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Biochemical and Biophysical Research Communications 303 (2003) 751–755

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Effect of chronic treatments with ghrelin on milk secretion in lactating rats

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Received 4 February 2003

Abstract

Ghrelin, a novel acylated peptide and endogenous ligand for growth hormone (GH) secretagogue receptor, was originally isolated from rat and human stomachs. In addition to its GH-releasing activity, ghrelin plays an important role in many physiological functions, including food intake, gastric acid secretion, neonatal development, and so on. In this study, the effect of daily treatment with ghrelin on milk production was investigated in lactating rats and the development of the pups was monitored. Daily subcutaneous injection of ghrelin into nursing dams for 8 days from parturition caused a significant increase in milk yield and litter weight gain. When litters nursed by ghrelin-treated and saline-treated dams were interchanged on day 4 of lactation, the growth curves were reversed. Daily injections of ghrelin also increased plasma GH levels. Northern blot analysis revealed that daily injection of ghrelin significantly increased mammary casein mRNA expression. In addition, RT-PCR analysis showed that a ghrelin receptor was present in the mammary glands of lactating rats. These results suggest that ghrelin may play an important role in milk production in lactating dams.

Keywords: Casein mRNA; Ghrelin; GH; Lactation; Milk secretion; Prolactin

Ghrelin has recently been purified from rat and human stomachs as an endogenous ligand for growth hormone (GH) secretagogue receptor (GHS-R) and is a 28-amino-acid peptide with *n*-octanoylation at Ser3 residue [1]. This octanoylation is important for stimulation of GH secretion from the pituitary gland [1]. Although cells immunostained for ghrelin are distributed widely in the stomach, hypothalamus, pituitary gland, liver, kidney, pancreas, and placenta, the main source of circulating ghrelin is considered to be the gastrointestinal tract [2–6]. Indeed, both acyl and desacyl ghrelin levels were significantly higher in the gastric vein than in the trunk [7]. When the concentration of ghrelin in peripheral blood increases, stomach ghrelin levels decrease. Several studies have been conducted on the physiological function of ghrelin. In addition to stimulation of GH secretion in vivo and in vitro [8–10], ghrelin was reported to stimulate food intake and body weight gain when administered peripherally or centrally to rodents [11,12]. It is likely that the appetite-stimulating effect of peripheral ghrelin is due to action via the afferent vagal nerve [13]. On the other hand, the central effect is thought to be via neuropeptide Y and aguti-related peptide secretion from the arcuate nucleus in the hypothalamus [11,14]. Administration of ghrelin continuously to rodents resulted in fat deposition and obesity [12]. These effects of ghrelin on appetite and fat deposition are counteracted by leptin [11]. These results imply that ghrelin may play an important role in the regulation of food intake and energy expenditure.

It has been also demonstrated that ghrelin might be involved in stomach motility [15], gastric acid secretion [16], insulin and gastrin release [17], and the

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0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00414-5

cardiovascular system and stress reactions [18]. In addition, ghrelin might be involved in the regulation of sexual maturation in female rats and in neonatal development [19]. It is likely that some of these effects are not due to the direct action of ghrelin but to indirect action through GH secretion. As GH seems to play an important role in mammogenesis and lactogenesis in laboratory animals and humans [20–22], circulating ghrelin may affect milk production in lactating rats by increasing GH secretion. If milk secretion is stimulated by ghrelin, the body weight gain of pups nursed by ghrelin-treated dams would be expected to be higher than that of pups nursed by untreated dams. Therefore, the effect of chronic treatment with ghrelin on milk secretion and body weight gain was investigated in rats. Circulating plasma GH and prolactin levels, and mammary casein mRNA were compared in ghrelin-treated and saline-treated dams. Ghrelin receptor mRNA expression in the mammary gland was also investigated.

Materials and methods

Animal experiments. Wistar rats (Charles River Japan, Shiga, Japan) were kept under a regimen of 12 h light and 12 h darkness (lights on at 07:00 h) and a temperature of 23 ± 1 °C. Animals were supplied with standard laboratory chow and water ad libitum. The rats were mated at about 3 months of age. After parturition, the number of pups per mother was adjusted to 10 (five males and five females) within 24 h. The day of parturition was counted as day 0 of lactation. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

To examine the effect of daily injection of ghrelin on milk yield and litter weight gain, ghrelin (1.5 nmol/100 μl saline, $n = 8$) or saline ($n = 8$) was injected subcutaneously, twice a day (08:00 and 17:00 h) for 8 days, beginning on day 1 of lactation. Half of the pups were interchanged between ghrelin-treated and saline-treated dams on day 4 of lactation. The body weights of the pups and dams and the food intake of the dams were measured at 08:00 h every day. The daily milk yield was calculated as described previously by Sampson and Jansen [23]. Each experiment was repeated twice independently.

Ghrelin or saline was also injected into nursing dams as described above for measurement of plasma GH and prolactin levels. Blood samples were obtained from the dams after decapitation at 09:00 h on day 4 or day 8 of lactation for determination of plasma GH and prolactin, and lumbar mammary gland samples were obtained for Northern blot analysis of casein mRNA and RT-PCR analysis of GHS-R mRNA. Stomach samples were collected simultaneously from all pups and the stomach contents were removed carefully for weighing. Plasma GH and prolactin concentrations were determined with a Biotrak Rat GH RIA kit (Amersham, Buckinghamshire, UK) and rat prolactin RIA kit (Biocode S.A, Liege, Belgium). The data were expressed as means ± SEM and were analyzed by ANOVA and post hoc Fisher's test.

Northern blot analysis of casein mRNA. After sacrifice, mammary glands were collected immediately. Total RNA was extracted with Trizol (Life Technologies, Grand Island, NY). One microgram of total RNA was denatured with 16 ml of 1 M glyoxal and 50% dimethyl sulfoxide and electrophoresed on a 0.8% agarose gel (FMC Bio Products, Rockland, ME) in 10 mM sodium phosphate buffer (pH 7.0). The sample was then transferred to a Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA) and fixed by UV irradiation. The probes used for Northern blot analysis were a cDNA fragment of rat casein (sense primer; 5′-ATAAATTTCACTCCGGCATTCAGTCA GA-3′ and antisense primer; 5′-GATTTGAGAGTTAAAGAGGC GGAGCAC-3′), and a 203-bp cDNA of GAPDH (sense primer; 5′-A CCACAGTCCATGCCATCAC-3′ and antisense primer; 5′-TCCACC ACCCTGTTGCTGTA-3′). The membrane was first treated for 2 h at 37 °C in 6× SSC (900 mM NaCl, 60 mM NaH_2PO_4/H_2O, and 7 mM EDTA, pH 7.4) containing 40% formamide, 1× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml denatured salmon sperm DNA, and then hybridized for 18 h at 42 °C in identical solution containing ^{32}P-labeled casein and GAPDH cDNA probes. The RNA blot was washed with 2× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0)/0.1% SDS solution at 5 °C and exposed to film. Hybridization signals were measured in a Fujix Bio-image analyzer, FLA3000 (Fuji Photo Film, Tokyo, Japan). The amount of casein mRNA was calculated from the relative radioactivity of GAPDH.

RT-PCR for GHS-R. Total RNA was extracted from the mammary glands and stomach of three lactating rats on day 8 using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized from 2 μg of total RNA by random primer reverse transcription. The resulting cDNA was subjected to PCR amplification with the sense and antisense primers each at 2 μM and 2.5 U Taq polymerase (Promega, Madison, WI). The PCR primers specific for GHS-R were: sense; 59-GAGATCGCTCAGATCAGCCAGTAC-39 and antisense; 59-TAATCCCCAAACTGAGGTTCTGC-39 (nucleotides 880–903 and 1170–1192, Accession No. AB001982, GenBank). The reaction volume was 25 μl and the PCR conditions were 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 65 °C, and extension for 1 min at 72 °C. The PCR products were electrophoresed on a 2% agarose gel.

Results and discussion

Daily administration of ghrelin into dams in the early stages of lactation stimulated the body weight gain of pups (Fig. 1A) and suppressed that of the dams (Fig. 1B). When half of the pups were interchanged between ghrelin-treated and saline-treated dams on day 4 of lactation, the body weight gain of pups newly nursed by ghrelin-treated dams from day 4 was greater than that of pups that had not been interchanged. Conversely, the body weight gain of pups newly nursed by saline-treated dams from day 4 was less than that of pups that had not been interchanged and had been nursed by ghrelin-treated dams (Fig. 1A). Daily administration of ghrelin also significantly increased the food intake of lactating dams (Fig. 1C). The weights of the stomach and its contents of pups nursed by ghrelin-treated dams were significantly greater at day 4 and day 8 than those of pups nursed by saline-treated dams (Fig. 2A). Milk yield was also stimulated significantly in ghrelin-treated dams compared with that in saline-treated dams (Fig. 2B).

The relative levels of casein mRNA in the lumbar mammary glands of lactating rats injected with ghrelin for 8 days increased to almost double that in rats treated with saline (Fig. 3A). The GHS-R transcript product corresponding to the predicted 313-bp size was present in the mammary glands as well as in the stomach of lactating rats (Fig. 3B).

Circulating GH levels in dams treated with ghrelin were significantly higher than those in dams treated with

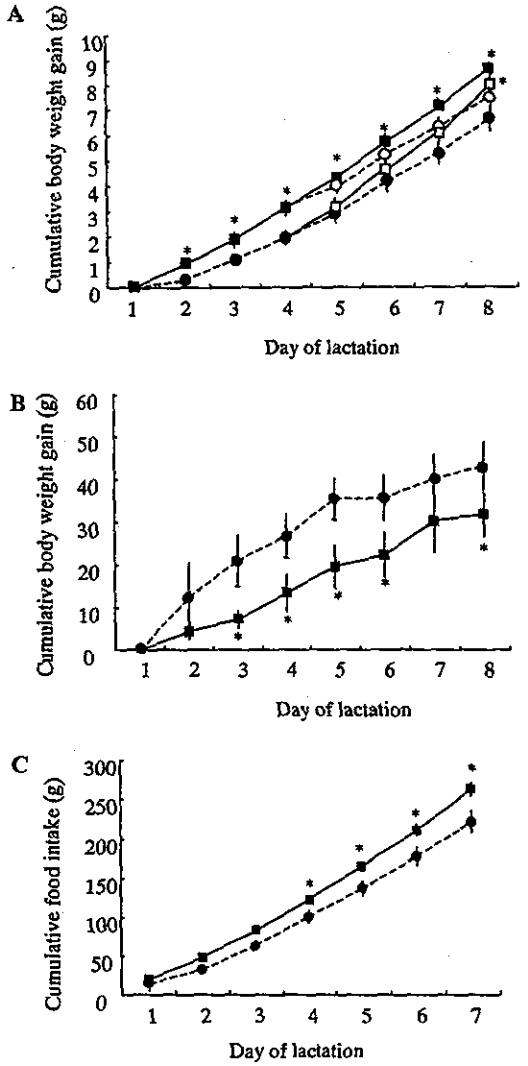

Fig. 1. Effect of daily treatment of nursing dams with ghrelin on body weight gain of pups and dams, and on food intake. (A) Weight of pups nursed with ghrelin-treated (■) or saline-treated dams (●) from day 1 to day 8 of lactation. Half of the pups were interchanged between ghrelin-treated and saline-treated dams on day 4 of lactation. Pups were transferred from saline-treated to ghrelin-treated dams (□) or ghrelin-treated to saline-treated dams (○). (B) Weight of ghrelin-treated (■) or saline-treated dams (●). (C) Food intake of ghrelin-treated (■) or saline-treated dams (●). Each symbol represents the mean value, vertical lines are ±SEM ((A) days 1–4, $n = 80$, days 5–8, $n = 40$; (B) $n = 8$; and (C) $n = 8$), and asterisks indicate significant differences ($P < 0.05$ vs. ●).

