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Ghrelin alleviates paclitaxel-induced peripheral neuropathy by reducing oxidative stress and enhancing mitochondrial anti-oxidant functions in mice

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ABSTRACT

Paclitaxel is an effective chemotherapeutic agent, but has some treatment-limiting adverse effects that markedly decrease patients' quality of life. Peripheral neuropathy is one of these, and no treatment for it has been established yet. Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is secreted from the stomach and has widespread effects on multiple systems. We investigated the pharmacological potential of ghrelin in preventing paclitaxel-induced peripheral neuropathy using wild-type mice, ghrelin-null mice, and growth hormone secretagogue receptor-null mice. In wild-type mice, ghrelin administration alleviated mechanical and thermal hypersensitivity, and partially prevented neuronal loss of small unmyelinated intraepidermal nerve fibers but not large myelinated nerve fibers. Moreover, ghrelin administration decreased plasma oxidative and nitrosative stress and increased the expression of uncoupling protein 2 (UCP2) and superoxide dismutase 2 (SOD2) in the dorsal root ganglia, which are mitochondrial antioxidant proteins, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a regulator of mitochondrial number. Both ghrelin-null mice and growth hormone secretagogue receptor-null mice developed more severe nerve injuries than wild-type mice. Our results suggest that ghrelin administration exerts a protective effect against paclitaxel-induced neuropathy by reducing oxidative stress and enhancing mitochondrial anti-oxidant functions, and that endogenous ghrelin has a neuroprotective effect that is mediated by ghrelin/growth hormone secretagogue receptor signaling. Ghrelin could be a promising therapeutic agent for the management of this intractable disease.

1. Introduction

Paclitaxel is a taxane-derived anti-neoplastic agent that is commonly used for solid tumors such as ovarian, breast, and lung cancers. It is highly effective against these cancers but has several treatment-limiting adverse effects (reviewed in (Rowinsky and Donehower, 1995)). Clinical symptoms of paclitaxel-induced peripheral neuropathy include numbness, tingling, and burning pain in a glove-and-stocking distribution, leading to markedly decreased quality of life (Tofthagen, 2010). However, prophylactic therapies for paclitaxel-induced neuropathy have not been established thus far.

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is secreted from the stomach. It acts on the pituitary to stimulate growth hormone release, and on the hypothalamus to enhance food intake (Kojima et al., 1999; Nakazato et al., 2001). Ghrelin also has widespread effects on multiple systems, influencing glucose metabolism, cell proliferation, and gastrointestinal, cardiovascular, and immune function (reviewed in (Kojima and Kangawa, 2006)). In addition, ghrelin has been reported to exert a neuroprotective effect on the peripheral nervous system against conditions such as diabetic neuropathy (Kyoraku et al., 2009; Tsuchimochi et al., 2013) and cisplatin-induced peripheral neuropathy (Garcia et al., 2008). However, it remains unclear whether ghrelin attenuates paclitaxel-induced neuropathy and which portions of the peripheral neurons it protects. Moreover, the mechanism by which ghrelin exerts a neuroprotective effect on the peripheral nervous system is unclear.

In this study, we examined ghrelin's effects in a murine model of paclitaxel-induced peripheral neuropathy, focusing on histology and neuroprotective mechanisms in the peripheral nerves. We further investigated the neuroprotective effect of the endogenous ghrelin/growth hormone secretagogue receptor system using ghrelin-null and growth hormone secretagogue receptor-null mice.

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2. Methods and materials

2.1. Animals

Male 8- to 10-week-old C57BL/6 J mice (wild-type [WT] mice) were purchased from Charles River Japan (Yokohama, Japan). Eight-week-old ghrelin-null (Sato et al., 2008) and growth hormone secretagogue receptor-null mice (Sun et al., 2004) were generously provided by Dr. M. Kojima (Kurume University, Fukuoka, Japan) and Dr. R. G. Smith (Baylor College of Medicine, Houston, TX), respectively. Growth hormone secretagogue receptor-enhanced green fluorescent protein (eGFP) reporter mice were obtained from Mutant Mouse Resource & Research Centers at University of California, Davis. All mice were housed under controlled temperature (21–23 °C) on a 12-h light (08:00–20:00)/12-h dark cycle, and fed standard laboratory chow with ad libitum access to food. All experimental procedures were approved by the Animal Care and Use Committee of the University of Miyazaki.

2.2. Administration of paclitaxel and ghrelin

Paclitaxel 2 mg/kg (Nippon Kayaku, Tokyo, Japan) or vehicle (Cremophor EL and 99.9% ethanol at a 1:1 ratio) were administered to mice intraperitoneally (i.p.), in a volume of 0.2 ml, once per day for 5 consecutive days (cumulative dose of 10 mg/kg) (Masocha, 2014). Concurrently with paclitaxel or vehicle administration, 300 nmol/kg/0.2 ml ghrelin or 0.2 ml PBS were intraperitoneally injected into the mice once per day for 5 consecutive days. This ghrelin administration protocol was modified from that of our previous work (Kyoraku et al., 2009; Tsuchimochi et al., 2013). WT mice were divided into three groups: “control” (vehicle + PBS), “PTX + PBS” (paclitaxel + PBS), and “PTX + ghrelin” (paclitaxel + ghrelin). Ghrelin-null and growth hormone secretagogue receptor-null mice were injected with paclitaxel 2 mg/kg and PBS for 5 consecutive days. Body weights and behavioral analyses were evaluated on day 1 before drug administration and on day 7. In addition, histological analysis, measurement of oxidative and nitrosative stress, and quantitative PCR were conducted after mice were euthanized on day 7.

