Circulating leptin concentrations do not distinguish menstrual status in exercising women

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BACKGROUND: Low concentrations of leptin secondary to low body fat or other modulators are thought to be a key signal whereby an energy deficit suppresses the reproductive axis in exercising women resulting in functional hypothalamic amenorrhea (FHA). The purpose of this study was to first examine leptin concentrations in exercising women with and without FHA to address whether there is a threshold concentration of leptin below which reproductive function is suppressed. Secondly, we examined the role of adiposity and other possible modulators of leptin to ascertain whether leptin regulation differs depending on reproductive status.

METHODS: This study assessed 50 exercising, premenopausal women (aged 18–30 years) over the course of one menstrual cycle (eumenorrheic women) or one 28-day monitoring period (amenorrheic women). Quantification of daily urinary ovarian steroids and menstrual history were used to determine menstrual status. Body composition was assessed using dual energy X-ray absorptiometry, and leptin was determined by enzyme-linked immunosorbent assay. Key modulators of leptin such as serum insulin concentration, carbohydrate intake, glucose availability, indirect indices of sympathetic nervous activity and other factors were assessed using linear regression.

RESULTS: Percentage body fat (%BF) (21.0 ± 1.0 versus 26.8 ± 0.7%; P < 0.001) and leptin concentration (4.8 ± 0.8 versus 9.6 ± 0.9 ng/ml; P < 0.001) were lower in the exercising women with amenorrhea (ExAmen; n = 24) compared with the exercising ovulatory women (ExOvul; n = 26). However, the ranges in leptin were similar for each group (ExAmen: 0.30–16.98 ng/ml; ExOvul: 2.57–18.28 ng/ml), and after adjusting for adiposity the difference in leptin concentration was no longer significant. Significant predictors of log leptin in ExAmen included %BF (β = 0.826, P < 0.001), log insulin (β = 0.308, P = 0.012) and log glycerol (β = 0.258, P = 0.030), but in ExOvul only %BF predicted leptin.

CONCLUSIONS: These data suggest that leptin concentrations per se are not associated with FHA in exercising women, but the modulation of leptin concentrations may differ depending on reproductive status.

Key words: leptin / reproductive status / amenorrhea / adiposity / exercise

Introduction

Evidence suggests that functional hypothalamic amenorrhea (FHA) is associated with a relative energy deficiency (Williams et al., 2001; Loucks and Thuma, 2003; Wade and Jones, 2004) or other stressors (Tilbrook et al., 2002) that results in the disruption of the hypothalamic–pituitary–gonadal axis and other neuroendocrine axes (Laughlin and Yen, 1996). Leptin, the hormone product of the obesity (ob) gene, is known as a key signal whereby nutritive status is relayed from the periphery to the hypothalamus (Barash et al., 1996) where it acts to regulate appetite (Weigle et al., 1995), energy balance (Halaas et al., 1995) and reproduction (Chehab et al., 1996; Cunningham et al., 1999). Leptin is secreted by adipocytes and circulates at concentrations highly correlated to adiposity (Ostlund et al., 1996), which explains why women with very low body fat, and presumably low leptin concentrations, are at risk for the development of FHA (Frisch and McArthur, 1974). Some investigators have shown that exercising women with FHA display leptin concentrations that are significantly lower than those of their ovulatory counterparts even after controlling for body fat (Miller et al., 1998; Warren et al., 1999). The association of this relative ‘hypo leptinemia’ with reproductive dysfunction suggests that other modulators of leptin concentration may play a role in the etiology of FHA. Other key factors shown to modulate leptin include plasma insulin concentration (Malmstrom et al., 1996), carbohydrate intake (Jenkins et al., 1997), glucose availability (Grinspoon and Trayhurn, 2001) and gonadal steroid milieu (Elbers et al., 1997; Wabitsch et al., 1997). Currently there are only a few studies that...
have carefully characterized leptin concentrations in FHA subjects, and data from these studies have yielded conflicting results (Tataranni et al., 1997; Warren et al., 1999; Thong et al., 2000). Similarly, the specific pathways whereby leptin disrupts gonadotropin releasing hormone (GnRH) pulsatility remain largely unknown. The rationale behind this study was therefore to further explore the role of leptin in the etiology of FHA and to investigate whether the modulation of leptin synthesis differs based on reproductive status. The findings from this study could lend further insight into the viability of leptin administration as a treatment option for women with FHA.

The purpose of this study was to examine the range of leptin concentrations observed in exercising women with FHA in comparison with their exercising ovulatory peers, with and without adjustment for body fat. It was hypothesized that exercising women with FHA would display lower serum leptin concentrations when compared with their ovulatory counterparts, even after adjusting for body fat. A secondary purpose was to determine whether factors other than body fat predict leptin concentrations in exercising women, and whether these factors differ depending on reproductive status. It was hypothesized that leptin would be differentially regulated in exercising women with FHA compared with exercising ovulatory women.

