Title

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Improvement of peripheral vascular impairment by a phosphodiesterase type 5 inhibitor tadalafl prevents oxaliplatin-induced peripheral neuropathy in mice

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Abstract
Oxaliplatin, a platinum-based chemotherapeutic drug, frequently induces peripheral neuropathy. Accumulating evidences suggest a possible relationship between peripheral vascular impairment and peripheral neuropathy. In this study, we investigated the effects of vasodilators on cumulative peripheral neuropathy induced by repeated injections of oxaliplatin (10 mg/kg) once a week for 8 weeks in mice. Single injections of vasodilators, including a phosphodiesterase type 5 inhibitor tadalafl acutely alleviated oxaliplatin-induced cold hypersensitivity, while tadalafl had no effect on the mechanical hypersensitivity. By contrast, long-term administration of tadalafl (0.1% in chow diets) during the oxaliplatin injection period reduced the oxaliplatin-induced decreases in skin temperature and blood flow without affecting platinum concentrations in blood, sciatic nerves, and dorsal root ganglion. The long-term administration significantly suppressed cold, mechanical, and electrical current hypersensitivities as well as thermal hypoesthesia. Furthermore, it prevented the decreases in sensory nerve conductance velocity and the number of endoneurial microvessels, and axon degeneration in the sciatic nerves. In vitro studies confirmed that tadalafl does not interfere with the cytotoxicity of oxaliplatin against human cancer cell lines. Altogether, these results suggest that improvement of peripheral vascular impairment by tadalafl could alleviate and prevent oxaliplatin-induced peripheral neuropathy.

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1. Introduction

Oxaliplatin (L-OHP) is a platinum-based chemotherapeutic drug widely used to treat colorectal, gastric, and pancreatic cancers. However, it rapidly induces acute peripheral neuropathy in 85–96% of patients, although it is usually improved within a week. Furthermore, cumulative peripheral neuropathy, with paresthesia, dysesthesia, numbness and pain in the hands and/or feet, occurs in 40–93% of patients who have undergone multiple chemotherapy cycles.1 This cumulative L-OHP-induced peripheral neuropathy (OIPN) diminishes the activities of daily living and quality of life in patients with cancer, often resulting in dosage reduction and delay or even discontinuation of chemotherapy in severe cases.1–3 Nevertheless, there are no or little effective strategies for preventing or treating OIPN.1,4

Endoneurial microvascular dysfunction is observed in patients and animal models with peripheral neuropathy.5,6 Moreover, peripheral nerve dysfunction in diabetic patients and animal models is preceded by impaired vascular reactivity, which is thus considered an early diagnostic marker and predictor of the severity of diabetic neuropathy.7–9 Vascular impairment of peripheral arterial diseases is responsible for the induction of sensory disturbance in the early stage, with increasing pain as disease progresses.10–12 Similarly, experimental animals in which peripheral blood flow in

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the hindlimbs is restricted exhibit spontaneous and evoked pain behaviors as well as tactile hypoesthesia. The association between peripheral vascular impairment and peripheral neuropathy is further supported by studies showing that vasodilators, such as phosphodiesterase (PDE) inhibitors, prostaglandin analogs, and endothelin (ET) receptor antagonists, improve peripheral vascular impairment but also attenuate neural dysfunction, nerve degeneration, hypoesthesia, and painful behaviors in animal models of diabetes. These findings propose the possibility that improvement of peripheral vascular impairment by vasodilators may be a potential preventive and/or therapeutic strategy for peripheral neuropathy accompanying peripheral vascular impairment.

Peripheral blood flow is reduced also in L-OHP-treated animals. Gauchan et al. further reported that persistent improvement of peripheral vascular impairment by a prostaglandin E1 analog attenuated the mechanical hypersensitivity evoked by a single administration of L-OHP in mice. However, detailed analyses for the efficacy of vasodilators on the cumulative OIPN are still lacking. In the present study, we examined the acute alleviative and preventive effects of vasodilators on the cumulative OIPN in mice. Here, we found that tadalafil, a PDE type 5 inhibitor, has good efficacy against cumulative OIPN. This drug alleviated cold hypersensitivity acutely, but prevented a wide range of symptoms, sensory nerve dysfunction and degeneration observed in cumulative OIPN.