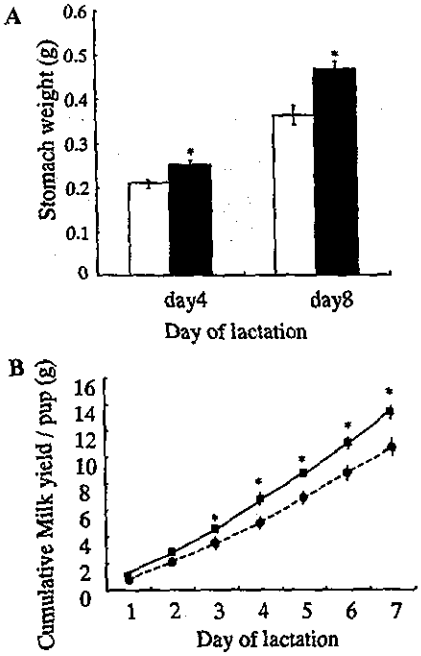

Fig. 2. Effect of daily treatment of nursing dams with ghrelin on the stomach weight of pups and milk yield. (A) Stomach weight of pups on day 4 and day 8 of lactation. Black and white bars represent the average stomach weight of pups nursed by ghrelin-treated and saline-treated dams, respectively. (B) Cumulative milk yield per pup, (■) ghrelin-treated dam, (●) saline-treated dam. Each symbol represents the mean value, vertical lines are ±SEM ((A) day 4, $n = 16$, day 8, $n = 24$; (B) $n = 40$), and asterisks indicate significant differences ($P < 0.05$).

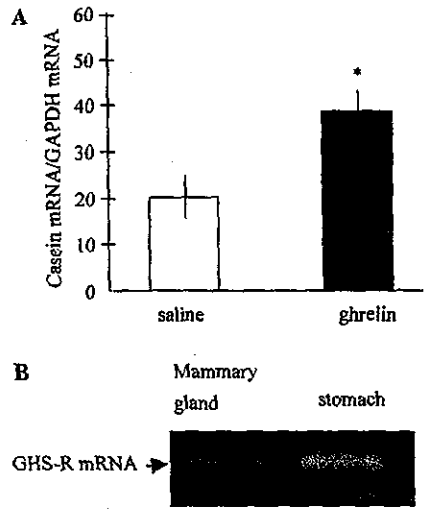

Fig. 3. (A) Effect of daily treatment with ghrelin or saline on the expression of mammary casein mRNA in nursing dams. The data are represented as values relative to GAPDH mRNA. Each bar and vertical lines represent the mean values ± SEM ($n = 8$). (B) Mammary gland cDNA fragments amplified by PCR in the presence of oligonucleotide primers specific for ghrelin receptor (GHS-R).

saline on days 4 and 8 of lactation (Fig. 4A). Although prolactin levels were increased by daily administration of ghrelin, the difference between the two groups was not significant (Fig. 4B).

In the present study, daily subcutaneous injections of ghrelin into nursing dams significantly increased milk secretion and litter weight gain from day 3 of lactation. As casein mRNA levels in the mammary gland were also increased by daily treatment with ghrelin, ghrelin may stimulate the de novo synthesis of milk. The weight of the stomach and its milk content also increased significantly in pups nursed by ghrelin-treated dams. Part of this significant increase in body weight gain seen in pups

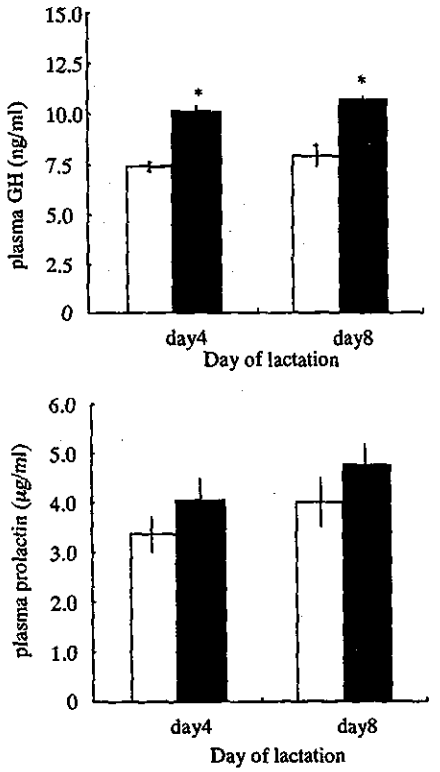

Fig. 4. Effect of daily treatment of nursing dams with ghrelin or saline on plasma GH (upper panel) and prolactin (lower panel) levels in the dams. Black and white bars represent the mean values for ghrelin-treated and saline-treated dams, respectively. Each bar and vertical lines represent mean values ±SEM ($n = 8$). The asterisks indicate significant differences ($P < 0.05$ vs. saline-treated dams).

nursed by ghrelin-treated dams may be attributable to the significant increase in their stomach contents [23]. In spite of the increased food intake, the body weight gain of ghrelin-treated dams was suppressed significantly compared with saline-treated dams. In general, the body weight gain of dams decreases during lactation, due to the consumption of large amount of nutrients to produce milk [24]. To maintain the increased milk production, ghrelin-treated dams may consume larger amounts of nutrients than saline-treated dams. In addition, it has been reported that GH regulates lipid metabolism in adipose tissue to utilize nutrients in the mammary glands of lactating rats [25]. Therefore, ghrelin-induced GH may act indirectly to decrease body weight gain during lactation.

Ghrelin-treated dams showed high levels of plasma GH. RT-PCR analysis revealed that GHS-R was present in the mammary glands of lactating rats. These two results imply two possible mechanisms of action of ghrelin on milk synthesis, a direct and an indirect action.

It is well known that exogenous GH administration increases milk secretion [20–23]. When antiserum to rat GH was administered to lactating rats on day 14 of lactation, litter weight gain decreased through a decrease in milk secretion and then recovered after GH treatment [20]. In domestic animals, such as pigs and ruminants, GH has often been used as a galactopoietics hormone [21,26]. Daily subcutaneous injection of GH-stimulated milk production in lactating women [22]. In addition, IGF-1, a mediator of action of GH, has a mammogenic and inhibitory function on mammary cell apoptosis [27,28]. These findings imply that ghrelin-induced GH could stimulate milk production in nursing dams. It has been demonstrated that the lactogenic action of GH may be indirect in the early lactation of rats and may be ancillary to prolactin [20]. In this study, however, daily subcutaneous injections of ghrelin dramatically increased litter weight without a significant increase in prolactin release. Therefore, ghrelin-stimulated milk production and litter weight gain may not only be due to stimulation of GH.

As it is unknown whether the mother's ghrelin or GH is secreted into the milk, the possibility that administered ghrelin itself or ghrelin-stimulated GH in the milk acted on the development of the pups cannot be excluded. It has already been reported that administration of ghrelin increases plasma GH in neonatal rats [19], therefore pup weight gain might be increased if ghrelin was secreted into the milk.

It is well known that mammary blood vessels develop and extend during lactation as milk production requires a large blood supply. Ghrelin administered into lactating dams may stimulate milk synthesis by increasing blood flow. Ghrelin is involved in regulation of the cardiovascular system in rats and humans. Ghrelin peptide and its binding sites have been detected in cardiovascular systems, including the heart, placenta, kidney, and blood vessels [18,29,30]. Hexarelin, an analogue of GHRP-6 and a potent GH secretagogue, decreased peripheral blood vessel resistance and increased cardiac output in rats [31]. Nagaya et al. [18] reported that ghrelin decreased mean arterial pressure without any significant change in the heart rate of healthy volunteers. In addition these authors also demonstrated the expression of GHS-R mRNA in the rat aorta and human heart [29]. These findings imply that ghrelin may have a vasodilative effect through a GH-independent mechanism and that it may increase blood inflow into the mammary gland.

Acknowledgments

This research was supported by the Japan Livestock Technology Association and Danon Institute for Promotion of Health and Nutrition.

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Optimum Collection and Storage Conditions for Ghrelin Measurements: Octanoyl Modification of Ghrelin Is Rapidly Hydrolyzed to Desacyl Ghrelin in Blood Samples, *Hiroshi Hosoda,[1,3] Kentaro Doi,[1] Noritoshi Nagaya,[2] Hiroyuki Okumura,[2] Eiichiro Nakagawa,[2] Mitsunobu Enomoto,[2] Fumiaki Ono,[2] and Kenji Kangawa[1,3*]* ([1] Department of Biochemistry, National Cardiovascular Center Research Institute, and [2] Department of Internal Medicine, National Cardiovascular Center, Osaka 565-8565, Japan; [3] Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan; * address correspondence to this author at: Department of Biochemistry, National Cardiovascular Center Research Institute, National Cardiovascular Center, Osaka 565-8565, Japan; fax 81-6-6835-5402, e-mail kangawa@ri.ncvc.go.jp)

Ghrelin is an acylated peptide with growth-hormone-releasing activity *(1)*. It was first isolated from rat and human stomach during the search for an endogenous ligand to the "orphan" G-protein-coupled receptor, growth hormone secretagogue receptor *(2)*. The peptide contains 28 amino acids, and n-octanoylation of the Ser-3 hydroxyl group is necessary for biological activity. Most studies have focused on the somatotropic and orexigenic roles of ghrelin; therefore, little is known about the kinetics of this peptide. Because the ester bond is both chemically and enzymatically unstable, elimination of the octanoyl modification of ghrelin can occur during storage, handling, and/or dissolution in culture medium *(3)*. Because of increased interest in ghrelin measurements, a standardized method of sample collection is required.

In the present study, which focused on the active form of ghrelin, we investigated the effects of anticoagulants and storage conditions on ghrelin stability. To distinguish the active form of ghrelin, we established two ghrelin-specific RIAs; N-RIA recognizes the N-terminal, octanoyl-modified portion of the peptide, whereas C-RIA recognizes the C-terminal portion. Thus, the value determined by N-RIA specifically measures active ghrelin, whereas the value determined by C-RIA gives the total ghrelin immunoreactivity, including both active and desacyl ghrelin *(4–6)*. The minimum detectable quantities in the N- and C-RIAs were 5.0 and 50 pmol/L, respectively. The respective intra- and interassay CV were 3% and 6% for the N-RIA and 6% and 9% for the C-RIA (n = 8 assays). Data are reported as the mean (SD). Comparisons of the time course of ghrelin concentrations between subgroups were made by two-way ANOVA for repeated measures, followed by the Scheffé test. $P < 0.05$ was considered statistically significant.

