Full Paper

The Contribution of Gi/o Protein to Opioid Antinociception in an Oxaliplatin-Induced Neuropathy Rat Model

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Abstract. Oxaliplatin is a chemotherapeutic agent that induces chronic refractory neuropathy. To determine whether opioids effectively relieve this chronic neuropathy, we investigated the efficacies of morphine, oxycodone, and fentanyl, and the mechanisms underlying opioid antinociception, in oxaliplatin-induced neuropathy in rats. Rats exhibited significant mechanical allodynia following 2 weeks of chronic oxaliplatin administration. Within the range of doses that did not induce sedation and/or muscle rigidity, morphine (3 mg/kg, subcutaneously, s.c.) and oxycodone (0.3 – 0.56 mg/kg, s.c.) completely reversed oxaliplatin-induced mechanical allodynia, whereas fentanyl (0.017 – 0.03 mg/kg, s.c.) showed partial antinociception. The antinociception of the optimal doses of morphine and oxycodone were completely inhibited by pertussis toxin (PTX; 0.5 µg/rat, i.c.v.), a Gi/o protein inhibitor, while the partial effect of fentanyl was not affected in the oxaliplatin model. In the [³⁵S]-GTPγS binding assay, activation of µ-opioid receptor by fentanyl, but not by morphine or oxycodone, in the mediodorsal thalamus was significantly reduced in oxaliplatin-treated rats. These results indicate that the lower antinociceptive potency of fentanyl in the oxaliplatin model might in part result from the loss of PTX-sensitive Gi/o protein activation, and the degree of Gi/o protein activation might be related to the potency of antinociception by opioids in this model.

Keywords: opioid, antinociception, oxaliplatin, neuropathy, Gi/o protein

Introduction

Antineoplastic agents are used for cancer therapy, often causing adverse effects such as diarrhea, emesis, muscle pain, joint pain, and peripheral neuropathy. Among these adverse effects, peripheral neuropathy is one of the most serious side effects during long-term cancer therapy (1, 2). Moreover, uncontrollable pain management limits the amount of chemotherapy drugs that can be prescribed for effective anticancer activity.

Oxaliplatin, a platinum-based chemotherapeutic agent, is used widely to treat colorectal cancer. Although oxali-

platin is effective in treating malignant advanced cancer, patients receiving oxaliplatin suffer from acute and chronic peripheral neuropathies. Acute neuropathy is peculiar to oxaliplatin and is characterized by cold-induced dysesthesia or paresthesia (3, 4). Chronic neuropathy results from the accumulation of oxaliplatin after long-term treatment. It is characterized by loss of sensory perception and is frequently associated with painful sensations (4, 5). Adequate control of chronic neuropathy is important for the continuation of long-term chemotherapy, as it is often a factor limiting treatment (6). In animal models, oxaliplatin provokes various pain-like behaviors such as mechanical allodynia, mechanical hyperalgesia, and cold allodynia (7 – 9). The decreased cellular metabolism and axoplasmic transport resulting from the accumulation of oxaliplatin in dorsal root

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ganglia (DRG) cells is thought to be a mechanism mediating chronic neuropathy (10). However, in comparison with general neuropathy, the mechanisms underlying oxaliplatin-induced neuropathy have not been well characterized.

Anticonvulsant drugs are used for oxaliplatin-induced neuropathy in clinical trials, but neuropathy symptoms are difficult to control completely with these therapies (11, 12). Recent evidence suggests that oxycodone, an opioid analgesic, is effective in patients with oxaliplatin-induced neuropathy (13), although no reports have described the efficacies of other opioid analgesics, such as morphine and fentanyl, for oxaliplatin-induced neuropathy in clinical trials. Oxycodone may have a unique analgesic profile in oxaliplatin-related pain conditions.

Opioid analgesics are prescribed clinically to manage moderate-to-severe pain due to cancerous and non-cancerous conditions such as chronic inflammatory and postoperative pain. Clinically used opioid analgesics, such as morphine, oxycodone and fentanyl, bind to μ -opioid receptor (MOR) as agonists, resulting in analgesia. After binding to MOR, MOR agonists mainly activate Gi/o protein, followed by multiple mechanisms such as inhibition of voltage-gated Ca^{2+} channels, inhibition of adenylyl cyclase, and activation of G protein-gated inwardly rectifying potassium (GIRK/Kir3) channels. The activity of G protein might be related to the analgesic potencies of MOR agonists (14, 15). In fact, we previously reported that a reduction in the rate of G protein activation in pain-related brain regions depending on MOR agonists in the femur bone cancer (FBC) model is related to antinociceptive potencies (16). Therefore, measuring G protein activity in pain-related brain regions is valuable for assessing functional MOR changes induced by MOR agonists. In addition, morphine, oxycodone, and fentanyl exhibit different antinociceptive profiles in several pain models (17). Thus, the individual pharmacological characterization of MOR agonists in various pain conditions is important.

