Inhibition of Paclitaxel-Induced A-Fiber Hypersensitization by Gabapentin

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ABSTRACT

Paclitaxel (Taxol) is widely used in cancer chemotherapy for the treatment of solid carcinomas (Kohler and Goldspiel, 1994; Socinski, 1999). Its mechanism is known to involve an increase in the stability of tubulin polymers and to inhibit cellular replication (Schiff et al., 1979; Horwitz, 1992). However, it has serious side effects such as peripheral neuropathic pain (Rowinsky et al., 1993; Chaudhry et al., 1994; Cavaletti et al., 1995; Forsyth et al., 1997), which become more marked with continued therapy. In some cases, the side effects may outweigh the beneficial effects on life quality, associating with the generation of peripheral neuropathic pain. We found that a single treatment with paclitaxel (4 mg/kg i.p.) led to a decrease in both thermal and mechanical nociceptive thresholds as well as a reduction in the thresholds for 250-Hz (Aδ-fiber) and 2000 Hz (Aβ-fiber) but not 5-Hz (C-fiber) sine wave electrical stimuli-induced paw withdrawal. The paclitaxel-induced neuropathic pain was completely abrogated by gabapentin (30 mg/kg i.p.) treatment. Furthermore, we found that mRNA and protein levels of the voltage-gated calcium channel α2δ-1 subunit (Caαδ-1), one of the putative targets for gabapentin, was up-regulated in dorsal root gangliaons (DRG) as well as increased expression of Caαδ-1 protein in medium/large-sized DRG neurons by immunohistochemistry, following paclitaxel treatment. This suggests that paclitaxel induces A-fiber-specific hypersensitization, which may contribute to the functional mechanical allodynia and hyperalgesia, and that gabapentin could be a potential therapeutic agent for paclitaxel-induced neuropathic pain.

Paclitaxel (Taxol) is a widely used chemotherapeutic agent in the treatment of several tumors. However, its use is often associated with the generation of peripheral neuropathic pain expressed as mechanical allodynia and thermal hyperalgesia. The molecular mechanism behind this debilitating side effect is obscure, and efficient drugs for its prevention are required. We sought to clarify the cellular changes in the involved nociceptor types underlying paclitaxel-induced neuropathic pain and to test for an alleviating effect of gabapentin treatment in a murine model of paclitaxel-induced neuropathic pain. We found that a single treatment with paclitaxel (4 mg/kg i.p.) led to a decrease in both thermal and mechanical nociceptive thresholds as well as a reduction in the thresholds for 250-Hz (Aδ-fiber) and 2000 Hz (Aβ-fiber) but not 5-Hz (C-fiber) sine wave electrical stimuli-induced paw withdrawal. The paclitaxel-induced neuropathic pain was completely abrogated by gabapentin (30 mg/kg i.p.) treatment. Furthermore, we found that mRNA and protein levels of the voltage-gated calcium channel α2δ-1 subunit (Caαδ-1), one of the putative targets for gabapentin, was up-regulated in dorsal root gangliaons (DRG) as well as increased expression of Caαδ-1 protein in medium/large-sized DRG neurons by immunohistochemistry, following paclitaxel treatment. This suggests that paclitaxel induces A-fiber-specific hypersensitization, which may contribute to the functional mechanical allodynia and hyperalgesia, and that gabapentin could be a potential therapeutic agent for paclitaxel-induced neuropathic pain.

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ABBREVIATIONS: Caαδ-1, voltage-gated calcium channel α2δ-1 subunit; DRG, dorsal root ganglion; AUC, area under the curve; EPW, electrical-stimulus induced paw withdrawal; PCR, polymerase chain reaction; RT, real time; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline.
tion following paclitaxel treatment. Finally, we report an increased expression of Ca$_{\text{v}}$3.1-1 in medium/large-sized DRG neurons after paclitaxel treatment.