All blood samples were taken from three healthy male volunteers who gave written informed consent. Blood was taken from the forearm vein and immediately divided into tubes for serum and plasma preparation using (*a*) disodium EDTA (1 g/L) with aprotinin (500 000 kIU/L), (*b*) disodium EDTA alone, (*c*) heparin sodium, or (*d*) no anticoagulant. Synthetic human ghrelin was added to each blood sample at a final concentration of 40 μg/L; each sample was then sequentially divided into two aliquots for incubation at either 4 or 37 °C. After incubation for 0, 30, and 60 min, blood samples were centrifuged, diluted 1:200 in RIA buffer, and subjected to ghrelin-specific RIAs. A comparison of the effects of different anticoagulants on the detected ghrelin concentrations is shown in Table 1A. Although the serum and three different plasma samples tested gave comparable results for total ghrelin by C-RIA, the N-RIA gave ghrelin concentrations that were significantly decreased at 37 °C. When the ghrelin was measured by N-RIA, serum samples were highly affected by such treatment; samples stored for 60 min at 37 °C lost ~35% of the ghrelin compared with the basal values at 0 min ($P < 0.05$). The ghrelin concentrations in samples containing heparin as an anticoagulant were also significantly decreased ($P < 0.05$). When EDTA–aprotinin was used as the anticoagulant for plasma treatment, the decreases in ghrelin stability were smaller than for other procedures. Storage at 4 °C also improved ghrelin stability.

To explore optimum storage conditions, we examined the effect of plasma pH on ghrelin stability. The EDTA–aprotinin-treated plasma (n = 3) was divided into five samples; the pH was then adjusted to 3, 4, 5, 6, or 7.4 with 1 mol/L HCl. Synthetic human ghrelin was then added to each sample aliquot at a final concentration of 75 μg/L. Each of the five plasma aliquots was then subdivided into two, with one stored at 4 °C and the other stored at 37 °C. The effects of acidification on ghrelin stability in plasma are summarized in Table 1B. When stored at 37 °C, ghrelin concentrations measured by N-RIA gradually decreased at all pH values tested. However, ghrelin was most stable in highly acidified plasma samples (pH 3–4). At pH 3–5 and a storage temperature of 4 °C, the stability of ghrelin in plasma did not change significantly over a 6-h period. By C-RIA, ghrelin concentrations remained stable across the different pH and storage temperature conditions.

We then evaluated the effects of repeated freezing and thawing on the stability of ghrelin. EDTA–aprotinin-treated plasma samples were divided into two pH groups; one was acidified to pH 4, whereas the other was not acidified (pH 7.4). After the addition of synthetic human ghrelin (75 μg/L), we subjected the samples to four freeze–thaw cycles. Repeated freezing and thawing also influenced ghrelin stability (Table 1C). As in the N-RIA, ghrelin concentrations in untreated plasma samples decreased significantly with each successive freeze–thaw cycle, whereas the ghrelin remained relatively stable after acidification. Ghrelin concentrations by C-RIA were unchanged despite repeated freeze–thaw treatments in both acidified and untreated plasma samples.

As well as differences in assay methodologies, differences in sample handling, such as the method of storage, effects of anticoagulants, or previous freezing and thawing of the samples, could influence the reported values *(7–10)*. Instability of peptides and proteins can be divided into two forms: chemical and physical instability *(11, 12)*.

Table 1. Effect of anticoagulants and storage conditions on ghrelin stability.[a]

A. Ghrelin measurements in serum and different plasma samples

		Mean (SD) percentage of baseline					
		C-RIA			N-RIA		
		0 min	30 min	60 min	0 min	30 min	60 min
EDTA–aprotinin	37°C	100.0 (6.2)	101.0 (4.4)	102.9 (10.4)	100.0 (9.1)	102.6 (5.1)	89.6 (1.8)
	4°C		100.4 (4.0)	101.1 (4.5)		97.4 (1.3)	99.9 (12.3)
EDTA	37°C	100.0 (7.4)	98.5 (5.4)	98.4 (3.8)	100.0 (3.7)	83.6 (9.9)	85.3 (3.3)
	4°C		98.2 (2.6)	98.6 (7.6)		96.5 (1.8)	88.3 (6.5)
Heparin	37°C	100.0 (10.0)	104.3 (6.6)	91.1 (13.1)	100.0 (5.0)	88.1 (2.3)	77.4 (2.2)[b]
	4°C		104.4 (6.2)	102.1 (12.2)		92.5 (0.8)	86.9 (4.3)
Serum	37°C	100.0 (9.9)	100.4 (10.3)	98.2 (11.8)	100.0 (10.9)	87.5 (1.3)	65.1 (6.9)[b]
	4°C		96.2 (7.1)	98.4 (8.7)		96.5 (0.8)	94.6 (7.4)

B. Effects of storage pH, duration, and temperature on ghrelin stability

			Mean (SD) percentage of baseline					
		pH	0 h	1 h	2 h	3 h	4 h	6 h
C-RIA	RT	7.5	100.0 (6.6)	95.7 (5.0)	95.5 (6.3)		98.5 (5.4)	102.1 (6.5)
		6	100.0 (5.2)	96.0 (5.3)	91.3 (8.2)		96.6 (6.1)	97.9 (4.6)
		5	100.0 (6.1)	101.9 (8.2)	99.0 (6.0)		100.3 (8.4)	104.1 (5.2)
		4	100.0 (9.2)	96.0 (6.4)	98.0 (3.5)		100.2 (2.9)	97.1 (3.4)
		3	100.0 (2.2)	96.1 (4.9)	95.6 (4.7)		95.2 (2.7)	89.8 (3.9)
	4°C	7.5	100.0 (2.0)			100.9 (8.3)		99.3 (3.3)
		6	100.0 (3.6)			95.1 (4.1)		98.1 (1.8)
		5	100.0 (4.0)			101.2 (7.8)		105.9 (5.1)
		4	100.0 (7.1)			99.1 (3.3)		99.1 (4.1)
		3	100.0 (4.2)			99.4 (1.7)		101.3 (5.0)
N-RIA	RT	7.5	100.0 (5.0)	81.8 (1.0)[c]	72.0 (3.1)[d]		50.6 (2.9)[d]	37.5 (2.6)[d]
		6	100.0 (8.8)	93.3 (5.7)	78.8 (2.7)[c]		54.7 (1.2)[d]	38.8 (2.9)[d]
		5	100.0 (12.8)	92.8 (4.1)	86.4 (2.2)		74.1 (3.8)[b]	59.2 (7.6)[c]
		4	100.0 (12.5)	94.5 (7.4)	90.8 (5.2)		78.8 (3.1)[b]	74.0 (9.8)[c]
		3	100.0 (6.0)	98.4 (1.9)	96.9 (0.9)		82.2 (2.4)[b]	76.4 (6.1)[c]
	4°C	7.5	100.0 (6.9)			66.5 (4.4)		40.4 (3.5)[b]
		6	100.0 (6.1)			96.3 (0.2)		83.0 (1.2)[b]
		5	100.0 (5.5)			103.2 (2.0)		87.7 (11.7)
		4	100.0 (0.8)			102.2 (0.4)		99.6 (5.7)
		3	100.0 (12.8)			105.8 (0.3)		103.5 (1.6)

C. Effects of repeated freeze–thaw cycles on plasma ghrelin stability

		Cycles				
		1	2	3	4	5
C-RIA	HCl (−)	100.0 (8.8)	94.7 (7.2)	92.8 (6.6)	95.5 (5.1)	91.2 (7.3)
	HCl (+)	100.0 (5.2)	96.0 (5.3)	91.3 (8.2)	96.6 (6.1)	97.9 (4.6)
N-RIA	HCl (−)	100.0 (4.0)	89.8 (2.7)[b]	59.9 (6.3)[d]	28.1 (5.2)[d]	14.5 (3.3)[d]
	HCl (+)	100.0 (4.3)	94.1 (4.2)	94.4 (5.7)	95.4 (4.2)	93.8 (7.0)

[a] Results are for triplicate measurements. Values measured at 0 min, 0 h, or zero cycles are the baseline values.
[b–d] Compared with baseline: [b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$.

The chemical degradation of peptides is influenced by the pH of the aqueous solution; human parathyroid hormone and luteinizing-hormone-releasing hormone derivatives are examples *(13–15)*. We demonstrated that in whole blood and plasma, ghrelin is unstable. The degradation of octanoylated ghrelin was shown to be attributable to hydrolysis to desacyl ghrelin (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6/). Acidification is a simple, reliable procedure that protected against degradation of the acylated modification and dramatically improved stability at pH 4. On the other hand, the stability of the octanoyl modification of ghrelin was markedly decreased in strongly acidic (below pH 2), neutral, and alkaline solutions (data not shown).

We evaluated the effectiveness of measuring active ghrelin compared with total ghrelin in response to oral glucose tolerance tests (OGTTs). Four healthy male volunteers (age range, 28–35 years; body mass index, 21.5–23.7 kg/m^2) were examined on 2 separate days (100 g of glucose administered on 1 day, and 50 g of glucose administered on the other day) at least 2 weeks apart in a randomized, crossover study. After the volunteers fasted overnight, 50 or 100 g of glucose was administered orally between 0930–1000. Blood samples were obtained at 0, 1, 2, 3, and 4 h after glucose ingestion. To each plasma sample was added 1 mol/L HCl (10% of plasma volume), which acidified the sample to pH ~4; samples were then treated with Sep-Pak C_{18} cartridges for ghrelin RIAs. After glucose ingestion, the mean plasma ghrelin concentrations as determined by N-RIA and C-RIA decreased to a nadir at 1 h (Fig. 1). At this point, 60.3% and 73.0% of the basal concentration was detected by the N-RIA and C-RIA, respectively, after the 100-g OGTT, and 64.2% and 78.7% of the basal concentration was detected after the 50-g OGTT. Plasma ghrelin values increased thereafter, although plasma ghrelin concentrations measured by the C-RIA were significantly lower for up to 2 h after the 100-g glucose load. The N-RIA for ghrelin could detect differences in the changes in ghrelin concentrations between the 50-g and 100-g OGTTs at 3 h. The ghrelin values observed with the C-RIA exhibited changes similar those in the N-RIA, but the changes were small and delayed. These effects may be attributable to the differential rates of metabolic turnover for octanoylated and desacylated ghrelin in circulating blood (see Fig. 2 in the online Date Supplement).

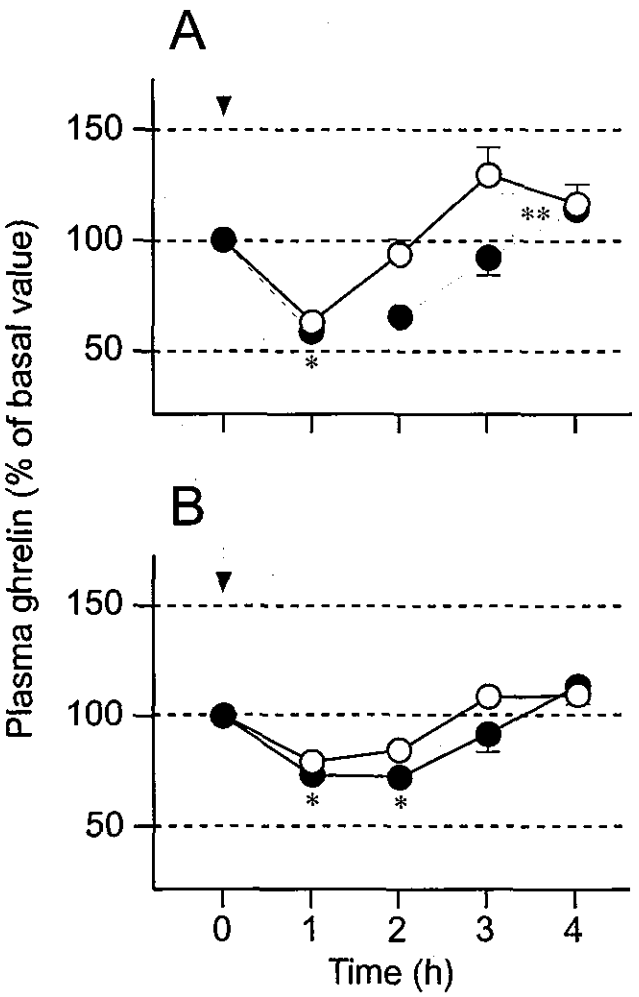

Fig. 1. Plasma ghrelin response to 50-g (○) and 100-g (●) OGTTs in four healthy individuals.