The present study investigated the pharmacological efficacies of morphine, oxycodone, and fentanyl in oxaliplatin-induced neuropathy in rats. Differential antinociceptive mechanisms of these MOR agonists were observed in this chronic pain model.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats (160 – 180 g, 6 weeks of age) (Charles River Laboratories Japan, Yokohama) were used in this study. Animals were housed in a room maintained at $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a 12-h light/dark cycle. Food and water were available ad libitum. The Institu-

tional Animal Care and Use Committee of Shionogi Research Laboratories, Osaka, Japan approved all animal protocols.

Drugs

Oxycodone hydrochloride and morphine hydrochloride were obtained from Shionogi Pharmaceutical (Osaka), and fentanyl citrate was obtained from Tyco Healthcare (Tokyo). Oxaliplatin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka), and pertussis toxin (PTX) and [D-Ala2, N-Me-Phe4, Gly5-ol]-encephalin (DAMGO) were purchased from Sigma-Aldrich (Tokyo). β -Funaltrexamine hydrochloride (β -FNA) was purchased from Tocris Bioscience (Bristol, UK). Oxaliplatin was dissolved in a 5% glucose solution (Otsuka Pharmaceutical, Tokyo), and other drugs were dissolved in 0.9% physiological saline (Otsuka Pharmaceutical) for in vivo experiments and in assay buffer for in vitro experiments.

Oxaliplatin-induced peripheral neuropathy

Oxaliplatin-induced peripheral neuropathy was initiated as described previously (8, 18). Body weight measurements were taken twice per week for 4 weeks (on days 1, 2, 8, 9, 15, 16, 22, and 23; Fig. 1B) and were followed by intraperitoneal (i.p.) injection of oxaliplatin (2 or 4 mg/kg) or vehicle (Fig. 1A). Paw withdrawal threshold was assessed before the first oxaliplatin injection and on day 1 and on days 8, 15, 22, 29, and 36 (Fig. 1C). Sciatic nerve conduction velocity (Fig. 2), drug efficacies (Figs. 3 and 4), [^{35}S]-GTP γ S-binding assays (Table 1), and receptor-binding assays (Fig. 5) were performed 36 – 38 days after the first administration of oxaliplatin (2 mg/kg).

von Frey hair test for mechanical allodynia

Mechanical allodynia was assessed using the up-down method of von Frey filament test. Briefly, von Frey filaments (0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g) were applied to each hind paw for a maximum period of 4 s, and the withdrawal response was observed. The 8 g stimulus was applied first. When a withdrawal response to a given filament was observed, weaker stimulation with a one-step-thinner filament was applied. The same procedure was repeated until monofilament stimulation no longer induced the behavioral response. When no response to monofilament stimulation was observed, stronger stimulation with a one-step-thicker monofilament was applied again to confirm a positive response. This was followed by reconfirmation of no response to the one-step-thinner monofilament to complete the test. The weakest stimulation that caused a positive response was taken as the threshold value. Rats that exhibited a

threshold change from 8–15 g before oxaliplatin injection to <4 g 36–38 days after oxaliplatin injection were used in the experiments. When sedation and/or muscle rigidity were observed after treatment with MOR agonists, such an individual was excluded from the result.

To compare the analgesic effects of the three MOR agonists, the antinociceptive effect was expressed as a percentage of the maximal possible effect (% MPE), calculated as $(T1 - T0) \times 100 / (T2 - T0)$, where T0 is the mean paw withdrawal threshold in the oxaliplatin-saline group, T1 is the paw withdrawal threshold of each animal treated with the drug, and T2 is the mean paw withdrawal threshold in the sham-saline group.

