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Acylated ghrelin and leptin in adolescent athletes with amenorrhea, eumenorrheic athletes and controls: a cross-sectional study

Karla Christo*, Jennalee Cord*, Nara Mendes*, Karen K. Miller*, Mark A. Goldstein†, Anne Klibanski*, and Madhusmita Misra*,‡

*Neuroendocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

†Adolescent Medicine Unit, Massachusetts General Hospital for Children and Harvard Medical School, Boston, Massachusetts, USA

‡Pediatric Endocrine Unit, Massachusetts General Hospital for Children and Harvard Medical School, Boston, Massachusetts, USA

Summary

Objectives—Neuroendocrine factors may predict which athletes develop amenorrhea and which athletes remain eugonadal. Specifically, ghrelin and leptin have been implicated in regulation of GnRH secretion, with ghrelin having inhibitory and leptin, facilitatory effects. We hypothesized that adolescent athletes with amenorrhea (AA) would have higher ghrelin and lower leptin levels than eumenorrheic athletes (EA) and would predict levels of gonadal steroids.

Design—Cross-sectional

Subjects and measurements—We enrolled 58 girls, 21 AA, 19 EA and 18 nonathletic controls 12–18 years old. Fasting blood was drawn for active ghrelin, leptin, E₂ and testosterone. Athletes were > 85% of ideal body weight for age based on body mass index (BMI).

Results—AA girls had lower BMI than EA and controls ($P = 0.003$). Log ghrelin was higher in AA than in EA and controls ($P < 0.0001$), and remained higher after controlling for BMI Z-scores. Leptin was lower in AA than in the other groups ($P < 0.0001$), however, the differences did not persist after controlling for BMI Z-scores. Testosterone was lower in AA than in EA and controls ($P = 0.002$) and log E₂ trended lower in AA ($P = 0.07$). We observed inverse associations of log active ghrelin with testosterone ($P = 0.01$), and positive associations of leptin with testosterone and log E₂ ($P = 0.02$ and 0.009).

Conclusion—Higher ghrelin levels, even after controlling for BMI, and lower leptin in AA compared with EA and controls, and their inverse and positive associations, respectively, with gonadal steroids suggest endocrine perturbations that may explain why hypogonadism occurs in some but not all athletes.

Introduction

Amenorrhea is reported in as many as 24% of adolescent athletes, and the prevalence depends on the nature and duration of exercise as well as the athlete's nutritional status.^{1–4}

Particularly, endurance athletes such as gymnasts, track runners, ballet dancers and swimmers are reported to be at increased risk of amenorrhea. However, not all endurance athletes develop amenorrhea, and neuroendocrine factors that differentiate athletes with amenorrhea (AA) from eumenorrheic athletes (EA) are not completely understood. In adults, a negative state of energy balance in AA has been attributed to cause disruption of gonadotrophin pulsatility^{5,6} and therefore amenorrhea. In addition, low leptin levels have been implicated;^{7,8} yet, administration of recombinant human leptin in one study in adults with hypothalamic amenorrhea led to normalization of the hypothalamo–pituitary–gonadal (H–P–G) axis in some, but not all, subjects.⁹ The adolescent years are characterized by maturation of the H–P–G axis and changes in body composition that may affect leptin levels. However, fat mass and leptin levels have not been compared in adolescent AA vs. EA and nonathletic controls, and neither has the relationship of these parameters with levels of gonadal steroids been assessed.

Ghrelin, an orexigenic peptide, has been implicated in the regulation of the H–P–G axis, and administration of ghrelin causes decreased gonadotrophin pulsatility in rodents^{10,11} monkeys¹² and humans.¹³ Ghrelin levels are high in adult athletes after exercise¹⁴ and are also high in anorexia nervosa¹⁵ another condition associated with a negative energy balance state and amenorrhea.¹⁴ However, ghrelin levels have not been examined in adolescent athletes, and it is not known whether levels are higher in adolescent AA compared with EA and controls, or whether ghrelin levels predict levels of gonadal steroids.

Hypogonadism in athletes is associated with decreased bone density and increased fracture risk.^{16–19} A greater understanding of the neuroendocrine factors that predispose to hypogonadism in some but not all athletes could lead to development of therapeutic strategies that target menses resumption as a means of increasing bone mass accrual. We hypothesized that fat mass and leptin levels would be lower and ghrelin levels higher in adolescent AA compared with EA and controls, and would be associated with lower levels of gonadal steroids.

