Ghrelin Receptor Expression in Lymphocytes Isolated from Adult Cystic Fibrosis Patients

Rubin I. Cohen Subani Chandra Seth Koenig Donna Tsang David Wilson
Thomas McCloskey

Adult Cystic Fibrosis Center, Division of Pulmonary, Critical Care and Sleep Medicine, Long Island Jewish Medical Center, Feinstein Institute for Medical Research, Albert Einstein College of Medicine, New Hyde Park, N.Y., USA

Key Words

Ghrelin · Ghrelin receptor · Cystic fibrosis · Weight loss

Abstract

Background: To explore mechanisms of weight loss in cystic fibrosis (CF), we studied ghrelin receptor expression on isolated lymphocytes from CF subjects with different body mass indices (BMIs). Eating behavior is influenced by hormone peptides such as ghrelin, a potent appetite stimulator. However, studies on ghrelin plasma levels in CF showed it to be increased in cachectic subjects, the expected physiological response. **Objectives:** (1) To compare ghrelin receptor expression between clinically stable CF subjects with normal BMI, CF subjects with cachexia and healthy controls. (2) To investigate ghrelin receptor expression in the same CF subjects before and after treatment for an acute exacerbation. **Methods:** Lymphocytes were isolated from CF patients with normal BMI and low BMI and from controls. Ghrelin receptor quantification was determined via flow cytometry. Body composition was determined by bioelectrical impedance, and plasma levels of ghrelin, TNF- α , IL-1 and IL-6 were determined. **Results:** CF subjects with low BMI had increased inflammation evidenced by increased plasma cytokines and showed decreased lymphocytic ghrelin receptor expression. Ghrelin receptor expression in the CF group with normal BMI was similar to controls; it decreased during an acute

exacerbation associated with weight loss and returned to baseline following treatment and recovery of the weight loss. **Conclusions:** Differences exist in ghrelin receptor expression in lymphocytes isolated from stable CF patients with different BMIs. These changes may be due to a disordered pathological response to weight loss.

Copyright © 2009 S. Karger AG, Basel

Introduction

Cystic fibrosis (CF) is a common genetic disease that arises from mutations in the CF transmembrane conductance regulator gene, resulting in improper functioning of chloride channels. Poor nutrition and weight loss have long been associated with poor survival outcomes in CF, and nutritional status often deteriorates in older patients as the pulmonary disease progresses. CF patients are chronically infected with bacteria even when clinically stable and develop acute pulmonary exacerbations necessitating treatment with antibiotics. These acute exacerbations are accompanied by decreased appetite and weight loss. The etiology of weight loss in CF remains poorly understood, but is likely multifactorial, involving altered metabolism, increased work of breathing, increased inflammation and decreased appetite [1, 2].

In recent years, it has become apparent that appetite is mediated, in part, by several peptide hormones through their action on hypothalamic pathways. Ghrelin, a potent appetite stimulator, is secreted by enteroendocrine cells of the stomach [3, 4]. It would then stand to reason that pathological alterations such as a decrease in plasma levels of ghrelin in CF patients would contribute to CF-associated anorexia. However, in adult CF subjects with low body mass index (BMI), we found that plasma ghrelin levels are elevated, indicating that changes in the plasma levels of this peptide are physiological rather than pathological, i.e., a response to the low food intake rather than its cause [5].

The effects of ghrelin are mediated via a 7-transmembrane G protein-coupled receptor called growth hormone secretagogue receptor (GHS-R), which has been identified within a variety of tissues in addition to the stomach [6]. Specifically, lymphocytes have been shown to express functional GHS-R. Such findings indicate that ghrelin could have other effects beyond regulating eating behavior. Indeed, studies have revealed that ghrelin is involved in the downregulation of the inflammatory system, and the constitutive expression of GHS-R on human lymphocytes attests to the link between eating behavior and inflammation. Ghrelin exerts potent inhibitory effects on proinflammatory mediators via its action on T cells, monocytes and endothelial cells, and ghrelin expression in T cells may also play a role in downregulation of NF κ B activation [7–9].