2. Materials and methods

2.1. Details are presented in supplementary materials and methods

2.1.1. Animals

C57BL/6j mice (Japan SLC, Shizuoka, Japan) aged 6–7 weeks were housed at 24 ± 1 °C and 55 ± 10% humidity under a 12 h light/dark cycle with ad libitum access to water and MF chow (Oriental Yeast, Tokyo, Japan). The experimental protocols used in this study were approved by the Kyoto University Animal Research Committee (permit number 13–38) and performed in accordance with their ethical guidelines.

2.1.2. Experimental design

To investigate the acute effects of vasodilators, mice received intraperitoneal (i.p.) injections of L-OHP (10 mg/kg body weight [Wako Pure Chemical Industries, Osaka, Japan]) or vehicle (5% glucose solution) once a week for 4–6 weeks. Skin blood flow and cold and mechanical sensitivities (see below) were measured before and after the following drugs were administered: tadalafil (10 mg/kg [Combi-Blocks, San Diego, CA]), dissolved in 10% DMSO and 20% polyethylene glycol 400 in distilled water, i.p., 1 h before testing; limaprost alfadex (0.3 mg/kg [Ono Pharmaceutical Co., Osaka, Japan]) saline per os, 0.5 h before testing; bosentan (30 mg/kg [Toronto Research Chemicals, Toronto, Canada]) in saline, i.p., 4 h before testing; vehicle, i.p., 1 h before testing.

To investigate the preventive effects of tadalafil, mice receiving i.p. injections of L-OHP (10 mg/kg) or vehicle once a week for 8 weeks were fed Chow containing 0.1% (w/w) tadalafil (Oriental Yeast) ad libitum from the day before starting to the end of repeated injections (8 weeks). Skin blood flow and temperature, cold, mechanical, and current stimulation sensitivities, and nerve conduction velocities (NCVs) were measured after 8 weeks (see below).

2.1.3. Physiological analyses

Skin blood flow through the plantar surface of the hindpaw was measured using a small laser Doppler blood flow sensor (RBF-101; Pioneer, Tokyo, Japan), and the temperature was measured with a skin surface probe connected to a thermometer (BAT-10 multipurpose thermometer; Physitemp Instruments, Clifton NJ). Sensory and motor NCVs in the tail were recorded with an amplifier (EX-1; AD Instruments, Milford, MA) in response to electrical stimulation (STG4002; Multi Channel Systems, Reutlingen, Germany) and processed with LabChart software (AD Instruments).

2.1.4. Behavioral tests

Cold sensitivity was assessed by quantifying cold-escape behaviors for 60 s after acetone (10 μl) was applied to the plantar skin of the hind paw. Mechanical sensitivity was assessed by measuring the paw withdrawal threshold against stimulation with von Frey filaments (Stoelting, Wood Dale, IL). Thermal sensitivity was assessed by measuring the time to withdraw a paw exposed to a radiant heat source from a Hargreaves apparatus (Ugo Basile, Milan, Italy). Paw withdrawal thresholds to transcutaneous current stimuli were measured using a Neurometer CPT/C (Neurotron Inc., Baltimore, MD).

2.1.5. Immunohistochemistry

Immunohistochemistry for CD31, a platelet-endothelial cell adhesion molecule highly expressed in the vascular endothelial cells, was performed with 6 μm-thick sciatic nerves sections incubated with CD31 antibody (1:100; BD Bioscience, Franklin Lakes, NJ). The density of CD31 vessels (count/mm²) was calculated.

2.1.6. Electron microscopy

Sciatic nerves fixed in 2% glutaraldehyde and 4% paraformaldehyde and postfixed in 1% osmium tetroxide were sectioned (70 nm) and imaged with a transmission electron microscope (H7650; Hitachi, Tokyo, Japan). The axons were classified by calculating the circularity and analyzed with MetaMorph software.

2.1.7. Platinum concentration

The concentrations of platinum in blood, sciatic nerve, and dorsal root ganglia (DRG) samples were measured with an Agilent 7700x ICP-MS system (Agilent Technologies, Santa Clara, CA).

2.1.8. Cytotoxicity assay

The cytotoxicity of L-OHP to human colon cancer (HCT116) and stomach adenocarcinoma (AGS) cell lines (ATCC, Manassas, VA) was assessed with an MTT assay.

2.1.9. Statistical analysis

Differences were compared using Student’s t tests for two groups and one-way or two-way analyses of variance (ANOVA) followed by an appropriate post hoc test for more than two groups. Statistical analyses of the 50% withdrawal thresholds in von Frey filament testing were performed using Mann–Whitney U test or Kruskal–Wallis test followed by Dunn’s post hoc test for each day. Other time-course data were analyzed by two-way ANOVA for repeated measures followed by Tukey’s post hoc test. In all cases, differences of P < 0.05 were considered statistically significant.