**Materials and Methods**

**Experimental design**

This investigation was part of a larger, prospective study at the University of Toronto and Penn State University that was designed to determine whether a 12-month intervention of increased caloric intake would improve indices of bone health and menstrual status in premenopausal women who suffer from severe exercise-associated menstrual disturbances (EAMD), including oligomenorrhea (long and inconsistent menstrual cycles of 36–90 days) and amenorrhea (the absence of menses for >90 days). The current investigation includes baseline data from two experimental groups, including an exercising amenorrheic group (ExAmen, n = 24) and an exercising ovulatory control group (ExOvul, n = 26). The baseline assessment period was equivalent to either one menstrual cycle, for women entering the study with regular menstrual cycles, or one 28-day monitoring period, for women entering the study with regular menstrual histories, the results of a physical examination and other endocrine and metabolic parameters.

**Subjects**

Volunteers were recruited by posters targeting physically active women for a study on the impact of increased caloric intake on bone health and menstrual cycle in energy deficient exercising women. Inclusion criteria for this study were: (i) no history of any serious medical conditions; (ii) no current clinical diagnosis of an eating or psychiatric disorder based on both self-report and an interview with a clinical psychologist or licensed clinical social worker; (iii) age 18–30 years; (iv) body mass index (BMI) 16–25 kg/m²; (v) weight stable (+2 kg) for the past 6 months; (vi) non-smoking; (vii) no medication use that would alter metabolic or reproductive hormone concentrations; (viii) ≥150 min/week self-reported purposeful exercise; (ix) no menses within the past 3 months or six or fewer menses within the past year, or regular menses within the past 6 months; (x) no history of a clinical diagnosis of polycystic ovarian syndrome (PCOS) or a free androgen index (FAI) ≥3 with other corroborative symptoms of PCOS, such as hirsutism and acne; (xi) no evidence of hyperprolactinemia or thyroid dysfunction; (xii) not currently pregnant, as assessed by a human chorionic gonadotrophin test. Each subject was informed of the purpose, procedures and potential risks of participation in the study before signing an informed consent approved by the University of Toronto and Penn State University Institutional Review Boards.

**Screening procedures**

During an initial visit, study details and participation requirements were explained, and written informed consent was obtained. Once consent was obtained, height and weight were measured, and subjects completed questionnaires to assess demographics, medical history, exercise history, menstrual history, eating behaviors, bone health and mental health. A physical exam was performed on all subjects by an on-site clinician to determine overall health and check for physical symptoms of PCOS such as acne or hirsutism. In addition, a fasting blood sample was analyzed for a complete blood count, basic chemistry panel, and an endocrine panel which included measures of luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid stimulating hormone (TSH), thyroxine (T4), prolactin (PRL) and dehydroepiandrosterone (DHEA) (Quest Diagnostics, Pittsburgh, PA), total testosterone and sex hormone-binding globulin (SHBG) to rule out endocrine or metabolic disease or other illnesses. A clinical psychologist or licensed clinical social worker interviewed each subject to determine if she was suffering from major psychiatric disorders including depression or clinical eating disorders. Subjects met with a General Clinical Research Center (GCRC) registered dietitian after completing a 3-day diet log (2 weekdays and 1 weekend day) to discuss eating patterns and preferences. Additionally, dual-energy X-ray absorptiometry (DXA) scans of the total body, lumbar spine and dual femur were performed to assess bone mineral density (BMD) and body composition (GE Lunar Prodigy and iDXA, Madison, WI, USA).

**Classification of menstrual status**

Subjects collected first morning urine samples throughout the 4 week baseline monitoring period. Day 1 of baseline was the first day of menses for women who reported eumenorrheic menstrual status and an arbitrary day for women who reported either no menses within the past 3 months or six or fewer menses within the past year. Our classification of menstrual status was based on urinary estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG) and LH profiles, self-reported menstrual histories, the results of a physical examination and other endocrine measures described below.

Subjects who reported no menses within the last 3 months or six or fewer menses within the past year, and whose baseline menstrual cycle confirmed diagnosis of amenorrhea were assigned to the ExAmen group. We assessed FHA by confirming a negative pregnancy test, chronically suppressed E1G and PdG profiles, the absence of biochemical (elevated FAI) or physical signs of PCOS, the absence of elevated LH and FSH and the absence of signs of other metabolic (thyroid) or endocrine (hyperprolactinemia) disease (Current evaluation of amenorrhea, 2008). FAI was calculated according to the following equation: FAI = (total testosterone (nmol/l)/sex hormone-binding globulin (nmol/l)) * 100 (Rosner et al., 2007). Subjects who exhibited an FAI of ≥3 were eliminated from the study as values in this range have been reported to be consistent with PCOS (Barber et al., 2007; Lindholm et al., 2008; Nardo et al., 2009; Stener-Victorin et al., 2010).