Dixit et al. [7] showed that lymphocytes isolated from normal volunteers and activated in vitro expressed a greater number of GHS-Rs compared to resting lymphocytes from the same volunteers. These authors postulated that the increased GHS-R expression in activated lymphocytes was an anti-inflammatory reaction to the activation of the lymphocytes. Since cachectic CF subjects have increased plasma levels of inflammatory cytokines [5], we postulated that such subjects would have increased GHS-R expression as a physiological response to the increased inflammatory milieu.

To assess whether GHS-R receptor expression on lymphocytes differs between CF subjects and normals, we conducted a 2-part study. Initially, we performed an observational study of the expression of GHS-R in lymphocytes isolated from cachectic CF patients and compared this to receptor expression in CF patients with normal BMI and healthy controls. After establishing that ghrelin receptor expression differs between CF subjects and controls, we studied lymphocytic GHS-R expression at the onset of an acute pulmonary exacerbation and once pa-

tients had recovered. Since increased inflammation is related to cachexia, we also determined plasma levels of the inflammatory cytokines associated with anorexia, TNF- α , IL-1 and IL-6.

Methods

Subjects

All subjects gave written informed consent, and the protocol was approved by the medical center's institutional review board.

Twelve adult CF patients with low BMI and 12 with normal BMI were studied (6 females and 6 males in each group). All were studied when clinically stable and without evidence of an acute exacerbation as defined previously [10, 11]. All had been weight stable (<5% change within prior 2 months) and none were using an appetite stimulant. All enrolled patients were chronically infected with *Pseudomonas*, but none were chronically infected with *Burkholderia cepacia*, methicillin-resistant *Staphylococcus aureus* or atypical mycobacteria. All were pancreatic insufficient, using pancreatic enzyme supplementation and taking daily vitamin supplements (A, D, E and K). None of the CF subjects had had any changes in their pancreatic enzyme dosage within the past several years or had clinical evidence of malabsorption. None of the CF subjects were, at the time of blood collection or within the previous 4 weeks, using supplemental nutrition via either a nasogastric or percutaneous enteral feeding tube. None of the CF patients had ascites, cor pulmonale, allergic bronchopulmonary aspergillosis or CF-related diabetes mellitus. None had been on oral steroids within 4 weeks prior to the study.

Seven CF subjects from the normal BMI group were studied prior to initiating treatment for an acute pulmonary exacerbation. Patients with an acute pulmonary exacerbation had the following: increased cough accompanied by a change in density and color of the sputum, along with increased dyspnea, malaise, anorexia and weight loss, change in physical examination of the chest and at least a 10% decrease in forced expiratory volume in 1 s (FEV₁) from baseline [10, 11]. These 7 subjects required 2–3 weeks of antipseudomonal intravenous antibiotics based on the bacterial sensitivity from sputum cultures, and none required the use of oral steroids. These 7 subjects were studied again 2–3 weeks following the completion of the intravenous antibiotics regimen.

Six healthy males and 6 females with normal BMI were also studied as control subjects. None of the controls had ever smoked, and all had been weight stable within the past 6 months.

Investigations and Analyses

Spirometry data were expressed as percentages of predicted values according to the equations of Knudson et al. [12]. Weight was measured with a beam scale to the nearest 0.25 kg with the subjects barefoot and lightly clothed. Height was determined to the nearest 0.5 cm, and the BMI was calculated. Body composition was assessed by bioelectrical impedance (BIA 101 Impedance Analyzer; RJL Systems, Detroit, Mich., USA). Fat-free mass (FFM) was calculated using a specific regression equation [13] and was expressed in kilograms. Fat mass (FM) was calculated as total weight minus FFM and expressed in kilograms.

Blood was collected by venipuncture between the hours of 7.00 and 10.00 a.m. following an overnight fast.