Subjects and methods

Subject selection

We enrolled 58 adolescent girls, 12–18 years old, in this study. This included 21 girls who met the criteria for diagnosis of AA, 19 EA and 18 nonathletic controls. Baseline and bone density data, but not hormonal characteristics have been previously reported for these subjects.²⁰ Girls with AA and EA were endurance athletes, and self reported a history of one of the following for at least 6 months (i) at least 4 h of aerobic weight-bearing training of the legs weekly (ii) at least 30 miles of running weekly, or (iii) at least 4 h of specific endurance training weekly (modified from adult criteria,²¹). Amenorrhea was defined as absence of menses for at least three consecutive cycles²¹ after initiation of menses and regular menses for at least 6 months, or absence of menarche at 15.3 years (mean age at menarche +2 SDs for girls in the United States).²² Nonathletic controls did not meet endurance criteria and had no history of amenorrhea or menarchal delay. Although our subjects did not meet DSM-IV criteria for diagnosis of anorexia nervosa or bulimia nervosa (based on self-report, reported history from their care providers, and an interview with our study psychiatrist), some form of disordered eating was reported in 15 athletes (13 AA and 2 EA). Disordered eating included various degrees of restrictive behaviour and dieting. None of our subjects admitted to bingeing or purging. Subjects were recruited by advertisements in area newspapers, and mailings to physician offices in the New England area. The Institutional Review Board of Partners Health Care approved the study, and informed consent and assent were obtained from all subjects and their parents.

Experimental protocol

Subjects were evaluated during an outpatient visit to the General Clinical Research Center (GCRC) of Massachusetts General Hospital. Subjects receiving hormonal medications, and those with an abnormal TSH or elevated FSH (indicative of hypergonadotropic hypogonadism) were excluded from study participation. Body mass index (BMI) was calculated using the formula: [weight (in kg)]/[height (in m)²]. We report both absolute BMI values, and BMI Z-scores (BMI-SDS, based on the data compiled by the Centers for Disease Control²³). Bone age was assessed by a single paediatric endocrinologist using methods of Greulich and Pyle.²⁴ In addition to a complete history of our subjects' exercise activities, a Modifiable Activity Questionnaire validated for use in adolescents²⁵ was administered to quantify activity into a composite score for comparison across groups. Fasting labs were obtained for active ghrelin, leptin, E₂, testosterone and sex hormone binding globulin (SHBG). EA and nonathletic controls were examined in the early follicular phase of their cycles. In addition, we used duration of amenorrhea in postmenarchal AA girls [8.3 ± 6.7 months (3–29 months)] as a surrogate of gonadal status. None of our subjects admitted to using performance enhancing drugs, however, we did not screen for this as part of the study. This history was reviewed by primary care physicians with study subjects and screening performed when considered necessary. There was no report of use of such agents based on information obtained from primary care physicians of our study subjects.

Biochemical measurements

RIA was used to measure leptin (Linco Research; St Charles, MO; intra-assay CV 3.4–8.3%, sensitivity 0.5 ng/ml) and active ghrelin (Linco, St Louis, MO; intra-assay coefficient of variation (CV) 6.5–9.5%, and sensitivity 7.8 pg/ml). Testosterone was measured by RIA (Diagnostic Products Corp, Los Angeles, CA; intra-assay CV 5.1–9.8% and sensitivity 139 pmol/l), SHBG by IRMA (Diagnostic Products Corp, intra-assay CV 2.8–5.3%; sensitivity 0.04 nmol/l), and E₂ by RIA (Diagnostic Systems Laboratories, Webster, TX; intra-assay CV 6.5–8.9%, sensitivity 8 pmol/l). Free androgen index (FAI) was calculated using the following formula: [total testosterone (nmol/l) $\times$ 100]/SHBG (nmol/l).²⁶ Samples were stored at -80°C until analysis, and were run in duplicate.

Statistical methods

Data are presented as mean $\pm$ SD, and were analysed using the JMP program (version 4, SAS Institute Inc., Cary, NC). Logarithmic conversions were performed to approximate a normal distribution when data were not normally distributed. This was necessary for active ghrelin, E₂, FAI and FSH. We used ANOVA to determine differences between groups followed by the Tukey Kramer test to correct for multiple comparisons. We also used ANOVA to assess differences between diagnostic subtypes after controlling for BMI Z-scores. A *P*-value of < 0.05 was considered significant, and trends (*P*-values between 0.05 and 0.10) are also reported. Univariate and mixed model stepwise regression analyses (*P* = 0.15 for entry into the model, and *P* = 0.10 to leave the model) were used to determine predictors of gonadal steroids and FSH.