Materials and Methods

Animals. Male ddY mice weighing 20 to 22 g were used. They were kept in a room maintained at 21 ± 2°C with free access to standard laboratory diet and tap water. Procedures were approved by Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1993).

Drugs. The following drugs were used: Paclitaxel was generously provided by Bristol-Myers Squibb Co. (Paris, France). Paclitaxel was dissolved in saline just before administration. 1-(Aminomethyl)-cyclohexanecarboxylic acid (gabapentin) was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in saline.

Drug Injection and Analysis. We used three types of paclitaxel (4 mg/kg)-injection schedules as follows: single i.p. injection, multiple (four times on alternate days; days 0, 2, 4, and 6); i.p. injection; and single i.v. injection. In the “area under the curve” (AUC) analysis for paclitaxel effect on pain thresholds, we calculated the area under the curve generated by the paw withdrawal threshold-time course from week 1 to 4 after paclitaxel treatment using a trapezoidal method. Gabapentin (3–30 mg/kg) was injected (i.p.) 30 min before behavioral tests.

Conventional Nociceptive Tests. In thermal paw withdrawal tests, nociception was measured as the latency to paw withdrawal evoked by exposure to a thermal stimulus (Hargreaves et al., 1988; Inoue et al., 2004). Unanesthetized animals were placed in Plexiglas cages on top of a glass sheet, and an adaptation period of 1 h was allowed. The thermal stimulus (IITC Inc., Woodland Hills, CA) was positioned under the glass sheet, and the focus of the projection bulb was aimed exactly on the middle of the plantar surface of the animal. A mirror attached to the stimulator permitted visualization of the plantar surface. A cut-off time of 20 s was set to prevent tissue damage. The paw pressure test was performed as described previously (Rashid et al., 2003; Inoue et al., 2004). In brief, mice were placed into a Plexiglas chamber on a 6-× 6-mm wire mesh grid floor and were allowed to acclimatize for a period of 1 h. The mechanical stimulus was then delivered onto the middle of the plantar surface of the right hind paw using a transducer indicator (model 1601; IITC Inc.). The pressure needed to induce a flexor response was defined as the pain threshold. All behavioral experiments were carried out by investigators blinded to the drug treatment.

Electrical Stimulation-Induced Paw Withdrawal Test. Electrodes (Neurotron Inc., Baltimore, MD) were fastened to the right plantar surface and instep of the mice. Transcutaneous nerve stimuli with each of the three sine wave pulses (0.1, 2, and 2000 Hz) were applied using the Neurometer CPT/C (Neurotron Inc.). The minimal intensity (microampere) at which each mouse withdrew its paw was defined as the current stimulus threshold. Stimuli were applied at 10-min intervals. All behavioral experiments were carried out by investigators blinded to the drug treatment.

Quantitative Real-Time Polymerase Chain Reaction. On days 3, 7, and 14 after paclitaxel treatment, the L4-6 DRGs were removed for Western blot analysis. Mixed L4-6 DRG protein (30 μg) was applied to an SDS-polyacrylamide gel (8%). The anti-Ca$_{\text{v}}$3.1-1 antibody (Sigma-Aldrich) diluted 1:200 (Inoue et al., 2004) was used, and anti-β-tubulin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 was used as an internal control. Horseradish peroxidase-labeled rabbit antibody was used as second antibody at 1:2000. Visualization of immunoreactive bands was performed by Light Capture (AE-6960/FC; Atto, Tokyo, Japan) with an enhanced chemiluminescent substrate for the detection of horseradish peroxidase, Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL). The intensities of immunoreactive bands were analyzed by NIH Image Software (Bethesda, MD) for Macintosh.