Subjects who reported regular menses within the last 6 months, confirmed by an ovulatory baseline cycle of 26–35 days were assigned to the ExOvul group. Ovulatory status was confirmed by the presence of a urinary LH peak, identified as a peak concentration above 25 mU/ml after a mid-cycle E1G peak >35 ng/ml, and followed by a peak luteal phase PdG concentration above 5 μg/ml (De Souza et al., 1998, 2010).
To determine estrogen and progesterone exposure, E1G and PdG urinary metabolites were compared between the groups using a modified trapezoidal integrated area under the curve (AUC) technique. Menstrual calendars were used to chart menstrual symptoms, such as cramps, bleeding, spotting, discharge, etc. for both ExOvul and ExAmen subjects. 

**Blood sampling**

Fasting morning venous blood samples were collected once during Week 3 of baseline and once at the end of baseline for all subjects. The latter two samples were pooled for all baseline hormone analyses. Blood was always collected before 10:00 h following an overnight fast and after subjects refrained from exercise and caffeine for at least 12 h. All samples were allowed to clot for 30 min and then were centrifuged at 4°C for 15 min at 3225.6 g-force (3000 rpm). The serum was then aliquoted into 2-ml polyethylene storage tubes and stored frozen at −80°C until analysis.

**Serum hormone analysis**

Serum leptin concentration was measured using a solid-phase sandwich enzyme-linked immunoassay (ELISA) for total leptin (Millipore, St Charles, MI, USA). All samples were measured in duplicate and the content of leptin in samples was calculated from a standard curve generated in each assay with recombinant human leptin. The inter-assay and intra-assay coefficients of variation for the low control were 6.2 and 4.6%, respectively. This assay is sensitive to leptin concentrations of 0.5 ng/ml. Serum triiodothyronine (TT3) was analyzed using a chemiluminescence immunoassay analyzer (Immulite, Diagnostic Products Corporation, Los Angeles, CA) through competitive immunoassay. Analytical sensitivity for the TT3 assay was 0.54 nmol/l (35 ng/dl). The intra-assay and inter-assay coefficients of variation were 13.2 and 15.6%, respectively.

Serum testosterone was measured using a radioimmunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The intra-assay and inter-assay coefficients of variation were 7.9 and 8.0%, respectively. This assay was sensitive to total testosterone concentrations of 21.7 μmol/l (0.20 mg/dl). Serum insulin concentration was analyzed using a chemiluminescence immunoassay analyzer (Immulite, Euro Diagnostic Products Corporation, Lianberis, UK) through competitive immunoassays (Siemens Medical Solutions Diagnostics). Analytical sensitivity for the TT3 assay was 0.54 nmol/l (35 ng/dl). The intra-assay and inter-assay coefficients of variation were 6.4 and 8.0%, respectively. Total testosterone was measured using a radioimmuno assay kit (Siemens, Los Angeles, CA, USA) through competitive immunoassay. Analytical sensitivity for the testosterone assay was 0.14 nmol/l (4.0 ng/dl). The intra-assay and inter-assay coefficients of variation were 6.4 and 7.5%, respectively. Sex hormone-binding globulin (SHBG) was analyzed using a chemiluminescence immunoassay analyzer (Immulite, Euro Diagnostic Products Corporation, Lianberis, UK) through competitive immunoassay. Analytical sensitivity for the SHBG was (0.2 mmol/l) 5.76 ng/dl. The intra-assay and inter-assay coefficients of variation were 6.4 and 8.7%, respectively. TSH, T4, T3, PRL, DHEA, FSH and LH were sent out for analysis (Quest Diagnostics, Pittsburgh, PA, USA).

**Urinary hormone measurements**

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (Miller *et al.*, 2004) which has been recommended to perform as well as creatinine correction for adjusting urinary hormone concentrations (Miller *et al.*, 2004). Microtiter plate competitive enzymelinked immunoassays were used to measure the urinary metabolites E1G and PdG as previously reported (De Souza *et al.*, 1998, 2010). Urinary LH was determined by coat-a-count immuno-adiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The sensitivity of the LH assay was 0.15 mU/ml. The intra-assay and inter-assay coefficients of variation were 1.6 and 7.1%, respectively.

**Energy status measurements**

Resting energy expenditure (REE) and resting respiratory exchange ratio (RER) were determined by indirect calorimetry using a ventilated hood (Sensormedics Vmax Series, Yorba Linda, CA, USA). Subjects were tested in the morning, between 06:00 and 10:00 h in a fasted state, having refrained from exercise and caffeine for 24 h. Subjects were also instructed not to take any medications the morning of the procedure and to do as little physical activity that morning as possible. Upon arrival at the laboratory, the subject rested in a supine position for 30–45 min to establish resting conditions and acclimate to room temperature. The ventilated hood was then placed over the subject, and REE measurements were collected for a minimum of 30 min. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were collected every 30 s. The first 5 min were automatically excluded, and steady state was defined as VO2 and VCO2 varying by <10% and RER varying by <5% from minute to minute. Steady state data for VO2 and VCO2 were averaged and RER was calculated using the Weir equation (Weir, 1990). Resting RER was calculated as the average ratio of VCO2 to VO2 during steady state.

