein. Previous studies have shown that PBR mRNA, protein expression, and ligand binding are undetectable in the DRG and spinal cord of naïve rat (23). Furthermore, it was shown that PBR mRNA, protein expression, and ligand binding in the DRG became detectable after sciatic nerve transaction and so did the PBR mRNA and ligand binding in the spinal cord. However, PBR protein expression in the spinal cord was not shown (23). Thus it is possible that PBR protein expression in SNL-operated mice spinal cord might also be very low and undetectable by antibody staining. On the other hand, we could confirm that the number of microglia identified by the antibody against Iba1 was enhanced on day 3 and gradually decreased two weeks after SNL operation (Fig. 2: A and B) as has been observed previously (11).

Effects of the PBR antagonist PK11195 on pain response

To assess whether the marginally enhanced expression of PBR is responsible for neuropathic pain, we tested the effect of a PBR antagonist. Intrathecal injection of PK11195 (10 and 30 nmol) produced a significant, dose-dependent inhibition of mechanical allodynia and thermal hyperalgesia induced by spinal nerve injury (Fig. 3: A and B). PK11195 did not change mechanical and thermal nociceptive thresholds of the contralateral hind paw (Fig. 3: A and B). In the present study, the maximum concentration of DMSO used as a solvent for the drugs is 6%. Although this seems rather high, 6% of DMSO by itself does not affect the pain behavior of SNL-operated mice up to three hours after injection (Fig. 3: C and D).

Effects of co-administration of progesterone on the antinociceptive effect of PK11195

Our previous study showed that the progesterone receptor antagonist ICI 182,780 is effective in blocking neuropathic pain (9). Progesterone is an intermediate metabolite of the neurosteroid biosynthesis. To test whether the possible augmented production of progesterone by the marginally enhanced expression of PBR is a cause of neuropathic pain, the effect of co-administration of progesterone on PK11195-induced
antinociception was examined. Co-administration of progesterone did not reduce the antinociceptive effects of PK11195 on mechanical allodynia and thermal hyperalgesia induced by spinal nerve injury (Fig. 4: A and B).

Effects of co-administration of allopregnanolone or 3α,5α-THDOC on the antinociceptive effect of PK11195

Allopregnanolone and 3α,5α-THDOC are neurosteroids synthesized from cholesterol transported by...
PBR and function as positive allosteric modulators of GABA$_A$ receptors. To test the possibility that the reduction of this neurosteroid synthesis is responsible for the PK11195-induced antinociception, the effect of co-administration of allopregnanolone or 3α,5α-THDOC was investigated. First to determine the appropriate concentration of allopregnanolone and 3α,5α-THDOC, we conducted preliminary experiments. Intrathecal injection of the GABA$_A$-receptor antagonist bicuculline is known to induce thermal hyperalgesia (24). We found that injection of 1 nmol of bicuculline induced thermal hyperalgesia and co-injection of 3 nmol of allopregnanolone or 3α,5α-THDOC completely inhibited the bicuculline-induced thermal hyperalgesia (data not shown). Next we tested the effect of co-administration of 3 nmol of these neurosteroids. Allopregnanolone or 3α,5α-THDOC did not reduce the antinociceptive effects of PK 11195 on mechanical allodynia (Figs. 5A and 6A). However, these agents antagonized the antinociceptive effect of PK11195 on thermal hyperalgesia (Figs. 5B and 6B). Limitation of the volume of the intrathecal injection and the solubility of these agents in DMSO does not allow us to administer very high doses of these agents. Nonetheless, in some experiments, we could also test 10 nmol of these neurosteroids. Recently allopregnanolone was demonstrated to be effective for increasing the thermal and mechanical nociceptive thresholds in naïve rats and it induced analgesia in neuropathic rats (25). However, we did not see any threshold-increasing effects on the contralateral side and analgesic effects on the ipsilateral side of the SNL-model mouse hindpaw by the administration of 10 nmol allopregnanolone (Fig. 5: C and D). The reason

