THE ROLE OF LEPTIN IN THE ETIOLOGY OF
EXERCISE-ASSOCIATED MENSTRUAL DISTURBANCES

A Thesis in
Kinesiology

by
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ABSTRACT

Background: The exact etiology of functional hypothalamic amenorrhea (FHA) is still unknown, yet is often associated with a relative energy deficit. Low concentrations of the adipocyte-secreted hormone leptin are thought to be the peripheral signal whereby an energy deficit disrupts the hypothalamic-pituitary-gonadal (HPG) axis. Objectives: To further assess the role of leptin in the regulation of reproductive function, the first study was designed to identify the association between circulating leptin concentration and FHA, hypothesizing that suppression of the HPG axis results from either having low leptin per se or having a lower concentration of leptin than can be solely accounted for by adiposity. The second study assessed the role of leptin in the resumption of menses in exercising women with FHA. We hypothesized that an increase in leptin concentration would be associated with the resumption of menses in women with FHA. Lastly, the third study was designed to test the hypothesis that significant predictors of leptin concentration, after adjusting for adiposity, are different in women with and without FHA. A second aim was to explore whether changes in modulators of leptin production are found in association with changes in leptin concentration observed in women with FHA who resume menses. Design: Menstrual status, body composition, and fasting serum leptin concentration were assessed in fifty-two exercising, premenopausal women over the course of one baseline menstrual cycle for ovulatory women (OV; n=26) or for one 28-day monitoring period for amenorrheic women (Amen; n=24) (Study 1 and 3). Fourteen volunteers who were categorized as Amen at baseline completed a 6-month monitoring period, during which repeated measures of body composition, leptin concentration, and other metabolic and dietary parameters were assessed. These women
were retrospectively categorized into 2 groups: 1) those who resumed menses between
months one and six of the study (Amen-R; n=5), and 2) those who remained amenorrheic
during the 6-month monitoring period (Amen-NR; n=7). Two women resumed menses
within the first month and were thus excluded because the concentration of leptin with
the resumption of menses could not be determined (Study 2 and 3). **Results:** Percentage
body fat (21±1% vs. 27±0.7%; *P*<0.001) and leptin concentration (4.8±0.8ng/ml vs.
9.6±0.9ng/ml; *P*<0.001) were significantly lower in Amen vs. OV. However, the ranges
observed in serum leptin concentration for Amen and for OV were similar (range for
Amen: 2.61-7.24ng/ml; range for OV: 2.60-7.25ng/ml), and after adjusting for adiposity
the difference in leptin concentration was no longer significant. Percentage body fat
(17±2% vs. 24±2%; *P*=0.033), but not serum leptin concentration (2.3±0.7ng/ml vs.
6.5±2.8ng/ml; *P*=0.207), was significantly lower in Amen-NR vs. Amen-R at baseline.
The increase in fasting serum leptin concentration, but not percentage body fat, from pre-
to post-study was significantly greater in Amen-R vs. Amen-NR (Interaction Effect:
*P*=0.002). A significant increase in resting respiratory exchange ratio was also observed
in Amen-R vs. Amen-NR from pre- to post-study (Interaction Effect: *P*=0.006). The
model that best predicted baseline serum leptin concentration in the OV women included
percentage body fat only (*r*²=0.522), and in the Amen women included percentage body
fat, serum insulin concentration, and serum glycerol concentration (*r*²=0.775).

**Conclusions:** These data suggest that the reproductive system senses and responds to
relative changes in leptin concentration rather than to a critical concentration of leptin.
Changes in leptin concentration may not always reflect changes in adiposity and in such
cases may be due to the influence of other modulators of leptin synthesis.
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CHAPTER I

INTRODUCTION

Statement of the Problem

The mechanism whereby the reproductive axis is suppressed in women with functional hypothalamic amenorrhea (FHA) is not well understood, yet it is most often directly related to chronic energy deficiency (1-4). Female athletes are therefore at greater risk for the development of FHA owing to their high exercise energy expenditure, which is often coupled with insufficient energy intake. Not surprisingly, the prevalence of FHA in physically active women has recently been reported to range up to 34% (5). Stress, and the subsequent activation of the hypothalamic-pituitary-adrenal (HPA) and sympathoadrenal axes, has also been implicated in the mechanism whereby the reproductive axis is suppressed (6-9). Infertility, although transient, is the most salient clinical consequence of FHA, yet compromised endothelial function (10), failure to achieve peak bone mass (11), as well as accelerated bone loss (12) are additional concerns for amenorrheic women and pose increased risks for cardiovascular disease, stress fractures and premature osteoporosis (13, 14).

