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Prevention of oxaliplatin-induced mechanical allodynia and neurodegeneration by neurotropin in the rat model

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ABSTRACT

Oxaliplatin is a key drug for colorectal cancer, but it causes acute peripheral neuropathy (triggered by cold) and chronic neuropathy (sensory and motor neuropathy) in patients. Neurotropin, a non-protein extract from the inflamed rabbit skin inoculated with vaccinia virus, has been used to treat various chronic pains. In the present study, we investigated the effect of neurotropin on the oxaliplatin-induced neuropathy in rats.

Repeated administration of oxaliplatin caused cold hyperalgesia from Day 5 to Day 29 and mechanical allodynia from Day 15 to Day 47. Repeated administration of neurotropin relieved the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia, and inhibited the oxaliplatin-induced axonal degeneration in rat sciatic nerve. Neurotropin also inhibited the oxaliplatin-induced neurite degeneration in cultured pheochromocytoma 12 (PC12) and rat dorsal root ganglion (DRG) cells. On the other hand, neurotropin did not affect the oxaliplatin-induced cell injury in rat DRG cells. These results suggest that repeated administration of neurotropin relieves the oxaliplatin-induced mechanical allodynia by inhibiting the axonal degeneration and it is useful for the treatment of oxaliplatin-induced neuropathy clinically.

1. Introduction

Oxaliplatin, a platinum-based chemotherapeutic agent, is widely used for colorectal cancer. However, it causes severe acute and chronic peripheral neuropathies. Acute neuropathy is peculiar to oxaliplatin and includes acral paresthesias enhanced by exposure to cold (Cassidy and Misset, 2002; Extra et al., 1998; Pasetto et al., 2006; Quasthoff and Hartung, 2002). It has been thought that the acute neuropathy is not due to morphological damage of the nerve (de Gramont et al., 2002; Wilson et al., 2002) and is due to alternations of voltage-gated Ca^{2+} and K^+ channels (Adelsberger et al., 2000; Benoit et al., 2006; Grolleau et al., 2001; Kagiava et al., 2008). On the other hand, the chronic neuropathy is characterized by loss of sensory and motor neuropathy after long-term treatment of oxaliplatin and it is similar to cisplatin-induced neurological symptom (Pasetto et al., 2006). Oxaliplatin causes the damage of the cell bodies (Cavaletti et al., 2001; Donzelli et al., 2004; McKeage et al., 2001; Scuteri et al., 2009; Ta et al., 2006), inhibition of neurite outgrowth (Luo et al., 1999), alterations in nuclearus morphology (Cavaletti et al., 2001), and selective atrophy of subpopulation of dorsal root ganglion (DRG) neurons (Jamieson et al., 2005). Oxaliplatin is metabolized to oxalate and dichloro(1,2-diaminocyclohexane) platinum [Pt(dach)Cl_2] (Graham et al., 2000). Recently, we demonstrated the involvement of oxalate in oxaliplatin-induced cold hyperalgesia but not mechanical allodynia, and preventive effect of pre-administration of Ca^{2+} or Mg^{2+} on the cold hyperalgesia in rats (Sakurai et al., 2009). On the other hand, pre-administration of Ca^{2+} or Mg^{2+} could not attenuate the mechanical allodynia which is related to Pt(dach)Cl_2 (Sakurai et al., 2009).

Neurotropin is a non-protein extract derived from the inflamed skin of rabbits inoculated with vaccinia virus. Neurotropin is clinically used to treat various chronic pain conditions, including post herpetic neuralgia and hyperesthesia of subacute myelo-optic neuropathy (SMON). Moreover, National Institute of Nursing Research (NINR) in the United States is now examining the safety and effectiveness of neurotropin for preventing or easing pain associated with fibromyalgia and treating chronic pain after injury to a limb or a large nerve. We previously reported that repeated administration of neurotropin reverses the paclitaxel-induced neuropathy without affecting anticancer activity in rats (Kawashiri et al.,

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2009). However, the effect of neurotropin on the oxaliplatin-induced neuropathy remains unexplored. Accordingly, we examined the effect of neurotropin on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia in rats.

2. Methods

2.1. Animals

Male Sprague–Dawley rats weighing 200–250 g (Kyudo Co., Saga, Japan) were used in the present study. Rats were housed in groups of four to five per cage, with lights on from 800 to 2000 h. Animals had free access to food and water in their home cages. All experiments were approved by the Experimental Animal Care and Use Committee of Kyushu University according to the National Institutes of Health guidelines, and we followed International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research (Zimmermann, 1983).

2.2. Drug administration

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan). Sodium oxalate was provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Neurotropin was a generous gift from Nippon Zoki Pharmaceutical Co. (Osaka, Japan). Oxaliplatin and sodium oxalate were dissolved in 5% glucose solution. The vehicle-treated rats were injected with 5% glucose solution. Oxaliplatin (4 mg/kg), sodium oxalate (1.3 mg/kg) or vehicle was injected i.p. in volumes of 1 mL/kg twice a week for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23). Neurotropin [200 Neurotropin Unit (NU)/kg] was administered p.o. in volume of 10 mL/kg three times a week for 4 weeks (Days 1, 2, 3, 8, 9, 10, 15, 16, 17, 22, 23 and 24). Behavioral tests were performed blind with respect to drug administration. The doses of oxaliplatin, sodium oxalate and neurotropin followed previous reports (Kawashiri et al., 2009; Ling et al., 2007; Sakurai et al., 2009).

2.3. von Frey test for mechanical allodynia

The mechanical allodynia was assessed by von Frey test. The von Frey test was performed before the first drug administration (on Day 1) and on Days 5, 8, 12, 15, 19, 22, 26, 29, 33, 40, 47 and 54. On Days 5, 15 and 22, test was performed before drug administration. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. von Frey filaments (The Touch Test Sensory Evaluator Set; Linton Instrumentation, Norfolk, UK) ranging 1–15 g bending force were applied to the midplantar skin of each hind paw with each application held for 6 s. Fifty percent paw withdrawal thresholds were determined by a modification of up-down method that described by Chaplan et al. (1994). First, each hind paw was touched with some filaments from 1 g up to the force that rat exhibited the withdrawal response, in ascending order. Next, the paw was touched with some filaments from 15 g down to the force that rat did not exhibit the response, in descending order. These up and down steps were repeated three times. Fifty percent thresholds were determined by average of the weakest force in each up or down step.

2.4. Acetone test for cold hyperalgesia

The cold hyperalgesia was assessed by acetone test. The acetone test was performed before the first drug administration (on Day 1) and on Days 5, 8, 12, 15, 19, 22, 26, 29, 33, 40, 47 and 54 according to the method described by Flatters and Bennett (2004). On Days 5, 15 and 22, test was performed before drug administration. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. Fifty microlitre of acetone (Wako Pure Chemical Ltd., Osaka, Japan) was sprayed onto the plantar skin of each hind paw three times with a Micro Sprayer® (Penn Century Inc., PA, USA), and rats were observed for 40 s from the start of the acetone spray. The number of elevation of each hind paw was recorded.

2.5. Assay of sciatic nerve axonal degeneration

On Day 5 or 25, sciatic nerves were harvested from rats anesthetized with sodium pentobarbital. Nerves were fixed in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 4 °C) for 4 h followed by wash with 0.1 M phosphate buffer. After 8% (w/v) sucrose-substitution, samples were embedded in Epon. Each section was stained with toluidine blue. Sample sections were evaluated using light microscopy (BX51; Olympus Corp., Tokyo, Japan).

2.6. Assay of cell injury in DRG neurons

The cell injury, especially nuclear damage, was stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). On Day 5 or 25, L5 DRG neurons were harvested from rats anesthetized with sodium pentobarbital. DRG neurons were fixed in 4% (w/v) paraformaldehyde for 30 min. Samples embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) were frozen and sliced. Each section was permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate on ice. TUNEL stain was carried out using a commercial assay kit (Cell Death Detection kit, Roche Applied Science, Tokyo, Japan), according to the manufacturer's instructions. Sample sections were evaluated using fluorescence microscopy (BX51; Olympus Corp., Tokyo, Japan).

2.7. Cell lines and cultures

PC12 was obtained from the American Type Culture Collection (Walkersville, MD, USA). L 4-5 DRG cells were removed from male Sprague–Dawley rats (6 weeks old), which anesthetize with sodium pentobarbital, and primary cultured. Ganglia was incubated with 0.125% (w/v) collagenase type 1 (Worthington Biochemical Corp., NJ, USA) at 37 °C for 90 min followed by incubation with 0.25% (w/v) trypsin–EDTA (Gibco BRL, USA) for 30 min. PC12 cells were grown in RPMI 1640 medium (MP Biomedicals Inc., CA, USA) supplemented with 2 mM L-glutamine, 10% horse serum, and 5% fetal bovine serum (FBS). DRG cells were grown in Dulbecco's modified Eagle's medium (MP Biomedicals Inc.) with 2 mM L-glutamine and 10% FBS. Both cell lines were cultured on 80 cm^2 tissue culture flasks (Nunc Apogent Co., Roskilde, Denmark) at 37 °C in an air supplemented with 5% CO_2 under humidified conditions.

2.8. Assay of cultured PC12 and DRG neurite outgrowths

PC12 is the rat pheochromocytoma and characterized by neuronal differentiation by nerve growth factor (NGF). PC12 cells have been used as the model of neurodegeneration such as paclitaxel- or cisplatin-induced neurite degeneration (Pisano et al., 2003; Kawashiri et al., 2009). PC12 cells were seeded at a density of 1×10^4 cells/cm^2 onto 24 well plates (Falcon, Becton Dickinson Co., Ltd., NJ, USA) and were used for experiments on the following day. Neurite outgrowth in PC12 cells was induced by 10 μM forskolin (Carbiochem, EMD Chemicals Inc., Darmstadt, Germany) at 21 h before drug exposures. DRG cells were seeded onto 24 well plates and were cultured for a week so that neurites were extended. Both cell types were exposed to oxaliplatin (0.3, 1, 3, 10

or 30 μM), sodium oxalate (0.3, 1, 3, 10 or 30 μM) and neurotropin (0.001, 0.003, 0.01 or 0.03 NU/mL) for 168 h. After incubation with drugs, dead cells were stained with trypan blue (Gibco BRL, NY, USA). Cells were monitored by a phase contrast microscope and neurite lengths in living cells were measured by analysis software (Image J 1.36; Wayne Rasband, National Institutes of Health, MD, USA).

2.9. Assay of cell viability in cultured DRG cells

DRG cells were seeded onto 24 well plates and were cultured for a week so that neurites were extended. Cells were exposed to oxaliplatin (0.3, 1, 3, 10 or 30 μM), sodium oxalate (0.3, 1, 3, 10 or 30 μM) and neurotropin (0.001, 0.003, 0.01 or 0.03 NU/mL) for 168 h. The cell viability was assessed by the mitochondrial activity in reducing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to formazan. At 168 h after incubation with oxaliplatin and neurotropin, the cells were washed with phosphate-buffered saline, then 210 μL of serum-free medium and 10 μL of WST-8 assay solution (Cell Counting Kit-8; Dojindo Laboratory, Kumamoto, Japan) were added and incubated for 1 h at 37 °C in humidified air supplemented with 5% CO_2. The incubation medium was carefully taken and transferred to 96 well flat-bottom plastic plates (Corning Costar, Corning, NY, USA). The amount of formed formazan dye was measured from the absorbance at 450 nm with a reference wavelength of 620 nm using a microplate reader (Immuno-mini NJ-2300; Inter Medical, Tokyo, Japan).

2.10. Statistical analyses

Values were expressed as the mean ± SEM. The values were analysed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc test to determine differences among the groups. A probability level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Pain behavior

No deterioration in general status was observed, and no rats in all groups died during the course of the experiment.

Before the first oxaliplatin injection, there were no significant differences in withdrawal thresholds in all groups in the von Frey test (Fig. 1A). Oxaliplatin (4 mg/kg, i.p.) significantly reduced the withdrawal threshold compared with vehicle on Days 15, 19, 22, 26, 29, 33, 40 and 47 ($P < 0.01$). However, no significant difference in withdrawal threshold compared to the vehicle group was observed on Day 54. Repeated administration of neurotropin (200 NU/kg) significantly inhibited the oxaliplatin-induced reduction of the withdrawal threshold on Days 15, 19, 22, 26, 29, 33 and 40 ($P < 0.01$).

In the acetone test, there were no significant differences in number of withdrawal responses in all groups before the first oxaliplatin injection (Fig. 1B). Oxaliplatin significantly increased the number of withdrawal responses compared with vehicle on Days 5, 8, 12, 15, 19, 22, 26 and 29 ($P < 0.01$). However, no significant difference in withdrawal responses compared to the vehicle group was observed on Day 33. Repeated administration of neurotropin significantly inhibited the oxaliplatin-induced increase of the number of withdrawal responses on Day 5 ($P < 0.05$). However, neurotropin did not affect the oxaliplatin-induced increase of the number of withdrawal responses on Days 8, 12, 15, 19, 22, 26 and 29.