Plasma ghrelin concentrations assayed by N-RIA (*A*) and C-RIA (*B*) are given as the mean (SD; *error bars*) percentage change from basal values. *, $P < 0.05$ compared with basal values; **, $P < 0.05$ for difference in plasma ghrelin between 50-g and 100-g glucose loads.

The results for the plasma ghrelin response to the OGTTs show that measuring the concentration of active ghrelin is useful for studying plasma ghrelin changes over short time periods. Plasma concentrations of active ghrelin changed more rapidly and dynamically than those of total ghrelin immunoreactivity. Fasting led to markedly increased plasma ghrelin values as measured by N-RIA, and the values decreased in a clearer dose-dependent manner in rats after glucose injection compared with those measured by C-RIA *(16)*. The proportion of active ghrelin in plasma was 2–5% of total ghrelin in rodents. In this study, the quantity of active ghrelin was ~10% of the total ghrelin in human plasma (data not shown). These findings imply that inactive desacyl ghrelin circulates in the bloodstream at much higher concentrations than active ghrelin. Similar to previous studies in which ghrelin concentrations were measured by C-RIA *(17)*, desacyl ghrelin is relatively stable, and its stability is not altered by different storage conditions. An analogous situation has been reported for the activity of pancreatic beta cells, which secrete insulin and C-peptide in a 1:1 molar ratio. However, the half-life of C-peptide is much longer than that of insulin, leaving more C-peptide available in the circulation for quantification *(18, 19)*. Measurement of C-peptide provides an assessment of β-cell secretory activity. Similarly, desacyl ghrelin concentrations may serve as an indicator of ghrelin secretory function *(20)*.

To acquire accurate data on ghrelin concentrations, this study recommends a standard procedure for the collection of blood samples: (*a*) the collection of blood samples with EDTA–aprotinin is preferred; (*b*) blood samples should be chilled and centrifuged as soon as possible, at least within 30 min after collection; and (*c*) because acidification is the best method for the preservation of plasma ghrelin, 1 mol/L HCl (10% of sample volume) can be added to the plasma sample for adjustment to pH 4.

We thank H. Mondo and M. Miyazaki for technical assistance. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan; the Ministry of Health, Labor and Welfare of Japan; the Promotion of Fundamental Studies in Health Science

from the Organization for Pharmaceutical Safety and Research of Japan; and the Takeda Science Foundation.

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DOI: 10.1373/clinchem.2003.025841

Substitution of 3′-Phosphate Cap with a Carbon-Based Blocker Reduces the Possibility of Fluorescence Resonance Energy Transfer Probe Failure in Real-Time PCR Assays, *Kendall W. Cradic,[1] Jason E. Wells,[2] Lindsay Allen,[2] Kent E. Kruckeberg,[1] Ravinder J. Singh,[1] and Stefan K.G. Grebe[1,3]** (Departments of [1] Laboratory Medicine and Pathology and [3] Medicine, Mayo Clinic, Rochester, MN; [2] Idaho Technology Inc., Salt Lake City, UT; * address correspondence to this author at: Endocrine Laboratory, Hilton 730C, Mayo Clinic, 200 1st St. SW, Rochester, MN 55905; fax 507-284-9758, e-mail grebs@mayo.edu)

During the last decade, research and clinical use of real-time PCR applications has continued to grow in importance *(1)*. Many laboratories that use real-time PCR with fluorescent probes experience an unexplained loss of probe fluorescence at some stage, in particular with pairs of fluorescence resonance energy transfer (FRET) probes. Photobleaching is often assumed to be the cause. Structural integrity of the oligonucleotides is also a major factor, and its loss has been shown to correlate with repeated freeze–thaw cycles *(2)*. Laboratories guard against these two problems by aliquoting probes and protecting them from light. Despite these precautions, inexplicable FRET probe failures are still observed. In one recent such case, we were able to determine an additional mechanism for FRET probe failure: loss of the phosphate cap from the 3′ end of a probe. To our knowledge, this has not been described previously. Our studies revealed that this may be a common and important problem, intrinsic to 3′-phosphate-blocking chemistry. We also found that alternative terminating groups may be a preferable option to 3′-phosphate blocking.

A 3-nmol/L synthesis-scale LightCycler™ hybridization probe set was purchased from Idaho Technology Inc. Biochem in April 2003. As is common practice, the manufacturer produced a large-scale synthesis and, after shipping our order, archived the remainder for a possible future reorder. The first half of the batch (α-probe set) was sent immediately, whereas the second half (β-probe set) was stored lyophilized for 6 months at −20 °C and then shipped with our next order.

Oligonucleotides from the first shipment were used in PCR reactions in a LightCycler with satisfactory results. PCR conditions were as follows: 1× LightCycler FastStart DNA Master Hybridization Probe Mix (Roche Diagnostics), $MgCl_2$ (final concentration, 3.5 mM), 0.5 μM each of the forward (5′-GGCCTTTCTGAAGCAAG-3′) and reverse (5′-GACGATTTCTTATTTCACAGCTCC-3′) primers, 0.2 μM each of the donor (5′-GGACGCAGAGGGGATGG-FITC-3′, where FITC is fluorescein isothiocyanate) and acceptor (LCRed640-GTGTATGGGACCCGCCAG-phosphate) probes, and 2 μL of cDNA template mixture. The final reaction volume was 10 μL. The reaction started with an initial melting step at 95 °C for 10 min followed by 45 cycles of 95 °C for 2 s, 57 °C for 10 s, and 72 °C for 5 s.

Loss of fluorescence activity was first observed when we received the β-probe set. PCR reactions were carried out under the same conditions, but the amplification

Effects of Ghrelin Administration on Left Ventricular Function, Exercise Capacity, and Muscle Wasting in Patients With Chronic Heart Failure

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Background—Ghrelin is a novel growth hormone–releasing peptide that also induces vasodilation, inhibits sympathetic nerve activity, and stimulates feeding through growth hormone–independent mechanisms. We investigated the effects of ghrelin on left ventricular (LV) function, exercise capacity, and muscle wasting in patients with chronic heart failure (CHF).

Methods and Results—Human synthetic ghrelin (2 μg/kg twice a day) was intravenously administered to 10 patients with CHF for 3 weeks. Echocardiography, cardiopulmonary exercise testing, dual x-ray absorptiometry, and blood sampling were performed before and after ghrelin therapy. A single administration of ghrelin elicited a marked increase in serum GH (25-fold). Three-week administration of ghrelin resulted in a significant decrease in plasma norepinephrine (1132±188 to 655±134 pg/mL; $P<0.001$). Ghrelin increased LV ejection fraction (27±2% to 31±2%; $P<0.05$) in association with an increase in LV mass and a decrease in LV end-systolic volume. Treatment with ghrelin increased peak workload and peak oxygen consumption during exercise. Ghrelin improved muscle wasting, as indicated by increases in muscle strength and lean body mass. These parameters remained unchanged in 8 patients with CHF who did not receive ghrelin therapy.

Conclusions—These preliminary results suggest that repeated administration of ghrelin improves LV function, exercise capacity, and muscle wasting in patients with CHF. (***Circulation.* 2004;110:3674-3679.**)

Key Words: growth substances ■ heart failure ■ hormones ■ nutrition

Left ventricular (LV) remodeling (dilatation and wall thinning) and cardiac cachexia (body weight loss and muscle wasting) often are observed in patients with end-stage chronic heart failure (CHF).[1,2] Growth hormone (GH) and its mediator, insulinlike growth factor-1 (IGF-1), are anabolic hormones that are essential for skeletal and myocardial growth and metabolic homeostasis.[3,4] Earlier studies have shown that GH supplementation may have beneficial effects on LV myocardial structure and function in some patients with CHF,[5] although the importance of GH resistance[6] and neutral results of randomized trials also have been reported.[7,8]

Ghrelin is a novel GH-releasing peptide that was isolated from the stomach and has been identified as an endogenous ligand for the growth hormone secretagogue receptor.[9] Therefore, we believed that administration of ghrelin may induce beneficial changes in LV function and energy metabolism in patients with CHF via a GH-dependent mechanism. On the other hand, growth hormone secretagogue receptor mRNA is detected not only in the hypothalamus and pituitary but also in the heart and blood vessels,[10] implying direct cardiovascular effects of ghrelin. Wiley and Davenport[11] have demonstrated that ghrelin is an endothelium-independent vasodilator in isolated human arteries. We have shown that intravenous administration of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with CHF.[12] Furthermore, ghrelin induces a positive energy balance by stimulating food intake[13,14] and adiposity[15] through GH-independent mechanisms. These findings raise the possibility that ghrelin administration may have beneficial effects in cachectic patients with CHF. In fact, we recently have demonstrated that treatment with ghrelin improves not only LV function but also cardiac cachexia in rats with CHF.[16] In humans, however, the potential effects of ghrelin as a therapeutic agent for CHF remain unknown.

Thus, the purposes of this study were as follows: (1) to investigate whether repeated administration of ghrelin im-

Received April 30, 2004; revision received July 6, 2004; accepted August 23, 2004.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000149746.62908.BB

TABLE 1. Patient Characteristics

	Control Group (n=8)	Ghrelin Group (n=10)
Age, y	74±2	75±2
Sex, M/F	6/2	7/3
Body mass index, kg/m²	19.0±1.1	19.0±0.9
Cause of CHF, n		
Dilated cardiomyopathy	4	4
Ischemic cardiomyopathy	1	3
Hypertensive heart disease	2	1
Valvular heart disease	1	2
NYHA functional class, n		
III	8	9
IV	0	1
LVEF, %	28±2	27±2
Presence of cardiac cachexia, n	6	8
Medication use, n		
Digoxin	6	9
ACE inhibitors	7	9
A II blockers	2	2
β-Blockers	6	7
Diuretics	7	10

LVEF indicates LV ejection fraction; A II, angiotensin II. Data are mean±SEM.

proves LV myocardial structure and function in patients with CHF, (2) to examine whether ghrelin improves exercise capacity in such patients, and (3) to examine whether ghrelin induces anabolic effects in patients with CHF.

Methods

Study Subjects

Eighteen patients with CHF (13 men, 5 women; mean age, 75 years; range, 63 to 80 years) were included in this study. Inclusion criteria were as follows: (1) LV ejection fraction <35% as assessed by cardiac catheterization, (2) a stable clinical condition, and (3) clinical evidence of heart failure despite conventional therapy. Exclusion criteria were the presence of any of the following: (1) chronic renal impairment (serum creatinine level ≥2.0 mg/dL), (2) significant liver dysfunction, (3) evidence of malignant diseases, (4) active infection, (5) hematologic abnormalities, or (6) systolic blood pressure <90 mm Hg. Ten patients with CHF (ghrelin group) received repeated administrations of ghrelin. Although this study was neither randomized nor placebo controlled, 8 patients with CHF who did not receive ghrelin (control group) were studied to exclude time-course effects during hospitalization. Patients in the ghrelin group were admitted only for the study. Those in the control group had been in hospital for diagnostic examination and stayed for 3 weeks for the study. There was no significant difference in demographic, clinical, or hemodynamic data at baseline between the ghrelin and control groups (Table 1). Eight patients in the ghrelin group and 6 patients in the control group were defined as exhibiting cardiac cachexia, as reported previously.[17] The weight loss in cachectic patients amounted to 6.4±0.4 kg or 11.8±0.7% loss of previous body weight during 14±2 months. The ethics committee of the National Cardiovascular Center approved the study, and all patients gave written informed consent.