**Dietary energy intake**

Dietary energy intake and macronutrient composition were estimated using a 3-day diet log (2 weekdays and 1 weekend day) once during baseline in all subjects. Subjects were provided with detailed instructions on how to record types and quantities of foods eaten. Diet analyses of the 3-day food logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN, USA).

**Anthropometric testing**

All subjects completed a health history questionnaire, which included information on menstrual history and 6-month exercise history (average minutes per week). Total body mass was measured to the nearest 0.1 kg on a physician’s scale (Seca, Model 770, Hamburg, Germany) on five occasions (at screening and four times during baseline), and the mean of these measurements is presented for baseline weight. Height was measured to the nearest 1.0 cm during screening. Baseline BMI was calculated as the baseline weight divided by screening height squared (kg/m²).

%BF, fat mass and fat free mass (FFM) were assessed using DXA. Of the 50 subjects, 45 were scanned on one of two scanners, either a GE Lunar Prodigy (n = 29, enCORE 2002 software version 6.50.069) or a GE Lunar iDXA (n = 16, enCORE 2008 software version 12.10.113). Consistent with the International Society of Clinical Densitometry guidelines, a cross-calibration study was performed to remove systematic bias between the systems. For the cross-calibration study, 14 participants were scanned in triplicate on both machines. The majority (n = 8) were scanned on both machines within 5 days; however, there was ~1 month between scans for some subjects (n = 6). The values were found to be highly correlated with no significant difference between the population mean values. However, we did find some biases in the total BMD, total bone mineral content, fat mass and %BF relative to the magnitude of the variable. Equations were derived using simple linear regression to remove these biases and report the Prodigy values calibrated to the iDXA. The remaining subjects (n = 5) were scanned on a Hologic QDR 4500 W.

**Training status**

Peak aerobic capacity (VO2 peak) was measured once during Week 3 of Baseline during a progressive treadmill test to volitional exhaustion with open-circuit spirometry using methods that have been previously reported (De Souza *et al.*, 2007; Scheid *et al.*, 2009). Subjects recorded daily
exercise activities throughout study participation on weekly exercise logs which were used to quantify purposeful exercise in minutes per week.

**Statistical analysis**

Logarithmic conversions were performed to approximate normal distribution when data were not normally distributed. This was necessary for leptin, glycerol and insulin. We used independent t-tests to determine differences between groups. Bivariate analysis was used to describe relationships between log leptin and variables that have been shown to either directly or indirectly modulate leptin. The variables considered were: (i) %BF (Ostlund et al., 1996), (ii) serum insulin concentration (Leroy et al., 1996), (iii) weekly exercise minutes and peak VO2, as indicators of training status (Hickey et al., 1997), (iv) dietary carbohydrate intake and RER, as indicators of carbohydrate availability (Jenkins et al., 1997), (v) RER/kgFFM and total kilocalories consumed per day, as indicators of acute energy status (Hilton and Loucks, 2000), (vi) E1G AUC as well as the E1G concentration coinciding with the day of the leptin sample, as indicators of gonadal steroid milieu (Elbers et al., 1997; Shimizu et al., 1997) and (vii) glycerol, as an indicator of lipolysis to reflect sympathetic activation of adipose tissue (Rayner and Trayhurn, 2001). These variables were then used in multivariate analysis, by use of stepwise regression, to determine the model which best predicted leptin in each group. An ANCOVA was also used to generate adjusted means for serum leptin concentration after correcting for adiposity. A specific power calculator was used to determine sample sizes required to detect a meaningful difference in leptin concentration using an independent t-test between ExOvul women and ExAmen. A sample size of 23 subjects per group was determined to be required to detect a meaningful difference in leptin concentrations between ExOvul women and ExAmen women. Statistical analyses were performed using SPSS for Windows (Version 18.0; Chicago, IL, USA) statistical software. For all analyses, P < 0.05 was considered statistically significant. Data are reported as mean ± SEM.

**Results**

**Descriptive characteristics**

Baseline descriptive characteristics for the ExOvul women and the ExAmen women are presented in Table I. The exercising groups of women were similar with regard to age, height and weight, yet signifi-

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### Table I Baseline descriptive data.

