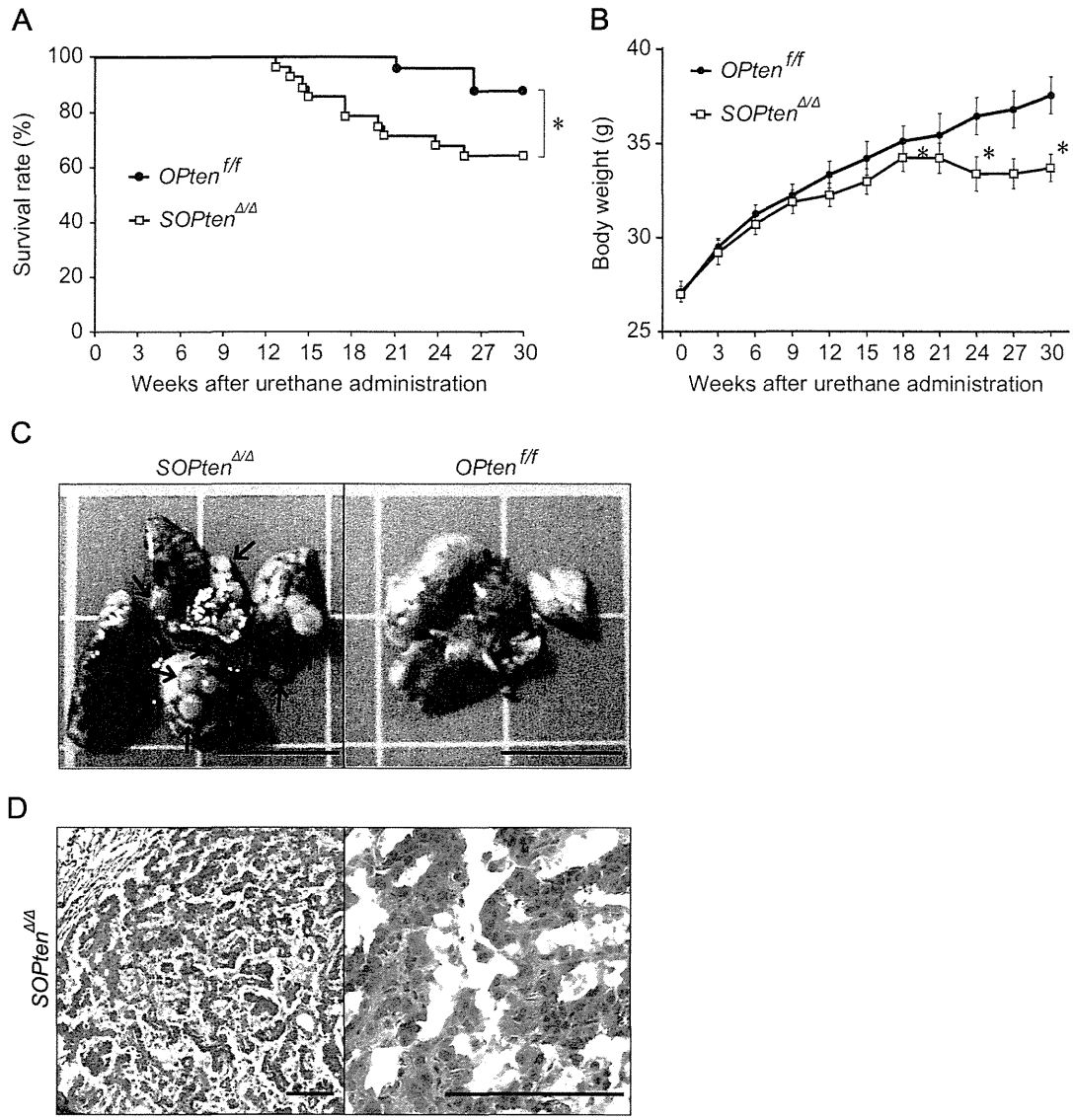

Fig. 2. Urethane-induced lung carcinogenesis and loss of body weights in lung epithelium-specific Pten-deficient mice. (A) Kaplan–Meier survival curves for the *SOPten*$^{\Delta/\Delta}$ mice ($n=28$) and *OPten*$^{f/f}$ mice ($n=25$) for 30 weeks after urethane administration. *$P<0.05$. (B) Alterations in body weights of *SOPten*$^{\Delta/\Delta}$ mice ($n=15$) and *OPten*$^{f/f}$ mice ($n=24$) over 30 weeks after administration of urethane. Data are shown as the mean ± S.E.M. *$P<0.05$. (C) Gross appearance of urethane-induced lung tumors (arrows) in *SOPten*$^{\Delta/\Delta}$ mice. The images shown are representative lung sections of 10 mice per group. Scale bars: 1 cm. (D) Histology of lung adenocarcinoma in the lungs of urethane-injected *SOPten*$^{\Delta/\Delta}$ mice, representative of adenocarcinomas observed in 9 of 10 of these mice. Scale bars: 100 μm.

(Fig. 2D), whereas all other tumors that formed in both *OPten*$^{f/f}$ and *SOPten*$^{\Delta/\Delta}$ lungs were lung adenomas.

3.2. The plasma ghrelin levels after ghrelin treatment

To determine the experimental dose and the administration interval of ghrelin, we measured the plasma ghrelin levels in 10-week-old *OPten*$^{f/f}$ mice at 2, 3, or 12 h after 1 or 10 nmol/mouse of ghrelin or PBS administration. At 12 h after ghrelin or PBS administration, the plasma ghrelin levels were comparable between the mice that were treated with 1 nmol/mouse of ghrelin and the mice that were treated with PBS (Fig. 3A). On the other hand, the plasma ghrelin levels of mice that were treated with 10 nmol/mouse of ghrelin were significantly higher than those in the PBS-treated mice or the mice treated with 1 nmol/mouse of ghrelin. In addition, the plasma ghrelin levels of mice that were administered 10 nmol/mouse of ghrelin decreased to approximately one tenth of the plasma ghrelin levels of mice at 2 h after the same dose of ghrelin treatment (Fig. 3B). Thus, we administered the 10 nmol/mouse of ghrelin or PBS alone at 12-h administration intervals in the following experiments.

3.3. Effects of ghrelin on body weight, food intake, and fat mass in urethane injected-SOPten$^{\Delta/\Delta}$ mice

To confirm the effects of ghrelin administration on cachectic conditions in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice, we started daily administration of ghrelin or PBS at 30 weeks after urethane injection. *SOPten*$^{\Delta/\Delta}$/PBS mice showed significant reduction of body weight, food intake, and intra-abdominal fat mass compared to *OPten*$^{f/f}$/PBS mice (Fig. 4A–C). Ghrelin administration significantly attenuated the weight loss, reduction of food intake and loss of intra-abdominal fat mass in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice.

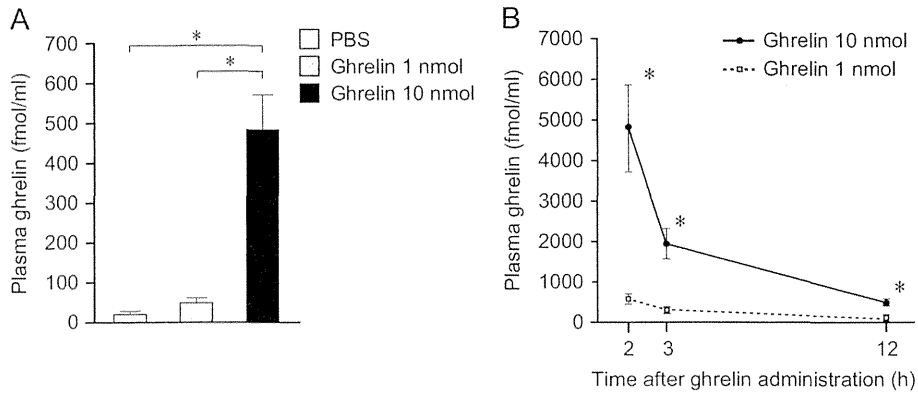

Fig. 3. The plasma ghrelin levels after ghrelin treatment. (A) The plasma ghrelin levels at 12 h after ghrelin administration (1 or 10 nmol/mouse) or phosphate buffered saline (PBS) are shown. Plasma ghrelin levels of mice that received ghrelin or PBS treatment were 53.0 ± 10.7, 489.4 ± 86.1, and 23.3 ± 3.9 fmol/ml, corresponding to 1 nmol/mouse ghrelin, 10 nmol/mouse ghrelin and PBS, respectively. Data are shown as the mean ± S.E.M. of 3 mice per group. $^{*}P < 0.01$. (B) The plasma ghrelin levels at 2, 3, and 12 h after ghrelin administration (1 or 10 nmol/mouse) are shown. Data are shown as the mean ± S.E.M. of 3 mice per group. $^{*}P < 0.05$.

3.4. Effects of ghrelin on plasma levels of proinflammatory cytokines, C-reactive protein, and IGF1 in urethane injected-SOPten$^{\Delta/\Delta}$ mice

The plasma levels of IL-1β, IL-6, TNF-α, and C-reactive protein in *SOPten*$^{\Delta/\Delta}$/PBS mice were significantly higher than those of *OPten*$^{f/f}$/PBS mice. Ghrelin administration significantly reduced the levels of these cytokines and C-reactive protein in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice (Fig. 5). Meanwhile, the plasma level of IGF1 was similar among the three groups.

3.5. Effects of ghrelin on the skeletal muscle mass, contractile force of skeletal muscle and levels of catabolic factors in urethane-injected SOPten$^{\Delta/\Delta}$ mice

We next explored whether ghrelin treatment mitigates the skeletal muscle wasting in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice. The gastrocnemius muscles of *SOPten*$^{\Delta/\Delta}$/PBS mice exhibited excessive shrinkage of muscle fibers and reduction of muscle weights compared with the *OPten*$^{f/f}$/PBS muscles (Fig. 6A–C). Ghrelin administration suppressed the reduction of muscle fiber size and loss of muscle weights, including those of the gastrocnemius muscle, soleus muscle and tibialis anterior muscle in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice (Fig. 6A–C). Ghrelin treatment also retained the muscle contraction force of both soleus muscle and tibialis anterior muscle (Fig. 6D and E). We next examined whether ghrelin administration affected the levels of catabolic factors in the skeletal muscles by measuring the mRNA levels of E3 ubiquitin ligases. We found that the mRNA levels of Atrogin1 and MuRF1 in the gastrocnemius muscles of urethane-injected *SOPten*$^{\Delta/\Delta}$/PBS mice were significantly higher than those of *OPten*$^{f/f}$/PBS mice (Fig. 7A). Ghrelin administration decreased these parameters in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice. In addition, ghrelin restored the reduced level of IGF1mRNA in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice. The gastrocnemius muscle lysates from *SOPten*$^{\Delta/\Delta}$/PBS mice showed decreased expressions of phosphorylated-Akt and phosphorylated-FoxO1 and increased expressions of phosphorylated-p38 MAPK and phosphorylated-NF-κB compared with their counterparts in *OPten*$^{f/f}$/PBS mice (Fig. 7B and C). Ghrelin administration restored the expressions of the phosphorylated-Akt and phosphorylated-FoxO1, and decreased the expressions of phosphorylated-p38 MAPK and phosphorylated-NF-κB in the lysates of gastrocnemius muscle isolated from the urethane-injected *SOPten*$^{\Delta/\Delta}$ mice.