Evaluation of sciatic nerve conduction velocity

Thirty-six days after i.p. administration of vehicle or oxaliplatin (2 mg/kg), rats were decapitated under anesthesia with pentobarbital sodium (60 mg/kg, i.p.). Both the left and right sciatic nerves were immediately dissected and their connective tissues removed in HEPES buffer (145 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 11 mM D-glucose [pH 7.4]). Each sciatic nerve was placed on a recording chamber. Using an electric stimulator (SEN-7203; Nihon Kohden, Tokyo) and isolator (SS-203J, Nihon Kohden), square-shaped stimulating pulses of 0.01-ms duration at 0.1 Hz were applied at supra-threshold intensity ($1.5 \times$ threshold) to the distal end of the nerve. Bipolar sciatic nerve action potentials (NAPs) were recorded at the proximal end of the nerve (20 mm from the stimulus point) using an AC differential amplifier (DAM80; World Precision Instruments, Sarasota, FL, USA) with a low-cut filter at 1 Hz and a high-cut filter at 1 kHz. The signal was digitized at 200 kHz with the Scope3 software using PowerLab (AD Instruments, Colorado Springs, CO, USA). Ten consecutive responses were averaged, and nerve conduction velocity and NAP amplitude were calculated for each rat, as an average of the left and right sciatic nerves, using Chart5 software (AD Instruments). Nerve conduction velocity was calculated using the latency between the peak stimulus artifact and NAP onset or the NAP peak. To determine onset time, the voltage trace was differentiated with respect to time. Onset time was defined as the time of >1 mV/ms before the beginning of the NAP. Peak time was determined as the time showing a maximal response. Peak amplitude was calculated by subtracting the voltage at onset time from the voltage at peak time.

Intracerebroventricular (i.c.v.) administration

Rats were anesthetized with 3% isoflurane and placed in a stereotaxic frame (SR-6; Narishige Instruments,

Tokyo) with a horizontal skull position. A guide cannula (22 G needle; Yamashita Seisakusho, Osaka) was positioned using the following coordinates: 0.5 mm posterior to the bregma, 1.5 mm lateral to the midline on the left side, and 3.0 mm ventral to the skull surface. All coordinates were derived from the atlas of Paxinos and Watson (19). The guide cannula was fixed with a stainless steel screw placed in the calvaria surrounding the cannula, and resin cement (ADFA; Shofu, Kyoto) was applied to the screw and guide cannula. Animals were allowed to recover for 1 week before further experimentation. A cannula dummy (30 G needle, Yamashita Seisakusho) was inserted to seal the top of the guide cannula and prevent tissue entry into the bottom of the guide cannula during the 1 week recovery period. When drugs were administered, the injection volume was set at 5 μ l per rat.

For PTX treatment, rats were pretreated with PTX (0.5 μ g/rat, i.c.v.) 6 days before opioid administration, and morphine, oxycodone, or fentanyl was administered 15 or 30 min before measurement of the paw withdrawal response.

Guanosine-5'-o-(3-thio) triphosphate ([³⁵S]-GTP γ S) binding assay

Tissue samples were prepared from sham and oxaliplatin-treated rats 36–38 days after the first oxaliplatin injection. The mediodorsal thalamus (mTH), periaqueductal gray matter (PAG), and spinal cord were removed quickly after decapitation. Briefly, referring to the rat brain atlas (19), a coronal block of the mTH was obtained 1.5–4.5-mm posterior to the bregma, and a coronal block of the PAG was obtained 4.5–7.5-mm posterior to the bregma. The collected tissues were then transferred to tubes filled with ice-cold 50 mM Tris-HCl buffer (pH 7.4) supplemented with 5 mM MgCl₂ and 1 mM EGTA. A membrane homogenate (10–20 μ g/assay) was prepared as described previously (20) with some modifications and incubated at 25°C for 2 h in 0.5 ml of assay buffer [50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 1 mM EGTA, 100 mM NaCl] with various concentrations of the MOR agonist, 30 μ M guanosine-5'-diphosphate (GDP), and 60 pM [³⁵S]-GTP γ S (specific activity, 1250 Ci/mmol; PerkinElmer Life Science, Arlington Heights, IL, USA). Agonist (morphine, oxycodone, or fentanyl) was applied simultaneously and co-incubated. The reaction was terminated by filtration using Whatman GF/C glass filters (Brandel, Gaithersburg, MD, USA) that had been presoaked in 50 mM Tris-HCl (pH 7.4) at 4°C for 2 h. The filters were washed three times with 2.5 ml of ice-cold water and then transferred to scintillation-counting vials. Then, 5 ml of Clear-sol 2 (Nacalai Tesque, Kyoto) were added to the vials. Radioactivity in the samples was determined using

a liquid scintillation analyzer. Nonspecific binding was measured in the presence of unlabeled GTP γ S (10 μ M). G-protein activation by each agonist was expressed as percent stimulation, calculated as $[100 \times (A1 - A0) / (A2 - A0)] - 100$, where A0 is the nonspecific activity, A1 is the [35 S]-GTP γ S activity in the presence of various concentrations of morphine, oxycodone, or fentanyl, and A2 is the [35 S]-GTP γ S activity in the absence of agonist.