Results

Baseline characteristics

Baseline characteristics have been previously reported²⁰ and are summarized in Table 1. AA girls did not differ from EA girls and controls with respect to chronological or bone age. We observed significantly lower BMI, BMI Z-scores and fat mass in AA than in EA and controls. As expected, activity scores were greater in AA and EA girls compared with controls. Menarchal age trended higher in AA than in EA and controls.

Hormonal characteristics

Log active ghrelin levels were significantly higher and leptin levels lower in AA than in the other two groups (Table 2). In AA, active ghrelin levels were twice as high as in EA, whereas leptin levels were about half those in EA. In addition, log E₂ levels trended lower in AA than in EA, total testosterone levels were lower in AA than in EA, and log FAI lower in AA than in controls. After controlling for BMI Z-scores, log active ghrelin levels were higher ($P = 0.0007$), and total testosterone ($P = 0.03$) levels lower in AA compared with the other groups, but differences in leptin and log E₂ were no longer significant.

We also examined ghrelin and leptin levels in AA girls who had primary amenorrhea ($n = 5$) vs. those with secondary amenorrhea ($n = 15$). Our study was not powered for this subset analysis, however, we observed lower leptin levels in AA girls with secondary amenorrhea vs. those with primary amenorrhea/menarchal delay (5.8 ± 2.8 vs. 10.1 ± 4.0 ng/ml, $P = 0.01$), whereas log ghrelin levels did not differ between the groups (4.6 ± 0.5 vs. 4.4 ± 0.4 pg/ml, $P = 0.45$). BMI Z-scores, the prevalence of disordered eating and activity scores did not differ between the two AA groups, however, percent body fat was lower in the group with secondary amenorrhea (20.3 ± 4.6 vs. $25.5 \pm 3.9\%$, $P = 0.04$). In addition, we examined ghrelin and leptin levels in AA girls with ($n = 12$) and without ($n = 8$) a history of disordered eating. Ghrelin and leptin levels did not differ between these groups of AA girls.

For the group as a whole, BMI Z-scores and fat mass inversely predicted log active ghrelin levels ($r = -0.33$, $P = 0.01$; and $r = -0.27$, $P = 0.048$, respectively), and positively predicted leptin levels ($r = 0.68$, $P < 0.0001$; and $r = 0.83$, $P < 0.0001$, respectively). Strong positive associations of leptin with fat mass were observed in AA ($r = 0.73$, $P = 0.0002$), EA ($r = 0.78$, $P = 0.0001$) and controls ($r = 0.84$, $P < 0.0001$).

Associations of body composition, active ghrelin and leptin with gonadal steroids and gonadotrophins

BMI and fat mass were positive predictors of gonadal steroids. Log active ghrelin correlated inversely with testosterone and log FAI, whereas leptin correlated directly with gonadal steroids (Table 3). Activity scores inversely predicted log FAI. To determine independent predictors and to eliminate confounders, we next performed regression modelling using these variables. BMI Z-scores and fat mass correlated very strongly with leptin levels ($r > 0.85$) and to avoid collinearity, the variables we entered into the regression model included log ghrelin, leptin and activity scores (but not BMI Z-scores or fat mass). Significant and independent predictors of testosterone were log ghrelin and leptin (11.6% and 4.5% of the variability explained, respectively); predictors of log FAI were leptin and activity score (11.9% and 8.2% of variability explained, respectively); and log E₂ was predicted by leptin (12% of variability explained). Even after adding either BMI Z-score or fat mass to the regression model, log ghrelin remained an independent predictor of testosterone. Leptin remained an independent predictor of log E₂ when BMI Z-score was added to the model, but when fat mass was added to the model instead of BMI Z-score, fat mass rather than leptin was the sole independent predictor of log E₂ levels. We found no associations of active ghrelin, leptin or fat mass with duration of amenorrhea.

Discussion

We demonstrate higher active ghrelin and lower leptin levels in AA girls compared with EA girls and controls, and also demonstrate that active ghrelin and leptin are independent predictors of gonadal steroids. Our data are consistent with reports of ghrelin administration causing a decrease in gonadotrophin secretion in animal^{10,12} and human studies¹³ and leptin administration causing reversal to an eugonadal state in at least some women with

hypothalamic amenorrhea.⁹ These data suggest that in addition to lower fat mass and leptin, higher ghrelin levels may predict which athletes will develop amenorrhea.