<table>
<thead>
<tr>
<th></th>
<th>ExOvul (n = 26)</th>
<th>ExAmen (n = 24)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td>23.2 ± 0.7</td>
<td>21.6 ± 0.7</td>
<td>0.120</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.9 ± 1.3</td>
<td>167.2 ± 1.3</td>
<td>0.220</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>59.0 ± 1.0</td>
<td>55.9 ± 1.4</td>
<td>0.076</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.7 ± 0.3</td>
<td>20.0 ± 0.4</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>26.8 ± 0.7</td>
<td>21.0 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>15.8 ± 0.5</td>
<td>11.8 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>43.1 ± 0.8</td>
<td>44.3 ± 1.0</td>
<td>0.349</td>
</tr>
<tr>
<td><strong>Menstrual characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of menarche</td>
<td>12.5 ± 0.2</td>
<td>13.4 ± 0.3</td>
<td>0.017</td>
</tr>
<tr>
<td>Gynecological age (year)</td>
<td>10.7 ± 0.7</td>
<td>8.1 ± 0.7</td>
<td>0.012</td>
</tr>
<tr>
<td>E1G AUC (ng/m²)</td>
<td>1010.6 ± 86.1</td>
<td>468.5 ± 46.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PdG AUC (µg/m²)</td>
<td>74.9 ± 5.5</td>
<td>23.6 ± 3.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>History of amenorrhea (days)</td>
<td></td>
<td>321 ± 59</td>
<td>n/a</td>
</tr>
<tr>
<td>Average cycle length (days)</td>
<td>30 ± 0.5</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td><strong>Training characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO2 peak (ml/kg/ min)</td>
<td>46.0 ± 1.3</td>
<td>50.3 ± 1.7</td>
<td>0.049</td>
</tr>
<tr>
<td>Exercise history (min/ week)</td>
<td>468 ± 71</td>
<td>662 ± 85</td>
<td>0.084</td>
</tr>
<tr>
<td>Exercise activity (min/ week)</td>
<td>236 ± 26</td>
<td>388 ± 53</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Serum hormones</strong></td>
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<td></td>
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</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>9.6 ± 0.9</td>
<td>4.8 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glycerol (µmol/l)</td>
<td>380.1 ± 12.7</td>
<td>456.1 ± 43.4</td>
<td>0.155</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>27.8 ± 2.9</td>
<td>27.0 ± 4.6</td>
<td>0.876</td>
</tr>
<tr>
<td>TT3 (nmol/l)</td>
<td>1.42 ± 0.05</td>
<td>1.22 ± 0.07</td>
<td>0.023</td>
</tr>
<tr>
<td>TSH (mU/l)</td>
<td>1.6 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>0.264</td>
</tr>
<tr>
<td>PRL (pmol/l)</td>
<td>468.9 ± 38.9</td>
<td>293.3 ± 28.8</td>
<td>0.001</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>10.7 ± 3.1</td>
<td>4.1 ± 1.1</td>
<td>0.051</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>4.0 ± 0.5</td>
<td>5.7 ± 1.4</td>
<td>0.248</td>
</tr>
<tr>
<td><strong>Metabolic characteristics</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RER</td>
<td>0.81 ± 0.01</td>
<td>0.87 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>REE (kJ/day)</td>
<td>5321 ± 109</td>
<td>5162 ± 163</td>
<td>0.430</td>
</tr>
<tr>
<td>REE (kJ/kgFFM/day)</td>
<td>123.9 ± 2.1</td>
<td>116.8 ± 2.9</td>
<td>0.053</td>
</tr>
<tr>
<td><strong>Dietary intake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy intake (kJ/day)</td>
<td>7942 ± 456</td>
<td>9056 ± 1017</td>
<td>0.310</td>
</tr>
<tr>
<td>Carbohydrate intake (g/day)</td>
<td>259 ± 18</td>
<td>315 ± 38</td>
<td>0.330</td>
</tr>
<tr>
<td>Carbohydrate intake (%)</td>
<td>53.6 ± 1.6</td>
<td>57.4 ± 2.0</td>
<td>0.144</td>
</tr>
<tr>
<td>Protein intake (g/day)</td>
<td>76 ± 4</td>
<td>86 ± 9</td>
<td>0.330</td>
</tr>
<tr>
<td>Protein intake (%)</td>
<td>16.2 ± 0.6</td>
<td>16.2 ± 0.8</td>
<td>0.999</td>
</tr>
</tbody>
</table>

*ExOvul (n = 21), ExAmen (n = 20).