The [tylosil-3,5-(3)H(N)]-[D-Ala(2),N-Me-Phe(4),Gly-ol(5)]enkephalin ([3 H]-DAMGO) receptor binding assay

Tissue samples were prepared from sham and oxaliplatin-treated rats 36–38 days after the first oxaliplatin injection. The mTH was removed immediately after decapitation, as described above. The collected tissues were then transferred to a tube filled with ice-cold sucrose (320 mM). The tissues were homogenized using a high-velocity revolution homogenizer. The resulting homogenate was centrifuged at 4°C for 10 min at $2,000 \times g$, and the supernatant was centrifuged at 4°C for 20 min at $48,000 \times g$. The pellet obtained was resuspended in ice-cold assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, and 1 mM EGTA as the membrane fraction. Membrane proteins were quantified using a bicinchoninic acid (BCA)-compatible protein assay kit (Pierce) with bovine serum albumin (BSA) as the standard. The amount of protein used in the assay was adjusted to 80 μ g.

A displacement binding experiment was performed in duplicate. The membrane homogenate was incubated at 25°C for 2 h in 0.5 ml of assay buffer with various concentrations of fentanyl (10^{-9} – 10^{-5} M) and 2 nM [3 H]-DAMGO (specific activity, 51.5 Ci/mmol; PerkinElmer Life Science). Fentanyl was applied simultaneously and co-incubated. Nonspecific binding was determined in the presence of 10 μ M DAMGO. The binding was terminated by rapid filtration through Whatman GF/C glass filters (Brandel). The filters were then washed three times with 50 mM Tris-HCl (pH 7.4) and transferred to scintillation vials. Next, 5 ml of Clear-sol 2 (Nacalai Tesque) were added to the vials. Radioactivity in the samples was determined using a liquid scintillation analyzer.

Statistical analyses

The data are shown as the means $\pm$ standard error of the mean (S.E.M.). Statistical analyses were performed using the SAS software (ver. 8; SAS Institute, Cary, NC, USA). Dose-dependent antinociceptive response and receptor-binding curves were fitted using GraphPad Prism ver. 6.0. The concentration causing 50% inhibition of the [3 H]-DAMGO response (IC₅₀) in the receptor-

binding assay were calculated using GraphPad Prism ver. 6.0. The significance of differences among groups was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test (Figs. 3 and 4) or two-way ANOVA followed by the Bonferroni multiple comparison test (Fig. 1 and Table 1). Statistical analysis of differences between two groups was carried out by Student's *t*-test (Fig. 2). In all analyses, $P < 0.05$ indicated statistical significance.

Results

Characterization of chronically administered oxaliplatin-induced neuropathy

We initially established the optimal dose of oxaliplatin as used in a previous report (8), by monitoring changes in body weight (Fig. 1B) and expression of mechanical allodynia-like behavior (Fig. 1C). The rats were given oxaliplatin (2 or 4 mg/kg, i.p.) twice weekly for 4 weeks. Chronic oxaliplatin treatment resulted in a significant decrease in weight gain compared with the sham group, beginning at 16 days with 2 mg/kg oxaliplatin and at 9 days with 4 mg/kg oxaliplatin (Fig. 1B). Both doses of oxaliplatin decreased weight gain, and animals receiving 4 mg/kg of oxaliplatin showed a larger weight loss than that of 2 mg/kg group. Deteriorations in general condition were not observed in the 2 mg/kg group, while a fatal incident was observed in the 4 mg/kg group (data not shown). Using the von Frey test, we confirmed the development of chronic neuropathy in oxaliplatin-treated rats. Paw withdrawal thresholds started to decrease significantly compared with the sham group 15 days after administration of 2 mg/kg oxaliplatin and 29 days after administration of 4 mg/kg oxaliplatin (Fig. 1C). Based on the weight changes, effects on general condition, and strength of mechanical allodynia, we selected 2 mg/kg for the oxaliplatin-induced chronic pain model.