Preparation of Human Ghrelin

Human synthetic ghrelin was obtained from the Peptide Institute Inc. This peptide is not commercially available. Ghrelin was dissolved in distilled water with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Co). Ghrelin was stored in 2-mL volumes, each containing 200 μg ghrelin. The chemical nature and content of the human ghrelin in vials were verified by high-performance liquid chromatography and radioimmunoassay. All vials were stored frozen at −80°C from the time of dispensing until the time of preparation for administration.

Study Protocol

This study was performed while patients were in a stable clinical condition during hospitalization. Ghrelin (2 μg/kg, 10 mL solution) was administered intravenously over 30 minutes at a constant rate. The infusion was repeated twice a day (before breakfast and before dinner) for 3 weeks. Study patients in both groups remained hospitalized for 3 weeks. Echocardiography, cardiopulmonary exercise testing, dual x-ray absorptiometry, hand-grip test, and blood sampling were performed at baseline and after 3 weeks of treatment with ghrelin (ghrelin group) or without ghrelin (control group). Long-term medication, including digitalis, diuretics, ACE inhibitors, and β-blockers, was kept constant during this study protocol.

Echocardiographic Studies

Echocardiography was performed by an investigator blinded to treatment allocation. Two-dimensional targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 3.5-MHz sector scan probe (SONOS 2000, Hewlett Packard). LV wall thickness, dimensions, and fractional shortening were measured according to the recommendations of the American Society of Echocardiology from at least 3 consecutive cardiac cycles. LV end-diastolic volume, end-systolic volume, and ejection fraction were calculated with a modified version of Simpson's method.[18]

Cardiopulmonary Exercise Testing

Cardiopulmonary exercise testing was performed in all patients except 1, who underwent a 6-minute walk test as recommended by attending physicians. The patients exercised seated on a cycle ergometer. The work rate was then increased by 15 W/min up to their symptom-limited maximum. Breath-by-breath gas analysis was performed with an AE280 (Minato Medical Science).[19] Exercise capacity was evaluated by peak oxygen consumption (peak $\dot{V}o$). Ventilatory efficiency during exercise was represented by the $\dot{V}_E$–$\dot{V}_{CO_2}$ slope.[19]

Food Intake and Body Mass Analyses

Food intake for 3 consecutive days was assessed before ghrelin administration and during the last week of ghrelin therapy. Food intake was semiquantitatively assessed by a calorie count based on a 10-point scale method (0=null intake, 10=full intake or 1800 kcal), which was averaged for 3 days. Dual x-ray absorptiometry (DPX-L, Lunar Radiation) was repeated in all patients to examine changes in lean body mass, fat mass, and bone mineral content. Hand-grip strength was determined with a dynamometer.

Blood Sampling and Assay

Blood samples were taken from the antecubital vein the morning after an overnight fast. Serum GH and IGF-1 were measured by immunoradiometric assay (Ab Bead HGH Eiken, Eiken Chemical Co, Ltd, sensitivity=0.1 ng/mL; Somatomedin CII Bayer, Bayer Medical Ltd, sensitivity=0.3 ng/mL). Plasma norepinephrine and epinephrine were measured by high-performance liquid chromatography (HLC8030, Tosoh Co, sensitivity=6 pg/mL). Serum cortisol and insulin were measured by enzyme immunoassay (AIA-PACK CORT, sensitivity=0.2 μg/dL; AIA-PACK IRI, sensitivity=2.0 μU/mL, Tosoh Co). Serum tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were measured by enzyme immunoassay (Quantikine HS, R&D Systems Inc, sensitivity=0.18 pg/mL; TFB kit, TFB Co, Ltd, sensitivity=0.3 pg/mL). Plasma renin and aldosterone were measured with radioimmunoassay kits (RENIN RIABEAD, sensitivity=0.1 ng/mL; ALDOSTERONE RIAKIT II, sensitivity=2.0

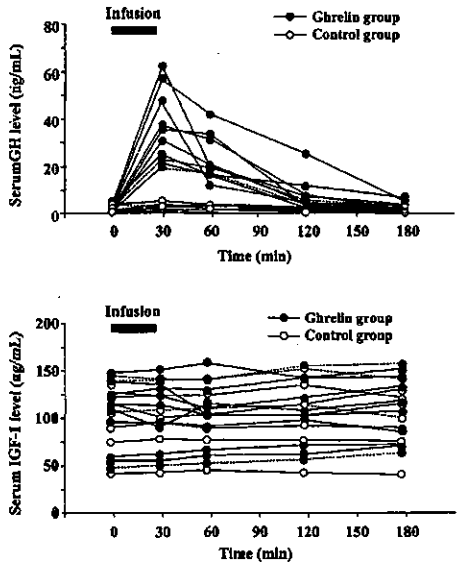

Figure 1. Changes in serum GH and IGF-1 after single administration of ghrelin. Solid line indicates cachectic patients; dotted line, noncachectic patients.

ng/dL, DAINABOT Co). Plasma brain natriuretic peptide (BNP) was measured by immunoradiometric assay (SHIONORIA BNP, sensitivity=4.0 pg/mL).

Statistical Analysis

Numerical values are expressed as mean±SEM. Comparisons of parameters between the 2 groups were made by unpaired Student's *t* test. Comparisons of the time course of serum GH and IGF-1 between the 2 groups were made by 2-way ANOVA for repeated measures, followed by the Newman-Keuls test. Comparisons of changes in parameters during the 3-week follow-up between the 2 groups were also made by 2-way ANOVA for repeated measures, followed by the Newman-Keuls test. A value of $P<0.05$ was considered significant.

Results

Administration of ghrelin transiently caused stomach rumbles in 6 patients and a slight feeling of being warm and sleepy in 4 subjects. Two patients felt slightly thirsty during ghrelin infusion. Other than these minor complaints, all subjects tolerated 3-week administration of ghrelin without incident. After 3-week administration of ghrelin, NYHA functional class improved in 4 patients and was unchanged in 6 patients. No change in NYHA functional class was observed in patients who did not receive ghrelin.

Effects of Ghrelin on Somatotropic Function

A single administration of ghrelin markedly increased serum GH level (baseline, 1.4±0.4; peak, 35.0±5.0 ng/mL; $P<0.001$; Figure 1). This elevation lasted >60 minutes after the start of ghrelin infusion. Serum IGF-1 level tended to increase 3 hours after the start of ghrelin infusion (101±12 to 110±12 ng/mL; $P=0.08$). Three-week administration of ghrelin tended to increase basal serum IGF-1 level (99±13 to 116±13 ng/mL; $P=0.07$). There was no significant difference in basal serum GH level between before and after 3 weeks of ghrelin therapy (2.0±0.8 to 1.2±0.3 ng/mL; P=NS).

Effects of Ghrelin on Food Intake, Body Weight, and Lean Body Mass

Administration of ghrelin significantly increased food intake (Figure 2). Three-week administration of ghrelin tended to increase body weight (49.6±2.7 to 50.4±2.7 kg; $P=0.09$). No development of edema was observed during ghrelin therapy. Dual x-ray absorptiometry demonstrated that treatment with ghrelin significantly increased lean body mass in patients with CHF (38.3±2.1 to 39.1±2.1 kg; $P<0.05$). Ghrelin did not significantly alter bone mineral content (2243±191 to 2265±189 g; P=NS) or fat mass (8877±1353 to 8748±1311 g; P=NS). Hand-grip strength was increased significantly by ghrelin therapy (20.5±1.7 to 22.7±2.0 kg; $P<0.01$). All of these parameters remained unchanged in patients who did not receive ghrelin.

Effects of Ghrelin on Cardiac Structure and Function

Neither heart rate nor blood pressure was significantly changed by 3-week administration of ghrelin (Table 2). Ghrelin increased LV ejection fraction (27±2% to 31±2%; $P<0.05$) in association with a decrease in LV end-systolic volume and an increase in LV mass (Figure 3), although ghrelin did not significantly alter LV end-diastolic volume. All of these parameters remained unchanged in patients who did not receive ghrelin.

Effects of Ghrelin on Exercise Capacity and Ventilatory Efficiency

Three-week administration of ghrelin significantly increased peak workload and peak $\dot{V}o$ during exercise (739±127 to 801±126 mL/min; $P<0.05$; Figure 4). Treatment with ghrelin did not significantly alter the $\dot{V}_E$–$\dot{V}co_2$ slope. In 1 patient

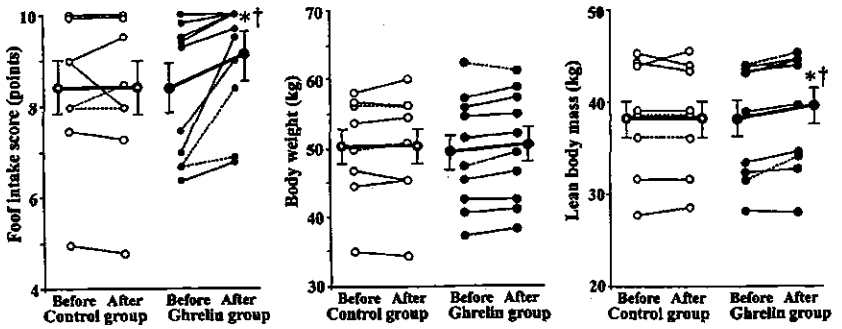

Figure 2. Food intake, body weight, and lean body mass before and after 3-week administration of ghrelin. Food intake was described semiquantitatively with 10-point scale method (0=null intake, 10=full intake). Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

TABLE 2. Physiological and Echocardiographic Measurements

	Control Group	Ghrelin Group
Heart rate, bpm		
Before	77±3	78±3
After	76±3	74±3
Mean arterial pressure, mm Hg		
Before	79±4	81±2
After	80±3	78±3
LVDd, mm		
Before	65.6±3.2	66.6±2.5
After	64.4±3.7	63.7±3.3
LVDs, mm		
Before	55.1±3.0	56.9 ± 2.9
After	53.9±3.6	52.8 ± 3.4*
FS, %		
Before	16.1±1.2	14.8±1.7
After	16.0±1.3	17.3±2.3
AWT diastole, mm		
Before	10.0±0.8	9.5±1.0
After	10.1±0.9	10.0±1.0*
PWT diastole, mm		
Before	9.2±0.4	9.3±0.6
After	9.4±0.4	9.9±0.5*†

LVDd indicates LV end-diastolic dimension; LVDs, LV end-systolic dimension; FS, fractional shortening; AWT, anterior wall thickness; and PWT, posterior wall thickness. Data are mean±SEM.

*$P<0.05$ vs before; †$P<0.05$ vs respective control group.

who did not undergo cardiopulmonary exercise testing, the distance walked in 6 minutes increased from 300 m to 410 m with ghrelin treatment. Exercise parameters remained unchanged without ghrelin.