3. Results

3.1. Acute alleviative effects of vasodilators on L-OHP-induced peripheral vascular impairment and cold hypersensitivity

Skin blood flow through the hindpaw was measured before and after various vasodilators were administered to mice treated with L-OHP or vehicle for 4 weeks (Fig. 1A). Skin blood flow was significantly reduced by repeated injections of L-OHP. A single administration of the PDE5 inhibitor tadalafil (10 mg/kg), the
prostaglandin E₁ analog limaprost alfadex (0.3 mg/kg), and the ET receptor antagonist bosentan (30 mg/kg) significantly recovered the decreased skin blood flow in L-OHP-treated mice. A single administration of tadalafl also significantly increased the skin blood flow in the vehicle-treated mice.

The frequency of licking and shaking in response to an acetone application was increased in mice treated with L-OHP for 5 weeks. The cold hypersensitivity was significantly attenuated by a single administration of tadalafl, limaprost alfadex, and bosentan (Fig. 1B). The 50% withdrawal threshold to von Frey filaments were decreased in mice treated with L-OHP for 6 weeks. However, the mechanical hypersensitivity was not affected by a single administration of tadalafl (Fig. 1C).

3.2. Long-term administration of tadalafl improves peripheral vascular impairment in L-OHP-treated mice

We selected tadalafl for subsequent experiments because of its relatively long half-life and vasodilatory effects among the tested vasodilator drugs. We first verified that oral long-term administration of tadalafl did not affect the blood concentration and tissue accumulation of L-OHP in mice. The concentrations of platinum in blood and tissue samples, sciatic nerves and DRG, from mice fed a chow for 8 weeks containing 0.1% [w/w] tadalafl did not differ from those on the control (normal) diet (Table 1).

Repeated injections of L-OHP significantly reduced body weight. Long-term administration of tadalafl did not influence the body weight in repeated vehicle- and L-OHP-treated mice (Fig. 2A). Repeated L-OHP injections gradually and significantly reduced skin blood flow through the hindpaw. Significant reductions were observed between 4 and 8 weeks after the L-OHP injections. Tadalafl tended to increase the skin blood flow in the repeated vehicle-treated mice, and it significantly prevented the reduction in the repeated L-OHP-treated mice (Fig. 2B). Accordingly, skin temperature in the hindpaw was significantly lowered 8 weeks after the repeated L-OHP injections. The reduction of skin temperature was significantly prevented by tadalafl (Fig. 2C).

3.3. Long-term tadalafl prevents L-OHP-induced cold, mechanical, and current hypersensitivities and thermal hypoesthesia in L-OHP-treated mice

Repeated injections of L-OHP significantly increased the frequency of licking and shaking in response to acetone application, which developed within 2 weeks. The L-OHP-induced cold hypersensitivity was significantly prevented by long-term administration of tadalafl (Fig. 3A). Similarly, repeated L-OHP injections significantly decreased the 50% withdrawal threshold to mechanical stimulation between 2 and 8 week. The mechanical hypersensitivity was significantly prevented by tadalafl (Fig. 3B).

### Table 1

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Platinum concentration (nmol/mL or nmol/g)</th>
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<tbody>
<tr>
<td></td>
<td>L-OHP</td>
</tr>
<tr>
<td>Blood plasma</td>
<td>0.85 ± 0.15</td>
</tr>
<tr>
<td>Sciatic nerves</td>
<td>4.21 ± 0.72</td>
</tr>
<tr>
<td>DRG</td>
<td>5.55 ± 0.69</td>
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L-OHP (10 mg/kg) was injected i.p. once a week for 8 weeks, during which mice were fed normal chow or a Chow diet containing 0.1% tadalafl (TDF). Blood samples, sciatic nerves, and dorsal root ganglia (DRG) at L4–L6 were isolated from each group, and platinum concentrations were analyzed using an ICP-MS system. The results are expressed as means ± S.E.M (n = 4–5).
hypersensitivity was significantly attenuated by long-term tadalafl administration (Fig. 3B). By contrast, repeated injections of L-OHP for 8 weeks significantly prolonged the latency of thermal nociceptive responses. The thermal hypoesthesia, a symptom of cumulative peripheral neuropathy,22 was significantly prevented by long-term tadalafl administration (Fig. 3C).