4. Discussion

In this study, we reported for the first time a rodent model of cancer cachexia associated with the development of lung adenocarcinoma, and the protective effect of ghrelin against this inexorable condition. Ghrelin inhibited the induction of proinflammatory cytokines, mitigated the reduction of food intake, and consequently ameliorated body weight loss in the mouse model of lung adenocarcinoma. We also demonstrated that the skeletal muscle mass and muscle contraction force were retained in tumor-bearing, ghrelin-treated mice with upregulation of local IGF1 signaling and downregulation of the FoxO1/MuRF1/Atrogin1 pathway. Muscle atrophy in cancer patients is a life-threatening condition because of the impairment of normal activity and respiratory failure. The most essential thing for control of the cachectic status is prevention of skeletal muscle wasting; however, current therapeutic strategies against muscle atrophy are quite limited. Ghrelin and its mimetic drugs have been tested in patients with cachexia associated with chronic heart failure (Nagaya et al., 2004), chronic obstructive pulmonary disease (Nagaya et al., 2005), and cancer (Garcia et al., 2013; Strasser et al., 2008). Although these studies demonstrated that ghrelin administration increased food intake (Nagaya et al., 2005, 2004), body weight (Garcia et al., 2013; Nagaya et al., 2005), and lean body mass (Nagaya et al., 2005, 2004), the effect of ghrelin on muscle atrophy, including its influence on both skeletal muscle mass and skeletal muscle function, and its molecular mechanisms have not been well documented. Our results suggest that ghrelin could provide a hopeful therapeutic strategy for cancer cachexia patients by exerting a protective effect against muscle wasting.

In the present study, the cachectic condition of urethane-injected *SOPten*$^{\Delta/\Delta}$ mice was demonstrated not only through the development of lung adenocarcinoma, but also through decreases in body weight gain, food intake, fat and skeletal muscle mass and through increases in circulating proinflammatory cytokine levels and in mRNA expression levels of muscle-specific E3 ubiquitin ligases. Since these conditions are also the main characteristics of cancer cachexia observed in patients with various cancers (Fearon et al., 2012), the present results suggested that lung adenocarcinoma-bearing mice could be an ideal cancer cachexia model for estimating effects in humans. Relative to their wild-type counterparts, the body weights of *SOPten*$^{\Delta/\Delta}$ mice began to decrease at five months after urethane injection, which corresponds to the time point of lung adenocarcinoma formation (Yanagi et al., 2007). We therefore started the daily administration of ghrelin to *SOPten*$^{\Delta/\Delta}$ mice five months after urethane injection.

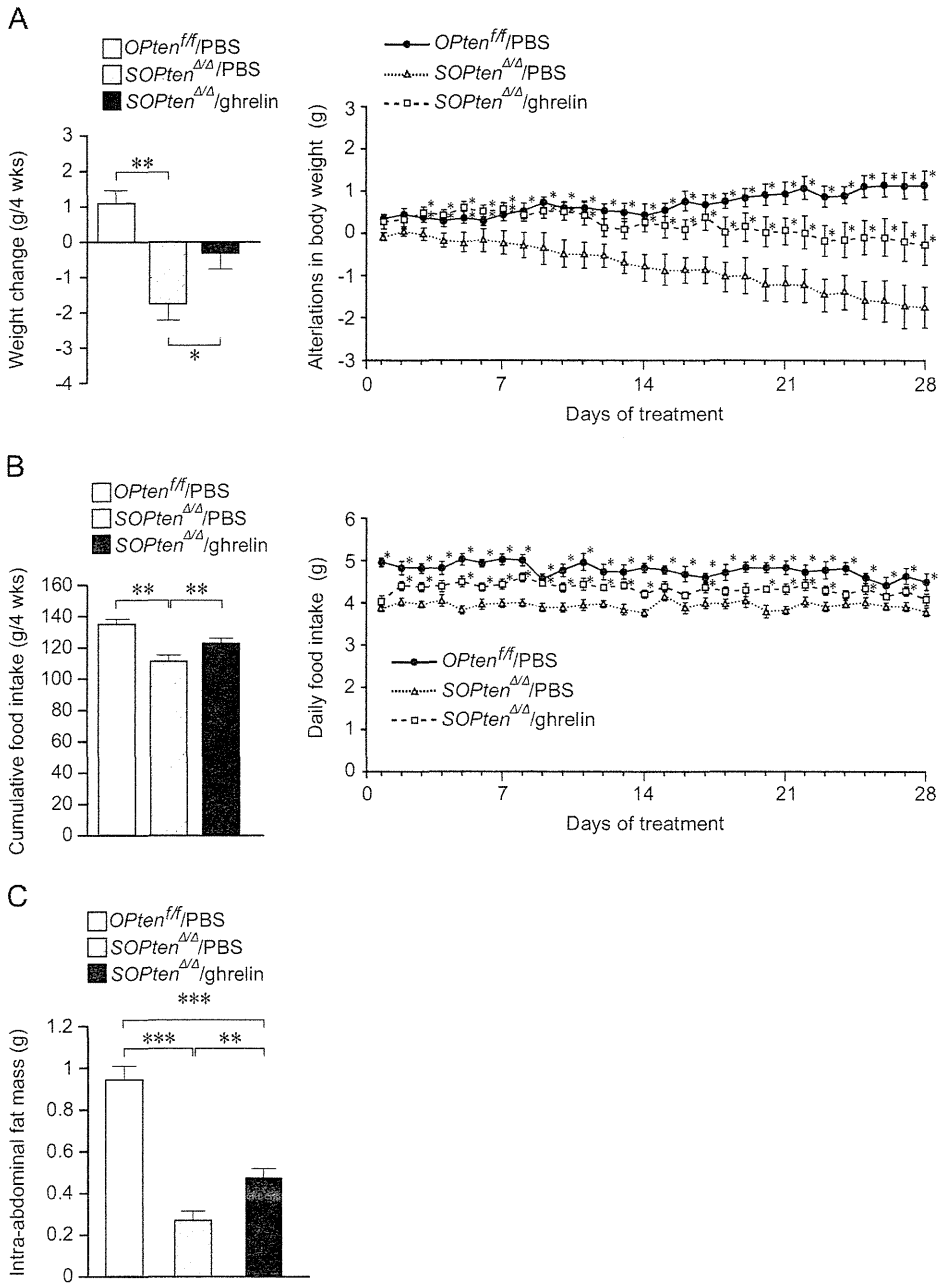

Fig. 4. Effects of ghrelin administration on weight change, food intake, and fat mass in urethane-injected SOPten$^{\Delta/\Delta}$ mice. (A) Overall changes of body weight over the 28-day study interval (left panel) and daily body weight change from the baseline (body weight at day 0, right panel) in the *SOPten*$^{\Delta/\Delta}$/phosphate-buffered saline (PBS) group, *SOPten*$^{\Delta/\Delta}$/ghrelin group and *OPten*$^{f/f}$/PBS group are shown (*SOPten*$^{\Delta/\Delta}$/PBS group, $n=12$; *SOPten*$^{\Delta/\Delta}$/ghrelin group, $n=13$; *OPten*$^{f/f}$/PBS group, $n=12$). Data are shown as the mean ± S.E.M. Differences between groups were evaluated using the Tukey–Kramer honestly significant difference test (left panel) and Student's *t*-test (right panel). $^{*}P<0.05$, $^{**}P<0.01$. vs. the *SOPten*$^{\Delta/\Delta}$/PBS group. (B) Cumulative food intake (left panel) and daily food intake (right panel) of the *SOPten*$^{\Delta/\Delta}$/ PBS group, *SOPten*$^{\Delta/\Delta}$/ghrelin group and *OPten*$^{f/f}$/PBS group over the 28-day study interval are shown (*SOPten*$^{\Delta/\Delta}$/PBS group, $n=12$; *SOPten*$^{\Delta/\Delta}$/ghrelin group, $n=13$; *OPten*$^{f/f}$/PBS group, $n=12$). Data are shown as the mean ± S.E.M. Differences between groups were evaluated using the Tukey–Kramer honestly significant difference test (left panel) and Student's *t*-test (right panel). $^{*}P<0.05$, $^{**}P<0.01$. vs. the *SOPten*$^{\Delta/\Delta}$/PBS group. (C) Intra-abdominal fat mass at 28 days after administration of ghrelin or PBS is shown (*SOPten*$^{\Delta/\Delta}$/PBS group, $n=22$; *SOPten*$^{\Delta/\Delta}$/ghrelin group, $n=23$; *OPten*$^{f/f}$/PBS group, $n=12$). Data are shown as the mean ± S.E.M. Differences between groups were evaluated using the Tukey–Kramer honestly significant difference test. $^{**}P<0.01$. $^{***}P<0.001$.

Ghrelin administration to the urethane-injected *SOPten*$^{\Delta/\Delta}$ mice suppressed the expressions of Atrogin1 and MuRF1 in skeletal muscle to levels comparable to those seen in urethane-injected *OPten*$^{f/f}$ mice, in which cancer did not occur. Expression of these two muscle-specific E3 ligases is essential for ubiquitination and subsequent degradation of myofiber proteins, and many studies have shown that the ubiquitin-proteasome proteolytic pathway plays a major role in the degradation of muscle proteins during cachexia. With regard to the mechanisms of downregulation of E3 ligases in skeletal muscle in ghrelin-treated mice, we demonstrated significant reductions of IL-1β, IL-6, and TNF-α in plasma as well as decreased expressions of p38 MAPK and NF-κB in skeletal muscle. One prominent subset of the procachectic mechanism is induction of proinflammatory cytokines which signal activation of the NF-κB

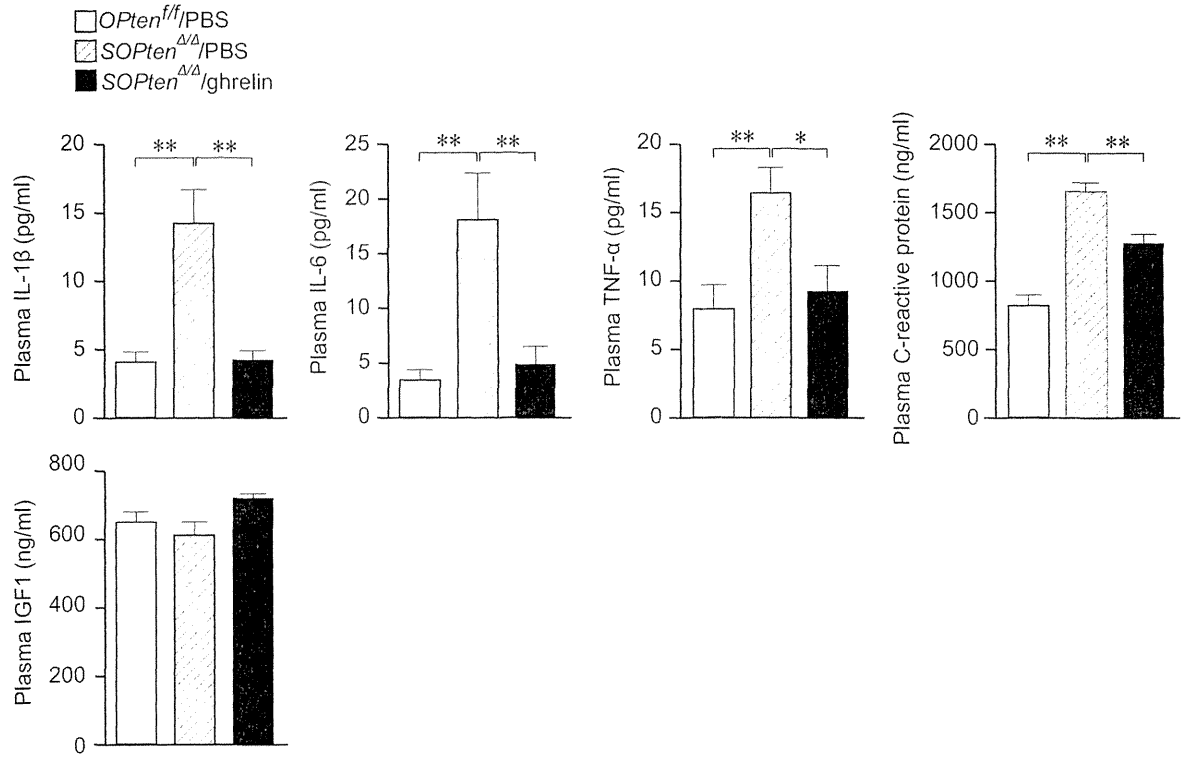

Fig. 5. Effects of ghrelin administration on plasma levels of inflammatory cytokines, C-reactive protein and insulin-like growth factor in urethane-injected SOPten$^{\Delta/\Delta}$ mice. The plasma levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein, and insulin-like growth factor 1 (IGF1) in the SOPten$^{\Delta/\Delta}$/phosphate buffered saline (PBS) group, SOPten$^{\Delta/\Delta}$/ghrelin group, and OPten$^{f/f}$/PBS group at 28 days after ghrelin or PBS treatment. Data are the mean ± S.E.M. of 8–15 mice per group. $^{*}P < 0.05$. $^{**}P < 0.01$.