Purpose

Leptin, the hormone product of the obesity (ob) gene, has been implicated as the peripheral signal whereby a relative energy deficiency is relayed to the hypothalamus, allowing for the coordinated control of nutritional status and reproduction (15). Leptin is secreted from adipocytes in proportion to adiposity (16), and low circulating leptin is thought to be a key signal whereby an energy deficiency causes reproductive dysfunction (17, 18). Although body fatness is the primary determinant of circulating leptin
concentrations, and as such explains why women with low body fat are at risk for the development of FHA, other factors also modulate its production and may therefore influence reproductive status. Sympathetic nervous activity (SNA), for example, inhibits leptin production and may thus contribute to the mechanism whereby stress down-regulates reproductive function (19). Although women with FHA have consistently been shown to exhibit circulating leptin concentrations that are lower than those observed in their normally cycling peers (20-25), the precise role of leptin in the etiology of FHA remains unclear. The purpose of this investigation was therefore twofold: 1) to explore the mechanism whereby leptin acts to regulate reproductive function, and to 2) examine whether modulators of leptin production can influence the reproductive system via their impact on circulating leptin concentration.

**Specific Aim 1:** To explore the relationship between fasting serum leptin concentration and exercise-associated FHA.

**Hypothesis 1a:** Lower serum leptin concentrations will be observed in exercising women with FHA in comparison to ovulatory women.

**Hypothesis 1b:** A positive correlation between percentage body fat and serum leptin concentration will be observed in women with and without FHA, yet women with FHA will exhibit leptin concentrations that are lower than those observed in ovulatory women for any given value of adiposity.

**Specific Aim 2:** To determine whether an increase in circulating leptin concentration is associated with the resumption of menses in women with exercise-associated FHA.

**Hypothesis 2a:** An increase in fasting serum leptin concentration will be observed in women with FHA who resume menses.
**Specific Aim 3:** To explore whether the mechanism underlying the association between hypoleptinemia and exercise-associated FHA includes evidence of elevated lipolysis. Elevated lipolysis in this case may serve as a proxy indicator of elevated sympathetic activation of adipose tissue and a consequent inhibition of leptin production.

**Hypothesis 3a:** After adjusting for adiposity, fasting serum glycerol concentration will be inversely related to fasting serum leptin concentration in women with FHA, but not in ovulatory women, such that greater serum glycerol concentrations will be associated with lower serum leptin concentrations.

**Hypothesis 3b:** After adjusting for adiposity, respiratory exchange ratio (RER) will be directly related to fasting serum leptin concentration in women with FHA, but not in ovulatory women, such that a lower RER will be associated with a lower serum leptin concentration.

**Specific Aim 4:** If specific aim 2 is found to be true, a further aim is to determine whether the increase in leptin observed with the resumption of menses in women with FHA is associated with changes in indirect measures of sympathetic nervous activation of adipose tissue.

**Hypothesis 4a:** Increases in resting RER will be observed in association with increase in fasting serum leptin concentration in women with FHA who resume menses.

**Hypothesis 4b:** Decreases in fasting serum glycerol concentration will be observed in association with increases in fasting serum leptin concentration in women with FHA who resume menses.
CHAPTER II

REVIEW OF THE LITERATURE

Reproductive disturbances

Figure 1. Schematic representation of the hypothalamic-pituitary-gonadal (HPG) axis, demonstrating how the release of gonadotropin releasing-hormone (GnRH) from the hypothalamus regulates reproduction by stimulating the release of the gonadotropins—luteinizing hormone (LH) and follicle stimulating hormone (FSH)—from the anterior pituitary, which then stimulate the synthesis and secretion of estrogen and progesterone from the gonads. Estrogen and progesterone then feed back to regulate the secretion of GnRH and the gonadotropins from the hypothalamus and anterior pituitary, respectively. Factors that chronically down-regulate GnRH release, such as energy deficiency and stress, disrupt the HPG axis and cause reproductive disturbances.
Reproductive function critically depends on the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. As seen in Figure 1, GnRH release stimulates the secretion of the pituitary gonadotropins—follicle-stimulating hormone (FSH) and luteinizing hormone (LH)—which drive gonadal steroid production (26, 27). Disruptions in GnRH signaling to the pituitary, as in functional hypothalamic amenorrhea (GnRH pulse frequency is reduced (28)) or polycystic ovarian syndrome (GnRH pulse frequency is accelerated (29)), are associated with compromised ovarian function (30). The severity of menstrual disturbance associated with exercise is likely related to the degree of suppression of the GnRH pulse generator. Disturbances documented in exercising women range from subtle defects in luteal function to anovulation and amenorrhea (31). Exercise-associated amenorrhea is the most severe of these menstrual disturbances, and is the cessation of menses for a minimum of three months.

Although the prevalence of exercise-associated amenorrhea has been reported to range anywhere from 1 to 44% (32), there is no question that the prevalence of both subtle and severe reproductive disturbances is greater in physically active women than in their sedentary counterparts (5). Only 50% of the observed menstrual cycles in exercising women were ovulatory, in comparison to 95.8% of the observed cycles in sedentary women (5). The occurrence of luteal phase defects and anovulatory cycles, for example, has been reported to be 48% and 12%, respectively, in exercising women (33). Infertility, although transient, is the most significant clinical consequence, yet failure to achieve peak bone mass (11) as well as accelerated bone loss (12) are additional concerns for women with exercise-associated menstrual disturbances due to the increased risk for stress fractures and premature osteoporosis (13, 14). Although amenorrheic women are
aware of their disturbance, subtle ovarian deficits such as anovulation and shortened luteal phases are not as noticeable, yet have also been shown to contribute to premenopausal bone loss (34).