Immunohistochemistry. The immunohistochemical analyses were performed as described previously (Inoue et al., 2004). In brief, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with 20 ml of potassium-free phosphate-buffered saline (K$^+$/free PBS; pH 7.4) followed by 50 ml of 4% paraformaldehyde solution. The L4-6 DRGs were removed, postfixed for 3 h, and cryoprotected overnight in 25% sucrose solution. The DRGs were fast-frozen in cryo-embedding compound on a mixture of ethanol and dry ice and stored at −80°C until use. DRGs were cut at 10-μm thickness, thaw-mounted on a silane-coated glass slides, and air-dried overnight at room temperature. Before immunolabeling, antigen unmasking was performed by microwave treatment for 15 min in 10 mM citrate buffer, pH 6.0. DRG sections were incubated with excess blocking buffer containing 2% skim milk in 0.1% Triton X-100 in K$^+$/free PBS and subsequently reacted overnight at 4°C with anti-Ca$_{\text{v}}$3.1-1 antibodies (1:200; Sigma-Aldrich) in 2% bovine serum albumin/0.1% Triton X-100 in K$^+$/free PBS. The sections were then placed in fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:200; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. All sections were treated with Permafluor (Thermo Shandon, Pittsburgh, PA) and coverslipped and evaluated by microscopy (Keyence, Tokyo, Japan).

Measurements of DRG neuron diameters were done using the BZ Image Measurement software (Keyence). Total cell counts were carried out in bright field images as reported previously (Josephson et al., 2001; Dai et al., 2004). Therefore, the quantification of the data may represent a biased estimate of the actual number of neurons. However, to determine the Ca$_{\text{v}}$3.1-1 positive neuron profile, we have carried out the counts using only neurons with clearly visible nuclei. In this experiment, we divided neurons into two groups, small- and medium/large-sized neurons. We used 24 μm in diameter as dividing line between small- and medium/large-sized neurons (Gold et al., 1996; Hiruma et al., 2000). In total, 1684 DRG neurons from vehicle-treated mice (n = 3) and 2724 DRG neurons from paclitaxel-treated mice (n = 4) were measured.

Statistical Analysis. The behavioral time-course data were analyzed using a Student’s t test to detect differences between vehicle- and paclitaxel-treated groups. The behavioral data on gabapentin and the RT-PCR data as well as the Western blot data of Ca$_{\text{v}}$3.1-1 were analyzed using a one-way and Tukey multiple comparison post
Results

Paclitaxel-Induced Thermal Hyperalgesia and Mechanical Alldynia. We treated mice with 4 mg/kg paclitaxel single (i.p. or i.v.) or multiple times (four i.p. injections on alternate days; days 0, 2, 4, and 6) and evaluated the threshold in several nociceptive tests from preinjection (control) to 4 weeks after the first treatment. No significant side effects such as ascites, loss of body weight, or alopecia was observed in this experimental schedule. A single i.p. injection of 4 mg/kg paclitaxel led to a decreased threshold in the paw pressure test, which was significant at 1 and 2 weeks after treatment but increased to baseline level at week 4 post-treatment (Fig. 1A). When comparing the mechanical allodynia after a single i.p. injection, multiple i.p. injections, or single i.v. injection of paclitaxel, no significantly different outcomes were observed, between the numbers of administrations or the route, as shown as the AUC values for the paw pressure test from week 1 to 4 after treatment (Fig. 1B). The thermal nociceptive threshold level decreased in a manner similar to the decrease in threshold for mechanical allodynia, although the effect was less pronounced. However, the effect was significant 2 weeks after a single i.p. injection of paclitaxel (Fig. 1C). Based on these results, we determined to adopt the single i.p. injection for the paclitaxel-induced neuropathic pain model in mice and to evaluate the effect of gabapentin 2 weeks after treatment, which was the peak time point of neuropathic pain.