Values are mean ± SEM. ExAmen, exercising women with amenorrhea; ExOvul, exercising women with ovulatory menstrual cycles; BMI, body mass index; FFM, fat free mass; AUC, area under the curve; E1G, estrone glucuronide; PdG, pregnanediol glucuronide; TT3, triiodothyronine; TSH, thyroid stimulating hormone; PRL, prolactin; LH, luteinizing hormone; FSH, follicle stimulating hormone; RER, respiratory exchange ratio; REE, resting energy expenditure. Glycerol conversion: mg/dl × 108.59 = µmol/l. TT3 conversion: ng/dl × 0.0154 = nmol/l. PRL conversion: ng/ml × 43.478 = pmol/l. REE and dietary intake conversion: kcal/day × 4.1868 = kJ/day.
Significantly different for BMI. However, the ExOvul group had a significantly greater BMI and %BF and fat mass than the ExAmen group. The ExAmen group had a greater VO2 peak and current weekly training volume than the ExOvul group. No participants presented with abnormally high PRL levels, and all participants were euthyroid as evidenced by TSH values in the normal range and total T4 (n = 21) or free T4 (n = 29) values also within normal ranges. There were no differences between the exercising groups of women with respect to dietary characteristics or total REE; however, the ExAmen women exhibited a trend toward a lower REE/kgFFM and a significantly greater resting RER than the ExOvul women.

Menstrual characteristics and urinary ovarian steroid data

Menstrual characteristics for ExOvul and ExAmen women are also shown in Table I. ExAmen women had a significantly older age of menarche, younger gynecological age and lower estrogen and progesterone exposure in comparison to the ExOvul women. Composite graphs of the daily concentrations of E1G and PdG are presented in Fig. 1. In exercising ovulatory women (top panel), a mid-cycle E1G peak followed by a rise in PdG during the luteal phase is observed. In exercising amenorrheic women (bottom panel), E1G and PdG concentrations are suppressed.

**Figure 1** Composite graphs of daily E1G and PdG in exercising women with ovulatory menstrual cycles (top panel) and exercising women with amenorrhea (bottom panel). The E1G and PdG data for ovulatory cycles are aligned by day of the LH peak defined as Day 0. The E1G and PdG data for amenorrheic cycles are aligned by chronological day of daily urinary hormone collections. E1G, estrone-1-glucuronide; PdG, pregnanediol glucuronide; LH, luteinizing hormone. Values are mean ± SEM.

**Figure 2** Individual (open circles) and mean (closed circles) serum leptin concentrations for 24 exercising amenorrheic (ExAmen) and 26 exercising ovulatory (ExOvul) women.

**Leptin measures**

Fasting serum leptin concentration was significantly lower in ExAmen compared with ExOvul exercising women (Table I). However, as seen in Fig. 2, the ranges in serum leptin concentration for ExAmen and for ExOvul women were similar (Range for ExAmen women: 0.30–16.98 ng/ml; Range for ExOvul women: 2.57–18.28 ng/ml). Regression analyses demonstrated a positive correlation between leptin and percent body fat (r² = 0.733, P < 0.001), regardless of menstrual status (Fig. 3). Using regression analyses performed on each group separately (Table II), the slopes of the regression equation were not significantly different (P = 0.732), as assessed by ANCOVA, which demonstrates that the %BF versus leptin relationship is similar for both groups. The difference in serum leptin concentration between groups was no longer significant after adjusting for adiposity (Fig. 4).

**Correlations with log leptin**

When both groups were combined, log leptin was positively correlated with %BF (r = 0.848, P < 0.001), REE/kgFFM (r = 0.426, P = 0.003), log TT3 (r = 0.429, P = 0.002), E1G AUC (r = 0.374, P = 0.008) and the E1G concentration from the morning urine sample that coincided with the day of the blood draw (r = 0.481, P = 0.001). Weekly exercise minutes (r = −0.285, P = 0.050), dietary energy intake (r = −0.363, P = 0.014) and carbohydrate intake (r = −0.288, P = 0.045) were negatively correlated with log leptin when both groups were combined. However, log leptin was not observed to be significantly correlated with LH or FSH.
Determinants of leptin

The statistical models that best predicted log leptin concentration in the ExOvul and ExAmen groups, respectively, are presented in Table III. Log leptin was best predicted by %BF in both groups. For the ExAmen group, %BF explained 65.7% of the total variability in leptin concentration. Log insulin and log glycerol concentrations explained an additional 5.6 and 6.1%, respectively, of the total variability observed in circulating leptin concentration. In contrast to the ExAmen group, %BF was the only variable that significantly predicted log leptin concentration in the ExOvul group, accounting for 52.2% of the total variability.

Discussion

Although strong evidence supports the view that leptin is a key signal allowing for the integrated control of nutrition and reproduction (Cunningham et al., 1999), no studies to date confirm the precise mechanism whereby leptin is sensed in the hypothalamus to regulate reproductive function, and none have determined the specific role leptin plays in the induction of EAMD in exercising women. We assessed reproductive status and measured fasting serum leptin concentration and body composition in 50 premenopausal women to explore whether low leptin per se mediates reproductive suppression, or whether hypoleptinemia (i.e. having lower leptin than what can be accounted for by adiposity) is the signal whereby the reproductive axis is suppressed. Corroborating the findings of others (Tataranni et al., 1997; Thong et al., 2000), we found that FHA was associated with low %BF and low fasting serum leptin concentration in comparison to regularly menstruating women. However, the findings from this study suggest that it is not low leptin concentrations per se that are suppressing reproductive function in exercising amenorrheic women. Although many of the exercising amenorrheic women exhibited circulating leptin concentrations that were lower than those observed in their ovulating peers, we observed a wide range of circulating leptin concentrations in association with FHA. Our data, therefore, do not support the hypothesis proposed by others (Matkovic et al., 1997) that a threshold concentration of leptin, below which reproductive function is suppressed, is the primary mechanism underlying reproductive suppression.