Effects of Ghrelin on Sympathetic Nerve Activity

Three-week administration of ghrelin significantly decreased plasma norepinephrine and epinephrine (Figure 5). Treatment with ghrelin significantly decreased plasma BNP level (Table 3). Ghrelin did not significantly alter circulating glucose, insulin, cortisol, TNF-α, or IL-6. Neither plasma renin activity nor plasma aldosterone level was changed significantly. All of these parameters remained unchanged in patients who did not receive ghrelin.

Discussion

Ghrelin is a novel GH-releasing peptide that acts through a mechanism independent of that of hypothalamic GH-releasing hormone.[9] The GH-releasing effect of ghrelin has been shown to be more potent than that of GH-releasing hormone.[20] In fact, in the present study, ghrelin infusion elicited potent GH release in patients with CHF. Three-week administration of ghrelin increased LV ejection fraction in association with an increase in LV mass, which is consistent with findings from a previous experimental study in rats.[16] Plasma BNP level, a marker for LV function and wall stress, was decreased by ghrelin therapy. GH and its mediator, IGF-1, have been shown to enhance physiological compensatory hypertrophy in rats with CHF, resulting in a decrease in LV wall stress, leading to improvement in cardiac function.[21] Thus, ghrelin may also improve cardiac function partly through GH-dependent mechanisms. On the other hand, Baldanzi et al[22] have shown that ghrelin inhibits apoptosis of cardiomyocytes and endothelial cells through activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases. Furthermore, stimulation of GHS-R by hexarelin has been shown to prevent cardiac damage after ischemia-reperfusion in hypophysectomized rats.[23] When these results are considered together, improvement in cardiac function by ghrelin therapy may be related to direct effects of ghrelin on myocardium. Importantly, ghrelin significantly decreased plasma norepinephrine levels in the present study. It is possible that improvement in cardiac function may lead to attenuation of sympathetic nerve activity. Interestingly, a recent study has demonstrated that ghrelin acts directly on the central nerve system to decrease sympathetic nerve activity.[24] Thus, inhibitory effects of ghrelin on sympathetic nerve activity may contribute to a decrease in plasma norepinephrine, which may have beneficial effects on cardiac performance in patients with CHF.

In the present study, 3-week administration of ghrelin improved exercise capacity in patients with CHF, as indicated by an increase in peak workload and peak $\dot{V}o$. A decrease in peak $\dot{V}o$ in patients with CHF is attributable not only to an inadequate increase in cardiac output during exercise, which is a central effect, but also to muscle wasting, a peripheral effect. Recently, we have shown that infusion of ghrelin increases cardiac output in patients with CHF.[12] In the present study, ghrelin increased lean body mass and muscle strength. These results suggest that ghrelin may improve exercise capacity through both central and peripheral effects.

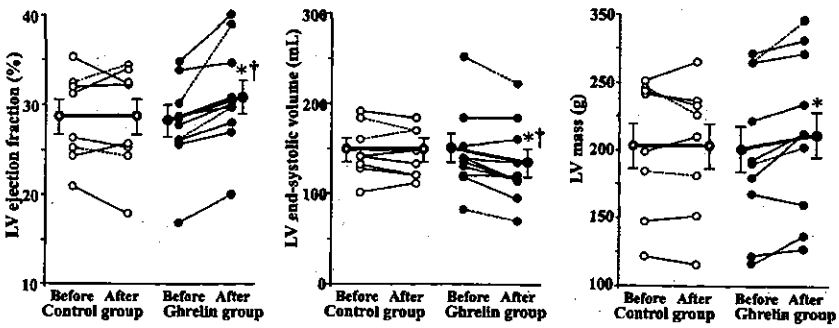

Figure 3. LV geometry and function before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

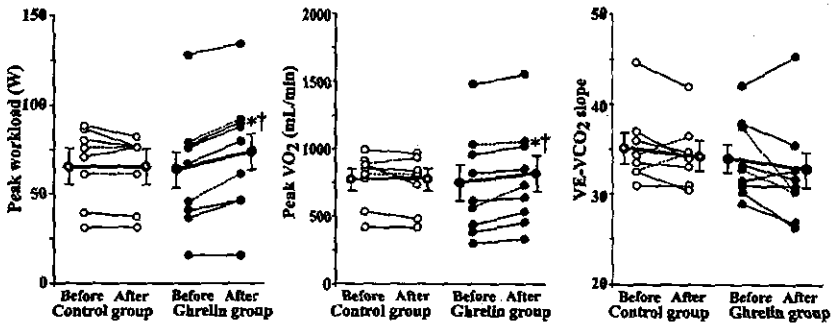

Figure 4. Exercise capacity and ventilatory efficiency before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

Cardiac cachexia, a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with end-stage CHF[25] and is a strong independent risk factor for mortality in such patients.[26] Recently, we have shown that plasma ghrelin level is increased in cachectic patients with CHF as a compensatory mechanism in response to anabolic-catabolic imbalance.[17] In the present study, 3-week administration of ghrelin tended to increase body weight and significantly increased lean body mass and muscle strength. These results suggest that treatment with ghrelin improves muscle wasting in patients with CHF. These effects may be mediated, at least in part, by GH/IGF-1, which is considered essential for skeletal muscle. Earlier studies have shown that ghrelin induces orexigenic effects via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus.[13,14] In the present study, intravenous administration of ghrelin increased food intake in patients with CHF, which may contribute to anabolic effects of ghrelin. Tschop et al[15] have shown that administration of ghrelin induces adiposity through a GH-independent mechanism. In the present study, however, ghrelin did not significantly increase fat mass. This difference may be explained by the high dose of ghrelin (>2000-fold) used by Tschop et al. Ghrelin itself decreases fat utilization and increases fat, whereas GH decreases fat tissue and increases lean tissue. Thus, in the present study, ghrelin-induced GH may have attenuated an increase in fat and enhanced an increase in lean tissue.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group. Patients in the control group were not treated identically because a placebo infusion was not performed. Nonetheless, this study was performed while patients were in a stable clinical condition during hospitalization. In addition, 8 patients in the control group were studied to exclude time-course effects during hospitalization. On the basis of the results of this study, a double-blind, randomized, and placebo-controlled study should be conducted. Second, this clinical study did not clarify mechanisms of increased LV ejection fraction by ghrelin therapy. Further studies are necessary to examine which mechanism predominantly contributes to improvement in LV ejection fraction.

Except for a few minor complications, long-term treatment with ghrelin was tolerated well in patients with CHF. Although a preliminary study documented the beneficial effects

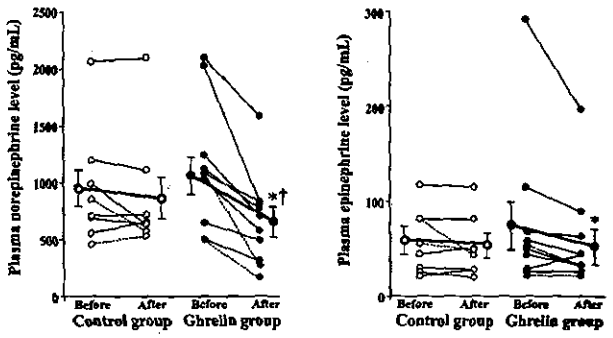

Figure 5. Plasma levels of norepinephrine and epinephrine before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

TABLE 3. Hormone Analysis in Patients With CHF

	Control Group	Ghrelin Group
BNP, pg/mL		
Before	180±53	238±59
After	181±62	190±60*
Fasting glucose, mg/dL		
Before	105±5	101±4
After	102±6	102±7
Insulin, μU/mL		
Before	6.0±1.4	3.9±0.7
After	6.8±2.0	5.5±1.2
Cortisol, μg/dL		
Before	15.5±1.9	17.9±1.6
After	14.5±2.6	17.2±1.5
TNF-α, pg/mL		
Before	5.3±0.9	5.7±0.8
After	5.4±0.9	5.6±0.8
IL-6, pg/mL		
Before	3.2±0.5	3.8±0.7
After	3.4±0.5	3.6±0.7
Renin, $ng \cdot mL^{-1} \cdot h^{-1}$		
Before	9.3±4.6	7.3±3.0
After	10.1±4.1	6.9±3.7
Aldosterone, ng/dL		
Before	11.6±4.1	15.0±4.7
After	12.7±4.1	11.9±4.2

Data are mean±SEM.
*$P<0.05$ vs before.

of GH,[5] controlled studies in humans have been predominantly negative.[7,8] Nevertheless, ghrelin has been shown to have GH-independent effects, stimulating vasodilation,[10–12] reversing cachexia,[13–15] and inhibiting sympathetic nerve activity[24] and myocyte apoptosis.[22] Thus, ghrelin may have additional therapeutic potential compared with GH supplementation. Ghrelin improved cardiac function and exercise capacity in not only cachectic CHF patients but also noncachectic ones. Nevertheless, the best candidates may be cachectic CHF patients because ghrelin stimulates feeding and improves muscle wasting.

Conclusions

These preliminary results suggest that repeated administration of ghrelin improves LV structure and function, exercise capacity, and muscle wasting in patients with CHF. Thus, administration of ghrelin may be a new therapeutic approach for the treatment of CHF.

Acknowledgments

This work was supported by Research Grant for Cardiovascular Disease 16C-6 from the Ministry of Health, Labor and Welfare; Industrial Technology Research Grant Program in 2003 from the New Energy and Industrial Technology Development Organization of Japan; Health and Labor Sciences Research Grants–Genome 005, Mochida Memorial Foundation for Medical and Pharmaceutical Research; and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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Elevated Plasma Ghrelin Level in Underweight Patients with Chronic Obstructive Pulmonary Disease

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Ghrelin, a novel growth hormone–releasing peptide, has been shown to cause a positive energy balance by reducing fat use and stimulating food intake. This study investigated whether plasma ghrelin is associated with clinical parameters in patients with chronic obstructive pulmonary disease. Plasma ghrelin was measured in 50 patients and 13 control subjects, together with anabolic and catabolic factors. Patients were divided into two groups based on body mass index: underweight patients (n = 26) or normal weight patients (n = 24). Plasma ghrelin was significantly higher in underweight patients than in normal weight patients and healthy control subjects. Circulating tumor necrosis factor-α, interleukin-6, and norepinephrine were significantly higher in underweight patients than in normal weight patients. Plasma ghrelin correlated negatively with body mass index and correlated positively with catabolic factors such as tumor necrosis factor-α and norepinephrine. In addition, plasma ghrelin correlated positively with percent predicted residual volume and residual volume-to-total lung capacity ratio. In conclusion, plasma ghrelin was elevated in underweight patients with chronic obstructive pulmonary disease, and the level was associated with a cachectic state and abnormality of pulmonary function.

Keywords: cachexia; ghrelin; hormone; pulmonary disease, chronic obstructive

Patients with chronic obstructive pulmonary disease (COPD) often show a certain degree of cachexia. Cachexia is an independent risk factor for mortality in such patients (1–3). Studies have shown that changes in endocrine hormones such as orexin and leptin have close relationships with cachexia associated with COPD (4–6). Growth hormone (GH) and its mediator, insulin-like growth factor (IGF)-I, are anabolic hormones that are essential for skeletal growth and metabolic homeostasis (7, 8). GH treatment has been shown to increase muscle mass in patients with COPD (9), although it has adverse effects including edema and abnormal glucose tolerance. These findings suggest a role of the GH/IGF-I axis in cachexia associated with COPD.