Blockade of Paclitaxel-Induced Neuropathic Pain by Gabapentin. Gabapentin, injected i.p. 30 min before behavioral tests, inhibited mechanical allodynia 2 weeks after paclitaxel treatment (4 mg/kg; single i.p. injection) in a dose-dependent manner from 3 to 30 mg/kg, whereas 30 mg/kg gabapentin did not cause any significant effects in vehicle-treated mice (Fig. 1D). The duration of the analgesic effect from treatment with 30 mg/kg gabapentin lasted for more than 24 h, but the significant effect had vanished by 48 h after treatment (Fig. 1E). Moreover, gabapentin injection (30 mg/kg i.p.) 2 weeks after paclitaxel treatment reversed thermal hyperalgesia within 30 min. However, gabapentin treatment of vehicle-treated mice did not affect the thresholds of thermal paw withdrawal latency (Fig. 1F).

Paclitaxel-Induced Myelinated A-Fiber-Specific Hypersensitization. Transcutaneous nerve stimuli with each of the three sine wave pulses of different frequencies of 5, 250, and 2000 Hz to activate C-, Aδ-, and Aβ-fibers, respectively (Katims, 1997; Lengyel et al., 1998), were applied to the right hind paw of the mice, and the intensity (microampere) was gradually increased. Thresholds were determined, at which paw withdrawal was observed, from preinjection (control) to 4 weeks after paclitaxel treatment (Fig. 2A). The reduction in threshold by the 250-Hz (Aδ) and 2000-Hz (Aβ) stimuli at 1 and 2 weeks after paclitaxel treatment (4 mg/kg; single i.p. injection) had vanished 4 weeks after treatment. No significant change in the threshold by 5-Hz (C) stimuli was observed at any time. These results suggest that paclitaxel induces myelinated Aδ- and Aβ-fiber-specific hypersensitization.

Increased Expression of Ca2+/δ-1 mRNA and Protein in DRG after Paclitaxel Treatment. We removed L4-6 DRGs at days 3, 7, and 14 after paclitaxel treatment, and Ca2+/δ-1 mRNA expression was quantified by RT-PCR analysis and compared with GAPDH mRNA expression. The specificity of the PCR primers was confirmed by the occurrence of a single peak in the melting curves for the PCR.
products, and the efficiency of the reaction was found to be approximately 100% shown by a threshold cycle versus sample-dilution plot (Fig. 3A). Caα2-δ-1 mRNA expression was slightly but not significantly increased at day 3, but a larger and significant increase was observed at day 7 in paclitaxel-treated mice compared with controls (Fig. 3A). At day 14, the Caα2-δ-1 mRNA expression was reduced to control level.

Moreover, we removed L4-6 DRGs at days 7, 14, and 28 after paclitaxel treatment, and Caα2-δ-1 protein expression was quantified by Western blot analysis. The result indicated that Caα2-δ-1 up-regulation was significant at day 7, maximum at day 14, and returned to baseline level 28 days post-paclitaxel injection (Fig. 3B).

**Increased Expression of Caα2-δ-1 in Medium/Large-Sized DRG Neurons following Paclitaxel Treatment.** We detected Caα2-δ-1 protein expression in DRGs at day 14 after treatment using immunohistochemistry. In vehicle-treated mice, Caα2-δ-1 expression was mainly localized to small-diameter DRG neurons (Fig. 4A), whereas in paclitaxel-treated mice, Caα2-δ-1 protein expression was both observed in small-diameter DRG neurons and in medium/large-diameter DRG neurons (Fig. 4A). The total amount of neurons in the DRGs, which stained positive for Caα2-δ-1 protein, was counted and their individual sizes measured both in paclitaxel- and vehicle-treated mice. A significant increase in the frequency of stained neurons was found in the

![Fig. 2. Paclitaxel-induced A-fiber hypersensitization and inhibitory effects of gabapentin. A, time courses of paclitaxel (4 mg/kg i.p.)-induced sensory fiber sensitizations determined by EPW tests using Neurometer from preinjection to 4 weeks after treatment. B, alleviating effects of gabapentin on paclitaxel-induced A-fiber hypersensitivity determined by EPW tests using 250- and 2000-Hz stimulations 2 weeks after paclitaxel treatment. All animals were analyzed 30 min after gabapentin treatment. C, control; Sal, saline; and GBP, gabapentin. *p < 0.05; **p < 0.01 versus control or vehicle/saline. ##p < 0.01 versus paclitaxel/saline. Data are presented as mean ± S.E.M. from experiments using at least six mice.](https://jpet.aspetjournals.org)

