Association of Nesfatin-1 and Fat Mass in Cystic Fibrosis

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Key Words

Nesfatin-1 · Cystic fibrosis · Anorexia · Fat mass · Cytokines

Abstract

Background: The mechanisms of fat mass (FM) loss in cystic fibrosis (CF) are poorly understood but could represent complex pathways involving dysregulation of appetite-modulating peptides and an amplified inflammatory response. Nesfatin-1 is a newly described peptide that decreases food intake and FM but has not been studied in CF. **Objectives:** We hypothesized that changes in the appetite-suppressing hormone nesfatin-1 would be physiological, and levels would be lower in advanced CF patients with lower FM compared to those with milder disease and healthy controls. We determined the levels of the cytokines TNF- α , IL-1 β , and IL-6 as they have been associated with weight loss in disease states. **Methods:** Fifty-four adult CF subjects, i.e. 17 with severe, 22 with moderate, and 15 with mild disease, as well as 18 controls were recruited. PFT and body composition analysis (via bioelectrical impedance) were performed. Nesfatin-1 and cytokine levels were determined by ELISA. **Results:** Contrary to our proposed hypothesis, nesfatin-1 levels were highest in CF patients with severe disease and the lowest FM. A significant negative correlation between nesfatin-1 levels and FM was found only in the severe CF group ($r = -0.7$, $p = 0.003$). In forward stepwise regression analysis, only FM was significantly associated with nesfatin-1 levels. Levels of TNF- α and

IL-6 were elevated in the severe CF group, but there was no association with either FM or nesfatin-1. **Conclusion:** In advanced CF and low FM, nesfatin-1 plasma levels are significantly increased and inversely correlated with the FM. Our results further suggest that nesfatin-1 exerts its effects independently of TNF- α or IL-6.

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Introduction

Advanced cystic fibrosis (CF) is often associated with decreased appetite and weight loss, both of which are recognized risk factors for increased morbidity and mortality. The mechanisms associated with this weight loss are not fully understood but likely represent complex and multifactorial disorders that involve nutrient malabsorption, increased metabolic demands, an elevated inflammatory response, and perhaps dysregulation of appetite-modulating peptides and hormones [1, 2].

Physiologically, feeding behavior is regulated by the central nervous system, with the hypothalamus playing a central role and multiple pathways contributing to appetite regulation and energy balance [2, 3]. Multiple peptides are involved in the hypothalamic control of feeding behavior and some of these, such as leptin and ghrelin, have been studied in CF. Leptin, a hormone produced mainly by adipose tissue, enters the brain to regulate food

intake according to changes in energy balance. Ghrelin, an orexigenic hormone, influences the neuropeptide Y pathway, the most powerful central enhancer of appetite. However, the changes observed in these two peptides in CF-associated weight loss appear to be physiological rather than pathological, that is the abnormal levels of leptin and ghrelin in CF-associated weight loss are a response to the weight loss and not the cause of it [3].

Nesfatin-1 was discovered in 2006 by Oh-I et al. [4] as an 82-amino acid polypeptide derived from the calcium and DNA-binding protein nucleobindin2 (NUCB2). Due to its inhibitory effect on food intake and its associated decrease in fat mass (FM), the first cleavage product of NUCB2 was named nesfatin-1, an acronym for NUCB2-encoded satiety- and fat-influencing protein [4]. A number of studies have implicated nesfatin-1 as an anorexigenic molecule and an FM-reducing peptide. In a pioneering study, the injection of nesfatin-1 into the brains of rodents inhibited food intake [5]. Nesfatin-1 is able to cross the blood-brain barrier (BBB) without saturation [6, 7], and when nesfatin-1 was injected peripherally into mice a reduction in food intake was documented. A concomitant reduction in body weight gain and fat pads was detectable upon subchronic peripheral administration of nesfatin-1 to rats [8].

In humans, the effects of nesfatin-1 are poorly understood. The plasma levels of nesfatin-1 have been determined in various conditions associated with abnormal food intake as well as in diabetes and epilepsy [9–13]. To our knowledge, there are no studies on nesfatin-1 levels in CF. Furthermore, accumulating evidence suggests that the immune system, in particular the production of inflammatory cytokines, plays an important role in the development of weight loss in disease states [2, 3]. The cytokines considered to be the most relevant to inflammatory anorexia are IL-1 β , IL-6, and TNF- α . We assessed the plasma levels of these cytokines to determine any association between them and nesfatin-1. We hypothesized that, similar to other previously studied peptides in CF, such as leptin and ghrelin, any changes in the appetite-suppressing hormone nesfatin-1 would be physiological, that is levels would be lower in advanced CF compared to those with milder disease and healthy controls. In this paper we present our preliminary findings.