Ghrelin, a novel endogenous GH-releasing peptide, was isolated from the stomach (10). Ghrelin stimulates the secretion of GH through a mechanism independent from that of hypothalamic GH-releasing hormone. Ghrelin has been shown to cause a positive energy balance by reducing fat utilization through GH-independent mechanisms (11). In addition, both intracerebroventricular and peripheral administration of ghrelin have been shown to elicit potent, long-lasting stimulation of food intake via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus in animals (12–14). The plasma ghrelin level has been reported to be elevated in cachectic states (15, 16). However, little information is available regarding the pathophysiology of ghrelin in COPD.

Thus, the purposes of this study were to investigate (1) whether the plasma ghrelin level is elevated in patients with COPD, and (2) whether the plasma ghrelin level is related to a cachectic state and pulmonary function in patients with COPD.

METHODS

Subjects

We studied 50 patients with COPD (46 men and 4 women; mean age, 71 years; range, 41 to 83 years). COPD was diagnosed according to Global Initiative for Chronic Obstructive Lung Disease criteria. All patients were clinically stable at the time of evaluation. This study included 13 control subjects who had normal pulmonary function. The age and sex of the control subjects were similar to those of the 50 patients. The Institutional Review Board of Nara Medical University (Nara, Japan) approved this study. All subjects provided informed consent.

Patients with COPD were divided into two groups based on body mass index (BMI): underweight patients (BMI < 20, $n = 26$), or normal weight patients (BMI ≥ 20, $n = 24$). There was no significant difference in age, sex, smoking history, disease severity, or medication use between underweight and normal weight patients with COPD (Table 1). The mean smoking history was significantly higher in patients with COPD than in control subjects.

Fat-free mass (lean body mass) was measured by bioelectrical impedance analysis to investigate the relationship between plasma ghrelin and body composition in a subsample of 16 patients (underweight patients, $n = 8$; normal weight patients, $n = 8$). Lean body mass was significantly lower in underweight patients than in normal weight patients (39.3 ± 1.4 versus 46.5 ± 2.1 kg, $p < 0.05$).

Pulmonary Function Testing

Lung volumes were measured by the helium gas dilution method, and forced expiratory flow rates were measured with a mass flow anemometer (FUDAC 70; Fukuda Denshi, Tokyo, Japan). Carbon monoxide transfer factor was measured by the single-breath method. Pulmonary function values were expressed as a percentage of predicted values (17). Arterial blood gases were measured at rest with a blood gas analyzer (ABL 720; Radiometer, Brønshøj, Denmark).

Blood Sampling and Analysis

Blood samples were taken from the antecubital vein in the morning between 7:00 and 8:00 A.M. after an overnight fast. The blood was centrifuged immediately at 4°C and stored at −80°C. Plasma ghrelin was measured by radioimmunoassay as described previously (18).

Serum IGF-I was measured by radioimmunoassay (Somatomedin CII Bayer; Bayer Medical, Tokyo, Japan). Serum tumor necrosis

(Received in original form October 14, 2003; accepted in final form July 20, 2004)

Supported by the Mochida Memorial Foundation for Medical and Pharmaceutical Research and by grants from the Japan Cardiovascular Research Foundation, the New Energy and Industrial Technology Development Organization (NEDO), the Organization for Pharmaceutical Safety and Research (OPSR) of Japan (Promotion of Fundamental Studies in Health Science), and the Research Committee, Intractable Respiratory Failure, Ministry of Health, Labor, and Welfare of Japan.

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Am J Respir Crit Care Med Vol 170. pp 879–882, 2004
Originally Published in Press as DOI: 10.1164/rccm.200310-1404OC on July 21, 2004
Internet address: www.atsjournals.org

TABLE 1. PATIENT CHARACTERISTICS

	Control (*n* = 13)	COPD	
		Normal Weight (*n* = 24)	Underweight (*n* = 26)
Age, yr	69 ± 2	71 ± 2	71 ± 2
Sex, male/female	11/2	23/1	23/3
Body mass index, kg/m²	24.2 ± 0.7	24.2 ± 0.6	18.0 ± 0.3*,†
Smoking history, pack-years	17.6 ± 5.8	69.4 ± 6.2*	53.9 ± 5.9*
Severity stage, n			
I	NA	6	2
II	NA	5	7
III	NA	9	11
IV	NA	4	6
Medication use, n			
Anticholinergics	NA	11	16
β-Agonists	NA	15	14
Inhaled corticosteroids	NA	8	7
Xanthines	NA	14	14
Pulmonary function			
FEV_1, % predicted	93.0 ± 3.9	47.7 ± 4.4*	47.6 ± 3.5*
FEV_1/FVC, %	84.0 ± 2.3	41.4 ± 2.5*	41.7 ± 2.8*
VC, % predicted	95.7 ± 2.0	90.6 ± 5.5	84.8 ± 3.4
RV, % predicted		132.3 ± 7.8	152.0 ± 9.2
TLC, % predicted		101.2 ± 2.9	105.6 ± 3.9
RV/TLC, %		48.7 ± 2.3	52.8 ± 1.7
D_{LCO}, % predicted		61.6 ± 7.8	41.9 ± 5.5†
Pa_{O_2}, mm Hg		72.1 ± 1.7	71.4 ± 2.0
Pa_{CO_2}, mm Hg		44.5 ± 1.4	44.1 ± 1.6

Definition of abbreviations: D_{LCO} = diffusing capacity of the lung for carbon monoxide; NA = not applicable; RV = residual volume; RV/TLC, RV-to-TLC ratio; TLC = total lung capacity; VC = vital capacity.

Data represent means ± SEM.

*$p < 0.05$ versus control.

†$p < 0.05$ versus normal weight.

factor-α, interleukin-6, and insulin were measured by enzyme immunoassay (Quantikine HS [R&D Systems, Minneapolis, MN]; TFB kit [TFB, Tokyo, Japan]; and AIA-PACK IRI [Tosoh, Tokyo, Japan], respectively). Plasma epinephrine and norepinephrine were measured by high-performance liquid chromatography (HLC8030; Tosoh). Serum testosterone in male subjects was measured by radioimmunoassay (DPC testosterone kit; DPC, Los Angeles, CA). Serum prealbumin, retinol-binding protein, and transferrin were measured by nephelometry (Dade Behring, Deerfield, IL).

Statistical Analysis

Data are expressed as means ± SEM. Comparisons of parameters between the two groups were made by Fishes exact test or unpaired Student *t* test. Comparisons of parameters among three groups were made by one-way analysis of variance followed by the Scheffé multiple comparison test. Five groups (control subjects and patients with Stage I, II, III, and IV COPD) were compared by one-way analysis of variance followed by the Scheffé multiple comparison test. Independent relations between plasma ghrelin and pulmonary function parameters were examined by multivariate regression analyses. A p value less than 0.05 was considered statistically significant.

RESULTS

Biochemical Factors

Serum total protein and total cholesterol were significantly lower in underweight patients with COPD than in control subjects (Table 2). In addition, serum triglyceride, prealbumin, retinol-binding protein, and transferrin were significantly lower in underweight patients than in normal weight patients and control subjects.

TABLE 2. CIRCULATING LEVELS OF HORMONAL AND BIOCHEMICAL FACTORS

	Control (*n* = 13)	COPD	
		Normal Weight (*n* = 24)	Underweight (*n* = 26)
Total protein, g/dl	7.5 ± 0.1	7.2 ± 0.1	7.0 ± 0.1*
Albumin, g/dl	4.6 ± 0.1	4.5 ± 0.1	4.4 ± 0.1
Total cholesterol, mg/dl	221 ± 7	206 ± 6	192 ± 5*
Triglyceride, mg/dl	119 ± 9	106 ± 11	68 ± 4†‡
Fasting glucose, mg/dl	109 ± 6	99 ± 4	97 ± 3
Prealbumin, mg/dl	30.3 ± 1.8	27.2 ± 0.8	23.0 ± 0.7†‡
Retinol-binding protein, g/dl	4.6 ± 0.4	3.9 ± 0.2	3.0 ± 0.1†‡
Transferrin, mg/dl	262 ± 7	228 ± 7*	202 ± 7†§
Tumor necrosis factor-α, pg/ml	1.4 ± 0.1	4.3 ± 0.3†	6.8 ± 0.8†‡
Interleukin-6, pg/ml	1.6 ± 0.3	2.3 ± 0.5	4.2 ± 0.7*§
Epinephrine, pg/ml	43 ± 8	45 ± 6	59 ± 7
Norepinephrine, pg/ml	308 ± 19	674 ± 76*	982 ± 97†§
Insulin-like growth factor-1, ng/ml	107 ± 6	137 ± 7*	114 ± 6§
Insulin, μU/ml	8.2 ± 0.8	7.0 ± 0.8	3.9 ± 0.6†‡
Testosterone, ng/dl	419 ± 32	421 ± 19	484 ± 24

Data represent means ± SEM.

*$p < 0.05$ versus control.

†$p < 0.01$ versus control.

‡$p < 0.01$ versus normal weight.

§$p < 0.05$ versus normal weight.

Plasma Ghrelin and Cachectic State in Patients with COPD

The plasma ghrelin level was significantly higher in patients with COPD than in control subjects (237 ± 13 versus 157 ± 10 fmol/ml, $p < 0.01$). In particular, the plasma ghrelin level was higher in underweight patients than in normal weight patients and control subjects (272 ± 20 versus 195 ± 11 and 157 ± 10 fmol/ml, respectively, $p < 0.01$; Figure 1). The level did not significantly differ between normal weight patients and control subjects. The plasma ghrelin level correlated negatively with BMI ($r = -0.38$, $p < 0.01$; Figure 2). In addition, plasma ghrelin level correlated negatively with fat-free mass (lean body mass) ($r = -0.49$, $p < 0.05$) in a subsample of 16 patients.

Circulating levels of catabolic factors such as tumor necrosis factor-α and norepinephrine were significantly higher in both COPD groups than in control subjects (Table 2). Furthermore, the increases in these catabolic factors were marked in underweight patients compared with normal weight patients. On the other hand, circulating levels of anabolic factors such as IGF-I and insulin were significantly lower in underweight patients than in normal weight patients, although these anabolic factors in normal weight patients ware increased (IGF-I) or unchanged (insulin) compared with those in control subjects. The plasma ghrelin level correlated positively with serum tumor necrosis

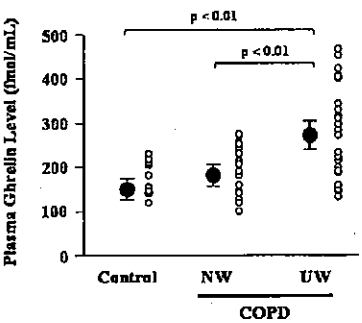

Figure 1. Plasma level of ghrelin in control subjects (Control), normal weight patients with chronic obstructive pulmonary disease (COPD) (NW), and underweight patients with COPD (UW).