pathway and p38 MAPK pathway. Upregulation of Atrogin1 occurs via p38 MAPK activation (Li et al., 2005), and inhibition of the NF-κB pathway is sufficient to decrease tumor-induced muscle loss by inhibiting the upregulation of MuRF1 (Cai et al., 2004; Moore-Carrasco et al., 2007). Ghrelin may therefore exert an anti-catabolic effect through the reduction of systemic proinflammatory cytokines. In addition, Yamamoto et al. (2008) reported that GH-releasing peptide-2, a GHS-receptor agonist, directly attenuated Atrogin1 and MuRF1 mRNA levels through the GHS-receptor in C2C12 myotubes. Taken together, these findings suggest that ghrelin may have an anti-catabolic effect through a direct blockade of the proteolytic pathways in skeletal muscle, in addition to its inhibitory effect on the systemic inflammation.

We also demonstrated that systemic ghrelin administration enhanced the anabolic pathway in skeletal muscle in tumor-bearing mice. While ghrelin administration did not affect plasma IGF1 levels, ghrelin-treated mice showed increases in IGF1 mRNA and phosphorylated-Akt expression in gastrocnemius muscle as compared to their control counterparts. It has been reported that systemic IGF1 infusion did not reverse muscle atrophy (Brink et al., 2001), while muscle-specific overexpression of IGF1 reversed muscle wasting (Song et al., 2005). These findings suggest that the local IGF1 expression induced by ghrelin in the skeletal muscle is important for muscle regeneration and hypertrophy. A previous study showed that transgenic mice in which Akt was inducibly activated in skeletal muscle demonstrated dramatic muscle hypertrophy (Lai et al., 2004), further supporting that this pathway is sufficient to mediate hypertrophy downstream of IGF1 upregulation. Akt induces activation of protein synthesis by upregulation of the mammalian target of rapamycin complex 1 signaling, which in turn activates p70-S6-kinase (Fearon et al., 2012). Akt also mediates FoxO phosphorylation and subsequent inhibition of FoxO transport to the nucleus that is sufficient to block upregulation of the E3 ligases, such as Atrogin1 and MuRF1. Therefore, the activating action of ghrelin on the local IGF1/Akt axis in skeletal muscle might also be involved in the amelioration of muscle wasting in tumor-bearing mice.

In regard to ghrelin's amelioration of skeletal muscle atrophy in tumor-bearing mice, we demonstrated that ghrelin administration retained not only the skeletal muscle mass but also the muscle contraction force in both soleus muscle and tibialis anterior muscle, which are slow-twitch muscle and fast-twitch muscle, respectively (Baldwin and Tipton, 1972; Pullen, 1977). These findings suggested that ghrelin treatment might improve the skeletal muscle function, not just retain the skeletal muscle mass and correct the levels of catabolic factors, in cancer cachexia. In regard to the effects of ghrelin on different types of skeletal muscle, our results differ from the previous study in which ghrelin administration attenuated the atrophy of plantaris muscle (one of the fast-twitch muscles), but did not mitigate the atrophy of soleus muscle in a mouse model of unloading-induced muscle atrophy (Koshinaka et al., 2011). In that study, the dose administered was one-eighth of that used in the present study (approximately 2.5 nmol/mouse/day vs. 20 nmol/mouse/day), and the duration of ghrelin treatment was one-half of that used in the present study (2 weeks vs. 4 weeks) (Koshinaka et al., 2011). Therefore, in addition to the differences in the rodent models, the discrepant finding of our present study that ghrelin affected both slow-twitch muscle and fast-twitch muscle may be related to the experimental dose and/or the duration of ghrelin treatment.

Weight loss is an important prognostic indicator for cancer patients (Tisdale, 2002), and one that has often been ascribed to accompanying anorexia. The pathogenesis of cancer anorexia is multifactorial, and involves the hypothalamic and energy intake-modulating signaling

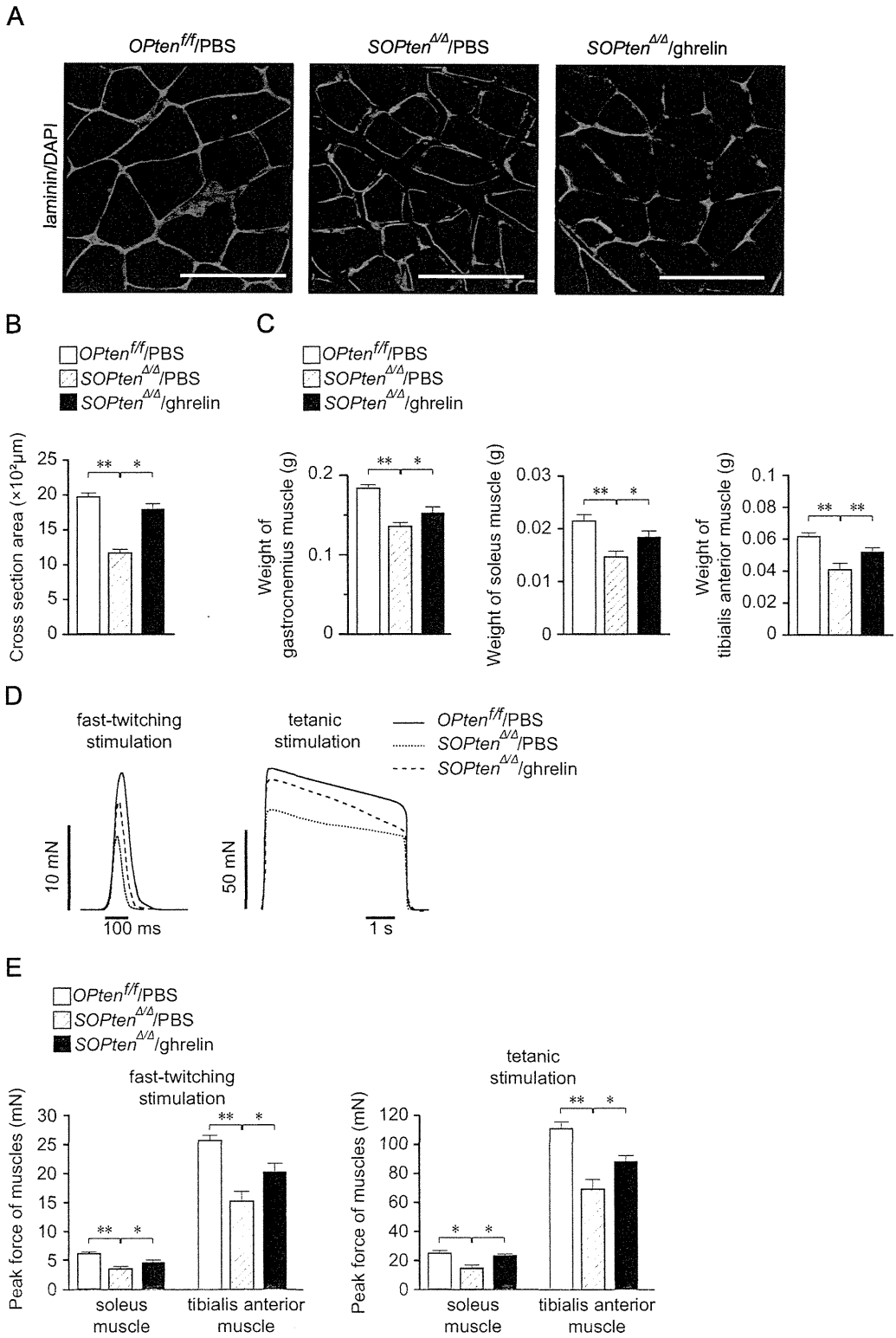

Fig. 6. Effects of ghrelin administration on muscle atrophy in urethane-injected SOPten$^{\Delta/\Delta}$ mice. (A) The sections of gastrocnemius muscles from *SOPten*$^{\Delta/\Delta}$/phosphate buffered saline (PBS) mice, *SOPten*$^{\Delta/\Delta}$/ghrelin mice, and *OPten*$^{f/f}$/PBS mice at 28 days after ghrelin or PBS treatment. The myofiber sizes of lung adenocarcinoma-bearing *SOPten*$^{\Delta/\Delta}$/PBS mice were smaller than those of *OPten*$^{f/f}$/PBS mice, and ghrelin administration suppressed the shrinkage of myofibers in urethane-injected *SOPten*$^{\Delta/\Delta}$ mice. The sections were immunostained with anti-laminin (red) and 4′,6-diamidino-2-phenylindole (DAPI, blue). The images shown are representative gastrocnemius muscle sections from 5 mice per group. Scale bars: 200 μm. The mean cross section areas of gastrocnemius myofibers (B) (n=5 per group) and the mean weights of gastrocnemius muscles (n=12–23 per group), soleus muscles (n=12–13 per group), and tibialis anterior muscles (n=12–13 per group) (C) are shown. The cross section areas were measured as described in the Section 2. Data are shown as the mean ± S.E.M. $^{*}P<0.05$. $^{**}P<0.01$. (D) Representative graph of the contraction force trace of tibialis anterior muscles. (E) The peak contraction forces from soleus muscles and tibialis anterior muscles under fast-twitching stimulation (5 mA/1 Hz) or tetanic stimulation (5 mA/75 Hz) in *SOPten*$^{\Delta/\Delta}$/PBS mice, *SOPten*$^{\Delta/\Delta}$/ghrelin mice and *OPten*$^{f/f}$/PBS mice are shown (n=6–8 per group). Data are shown as the mean ± S.E.M. $^{*}P<0.05$. $^{**}P<0.01$.