To characterize oxaliplatin-induced neuropathy in rats, we also examined changes in sciatic nerve conduction velocity and amplitude (Fig. 2). Because previous studies have demonstrated that oxaliplatin treatment slows nerve conduction in rats and humans (21–24), we examined whether nerve conduction velocity was reduced by treatment with oxaliplatin (2 mg/kg, i.p.). NAP conduction velocity and NAP peak amplitude were measured using sciatic nerves prepared from the sham and oxaliplatin groups. Figure 2A shows representative traces of sciatic NAPs from the sham and oxaliplatin groups. Mean conduction velocity at the onset time was reduced significantly in the oxaliplatin group (35.7 ± 1.3 m/s) compared with the sham group (44.3 ± 2.7 m/s) (Fig. 2B). Conduction velocity at peak in the oxaliplatin

Fig. 1. Effects of oxaliplatin on body weight and mechanical allodynia. A) Drug administration schedule. B) Rats were weighed before administration of vehicle or oxaliplatin (2 or 4 mg/kg, i.p.) twice per week for 4 weeks. C) The von Frey hair test was performed before oxaliplatin administration on day 1 and on days 8, 15, 22, 29, and 36. The y-axis represents the paw withdrawal threshold, expressed as the sum of the right and left paw latencies (g). Data represent the mean $\pm$ S.E.M. of 10–16 rats. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the vehicle group (two-way ANOVA, Bonferroni multiple comparison test).

Fig. 2. Effect of oxaliplatin on sciatic nerve conduction. A) Typical recording of action potentials of sciatic nerves prepared from rats treated with vehicle or 2 mg/kg, along with the effects of oxaliplatin on conduction velocity at onset (B), peak conduction velocity (C), and peak amplitude (D). Data represent the means $\pm$ S.E.M. of 15 rats. * P < 0.05, ** P < 0.01 vs. the sham group (Student's t -test; B, C, D).

group (21.25 ± 0.89 m/s) was also significantly lower than that in the sham group (18.8 ± 0.59 m/s) (Fig. 2C). However, amplitudes were similar in the sham and oxaliplatin groups (4.5 ± 0.21 mV vs. 4.5 ± 0.31 mV, Fig. 2D). Consistent with previous studies (9, 25), conduction velocity at onset and at peak were reduced significantly, and the peak amplitude was unchanged, in our oxaliplatin-induced chronic pain model.

Effects of MOR agonists on oxaliplatin-induced mechanical allodynia

We next investigated the effects of MOR agonists on the oxaliplatin-induced neuropathy in rats using the von Frey test. Based on our previous report (26), the doses of MOR agonists were selected. Maximal drug effects were established 15 min after subcutaneous (s.c.) administration of oxycodone or fentanyl and 30 min after s.c.

Fig. 3. Effects of opioid analgesics on oxaliplatin-induced neuropathy. Groups of rats were treated with A) morphine (0.3–3 mg/kg, s.c.), B) oxycodone (0.1–0.56 mg/kg, s.c.), or C) fentanyl (0.0056–0.03 mg/kg, s.c.). Antinociceptive effects were evaluated 30 min after morphine administration and 15 min after oxycodone or fentanyl administration and were expressed as the sum of the right and left paw withdrawal thresholds. D) Dose–response curves for opioid antinociception in rats based on the von Frey hair test. The antinociceptive effects were calculated as % MPE. Data represent the means $\pm$ S.E.M. of 5–13 rats. # P < 0.05, ## P < 0.01, ### P < 0.001 vs. the sham-saline group (one-way ANOVA, Dunnett's multiple comparison post hoc test; A, B, C). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the oxaliplatin-saline group (one-way ANOVA, Dunnett's multiple comparison post hoc test; A, B, C).

administration of morphine (data not shown), and these time points were used in further analyses. Both morphine (1, 3 mg/kg, s.c.) and oxycodone (0.3–0.56 mg/kg, s.c.) significantly reversed the decline in the paw withdrawal threshold compared with the oxaliplatin-saline group; the withdrawal thresholds of morphine (3 mg/kg) and oxycodone (0.3–0.56 mg/kg) groups did not differ significantly from that of the sham-saline group (Fig. 3: A, B). Consistent with our previous report (26), the highest doses of morphine (3 mg/kg) and oxycodone (0.56 mg/kg) in this study did not produce sedation and/or muscle rigidity. On the other hand, sedation and/or strong muscle rigidity were observed in about half of rats receiving the highest dose of fentanyl (0.03 mg/kg). Therefore, rats which did not show sedation and/or muscle rigidity were chosen to calculate the paw withdrawal threshold. Fentanyl (0.017–0.03 mg/kg, s.c.) significantly reversed the decrease in the paw withdrawal threshold (Fig. 3C), but the withdrawal thresholds of higher doses of fentanyl (0.017 or 0.03 mg/kg) groups significantly differed from that of the sham-saline group (Fig. 3C), indicating that the antinociceptive effect of fentanyl was partial. Figure 3D shows the relative antinociceptive efficacies of MOR agonists in the oxaliplatin-induced neuropathy in rats, expressed as the % MPE. When the highest dose of MOR agonist that did not induce sedation was administered, the antinociceptive