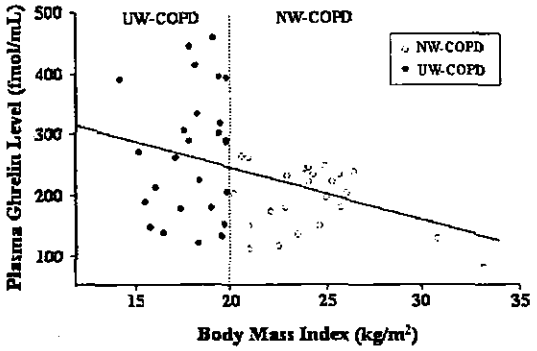

Figure 2. Correlation between plasma ghrelin level and body mass index in patients with COPD. Patients with COPD were divided into two groups: normal weight patients (NW-COPD) and underweight patients (UW-COPD). $r = -0.38$, $p < 0.01$.

factor-α ($r = 0.47$, $p < 0.01$) and plasma norepinephrine ($r = 0.40$, $p < 0.01$), but not serum IGF-I ($r = 0.12$, $p = 0.83$) and insulin ($r = -0.25$, $p = 0.27$). The plasma ghrelin level did not significantly differ between COPD patients with (n = 15) and without (n = 35) corticosteroid therapy (255 ± 27 versus 225 ± 14 fmol/ml, p = NS).

Plasma Ghrelin and Pulmonary Function in Patients with COPD

The plasma ghrelin level was higher in COPD patients with Stage IV disease than in control subjects (283 ± 31 versus 157 ± 10 fmol/ml, $p < 0.05$; Figure 3). Plasma ghrelin level tended to correlate negatively with percent predicted forced expiratory volume in one second ($r = -0.28$, $p = 0.07$), although the correlation did not reach statistical significance. Interestingly, plasma ghrelin level correlated positively with percent predicted residual volume ($r = 0.34$, $p < 0.05$) and residual volume-to-total lung capacity ratio ($r = 0.33$, $p < 0.05$) (Figure 4). Multiple regression analysis demonstrated that percent predicted residual volume or the residual volume-to-total lung capacity ratio was an independent determinant of plasma ghrelin level (each $p < 0.05$) even after adjustment for age, sex, and BMI. On the other hand, the plasma ghrelin level did not significantly correlate with any other pulmonary function parameters.

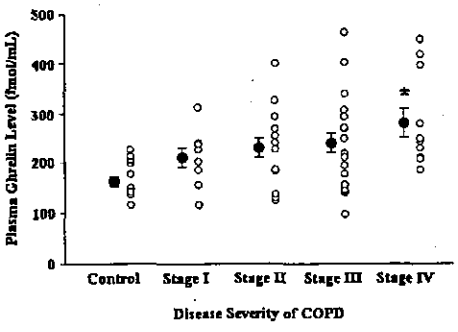

Figure 3. Plasma ghrelin level in patients with COPD according to disease severity based on Global Initiative for Chronic Obstructive Lung Disease guidelines. *$p < 0.05$ versus control.

DISCUSSION

In the present study, we demonstrated that (*1*) the plasma ghrelin level was elevated in underweight patients with COPD, and that (*2*) plasma ghrelin correlated negatively with BMI and correlated positively with circulating levels of tumor necrosis factor-α and norepinephrine. We also demonstrated that (*3*) the plasma ghrelin level was associated with indexes of hyperinflation including percent predicted residual volume and residual volume-to-total lung capacity ratio.

Ghrelin strongly stimulates GH release through a mechanism independent from that of hypothalamic GH-releasing hormone (10). Ghrelin has also been shown to cause a positive energy balance by reducing fat utilization and stimulating food intake (11–14). These findings suggest that ghrelin induces anabolic effects through GH-dependent and independent mechanisms. Thus, we investigated the pathophysiological significance of ghrelin in pulmonary cachexia. In the present study, we defined underweight as BMI < 20 kg/m². Some nutritional parameters including serum triglyceride, prealbumin, retinol-binding protein, and transferrin were also lower in underweight patients than in normal weight patients. These results suggest that "underweight" defined in the present study is accompanied by malnutrition. We demonstrated that plasma ghrelin level was higher in underweight patients than in normal weight patients. Furthermore, the plasma ghrelin level correlated negatively with BMI and lean body mass. These results suggest that the plasma ghrelin level is elevated in response to a cachectic state. Earlier studies have shown that hormonal changes and cytokine activation induce a catabolic state in patients with COPD, resulting in the development of cachexia (4–6, 19). In fact, some catabolic factors such as tumor necrosis factor-α, interleukin-6, and norepinephrine were significantly higher in underweight patients than in normal weight patients, whereas anabolic factors including IGF-I and insulin were significantly lower in underweight patients than in normal weight patients. The plasma ghrelin level correlated positively with catabolic factors such as tumor necrosis factor-α and norepinephrine. Considering the positive energy effects induced by ghrelin, increased ghrelin may represent a compensatory mechanism under catabolic–anabolic imbalance in cachectic patients with COPD. Unexpectedly, the serum IGF-I level was significantly higher in normal weight patients than in control subjects. Catabolic factors including tumor necrosis factor-α and norepinephrine were significantly higher in normal weight patients than in control subjects, although the increases were marked in underweight patients. These findings raise the possibility that increased IGF-I in normal weight patients may represent a compensatory mechanism under conditions of energy imbalance.

In the present study, the plasma ghrelin level showed significantly positive correlation with indexes of hyperinflation such as percent predicted residual volume and residual volume-to-total lung capacity ratio. In addition, the plasma ghrelin level tended to correlate negatively with percent predicted forced expiratory volume in 1 second. Thus, elevated ghrelin may be associated with abnormality of pulmonary function in patients with COPD. Because GH secretagogues receptor, a receptor for ghrelin, is expressed in the lung (20), further studies are to investigate a role of ghrelin in the lung. Although the present study demonstrated that body composition and indexes of hyperinflation were among the determinants of the plasma ghrelin level, further work will be required to determine the factors that contribute to the wide range of ghrelin levels among underweight patients with COPD.

In conclusion, the plasma ghrelin level was elevated in underweight patients with COPD, and the level was associated with a cachectic state and abnormality of pulmonary function.

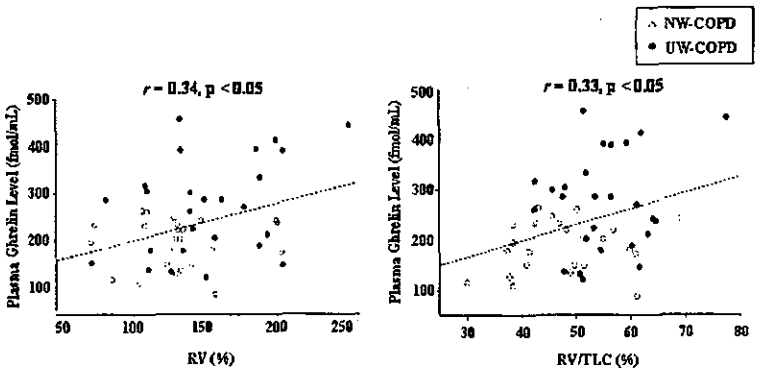

Figure 4. Correlations between plasma ghrelin level and percent predicted residual volume (RV, *left*), and between plasma ghrelin level and residual volume-to-total lung capacity ratio (RV/TLC, *right*) in patients with COPD. NW-COPD indicates normal weight patients with COPD; UW-COPD, underweight patients with COPD.

Conflict of Interest Statement: T.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; N.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(12):5365–5371

doi: 10.1210/en.2003-0427

Alterations of Plasma Ghrelin Levels in Rats with Lipopolysaccharide-Induced Wasting Syndrome and Effects of Ghrelin Treatment on the Syndrome

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Ghrelin not only strongly stimulates GH secretion, but is also involved in energy homeostasis by stimulating food intake and promoting adiposity through a GH-independent mechanism. These effects of ghrelin may play an important role in the pathophysiology of inflammatory wasting syndrome, in which both the somatotropic axis and energy balance are altered. In this study we investigated plasma ghrelin concentrations after lipopolysaccharide (LPS) administration to rats, a model of the wasting syndrome and critical illness. In addition, the therapeutic potential of the antiwasting effects of ghrelin was explored using LPS-injected rats. A single LPS injection suppressed plasma ghrelin levels 6 and 12 h later. Maximal reduction was observed 12 h after LPS injection, in a dose-dependent manner. In contrast, plasma ghrelin levels were elevated after repeated LPS injections on d 2 and 5. Peripheral administration of ghrelin twice daily (10 nmol/rat) for 5 d increased body weight gain in repeated LPS-injected rats. Furthermore, both adipose tissue weight and plasma leptin concentrations were increased after ghrelin administration in these rats. In conclusion, plasma ghrelin levels are altered in LPS-injected rats, and ghrelin treatment may provide a new therapeutic approach to the wasting syndrome and critical illness. (*Endocrinology* 144: 5365–5371, 2003)

WASTING SYNDROME may be defined as a severe state of malnutrition, weight loss, muscle atrophy, and anemia. This condition is characterized by continued protein loss from vital organs and tissues due to both activated degradation and suppressed synthesis, and it contributes to increased mortality, accelerated disease progression, and impaired strength and functional status (1, 2). GH is an anabolic hormone, sparing protein stores at the expense of fat in situations of caloric restriction. GH stimulates the synthesis and secretion of IGF-I from various tissues (3), and many GH effects are mediated by IGF-I (4). In addition to its use for treatment of short-statured children with impaired production or complete lack of GH and of GH deficiency in adults, GH has been used to improve protein metabolism in critical illness (5). However, recent large, multicenter-randomized, placebo-controlled clinical trials in critically ill adults have shown significant increases in morbidity and mortality with GH treatment (6). Therefore, the effects of GH treatment are controversial.

Ghrelin is the natural ligand of the GH secretagogue receptor and strongly stimulates GH secretion (7–9). In addition to this action, recent studies in rodents suggest that ghrelin is involved in energy homeostasis, acting as a peripheral signal stimulating food intake and promoting adiposity through a GH-independent mechanism (10, 11). GH-releasing peptide-2 was reported to be effective in improving alterations in the somatotropic axis and protein catabolism in patients with prolonged severe illness, and the nonpeptidyl GH-releasing peptide mimetic, MK-0677, reverses diet-induced catabolism (12, 13). Furthermore, peripheral administration of ghrelin attenuated body weight loss in a rat model of cachexia with chronic heart failure (14), in mice given IL-1β (15) and in a rat model of cancer cachexia (16). These findings suggest that ghrelin may play an important role in the regulation of metabolic balance and improve the wasting syndrome through GH-dependent or independent effects.

Ghrelin is a unique 28-amino acid peptide hormone that contains an *n*-octanoyl modification on Ser^3. This lipid modification is essential for ghrelin-mediated stimulation of GH release, and des-acyl ghrelin, the des-*n*-octanoyl form of ghrelin, has almost no biological activity (17). Two kinds of RIAs, namely C-RIA, for the carboxyl terminal, and N-RIA, for the amino terminal, of ghrelin, were previously reported to measure plasma ghrelin concentrations (17–19). However, little is known about the physiological conditions in which plasma levels of these two forms of ghrelin differ.

Bacterial lipopolysaccharide (LPS) is a component of the Gram-negative bacterial cell wall and is believed to mediate many of the sequelae of infection. LPS administration induces anorexia, body weight loss, and other catabolic responses and is commonly used to generate animal models of excessive inflammation, septic shock, and wasting syndrome (20–22). Concerning the somatotropic axis, acute endotoxin administration in rats decreased circulating GH and IGF-I (3, 23), and chronic LPS administration decreased pituitary GH content and circulating IGF-I (23). However, neither plasma ghrelin levels nor ghrelin treatment has been tested in this model.

The aim of this study was to analyze the effects of LPS

Abbreviations: LPS, Lipopolysaccharide; PF, pair-fed.