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Circulating fetuin-A levels are not affected by short and long-term energy deprivation and/or by leptin administration☆,,☆☆

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Abstract

Objective—Fetuin-A may mediate cross-talk between the liver and adipose tissue. We studied the physiologic regulation of fetuin-A and explored its potential regulation by leptin.

Design and Methods—Fetuin-A levels were measured in three interventional studies as well as in *in vitro* experiments. Study 1: 15 lean subjects received placebo or physiologic replacement-dose recombinant human leptin (metreleptin) following short term complete caloric deprivation to induce severe hypoleptinemia; Study 2: 7 women with relative leptin deficiency due to strenuous exercise or low weight received 3 months of metreleptin; Study 3: 17 women with relative leptin deficiency were randomized to receive metreleptin or placebo over 9 months. In study 4 human hepatoma Hep G2 cells were treated with leptin. Fetuin-A mRNA expression and secretion were measured.

Results—Complete caloric deprivation significantly decreased leptin but had no effect on fetuin-A levels. Normalizing leptin levels with metreleptin in hypoleptinemic subjects had no effect on

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Author contributions

CSM derived the hypothesis and conceived the study design. JJH, BT, JC and CSM contributed to data collection and analysis and to the interpretation and discussion of the results. JJH and BT wrote the manuscript with guidance from CSM and input from JC.

Conflict of interest

Dr. CS Mantzoros has received research support for investigator initiated trials from Amylin Pharmaceuticals, Inc. through Beth Israel Deaconess Medical Center. All other authors have no relevant conflicts of interest related to this research.

circulating fetuin-A levels. Leptin treatment had no effect on fetuin-A mRNA expression and secretion *in vitro*.

Conclusions—Circulating fetuin-A levels are not affected by short and long-term energy deprivation. Furthermore, both in vivo and *in vitro* experiments confirm that fetuin-A is not regulated by leptin.

Keywords

Fetuin-A; Leptin; Alpha-2-Heremans-Schmid glycoprotein

1. Introduction

As the epidemic of obesity continues to spread worldwide, understanding the underlying mechanisms which regulate energy homeostasis is of critical importance. In particular, there is a strong need for increased understanding of the interactions between the liver and adipose tissue as disruptions in these interactions can lead to non-alcoholic fatty liver disease (NAFLD), insulin resistance and obesity.

Recently, fetuin-A (α2-Heremans-Schmid Glycoprotein, AHSG) has garnered significant scientific interest as an important factor in the association between fatty liver disease, obesity and insulin resistance. Fetuin-A is secreted predominantly from the liver and mRNA expression is elevated in patients with NASH (Non-alcoholic steatohepatitis) and NAFLD [1]. In rodents, fetuin-A knock-out animals have improved insulin sensitivity driven by increased insulin stimulated phosphorylation of the insulin receptor in both liver and muscle [2], and fetuin-A infusion results in increased insulin resistance, decreased adipose tissue expression of adiponectin and increased proinflammatory cytokine expression [3]. In humans, numerous studies have shown that higher fetuin-A levels are predictive of type 2 DM [4–7]. Furthermore, one recent study found that fetuin-A levels decreased significantly following 12 weeks of caloric restriction in overweight women with type 2 DM [8].

Leptin, one of the most important adipokines, regulates energy homeostasis by serving as a signal between the adipose tissue and the brain. Furthermore, leptin plays an important role in orchestrating the cross-talk between fat and liver [9]. In mice, fasting leads to suppression of hepatic sympathetic activity and elevated hepatic triglyceride content and steatosis. These effects are blunted in mice with deficient leptin signaling in the AGRP neurons in the hypothalamus [10]. In humans, congenital leptin deficiency is associated with severe hepatic steatosis, which can be treated with metreleptin [11].

Given the associations between fetuin-A, hepatic steatosis and insulin resistance, in this study, we seek to investigate the interactions between adipose tissue and liver by examining the interactions between leptin and fetuin-A in healthy individuals with either short-term (72-h of complete caloric deprivation) or longer term (weeks or months) energy deprivation.

2. Methods and procedures

The study protocols were approved by the Institutional Review Board of Beth Israel Deaconess Medical Center (BIDMC) and written informed consent was obtained from all

participants. Clinical quality metreleptin was supplied by Amylin Pharmaceuticals (a wholly-owned subsidiary of Bristol-Myers Squibb) and administered under an Investigational New Drug application submitted to the Food and Drug Administration by Christos Socrates Mantzoros.