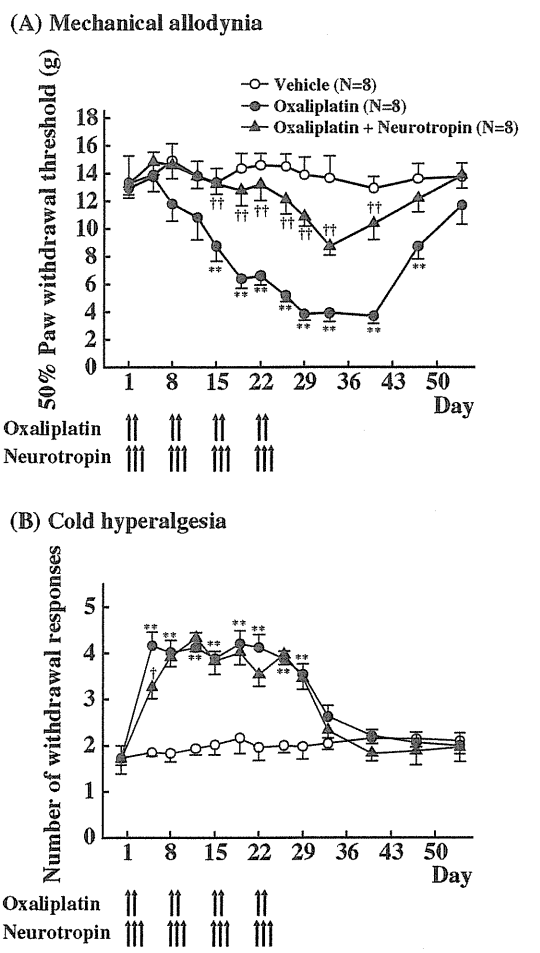

Fig. 1. Effect of neurotropin on oxaliplatin-induced mechanical allodynia in von Frey test (A) and cold hyperalgesia in acetone test (B) in rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks. Neurotropin (200 NU/kg) was administered p.o. 3 times a week for 4 weeks. Both tests were performed before the first drug administration and on Days 5, 8, 12, 15, 19, 22, 26, 29, 33, 40, 47 and 54. Values are expressed as the mean ± SEM. $^{**}P < 0.01$ compared with vehicle. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with oxaliplatin alone. The number of animals is shown in each parenthesis.

3.2. Rat sciatic nerve and DRG neurons

No histological abnormalities in sciatic nerve were observed in vehicle-, oxaliplatin- or sodium oxalate-treated rats on Day 5 (Fig. 2). Oxaliplatin (4 mg/kg, i.p.) caused the decrease in the density of myelinated fibers and the degeneration of myelinated fibers in rat sciatic nerve on Day 25. These histological changes were not observed in the tissue of rat treated with co-administration of oxaliplatin and neurotropin.

In DRG neurons, TUNEL-positive cells were hardly observed in the vehicle-, oxaliplatin- or sodium oxalate-treated rats on Day 5 (Fig. 3). Also, TUNEL-positive cells were hardly observed in the vehicle-treated rats on Day 25. Oxaliplatin markedly increased the TUNEL-positive cells on Day 25. Neurotropin had no effect on the oxaliplatin-induced increase of the TUNEL-positive cells.

3.3. Cultured PC12 and DRG cells

The exposure to oxaliplatin (0.3–30 μM) for 168 h dose-dependently shortened the length of neurites in cultured PC12 cells (0.3 μM, $P < 0.05$; 1–30 μM, $P < 0.01$, Fig. 4A). On the other hand, the exposure to sodium oxalate (0.3–30 μM) for 168 h had no effect on the length of neurites (Fig. 4B). The exposure to

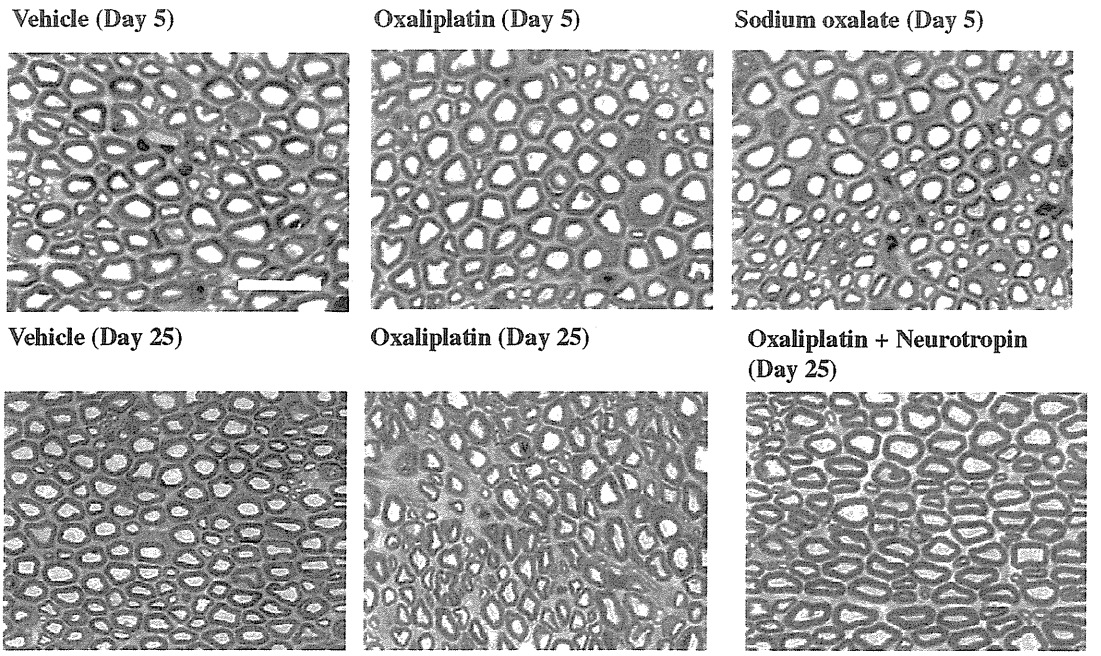

Fig. 2. Effect of neurotropin on histological change induced by oxaliplatin in rat sciatic nerve. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. Sodium oxalate (1.3 mg/kg, i.p.) was administered twice on Days 1 and 2. Neurotropin (200 NU/kg, p.o.) was administered 3 times a week for 4 weeks. On Day 5 or 25, the sciatic nerve was harvested, and samples were stained with toluidine blue. Photographs were originally magnified 1600×. Scale bar 20 μm.

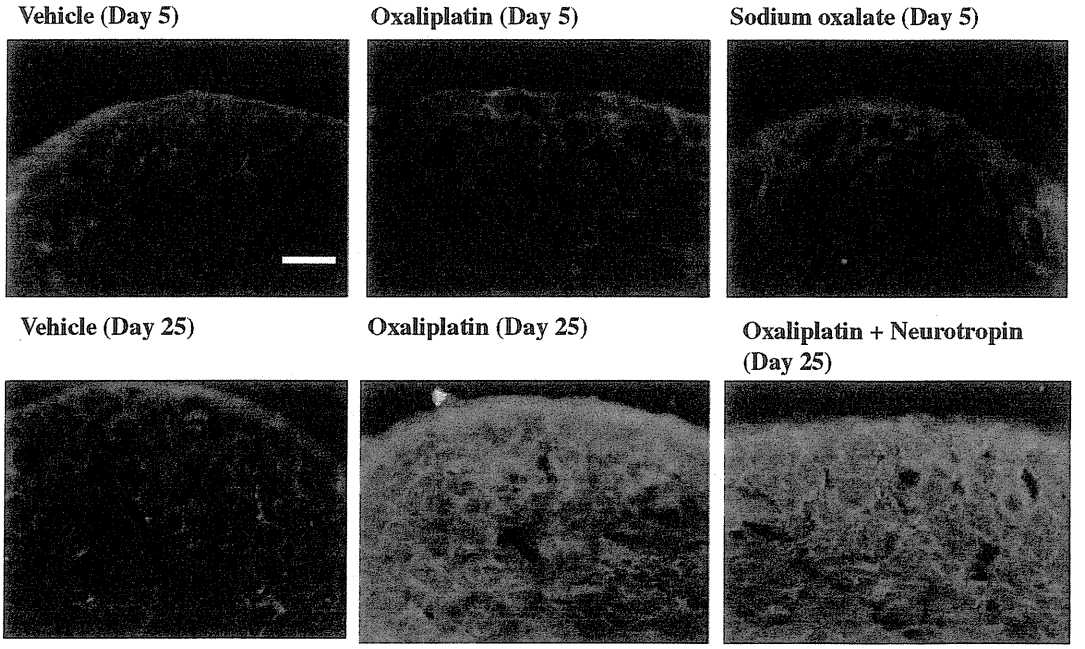

Fig. 3. Effect of neurotropin on cell injury induced by oxaliplatin in DRG neurons. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. Sodium oxalate (1.3 mg/kg, i.p.) was administered twice on Days 1 and 2. Neurotropin (200 NU/kg, p.o.) was administered three times a week for 4 weeks. On Day 5 or 25, the L5 DRG was harvested, and samples were stained terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. Photographs were originally magnified 400×. Scale bar 50 μm.

oxaliplatin (3 μM) for 168 h also significantly shortened the length of neurites in DRG cells ($P < 0.01$, Fig. 5B). The co-exposure to neurotropin (0.001–0.03 NU/mL) for 168 h significantly extended the length of neurites compared with the oxaliplatin-treated group in the dose-dependent manner in both cultured PC12 and DRG cells (PC12: 0.003, 0.01 and 0.03 NU/mL, $P < 0.01$; DRG: 0.01 and 0.03 NU/mL, $P < 0.01$, Fig. 5).

The exposure to oxaliplatin (3–30 μM) for 168 h dose-dependently decreased the cell viability in DRG cells (3 μM, $P < 0.05$; 10, 30 μM, $P < 0.01$, Fig. 6A). On the other hand, the exposure to sodium oxalate (0.3–30 μM) for 168 h had no effect on the cell viability (Fig. 6B). The co-exposures to neurotropin (0.001–0.03 NU/mL) for 168 h had no effect on the oxaliplatin-induced decrease of the cell viability (Fig. 7).

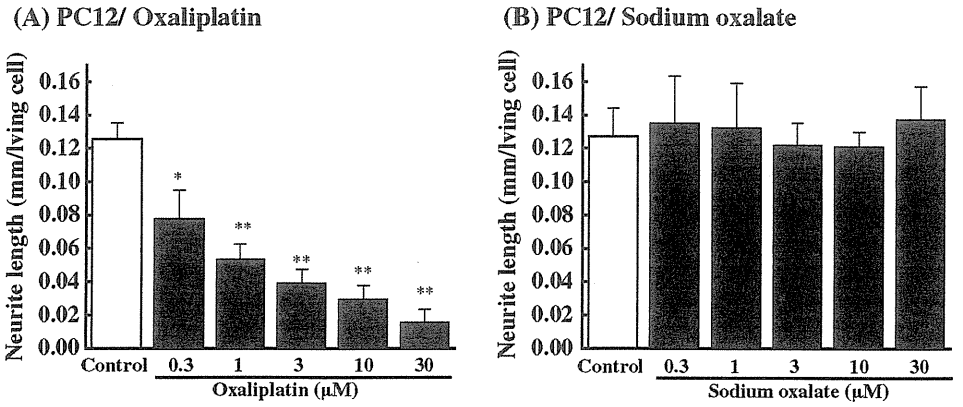

Fig. 4. Effects of oxaliplatin (A) and sodium oxalate (B) on neurite outgrowth in cultured PC12 cells. PC12 cells were incubated with oxaliplatin (0.3, 1, 3, 10 or 30 μM) or sodium oxalate (0.3, 1, 3, 10 or 30 μM) for 168 h. The neurite lengths were measured using image analysis software (Image J 1.36). Results are expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

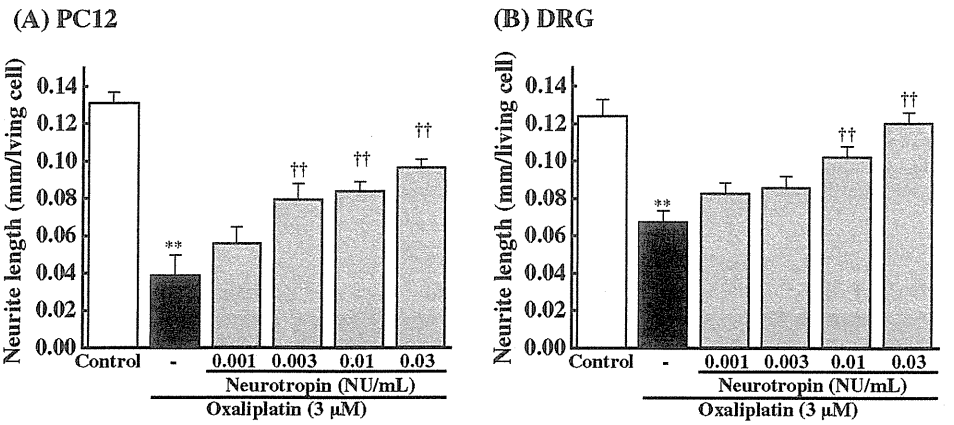

Fig. 5. Effect of neurotropin on neurite degeneration induced by oxaliplatin in cultured PC12 (A) and DRG (B) cells. Both cells were incubated with oxaliplatin (3 μM) for 168 h in the presence or absence of neurotropin (0.001–0.03 NU/mL). The neurite lengths were measured using image analysis software (Image J 1.36). Results are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ compared with control, $^{††}P < 0.01$ compared with oxaliplatin alone.