effects of morphine (3 mg/kg), oxycodone (0.56 mg/kg), and fentanyl (0.03 mg/kg) were 67.7%, 83.6%, and 42.3% MPE, respectively. The ED_{50} values for the antinociceptive effects of morphine and oxycodone were 1.60 and 0.18 mg/kg, respectively. The ED_{50} value of fentanyl was not determined because the antinociceptive efficacy at the highest dose of 0.03 mg/kg did not reach 50% MPE. In addition, the antinociceptive effects of morphine, oxycodone, and fentanyl were completely inhibited by β -FNA (10 mg/kg, s.c.), a MOR-selective antagonist (data not shown). These results indicate that although the antinociceptive components of all three MOR agonists involved MOR activation, the antinociceptive potencies in oxaliplatin-induced neuropathy in rats differed among the three MOR agonists.

The effect of PTX on antinociception of MOR agonists in rats with oxaliplatin-induced neuropathy

To reveal the mechanism underlying the partial antinociceptive effect of fentanyl in oxaliplatin-induced neuropathy in rats, we focused on G proteins. In general, MOR agonists are known to activate Gi/o protein to exert their effects. Many laboratories reported that the antinociceptive effects of morphine, oxycodone, and fentanyl in naïve animals were mainly mediated by Gi/o protein (27–30). Therefore, we next investigated the contribution of Gi/o protein to opioid antinociception

(A) Morphine

(B) Oxycodone

(C) Fentanyl

Fig. 4. Effects of PTX on opioid-induced antinociception. PTX (0.5 µg/rat, i.c.v.) was administered 6 days before opioid administration. A) Morphine (3 mg/kg, s.c.), B) oxycodone (0.56 mg/kg, s.c.), and C) fentanyl (0.017 mg/kg, s.c.) were administered to oxaliplatin-treated rats 30, 15, and 15 min, respectively, prior to evaluation with the von Frey hair test. The antinociceptive effect was expressed as the sum of the right and left paw withdrawal thresholds. Data represent the means $\pm$ S.E.M. of 5–13 rats. ** $P < 0.01$, *** $P < 0.001$ vs. the saline-opioid group (one-way ANOVA, Dunnett's multiple comparison post hoc test; B, C, D).

in rats with oxaliplatin-induced neuropathy. In this experiment, we used the doses of MOR agonists that exhibited the significant antinociceptive effects without showing excessive sedation and/or muscle rigidity. For fentanyl, we set at a dose of 0.017 mg/kg because this dose rarely produced sedation and/or muscle rigidity. Pretreatment with PTX (0.5 µg/rat, i.c.v.), a selective Gi/o protein inhibitor, completely inhibited the antinociceptive effects of morphine (3 mg/kg, s.c.) and oxycodone (0.56 mg/kg, s.c.) in oxaliplatin-treated rats (Fig. 4: A and B). By contrast, the partial antinociception by fentanyl (0.017 mg/kg, s.c.) was not blocked by PTX pretreatment (0.5 µg/rat, i.c.v.) in rats with oxaliplatin-induced neuropathy (Fig. 4C). These results show that a PTX-sensitive G protein mediates the antinociceptive effects of morphine and oxycodone, but not that of fentanyl, in rats with oxaliplatin-induced neuropathy.

MOR agonist-stimulated G protein activation in oxaliplatin-induced neuropathy in rats

In rats with oxaliplatin-induced neuropathy the contribution of Gi/o protein differed according to MOR agonists used. We next examined the MOR agonist activities of morphine, oxycodone, and fentanyl in pain-related brain regions in oxaliplatin-treated rats to assess