![Fig. 3. Expression of Caα2-δ-1 mRNA and protein in L4-6 DRGs after paclitaxel treatment. A, top panel, dissociation curves of PCR products from reactions using primers specific for mRNA encoding Caα2-δ-1 and GAPDH, and amplification efficiency analysis. Bottom panel, quantitative RT-PCR analysis. Caα2-δ-1 and GAPDH mRNAs in L4-6 DRGs on days 3, 7, and 14 after paclitaxel treatment were measured and Caα2-δ-1 was normalized to GAPDH. The graph shows the relative amount of Caα2-δ-1 mRNA in paclitaxel-treated mice compared with the controls. B, Western blot analysis. Caα2-δ-1 and β-tubulin protein in L4-6 DRGs 7, 14, and 28 days after paclitaxel treatment was measured, and Caα2-δ-1 was normalized to β-tubulin. The graph shows the relative amount of Caα2-δ-1 protein in paclitaxel-treated mice compared with the controls. Photograph shows representative data. *p < 0.05; **p < 0.01 versus control (C). Data are presented as mean ± S.E.M. from experiments using at least eight mice.](https://jpet.aspetjournals.org)
paclitaxel-treated mice compared with vehicle-treated controls (Fig. 4B). Furthermore, the cell size measurements showed that the increased number of Ca<sub>2+/-1</sub>-stained neurons reflected an increased expression of Ca<sub>2+/-1</sub> in medium/large-sized neurons, whereas the number of positively stained small neurons remained almost the same between vehicle- and paclitaxel-treated groups (Fig. 4, C and D). However, no significant difference in the general size distribution of DRG neurons was found between the two groups, supporting the notion that the increased Ca<sub>2+/-1</sub> protein expression indeed resulted from an up-regulation in medium/large-sized neurons (Fig. 4C).

**Discussion**

Here, we found that single administration of paclitaxel at a dose of 4 mg/kg i.p. caused peripheral neuropathic pain. According to the paclitaxel injection prescribing information sheet, the recommended dose is 135 to 175 mg/m<sup>2</sup> [TAXOL (paclitaxel) package insert, Bristol-Myers Squibb Company, 2003], which is converted into approximately 3.6 to 4.7 mg/kg (height, 1.7 m; weight, 65 kg; Du Bois formula). In addition, it states that paclitaxel should be given every 3 weeks in clinical states. However, in previous experimental studies, the paclitaxel administration has been performed multiple times on consecutive/alternate days or in a single but high dose (Authier et al., 2000; Dina et al., 2001; Polomano et al., 2001; Flatters and Bennett, 2004; Smith et al., 2004). Therefore, the present finding seems to have some advantages. We conclude that the protocol using single administration of 4 mg/kg paclitaxel is a proper and easy approach to study paclitaxel-induced neuropathic pain.

In our experimental model, we observed mechanical allodynia, thermal hyperalgesia, and A-fiber specific hypersensitization using the Neurometer test. The Neurometer test is widely used clinically in the evaluation of sensory function in humans with complex regional pain syndrome or other types of peripheral neuropathic pain, which can result from common diseases such as diabetes mellitus (Masson and Boullon, 1991; Katims, 1997; Lengyel et al., 1998). Our results are supported by previous data from animal models of paclitaxel-induced hypersensitivity (Smith et al., 2004) as well as Neurometer analysis of paclitaxel-treated patients, which also show A fiber sensitivity, as determined by stimulating the median nerve with 2000-Hz pulses (Doi et al., 2003). Therefore, we conclude that our model is useful for investigating the mechanism by which paclitaxel induces neuropathic pain and for testing new drug candidates.