2.1. Study 1: Short-term complete energy deprivation induced hypoleptinemia with and without metreleptin administration in replacement doses

15 healthy lean (BMI <25 kg/m^2) subjects, 8 men (age 23.3 ± 1.2 years) and 7 eumenorrheic women (age 23.7 ± 1.5 years) were admitted to BIDMC General Clinical Research Center and studied during three separate admissions [12,13]. All female subjects were studied in the early follicular phase (6th to 11th day of menstrual cycle). The subjects were not on any medications (including oral contraceptive pills in females for at least 6 months). Activities and light/dark intervals were standardized for all subjects. These subjects were studied during 3 separate admissions (baseline fed condition; fasting with administration of placebo; and fasting with administration of subcutaneous replacement-dose metreleptin designed to normalize the fasting-induced decline in leptin levels) as described below. The same subjects participated in all three admissions. Each admission was separated by at least 7 weeks to permit recovery of hematocrit and leptin levels, and to assure that weight was similar to weight at baseline. Blood samples were obtained at 0800 h on day 1 and 0800 h on day 4 for measurement of leptin and fetuin-A levels in all states.

In the baseline fed state study, subjects were placed on an isocaloric diet to maintain their admission body weight, with four standardized meals per day; 20% of the calories were from breakfast (8:00 am), 35% from lunch (1:00 pm), 35% from dinner (6:00 pm), and 10% from a snack (10:00 pm).

In the leptin/placebo replacement in fasting study, the same 8 male and 7 female subjects from the baseline fed study participated on two separate admissions, during which they were randomized to receive either metreleptin or placebo during a 72-h fasting. During both fasting studies, subjects received only calorie-free liquids, NaCl (500 mg), KCl (40 meq), and a standard multivitamin with minerals daily. Cross over of subjects now to the opposite arm occurred in the latter admission separated by at least 7 weeks. In the fasting/leptin admission metreleptin was administered at dose 0.04 mg/kg/day (men) or 0.08 mg/kg/day (women) on day 1, increased to 0.1 mg/kg/day (men) or 0.2 mg/kg/day (women) on days 2–3 to account for declining leptin levels with additional fasting, administered as 4 equal doses given subcutaneously every 6 h, starting at 8 a.m. on day 1. These replacement dose regimens for men and women have been validated in our previous pharmacokinetics studies [14]. During the fasting/placebo admission a buffer solution of similar volume was administered subcutaneously every 6 h, similar to the leptin/fasting arm. 2 male subjects and 1 female subject did not complete the placebo study and thus data for only 6 males and 6 females are presented.

2.2. Study 2: Open label clinical trial of metreleptin replacement in hypoleptinemic women with chronic energy deprivation

Seven otherwise healthy lean (BMI <25 kg/m^2 and age = 25.0 ± 2.2 years) women with mild chronic energy deficit related to either strenuous exercise (running >20 miles per week or equivalent) or low weight for at least 6 months (resulting in hypothalamic amenorrhea (HA)) and relative leptin deficiency (baseline leptin level < 5 ng/ml) were evaluated as part of a larger study on the effects of metreleptin on neuroendocrine function [15]. All had stable weight for six months, had no eating disorders and were not on any other medication including estrogens for three months.

It is known that as compared with controls matched for weight and body composition, women with HA have low leptin levels [16–18] and absence of diurnal variation [19]. Thus in this study subjects were given exogenous recombinant leptin replacement with the expectation that leptin would improve reproductive and neuroendocrine function in women with HA. Subjects self-administered metreleptin (0.08 mg/kg/day for 2 months, then 0.2 mg/kg/day for the third month) subcutaneously twice daily to mimic the normal diurnal variation of leptin levels. Blood samples for measurement of leptin and fetuin-A were obtained at an initial screening visit one month prior to the study and after 1, 3, 7, and 11 weeks of metreleptin treatment.

2.3. Study 3: Randomized, placebo controlled clinical trial of 17 hypoleptinemic women with chronic energy deprivation

Seventeen otherwise healthy lean (BMI <25 kg/m^2 and age = 26.1 ± 3.9 years) women with secondary HA for at least 6 months and with mild chronic energy deficit related to either strenuous exercise or low weight and relative leptin deficiency (baseline leptin level < 5 ng/ml) were evaluated as part of a larger study on the effects metreleptin on bone health, neuroendocrine and immunological function [20,21]. All had stable weight for six months, had no eating disorders and were not on any other medications known to affect bone health.