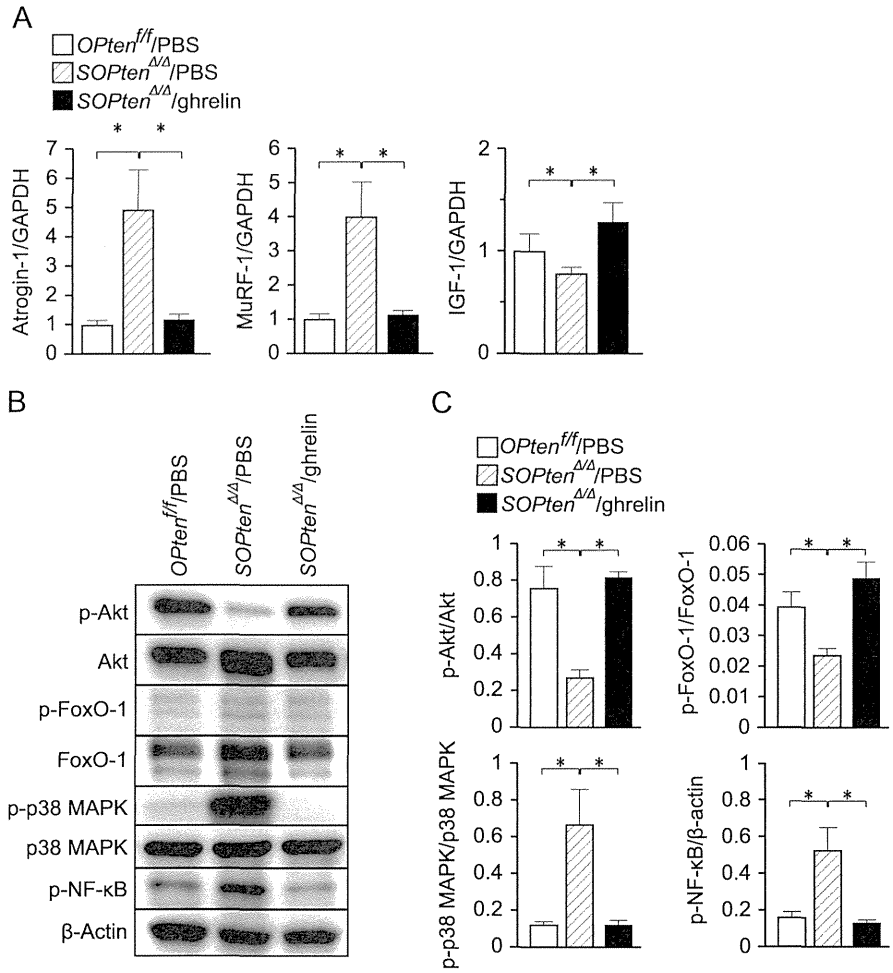

Fig. 7. Effects of ghrelin administration on the expression of catabolic factors and signal transduction molecules in urethane-injected SOPten$^{\Delta/\Delta}$ mice. (A) The mRNA levels of F-box protein-32 (Atrogin1), muscle-specific RING finger protein-1 (MuRF1) and insulin-like growth factor 1 (IGF1) in the lysates of gastrocnemius muscle from *SOPten*$^{\Delta/\Delta}$/phosphate buffered saline (PBS) mice, *SOPten*$^{\Delta/\Delta}$/ghrelin mice, and *OPten*$^{f/f}$/PBS mice at 28 days after ghrelin or PBS treatment. Results are expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are the mean ± S.E.M. of 5–7 mice per group. $^*P < 0.05$. (B and C) Immunoblots of the phosphorylated forms of Akt, Forkhead box protein O1 (FoxO1), p38 mitogen-activated protein kinase (p38 MAPK), and nuclear factor-kappa beta (NF-κB) protein in the lysates of gastrocnemius muscle from *SOPten*$^{\Delta/\Delta}$/PBS mice, *SOPten*$^{\Delta/\Delta}$/ghrelin mice and *OPten*$^{f/f}$/PBS mice. The quantitative comparisons were performed by using total Akt, total FoxO1, total p38 MAPK, and β-actin as loading controls. Data are the mean ± S.E.M. of 3 mice per group. $^*P < 0.05$.

pathways, including hormones, neuropeptides, neurotransmitters and cytokines. These mediators are produced by host immune cells in response to tumor cells or by tumor cells themselves (Fearon et al., 2012; Laviano et al., 2003). Because administration of proinflammatory cytokines (i.e., IL-1β and TNF-α) induces food intake reduction (Bodnar et al., 1989; Ling et al., 1997) and reproduces the characteristic features of the cancer anorexia syndrome (Ling et al., 1997; Mantovani et al., 1998), these cytokines are considered to have a pivotal role in the pathogenesis of cancer anorexia. Ghrelin has been reported to inhibit the expression of proinflammatory cytokines by monocytes (Dixit et al., 2004; Theil et al., 2009) and T cells (Dixit et al., 2004). In this study, we demonstrated not only amelioration of weight loss and food intake reduction but also decreased plasma levels of IL-1β, IL-6, and TNF-α in ghrelin-treated, urethane-injected *SOPten*$^{\Delta/\Delta}$ mice. The inhibitory effect of ghrelin against the expression of proinflammatory cytokines might also attenuate the reduction of food consumption in ghrelin-treated, tumor-bearing mice.

Ghrelin is an endogenous agonist of the GHS-receptor, and exogenous ghrelin administration transiently increases the levels of GH and IGF1 in humans (Nagaya et al., 2001). A previous study demonstrated that high sustained concentrations of IGF1 were associated with an increased risk of various cancers (Renehan et al., 2004). Although we cannot rule out the potential for increased tumor growth after longer-term treatment with ghrelin, there were no significant differences in plasma IGF1 levels or the number and size of the lung tumors between the ghrelin-treated group and PBS-treated group in this study (data not shown). Our data were consistent with a previous study that used a rodent xenograft model (DeBoer et al., 2007; Northrup et al., 2013). Since few studies have examined the effect of ghrelin administration on tumor growth (DeBoer et al., 2007; Northrup et al., 2013), and since IGF1 (Renehan et al., 2004), at least in theory, might stimulate cancer growth, further studies are needed to validate the long-term safety of ghrelin treatment on cancer cachexia.

5. Conclusion

Our results demonstrated the efficacy of ghrelin administration in a rodent model of cancer cachexia. Ghrelin administration ameliorated the body weight loss, suppression of food intake, reduction of fat mass, and skeletal muscle wasting that were

associated with development of lung adenocarcinoma in mice. The pleiotropic effects of ghrelin against cancer cachexia shown in this study may provide relief from the difficult pathological conditions in patients with cancer cachexia.

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Ghrelin Administration for Chronic Respiratory Failure: A Randomized Dose-Comparison Trial

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Abstract

Background Repeated ghrelin administration leads to improvements in symptoms, muscle wasting and exercise tolerance in cachectic patients with pulmonary disease. We investigated the optimal ghrelin dose for underweight patients with chronic respiratory failure.

Methods In this multicenter, randomized, dose-comparison exploratory study, 44 cachectic patients with chronic respiratory failure were randomly assigned pulmonary rehabilitation with intravenous twice-daily administration of 1 or 2 μg/kg ghrelin for 3 weeks. The primary endpoint was improvement in 6-min walking distance (6MWD). The secondary endpoint was change in peak $\dot{V}O_2$.

Results Twenty-one patients were assigned to the 1 μg/kg ghrelin group and 23 to the 2 μg/kg ghrelin group. Change from baseline 6MWD after treatment was similar between groups(1 μg/kg: 53.9 m, 2 μg/kg: 53.9 m, $p = 0.99$). Mean change in peak $\dot{V}O_2$ was significantly greater in the 2 μg/kg group (63.1 ml/min) than in the 1 μg/kg group (−63.8 ml/min, $p = 0.048$). Food intake and lean body mass significantly increased in both groups, and the St. George Respiratory Questionnaire score, body weight, and body mass index were remarkably improved in only the 2 μg/kg group, although there was no significant difference between groups. No treatment-related serious events were reported for either group.

Conclusion Improvements in the oxygen uptake capacity were greater in patients receiving 2 μg/kg ghrelin twice daily for 3 weeks than in those receiving 1 μg/kg, although exercise tolerance was similar between groups at the end of

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the 3-week treatment period. Thus, a twice daily dose of 2 μg/kg ghrelin is recommended over 1 μg/kg ghrelin for patients with chronic respiratory failure and weight loss.

Keywords Ghrelin · Chronic respiratory failure · Cachexia · 6MWD · Peak $\dot{V}O_2$

Introduction

Chronic respiratory failure results in the inability to effectively exchange carbon dioxide and oxygen. Chronic respiratory disease, such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, or pulmonary tuberculosis sequelae, often leads to chronic respiratory failure. When chronic respiratory failure progresses to advanced stages, excess energy expenditure due to respiratory movements induce severe wasting [1]. Wasting worsens the patient's quality of life and prognosis [1, 2].

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor, originally isolated from the stomach [3]. Findings from the past decade indicate that ghrelin induces a positive energy balance and weight gain by stimulating food intake and adiposity [4], controlling fat utilization and thermogenesis [5], increasing cardiac output [6], and attenuating sympathetic nerve activity [7]. In addition, ghrelin has anti-inflammatory effects by suppressing both the production of pro-inflammatory cytokines and the expression of adhesion molecules [8]. These various effects of ghrelin are ideal targets for the treatment of severely wasting chronic pulmonary disorder.

In an open-label pilot study, repeated administration of ghrelin improved walking distance and muscle wasting in cachectic patients with COPD [9]. We recently demonstrated that ghrelin treatment is associated with significant improvement in symptoms and respiratory muscle strength in underweight COPD patients with a body mass index (BMI) <21 [10]. The primary objective of this exploratory study was to investigate the optimal dose of ghrelin and to assess the safety of repeated ghrelin administration.

Materials and Methods

Study Design

This study was a multicenter, randomized, double-blind dose-comparison trial of ghrelin administration during pulmonary rehabilitation. The primary endpoint was change from the baseline 6-min walk distance (6MWD) at the end of the 3-week ghrelin treatment period (week 3). The secondary endpoint was change in the peak $\dot{V}O_2$ in a cardiopulmonary exercise test (CPET) from pre-treatment at week 3. This study was conducted at six medical centers in Japan from January 2009 through January 2012. The protocol was approved by the institutional review boards at all participating study centers, and all patients provided written informed consent. This study was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines. The study was registered with University Hospital Medical Information Network in Japan (UMIN, https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000001822&language=E), number of UMIN000001512.

Patient Selection

Inclusion criteria were as follows: (1) patients with hypoxemia (PO_2 of ≤70 Torr) during exercise or sleeping; (2) underlying disease of COPD, idiopathic pulmonary fibrosis, and/or pulmonary tuberculosis sequelae; (3) BMI <21 kg/m^2; (4) underlying disease stable within 2 months prior to this study; (5) patients who could participate in pulmonary rehabilitation; (6) under 80 years old; and (7) signed the agreement for participation in this study. Patients with any of the following were excluded: (1) cancer (and/or postoperative state); (2) HbA1c >8.4 %; (3) severe heart disease; (4) liver dysfunction (serum levels of aspartate aminotransferase and alanine aminotransferase at least twice the upper limit of normal); (5) renal dysfunction (serum creatinine levels ≥2.0 mg/dl); (6) definitely or possibly pregnant; (7) dementia; (8) judged to be unable to participate in this study by their physician.

Randomization and Intervention

The dose of ghrelin in our previous report was 2 μg/kg [10]. Enrolled patients were randomly assigned at a ratio of 1:1 to pulmonary rehabilitation with either 1 μg/kg or 2 μg/kg ghrelin administration twice daily for 3 weeks. Each dose of ghrelin, which was dissolved in 10 ml saline, was administered intravenously over 30 min at a constant rate. Enrolled patients were examined pre-treatment, at the end of the 3-week treatment (week 3), and at 4 weeks after the end of the treatment (week 7; Fig. 1).