Interestingly, leptin levels and fat mass were lower amongst AA who had secondary amenorrhea as opposed to those with primary amenorrhea, despite no differences in BMI measures, activity levels and prevalence of disordered eating between these groups. The implication of these findings is unclear, but may indicate that the threshold of leptin and fat mass required to initiate menses during puberty may differ from that required to maintain menses. More studies are necessary to confirm these data and to understand its implications. In addition, although this did not reach statistical significance, EA had somewhat higher weights than sedentary controls, and larger studies are needed to determine whether relatively higher weights in athletes protect against development of amenorrhea.

The pathophysiology underlying the decrease in leptin and increase in ghrelin is important to understand. Leptin levels in our subjects were strongly associated with fat mass, consistent with studies in adolescents with other conditions such as anorexia nervosa²⁷ and consistent with the fact that leptin is secreted by adipocytes. In fact, differences in leptin between the groups were no longer significant after controlling for weight measures. Leptin has facilitatory effects on GnRH secretion and is considered to be an important regulator of GnRH and gonadotrophin secretion.^{9,28} Low leptin levels would therefore contribute to a hypogonadal state and low levels of gonadal steroids. It is interesting that in our study, leptin was an independent predictor of E₂ but not of testosterone, and that when fat mass was added to the model, this replaced leptin as an independent predictor of E₂ levels. One may speculate that these findings reflect the secretion of leptin by adipocytes, that leptin levels reflect the amount of fat mass, and that aromatization of androgens to oestrogens occurs peripherally in adipose tissue. Thus, girls with lower fat mass would have lower peripheral conversion of androgens to oestrogens, with findings similar to those observed by us in this study. This could be even more relevant in AA girls, in whom gonadal contribution to circulating oestrogen levels is low, and in eumenorrheic controls studied in the early follicular phase of their cycles, when their gonadal oestrogen production would also be low.

High ghrelin levels have been related to lower BMI in other studies^{15,29} and we observed inverse associations of ghrelin with BMI Z-scores and to a lesser extent with fat mass, for the group as a whole. In addition, ghrelin levels were higher in AA girls even after controlling for body weight measures, suggesting that higher ghrelin levels in AA are driven by factors other than low BMI. One possibility is that ghrelin reflects the state of energy balance. Lower BMI and fat mass in AA than in EA girls suggest that AA girls may be in a state of negative energy balance as reported in studies of adult athletes.^{5,6} In addition, a recent study indicates that drive for thinness is a proxy measure of energy deficiency in exercising women.³⁰ Although we assessed presence or absence of disordered eating rather than drive for thinness in our subjects, and our subjects are adolescents rather than adults, the high prevalence of disordered eating in the AA group also suggests a state of energy deficiency in these girls. However, AA girls with a history of disordered eating did not differ from those without this history for ghrelin and leptin levels. Detailed food records and questionnaires that provide a composite measure of average daily energy expenditure based on the nature of exercise and assigned metabolic equivalents³¹ will be useful to determine whether AA girls are indeed in a state of negative energy balance, and whether this predicts high ghrelin levels in this group. In addition, it will be important to assess relationships based on training load. The lack of these tools is a limitation of our study. Interestingly, one recent study reported higher ghrelin levels in physically active vs. inactive girls.²⁹ In contrast, we observed no differences in ghrelin levels between EA and sedentary controls.

Ghrelin administration has been shown to decrease gonadotrophin pulsatility in human¹³ and animal studies¹² and high ghrelin levels resulting from a state of negative energy balance would similarly be expected to cause a decrease in gonadotrophin pulsatility and low levels of gonadal steroids. In our study, high ghrelin levels were associated with low levels of testosterone, and to a lesser extent E₂. However, after controlling for leptin levels and/or fat mass, this association persisted for testosterone, but not for E₂, maybe because of the reasons described above. Given the associative nature of this study, further and interventional studies are necessary to confirm our findings. High ghrelin levels have been reported in a study in adult exercising women with amenorrhea, however, this study did not examine ghrelin levels in relation to levels of gonadal steroids.³² In addition, the adolescent years are characterized by changing patterns of gonadotrophin pulsatility and ghrelin secretion³³ which differentiate adolescents from adults, and therefore, adult data cannot always be extrapolated to adolescents.

Another study limitation is that we do not have data on gonadotrophin pulsatility in relation to active ghrelin and leptin levels, and further studies are necessary to assess gonadotrophin pulsatility patterns in this population in relation to ghrelin. We have previously established that fasting ghrelin and leptin levels are very strongly associated with ghrelin and leptin levels obtained by frequent sampling overnight.^{15,27} Of note, we examined menstruating subjects in the early follicular phase of their menstrual cycles when gonadotrophin and gonadal steroid levels are the lowest. Reported E₂ levels therefore are not representative of the higher E₂ levels that would be seen in menstruating subjects in the late follicular and luteal phases, when a significant difference from AA would be obvious. Despite this, we observed differences in levels of testosterone and FAI, and a trend in E₂ levels, in comparison to girls with AA, suggesting that differences are likely even more marked in the latter half of the follicular phase.