the possible changes in the functional activation of G protein by MOR agonists. When [35 S]-GTP γ S binding assays were conducted in the mTH, PAG, and spinal cord from oxaliplatin-treated rats, the greatest change among the regions was observed in the mTH. In the mTH, morphine (10^{-8} – 10^{-5} M) and oxycodone (10^{-8} – 10^{-5} M) increased [35 S]-GTP γ S binding in a concentration-dependent manner (data not shown), and [35 S]GTP γ S binding at the highest concentration (10^{-5} M) of morphine and oxycodone was not significantly different between the sham group (morphine, 58.3%; oxycodone, 45.5%) and the oxaliplatin group (morphine, 71.6%; oxycodone, 49.7%) (Table 1). Fentanyl (10^{-8} – 10^{-5} M) also increased [35 S]-GTP γ S binding in a concentration-dependent manner (data not shown), but [35 S]GTP γ S binding at the highest concentration (10^{-5} M) in the oxaliplatin-treated group (83.2%) was significantly lower than that in the sham group (120.9%) (Table 1). In the PAG and spinal cord, morphine-, oxycodone-, and fentanyl-induced G protein activation did not differ between the sham group and the oxaliplatin group (Table 1). In summary, morphine- and oxycodone-stimulated G protein activation was not affected in the mTH in the oxaliplatin model, whereas fentanyl-stimulated G protein activation was attenuated.

Table 1. [35 S]GTP γ S binding at 10 μ M of morphine, oxycodone, and fentanyl

	Morphine		Oxycodone		Fentanyl	
	Sham	Oxaliplatin	Sham	Oxaliplatin	Sham	Oxaliplatin
mTH	58.3 $\pm$ 5.7	71.6 $\pm$ 8.7	45.5 $\pm$ 5.2	49.7 $\pm$ 7.6	120.9 $\pm$ 15.1	83.2 $\pm$ 7.4*
PAG	32.2 $\pm$ 9.3	43.2 $\pm$ 2.4	28.4 $\pm$ 4.9	31.9 $\pm$ 6.0	45.0 $\pm$ 6.2	41.5 $\pm$ 6.8
Spinal cord	19.6 $\pm$ 1.3	30.5 $\pm$ 4.8	16.7 $\pm$ 3.0	21.4 $\pm$ 2.5	31.2 $\pm$ 3.4	38.9 $\pm$ 4.9

Using the membranes from mTH, PAG, and spinal cord of sham and oxaliplatin-treated groups, membrane-bound [35 S]-GTP γ S was measured in the presence of various concentrations of morphine (10^{-8} – 10^{-5} M), oxycodone (10^{-8} – 10^{-5} M), and fentanyl (10^{-8} – 10^{-5} M). [35 S]GTP γ S binding was expressed as % stimulation compared to the basal level. Using concentration–response curves, the statistical significance of differences among groups was assessed by two-way ANOVA followed by the Bonferroni multiple comparison test. In this table, the values of % stimulation at 10^{-5} M of MOR agonists are shown. Each value represents the mean $\pm$ S.E.M. of 4 independent experiments (5 rats per group). Values in parenthesis indicate the 95% confidence range. * P < 0.05 vs. the sham group (two-way ANOVA, Bonferroni multiple comparison test).

Fig. 5. Displacement of [3 H]-DAMGO from mTH membranes by fentanyl. Tissue samples were collected 36–38 days after injection of vehicle (Sham) or oxaliplatin (Oxaliplatin), and membranes prepared from mTH were subjected to displacement assay. Experiments were performed in the presence of [3 H]-DAMGO (2 nM) and increasing concentrations of fentanyl (10^{-9} – 10^{-5} M). Specific binding was defined as the difference in binding in the absence and presence of unlabeled DAMGO (10 μ M). Two independent experiments (eight rats per group) were conducted. The graph shows representative data.

Binding affinity of fentanyl for MOR in rats with oxaliplatin-induced neuropathy

A possible mechanism underlying the decreased fentanyl-stimulated G protein activation was a reduction in the binding affinity of fentanyl for MOR. Therefore, we conducted a [3 H]-DAMGO displacement binding assay with fentanyl using membrane samples from mTH of sham and oxaliplatin-treated rats (Fig. 5). [3 H]-DAMGO was displaced by fentanyl in a concentration-dependent manner (Fig. 5). Displacement curves for the sham group (IC_{50} , 10.2 nM) and the oxaliplatin group (IC_{50} , 6.9 nM) were similar (Fig. 5), suggesting that the affinity of fentanyl for MOR was not altered in the oxaliplatin model. These results indicate that the change in the MOR binding affinity is not likely to be the mechanism underlying the attenuation of MOR activation by fentanyl in the oxaliplatin-induced neuropathic pain model.