Preparation of Synthetic Human Ghrelin

Synthetic human ghrelin was prepared as described previously [10]. Synthetic human ghrelin was obtained from the Peptide Institute Inc. (Japan), and the ghrelin was dissolved in distilled water with 3.75 % D-mannitol and sterilized by passage through a 0.22-μm filter (Millex; Millipore Co.,

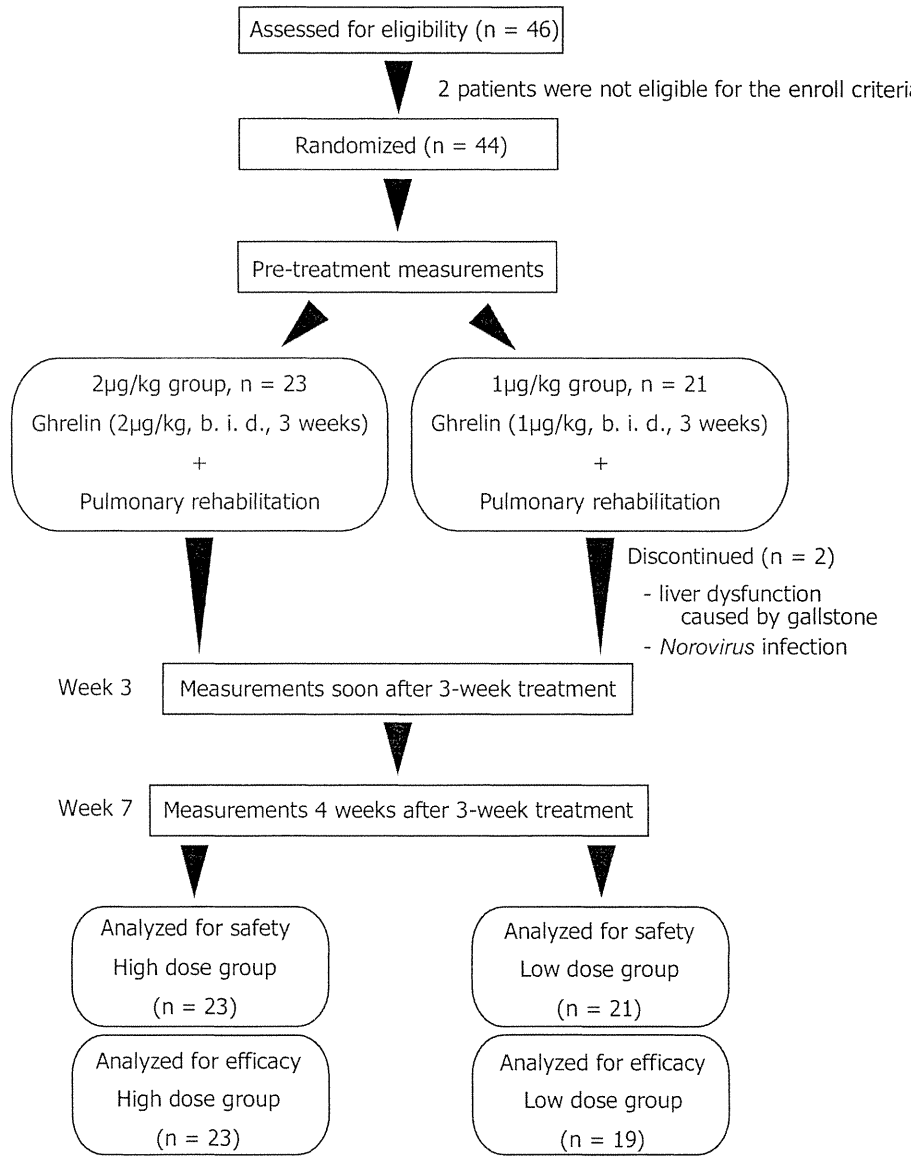

Fig. 1 Study flow chart

Bedford, MA). The ghrelin was stored in 2-ml volumes, each containing 120 μg ghrelin. All of the vials were stored frozen at −80 °C from the time of dispensing until the time of preparation for administration.

Pulmonary Rehabilitation

Pulmonary rehabilitation, which included exercise training and patient education, was conducted as described previously [10]. Exercise training was conducted in three sets daily, every weekday for 3 weeks using electromechanically braked cycle ergometers. The initial exercise level of each set was set for 6 min at a work rate corresponding to 60 % of the peak $\dot{V}O_2$ achieved at baseline. As tolerated by the subject, the exercise was initially increased to 10 min. After that, the training work rate was increased by 5 W and then extended to the work rate corresponding to 80 % of the baseline peak $\dot{V}O_2$.

Six-min Walking Test

The 6MWD test was performed in all of the patients according to a standardized protocol, as reported previously [11].

Symptom Scores

Respiratory symptoms and health-related quality of life were evaluated using the St. George Respiratory Questionnaire (SGRQ) [12] and the Medical Research Council (MRC) dyspnea scale [13].

CPET

While breathing room air with a mask, symptom-limited CPET was conducted on an electrically braked cycle ergometer using an incremental protocol (continuous ramp rate of 5 W/min). Expired gas data were measured breath-by-breath and collected as 30-s means at rest and during exercise. This test continued until subject exhaustion.

Food Intake

Dietary intake was measured as previously described [9]. Food intake for 3 consecutive days was assessed before ghrelin administration and during the last week of ghrelin treatment. Food intake was semiquantitatively assessed by staff nurses using a calorie count, based on a 10-point scale method (0 = no intake to 10 = full intake), which was averaged for 3 days.

Respiratory Muscle Strength

The respiratory muscle strength was examined during maximal voluntary efforts against occluded airways (Vitaropov KH-101; Chest Scientific Instruments Ltd; Westerham, UK), as reported previously [14]. The maximal inspiratory pressure, maximal expiratory pressure, vital capacity (VC), and percent-predicted VC (%VC) were measured from the functional residual capacity.

Dual-Energy X-ray Absorptiometry

All participating centers measured dual-energy X-ray absorptiometry to assess the total body composition, including lean body mass. The measurements were performed with the subject lying in a supine position.

Blood Samples and Analysis

Serum growth hormone and insulin levels were measured by chemiluminescent immunoassay (Beckman Coulter, Tokyo, Japan and Abbot Japan, Tokyo, Japan). Serum levels of brain natriuretic peptide were measured by chemiluminescent enzyme immunoassay (Shionogi, Osaka, Japan). Serum levels of insulin-like growth factor (IGF)-1 were measured by immunoradiometric assay (Siemens Healthcare Diagnostics, Tokyo, Japan). Serum levels of transthyretin, retinol-binding protein, and C-reactive protein were measured by nephelometric assay (Siemens Healthcare Diagnostics, Tokyo, Japan). Serum levels of transferrin were measured by turbidimetric immunoassay (Nippo, Osaka, Japan). Plasma levels of epinephrine, norepinephrine, and dopamine were measured by high-performance liquid chromatography (Tosoh, Tokyo, Japan).

Sample Size

Using an allocation ratio of 1:1 between 1 and 2 μg/kg ghrelin treatment, a fixed sample size of approximately 36 patients was needed to provide at least 70 % power at a significance level of 0.05 (two-sided hypothesis) to detect a 14.5 m between-treatment difference in the change from the baseline 6MWD, assuming a standard deviation (SD) of 17 m. We determined the target sample size of 25 patients in each treatment group at the time of this study design, assuming 28 % loss to finish this study. The trial was discontinued, however, when 44 patients were enrolled in this study because the number of dropout patients was much lower than anticipated.

Statistical Analysis

All data are expressed as mean ± SD unless otherwise indicated. Comparisons of baseline characteristics between the two groups were made using unpaired *t* tests. The results at weeks 3 and 7 were compared with the pre-treatment within each group, and between the two treatment groups using paired *t* tests and unpaired *t* tests, respectively. Treatment effects were examined at weeks 3 and 7. A comparative analysis of those subjects who completed this study was undertaken (not an intention-to-treat analysis). A *p* value of less than 0.05 was considered significant (JMP 9.0.0, SAS Institute Inc., Cary, NC, USA).

Results

Baseline Patient Characteristics

A total of 46 patients were screened at 6 centers from January 2009 through January 2012. Of the 46 patients, 2 patients were ineligible for the entry criteria and were thus excluded from the study. Among the 44 enrolled patients, 21 were assigned to receive 1 μg/kg synthetic human ghrelin and 23 were assigned to receive 2 μg/kg synthetic human ghrelin. Of the 44 randomized patients, 42 completed the 3-week study. One patient in the 1 μg/kg group discontinued the study due to *norovirus* infection and one patient in the 1 μg/kg group dropped out of the study because of liver dysfunction due to gallstones (Fig. 1).

The mean BMI of each treatment group was very low (1 μg/kg group: 17.5, 2 μg/kg group: 18.0). Although no statistical differences were detected, the difference in the mean 6MWD between treatment groups was large. Each treatment group was generally well-matched, except for the 6MWD (Table 1).

Table 1 Baseline patient characteristics

	1 μg/kg Group	2 μg/kg Group	Differences between groups (95 % CI)
Age (years)	69.6 (7.9)	67.8 (6.6)	−3.06 (−7.97 to 1.84)
Smoking (never/former/current/unknown)	2/14/1/2	3/18/2/0	
Sex (male/female)	16/2	16/6	
Body weight (kg)	45.9 (6.8)	46.7 (6.0)	0.20 (−3.94 to 4.35)
BMI (kg/m^2)	17.5 (1.9)	18.0 (1.8)	0.36 (−0.83 to 1.56)
Lean body mass (kg)	37.1 (5.2)	36.7 (5.4)	−0.52 (−39.9 to 29.6)
FEV1 (L)	0.9 (0.4)	1.0 (0.6)	0.15 (−0.15 to 0.45)
FEV1 % (%)	42.7 (21.6)	46.1 (23.0)	3.1 (−12.1 to 18.3)
VC (L)	2.4 (1.0)	2.4 (0.9)	0.04 (−0.58 to 0.64)
%VC (%)	74.2 (28.1)	78.2 (25.2)	5.1 (−12.3 to 22.5)
MIP (cmH_2O)	51.6 (27.1)	54.1 (22.1)	3.0 (−13.0 to 19.0)
MEP (cmH_2O)	64.8 (25.2)	72.5 (31.8)	7.7 (−11.1 to 26.5)
6MWD (m)	255 (115)	323 (93)	68.9 (−3.72 to 141.4)
Peak $\dot{V}O_2$ (ml/min)	545.4 (166.6)	614.4 (248.3)	69.0 (−88.7 to 226.7)
Food intake (kcal/day)	1590 (349)	1713 (301)	142.6 (−63.6 to 348.9)
GH (ng/ml)	0.96 (1.22)	0.60 (0.94)	−0.36 (−1.06 to 0.34)
IGF-1 (ng/ml)	115.8 (57.7)	131.8 (54.3)	16.0 (−19.6 to 51.6)
Insulin (μU/ml)	5.77 (7.23)	6.34 (7.78)	0.57 (−4.18 to 5.31)
BNP (pg/ml)	28.2 (29.1)	40.8 (124.8)	12.7 (−44.0 to 69.3)
Transthyretin (mg/dl)	24.0 (7.0)	24.1 (6.5)	0.16 (−4.16 to 4.48)
Retinol-binding protein (mg/dl)	3.38 (1.00)	3.42 (1.18)	0.04 (−0.65 to 0.72)
Transferrin (mg/dl)	209.2 (33.5)	222.5 (38.4)	13.3 (−9.4 to 36.0)
C-reactive protein (mg/dl)	0.14 (0.17)	0.12 (0.15)	−0.03 (−0.13 to 0.08)
Plasma epinephrine (ng/ml)	0.03 (0.02)	0.04 (0.03)	0.01 (−0.01 to 0.02)
Plasma norepinephrine (ng/ml)	0.40 (0.27)	1.77 (6.09)	1.4 (−1.33 to 4.06)
Plasma dopamine (ng/ml)	0.02 (0.002)	0.03 (0.02)	0.01 (−0.003 to 0.02)
SGRQ			
Total	60.1 (15.8)	54.4 (14.4)	−4.7 (−14.7 to 5.3)
Symptoms	63.4 (16.0)	60.7 (19.1)	−1.4 (−12.9 to 10.0)
Activity	72.5 (20.6)	71.6 (16.8)	−0.26 (−12.7 to 12.2)
Impacts	51.0 (17.3)	41.1 (17.6)	−8.6 (−20.1 to 2.9)
MRC	3.3 (0.8)	3.1 (1.2)	−0.2 (−0.8 to 0.5)
Underlying respiratory disease			
COPD	14	19	
Idiopathic pulmonary fibrosis	2	2	
Pulmonary TB sequelae	3	2	
Home oxygen therapy	11	13	
NPPV	1	1	