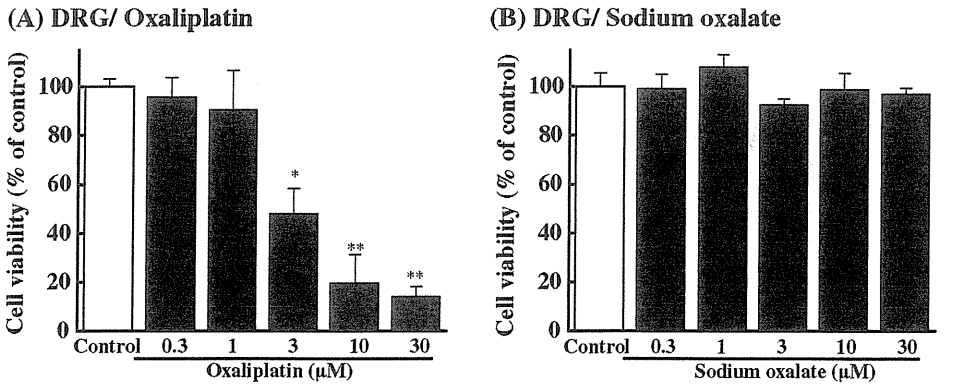

Fig. 6. Effects of oxaliplatin (A) and sodium oxalate (B) on cell viability in cultured DRG cells. DRG cells were incubated with oxaliplatin (0.3, 1, 3, 10 or 30 μM) or sodium oxalate (0.3, 1, 3, 10 or 30 μM) for 168 h. The cell viabilities were measured using WST-8 assay. Results are expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

4. Discussion

In the present study, oxaliplatin caused cold hyperalgesia from the early phase and mechanical allodynia in the late phase, consistently with our previous report (Sakurai et al., 2009). These oxaliplatin-induced neuropathies were gradually recovered after the end of oxaliplatin administration. Clinically, the reversibility of sensory neurotoxicity is observed in patients treated with oxaliplatin (de Gramont et al., 2000). Moreover, in this study, the repeated administration of neurotropin relieved the oxaliplatin-induced mechanical allodynia in the von Frey test. On the other hand, neurotropin was almost ineffective against the oxaliplatin-induced cold hyperalgesia in the acetone test. Therefore, neurotropin may be useful for treatment of the oxaliplatin-induced chronic peripheral neuropathy.

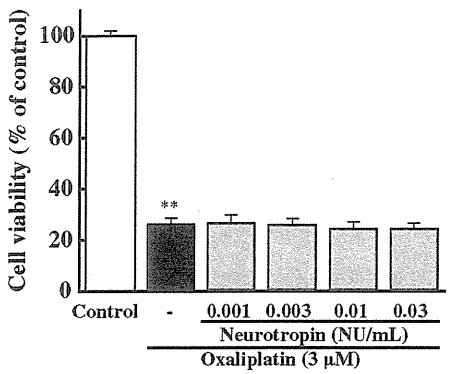

Fig. 7. Effect of neurotropin on cell injury induced by oxaliplatin in cultured DRG cells. The DRG cells were incubated with oxaliplatin (3 μM) for 168 h in the presence or absence of neurotropin (0.001–0.03 NU/mL). The cell viabilities were measured using WST-8 assay. Results are expressed as the mean ± SEM ($n = 8$). $^{**}P < 0.01$ compared with control.

In the present study, no histological abnormalities in sciatic nerve and TUNEL-positive apoptotic cell in L5 DRG were observed in oxaliplatin- or oxalate-treated rats on Day 5, although oxaliplatin caused cold hyperalgesia in the acetone test on that day. We previously reported that the oxaliplatin- and oxalate-induced hyperalgesia are completely prevented by the pre-administration of Ca^{2+} and Mg^{2+} before oxaliplatin or oxalate (Sakurai et al., 2009). Oxalate is well known as a chelator of both Ca^{2+} and Mg^{2+}. Some in vitro studies demonstrated that oxaliplatin modulates the voltage-gated Na^{+} and K^{+} channels (Adelsberger et al., 2000; Benoit et al., 2006; Kagiava et al., 2008) and that oxalate blockes the voltage-gated Na^{+} channel (Grolleau et al., 2001). Moreover, the effect of oxaliplatin on these channels has been thought to be involved in acute neuropathy (Boughattas et al., 1994; Kiernan, 2007; Saif and Reardon, 2005). Taken together with these findings, it is unlikely that the axonal degeneration in rat sciatic nerve and cell injury in DRG cells are involved in the oxaliplatin-induced cold hyperalgesia. Rather the oxaliplatin-induced cold hyperalgesia may be due to chelating of Ca^{2+} and Mg^{2+} by oxalate.

Oxaliplatin (4 mg/kg, i.p.) caused the degeneration and the decrease in the density of myelinated fibers in rat sciatic nerve on Day 25. These histological changes were not observed in the tissue of rats treated with co-administration of oxaliplatin and neurotropin. In pain behavior, neurotropin relieved the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia. Therefore, the protective effect of neurotropin on the axonal degeneration may partially contribute to the relief of the oxaliplatin-induced mechanical allodynia.

On the other hand, neurotropin had no effect on the oxaliplatin-induced increase of the TUNEL-positive cells in rat DRG neurons on Day 25. The oxaliplatin-induced cell death is completely protected by z-VAD-fmk, a caspase inhibitor, in DRG neurons (Ta et al., 2006), indicating to the involvement of apoptosis. Hence, neurotropin cannot protect the cell injury including apoptosis. We previously reported that Pt(dach)Cl_2 induces the mechanical allodynia in the late phase, but does not induce the cold hyperalgesia/allodynia (Sakurai et al., 2009). Several symptoms of oxaliplatin-induced chronic neuropathy are similar to those of cisplatin-induced chronic neuropathy (Pasetto et al., 2006). Oxaliplatin causes the damage of the cell bodies (Cavaletti et al., 2001; McKeage et al., 2001; Scuteri et al., 2009), the alterations in nucleus (Cavaletti et al., 2001) and nucleolus (Holmes et al., 1998; McKeage et al., 2001) and selective atrophy of subpopulation of DRG neurons (Jamieson et al., 2005) in animal models. Similarly, other platinum drugs such as cisplatin cause the damage of the cell bodies and the alterations of nucleolus (Holmes et al., 1998; McKeage et al., 2001). Moreover, oxaliplatin induces the cell death (Donzelli et al., 2004; Luo et al., 1999; Ta et al., 2006) and the inhibition of neurite outgrowth (Luo et al., 1999; Ta et al., 2006) in neuronal cells. The neurotoxicity of oxaliplatin and cisplatin for the DRG neurons correlates with platinum–DNA bindings (Ta et al., 2006). Taken together, Pt(dach)Cl_2 may be involved in the mechanical allodynia but not cold hyperalgesia/allodynia in the oxaliplatin-induced neuropathy, and the oxaliplatin-induced mechanical allodynia may be due to the neurotoxicity of platinum. In the present study, neurotropin is ineffective against the oxaliplatin-induced cell injury in rat DRG neurons. Therefore, the preventive effect of neurotropin on the oxaliplatin-induced mechanical allodynia might be not complete.

Ta et al. reported that oxaliplatin inhibits the neurite outgrowth and decreases the cell survival, whilst sodium oxalate does not affect the neurite outgrowth and cell survival in DRG neurons (2006). In the present study oxaliplatin shortened the length of neurites in both cultured PC12 and DRG cells and decreased the cell viability in DRG cells, whereas sodium oxalate did not affect the neurite length in PC12 cells. Hence, it is unlikely that oxalate is involved in the neurodegeneration induced by oxaliplatin. Moreover, we found that neurotropin reversed the oxaliplatin-induced neurite shortening in both cultured PC12 and DRG cells. On the other hand, neurotropin had no effect on the oxaliplatin-induced decrease of the cell viability. These results suggest that neurotropin ameliorates the neurodegeneration but not cell death induced by oxaliplatin. This hypothesis is confirmed from histological results in the tissue of rats treated with co-administration of oxaliplatin and neurotropin.

Nerve injury causes neuropathic pain via many molecular signaling including over expression of NGF (Pezet and McMahon, 2006), ion channels as voltage-gated sodium channels (Dib-Hajj et al. 2009), calcium channel alpha 2 delta subunit (Luo et al. 2001), and activation of microglia via P2X4 (Inoue and Tsuda, 2009; Tsuda et al. 2003). Especially, NGF is known to be involved in the release and upregulation of neurotransmitters such as substance P, calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor (BDNF), and sensitization and/or upreguration of ion channels and receptors including transient receptor potential cation channel V1 (TRPV1), voltage-gated sodium channel (Nav1.8/SCN10A), P2X3, acid-sensing ion channel 3 (ASIC3) (Watson et al., 2008). These molecular mechanisms might be involved in the oxaliplatin-induced mechanical allodynia.

On Day 5, neurotropin significantly attenuated the oxaliplatin-induced cold hyperalgesia in the acetone test. Previous reports indicated single administration of neurotropin has a transient analgesic effect via activation of descending pain inhibitory system in animal pain models (Kawamura et al., 1998; Okazaki et al., 2008). Therefore, this effect might be involved in the anti-cold hyperalgesia action by neurotropin on Day 5.

In conclusion, the present results suggest that repeated administration of neurotropin relieves the oxaliplatin-induced mechanical allodynia by inhibiting the axonal degeneration. Therefore, neurotropin may be useful for the treatment of oxaliplatin-induced neuropathy.

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References

Adelsberger H, Quasthoff S, Grosskreutz J, Lepier A, Eckel F, Lersch C. The chemotherapeutic oxaliplatin alters voltage-gated Na^+ channel kinetics on rat sensory neurons. Eur J Pharmacol 2000;406:25–32.

Benoit E, Brienza S, Dubois JM. Oxaliplatin, an anticancer agent that affects both Na^+ and K^+ channels in frog peripheral myelinated axons. Gen Physiol Biophys 2006;25:263–76.

Boughattas NA, Hecquet B, Fournier C, Bruguerolle B, Trabelsi H, Bouzouita K, et al. Comparative pharmacokinetics of oxaliplatin (L-OHP) and carboplatin (CBDCA) in mice with reference to circadian dosing time. Biopharm Drug Dispos 1994;15:761–73.

Cassidy J, Misset JL. Oxaliplatin-related side effects: characteristics and management. Semin Oncol 2002;29:11–20.

Cavaletti G, Tredici G, Petruccioli MG, Donde E, Tredici P, Marmiroli P, et al. Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat. Eur J Cancer 2001;37:2457–63.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994;53:55–63.

de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 2000;18:2938–47.

de Gramont A, Vignoud J, Tournigand C. Oxalipaltin/5FU/LV in adjuvant colon cancer: safety results of the international randomized MOSAIC trial. Proc Am Soc Clin Oncol 2002;21:132.

Dib-Hajj SD, Black JA, Waxman SG. Voltage-gated sodium channels: therapeutic targets for pain. Pain Med 2009;10:1260–9.

Donzelli E, Carfì M, Miloso M, Strada A, Galbiati S, Bayssas M, et al. Neurotoxicity of platinum compounds: comparison of the effects of cisplatin and oxaliplatin on the human neuroblastoma cell line SH-SY5Y. J Neurooncol 2004;67:65–73.

Extra JM, Marty M, Brienza S, Misset JL. Pharmacokinetics and safety profile of oxaliplatin. Semin Oncol 1998;25:13–22.

Flatters SJ, Bennett GJ. Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. Pain 2004;109:150–61.

Graham MA, Lockwood GF, Greenslade D, Brienza S, Bayssas M, Gamelin E. Clinical pharmacokinetics of oxaliplatin: a critical review. Clin Cancer Res 2000;6:1205–18.

Grolleau F, Gamelin L, Boisdron-Celle M, Lapied B, Pelhate M, Gamelin E. A possible explanation for a neurotoxic effect of the anticancer agent oxaliplatin on neuronal voltage-gated sodium channels. J Neurophysiol 2001;85:2293–7.

Holmes J, Stanko J, Varchenko M, Ding H, Madden VJ, Bagnell CR, et al. Comparative neurotoxicity of oxaliplatin, cisplatin, and ormaplatin in a Wistar rat model. Toxicol Sci 1998;46:342–51.

Inoue K, Tsuda M. Microglia and neuropathic pain. Glia 2009;57:1469–79.

Jamieson SM, Liu J, Connor B, McKeage MJ. Oxaliplatin causes selective atrophy of a subpopulation of dorsal root ganglion neurons without inducing cell loss. Cancer Chemother Pharmacol 2005;56:391–9.