Frequent reports of the alleviating effect of gabapentin treatment in various models of neuropathic pain such as the peripheral nerve ligation model and diabetic neuropathic pain models, have been published (Hunter et al., 1997; Hwang and Yaksh, 1997; Cesena and Calcutt, 1999; Miki et al., 2001; Luo et al., 2002). The present study is the first to report an antinociceptive effect of gabapentin on paclitaxel-induced neuropathic pain in mice. We evaluated the effect by EPW tests as well as conventional nociceptive tests measuring mechanical nociception. Gabapentin revealed specific analgesic effects against paclitaxel-induced mechanical allodynia, thermal hyperalgesia, and A-fiber hypersensitization without affecting the pain threshold of naive mice. These results suggest that gabapentin could be used to treat paclitaxel-induced neuropathic pain in the clinic.

Referring to the mechanism of antinociceptive effects of gabapentin, we measured the expression of Ca<sub>2+/-1</sub>-1, which is known to possess gabapentin-binding properties (Gee et al., 1996). We first demonstrated that the expression of Ca<sub>2+/-1</sub>-1
mRNA and protein was increased in the DRGs of paclitaxel-treated mice. The difference in the peak time point between mRNA and protein expression seems to be attributable to the longer life time of protein. It should be noted that the time course of Ca\textsubscript{\textit{v}}\textsubscript{2.1} protein expression was very close to that of paclitaxel-induced neuropathic pain (Figs. 1, A and C, and 2A). Interestingly, immunohistochemistry revealed that Ca\textsubscript{\textit{v}}\textsubscript{2.1} protein was increased in medium/large-diameter neurons following paclitaxel treatment. Because the function of the calcium channel containing Ca\textsubscript{\textit{v}}\textsubscript{2.1} is related to modulation of synaptic neurotransmission (Patel et al., 2000; Shimoyama et al., 2000; Maneuf et al., 2004), up-regulation of Ca\textsubscript{\textit{v}}\textsubscript{2.1} expression in medium/large-sized neurons may contribute to the paclitaxel-induced mechanical allodynia and A-fiber-dependent hypersensitization. Recently, we have reported that Ca\textsubscript{\textit{v}}\textsubscript{2.1} is up-regulated in medium/large-sized DRG neurons following peripheral nerve injury or intrathecal injection of lysophosphatidic acid, an initiator of neuropathic pain (Inoue et al., 2004). Luo et al. (2002) reported that inhibitory effects of gabapentin on neuropathic pain correlate to Ca\textsubscript{\textit{v}}\textsubscript{2.1}-expression. Gabapentin sensitivity and Ca\textsubscript{\textit{v}}\textsubscript{2.1}-1 up-regulation were observed in spinal nerve ligation, spinal nerve transection, sciatic nerve chronic constriction injury, and diabetic models but not in the vincristine-induced neuropathic pain model. In the present study, we confirmed the gabapentin sensitivity and Ca\textsubscript{\textit{v}}\textsubscript{2.1} up-regulation in the paclitaxel-induced neuropathic pain model. It is interesting that the gabapentin sensitivity and Ca\textsubscript{\textit{v}}\textsubscript{2.1} up-regulation differed between the vincristine and paclitaxel models, when taken into account that both drugs are known to act through tubulin assembly. Although the underlying mechanism remains to be determined, it might be attributed to different manners of action; disruption of tubulin assembly by vincristine and increasing stabilization by paclitaxel (Kohler and Goldspie, 1994).

In conclusion, we report that paclitaxel induces A-fiber hypersensitization and novel expression of Ca\textsubscript{\textit{v}}\textsubscript{2.1} in medium/large-sized DRG neurons, which may functionally contribute to mechanical allodynia and hyperalgesia. Furthermore, we identify gabapentin as a potential therapeutic agent for paclitaxel-induced peripheral neuropathic pain.

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References

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