2.3. Cell culture and administration of paclitaxel and ghrelin

Rat pheochromocytoma PC12 cells were obtained from the RIKEN Cell Bank (Ibaraki, Japan), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10% horse serum, and 1% penicillin-streptomycin at 37 °C in humidified 5% CO_2. These cells were exposed to paclitaxel (1 μM) in the presence or absence of ghrelin (10 μM) for 24 h, as described previously (Liu et al., 2013; Konaka et al., 2017), followed by immunocytochemistry and western blotting.

2.4. Behavioral analyses

Mechanical and thermal sensitivities were measured on day 1 before paclitaxel administration and on day 7. Mechanical sensitivity was assessed by measuring the 50% mechanical withdrawal threshold with calibrated von Frey filaments (Muromachi Kikai, Tokyo, Japan) using the up–down method (Chaplan et al., 1994; Sommer and Schafers, 1998). Briefly, mice were placed in plastic cages with an elevated wire mesh floor, and allowed to acclimate for 15 min before testing. Increasing strengths (0.4–8.0 g) of von Frey filaments were applied sequentially to the plantar surface of the hind paw of each mouse. The strength of the filament that caused paw withdrawal in 3 of the 6 applications was defined as the 50% mechanical withdrawal threshold.

Thermal sensitivity was evaluated by measuring the thermal withdrawal threshold using the hot plate test, as described previously (Kyoraku et al., 2009; Tsuchimochi et al., 2013; Masocha et al., 2016). Briefly, mice were placed on a hot plate (Muromachi Kikai, Tokyo, Japan) with the temperature maintained at 55 ± 1 °C after a 15-min acclimation period. The response latency to either a hind paw lick or to a jump was recorded. A cut-off time of 20 s was chosen to prevent tissue damage.

2.5. Immunohistochemistry

Mice were anesthetized by a mixed anesthetic agent (0.4 mg/kg of medetomidine, 2.0 mg/kg of midazolam, and 2.5 mg/kg of butorphanol) (Kawai et al., 2011) and transcardially perfused with ice-cold PBS followed by 4% PFA solution. Lumbar dorsal root ganglia and hind paw footpads were immersed in 4% paraformaldehyde/PBS overnight at 4 °C, and subsequently cryoprotected in 0.1 M phosphate buffer (PB) containing 20% sucrose. The dorsal root ganglia and footpads were embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan) and were cut into 8-μm slide sections and 30-μm free-floating sections, respectively, using a cryostat (Leica CM3050S; Leica, Nussloch, Germany).

Dorsal root ganglia sections were blocked in Serum-Free Protein Block (Dako, Carpinteria, CA) for 10 min, and then incubated overnight at 4 °C with rabbit anti-activating transcription factor 3 (ATF3) (1:500; Santa Cruz Biotechnology, Dallas, TX), a neuronal injury marker (Tsujino et al., 2000), and Alexa Fluor 488–conjugated mouse monoclonal anti-neuron specific nuclear protein (NeuN) (1:500; Merck Millipore, Billerica, MA), a neuronal marker to confirm that dorsal root ganglia sections actually contain neurons.

Footpad sections were incubated in blocking solution (0.01 M PBS containing 5% normal donkey serum, 2% bovine serum albumin, and 0.25% Triton X-100) for 1 h, then incubated overnight at 4 °C with rabbit anti-protein gene product 9.5 (PGP9.5) (1:2000; Abcam, Cambridge, UK).

Both dorsal root ganglia and footpad sections were treated with Alexa Fluor 594–labeled anti-rabbit secondary antibody (1:500; Invitrogen, Carlsbad, CA).

For quantification, 4 dorsal root ganglia sections per animal were randomly selected, and ATF3- or NeuN-positive cells in these sections were examined with a Confocal Microscope C2 (Nikon, Tokyo, Japan). The number of ATF3-positive cells was expressed as the percentage of the number of NeuN-positive cells. Intraepidermal nerve fibers were quantified by the method described previously (Ko et al., 2002). Briefly, nerve fibers crossing the basement membrane were counted as one. The density was determined as the number of nerve fibers per epidermal length.

2.6. Immunocytochemistry

After the plated PC12 cells were washed with PBS, they were fixed in 4% paraformaldehyde in 0.1 M PBS for 20 min. The cells were blocked with Serum-Free Protein Block (Dako, Carpinteria, CA) for 10 min, then incubated overnight at 4 °C with rabbit anti- superoxide dismutase 2 (SOD2) (1:500; Cell Signaling Technology, Danvers, MA). The cells were treated with Alexa Fluor 488–labeled anti-rabbit secondary antibody (1:500; Invitrogen, Carlsbad, CA). The sections were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan). Images were obtained with a Confocal Microscope C2 (Nikon, Tokyo, Japan). The quantification method was developed using ImageJ software (National Institutes of Health, Bethesda, MD), and involved assigning a value for green fluorescence intensity to every pixel in a cell area, and calculating the average fluorescence intensity in eight cells across each slide.

2.7. Morphometry of sciatic nerves

The sciatic nerves were dissected, postfixed in 3% glutaraldehyde, osmicated in 1% osmium tetroxide, dehydrated, and embedded in epoxide resin. The embedded nerves were cut into 1-μm sections, which