Kagiava A, Tsingotjidou A, Emmanouilides C, Theophilidis G. The effects of oxaliplatin, an anticancer drug, on potassium channels of the peripheral myelinated nerve fibres of the adult rat. Neurotoxicology 2008;29:1100–6.

Kawamura M, Ohara H, Go K, Koga Y, Ienaga K. Neurotropin induces antinociceptive effect by enhancing descending pain inhibitory systems involving 5-HT3 and noradrenergic alpha2 receptors in spinal dorsal horn. Life Sci 1998;62:2181–90.

Kawashiri T, Egashira N, Itoh Y, Shimazoe T, Ikegami Y, Yano T, et al. Neurotropin reverses paclitaxel-induced neuropathy without affecting anti-tumour efficacy. Eur J Cancer 2009;45:154–63.

Kiernan MC. The pain with platinum: oxaliplatin and neuropathy. Eur J Cancer 2007;43:2631–3.

Ling B, Authier N, Balayssac D, Eschalier A, Coudore F. Behavioral and pharmacological description of oxaliplatin-induced painful neuropathy in rat. Pain 2007;128:225–34.

Luo FR, Wyrick SD, Chaney SG. Comparative neurotoxicity of oxaliplatin, ormaplatin, and their biotransformation products utilizing a rat dorsal root ganglia in vitro explant culture model. Cancer Chemother Pharmacol 1999;44:29–38.

Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, et al. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. J Neurosci 2001;21:1868–75.

McKeage MJ, Hsu T, Screnci D, Haddad G, Baguley BC. Nucleolar damage correlates with neurotoxicity induced by different platinum drugs. Br J Cancer 2001;85:1219–25.

Okazaki R, Namba H, Yoshida H, Okai H, Miura T, Kawamura M. The antiallodynic effect of neurotropin is mediated via activation of descending pain inhibitory systems in rats with spinal nerve ligation. Anesth Analg 2008;107:1064–9.

Pasetto LM, D'Andrea MR, Rossi E, Monfardini S. Oxaliplatin-related neurotoxicity: how and why? Crit Rev Oncol Hematol 2006;59:159–68.

Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. Annu Rev Neurosci 2006;29:507–38.

Pisano C, Pratesi G, Laccabue D, Zunino F, Lo Giudice P, Bellucci A, et al. Paclitaxel and cisplatin-induced neurotoxicity: a protective role of acetyl-L-carnitine. Clin Cancer Res 2003;9:5756–67.

Quasthoff S, Hartung HP. Chemotherapy-induced peripheral neuropathy. J Neurol 2002;249:9–17.

Saif MW, Reardon J. Management of oxaliplatin-induced peripheral neuropathy. Ther Clin Risk Manage 2005;1:249–58.

Sakurai M, Egashira N, Kawashiri T, Yano T, Ikesue H, Oishi R. Oxaliplatin-induced neuropathy in the rat: involvement of oxalate in cold hyperalgesia but not mechanical allodynia. Pain 2009;147:165–74.

Scuteri A, Galimberti A, Maggioni D, Ravasi M, Pasini S, Nicolini G, et al. Role of MAPKs in platinum-induced neuronal apoptosis. Neurotoxicology 2009;30:312–9.

Ta LE, Espeset L, Podratz J, Windebank AJ. Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum-DNA binding. Neurotoxicology 2006;27:992–1002.

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, et al. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 2003;424:778–83.

Watson JJ, Allen SJ, Dawbarn D. Targeting nerve growth factor in pain: what is the therapeutic potential? BioDrugs 2008;22:349–59.

Wilson RH, Lehky T, Thomas RR, Quinn MG, Floeter MK, Grem JL. Acute oxaliplatin-induced peripheral nerve hyperexcitability. J Clin Oncol 2002;20:1767–74.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.

Kawashiri et al. Molecular Pain 2012, 8:7
http://www.molecularpain.com/content/8/1/7

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L type Ca^{2+} channel blockers prevent oxaliplatin-induced cold hyperalgesia and TRPM8 overexpression in rats

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Abstract

Background: Oxaliplatin is an important drug used in the treatment of colorectal cancer. However, it frequently causes severe acute and chronic peripheral neuropathies. We recently reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats, and that oxalate derived from oxaliplatin is involved in the cold hyperalgesia. In the present study, we examined the effects of Ca^{2+} channel blockers on oxaliplatin-induced cold hyperalgesia in rats.

Methods: Cold hyperalgesia was assessed by the acetone test. Oxaliplatin (4 mg/kg), sodium oxalate (1.3 mg/kg) or vehicle was injected i.p. on days 1 and 2. Ca^{2+} (diltiazem, nifedipine and ethosuximide) and Na^{+} (mexiletine) channel blockers were administered p.o. simultaneously with oxaliplatin or oxalate on days 1 and 2.

Results: Oxaliplatin (4 mg/kg) induced cold hyperalgesia and increased in the transient receptor potential melastatin 8 (TRPM8) mRNA levels in the dorsal root ganglia (DRG). Furthermore, oxalate (1.3 mg/kg) significantly induced the increase in TRPM8 protein in the DRG. Treatment with oxaliplatin and oxalate (500 μM for each) also increased the TRPM8 mRNA levels and induced Ca^{2+} influx and nuclear factor of activated T-cell (NFAT) nuclear translocation in cultured DRG cells. These changes induced by oxalate were inhibited by nifedipine, diltiazem and mexiletine. Interestingly, co-administration with nifedipine, diltiazem or mexiletine prevented the oxaliplatin-induced cold hyperalgesia and increase in the TRPM8 mRNA levels in the DRG.

Conclusions: These data suggest that the L type Ca^{2+} channels/NFAT/TRPM8 pathway is a downstream mediator for oxaliplatin-induced cold hyperalgesia, and that Ca^{2+} channel blockers have prophylactic potential for acute neuropathy.

Background

Oxaliplatin, a platinum-based chemotherapeutic agent, is widely used for treatment of colorectal cancer. However, oxaliplatin frequently causes severe acute and chronic peripheral neuropathies. Acute neuropathy is peculiar to oxaliplatin and includes acral paresthesias enhanced by exposure to cold [1-4]; the acute neuropathy is not attributed to morphological damage to the nerve [5,6]. On the other hand, the chronic neuropathy is characterized by loss of sensory and motor function after long-term oxaliplatin treatment, and it is similar to cisplatin-induced neurological symptoms [4]. Recently, we reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats [7]. Oxaliplatin is metabolized to oxalate and dichloro (1,2-diaminocyclohexane)platinum [Pt(dach)Cl_2] [8]. We reported that oxalate and platinum metabolites were involved in the cold hyperalgesia and mechanical allodynia, respectively, and that intravenous pre-administration of Ca^{2+} or Mg^{2+} prevented the cold hyperalgesia but not mechanical allodynia in rats [7].

Oxaliplatin-induced acute neuropathy is termed a 'channelopathy', as oxaliplatin and oxalate were shown to modulate voltage-gated Na^{+} and K^{+} channels in

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several types of neurons [9-12]. For example, oxaliplatin increases the amplitude and duration of compound action potentials interacting with voltage-gated Na^+ channels in rat sensory neurons [9]. Furthermore, oxaliplatin prolongs the duration of the A-fiber compound action potential related to K^+ channels [12]. Thus, the effect of oxaliplatin on Na^+ and K^+ channels is thought to be involved in acute neuropathy [13].

Transient receptor potential (TRP) melastatin 8 (TRPM8), an ion channel that belongs to the TRP family, is activated by innocuous cold (< 25°C) or menthol [14,15]. Recently, an increase in TRPM8 mRNA levels was reported to be involved in the oxaliplatin-induced cold hyperalgesia in mice [16]. However, the molecular mechanisms mediating the acute neuropathy remain unclear. In the present study, we investigated the involvement of voltage-gated Ca^{2+} channels, nuclear factor of activated T-cell (NFAT) and TRPM8 in the oxaliplatin-induced cold hyperalgesia, as up-regulation of TRP channel 1 (TRPC1) expression is induced by store-operated calcium (SOC) channel/NFAT, a transcription factor regulated by the calcium signaling pathway [17]. Furthermore, we investigated the effects of Ca^{2+} channel blockers on oxaliplatin-induced cold hyperalgesia in rats.

Methods

Drugs and chemicals

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan). Sodium oxalate was provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mexiletine hydrochloride, nifedipine, diltiazem hydrochloride and ethosuximide were purchased from Sigma-Aldrich, Co. (MO, USA). Vivit was purchased from Calbiochem (Darmstadt, Germany).

Animals

Male Sprague-Dawley rats weighing 200-250 g (Kyudo Co., Saga, Japan) were used. Rats were housed in groups of four to five per cage with lights on from 07:00 to 19:00 h. Animals had free access to food and water in their home cages. All experiments were approved by the Experimental Animal Care and Use Committee of Kyushu University according to the National Institutes of Health guidelines, and we followed the International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research [18].

Behavioral test

Cold hyperalgesia was assessed by the acetone test. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor, and were allowed to habituate for 30 min prior to testing. Fifty microliters of acetone (Wako Pure Chemical Industries, Ltd.) was sprayed onto the plantar skin of each hind paw 3 times with a Micro Sprayer® (Penn Century Inc., PA, USA), and the number of withdrawal response was counted for 40 s from the start of the acetone spray. Acetone tests were performed on days 0 (pre), 3, 5, 8 and 15. Oxaliplatin and sodium oxalate were dissolved in 5% glucose solution. Oxaliplatin (4 mg/kg), sodium oxalate (1.3 mg/kg) or vehicle was injected i.p. on days 1 and 2. The vehicle-treated rats were injected with 5% glucose solution. Mexiletine hydrochloride, diltiazem and ethosuximide were dissolved in 5% saline. Nifedipine was suspended in 1% carboxymethylcellulose sodium solution. Mexiletine, diltiazem, nifedipine and ethosuximide were administered p.o. simultaneously with oxaliplatin or oxalate on days 1 and 2.

Cell cultures

Male Sprague-Dawley rats (6 weeks old, Kyudo Co.) were anesthetized with sodium pentobarbital, and the L 4-6 dorsal root ganglia (DRG) cells were removed and primary cultured. Ganglia were incubated with 0.125% (w/v) collagenase type 1 (Worthington Biochemical Corp., NJ, USA) at 37°C for 90 min followed by incubation with 0.25% (w/v) trypsin-EDTA (Gibco BRL, CA, USA) for 30 min. DRG cells were grown in Dulbecco's modified Eagle's medium (MP Biomedicals, Inc., CA, USA) with 2 mM L-glutamine and 10% FBS. The cells were cultured at 37°C in air supplemented with 5% CO_2 under humidified conditions. Oxaliplatin, oxalate, mexiletine, diltiazem and ethosuximide were dissolved in medium. Nifedipine and vivit were dissolved in 0.2% DMSO.

Measurement of intracellular Ca^{2+} level

Cells were loaded with 5 μg/mL of Fura-2/AM (Dojindo Lab., Kumamoto, Japan) and then incubated for 1 h at 37°C in HEPES buffer. The Fura-2/AM-loaded cells were washed and placed in HEPES buffer. The intracellular Ca^{2+} levels were determined by emission fluorescence at 510 nm with excitation at 340 nm and 380 nm, using FlexStation3 (Molecular Devices, Inc., CA, USA).

Immunostaining of NFATc4

Immunofluorescent staining for NFATc4 was performed using a rabbit monoclonal antibody (Cell Signaling Technology, Inc., MA, USA). Briefly, cells were cultured on cover slips, and the cover slips then rinsed with ice-cold phosphate-buffered saline and fixed with 4% (w/v) ice-cold paraformaldehyde for 30 min at -20°C. The NFATc4 antibody was diluted (1:100) with phosphate-buffered saline (PBS) containing 5% (w/v) bovine serum albumin and 0.1% Triton X-100. Cells were incubated with diluted antibody solution overnight in a humidified

chamber at 4°C. After washing with PBS, cover slips were incubated at room temperature for 1 h with goat anti-rabbit IgG (1:500 dilution in PBS) that was conjugated with Alexa Fluor® 488 (Cell Signaling Technology, Inc.). The nucleus was stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo Lab.). NFATc4 and nuclear staining were visualized with a fluorescence microscope (BX51; Olympus Corp., Tokyo, Japan). The nuclear translocation of NFATc4 was calculated by comparing the ratio of nuclear NFATc4 immunofluorescence/total NFATc4 immunofluorescence using analysis software (Image J 1.36; Wayne Rasband, National Institutes of Health, MD, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA was isolated from L4-6 DRG using PolyATtract® System 1000 (Promega, Corp., WI, USA). cDNA was synthesized with PrimeScript® 1st strand cDNA Synthesis Kit (TaKaRa Bio, Inc., Shiga, Japan). PCR was performed with Gene Taq (Nippon Gene, Co., Ltd., Tokyo, Japan). The oligonucleotide primers for TRPM8 were designed based on the sequences described by Ta and colleagues [19]. The sequences of PCR primers were as follows: TRPM8, 5'-GCC CAG TGA TGT GGA CAG TA-3' (sense), 5'-GGA CTC ATT TCC CGA GAA GG-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-YGC CTG CTT CAC CAC CTT-3' (sense), 5'-TGC MTC CTG CAC CAC CAA CT-3' (antisense) (Sigma-Aldrich, Co.). Reactions were run for 35 cycles with 95°C denaturing cycle (30 s), 62°C annealing cycle (1 min) and 72°C extension cycle (20 s) for TRPM8, or for 30 cycles with 94°C denaturing cycle (45 s), 53°C annealing cycle (45 s) and 72°C extension cycle (1.5 min) for G3PDH. PCR products were resolved by electrophoresis on a 4% agarose gel, and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. The intensities of the PCR products were semi-quantified densitometrically using Alpha Imager 2200 (Cell Biosciences, Inc., California, USA).