Receptor quantification and flow cytometric analysis were performed as previously described with modifications. A whole-blood labeling method was employed so as to minimally perturb the in vivo physiological status of the receptor for ghrelin [14, 15]. Anti-ghrelin receptor (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) monoclonal antibody was incubated with 100 μ l of whole blood at previously determined optimal concentrations for 10 min at room temperature. Samples were washed, and the secondary antibody goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pa., USA), conjugated to the fluor allophycocyanin, was added. Samples were incubated for an additional 10 min at room temperature in the dark. Samples were washed and lysed with Immunolysate reagent (Coulter, Miami, Fla., USA) and then fixed with 1% paraformaldehyde and stored at 4°C until flow cytometric analysis.

Lymphocytes were electronically gated by their light scatter properties and their fluorescence intensity was measured. Background control utilized the allophycocyanin-conjugated secondary antibody alone to account for cellular autofluorescence and nonspecific binding. Data analysis allowed quantification of the percentage of lymphocytes demonstrating expression of the receptor.

Blood for active ghrelin assay was immediately centrifuged at 4°C, separated and acidified with 1 N HCl then stored at -80°C. Plasma active ghrelin levels were measured in duplicate via a non-radioactive ELISA-based method using anti-human ghrelin IgG raised against Ser(3)octanoylated ghrelin with no cross-reactivity to des-octanoyl ghrelin, according to the manufacturer's instructions (Millipore, Billerica, Mass., USA).

Plasma TNF- α , IL-1 and IL-6 were analyzed via a cytometric bead array system (BD Bioscience, San Diego, Calif., USA) which relies on bead populations with distinct fluorescence intensities that have been coated with capture antibodies specific for TNF- α , IL-1 and IL-6 proteins. The bead populations are mixed together to form the cytometric bead array. A flow cytometer (FACSCaliber, Becton Dickinson) was used to analyze the light intensity that reflects the concentration of each cytokine.

Statistical Analysis

Data were collected and expressed as means $\pm$ SD. Data were then analyzed using Sigma Stat 3.5 statistical software (SPSS, Chicago, Ill., USA). Data were tested for normality by the Kolmogorov-

Smirnov test, using $p < 0.05$ as the threshold for rejection of normality. Since data were normally distributed, one-way analysis of variance was used to compare differences among groups, followed by post hoc analysis (Tukey) to determine the source of the significance. p values < 0.05 were considered significant.

Results

There was no difference in the ages of the subjects in the 3 groups studied. There was no difference between the BMI of the control group and the BMI of the weight-stable CF group. The CF patients with low BMI were clearly underweight (BMI below 18.5), had advanced pulmonary disease and were severely depleted with regard to both FFM and FM compared to the other 2 groups. The CF group with normal BMI exhibited decreased FFM compared to the controls, but their FFM was higher than that of the underweight CF group (table 1).

Table 1. Subjects' characteristics

	Controls (n = 12)	CF subjects- normal BMI (n = 12)	CF subjects- low BMI (n = 12)	p value
Age, years	35 $\pm$ 8	35 $\pm$ 10	32 $\pm$ 5	NS
BMI	25.3 $\pm$ 1.7	23.6 $\pm$ 1.9	16.9 $\pm$ 0.7 ^{a, b}	0.0001
FFM, kg	54.3 $\pm$ 5	45.0 $\pm$ 4 ^a	38 $\pm$ 5 ^{a, b}	0.002
FM, kg	16.7 $\pm$ 6.1	17.9 $\pm$ 6.9	5.1 $\pm$ 2.9 ^{a, b}	0.006
FEV ₁ , % predicted	98 $\pm$ 14	62 $\pm$ 12 ^a	27 $\pm$ 9 ^{a, b}	<0.001
FVC, % predicted	95 $\pm$ 14	70 $\pm$ 8 ^a	41 $\pm$ 16 ^{a, b}	<0.001
FEV ₁ /FVC	86 $\pm$ 4	74 $\pm$ 8 ^a	59 $\pm$ 8 ^{a, b}	<0.001

FVC = Forced vital capacity; NS = not significant.

^a Significantly different from the control group.