Many of the exercising amenorrheic women in our study exhibited circulating leptin concentrations that were no different from those
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observed in the ovulatory women, which challenges the hypothesis that there is a critical leptin threshold, i.e. a leptin concentration below which reproduction function is disrupted (Tataranni et al., 1997). However, the lowest leptin concentration observed in the group of exercising ovulatory women was 2.57 ng/ml, and several of the exercising amenorrheic subjects exhibited leptin concentrations that were lower than 2.57 ng/ml. One could therefore argue that if there is a critical concentration of serum leptin, it would approximate 2.57 ng/ml, which is in close agreement with the plasma leptin threshold identified by others (Tataranni et al., 1997; Thong et al., 2000). Nevertheless, many women with FHA exhibited leptin concentrations that were above this ‘threshold’, which may suggest that relative or individual changes in leptin concentrations are sensed by, and influence, the reproductive system.

The primary regulator of leptin (ob) gene expression is adiposity (Ostlund et al., 1996); however, reports of hypoleptinemia, i.e. having a lower leptin concentration than can be accounted for by adiposity alone, led us to address whether hypoleptinemia explained reproductive suppression in our subjects, and whether other modulators of leptin might contribute to leptin concentrations. Our data did not support the hypothesis that hypoleptinemia as we defined it mediates reproductive suppression. After adjusting for adiposity there was no difference in mean fasting leptin concentration between the exercising women with and without amenorrhea. Others (Tataranni et al., 1997; Laughlin et al., 1998) have reported similar findings in women with amenorrhea, regardless of exercise status, and have concluded that significantly lower body fat accounts for much of the difference in leptin levels between amenorrheic and regularly menstruating women. These findings support the view that leptin acts as a peripheral signal to relay long-term energy status, i.e. size of energy stores, to the hypothalamus, and helps to explain why women with low adiposity are at greater risk for amenorrhea (Frisch and McArthur, 1974). However, fasting leptin concentrations do not always discriminate reproductive status, as many normal-weight women with impaired reproductive function exhibit leptin concentrations comparable to their ovulating peers.

We have previously reported that exercising women with exercise-induced amenorrhea present with an energy deficiency as evidenced by significantly lower concentrations of circulating TT3 and a suppressed resting metabolic rate when compared with their ovulating counterparts (De Souza et al., 2004, 2007; Scheid et al., 2009). Likewise, in this sample of exercising women, we observed significantly lower levels of TT3 and a trend toward suppressed REE (kJ/kg FFM/day) among ExAmen women, thus indicating the presence of an energy deficiency among this group (Table I). Our data showed that leptin was a significant positive predictor of both REE after adjustment for FFMI ($r^2 = 0.181, P = 0.002$) and circulating TT3 ($r^2 = 0.184, P = 0.003$). Other investigators (Polito et al., 2000) have also observed this significant positive association between circulating leptin and REE; however, results have been equivocal (Satoh et al., 2003; Johnstone et al., 2005).

An acute state of low energy availability, i.e. a day of insufficient caloric intake to compensate for energy expenditure, suppresses the nocturnal rise in leptin concentration and LH pulsatility, as demonstrated by Hilton and Loucks (2000), and may explain why impaired reproductive function is not always associated with low body fatness and low fasting serum leptin concentration. In the current study only fasting serum leptin was measured, and we were thus unable to detect the nocturnal rise in leptin and confirm whether it was absent in our normal-weight amenorrheic women who exhibited fasting leptin concentrations that were comparable to their ovulatory peers.

Although leptin production is primarily regulated by adiposity (Ostlund et al., 1996), it is also under the influence of other acute modulators, including, but not limited to, sympathetic nervous activity (Rayner and Trayhurn, 2001), carbohydrate availability (Jenkins et al., 1997) and training status (Zheng et al., 1996). Circulating leptin concentration reflects the integrated stimulatory and inhibitory effects of these and other modulators on its production. Although %BF was the primary predictor of log leptin concentration in both groups of exercising women, and the only significant predictor in exercising ovulatory women, log insulin and log glycerol concentrations were also significant predictors of log leptin concentration in exercising women with amenorrhea. While the added contributions of insulin and glycerol concentrations to the model predicting leptin concentration in exercising women with amenorrhea were modest, the results demonstrate that factors other than adiposity are likely influencing leptin concentrations in exercising women with amenorrhea and may play a role in the etiology of FHA.