Western blotting

The L4-6 DRG was quickly removed on day 5. The tissues were homogenized in a solubilization buffer containing 20 mM Tris-HCl (pH7.4, 2 mM EDTA, 0.5 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.32 M Sucrose, 2 mg/ml aprotinine, 2 mg/ml leupeptin), and the homogenates were subjected to 4% SDS-PAGE, and proteins were transferred electrophoretically to PVDF membranes. The membranes were blocked in Tris-buffered saline Tween-20 (TBST) containing 5% BSA (Sigma-Aldrich) for an additional 1 h at room temperature with agitation. The membrane was incubated overnight at 4°C with rabbit polyclonal TRPM8 antibody (Abcam, MA, USA) and then incubated for 1 h with anti-rabbit IgG horseradish peroxidase (Jackson Immuno Research Laboratories, Inc., PA, USA). The immunoreactivity was detected using Enhanced Chemiluminescence (Perkin Elmer, Massachusetts, USA).

Statistical analyses

Data are expressed as the mean ± SEM. Data were analyzed by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test to determine differences between the groups. A probability level of $p < 0.05$ was accepted as statistically significant.

Results

Oxaliplatin increases cold hyperalgesia and TRPM8 expression in the DRG in rats

Administration of oxaliplatin (4 mg/kg, p.o., on days 1 and 2) significantly increased the number of withdrawal responses to cold stimulation by acetone spray in rats (Figure 1A, days 3, 5 and 8: $p < 0.01$). This increase in withdrawal response had disappeared on day 15. On day 5, TRPM8 mRNA levels in the L4-6 DRG of oxaliplatin-treated rats markedly increased as compared with those of vehicle-treated rats (Figure 1B, $p < 0.01$). Also, oxalate treatment significantly induced the increase in TRPM8 protein in the L4-6 DRG (Figure 1C, $p < 0.05$).

Oxaliplatin and oxalate increase the TRPM8 mRNA levels in primary cultured DRG cells

Treatment with either oxaliplatin (Figure 1D) or oxalate (Figure 1E) for 12 h markedly increased the TRPM8 mRNA levels in primary cultured DRG cells ($p < 0.05$ for both).

Oxaliplatin and oxalate increase the intracellular Ca^{2+} levels in primary cultured DRG cells

Oxaliplatin and oxalate (100-500 μM) induced dose-dependent increases in intracellular Ca^{2+} levels in cultured DRG cells (Figure 2A, B). The percentages of DRG neurons that responded to oxaliplatin and oxalate were 69.2% and 64.0%, respectively. Nifedipine (30 μM), an L type Ca^{2+} channel blocker, and diltiazem (30 μM), an L/T type Ca^{2+} channel blocker, inhibited the increase in intracellular Ca^{2+} levels induced by oxalate (500 μM) (Figure 2C, D). Mexiletine, a Na^+ channel blocker, also dose-dependently inhibited the oxalate-induced increase in intracellular Ca^{2+} levels (Figure 2F). By contrast, ethosuximide (1 mM), a T type Ca^{2+} channel blocker, only weakly attenuated the oxalate-induced increase in intracellular Ca^{2+} levels (Figure 2E).

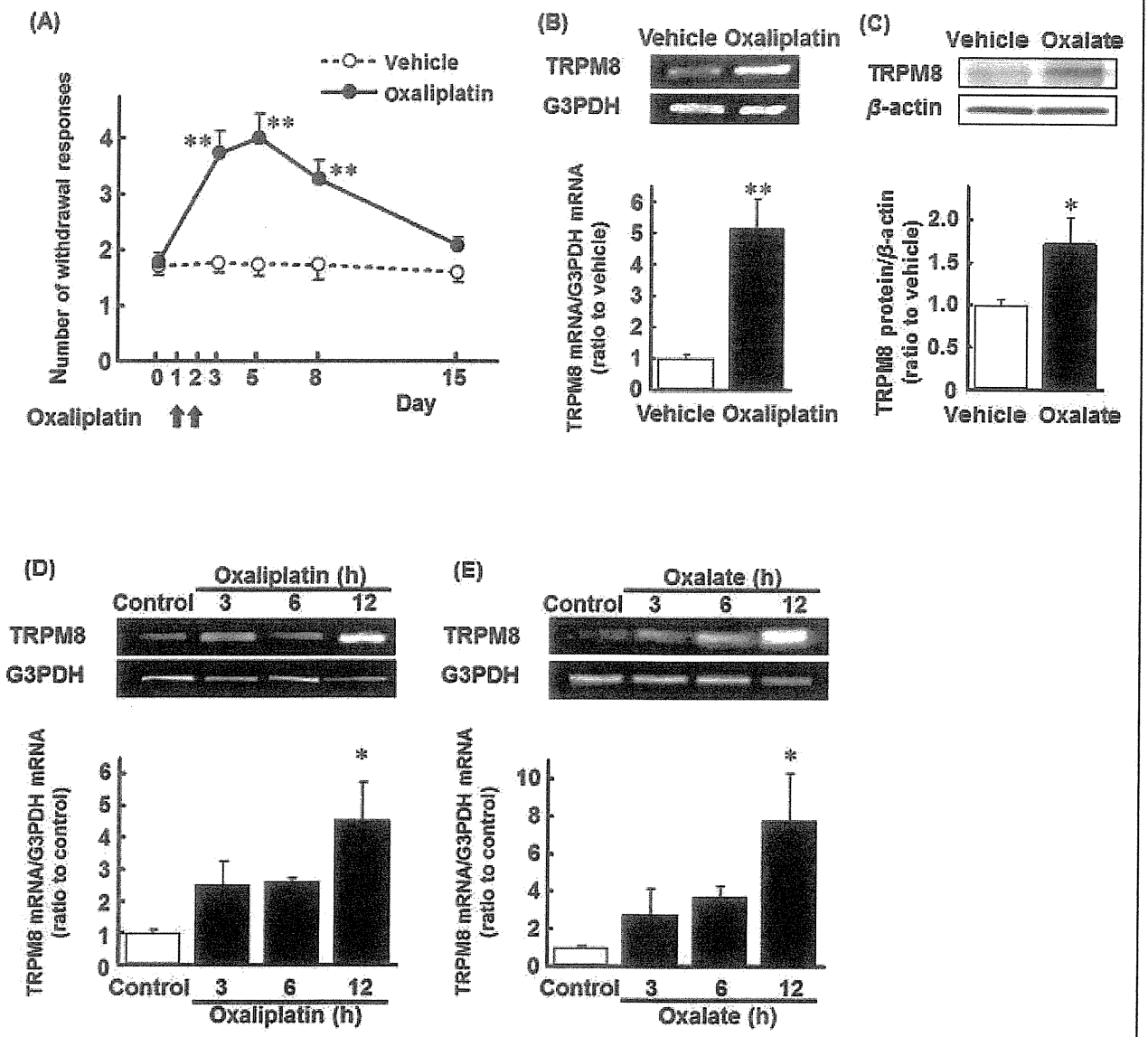

Figure 1 The incidence of cold hyperalgesia (A) and expression of TRPM8 (B-E) following oxaliplatin or sodium oxalate treatment. Oxaliplatin (4 mg/kg) or sodium oxalate (1.3 mg/kg) was administered i.p. on days 1 and 2. **A**: The acetone test was performed on days 0, 3, 5, 8 and 15. **B**: On day 5 the rat L4-6 DRG treated with oxaliplatin was harvested and the mRNA expression of TRPM8 and G3PDH were determined by PCR. **C**: On day 5 the rat L4-6 DRG treated with sodium oxalate was harvested and the protein of TRPM8 and β-actin were determined by Western boltting. **D, E**: 500 μM of oxaliplatin (D) or sodium oxalate (E) was administered to cultured DRG cells for 3, 6 or 12 h. mRNA expression of TRPM8 and G3PDH was determined by PCR. Values are expressed as the mean ± SEM of 4-6 animals (A, B) or 4-6 wells (C, D). $*p < 0.05$, $**p < 0.01$ compared with vehicle or control group.

Oxaliplatin and oxalate induce NFAT nuclear translocation in primary cultured DRG cells

Treatment with oxaliplatin (500 μM) for 6 h induced NFAT nuclear translocation (Figure 3A, B, 6 h: $p < 0.01$). Similarly, 500 μM oxalate caused NFAT nuclear translocation (Figure 3C, D, 6 h: $p < 0.01$). Mexiletine (1 mM), nifedipine (30 μM) and diltiazem (30 μM) completely blocked the oxalate-induced NFAT nuclear translocation (500 μM) (Figure 3E, F, $p < 0.01$). Similarly, vivit (2 μM), a selective NFAT inhibitor, completely blocked the oxalate-induced NFAT nuclear translocation (Figure 3E, F, $p < 0.01$).

Ca^{2+} and Na^{+} channel blockers inhibit the oxalate-induced increase of TRPM8 mRNA levels in cultured DRG cells

Mexiletine (1 mM), nifedipine (30 μM) and diltiazem (30 μM) reversed the increase in TRPM8 mRNA levels induced by oxalate (500 μM, 12 h) (Figure 4, mexiletine

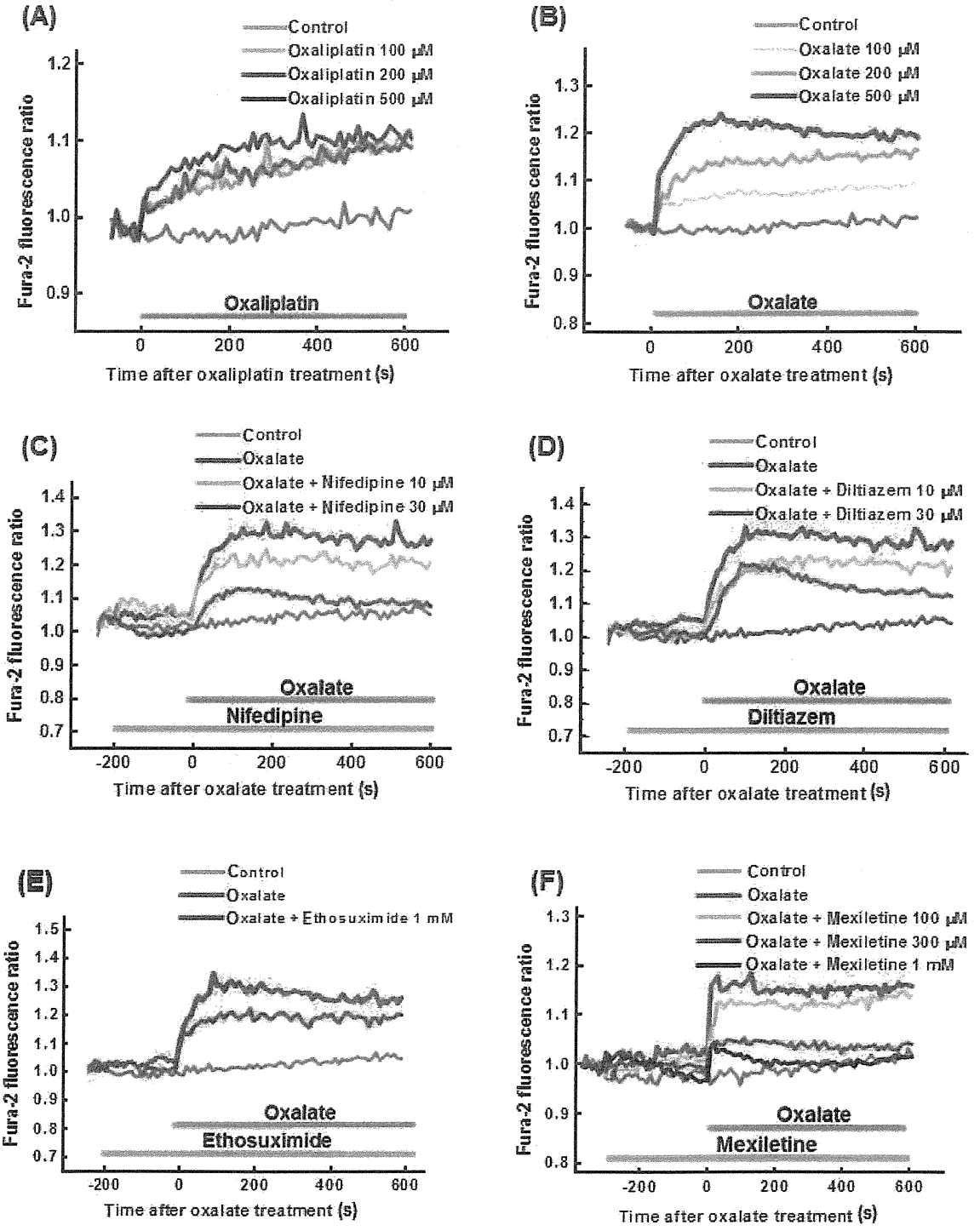

Figure 2 Increase in the intracellular Ca^{2+} following oxaliplatin or oxalate treatment in primary cultured DRG cells. Oxaliplatin (**A**: 100-500 μM) or sodium oxalate (**B**: 100-500 μM) was administered to cultured DRG cells. Nifedipine (**C**: 10-30 μM), diltiazem (**D**: 10-30 μM), ethosuximide (**E**: 1 mM) or mexiletine (**F**: 100 μ-1 mM) were co-administered with sodium oxalate (500 μM) to cells. Intracellular Ca^{2+} levels were determined based on Fura-2 fluorescence (340 nm/380 nm). Values are expressed as the mean of 4-8 wells.