^b Significantly different from the CF subjects-normal BMI group.

Table 2. Percentage of lymphocytes expressing ghrelin receptor (GHS-R) and corresponding lymphocyte count and plasma ghrelin level

	Controls	CF subjects- normal BMI	CF subjects- low BMI	p value
Lymphocytes expressing GHS-R, %	12.6 $\pm$ 2.0	12.7 $\pm$ 2.3	6.8 $\pm$ 1.9 ^{a, b}	0.001
Absolute lymphocyte count, $\times 10^3/\mu$ l	2 $\pm$ 0.5	1.7 $\pm$ 0.5	1.8 $\pm$ 0.6	NS
Absolute GHS-R-positive lymphocyte count, $\times 10^3/\mu$ l	0.25 $\pm$ 0.001	0.21 $\pm$ 0.001	0.12 $\pm$ 0.001	0.001
Plasma ghrelin, pg/ml	21 $\pm$ 4	25 $\pm$ 10	110 $\pm$ 28 ^{a, b}	0.001

NS = Not significant.

^a Significantly different from the control group.

^b Significantly different from the CF subjects-normal BMI group.

GHS-R expression on isolated lymphocytes from the cachectic CF group was lower than in the other 2 groups. There was no difference in GHS-R expression between the CF group with normal BMI and healthy controls. The ghrelin level was elevated in the CF group with low BMI (table 2).

Cytokines were significantly elevated in the CF group with low BMI compared to the other 2 groups. There was no difference in plasma cytokine levels between the controls and the CF group with normal BMI (table 3).

Table 4 shows the changes in the 7 patients who were studied at the beginning of an acute pulmonary exacerbation and following treatment for it. We found a significant decrease in GHS-R expression in isolated lymphocytes at the beginning of the exacerbation, which returned to baseline levels following treatment. However, there was no change in plasma ghrelin levels. The acute exacerbations were also marked by a decrease in weight and percentage of predicted FEV₁, both of which returned to baseline levels; we failed to detect changes in cytokine levels.

Discussion

Cachexia is a complex syndrome occurring in patients with advanced CF and is represented by weight loss, muscle wasting and an increased inflammatory response. In CF, the development of cachexia portends a poor outcome. Appetite-modulating peptides such as ghrelin may provide an important link between eating behavior, weight loss and the inflammatory response. It had been postulated that decreased BMI in CF may be related, in

part, to decreased appetite. Since ghrelin stimulates appetite, it would be expected that ghrelin levels are low in CF patients with low BMI. However, we found the opposite; that is, ghrelin levels were elevated in the group of CF subjects with low BMI [5]. This suggests that the elevated ghrelin levels may be a response to the low BMI and not its cause. In the present study, we explored the possibility that differences in receptor expression of this peptide rather than its plasma levels may be associated with weight loss and cachexia in CF. We found clear differences in receptor expression density between controls and CF subjects with normal and low BMI. We also found differences in receptor expression just before an acute pulmonary exacerbation and following its resolution.

We believe that this is the first report to assess GHS-R expression on lymphocytes in CF patients. We observed that lymphocytes isolated from anorexic CF subjects who had markedly elevated anorexia-associated cytokines and who were both FM and FFM depleted exhibited decreased GSH-R expression when compared to either lymphocytes from healthy controls or CF subjects with normal BMI.

Table 3. Cytokine plasma levels (pg/ml)

	Controls	CF subjects-normal BMI	CF subjects-low BMI	p value
TNF-α	1.2 ± 0.5	2.1 ± 0.8	4.8 ± 0.6 ^{a, b}	0.001
IL-1	0.2 ± 0.4	1.0 ± 0.8	6.3 ± 1 ^{a, b}	0.001
IL-6	2.0 ± 2	4.8 ± 3	21.7 ± 4 ^{a, b}	0.001

^a Significantly different from the control group.

^b Significantly different from the CF subjects-normal BMI group.