The subjects enrolled in the larger study on HA were randomized into either metreleptin or placebo treated groups. Metreleptin/placebo was self-administered subcutaneously once daily for 36 weeks. As this was a longer study, once daily regimen was chosen for ease of administration and this has been previously shown to achieve physiological or supra-physiological levels of leptin [14]. The initial dose for all subjects was 0.08 mg/kg/day for 12 weeks. At the end of 12 weeks, subjects who had started menstruation would continue on this dose, whereas subjects who had not started menstruating would increase the dose to 0.12 mg/kg/day. If weight dropped below 5% of baseline weight, the dose was decreased by 0.04 mg/kg. Blood samples for measurement of leptin and fetuin-A were obtained at a baseline visit (before initiation of metreleptin/placebo) and after 12, 24, and 36 weeks of metreleptin treatment.

2.4. Study 4: In Vitro Study: Leptin treatment of human HepG2 cells and Fetuin-A mRNA expression and secretion

a. Measurement of *in vitro* Fetuin-A secretion from HepG2 Cell Line Media:

HepG2 human hepatoma cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Treatments were carried out in minimal essential medium containing 10% FBS (= control) or containing 5 ng/ml,10 ng/ml, 20 ng/ml and100 ng/ml leptin (prospecbio) for 12 h, 24 h, 36 h and/or 48 h. All experiments were performed at 75% confluence. Supernatants were collected and stored at −80 °C until analysis.

b. Determination of Fetuin A mRNA via Real Time PCR (RT-qPCR)

Total RNA was extracted from HepG2 cells using Trizol reagent (Invitrogen, Grand Island, NY) and quantified spectrophotometrically at 260 nm. Integrity was confirmed by visualization of 18S and 28S rRNA on the Flash-Gel system (Lonza, Rockland, ME). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantification of mRNA expression was done by Real Time PCR (RT-qPCR) using human-specific TaqMan® Gene Expression Assays (Assay ID, AHSG (Fetuin-A) primer is: Hs00155659_m1 Applied Biosystems, Foster City, CA) in 7500 Fast Real-Time PCR system using Standard real-time 7500 protocol. Data were analyzed using 7500 system software (Applied Biosystems, Foster City, CA) and relative quantification was done using ΔΔCt method with 18S as the internal control and the 12-h control cells (without leptin) group as the reference control.

2.5. Hormone assays

All samples used were stored at −80 °C. Fetuin-A was measured in human serum and HepG2 supernatant by ELISA (BioVendor, Candler, NC) with sensitivity of 0.35 ng/ml. Leptin levels were measured by radioimmunoassay (Millipore; Billerica, MA) with a sensitivity of 0.5 ng/ml as previously described [22,23].

2.6. Statistical methods

Data are expressed as mean ± SEM. Statistical analyses were conducted using SPSS 11.5 (Chicago, IL) and SAS. Differences in hormone levels were analyzed across time points and between groups using repeated measures ANOVA in the chronic energy deficit studies. For the short-term complete energy deprivation study, nonparametric Wilcoxon rank sum and paired t-tests were used to assess change in hormone levels for each condition. One-way ANOVA was used to determine whether changes in hormone levels varied between conditions. Statistical analyses of *in vitro* experiments were performed using one-way ANOVA followed by post-hoc test for multiple comparisons.

3. Results

3.1. Fetuin-A levels are not affected by short-term complete energy deprivation or metreleptin replacement

Fetuin-A levels remained stable during the fed condition (first day 291.9 ± 17.3 μg/ml; final day 270.8 ± 29.4 μg/ml, $P = 0.31$). We then evaluated effects of 72 h of complete fasting on

fetuin-A levels and whether potential fasting-induced changes are mediated by leptin in lean subjects (BMI 23.5 ± 0.4 kg/m^2 [men], 21.7 ± 0.8 kg/m^2 [women]) (Fig. 1 A). There were no differences between men and women, and data are shown together. Furthermore, there was no difference across the first day of all three conditions, indicating that parameters had normalized to baseline between interventions ($P = 0.13$).

As previously reported, leptin levels decreased after 72 h of complete fasting, from 8.5 ± 2.3 ng/ml on the first day to 1.8 ± 0.4 ng/ml on the final day ($P = 0.002$) [22]. The fasting-induced decrease in leptin was normalized to within the physiologic range with administration of metreleptin (first day, 7.0 ± 1.7; final day, 15.9 ± 3.8 ng/ml, $P = 0.002$). After fasting alone, there was no change in fetuin-A levels (first day, 268.2 ± 24.8 μg/ml; final day, 265.0 ± 15.4 μg/ml, $P = 0.81$). Furthermore, administration of metreleptin to normalize the fasting-induced leptin levels had no effect on fetuin-A (first day, 316.2 ± 23.1 μg/ml; final day, 302.2 ± 29.7 μg/ml, $P = 0.60$).