**Fig. 5.** The effects of intrathecal co-administration of PK11195 (PK) (10 or 30 nmol) and allopregnanolone (Allo) on mechanical allodynia (n = 4 for 3 and 10 nmol of allopregnanolone) (A) and thermal hyperalgesia (n = 8 for 3 nmol of allopregnanolone) (B). For comparison, data of 10 or 30 nmol PK11195 alone and 10 nmol (n = 4 each) allopregnanolone alone (C, D) are also shown. *P<0.05, ***P<0.001, when compared between pre-drug (at 0 h) data and post-drug (at each time point) data. Paw-withdrawal threshold or latency is plotted against the time after injection of the agents. Antinociceptive effects of intrathecal injection of PK11195 alone or in combination with allopregnanolone are compared as described in the Fig. 3 legend. **P<0.01, when compared between PK11195 alone and PK11195 + allopregnanolone (at 30 min). Cont, contralateral to the nerve injury; Ipsi, ipsilateral to the nerve injury.
causing this apparent discrepancy is not clear at this moment. Major differences are that they used the CCI-model (26) rats and pain behavior was tested 7–11 days after operation. We used SNL-model (18) mice and pain behavior was tested 2–3 weeks after operation.

**Discussion**

Generally, activation of GABA<sub>A</sub> receptors will lead to the reduction of excitation in neurons and PBR is responsible for supplying the neurosteroidal GABA<sub>A</sub>-receptor activators allopgrenolone and 3α,5α-THDOC. Thus the enhanced expression of PBR could be considered as a part of the self defensive functions. Actually, the enhanced neurosteroid production found in the inflammatory pain state seems to be effective in reducing thermal pain through the mechanism of GABA<sub>A</sub>-receptor activation (27). It has also been shown that topical application (local intraplantar injection) of allopgrenolone is effective in alleviating neuropathic pain (CCI-model rats) (28). So we first investigated the effect of intrathecal injection of the PBR antagonist PK11195 on neuropathic pain symptoms of SNL-model mice. Interestingly, our results showed that inhibition of PBR activity alleviates neuropathic pain, suggesting activation of PBR may lead to neuropathic pain. These differential effects may suggest that the central and peripheral effect of neurosteroid may be quite diverse or the CCI-model rats and SNL-model mice might exhibit different phenotypic responses.

Peripheral nerve injury has been shown to activate spinal microglia through P2X4-receptor activation (11). Then the activated microglia release BDNF and the released-BDNF acts on its receptor TrkB in lamina I neurons of the dorsal horn. BDNF-TrkB signaling collapses the transmembrane anion gradient of lamina I neurons, possibly through the down regulation of potassium-chloride co-transporter KCC2. Thus in the lamina I neurons in the neuropathic pain state, GABA- and glycine-receptor signaling became excitatory instead of inhibitory (13, 14). Thus the following scenario may be probable. First, marginally enhanced PBR expression induces augmented allopgrenolone and/or 3α,5α-THDOC production. Second, these neurosteroids may activate excitatory GABA<sub>A</sub> receptors, which resulted in neuropathic pain. We found thermal hyperalgesia was significantly reduced by the co-administration of these neurosteroidal GABA<sub>A</sub>-receptor activators. However, these agents did not affect the mechanical allodynia, suggesting that the differential induction mechanism between thermal hyperalgesia and mechanical allodynia. There is substantial evidence suggesting that the thermal hyperalgesia was mediated through unmyelinated C fibers, whereas mechanical allodynia was mediated through myelinated, primary Aβ afferents (29). C and Aβ fibers are known to terminate at the different regions of the dorsal horn. Possible subtype variation, localization, and the expression level of GABA<sub>A</sub> receptors (15) in these regions may explain our differential effect of neurosteroids on thermal hyperalgesia and mechanical allodynia. The antiallodynic effect of PK11195 might be explained by another mechanism (12). However, further rigorous study is necessary to clarify these issues.

We have also tested the possible involvement of
progesterone-receptor activation in PBR-induced neuropathic pain. Previously we showed that administration of progesterone alone (3 nmol) did not have any effects and 3 nmol of progesterone is enough to block the antinociceptive effect of ICI 182,780 (9). However, we found that progesterone did not inhibit the antinociceptive effect of PK11195, indicating that the antinociceptive effect achieved by the blockade of PBR was not caused by the reduced production of progesterone. In other words, the mechanisms underlying the antinociceptive effects observed by the blockade of progesterone receptor and by the blockade of PBR may be different.

In conclusion, our results strongly suggest that the PBR plays an important role in the maintenance of neuropathic pain. The PBR in the dorsal horn area may be a useful target in the management of neuropathic pain. Thus, it may be worth testing the potency of PBR antagonists to inhibit pain in patients with neuropathic pain.

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