Discussion

In this study, we found that morphine and oxycodone completely reversed oxaliplatin-induced chronic neuropathic pain, whereas fentanyl showed a partial antinociceptive effect within the range of doses without showing sedation and/or muscle rigidity. Activation of Gi/o protein contributed to the antinociception by morphine and oxycodone in rats with oxaliplatin-induced neuropathy. However, the partial antinociceptive effect by fentanyl in rats with oxaliplatin-induced neuropathy seemed to result at least in part from the loss of PTX-sensitive Gi/o protein activation. Overall, Gi/o protein activation might be needed for robust antinociceptive effects of MOR agonists in oxaliplatin-induced neuropathy.

The limited contribution of Gi/o protein might explain the partial antinociception by fentanyl in rats with oxaliplatin-induced neuropathy. The attenuation of fentanyl-stimulated Gi/o protein activity might be responsible for this loss of the contribution of Gi/o protein to fentanyl antinociception because fentanyl-stimulated G protein activation was reduced in the mTH in oxaliplatin-treated rats, and Gi/o protein activation contributes to fentanyl antinociception in naïve rats (27). These results indicate that oxaliplatin stimulation altered the MOR-related G protein activation pattern of fentanyl.

Regardless of the loss of the Gi/o protein activation component of fentanyl antinociception in the oxaliplatin model, the antinociceptive effects of morphine and oxycodone, which are mediated by Gi/o protein activation, were not affected in rats with oxaliplatin-induced neuropathy. Both in vitro and in vivo studies indicated that structurally and selectively distinct MOR agonists showed a distinct pattern of G protein activation (31–33). Gi/o proteins are classified into four subtypes: Gi1, Gi2, Gi3, and Go. Notably, morphine activates Gi1 protein dominantly, while fentanyl partially activates both Gi1 and Go proteins in MOR-expressing cells (31).

Each MOR agonist showed a distinct pattern of G protein activation in MOR-mediated supraspinal antinociception. For example, Gi2 protein mainly contributed to the supraspinal antinociceptive effect of morphine in naïve animals (32, 33). Although no information of oxycodone is available, it is possible to consider that oxycodone also has a unique Gi/o protein activation profile. In addition, it might be possible to consider the changes in the expression and/or function of Gi/o proteins to explain the different antinociceptive potencies depending on MOR agonists in chronic pain models. In fact, our preliminary data showed that the mRNA level of Gi3 protein, but not those of Gi1, Gi2, or Go protein, was reduced by 30% in the mTH in the oxaliplatin group, compared with that in sham group (data not shown). Further studies are required to identify the effects of oxaliplatin treatment on the expression levels of Gi/o protein subtypes and the activation pattern of Gi/o proteins in response to MOR agonists.

Fentanyl-stimulated G protein activation in the mTH was attenuated in the oxaliplatin model. However, two possibilities should be considered. One is a reduction in the binding affinity of fentanyl for MOR. The present study showed that the change in the MOR binding affinity is not likely to be the mechanism for the fentanyl-dependent attenuation of MOR activation in oxaliplatin-induced neuropathy. The second possibility for decreased G protein activation by fentanyl is upregulation of G protein-coupled receptor kinases (GRKs) because upregulation of the expression of GRKs might reduce opioid-stimulated G protein activation in animal pain models (34), and fentanyl showed a stronger interaction with the arrestin-GRK pathway compared with morphine (35, 36). Therefore, we examined the expression level of GRK2, a major link to MOR, in the mTH in the oxaliplatin group by western blotting. The expression level of GRK2 in the mTH in the oxaliplatin group was similar to that in the sham group (data not shown). Although the expression levels of other GRKs and changes in GRK active forms were not investigated in the present study, these results suggest that the second possibility was also not responsible for the reduction of fentanyl-stimulated G protein activation in the mTH in the oxaliplatin model.

In conclusion, this study demonstrated that morphine and oxycodone completely reversed oxaliplatin-induced chronic neuropathic pain, whereas fentanyl showed a partial antinociceptive effect within the range of doses without showing sedation and/or muscle rigidity. The results in the present study suggested that the degree of agonist-stimulated Gi/o protein activation might be related to the potency of antinociception by MOR agonists. To our knowledge, this is the first study

demonstrating different antinociceptive effects among MOR agonists in rats with oxaliplatin-induced neuropathy. Although the efficacies of MOR agonists in the oxaliplatin animal model do not directly represent their analgesic effects in clinical settings, our findings provide a suggestion that some opioid analgesics can be useful for relieving oxaliplatin-induced neuropathy.

Conflicts of Interest

T. Kanbara, A. Nakamura, K. Takasu, K. Ogawa, M. Hasegawa, G. Sakaguchi, and T. Kanemasa are employees of Shionogi & Co., Ltd., the manufacturer of oxycodone and morphine.

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