Methods

Fifty-four adult CF patients (25 males and 29 females), i.e. 17 with severe disease (FEV₁ $\leq$ 40% predicted), 22 with moderate disease (FEV₁ % predicted >41 and <75), and 15 with mild disease (FEV₁ $\geq$ 75% predicted) were included. All were studied

when clinically stable and without evidence of an acute exacerbation as defined previously [14]. All had been weight stable (less than 5% change in body weight within the prior 4 weeks). All pancreatic insufficient subjects were taking pancreatic enzyme supplementation and 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 year 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 a percutaneous enteral feeding tube.

Nine healthy males and 9 females with normal BMI (range 19–25) were studied as controls. None of the controls had ever smoked, and all had been weight stable within the past 6 months.

Exclusion criteria for all subjects (CF and controls) included a history of diabetes, congestive heart failure, seizures, liver or kidney disease, malignancy, thyroid disease, and the use of an appetite stimulant. None had been on an oral steroid dose exceeding 10 mg daily for more than 1 week within 8 weeks prior to the study. Female subjects who were pregnant or breastfeeding were also excluded.

Spirometry was expressed as percentages of predicted values according to National Health and Nutrition Examination Survey III (NHANES-III) criteria. 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 [15] and was expressed in kilograms. FM was calculated as total weight minus FFM and expressed in kilograms. Serum nesfatin-1 levels were determined between 9:00 a.m. and 11:00 a.m. following an overnight fast and using a commercially available ELISA-based kit (Phoenix Pharmaceuticals, Burlingame, Calif., USA) according to the manufacturer's instructions. Similarly, cytokine levels were determined using separate ELISA kits for each cytokine (R&D Systems, Minneapolis, Minn., USA). All samples were performed in duplicate and the average taken. All plates included positive and negative controls.

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

Statistical Analysis

The program Sigma Plot 3.5 was used for analysis (Systat Software, Chicago, Ill., USA). Normality was determined by the Kolmogorov-Smirnov test. If data were normally distributed, results were expressed as means $\pm$ SD and one-way analysis of variance (ANOVA) was employed to ascertain differences between the four groups. All pairwise multiple comparisons were determined by the Holm-Sidak method. Nonnormally distributed data were expressed as medians (IQR) using the Kruskal-Wallis ANOVA on ranks to determine differences. To isolate the group or groups that differed from others, Dunn's method was then employed. Univariate analysis using Spearman's correlation coefficient was used to assess potential associations between nesfatin-1 and other variables. Factors that were associated with nesfatin-1 levels in the univariate analysis were included in a multiple linear regression model. $p < 0.05$ was considered statistically significant.

Table 1. Lung function and body mass composition of study participants

	Controls (n = 18)	Mild CF (n = 15)	Moderate CF (n = 22)	Severe CF (n = 17)
FEV ₁ , % predicted	92 ± 12 ^a	79 ± 9 ^a	51 ± 7 ^a	30 ± 7
BMI	23 (22–24)	22 (21–23)	23 (21–25)	20 (19–23)
FM, kg	17 (11–17) ^a	30 (26–36) ^a	16 (12–20) ^a	10 (8–14)
FFM, kg	55 (43–62)	40 (38–63)	49 (41–58)	43 (37–55)

Values are presented as medians (IQR) or means ± SD.

^a Significant in relation to the severe CF group.

Table 2. Serum cytokine levels

	Controls	Mild CF	Moderate CF	Severe CF
TNF-α	3.2 ± 2.7	7.1 ± 4.2 ^a	6.6 ± 3.3 ^a	5.6 ± 3.6 ^a
IL-1β	3.6 (2.3–4.6)	3.4 (2.9–5.0)	4.1 (3.1–5.7)	4.6 (3.6–7.4)
IL-6	1.9 (0.7–2.6)	5.7 (2.2–9.1) ^a	7.9 (4.5–15.9) ^a	9.7 (4.5–18.8) ^a

^a Significant in relation to controls.

Results

The median age of the CF population was 24 years (IQR: 23–38) with a range of 18–68 years. When data were examined according to sex, there were no differences between CF males and CF females with respect to age (median: 29 years for males and 27 years for females), FEV₁ % predicted (mean: 49% for males and 55% for females), and BMI (median: 23 for males and 21 for females).