Data are presented as mean (SD)

BMI body mass index, *FEV1* forced expiratory volume in 1 s, *VC* vital capacity, *% VC* % predicted vital capacity, *MIP* maximal inspiratory pressure, *MEP* maximal expiratory pressure, *6MWD* 6-min walking distance, *GH* growth hormone, *IGF-1* insulin-like growth factor, *BNP* brain natriuretic peptide, *SGRQ* St. George's Respiratory Questionnaire, *MRC* medical research council, *COPD* chronic obstructive pulmonary disease, *TB* tuberculosis, *NPPV* non-invasive positive pressure ventilation

Exercise Tolerance and Gas Exchange Measurements

In both treatment groups, the mean 6MWD at weeks 3 and 7 was significantly longer than that at baseline. The primary endpoint analysis showed that at week 3, the increase in the mean 6MWD from baseline was similar between groups (Table 2). At week 3, the mean 6MWD was significantly different between groups, but at week 7, the mean 6MWD did not differ significantly between groups (Online Data Supplement, Table S1).

Table 2 Changes from the baseline exercise capacity, SGRQ, pulmonary function, and other parameters at weeks 3 and 7

	At week 3			At week 7		
	1 μg/kg Group	2 μg/kg Group	Differences between groups (95 % CI)	1 μg/kg Group	2 μg/kg Group	Differences between groups (95 % CI)
6MWD (m)	53.9 (73.1)*	53.9 (39.5)*	0.02 (−39.5 to 39.5)	49.8 (64.6)*	49.1 (51.3)*	−0.68 (43.0 to −44.3)
Peak $\dot{V}O_2$ (ml/min)	−63.8 (221.7)	63.1 (111.9)*	126.9 (0.99 to 252.8)†	ND	ND	
Food intake (kcal/day)	157.6 (275.6)*	115.7 (257.4)*	−41.9 (−209.9 to 126.1)	ND	ND	
Body weight (kg)	0.76 (1.8)	0.69 (1.1)*	−0.07 (−1.1 to 0.92)	0.54 (1.9)	0.57 (1.3)*	0.02 (−1.1 to 1.2)
BMI (kg/m^2)	0.27 (0.69)	0.26 (0.4)*	−0.01 (−0.34 to 0.36)	0.22 (0.73)	0.23 (0.54)*	0.01 (−0.43 to 0.45)
Lean body mass (kg)	1,127.9 (1,347.7)*	714.1 (1,112.7)*	−413.9 (−1,227.2 to 399.5)	ND	ND	
VC (L)	0.01 (0.31)	0.15 (0.21)*	0.13 (−0.04 to 0.31)	−0.03 (0.25)	0.12 (0.26)*	0.16 (−0.01 to 0.33)
%VC (%)	−0.41 (10.2)	4.3 (6.0)*	4.7 (−0.86 to 10.3)	−2.1 (8.3)	3.1 (6.8)*	5.2 (−0.09 to 10.5)
FEV1 (L)	−0.04 (0.1)	0.02 (0.11)	0.06 (−0.01 to 0.12)	−0.06 (0.07)	0.01 (0.13)	0.07 (0.001 to 0.14)†
FEV1/FVC (%)	−0.83 (3.9)	0.14 (5.2)	1.5 (−2.0 to 3.9)	−2.1 (4.4)	2.0 (8.3)	2.1 (−0.17 to 8.4)
MIP (cmH_2O)	2.0 (12.7)	11.0 (15.4)*	9.0 (−0.24 to 18.3)	5.4 (12.0)	8.4 (16.7)*	3.0 (−7.2 to 13.2)
MEP (cmH_2O)	8.1 (16.5)	7.3 (17.1)	−0.82 (−11.9 to 10.3)	4.1 (12.9)	6.1 (18.4)	2.0 (−9.1 to 13.2)
SGRQ						
Total	−4.0 (8.1)	−4.0 (7.3)*	0.001 (−5.0 to 5.0)	−2.1 (8.2)	−1.3 (8.5)	0.80 (−4.8 to 6.4)
Symptom	−5.7 (17.2)	−2.5 (8.9)	3.2 (−6.3 to 12.6)	−5.8 (14.4)	−0.52 (10.6)	5.3 (−3.5 to 14.1)
Activity	2.3 (10.0)	−5.3 (14.6)	−7.6 (−15.5 to 3.9)	3.6 (10.4)	−0.31 (16.6)	−3.9 (−13.0 to 5.2)
Impact	−4.7 (10.0)	−3.8 (8.7)*	0.86 (−5.3 to 7.0)	−5.8 (11.2)	−2.2 (9.9)	3.6 (−3.6 to 10.9)
MRC	−0.16 (0.5)	−0.13 (0.81)	0.03 (−0.39 to 0.44)	0.13 (0.52)	−0.23 (0.61)	−0.36 (−0.74 to 0.02)

Data are presented as mean (SD)

6MWD 6-min walking distance, *BMI* body mass index, *VC* vital capacity, *% VC* % predicted vital capacity, *FEV1* forced expiratory volume in 1 s, *MIP* maximal inspiratory pressure, *MEP* maximal expiratory pressure, *SGRQ* St. George's Respiratory Questionnaire, *MRC* medical research council

* $p < 0.05$ change between pre-treatment and post-treatment within-group difference. † $p < 0.05$ between 1 and 2 μg/kg groups

In the 2 μg/kg group, the mean peak $\dot{V}O_2$ was significantly increased during the 3-week treatment, whereas that of the 1 μg/kg group was decreased. At week 3, the mean peak $\dot{V}O_2$ was higher in the 2 μg/kg group than in the 1 μg/kg group, although no significant differences were detected (1 μg/kg group: 509.4 ± 177.8 ml/min, 2 μg/kg group: 677.5 ± 282.0 ml/min, $p = 0.06$, Online Data Supplement, Table S1). The secondary endpoint analysis showed that at week 3, the mean change in the peak $\dot{V}O_2$ from pre-treatment was significantly higher in the 2 μg/kg group than in the 1 μg/kg group (Table 2).

Food Intake, Body Weight, and Nutritional Parameters

Dietary food intake at week 3 was significantly increased compared with that at baseline in both treatment groups. At week 3, no significant differences in the change from the baseline dietary food intake were detected between the two treatment groups. In the 2 μg/kg group, body weight and BMI were remarkably increased at weeks 3 and 7 compared with the baseline values, and in both groups, lean body mass at week 3 was markedly increased from baseline. Changes from the baseline body weight, BMI, and lean body mass were not significantly different between the two treatment groups (Table 2).

In both treatment groups, serum transferrin levels at weeks 3 and 7 were significantly increased compared with those at baseline, although the differences in the change from the baseline value between the two groups were not significant (Table 3).

SGRQ and MRC Scores

At weeks 3 and 7, the changes from the baseline SGRQ and MRC scores did not differ significantly between the two treatment groups. In the 2 μg/kg group, the SGRQ total and impact scores at week 3 were significantly improved compared with those at baseline (Table 2).

Pulmonary Function and Respiratory Muscle Strength

The improvement from the baseline forced expiratory volume in 1 s (FEV1) at week 7 was significantly different between the two treatment groups. Except for FEV1 at week 7, changes from the baseline pulmonary function and respiratory muscle strength were not significantly different

Table 3 Changes from baseline serum levels of hormones, rapid turnover proteins, and plasma levels of catecholamines at weeks 3 and 7

	At week 3			At week 7		
	1 μg/kg Group	2 μg/kg Group	Differences between groups (95 % CI)	1 μg/kg Group	2 μg/kg Group	Differences between groups (95 % CI)
GH (ng/ml)	−0.56 (1.0)*	0.2 (2.1)	0.71 (−0.28 to 1.7)	0.43 (2.8)	1.1 (2.2)*	0.64 (−0.92 to 2.2)
IGF-1 (ng/ml)	10.6 (19.4)*	−1.2 (19.5)	−11.7 (−24.0 to 0.63)	36.8 (159.5)	−1.7 (25.9)	−36.2 (−118.7 to 46.4)
Insulin (μU/ml)	−2.3 (7.0)	0.05 (10.8)	2.3 (−3.5 to 7.2)	2.5 (12.6)	4.0 (14.4)	1.3 (−6.7 to 9.3)
BNP (pg/ml)	0.05 (10.7)	−2.3 (51.6)	−2.3 (−24.4 to 7.9)	2.7 (13.5)	−7.5 (56.3)	−7.3 (−31.0 to 16.4)
Transthyretin (mg/dl)	3.8 (5.5)	1.22 (4.8)	−2.6 (−6.1 to 0.89)	3.8 (5.5)*	1.2 (4.8)	−2.6 (−6.1 to 0.89)
Retinol-binding protein (mg/dl)	0.11 (0.86)	−0.18 (1.0)	−0.29 (−0.92 to 0.35)	0.71 (1.0)*	0.06 (0.87)	−0.66 (−1.3 to −0.03)†
Transferrin (mg/dl)	25.1 (24.2)*	21.2 (20.3)*	−3.9 (−19.0 to 1.2)	25.1 (24.2)*	21.2 (20.3)*	−3.9 (−19.0 to 11.2)
C-reactive protein (mg/dl)	0.01 (0.2)	0.01 (0.14)*	0.001 (−0.12 to 0.12)	0.01 (0.22)	0.01 (0.14)	0.01 (−0.11 to 0.12)
Plasma epinephrine (ng/ml)	−0.003 (0.02)	−0.01 (0.03)	−0.005 (−0.02 to 0.01)	0.02 (0.03)*	0.02 (0.03)*	0.002 (−0.02 to 0.02)
Plasma norepinephrine (ng/ml)	0.01 (0.16)	−1.4 (6.4)	−1.3 (−3.9 to 1.3)	0.36 (0.43)*	−1.2 (6.5)	−1.3 (−4.0 to 1.3)
Plasma dopamine (ng/ml)	0.001 (0.002)	−0.002 (0.03)	−0.003 (−0.01 to 0.01)	0.01 (0.02)*	0 (0.03)	−0.01 (−0.02 to 0.004)

Data are presented as mean (SD)

GH growth hormone, *IGF-1* insulin-like growth factor, *BNP* brain natriuretic peptide

* $p < 0.05$ change between pre-treatment and post-treatment within-group difference

† $p < 0.05$ between 1 and 2 μg/kg groups

between the two treatment groups. In the 2 μg/kg group, VC, %VC, and maximal inspiratory pressure values at weeks 3 and 7 were significantly higher than the baseline values (Table 2).

Plasma Catecholamines and Other Hormone Levels

At weeks 3 and 7, the increase from the baseline plasma catecholamine levels, and the serum growth hormone, IGF-1, insulin, brain natriuretic peptide, and C-reactive protein levels were not significantly different between the treatment groups (Table 3).