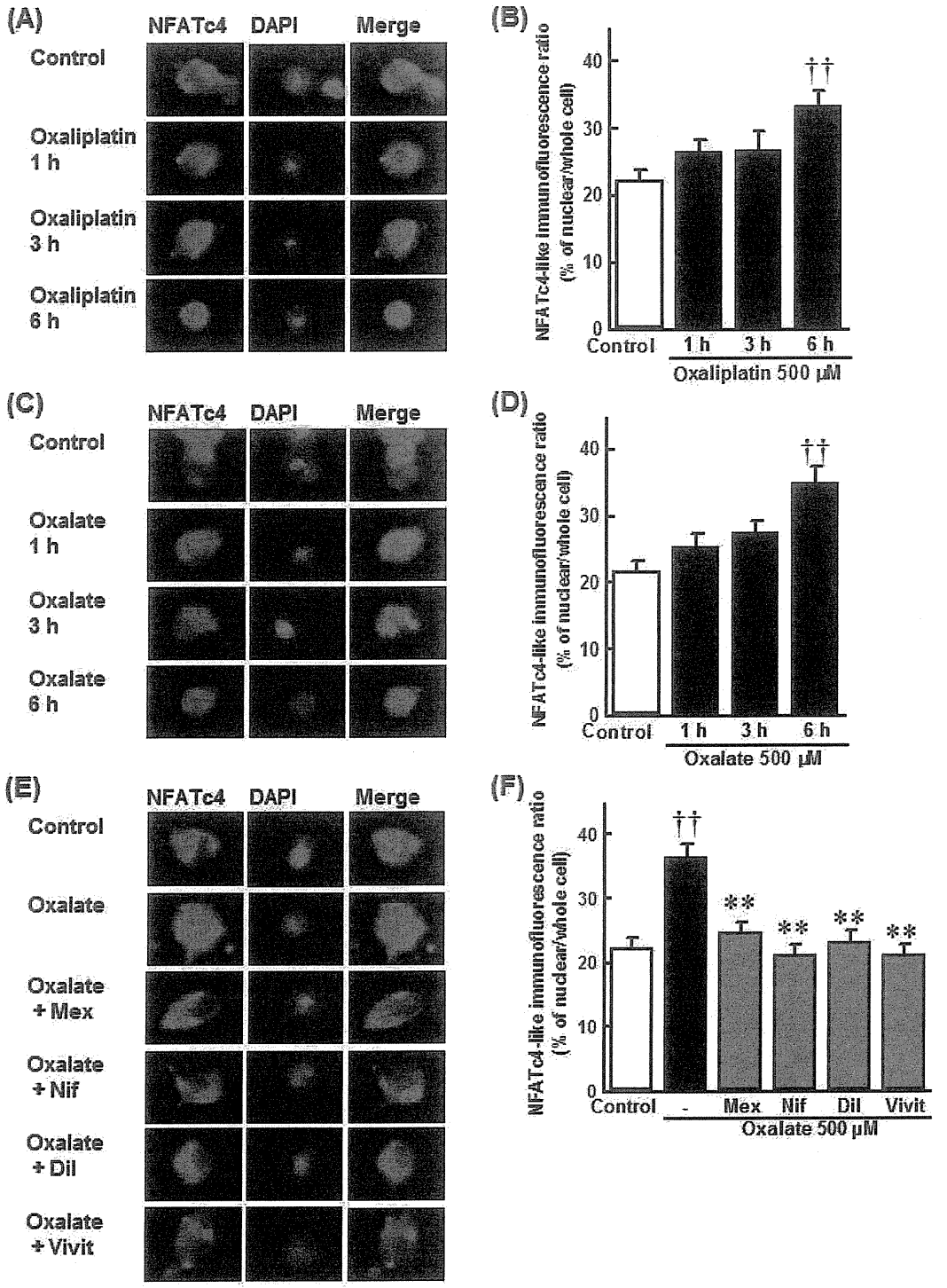

Figure 3 NFAT nuclear translocation in primary cultured DRG cells. Oxaliplatin (**A**, **B**: 500 μM for 1-6 h) or sodium oxalate (**C**, **D**: 500 μM for 1-6 h) was administered to cultured DRG cells. **E**, **F**: Mexiletine (Mex, 1 mM), nifedipine (Nif, 30 μM), diltiazem (Dil, 30 μM) or vivit (2 μM) was co-administered with sodium oxalate (500 μM) to cells for 6 h. NFATc4 immunostaining (green) and nuclear staining with DAPI (blue). NFATc4 and DAPI-positive nuclei were visualized by fluorescence microscopy (A, C, E). The nuclear translocation of NFATc4 was calculated by comparing the ratio of nuclear NFATc4 immunofluorescence/total NFATc4 immunofluorescence (B, D, F). Values are expressed as the mean ± SEM of 24-33 cells. ††$p < 0.01$ compared with control group, **$p < 0.01$ compared with oxalate group.

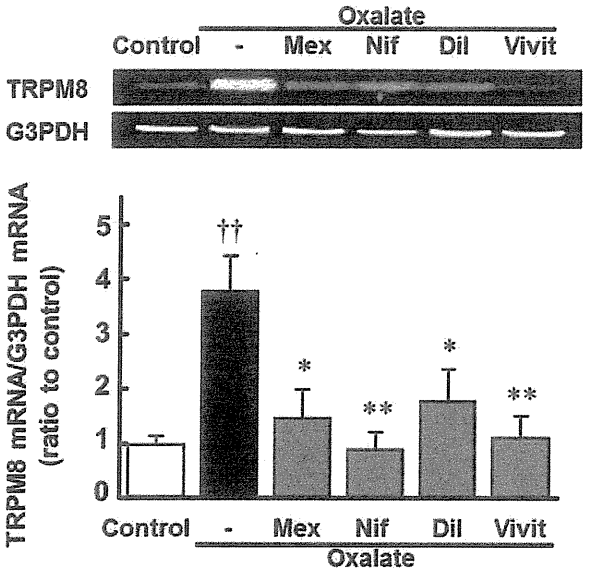

Figure 4 Reversal of the oxalate-induced increase in TRPM8 mRNA in cultured DRG cells by Ca^{2+} and Na^{+} channel blockers. Mexiletine (Mex, 1 mM), nifedipine (Nif, 30 μM), diltiazem (Dil, 30 μM) or vivit (2 μM) was co-administered with sodium oxalate (500 μM) to cells for 12 h. The mRNA expression of TRPM8 and G3PDH were determined by PCR. Values are expressed as the mean ± SEM of 6 wells. ††$p < 0.01$ compared with control group, *$p < 0.05$, **$p < 0.01$ compared with oxalate group.

and diltiazem: $p < 0.05$; nifedipine: $p < 0.01$). Similarly, vivit (2 μM) completely reversed the oxalate-induced increase in TRPM8 mRNA levels ($p < 0.01$).

Ca^{2+} and Na^{+} channel blockers inhibit the oxaliplatin-induced cold hyperalgesia and increase in TRPM8 mRNA levels in the DRG in rats

Co-administration with nifedipine (10, 30 mg/kg, p.o.) completely inhibited the oxaliplatin-induced increase in withdrawal responses to acetone spray in rats (Figure 5A, $p < 0.01$). Diltiazem (10, 30 mg/kg, p.o.) also strongly inhibited the oxaliplatin-induced increase in withdrawal responses (Figure 5B, $p < 0.01$). Similarly, mexiletine (10, 30 mg/kg, p.o.) attenuated the oxaliplatin-induced increase in withdrawal responses (Figure 5D, $p < 0.01$). By contrast, ethosuximide (300 mg/kg, p.o.) only weakly prevented the oxaliplatin-induced increase in withdrawal responses (Figure 5C, days 3 and 8: $p < 0.05$). Moreover, co-administration with mexiletine (30 mg/kg, p.o.), nifedipine (30 mg/kg, p.o.) or diltiazem (30 mg/kg, p.o.) completely inhibited the oxaliplatin-induced increase in TRPM8 mRNA levels on day 5 (Figure 6, $p < 0.01$).

Discussion

Oxaliplatin was previously reported to induce cold allodynia and increase in TRPM8 mRNA levels in the DRG after 3 days in mice [16] and increase the TRPM8 mRNA levels in cultured rat DRG cells [19]. Consistent with these reports, in the present study we demonstrated that oxaliplatin induced cold hyperalgesia in rats on days 3, 5 and 8 and increased the TRPM8 mRNA levels in the DRG on day 5, the peak of cold hyperalgesia. Furthermore, we found that oxalate significantly induced the increase in TRPM8 protein in the DRG on day 5. In addition, we confirmed that oxaliplatin markedly increased the TRPM8 mRNA levels in primary cultured DRG cells.

TRPM8 is known to be involved in cold sensitivity [20] and cold allodynia after chronic nerve injury [21]. Moreover, TRPM8-deficient mice attenuate behavioral response to cold stimulation [22,23]. Oxaliplatin-induced cold allodynia is reversed by capsazepine, a blocker of both TRPM8 and TRP vanilloid 1 (TRPV1), but not by 5'-iodoresiniferatoxin, a selective TRPV1 blocker [16]. Hence, the increase in TRPM8 expression in DRG neurons may be involved in oxaliplatin-induced cold hyperalgesia. Recently, Nassini et al. [24] have reported that oxaliplatin induces mechanical and cold allodynia via TRP ankyrin 1 (TRPA1) activation in rodents. Considering these collective findings, both up-regulation of TRPM8 and activation of TRPA1 may be involved in the cold hypersensitivity by oxaliplatin. We also found that treatment with oxalate, a metabolite of oxaliplatin, markedly increased the TRPM8 mRNA levels in primary cultured DRG cells. Furthermore, oxalate significantly induced the increase in TRPM8 protein in the DRG. Oxaliplatin is rapidly metabolized to $Pt(dach)Cl_2$ in rat blood *in vitro* [25], suggesting that oxalate is immediately derived from oxaliplatin. We previously reported that oxalate induced cold hyperalgesia/allodynia but not mechanical allodynia in rats [7]. Taken together, these data suggest that oxalate may be involved in the oxaliplatin-induced increase in TRPM8 expression, resulting in cold hyperalgesia.