We then examined the CF population according to disease severity. Table 1 shows that BMI and FFM were preserved among the CF groups; however, FM was different among groups, with the severe CF group having the lowest FM. Figure 1 illustrates the nesfatin-1 values among the control and CF groups. Nesfatin-1 was highest in the severe CF group when compared to all other groups, but there were no differences among the other groups.

Table 2 shows the cytokine levels in the four groups. Levels of TNF-α and IL-6 were elevated in all CF groups compared to controls; however, we found no differences in IL-1β levels between the groups.

Spearman's rank analyses demonstrated that plasma nesfatin-1 correlated negatively with FM. Figure 2 demonstrates this significant negative correlation between nesfatin-1 levels and FM found in the severe CF group ($r = -0.7$, $p < 0.003$). When subjected to multiple regression analysis, BMI, FFM, and FEV₁ % predicted in the

severe group were not predictive of nesfatin-1 levels. We found no association between levels of nesfatin-1 and any of the cytokines measured.

Discussion

Our study describes the novel observation on the levels of nesfatin-1 in CF and the relationship between nesfatin-1 levels and the corresponding body adiposity. The main findings of this study are that nesfatin-1 levels were elevated in the CF group with severe lung disease, a group that also has the lowest FM. Further, we found a negative correlation between FM and nesfatin-1 plasma levels in this group; that is as FM decreased the plasma levels of nesfatin-1 tended to increase. The lack of association between elevated levels of nesfatin and TNF-α and IL-6 suggests that nesfatin-1 may exert its effects through mechanisms independent of these cytokines.

Since eating behavior is most critical for the survival of any species, a complex circuitry of compensatory and overlapping mechanisms has evolved to protect the host against deficiency in one or more of these regulators. We now understand that eating behavior is a highly regulated multisystem process [16, 17]. The hypothalamus is critical in the regulation of food intake containing neural circuits, which produce a number of peptides that influence

Fig. 1. Plasma nesfatin-1 levels in normal controls, mild, moderate, and severe CF. The asterisk indicates significance compared to other groups.

Fig. 2. Correlation between plasma nesfatin-1 and FM.

food intake. For example, the hypothalamic arcuate nucleus produces both orexigenic peptides and anorectic peptides. Other hypothalamic factors implicated in appetite regulation include nesfatin-1 and the endocannabinoids. Circulating factors affect food intake and mediate their effects by signaling to the hypothalamus and brainstem. Several such factors are produced by peripheral or-

gans such as leptin by adipose tissue, insulin by the pancreas, and ghrelin by the stomach [17]. There is increasing evidence that the immune system, particularly the production of inflammatory cytokines by leukocytes, plays an important role in the development of anorexia-cachexia syndrome. The cytokines considered to be the most relevant to inflammatory anorexia include IL-1 β , IL-6, and TNF- α . Studies further demonstrate that factors regulating feeding behavior and those regulating the immune response are interlinked [3, 16]. Peripherally administered ghrelin, for example, blocks IL-1 β -induced anorexia and produces a positive energy balance by promoting food intake and decreasing energy expenditure [16]. The lack of adiponectin has been implicated in promoting inflammation in obesity-associated asthma [18]. Current data demonstrate that ghrelin downregulates proinflammatory cytokine expression while leptin is regarded as promoting inflammation [16]. Given this highly complex multisystem process, it is perhaps not surprising that not one appetite-modulating peptide has been fully implicated in either weight gain or weight loss. Indeed, clinical studies have demonstrated that the levels of ghrelin and leptin in CF-associated weight loss are physiological rather than pathological [2].

Nesfatin-1 is an 82-amino acid peptide recently discovered in the brain which is derived from NUCB2, a protein that is highly conserved across mammalian species. Nesfatin-1 has received much attention due to its reproducible food intake-reducing effects [10]. The molecular mechanisms through which nesfatin-1 exerts these effects have not been fully elucidated. Prior studies have shown that hypothalamic nesfatin-1 acts through a neural-mediated pathway that may contribute to increased peripheral and hepatic insulin sensitivity by decreasing gluconeogenesis and promoting peripheral glucose uptake. Nesfatin-1 has also been shown to exert at least a part of its physiological actions on the control of food intake by directly modulating the excitability of glucosensing neurons [19]. Alternatively, a direct effect of nesfatin-1 on mTOR signaling has been postulated [10]. Another study demonstrated that nesfatin-1 activates vagal afferent neurons by stimulating calcium influx through N-type channels [20]. Finally, data suggest that a portion of nesfatin-1 neurons in both the hypothalamus and the brainstem are sensitive to peripheral inflammatory signals, and centrally released nesfatin-1 may contribute to the neural mechanisms leading to endotoxemic anorexia [21].

