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Ghrelin prevents the development of experimental diabetic neuropathy in rodents

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ABSTRACT

Ghrelin is an acylated peptide discovered in gastric extracts as an endogenous ligand for the growth hormone secretagogue (GHS) receptor. This peptide increases food intake and growth hormone secretion, suppresses inflammation and oxidative stress, and promotes cell survival and proliferation. Our study investigated the pharmacological effect of ghrelin in the prevention of polyneuropathy in streptozotocin-induced diabetes mellitus in C57BL/6N mice, GHS receptor-deficient mice, and growth hormone-deficient rats. Ghrelin or desacyl-ghrelin was administered daily for four weeks immediately after disease onset. The effects of ghrelin on food intake, body weight, blood glucose and plasma insulin levels, nerve conduction velocities, temperature sensation, and 8-isoprostaglandin F2α (8-iso-PGF2α) levels were examined. We found that ghrelin administration did not change food intake, body weight gain, blood glucose levels, or plasma insulin levels in C57BL/6N mice in comparison with mice treated with saline or desacyl-ghrelin administration. Ghrelin administration, but not desacyl-ghrelin, prevented motor and sensory polyneuropathy and reduced the plasma concentrations of 8-iso-PGF2α in C57BL/6N mice. Ghrelin also prevented the reduction in nerve conduction velocities in growth hormone-deficient rats, but not in GHS receptor-knockout mice. In conclusion, ghrelin administration in a rodent model of diabetes prevented polyneuropathy, and this effect was mediated through the GHS receptor and was independent of growth hormone. The protection against the development of experimental diabetic polyneuropathy by ghrelin could be key in preventing this otherwise intractable disorder.

1. Introduction

Diabetic polyneuropathy is a common complication that occurs in large portion of both type 1 and type 2 diabetic patients (Boulton et al., 2005). Typical symptoms of diabetic polyneuropathy include chronic pain, numbness, weakness and difficulties with balance. Hyperglycemia is the definitive cause of polyneuropathy, whereas the vascular, glial, and neuronal damage underlying the progressive axonopathy in diabetic polyneuropathy have complex biochemical etiologies involving oxidative stress, protein glycation, protein kinase C activation, polyol synthesis, and the hexosamine pathway. Although favorable treatments of diabetic polyneuropathy have been suggested as a consequence of various pathogenic mechanisms, these treatments have generally produced disappointing results in clinical trials (Vincent et al., 2011).

Ghrelin, a peptide consisting of 28-amino-acids, was initially isolated from gastric extracts as an endogenous ligand for the growth hormone secretagogue (GHS) receptor (Kojima et al., 1999). Ghrelin 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 exist in two major forms, *n*-octanoyl–modified ghrelin and desacyl-ghrelin (a non-acylated form of ghrelin). Acylation at the third amino acid threonine is necessary for the binding of ghrelin to the GHS receptor. Desacyl-ghrelin can neither bind the GHS receptor nor exhibit growth hormone-releasing activity (Toshinai et al., 2006). The GHS receptor is expressed in the central nervous system, and moreover, in multiple peripheral organs such as the stomach, intestine, pancreas, thyroid, gonads, adrenal, kidney, heart and vasculature, and bone. This widespread expression suggests that ghrelin may have a variety of effects on multiple systems. In fact, ghrelin mediates glucose homeostasis, gastrointestinal, cardiovascular, pulmonary, and immune function, cell proliferation and differentiation, and bone physiology (Kojima and Kangawa, 2006). The diverse actions of

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ghrelin raise the possibility of its clinical application; indeed, clinical trials with ghrelin for the treatment of anorexia nervosa, chronic respiratory infection, diabetic gastroparesis, and cachexia associated with chronic obstructive pulmonary disease and cancer have commenced (Miki et al., 2012; Miljic et al., 2006; Murray et al., 2005; Neary et al., 2004; Strasser et al., 2008).

Ghrelin has been reported to promote cell proliferation and neurogenesis in the neurons of the hypothalamus, dorsal motor nucleus of the vagus, nucleus of the solitary tract, and spinal cord (Steculorum et al., 2011). However, there has been little data demonstrating an effect of ghrelin on peripheral nerve functions. We have demonstrated that four-week intraperitoneal administration of ghrelin ameliorated the reduction of both motor and sensory nerve velocities, and reduced thermal sensation induced by streptozotocin in mice (Kyoraku et al., 2009). Here we investigate the efficacy of ghrelin on the prevention of streptozotocin-induced diabetic polyneuropathy in addition to the roles of the GHS receptor and growth hormone in ghrelin's therapeutic mechanisms.