In the present study, both oxaliplatin and oxalate increased the intracellular Ca^{2+} levels in primary cultured DRG cells, and the oxalate-induced increase in intracellular Ca^{2+} level was inhibited by nifedipine (an L type Ca^{2+} channel blocker) and diltiazem (an L/T type Ca^{2+} channel blocker). By contrast, ethosuximide (a T type Ca^{2+} channel blocker) only weakly attenuated the oxalate-induced increase in intracellular Ca^{2+}. Thus, it is likely that oxaliplatin induces Ca^{2+} influx via mainly L type Ca^{2+} channels. Oxaliplatin was reported to increase the amplitude and duration of compound action potentials interacting with voltage-gated Na^{+} channels in rat sensory neurons [9], and prolong the duration of the A-fiber compound action potential related to K^{+} channels [12]. Thus, enhancement of action potentials via Na^{+} or K^{+} channels might result in Ca^{2+} influx through L type Ca^{2+} channels. This mechanism is supported by the

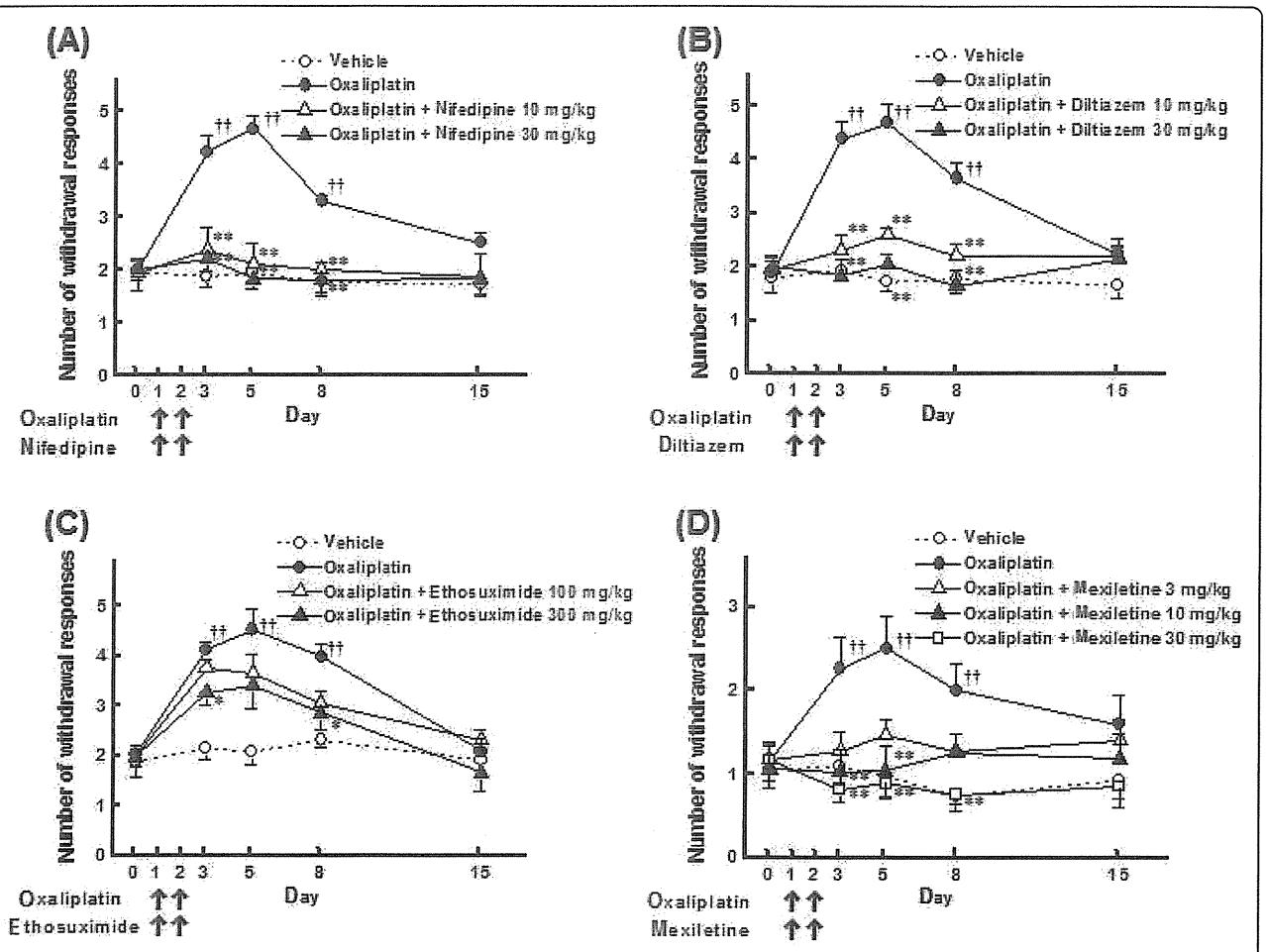

Figure 5 Reversal of the oxaliplatin-induced cold hyperalgesia in rats by Ca^{2+} and Na^{+} channel blockers. Oxaliplatin (4 mg/kg) was administered i.p. on days 1 and 2. Nifedipine (**A**: 10 and 30 mg/kg), diltiazem (**B**: 10 and 30 mg/kg), ethosuximide (**C**: 100 and 300 mg/kg) or mexiletine (**D**: 3-30 mg/kg) was orally co-administered with oxaliplatin. Acetone test was performed on days 0, 3, 5, 8 and 15. Values are expressed as the mean ± SEM of 6-10 animals. $\dagger\dagger p < 0.01$ compared with vehicle group, $*p < 0.05$, $**p < 0.01$ compared with oxaliplatin group.

present result that the Na^{+} channel blocker mexiletine completely reversed the oxalate-induced Ca^{2+} influx.

In general, NFAT is activated and translocated into the nucleus via Ca^{2+} signaling [26]. In the present study, both oxaliplatin and oxalate induced the nuclear translocation of NFAT in cultured DRG cells, and the oxalate-induced NFAT nuclear translocation was completely blocked by nifedipine, diltiazem and mexiletine, as well as vivit, a selective NFAT inhibitor. Furthermore, nifedipine, diltiazem, mexiletine and vivit reversed the oxalate-induced increase in TRPM8 mRNA levels in cultured DRG cells. Taken together, these data suggest that oxalate may induce up-regulation of TRPM8 expression via NFAT activation by Ca^{2+} influx through L/T type Ca^{2+} channels derived from Na^{+} channels activation. We also confirmed that co-administration with nifedipine, diltiazem or mexiletine inhibited the oxaliplatin-induced cold hyperalgesia and increase in TRPM8 mRNA levels in the DRG *in vivo* in rats. Thus, the oxaliplatin-induced cold hyperalgesia is mediated by up-regulation of TRPM8 expression via Na^{+} and Ca^{2+} influx.

In addition, Fajardo et al. [27] have reported that L-type Ca^{2+} channel blockers 1,4-dihydropyridines such as nifedipine activate TRPA1-mediated currents in CHO cells in electrophysiological study. However, they reported that no signs of behavioral pain were observed following local application of nifedipine to the hind paw of mice. Because nifedipine blocks electrically evoked Ca^{2+} transients in peripheral sensory nerves [28], it is possible that these potent inhibitory actions on L-type Ca^{2+} channels prevent the propagation of electrical impulses at nerve terminals, despite a powerful TRPA1 activation.

Conclusions

We demonstrated that L type Ca^{2+} channel/NFAT/TRPM8 pathway plays a crucial role in signaling the

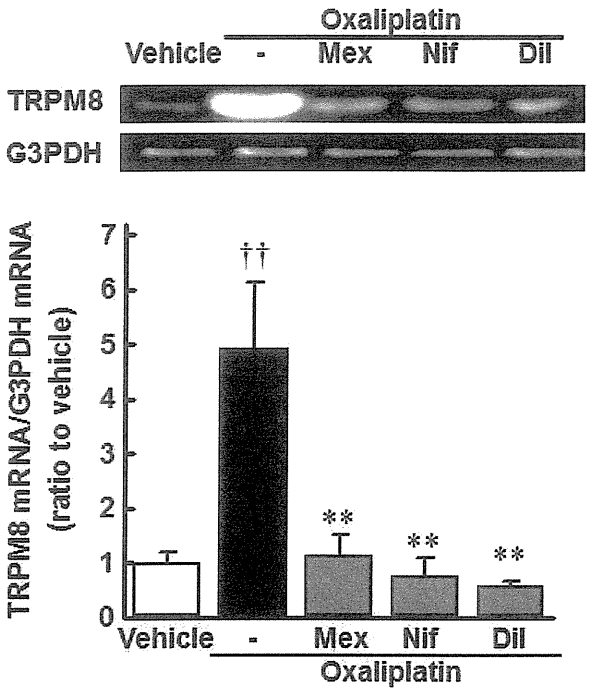

Figure 6 Reversal of the oxaliplatin-induced increase of TRPM8 mRNA in rat DRG neurons by Ca^{2+} and Na^{+} channel blockers. Oxaliplatin (4 mg/kg) was administered i.p. on days 1 and 2. Mexiletine (Mex, 30 mg/kg), nifedipine (Nif, 30 mg/kg) or diltiazem (Dil, 30 mg/kg) was orally co-administered with oxaliplatin. The expression of TRPM8 and G3PDH mRNAs were determined by PCR on day 5. Values are expressed as the mean ± SEM of 5 animals. $††p < 0.01$ compared with vehicle group, $**p < 0.01$ compared with oxaliplatin group.

oxaliplatin-induced cold hyperalgesia. Co-administration of L type Ca^{2+} channel blockers inhibited the oxaliplatin-induced cold hyperalgesia. Therefore, novel strategies involving Ca^{2+} channel blockers may be useful for prevention of oxaliplatin-induced acute neuropathy.

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Authors' contributions
TK, NE and RO are responsible for experimental design. TK and KK are responsible for performance of behavioral test. TK, KK, KT and YY are responsible for measurement of intracellular Ca^{2+} level, immunostaining and PCR. KT, SU and TY are responsible for performance of Western blotting. TK, NE and RO are responsible for writing the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References

1. Cassidy J, Misset JL: **Oxaliplatin-related side effects: characteristics and management.** *Semin Oncol* 2002, **29**:11-20.
2. Extra JM, Marty M, Brienza S, Misset JL: **Pharmacokinetics and safety profile of oxaliplatin.** *Semin Oncol* 1998, **25**:13-22.
3. Pasetto LM, D'Andrea MR, Rossi E, Monfardini S: **Oxaliplatin-related neurotoxicity: how and why?** *Crit Rev Oncol Hematol* 2006, **59**:159-168.
4. Quasthoff S, Hartung HP: **Chemotherapy-induced peripheral neuropathy.** *J Neurol* 2002, **249**:9-17.
5. de Gramont A, Vignoud J, Tournigand C: **Oxalipaltin/5FU/LV in adjuvant colon cancer: safety results of the international randomized MOSAIC trial.** *Proc Am Soc Clin Oncol* 2002, **21**:132.
6. Wilson RH, Lehky T, Thomas RR, Quinn MG, Floeter MK, Grem JL: **Acute oxaliplatin-induced peripheral nerve hyperexcitability.** *J Clin Oncol* 2002, **20**:1767-1774.
7. Sakurai M, Egashira N, Kawashiri T, Yano T, Ikesue H, Oishi R: **Oxaliplatin-induced neuropathy in the rat: involvement of oxalate in cold hyperalgesia but not mechanical allodynia.** *Pain* 2009, **147**:165-174.
8. Graham MA, Lockwood GF, Greenslade D, Brienza S, Bayssas M, Gamelin E: **Clinical pharmacokinetics of oxaliplatin: a critical review.** *Clin Cancer Res* 2000, **6**:1205-1218.
9. Adelsberger H, Quasthoff S, Grosskreutz J, Lepier A, Eckel F, Lersch C: **The chemotherapeutic oxaliplatin alters voltage-gated Na^{+} channel kinetics on rat sensory neurons.** *Eur J Pharmacol* 2000, **406**:25-32.
10. Grolleau F, Gamelin L, Boisdron-Celle M, Lapied B, Pelhate M, Gamelin E: **A possible explanation for a neurotoxic effect of the anticancer agent oxaliplatin on neuronal voltage-gated sodium channels.** *J Neurophysiol* 2001, **85**:2293-2297.
11. Kagiava A, Tsingotjidou A, Emmanouilides C, Theophilidis G: **The effects of oxaliplatin, an anticancer drug, on potassium channels of the peripheral myelinated nerve fibres of the adult rat.** *Neurotoxicology* 2008, **29**:1100-1106.
12. Sittl R, Carr RW, Fleckenstein J, Grafe P: **Enhancement of axonal potassium conductance reduces nerve hyperexcitability in an in vitro model of oxaliplatin-induced acute neuropathy.** *Neurotoxicology* 2010, **31**:694-700.
13. Saif MW, Reardon J: **Management of oxaliplatin-induced peripheral neuropathy.** *Ther Clin Risk Manage* 2005, 1:249-258.
14. McKemy DD, Neuhausser WM, Julius D: **Identification of a cold receptor reveals a general role for TRP channels in thermosensation.** *Nature* 2002, **416**:52-58.
15. Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A: **A TRP channel that senses cold stimuli and menthol.** *Cell* 2002, **108**:705-715.
16. Gauchan P, Andoh T, Kato A, Kuraishi Y: **Involvement of increased expression of transient receptor potential melastatin 8 in oxaliplatin-induced cold allodynia in mice.** *Neurosci Lett* 2009, **458**:93-95.
17. Wang C, Li JF, Zhao L, Liu J, Wan J, Wang YX, Wang J, Wang C: **Inhibition of SOC/Ca^{2+}/NFAT pathway is involved in the anti-proliferative effect of sildenafil on pulmonary artery smooth muscle cells.** *Respir Res* 2009, **10**:123.
18. Zimmermann M: **Ethical guidelines for investigations of experimental pain in conscious animals.** *Pain* 1983, **16**:109-110.
19. Ta LE, Bieber AJ, Carlton SM, Loprinzi CL, Low PA, Windebank AJ: **Transient Receptor Potential Vanilloid 1 is essential for cisplatin-induced heat hyperalgesia in mice.** *Mol Pain* 2010, **6**:15.
20. Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D: **The menthol receptor TRPM8 is the principal detector of environmental cold.** *Nature* 2007, **448**:204-208.
21. Xing H, Chen M, Ling J, Tan W, Gu JG: **TRPM8 mechanism of cold allodynia after chronic nerve injury.** *J Neurosci* 2007, **27**:13680-13690.
22. Colburn RW, Lubin ML, Stone DJ Jr, Wang Y, Lawrence D, D'Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N: **Attenuated cold sensitivity in TRPM8 null mice.** *Neuron* 2007, **54**:379-386.
23. Dhaka A, Murray AN, Mathur J, Earley TJ, Petrus MJ, Patapoutian A: **TRPM8 is required for cold sensation in mice.** *Neuron* 2007, **54**:371-378.
24. Nassini R, Gees M, Harrison S, De Siena G, Materazzi S, Moretto N, Failli P, Preti D, Marchetti N, Cavazzini A, Mancini F, Pedretti P, Nilius B, Patacchini R, Geppetti P: **Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation.** *Pain* 2011, **152**:1621-1631.
25. Luo FR, Wyrick SD, Chaney SG: **Biotransformations of oxaliplatin in rat blood in vitro.** *J Biochem Mol Toxicol* 1999, **13**:159-169.