Table 4. Changes before and after treatment for an acute CF exacerbation

	Baseline	Before treatment	After treatment	p value
FEV ₁ , % predicted	58.0 ± 7	41.3 ± 8 ^a	57.7 ± 7	<0.001
Weight, kg	61.4 ± 8	57.6 ± 8	61.3 ± 8	NS
Lymphocytes expressing GHS-R, %	12.3 ± 0.9	8.7 ± 2.1 ^a	12.1 ± 1.5	<0.001
Absolute lymphocyte count, × 10 ³ /μl	1.9 ± 0.4	1.8 ± 0.4	2.4 ± 0.9	NS
Plasma ghrelin, pg/ml	28 ± 6	31 ± 9	30 ± 8	NS
TNF-α, pg/ml	2.4 ± 1	4.2 ± 3	2.8 ± 2.1	NS
IL-1, pg/ml	1.5 ± 0.8	2.3 ± 3	1.9 ± 1.2	NS
IL-6, pg/ml	4.2 ± 3	5.8 ± 4	5.8 ± 4	NS

NS = Not significant.

^a Significantly different from values at baseline and after treatment.

phocytes from CF patients with normal BMI or from controls. We also demonstrate that an acute pulmonary exacerbation in CF is associated with decreased ghrelin lymphocytic expression which returned to baseline following clinical improvement.

Dixit et al. [7] showed that GHS-R expression increased significantly in lymphocytes isolated from healthy volunteers following in vitro exposure to an inflammatory milieu as compared to nonactivated lymphocytes isolated from the same volunteers. These authors concluded that such findings were physiological responses to increased inflammation, as ghrelin possesses anti-inflammatory properties [7]. We postulated that if receptor expression changes in response to changes in body mass, then the expected physiological change would be increased GHS-R expression in an acute exacerbation and in cachectic CF patients. However, we found a 46% decrease in GHS-R expression in lymphocytes isolated from cachectic CF subjects, a population in whom plasma inflammatory cytokine levels were markedly elevated. We also noted a 20% decrease in GHS-R expression in lymphocytes isolated from CF subjects at the beginning of an acute pulmonary exacerbation and following treatment. Our findings of decreased GHS-R expression suggest that these changes are pathological.

The cellular response following receptor-ligand interaction may be controlled in vivo by the total amount of ligand available or by the available receptor binding sites. For example, other groups have showed that by measuring the percentage of T lymphocytes expressing receptors for the cytokines IL-2, -7 and -15, a link could be demonstrated between cytokine receptor deficiency and immune deficiency in HIV-infected patients [16]. In an analogous manner, in the current study we demonstrate decreased GHS-R expression in CF subjects with low BMI. In addition, we report data which strengthens this finding by showing decreases in GHS-R expression during an acute exacerbation, with a return to baseline following treatment and recovery. Therefore, quantification of the percentage of lymphocytes expressing GHS-R is justified, because we demonstrate a link between changes in the percentage of cells expressing this receptor and clinical status, which is perhaps an important clue regarding the pathogenesis of weight loss in CF.

It is currently unclear to what extent the changes in GHS-R expression on lymphocytes affect appetite or the immune response and what is the nature of the signaling pathways in CF. To our knowledge, there are no published studies examining these issues. We also recognize that an association between peptide receptor expression

and BMI in CF may not imply cause and effect. However, unlike the results for plasma levels of ghrelin, our findings in isolated lymphocytes from CF patients suggest a disordered, pathological response to weight loss. Further studies are required to explore and clarify these important issues. We also considered the fact that decreased GHS-R expression in CF subjects with low BMI may be a response to the elevated ghrelin plasma levels (table 2). However, the decrease in GHS-R expression at the beginning of an exacerbation and its return to baseline following treatment was not associated with any changes in plasma ghrelin. Therefore, a change in GHS-R expression solely due to a change in ghrelin plasma levels cannot fully account for our findings, which implies that other mechanisms must be involved. We also note that we failed to detect a significant change in cytokine levels during the acute exacerbation as had been demonstrated by others [2]. This is likely due to the variability in the cytokine levels, as only 7 subjects were studied. However, the main purpose of this study was to examine GHS-R prior to, during and following an acute exacerbation, and this was accomplished.