Our data indicate that AA girls differ from EA girls and controls such that their active ghrelin levels are higher, and leptin levels lower. Higher ghrelin levels in AA persist after controlling for body weight measures. Ghrelin, leptin and activity scores are independent predictors of levels of gonadal steroids. We speculate that in addition to low leptin levels (from lower fat mass), high ghrelin levels resulting from a negative state of energy balance in adolescent AA girls may contribute to suppression of gonadotrophin pulsatility, and therefore hypothalamic amenorrhea.

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Table 1

Baseline characteristics in athletes with amenorrhea, eumenorrheic athletes and controls

	Athletes with amenorrhea <i>N</i> = 21	Eumenorrheic athletes <i>N</i> = 19	Controls <i>N</i> = 18	<i>P</i>
Age (years)	16.1 ± 1.5	15.6 ± 1.4	15.5 ± 1.4	NS
Bone age (years)	16.0 ± 1.0	16.0 ± 1.3	15.6 ± 1.6	NS
Weight (kg)	50.6 ± 4.3 [†]	58.1 ± 8.0	55.4 ± 7.0	0.003
Height (m)	163.0 ± 5.8	163.0 ± 5.9	163.7 ± 5.6	NS
BMI (kg/m ²)	19.1 ± 1.3 ^{*†}	21.9 ± 3.2	20.7 ± 2.5	0.003
BMI Z-score	-0.61 ± 0.29 [†]	0.16 ± 0.76	-0.14 ± 0.57	0.0003
Fat mass (kg)	11.2 ± 2.7 ^{*†}	16.2 ± 4.6	15.1 ± 5.3	0.001
Activity score	23.8 ± 6.6 [*]	28.1 ± 10.3 [*]	9.9 ± 4.5	< 0.0001
Age at menarche (years)	12.9 ± 1.5	12.1 ± 0.8	12.2 ± 1.0	0.09
Disordered eating	61.9%	10.5%	0%	< 0.0001

Mean ± SD.

^{*} *P* < 0.05 compared with controls;[†] *P* < 0.05 compared with EA (Tukey Kramer's test to adjust for multiple comparisons).

NS, not significant; BMI, body mass index.

Table 2

Hormonal parameters in amenorrheic athletes, eumenorrheic athletes and controls

	Athletes with amenorrhea <i>N</i> = 21	Eumenorrheic athletes <i>N</i> = 19	Controls <i>N</i> = 18	<i>P</i>
Active ghrelin (pg/ml)	109.6 ± 47.6 ^{†‡}	52.7 ± 37.6	67.0 ± 27.2	< 0.0001 [*]
Leptin (ng/ml)	6.8 ± 3.6 ^{†‡}	13.2 ± 8.4	14.0 ± 10.8	0.01
Total testosterone (pmol/l)	638 ± 264 [‡]	980 ± 318	842 ± 288	0.002
Free androgen index	1.6 ± 1.2 [†]	2.2 ± 1.7	2.4 ± 1.1	0.04 [*]
E ₂ (pmol/l)	80.9 ± 41.5 [§]	104.8 ± 38.8	82.0 ± 28.2	0.07 [*]

Mean ± SD.

^{*} Comparisons performed after log conversion of data to approximate a normal distribution.[†] *P* < 0.05 compared with controls,[‡] *P* < 0.05 compared with EA,[§] *P* < 0.10 compared with EA (Tukey Kramer's test to adjust for multiple comparisons).

Table 3

Correlation coefficients of associations of gonadal steroids and FSH with body composition parameters, active ghrelin and leptin in all subjects

	Total testosterone	Log free androgen index	Log E ₂
BMI	0.46 [§]	0.38 [‡]	0.33 [†]
BMI-Z score	0.42 [‡]	0.38 [‡]	0.31 [†]
Fat mass	0.43 [‡]	0.32 [†]	0.43 [‡]
% body fat	0.35 [‡]	0.25 [*]	0.37 [‡]
Activity score	0.10	-0.31 [†]	0.17
Log ghrelin	-0.34 [†]	-0.18	-0.25 [*]
Leptin	0.30 [†]	0.35 [‡]	0.34 [‡]

* $P < 0.1$,

[†] $P < 0.05$,

[‡] $P < 0.01$,

[§] $P < 0.001$.