3.2. Chronic relative leptin deficiency and/or leptin replacement has no effect on fetuin-A levels

In an open-label clinical trial, we then examined effects of metreleptin administration on fetuin-A levels in 7 women with strenuous exercise or low weight and chronic relative leptin deficiency. Over 7 weeks of metreleptin treatment, leptin levels returned to physiologic levels, and by week 11, levels were mildly supraphysiologic (baseline, 3.9 ± 0.8 ng/ml; week 1, 8.9 ± 1.4 ng/ml; week 3, 10.0 ± 1.8 ng/ml; week 7, 22.1 ± 7.3 ng/ml; week 11, 39.1 ± 13.1 ng/ml). There was no significant change in fetuin-A levels over the 11 weeks of metreleptin treatment ($P = 0.29$).

Furthermore, in a randomized, placebo controlled trial of 17 women with chronic leptin deficiency and strenuous exercise/low weight, randomized to receive metreleptin or placebo over 36 weeks, we found that treatment with metreleptin had no effect on serum fetuin-A levels over time (Fig. 1 B, baseline, 219.8 ± 22.9 μg/ml; week 12, 222.3 ± 32.5 μg/ml; week 24, 232.0 ± 43.1 μg/ml; week 36, 253.5 ± 63.3 μg/ml, $P = 0.19$). Leptin levels continued to rise throughout treatment (baseline, 4.55 ± 0.64 ng/ml; week 12, 44.51 ± 8.74 ng/ml; week 24, 57.26 ± 11.36 ng/ml; week 36, 59.33 ± 14.15 ng/ml, $P = <0.0001$).

3.3. Fetuin A secretion/mRNA expression from liver cells is not altered by leptin

We also conducted an *in vitro* experiment, exposing human hepatoma cells, Hep G2, with control (= 0 ng/ml), low (5 ng/ml, 10 ng/ml, 20 ng/ml) and high (100 ng/ml) dose leptin for 12, 24, 36 and 48 h. Quantification of fetuin-A expression from the HepG2 culture showed no statistically significant difference with any dose of leptin (0 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml and 100 ng/ml) as compared to different treatment times, 12 h ($p = 0.45$), 24 h ($p = 0.58$), 36 h ($p = 0.34$) and 48 h ($p = 0.31$) (data not shown). Similarly, leptin had no significant effect on HepG2 Fetuin-A secretion *in vitro* at any dose.

4. Discussion

In this study, we investigate the physiology of fetuin-A in states of short and long term energy deprivation resulting in leptin deficiency. Furthermore, we directly examine the

effects of leptin treatment (with metreleptin) on fetuin-A levels. We show that despite 72 h of complete caloric deprivation induced hypoleptinemia, there is no change in fetuin-A levels. Furthermore, we show that replacement of leptin over a few days to normalize levels in the serum does not affect fetuin-A levels. Similarly, mild hypoleptinemia (leptin levels <5 ng/ml) reflecting relative chronic energy deficiency and replacement of leptin levels over a few weeks (open-label clinical trial) or over a few months (randomized, placebo controlled trial) had no effect on fetuin-A levels despite supra-physiological levels of leptin achieved in the serum. These findings are also supported with our in vitro experiments, as fetuin-A secretion and mRNA expression in liver cells also remain unaltered for up to 48 h with either low or high dose metreleptin treatment.

Leptin has been shown to play an important role in orchestrating the cross-talk between fat and liver. In mice, deficient leptin signaling in the hypothalamus blunts the fasting lead suppression of hepatic sympathetic activity and elevation of hepatic triglyceride content and steatosis [10]. Furthermore, severe hepatic steatosis seen in congenital lipodystrophies can be treated with metreleptin [11,24]. Several studies have shown that fetuin-A levels decline along with improvement with hepatic steatosis; however, whether fetuin-A levels are also impacted by fat mass regardless of intra-hepatic fat remains unclear. One study involving obese but non-diabetic women showed that despite a significant decrease in BMI after 3-months of exercise fetuin-A levels did not change significantly [25]. Another recent study showed that fetuin-A levels fell after 12-weeks of caloric restriction in over-weight women with type-2 diabetes [8]. In this study visceral fat area was shown to decrease with weight loss [8]. Another longitudinal study with obese children showed that fetuin-A levels were higher only in obese children with NAFLD and decreased with substantial weight loss. The decrease in fetuin-A levels was accompanied by a reduced prevalence of NAFLD [26]. Our study eliminates the potential confounding effect of a potential decrease of fetuin-A due to alterations in liver fat in response to energy deprivation given that the study subjects are lean, do not lose weight and their baseline fat mass or liver fat is not expected to be altered. In this way we are able to directly examine the effect of metreleptin treatment on fetuin-A.