In conclusion, we conducted an observational, cross-sectional study which revealed different expression of ghrelin receptors in lymphocytes isolated from controls and CF patients with early and advanced disease. We also studied ghrelin receptor expression at the onset and following an acute CF exacerbation. GHS-R expression in lymphocytes isolated from cachectic CF patients and in subjects undergoing an acute pulmonary exacerbation was markedly decreased. Studying GHS-R expression may provide important clues regarding the mechanisms of weight loss in CF.

Acknowledgement

This work was funded by a research grant from the Division of Pulmonary, Critical Care and Sleep Medicine, Long Island Jewish Medical Center, Feinstein Institute for Medical Research, Albert Einstein College of Medicine, New Hyde Park, N.Y., USA.

References

- 1 Dinwiddie R: Pathogenesis of lung disease in cystic fibrosis. *Respiration* 2000;67:3–8.
- 2 Ionescu AA, Nixon LS, Evans WD, Stone MD, Lewis-Jenkins V, Chatham K, Shale DJ: Bone density, body composition, and inflammatory status in cystic fibrosis. *Am J Respir Crit Care Med* 2000;162:789–794.
- 3 Meier U, Gressner AM: Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin Chem* 2004;50:1511–1525.
- 4 Inui A: Ghrelin: an orexigenic and somatotropic signal from the stomach. *Nat Rev Neurosci* 2001;2:551–560.
- 5 Cohen RI, Tsang D, Koenig S, Wilson D, McCloskey T, Chandra S: Plasma ghrelin and leptin in adult cystic fibrosis patients. *J Cyst Fibros* 2008;7:398–402.
- 6 Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M: The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 2002;87:2988–2991.
- 7 Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R, Lillard JW, Taub DD: Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. *J Clin Invest* 2004;114:57–66.
- 8 Li A, Cheng G, Zhu GH, Tarnawski AS: Ghrelin stimulates angiogenesis in human microvascular endothelial cells: implications beyond GH release. *Biochem Biophys Res Commun* 2007;353:238–243.
- 9 Li WG, Gavrila D, Liu X, Wang L, Gunnlaugsson S, Stoll LL, McCormick ML, Sigmund CD, Tang C, Weintraub NL: Ghrelin inhibits proinflammatory responses and nuclear factor-kappaB activation in human endothelial cells. *Circulation* 2004;109:2221–2226.
- 10 Koch C, Hoiby N: Diagnosis and treatment of cystic fibrosis. *Respiration* 2000;67:239–247.
- 11 Goss CH, Burns JL: Exacerbations in cystic fibrosis. 1. Epidemiology and pathogenesis. *Thorax* 2007;62:360–367.
- 12 Knudson RJ, Lebowitz MD, Holberg CJ, Burrows B: Changes in the normal maximal expiratory flow-volume curve with growth and aging. *Am Rev Respir Dis* 1983;127:725–734.
- 13 Hollander FM, De Roos NM, De Vries JH, Van Berkhout FT: Assessment of nutritional status in adult patients with cystic fibrosis. Whole-body bioimpedance vs. body mass index, skin folds, and leg-to-leg bioimpedance. *J Am Diet Assoc* 2005;105:549–555.
- 14 Fleisher TA, Marti GE: Detection of unseparated human lymphocytes by flow cytometry. *Curr Protoc Immunol* 2001;chapter 7: unit 7.9.
- 15 McCloskey TW, Haridas V, Pahwa R, Pahwa S: Human immunodeficiency virus gag and pol-specific CD8 T cells in perinatal HIV infection. *Cytometry* 2001;46:265–270.
- 16 Pahwa R, McCloskey TW, Aroniadis OC, et al: CD8+ T cells in HIV disease exhibit cytokine receptor perturbation and poor TCR activation but are responsive to gamma-chain cytokine driven proliferation. *J Infect Dis* 2006;193:879–887.