Safety

We recorded 18 adverse events. Among these events, 12 were considered not clearly related to the ghrelin treatment and 6 were considered not related to the ghrelin treatment (Table 4). The severity of all 12 adverse events unclearly related to the ghrelin was very mild. Five patients in the 1 μg/kg group and seven patients in the 2 μg/kg group reported events unclearly related to the ghrelin, and the incidence rate was not significantly different between the groups.

Discussion

In the present study, 3-week treatment with either 1 or 2 μg/kg ghrelin and pulmonary rehabilitation in patients with chronic respiratory failure led to a significant increase in the 6WMD compared with the baseline. The primary endpoint of the increase in the mean 6MWD from the baseline was similar between the two treatment groups. Although the 6MWD showed a slight, but statistically significant improvement after 3-week treatment with 2 μg/kg ghrelin compared that with 1 μg/kg ghrelin, interpretation of the result is unclear. The difference in the mean 6MWD between the treatment groups was large at baseline, but not statistically significant.

In the 2 μg/kg treatment group, the peak $\dot{V}O_2$ at week 3 was significantly higher than that at baseline, where as the peak $\dot{V}O_2$ at week 3 in the 1 μg/kg treatment group was lower than that at baseline. The secondary endpoint of the mean change from the baseline in the peak $\dot{V}O_2$ was significantly different between groups (Table 2). There was a trend toward higher peak $\dot{V}O_2$ at week 3 in the 2 μg/kg ghrelin group compared with the 1 μg/kg ghrelin group, although the differences between the groups were not significant (1 μg/kg group: 509.4 ± 177.8 ml/min, 2 μg/kg group: 677.5 ± 282.0 ml/min, $p = 0.06$, Online Data Supplement, Table S1). Although improvements in exercise tolerance did not differ significantly between the treatment groups based on analysis of the 6MWD, amelioration of the symptom-limited oxygen uptake capacity of the 2 μg/kg ghrelin group was thought to be better than that in the 1 μg/kg ghrelin group. Twice daily administration of 2 μg/kg ghrelin appears to be more beneficial than 1 μg/kg. Of the 42 patients who completed this study, 35 were smokers. Of the 35 smokers, 33 had COPD. In this study, 78.6 % of the

Table 4 Adverse events

	1 μg/kg Group	2 μg/kg Group
Adverse events unclearly related to ghrelin		
Transient palpitation		1
Cough		1
Sputum		1
Eruption		1
Hair loss	1	
Low grade fever		2
Diarrhea		1
Vertigo	1	
Elevation of red blood cell counts	1	
Elevation of serum total protein levels	1	
Elevation of serum albumin levels	1	
Adverse events considered not to be related to ghrelin		
Pneumonia	1	
Acute bronchitis		1
Upper respiratory infection		1
Liver dysfunction due to gallstone	1	
Enteritis due to norovirus infection	1	
Elevation of white blood cell counts		1

analyzed patients had COPD. When only COPD patients or smokers were included in the analysis, the results of the primary or secondary endpoint analyses were similar to the all-patient analysis. As scores of the MRC scale during the 6MWD test demonstrated no significant changes from the baseline and the 6MWD of both groups improved, the enrolled patients had a decreased perception of dyspnea for a given exercise load. It is well known that high-intensity rehabilitative exercise training improves the aerobic function of muscles [15–17]. The improvement of exercise tolerance in both treatment groups is possibly due to an increase in skeletal muscle and improved muscle function. In this study, food intake and lean body mass of both groups at week 3 increased remarkably. Especially in the 2-μg/kg ghrelin group, body weight, BMI, maximal inspiratory pressure, and VC at weeks 3 and 7 increased significantly compared with the baseline values (Table 2). In our previous randomized controlled trial of ghrelin administration for underweight patients with COPD, both symptoms and exercise capacity also improved [10, 18]. A previous clinical study demonstrated that ghrelin administration improves cardiac function [19]. Recently, Miki et al. reported that ghrelin administration improves the ventilatory equivalent ratio for O_2 and the ratio of dead space over tidal volume, and increased O_2-pulse [18]. Further clinical investigations are necessary to elucidate the disease conditions suitable for ghrelin treatment.

Of the 42 patients who completed this study, 10 patients exhibited a decreased 6MWD at week 7 compared with the baseline (Online Data Supplement, Figure S1). In the patients with a decreased 6MWD at week 7, pre-treatment BMI (patients with increased 6MWD: 18.5 ± 1.3, patients with decreased 6MWD: 16.2 ± 1.3, $p = 0.002$) and pre-treatment lean body mass (patients with increased 6MWD: 38.0 ± 4.4 kg, patients with decreased 6MWD: 33.3 ± 5.3 kg, $p = 0.041$) were significantly lower than those of patients with an increased 6MWD at week 7 (Online Data Supplement, Figures S2, S3). Excessive exercise rehabilitation programs worsen muscle wasting in patients with COPD [20, 21]. Patients with decreased exercise capacity at week 7 are thought to be too cachectic to benefit from pulmonary rehabilitation. Among the underweight patients with respiratory failure enrolled in the present study, 1 μg/kg ghrelin might not have been sufficient for those with less lean body mass.

In this study, the difference in the ghrelin dose did not provide a significant treatment advantage with respect to the SGRQ and MRC scores. Only the SGRQ total and impact scores at week 3 in the 2 μg/kg group were significantly improved compared with the baseline values. In analysis limited to the COPD patients or smokers, improvements in SGRQ activity at week 3 were significantly greater in the 2 μg/kg group than in the 1 μg/kg group (1 μg/kg group: 2.3 ± 10.0, 2 μg/kg group: −6.3 ± 14.5, $p = 0.04$). Mean improvement was −6.3 points, indicating a clinically meaningful change. In our previous investigation, we found significant treatment effects in the ghrelin treatment group compared with a placebo treatment group [10]. In the present study, the serum growth hormone and IGF-1 levels at weeks 3 and 7 did not differ significantly between the treatment groups.

Throughout this clinical trial, 3-week administration of ghrelin was safe and tolerable. No treatment-related serious events were reported in either treatment group. Of the adverse events not clearly related to ghrelin administration, no significant differences were found between the 1 and 2 μg/kg ghrelin groups. These events were similar to those reported in previous clinical research of ghrelin treatment [9, 10, 19, 22].

This study has some limitations. First, the number of the enrolled patients was small. Second, the difference in the 6MWD at baseline between treatment groups was large, although not statistically significant. Third, this study had only two treatment arms and a dose greater than 2 μg/kg ghrelin might be more effective.

In conclusion, improvements in the oxygen uptake capacity after 3-week treatment with ghrelin and pulmonary rehabilitation were greater in those receiving a twice daily dose of 2 μg/kg ghrelin compared with a twice daily dose of 1 μg/kg. Serious adverse events related to ghrelin

administration did not occur and ghrelin treatment was well tolerated.

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Conflict of interest None of the authors have any conflict of interests to disclose.

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7. 呼吸器疾患におけるPTENの役割

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key words　PTEN, PI3K-AKT-mTOR, nucleus, lung cancer, epithelial integrity, lung fibrosis

動　向

癌抑制遺伝子*PTEN*は，1997年に2つの別個の研究グループによって同定された蛋白/脂質フォスファターゼである[1,2]．PI3K-AKT-mTOR経路を負に制御し，個体発生と細胞の生存と増殖とエネルギー代謝と細胞骨格を統制する[3]．*PTEN*のgermline mutationは，PTEN hamartoma tumor syndrome（PHTS）とよばれる癌高感受性症候群を引き起こす[3,4]．また，*PTEN*は悪性腫瘍においてp53に次いで2番目に高頻度に変異がみられる癌抑制遺伝子でもある[4]．Ptenヘテロ欠損マウスは様々な臓器で自然発癌がみられることから[5,6]，PTENは癌の発生と進展の抑制に必須の遺伝子であるといえる．最近PTENには，核内においてフォスファターゼ活性非依存性に染色体やゲノムの安定性維持に寄与したり，細胞外に分泌されて隣接する細胞の増殖を抑制するなど，細胞質内での作用にとどまらない新たな機能が明らかにされつつある．またPTENは，自己免疫性疾患や炎症性疾患，臓器線維化，感染症などの非腫瘍性疾患の病態にも重要な役割をもつ．本稿では，PTENの作用機序，PTENの制御機構に加えて，肺癌や急性呼吸促迫症候群，肺線維症などの呼吸器疾患におけるPTENの役割について概説する．

A. PTEN signaling pathway

PTENは403個のアミノ酸からなり，PIP_2結合領域，フォスファターゼ活性領域，C2領域，C-tail領域，PDZ結合領域から構成される（図1）．癌でのPTEN遺伝子変異の40%以上はC末端領域にみられることから[7]，PTENの機能には脂質フォスファターゼ活性以外の特性も重要であることが示唆される．C2領域はPTEN蛋白の安定化，

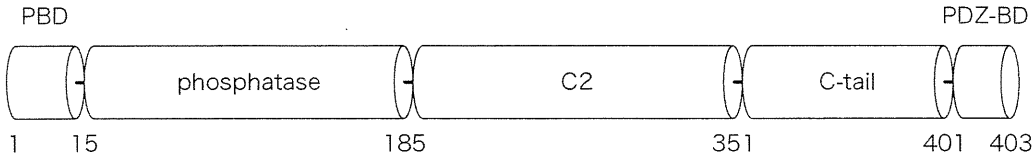

図1　PTENの構造
PBD：PIP_2-binding domain, PDZ-BD：PDZ-binding domain

細胞膜への局在，DNAやセントロメアや他の蛋白との相互作用に関与し，PDZ領域はMAGI2等の蛋白と結合することでPTEN蛋白の安定化とフォスファターゼ活性増強に関与する[8,9]．

1．PTEN-PI3K-AKT-mTOR経路

PTENのユニークな特性の中でも，PIP_3を基質とする脂質フォスファターゼ活性は，PTEN固有の癌抑制遺伝子としての機能に最も深く関与していると考えられている．種々の細胞増殖因子などの刺激によりPI3Kが活性化され，活性化PI3Kは細胞膜上のPIP_2をリン酸化しPIP_3を産生する．PIP_3はAKTとPDK1を細胞膜に動員し活性化する．AKTはPDK1によりThr308にてリン酸化，あるいはmTORC2によりSer473にてリン酸化されることで活性化する．活性化AKTは下流分子であるGSK3，FOXO，MDM2，p27をリン酸化することで，細胞生存，増殖，血管新生，細胞代謝を促進する[10]．また活性化AKTはmTORC1の負の制御因子であるTSC2とPRAS40を抑制することでmTORC1を活性化する．活性化mTORC1はp70S6Kの活性化と4EBP1の不活化により，MYCやHIF1，SREBP1Cなどの細胞増殖に極めて重要な蛋白の発現を亢進させる．PTENはPIP_3をPIP_2に脱リン酸化することでPI3K-AKT-mTOR経路を上流で負に制御し[11]，癌抑制遺伝子として機能する（図2）．

2．PTEN protein phosphatase activity

PTENはFAKとその下流分子であるp130Casを脱リン酸化し，細胞遊走と細胞浸潤を阻害する[12]．またPTENはShcの脱リン酸化とそれに引き続くMAPK経路の阻害により細胞運動性を制御する[13]．PTENはMAPKの上流分子であるRasとIRS-1の不活性化，Erk上流分子であるFAKの脱リン酸化，下流分子である転写因子ETS-2，Sp1の阻害により，MAPK経路を多段階的に抑制する[4]．