Animal studies have shown that excess nesfatin-1 in the brain leads to a loss of appetite and a drop in body fat. Similarly, a lack of nesfatin-1, induced by injecting an an-

ti-nesfatin-1 antibody into rodents' brains, led to an increase in both appetite and body fat [4, 5, 8]. Studies in mice have provided strong evidence that nesfatin-1 traverses the BBB bidirectionally in a nonsaturable way [6, 7]. However, there are very few studies assessing plasma nesfatin-1 levels in human diseases associated with either FM loss or gain. Ogiso et al. [12] measured plasma nesfatin-1 levels in 7 anorexia nervosa patients (average BMI 13.0) and 8 age-matched healthy controls (average BMI 21.6). Their results showed that plasma nesfatin-1 levels were significantly lower in the anorexia nervosa group than in controls. Another study showed that plasma nesfatin-1 levels were 1.9-fold higher in obese subjects compared to controls [13]. The data from these two studies suggest that although FM is associated with nesfatin-1, its levels appear to be regulated by the nutritional status and response to starvation or obesity.

In many aspects, CF is a unique disease that allows investigation of the weight loss associated with advanced disease. CF patients are cared for at specialized centers where their clinical status, lung function, and nutritional status are continuously monitored. While survival in CF has increased markedly in the last 2 decades, it remains an ultimately fatal disease whose course is punctuated by respiratory exacerbations leading to eventual respiratory failure. As the lung disease progresses, it is accompanied by a decrease in BMI allowing a comparison between those with mild, moderate, and severe disease. In this study, we postulated that changes in nesfatin-1 levels would be physiological and would be lower in those with the greatest FM loss (as was the case with ghrelin and leptin [2]). Further, we sought to study whether a relationship exists between nesfatin-1 and the inflammatory cytokines associated with anorexia.

Our results show the opposite, for in CF patients with advanced disease and reduced FM, nesfatin-1 was elevated compared to controls and other CF patients with higher FM. Our results suggest that the elevated nesfatin-1 levels in subjects with severe CF and the lowest FM cannot be a physiological response to the reduced FM but may play a role in the loss of FM.

Interestingly, overall BMI was maintained in our CF patients, and there were no differences in BMI between groups. The reasons for this are not entirely clear; however, our adult CF center has embarked on nutritional and exercise quality improvement initiatives with the aim of maintaining or increasing BMI. This could have contributed to the maintained BMI in our CF population. However, in spite of the normal BMI, FM was significantly reduced in the advanced CF group, and we found a

strong negative correlation between nesfatin-1 and FM in those with severe disease.

Assessment of body composition using BIA may be less precise than dual-energy X-ray absorptiometry (DEXA) scanning. Nevertheless, prior studies have shown good correlations between DEXA and BIA on a group basis, although such agreement between these two methods for individuals could vary [15, 22]. In the present study, we assessed FM in our subjects in groups (controls, mild disease, moderate disease, and severe disease) and our aim was not to follow individuals over time. Therefore, BIA is an acceptable method for the assessment of body composition in CF. We recognize this to be a descriptive study and we acknowledge that the elevated nesfatin-1 levels and the association with FM in those with advanced CF do not necessarily imply causation. However, not much is known about nesfatin-1 and human studies remain altogether descriptive as they are limited by what can ethically be performed in human subjects [9–13]. Moreover, we cannot discount the role that psychological stress plays in the weight loss associated with CF. Patients with advanced CF are quite ill and are documented to have worse quality of life, increased pain, and anxiety [23]. Whether physiological stress impacts on nesfatin-1 or vice versa remains to be investigated. The mechanisms of nesfatin-1 actions remain unclear, partly because nesfatin-1 receptors in the brain are not completely understood [10, 24]. Nevertheless, our results, while preliminary, are intriguing and require further investigation.

Conclusion

In patients with advanced CF and low FM, nesfatin-1 plasma levels are significantly elevated and correlate with a low FM but do not correlate with elevated levels of TNF- α or IL-6, suggesting that nesfatin-1 does not exert its effect through modulation of the two cytokines. Taken together, our original findings could have important implications with respect to the potential FM-reducing actions of nesfatin-1 in CF pathophysiology.

Financial Disclosure and Conflicts of Interest

The authors declare that they have no conflicts of interest.

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