In summary, we show for the first time that fetuin-A is not affected by short-term complete caloric/energy deprivation and long term energy deficiency which induce hypoleptinemia with metreleptin administration in replacement doses, in otherwise healthy lean humans with stable weight. Both obesity and insulin resistance involve an orchestrated action of a web of interacting adipokines [27,28] and other factors [29] such as fetuin-A. Understanding the interrelationships between these proteins may provide deeper insights into the body's physiological regulation of glucose metabolism and may pave the way for the development of future therapeutics and treatments for type 2 diabetes. This study contributes to the effort to map out the interrelationships between peripherally secreted molecules important in regulating metabolism by providing interventional evidence that leptin does not have a significant effect on the regulation of fetuin-A levels. Future studies need to focus on changes of fetuin-A levels with patients with lipodystrophy who are known to have ectopic fat deposition in the liver or obese subjects with visceral fat mass. In addition, future studies should be focusing on potential interactions between fetuin-A and/or leptin administration in such subjects with insulin resistance.

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Abbreviations

metreleptin	recombinant human leptin
NAFLD	non-alcoholic fatty liver disease
fetuin-A	AHSG, α2-Heremans-Schmid Glycoprotein
NASH	Non-alcoholic steatohepatitis
BIDMC	Beth Israel Deaconess Medical Center
HA	hypothalamic amenorrhea
ATCC	American Type Culture Collection
FBS	fetal bovine serum
RT-qPCR	Real Time PCR

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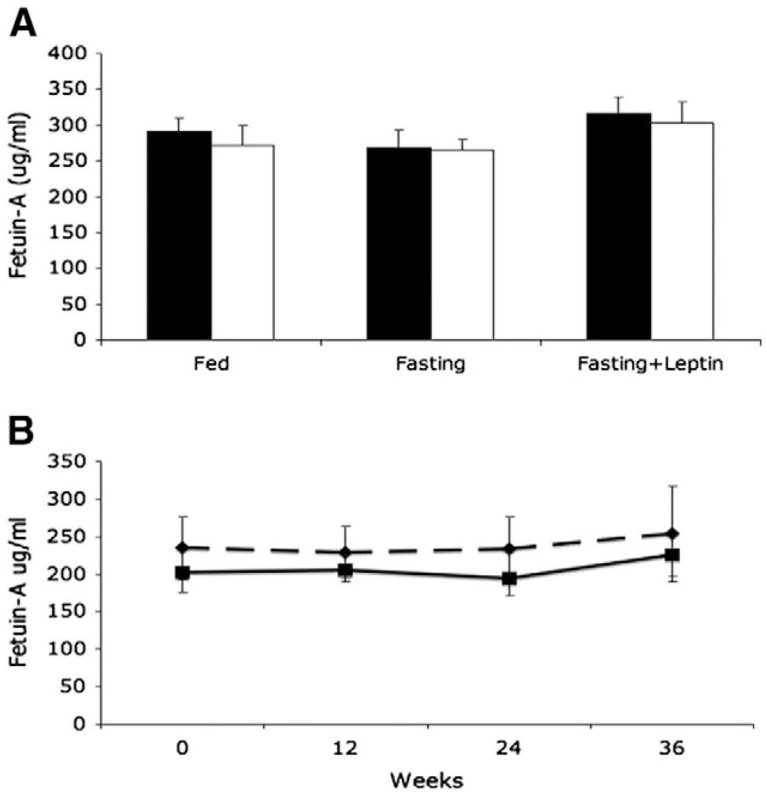

Fig. 1.

A: Measurement of Fetuin-A at the beginning (day 1) and end (day 3 or 4) of a baseline fed condition, 72-h complete fasting with administration of placebo, and 72-h complete fasting with administration of metreleptin (n = 8 normal-weight men and 7 normal-weight women). ■ day 1; □ final day, p = 0.13. B: Measurement of Fetuin-A in 17 women with chronic leptin deficiency and HA randomized to receive metreleptin or placebo for 36 weeks. — Placebo—Metreleptin, repeated measures ANOVA, p = 0.19.