3．核とPTEN

癌化の特徴の一つに，ゲノムと染色体の不安定化がある．多くの癌腫で核内のPTEN発現低下が悪性度と相関することが知られていたが[3]，核内PTENの役割については明らかにされていなかった．Pucらは，PTEN欠損ES細胞において，DNA二重らせん構造に間隙や破綻を伴う未修復の染色体が集積することを報告した[14]．PTEN欠損に伴いAKTが活性化され，活性化AKTはG2チェックポイント蛋白であるCHK1をリン酸化し核内移行を阻害することでDNA二重結合破綻を招く[14]（図3）．さらに最近，核内PTENはC2領域を介してセントロメアの動原体構成成分であるCENP-Cと結合し，フォスファターゼ活性非依存性に染色体統合性維持に機能すること[15]や，Rad51の発現促進を介してDNA二重結合破綻を抑制することが報告された[15]．またPTENは，c-MetやNF-κB，AP-1，HIF-1などの転写因子活性を抑制し[4,16]，さらにAPCと結合しAPC-CDH1複合体形成を促進することで，PLK-1やAURKsなどの腫瘍性蛋白質の分解を亢進させることが報告された[17]．PTENの核内局在制御機構について，NEDD4-1がPTENのC末領域をモノユビキチン化することで，PTENの核内移入を促進することが報告された[18]．以上のように，これまでPTENは細胞質において自身の脂質フォスファターゼ活性によりPI3K-AKT-mTOR経路を負に制御することが，癌抑制遺伝子として機能するための主徴であると考えられてきたが，核内PTENの新たな機能が発見されたことで，PTENの役割はさらに広範囲にわたることが明らかになった．

4．PTENと*p53*

*p53*は種々の癌腫で最も高頻度に変異のみられる癌抑制遺伝子で[19]，細胞周期制御，アポトー

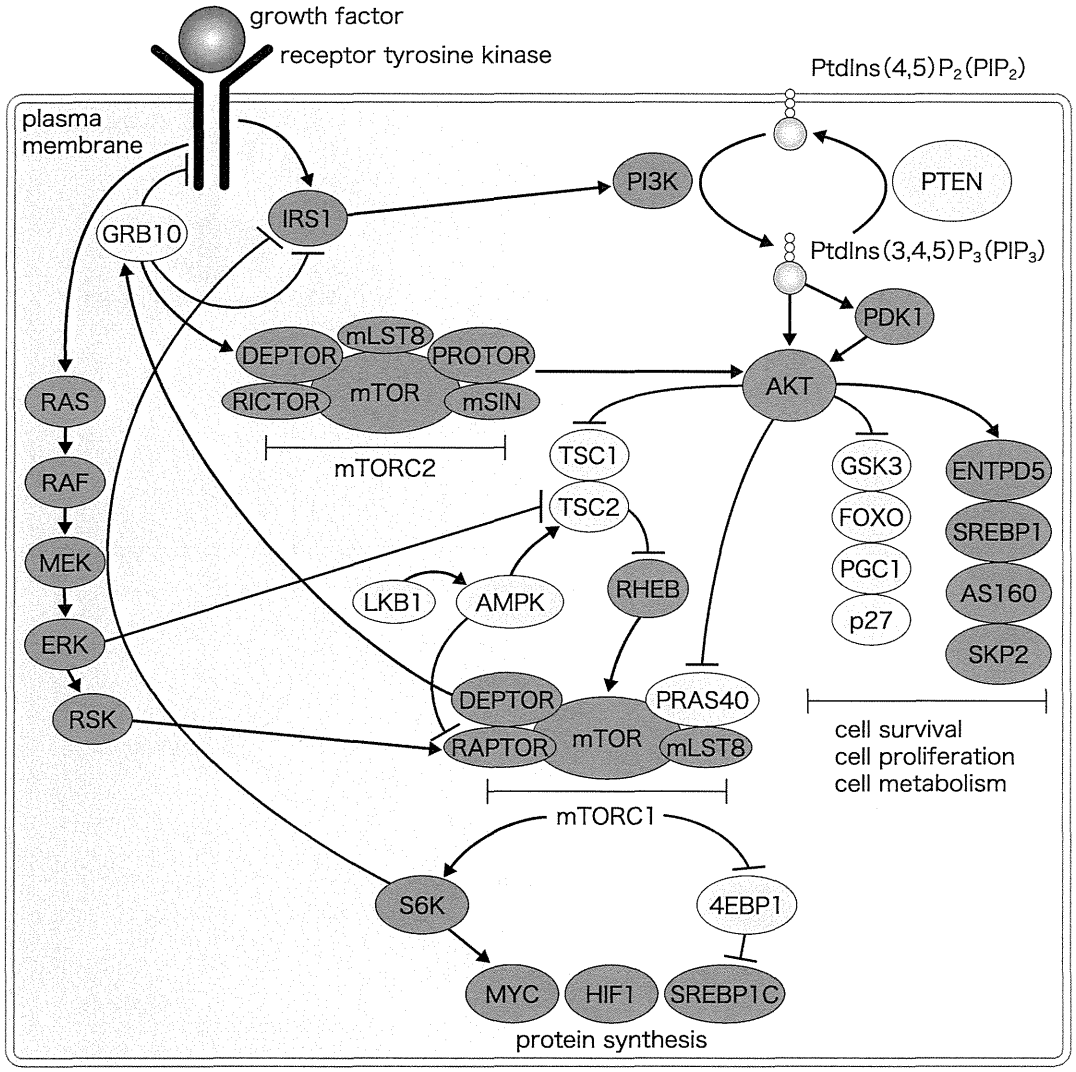

図2　PTEN-PI3K-AKT-mTOR経路

(Song MS, et al. Nat Rev Mol Cell Biol. 2012; 13: 283-96[3] より改変)

シス，ゲノム安定性を制御する．興味深いことに，癌腫での*p53*変異と*PTEN*変異は相互排他的であることから，これらの癌抑制遺伝子は相補的に機能していることが示唆される．また，p53は定常状態では発現量は低く，ストレス刺激下で発現が増加する一方，PTEN発現は定常状態で発現が高いことから，p53は動的に，PTENは静的・連続的に癌抑制遺伝子として機能することが示唆される．p53はPTENの発現を増加させる一方[20]，PTENはp53を安定化と転写活性亢進に働くことから[21,22]，これら2つの主要な癌抑制遺伝子は互いに正に制御し合うことが推察される．一方，PTENの完全欠損下ではp53発現が増加し細胞老化を誘導すること[23,24]，p53とPTEN二重欠損下では細胞老化が起こらず細胞増殖が亢進することから，p53とPTENは癌進展抑制とゲノム統合性維持に関して二重安全装置として機能することが示唆される．

5. PTENの細胞間作用

細胞は，自身の細胞内でのみ作用する酵素を合成すると考えられてきた．エキソソームは径30

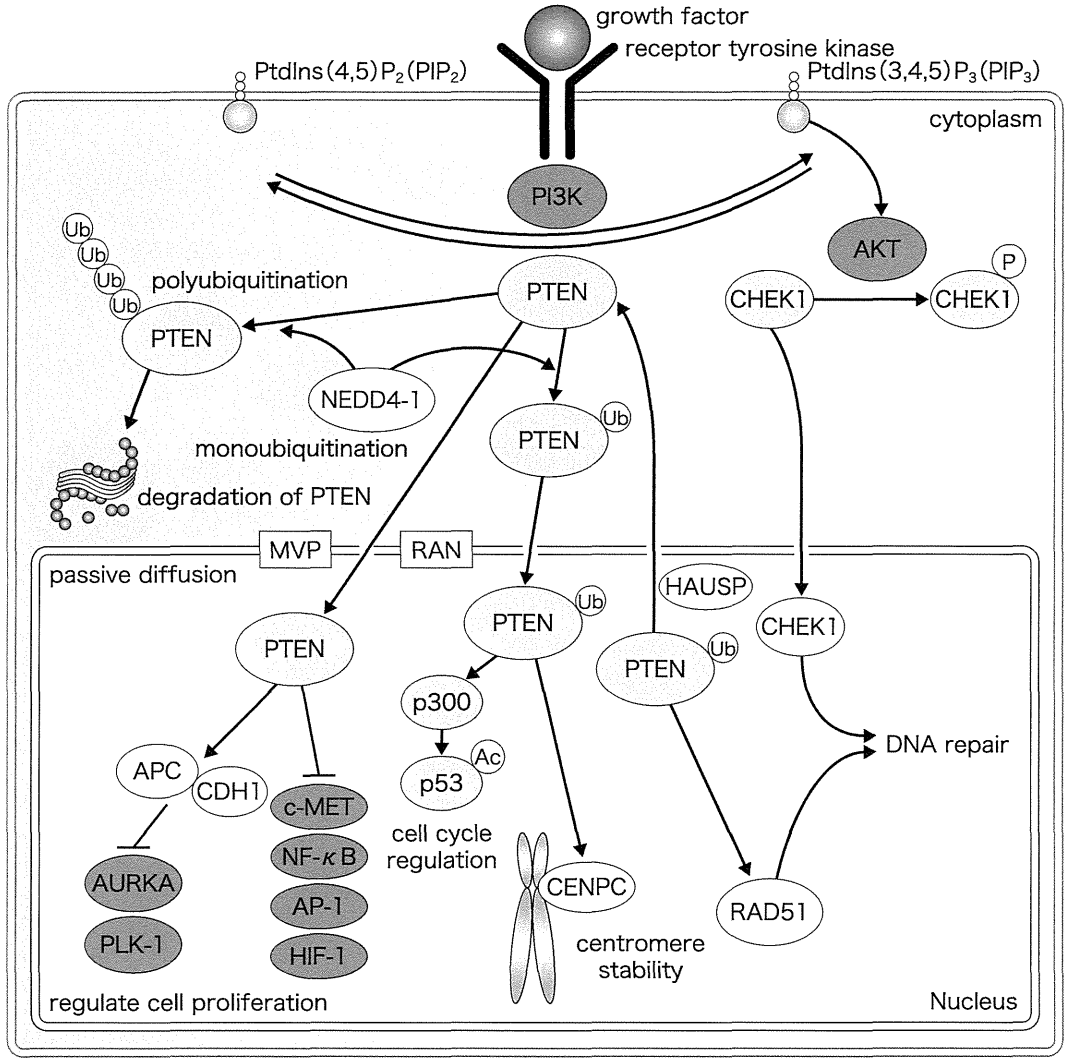

図3　核でのPTENの機能

(Song MS, et al. Nat Rev Mol Cell Biol. 2012; 13: 283-96[3]より改変)

～100nmのエンドソーム由来の微小胞で，細胞内から細胞外に分泌され，その内容物である蛋白，脂質，DNA，microRNAs（miRNA）などが隣接するレシピエント細胞に取り込まれ，その細胞の生理状態を変化させる．Putzらは，これまで自身が由来する細胞内での機能しか明らかにされていなかったPTENが，ドナー細胞からエキソソームによって細胞外に輸送され，レシピエント細胞にとり込まれた後にその細胞内のpAKTを低下させ，細胞増殖を抑制するという，PTENの非細胞自律的な作用様式を示す驚くべき結果を報告した[25]．さらにHopkinsらは，576アミノ酸残基からなり，細胞膜透過性を持つPTEN variant (PTEN-long) を同定した[26]．PTEN-longは細胞から分泌されレシピエント細胞に取り込まれることでPI3K-AKT経路を抑制し，腫瘍細胞死と*in vivo*での腫瘍縮小を導く[26]．これらの報告は，癌間質線維芽細胞特異的にPTENを欠損させた場合に，正常にPTENを発現する線維芽細胞の場合と比較して癌細胞増殖が亢進するという結果[27]の機序を説明するものである．PTENの細胞間作用機構という新たな概念と癌治療標的を提唱する