The regression model for the ExAmen group suggests that lower insulin concentrations are associated with decreased leptin production in exercising amenorrheic women. Insulin stimulates leptin synthesis (Gettys et al., 1996; Leroy et al., 1996), which supports this observed association. Similarly, circulating insulin concentrations are often lower in exercising women with FHA (Laughlin et al., 1998), which may contribute to the lower leptin concentrations observed in these women (Tataranni et al., 1997; Miller et al., 1998; Warren et al., 1999; Thong et al., 2000). The regression model demonstrates that exercising amenorrheic women who exhibit lower concentrations of insulin are more likely to have lower leptin concentrations, which may explain why exercising amenorrheic women often exhibit circulating leptin concentrations that are lower than can be solely accounted for by adiposity (Miller et al., 1998; Warren et al., 1999). Laughlin and Yen (1997) observed hypoinsulinemia in amenorrheic women in association with hypoinsulinemia, which further supports the hypothesis that acute modulators of leptin synthesis may play a role in the etiology of FHA.

For the ExAmen group, the regression model for log leptin also suggests that lower glycerol concentrations are associated with lower leptin concentrations. Circulating glycerol is a marker of lipolysis, and lipolysis is primarily stimulated by sympathetic stimulation of adipose tissue (Hjemdahl and Linde, 1983). Sympathetic nervous system activity has been shown to inhibit leptin synthesis via activation of beta-adrenergic receptors (Carulli et al., 1999; Scriba et al., 2000; Rayner and Trayhurn, 2001). The inhibition of leptin production due to sympathetic activation of adipose tissue would lead one to expect a negative association between glycerol and leptin concentrations; however, our regression model was not in agreement with this indirect relationship. A possible explanation is that those with low concentrations of glycerol may be relying less on fat utilization and more on carbohydrate metabolism, thus increasing carbohydrate usage and decreasing carbohydrate availability. Carbohydrate availability has been observed to be positively correlated with leptin production (Grinspoon et al., 1997; Jenkins et al., 1997), and may therefore explain why our regression model displayed lower leptin
concentrations in association with lower glycerol concentrations. We did observe a significantly greater RER among the ExAmen women who also displayed significantly lower leptin concentrations than the ExOvul women, suggesting a greater reliance on carbohydrate metabolism among the ExAmen women. However, resting RER has also been shown to negatively correlate with dietary fat intake (Goedecke et al., 2000), and although it was not statistically significant, the ExAmen group trended towards a lower fat diet (Table I). We acknowledge, however, that we did not see a significant difference in glycerol concentration between the two groups, thus highlighting the necessity of more research to better understand the link between substrate metabolism, availability of oxidizable fuels and leptin synthesis. The effect of stress hormones on leptin synthesis is also poorly understood, and although we were unable to measure cortisol, Laughlin and Yen (1997) observed a negative correlation between circulating cortisol and leptin in young women, and likewise observed elevated cortisol levels in exercising women with FHA. It seems likely that modulators of leptin other than adiposity play a role in the link between nutritional status and leptin production; however, more research is needed.

Taken together, our data indicate that women with exercise-associated FHA exhibit circulating leptin concentrations that are, on average, lower than those observed in exercising ovulatory women. However, the range of leptin concentrations associated with exercise-associated amenorrhea is wide. Although Welt et al. (2000) demonstrated that the administration of recombinant leptin restored normal ovulation and LH pulsatility in amenorrheic women, implying that an increase in leptin concentration can reverse the suppression of reproductive function, our findings suggest that increasing leptin concentrations may not be effective for all women with FHA because the majority of ExAmen women in this study exhibited leptin concentrations that fell within the same range as the ExOvul women. However, it is possible that the reproductive system is sensitive to relative changes in leptin concentration, and not just a critical concentration of leptin. Further research is needed to better understand the clinical value of leptin administration as a form of treatment for women with FHA who do not exhibit low leptin levels. Another possibility is that leptin signaling may be impaired in exercising women with FHA, independent of circulating levels. While we know of no reports of leptin resistance in association with FHA, exercise may alter receptor expression independent of changes in circulating concentrations (Kimura et al., 2004). Whether this is a possibility that would differentiate our groups is not known. The specific pathways whereby leptin is sensed by GnRH neurons are not fully understood, and thus more research is needed. Since our data also suggest that leptin production may be regulated differently in exercising women with and without hypothalamic amenorrhea, it may be interesting to investigate the role of other modulators of leptin production, such as metabolic substrates and stress hormones, in the etiology of hypothalamic amenorrhea.

**Authors’ roles**

M.C. was responsible for data collection, data analysis and interpretation, and the writing of the manuscript. N.I.W. and M.J.D. designed the study, supervised the data collection and data analysis, participated in data interpretation and N.I.W. supervised the writing of the manuscript. R.J.T. participated in data collection and writing of the manuscript.

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