We measured current perception thresholds to sine-wave pulses of 5, 250, and 2000 Hz produced by a Neurometer to stimulate C, Aδ, and Aβ fibers, respectively. Current perception thresholds at these frequencies were significantly decreased by repeated L-OHP injections for 8 weeks. Long-term tadalafl administration prevented these decreases at any frequencies (Fig. 3D–F).

3.4. Long-term tadalafl preserves sensory NCV in L-OHP-treated mice

We measured sensory and motor NCVs in the tails of mice receiving repeated L-OHP injections for 8 weeks. Sensory NCV was significantly reduced in mice treated with L-OHP. Long-term tadalafl administration prevented the decrease in sensory NCV (Fig. 4A). L-OHP treatment also reduced motor NCV, but this decrease and the effect of tadalafl were not significant (Fig. 4B).

3.5. Long-term tadalafl prevents L-OHP-induced decreases in endoneurial microvessel density in sciatic nerves

Immunostaining for CD-31, an endothelial marker, was performed on sciatic nerve sections from mice receiving repeated L-OHP injections for 8 weeks (Fig. 5A). Quantitative analysis of confocal images showed that the density of CD31-immunoreactive microvessels was significantly reduced by L-OHP, which was prevented by long-term administration of tadalafl (Fig. 5B).

3.6. Long-term tadalafl prevents L-OHP-induced axon degeneration in sciatic nerves

Electron microscopy of sciatic nerve sections revealed a degeneration of myelinated fibers with abnormal distorted morphology in mice receiving repeated L-OHP injections for 8 weeks (Fig. 6A). The percentage of axons with a circularity measure of >0.7 (indicative of normal axon morphology) was significantly reduced in L-OHP-treated mice. Consistently, the percentage of axons with a circularity of <0.5 (indicative of severe axonal degeneration) was significantly increased in these mice. Long-term tadalafl administration tended to prevent the changes in the distribution of axon circularities (Fig. 6B).

3.7. Tadalafl does not affect the cytotoxicity of L-OHP in human cancer cells

To verify that tadalafl did not impact the cytotoxicity of L-OHP on cancer cells, we performed in vitro MTT assays. The viability of HCT116 and AGS cells was not altered by exposure to various concentrations of tadalafl (up to 10 μM) for 24 h. Furthermore, tadalafl did not alter the reduced viability of cell lines exposed to 10 μM L-OHP for 24 h (Fig. 7). Thus, the above-described effects of tadalafl on L-OHP-induced peripheral neuropathy did not interfere with the ability of L-OHP to kill cancer cells.

4. Discussion

In the present study, we provided evidences that improvement of peripheral vascular impairment by tadalafl, a PDE5 inhibitor, showed acute alleviative and preventive effects on OIPN. A single administration of tadalafl exerted limited efficacy on cold hypersensitivity, but not on mechanical hypersensitivity. However, long-term administration of tadalafl had wide range effects on signs, symptoms and pathophysiological changes observed in OIPN model mice.

Patients treated with cumulative doses of L-OHP experience multiple sensory disturbances; cold hypersensitivity, but
concomitantly also hypoesthesia in warm, cool, touch and bump detection, accompanied by a reduction of NCV. However, there are no reliable animal models of OIPN, in which such sensory disturbances can be appropriately assessed. Mechanical and cold hypersensitivities have been frequently documented in animals receiving relatively low-doses of L-OHP (2–5 mg/kg) once or several times a week. However, mice treated with these doses in our preliminary experiments did not exhibit thermal hypoesthesia, reduced NCV, or axonal degeneration (data not shown). In this study, we selected relatively higher-dose of L-OHP (10 mg/kg) once a week for 4–8 weeks. It enabled us to examine the effects of test drugs on abnormal sensations (mechanical/cold/current hypersensitivity and thermal hypoesthesia), neurological dysfunction and axon degeneration, although it caused the decrease in body weight.