26. Hogan PG, Chen L, Nardone J, Rao A: **Transcriptional regulation by calcium, calcineurin, and NFAT.** *Genes Dev* 2003, 17:2205-2232.
27. Fajardo O, Meseguer V, Belmonte C, Viana F: **TRPA1 channels: novel targets of 1,4-dihydropyridines.** *Channels (Austin)* 2008, 2:429-438.
28. Gover TD, Kao JP, Weinreich D: **Calcium signaling in single peripheral sensory nerve terminals.** *J Neurosci* 2003, 23:4793-4797.

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Clinical and Experimental Gastroenterology Dovepress

ORIGINAL RESEARCH

Rikkunshito, a traditional Japanese medicine, suppresses cisplatin-induced anorexia in humans

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Background: The aim of this study was to investigate the effects of Rikkunshito on ghrelin secretion and on cisplatin-induced anorexia in humans.

Methods: The study was performed as a crossover design, and ten unresectable or relapsed gastric cancer patients were randomly divided into two groups. Group A (n = 5) was started on Rikkunshito (2.5 g three times daily, orally) from the first course of chemotherapy and followed by a second course without Rikkunshito. A treatment with reversed order was performed for Group B (n = 5). All patients received combined chemotherapy with S-1 plus cisplatin. The primary endpoint was the amount of oral intake, and the categories of scales of anorexia, nausea, and vomiting; secondary endpoints included the plasma concentration of acylated ghrelin.

Results: In the Rikkunshito-on period, no decrease of the plasma concentration of acylated ghrelin induced by cisplatin was observed. The average oral intake in the Rikkunshito-on period was significantly larger than that in the Rikkunshito-off period, and the grade of anorexia was significantly lower in the Rikkunshito-on period than in the Rikkunshito-off period.

Conclusion: Rikkunshito appeared to prevent anorexia induced by cisplatin, resulting in effective prophylactic administration of chemotherapy with cisplatin, and patients could continue their treatments on schedule.

Keywords: Rikkunshito, cisplatin, ghrelin, anorexia, stomach cancer

Introduction

Combined chemotherapy with S-1 plus cisplatin is an attractive chemotherapy regimen for gastric cancer. A previous Phase I/II trial of this regimen in metastatic gastric cancer reported a high response rate of 76% and acceptable toxicity.[1] Recently, a Phase III Japanese trial of chemotherapeutic regimens for metastatic gastric cancer (SPIRITS trial) demonstrated that S-1 plus cisplatin led to significantly longer median overall survival than S-1 alone (13 months versus 11 months).[2] Cisplatin is widely used in various chemotherapies, but undesirable side effects, such as nausea, vomiting, and anorexia, markedly affect the quality of life of patients and may make the continuation of chemotherapy difficult. While some antiemetic agents have been introduced as treatment for nausea and vomiting,[3,4] appetite loss is still present in many cancer patients. However, the mechanism of the resulting appetite loss during chemotherapy is not thoroughly understood.

Ghrelin is an endogenous ligand of the growth hormone secretagogue receptor; it consists of 28 amino acids and is secreted mainly from the stomach.[5] Ghrelin is known to have an intense appetite-enhancing effect in addition to a growth hormone secretion-promoting effect.[6] Ghrelin is the only hormone that exhibits an orexigenic

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effect following peripheral administration.[7] In addition, ghrelin exhibits a variety of actions, including stimulation of growth hormone secretion, gastric motility, and gastric acid secretion,[8] as well as induction of a positive energy balance.[9] The level of plasma ghrelin is thought to be related to gastrointestinal disorders, and ghrelin has been administered to patients with anorexia-related disorders as a new therapy.[10] However, the intravenous and repeated administration of ghrelin presented a considerable burden for the patients.

Rikkunshito, a traditional Japanese medicine, is used to treat various gastrointestinal tract disorders, such as functional dyspepsia, gastroesophageal reflux, dyspeptic symptoms of post-gastrointestinal surgery, and chemotherapy-induced nausea.[11–13] Takeda et al[14] showed that a flavonoid in Rikkunshito suppressed a cisplatin-induced decrease in plasma acylated ghrelin levels and increased food intake in rats, and was mediated by serotonin (5-HT_{2B} and 5-HT_{2C}) receptors. The aim of this study was to investigate the effect of Rikkunshito on ghrelin secretion and on cisplatin-induced anorexia in humans.

Materials and methods

Patient eligibility

Eligible patients had histologically proved unresectable or recurrent gastric cancer. Up to one regimen of prior chemotherapy was allowed (adjuvant chemotherapy was allowed provided that at least 28 days had elapsed since the last treatment), except for prior treatment with cisplatin.

Other inclusion criteria were as follows: age 20–75 years; Eastern Clinical Oncology Group performance status 0–1; estimated life expectancy more than 3 months; a white blood cell count between 4000 and 12,000 mm^3; an absolute neutrophil count of over 2000 mm^3, a platelet count of over 100,000 mm^3, and a hemoglobin level of over 8.0 g/dL; aspartate aminotransferase and alanine aminotransferase levels within two times the upper limit of normal for the institution; serum bilirubin level less than 1.5 mg/dL; serum creatinine level within the upper limit of the normal value for the institution; 24-hour creatinine clearance more than 50 mL/minute; and a normal electrocardiogram. Only patients who could swallow tablets were eligible. Patients were excluded if they had brain metastases, severe comorbid conditions, active double cancers, or a past history of drug allergy or were unable to comply with the protocol requirements. Pregnant women were also excluded. Written informed consent was obtained from all patients before study entry.

Treatment design

The study was performed as a crossover design because of the limited number of patients (Figure 1). Ten patients were randomly divided into two groups. The gender, age, tumor characteristics, and performance status of these 10 patients are listed in Table 1. Group A (n = 5) was started on Rikkunshito from the first course of chemotherapy followed by a second course of chemotherapy without Rikkunshito. Treatment with reversed order was performed for Group B (n = 5). Patients in the intervention period took Rikkunshito (Tsumura Co, Ltd, Tokyo, Japan) at a daily dose of 7.5 g (2.5 g three times daily, orally) before every meal through chemotherapy from days 1 to 21. On the other hand, patients in the control period took nothing. All patients received combined chemotherapy with S-1 plus cisplatin as follows. S-1 (Taiho Pharmaceutical Co, Ltd, Tokyo, Japan) was given orally twice daily after meals at a fixed dose of 80 mg/m^2/day for 21 consecutive days followed by a 14-day rest period; this cycle was repeated every 5 weeks. The dose of S-1,

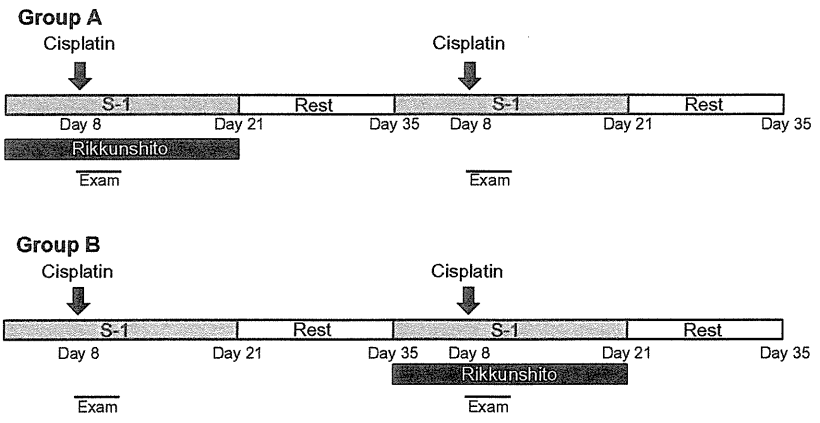

Figure 1 Crossover study design. Patients in group A initially took oral Rikkunshito before every meal for 3 weeks (on treatment). After a rest period of 2 weeks, Rikkunshito was discontinued for 3 weeks (off treatment). Conversely, patients in group B initially were off treatment for 3 weeks and then on treatment for 3 weeks after the rest period.

Table 1 Background of patients

Patient	Age (years)	Sex	Tumor	PS	Group
1	55	M	Recurrent	1	A
2	61	F	Unresectable	0	A
3	67	M	Recurrent	0	B
4	71	M	Unresectable	0	A
5	52	M	Unresectable	1	A
6	72	M	Recurrent	0	B
7	50	F	Unresectable	1	B
8	62	M	Unresectable	0	B
9	67	M	Recurrent	1	A
10	61	M	Unresectable	0	B

Abbreviation: PS, performance status.

decided on the basis of body surface area (BSA), was 80 mg (BSA <1.25 m^2), 100 mg (BSA 1.25–1.5 m^2), or 120 mg (BSA ≥ 1.5 m^2). Cisplatin (Landa™, Nippon Kayaku Co, Ltd, Tokyo, Japan) was administered intravenously on day 8. All patients received 16 mg of dexamethasone and 3 mg of granisetron intravenously one hour before cisplatin infusion and 8 mg of dexamethasone on days 9 and 10. The initially administered dose of cisplatin was 60 mg/m^2. Blood samples were obtained from each patient before the administration of cisplatin on day 8 and 3 hours after the administration of cisplatin was finished. The primary endpoint was the amount of oral intake, and the categories of the scales of anorexia, nausea, and vomiting; secondary endpoints included the plasma concentration of acylated ghrelin.

Measurement of acylated ghrelin

The blood samples were collected before and 3 hours after the administration of cisplatin. The sample collecting time was determined on the basis of our unpublished experimental data from dogs. The plasma samples for acylated ghrelin were promptly centrifuged at 4°C, and the supernatants were acidified with 1 mol/L HCl (1/10 volume), frozen, and kept below 40°C until measurement. The acylated ghrelin level was determined using the active ghrelin enzyme-linked immunosorbent assay kit (SCETI Co, Ltd, Tokyo, Japan).

Definition of response

Following administration of cisplatin, each patient was hospitalized and monitored by direct observation and patient interview for 5 days. The amount of oral intake of each meal was observed and scored by 11 stages from 0 to 10 by nurses, and the average oral intake during 5 days was calculated and analyzed. Categories of the scales for anorexia, nausea, and vomiting were graded according to the National Cancer Institute common toxicity criteria, version 3.0.[15] We defined the time to treatment failure as the period between the time that administration of cisplatin was finished and the time that vomiting or dry vomiting occurred or the time of administration of the antiemetic.

Statistical analysis

The results were expressed as the mean ± the standard error of the mean. The Student's *t*-test was used to test for the significance of differences between groups. For the comparison of time to treatment failure between two groups, the Kaplan-Meier product-limit method and log-rank test were used. A P value <0.05 was considered statistically significant. Statistical calculations were performed using StatView® software (version 5.0, Abacus Concepts Inc, Berkeley, CA).

Results

Plasma concentration of acylated ghrelin

In the Rikkunshito-off period, the average concentration of plasma acylated ghrelin 3 hours after the administration of cisplatin decreased from that before administration of cisplatin, but the difference was not significant. On the other hand, in the Rikkunshito-on period, no decrease in plasma concentration of acylated ghrelin was observed between before and after administration (Figure 2).

Amount of oral intake

The average oral intake in the Rikkunshito-on period was significantly larger than that in the Rikkunshito-off period (6.29 versus 3.94, $P = 0.0496$, Figure 3). This tendency was similarly seen in group A and group B, and neither an order effect nor a carry-over effect was seen.

Anorexia, nausea, vomiting

The grade of anorexia (0–4) was significantly lower in the Rikkunshito-on period than in the Rikkunshito-off period

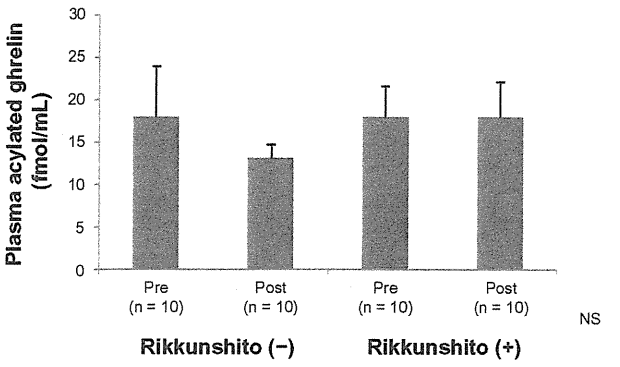

Figure 2 Plasma concentration of acylated ghrelin. In the Rikkunshito-on period (Rikkunshito [+]), no decrease of plasma concentration of acylated ghrelin was observed before and after administration.
Abbreviation: NS, not significant.