2. Materials and methods

2.1. Animals and induction of diabetes

We used several strains of rodents in these experiments: 6-week-old male C57BL/6N mice weighing 15–17 g (Charles River Japan Inc., Numazu, Japan); 6-week-old male GHS receptor-deficient mice weighing 15–17 g, which were generated by targeted mutation of embryonic stem cells as reported by Sun et al. (2004); and 10-week-old 58–70 g spontaneous dwarf rats (Japan SLC Inc., Hamamatsu, Japan), which were growth hormone deficient due to a point mutation in the *gh* gene (Okuma et al., 1980). Animals were housed individually at constant room temperature (23 ± 1 °C) under a 12-h light (08:00–20:00 h)/12-h dark cycle, and were provided standard laboratory chow and water ad libitum. After fasting for 24 h, C57BL/6N mice and GHS receptor-deficient mice were given a single intraperitoneal injection of streptozotocin (140 mg/kg body weight; Sigma-Aldrich Japan Inc., Tokyo, Japan), which was freshly dissolved in sodium citrate buffer (10 mmol/l, pH 5). Spontaneous dwarf rats were given a single intraperitoneal injection of streptozotocin (60 mg/kg body weight). Control animals received an intraperitoneal injection of citrate buffer only. Three days after streptozotocin administration, the animals exhibiting plasma glucose concentrations greater than 16 mmol/l were selected as diabetic animals (Matteucci and Giampietro, 2008; Roussel et al., 2004). Glucose levels were measured with a diagnostic kit (Ascensia Breeze 2, Bayer HealthCare AG, Leverkusen, Germany) using blood samples obtained from tail vein punctures. All experimental procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care and were approved by the Ethics Committee on Animal Experimentation of the University of Miyazaki.

2.2. Peptide administration

Four groups of 10C57BL/6N mice were examined: a 'saline' group receiving saline only, 'ghrelin' group receiving 300 nmol/kg body weight/200 μl saline, 'desacyl-ghrelin' group receiving 300 nmol/kg body weight/200 μl saline (Asubio Pharma Co., Tokyo, Japan), and 'control' group (without streptozotocin treatment). Three groups of 6 GHS receptor-deficient mice were examined: saline, ghrelin, and control groups. Finally, two groups of 6 spontaneous dwarf rats were examined: saline and ghrelin groups. Peptides or saline were administered intraperitoneally twice a day (06:00 and 18:00) for four weeks immediately following streptozotocin or control vehicle administration. We measured body weights, one-day food intake, and blood glucose concentrations of the animals at 10:00–12:00 at weekly or biweekly intervals after streptozotocin treatment.

2.3. Electrophysiology

Animals were anesthetized with pentobarbital (Nembutal, 0.1 ml/mouse, Abbott Co., North Chicago, IL, USA), and their body temperature was maintained at a rectal temperature of 37.5–37.9 °C via a heating pad. The right sciatic nerve was stimulated (5–10 V, 0.05 ms single square-wave pulses), proximally at the level of the sciatic notch and distally at the level of the ankle, with paired sub-dermal needle electrodes (NE-2235, NIHON KOHDEN CORP., Tokyo, Japan) as described previously (Stanley et al., 1981). Compound muscle action potentials (CMAPs) were recorded from the interosseous muscles of the ipsilateral foot with two needle electrodes, and were amplified, stored, and displayed on a computer. Sensory nerve conduction velocity (SCV) was determined in a similar manner, using the same stimulating and recording electrode pairs by measuring the latency difference of the H-reflex (Schratzberger et al., 2001). Averaged distal and proximal motor and sensory latencies from 10 separate recordings, together with the nerve length between the two stimulation sites, were used for determination of the motor nerve conduction velocity (MCV) and SCV. MCVs and SCVs were calculated by dividing the interelectrode distance between the two stimulation sites by the latency difference of the CMAPs. We determined the nerve conduction velocity of the sciatic nerve at weekly intervals for four weeks after streptozotocin treatment.

2.4. Hot plate test

A hot plate test was performed after the last administration of ghrelin at four weeks. Each animal was habituated to the test apparatus for three days before the test. The mice were placed on a hot plate maintained at 55 °C, and the latency to lick the front or hind paws was monitored with a video camera and recorded on videotape (Kakinoki et al., 2006). The latency time was then analyzed by two hidden examiners.

2.5. Insulin and 8-iso-prostaglandin F2α (8-iso-PGF2α) measurement

At the end of the experiments, we deprived the mice of food for 8 h and sacrificed them under anesthesia with Nembutal at 21:00–22:00. Blood was obtained for the measurement of plasma insulin with an enzyme immunoassay (EIA) kit (Funakoshi Chemical Co., Tokyo, Japan) and of 8-iso-prostaglandin F2α (8-iso-PGF2α) with an 8-isoprostan EIA kit (Funakoshi Chemical Co., Tokyo, Japan).

2.6. Statistical analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Differences among multiple groups were determined via a one-way or repeated-measures analysis of variance (ANOVA) with Bonferroni post-hoc *t*-tests. When two mean values were compared, the analysis was performed with an unpaired *t*-test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Body weights, blood glucose levels, and one-day food intake

Body weight gains in the three diabetic groups were suppressed; however, food intake in all diabetic groups increased one week after streptozotocin treatment and rose to nearly double that in controls two weeks after streptozotocin treatment