Consistent with the previous experiments in animals and patients, peripheral blood flow was gradually reduced along with increasing cumulative doses of L-OHP, and it was accompanied with the reduction in skin temperature. Although the mechanisms underlying the peripheral vascular impairment are largely unknown, L-OHP can directly and/or indirectly affect vascular endothelial function, and L-OHP-based chemotherapy induces vascular endothelial injury in colorectal cancer patients. It is possible that the vascular endothelial injury and dysfunction induced by L-OHP may lead to the peripheral vascular impairment. In the present study, mechanical hypersensitivity was invariably observed during the observation period. By contrast, cold hypersensitivity was gradually exacerbated along with increasing cumulative doses of L-OHP, which was likely paralleled by the progression of peripheral vascular impairment. L-OHP can induce rapid-onset cold hypersensitivity, a characteristic symptom of acute OIPN, within several hours after the injection. We and other groups previously found that it is mediated through an L-OHP metabolite, oxalate, which indirectly sensitizes a redox-sensitive nociceptor, transient receptor potential ankyrin 1 (TRPA1), against cold temperature. However, as the cold hypersensitivity usually improves within a week, cumulative doses of L-OHP escalate the cold hypersensitivity through other mechanisms. In this study, three different vasodilators, namely, tadalafil, limaprost alfadex, and bosentan, attenuated the cold hypersensitivity while improving skin blood flow. These findings suggest that
cold hypersensitivity can be mitigated by an increase in peripheral blood flow. We recently showed that hypoxia resulting from peripheral vascular impairment sensitizes TRPA1 in diabetic peripheral neuropathy and hindlimb ischemic mouse models.\textsuperscript{15,34} Thus, it is conceivable that the acute effect of vasodilators on cold hypersensitivity may be mediated by a suppression of hypoxia-induced TRPA1 sensitization. By contrast, a single administration of tadalafil had no effect on the L-OHP-induced mechanical hypersensitivity, consistent with our previous findings in diabetic neuropathy and hindlimb ischemic mouse models.\textsuperscript{34}

The key finding of this study is that the progressive improvements of peripheral vascular impairment and rewarming by tadalafil attenuated a variety of symptoms associated with OIPN, including mechanical, cold, and current hypersensitivities, thermal hypoesthesia, decreased sensory NCV and microvessel density, and axon degeneration in the sciatic nerves. These inhibitory effects are unlikely to be caused by the altered blood concentration and accumulation of L-OHP in peripheral neurons. Mechanical, cold, and current hypersensitivities are generally considered to be mediated through the sensitization of nociceptors and enhanced excitability of nociceptive primary sensory neurons (peripheral sensitization), as well as synaptic facilitation and enhanced responsiveness of nociceptive dorsal horn neurons (central sensitization). Peripheral and central sensitization in OIPN are

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**Fig. 5. Effect of tadalafil on L-OHP-induced decrease in the density of endoneurial microvessels in the sciatic nerve.** L-OHP (10 mg/kg) or its vehicle (Veh) was injected i.p. once a week for 8 weeks, during which mice were fed normal chow or a chow diet containing 0.1% tadalafil (TDF). (A) Representative confocal fluorescence photographs of CD31-immunoreactive staining of endoneurial microvessels in cross-sections of sciatic nerves. Scale bar = 100 μm (20 μm in an enlarged image). (B) Quantitative analysis of the density of CD31-immunoreactive vessels (number/mm\(^2\)) \((n = 5–11). ^* P < 0.05, ^{**} P < 0.01 \text{ (Tukey's post hoc test following one-way ANOVA). The results are expressed as means ± S.E.M.}**

**Fig. 6. Effect of tadalafil on L-OHP-induced axonal degeneration in the sciatic nerve.** L-OHP (10 mg/kg) or its vehicle was injected i.p. once a week for 8 weeks, during which mice were fed normal chow or a chow diet containing 0.1% tadalafil (TDF). (A) Representative electron light micrographs of cross-sections of the sciatic nerves from vehicle- and L-OHP-treated mice with or without tadalafil. Scale bar = 10 μm. (B) Quantitative analysis of the distribution of axon circularity in the sciatic nerves. The percentages of the myelinated fibers showing axon circularity >0.7, between 0.5 and 0.7, and <0.5 are presented \((n = 7–10). ^* P < 0.05 \text{ (Tukey's post hoc test following one-way ANOVA). The results are expressed as means ± S.E.M.}**
have no effect on hypoesthesia and no preventive effect on OIPN.\textsuperscript{35} gabapentin/pregabalin, while it also describes that these drugs moderately recommends a serotonin noradrenalin reuptake in-
to induction and/or progression of OIPN, resulting prevention of abnormal sensa-
tions, neurological dysfunction and morphological neuro-
degeneration. Furthermore, we confirmed that tadalafl do not impact the antitumor effect of L-OHP in vitro. Although it will be
needed to determine whether continuous increase in blood flow affects tumor growth and antitumor effect of L-OHP in tumor-
bearing animal experiments, our data suggest that vasodilators, such as tadalafl, are potential therapeutic and preventive options for OIPN.

Declarations of Competing Interest

The authors declare no potential conflicts of interest associated with this manuscript.

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