**Etiology of exercise-associated reproductive disturbances**

The mechanism whereby reproductive function becomes disrupted is not well understood (1, 4, 35), yet it is most often directly related to a chronic energy deficiency (36, 37), which is a metabolic stress, and which causes a down-regulation in GnRH pulsatility (Figure 1). When energy intake is insufficient to compensate for energy expenditure and consequent energy needs, essential functions involved in basic survival are maintained, while reproductive function is suppressed (38). In a prospective study, reproductive disturbances were induced in initially untrained regularly cycling college women by abruptly imposing a vigorous aerobic exercise program while controlling dietary intake (3). Similarly, Williams *et al* (39) demonstrated an abrupt transition to amenorrhea in eight cynomolgus monkeys who underwent a strenuous exercise program while food intake remained constant. Williams *et al* (40) then showed recovery of ovulatory cycles in the four monkeys who were re-fed, even though the daily exercise training was maintained. The suppression of reproductive function is thus commonly viewed as an adaptive response to chronically low energy availability, rather than to inadequate energy stores (38) or exercise, *per se*.

Although the evidence supporting the linkage between low energy availability and reproductive disturbances is strong, others have proposed the idea of a stress-related mechanism in which reproductive function is compromised secondary to the activation of
the stress-response systems—the hypothalamic-pituitary-adrenal (HPA) and sympathoadrenal axes (6, 7, 41). Metabolic stress (1, 40), psychosocial stress (8, 42, 43), environmental stress (44) and physical stress (45) have all been associated with alterations in GnRH secretion and subsequent reproductive disturbances. Although stress appears to exert an inhibitory effect on reproductive function, many claim that the impact of stress cannot be separated from its impact on energy balance, and that the final common pathway leading to compromised reproductive function is low energy availability (35, 46). The theory behind this hypothesis is that activation of the body’s stress response systems stimulates the synthesis and secretion of glucocorticoids and catecholamines into the bloodstream (6), which are counter-regulatory hormones that alter fuel regulation and signal adaptive metabolic changes that are characteristic of a catabolic state, i.e. hypo-metabolic state (47). In other words, stress has a metabolic impact, and the stress-induced metabolic profile is similar to that of chronic low energy availability, which is associated with reproductive disturbances. It therefore seems likely that the final pathway whereby reproductive function is suppressed, due to either metabolic and/or stress factors, is shared.

**Leptin and reproductive function**

The *ob* gene product leptin has been implicated as the signal whereby energy status is relayed from the periphery to the brain (48). Leptin is an adipocyte-secreted hormone that signals energy sufficiency and acts on the hypothalamus to influence feeding, metabolism (49), and reproduction (15, 50). The secretion of leptin is highly correlated with percent body fat (16) and body mass index (51) in lean and obese healthy subjects, such that higher leptin concentrations are observed in people with greater
adiposity. Serum leptin concentrations increase gradually over time in response to increases in fat mass, and fall rapidly in response to complete fasting in the absence of changes in fat mass (52, 53), which suggests leptin plays an important role in both long-term and acute energy-balance. Leptin appears to signal the amount of energy available in adipose stores as well as acute changes in immediate energy availability (i.e. oxidizable fuels) from the periphery to the brain (17, 54). It has therefore been postulated that leptin facilitates reproductive function and might mediate the neuroendocrine response to metabolic stress, i.e. low energy availability, which results in reduced GnRH release and subsequent reproductive dysfunction (17, 18).

The hypothesis that leptin facilitates reproductive function is supported by the finding that women with hypothalamic amenorrhea exhibit low circulating leptin concentrations in comparison to their height and weight matched cycling peers (20-24, 55), and that recombinant human leptin administration was shown to improve LH pulsatility and restore ovulation in women with hypothalamic amenorrhea (56). Similarly, ob/ob mice that lack the leptin gene are infertile and fail to go through puberty, yet treatment with recombinant leptin restored fertility (50, 57). Injection of leptin also brought forward the age of sexual maturation in normal weanling mice, as well as the timing of their first litter (58, 59). These findings, as well as those reported by Hoggard and colleagues (60), who demonstrated that leptin stimulates the release of FSH and LH from tissue explants in an ex vivo study, strongly suggest that leptin facilitates reproductive function, and that low leptin concentrations may play a role in the development of exercise-associated reproductive disturbances.
Although the primary determinant of leptin concentration is adiposity, women with exercise-associated amenorrhea exhibit leptin concentrations that are lower than can be explained by body fat alone, and also display altered leptin secretion patterns (22, 55). Miller and colleagues (24) and Thong et al (21) have shown that amenorrheic women have reduced plasma leptin concentrations after an overnight fast, independent of changes in weight or fat mass, in comparison to cycling women. Similarly, Warren and colleagues (20) reported the same findings after comparing mid-afternoon leptin concentrations in amenorrheic and cycling women. Laughlin and Yen (22) found that amenorrheic athletes had reduced 24-hour mean leptin concentrations in comparison to both active and sedentary cycling women after controlling for adiposity, while also lacking the diurnal secretion pattern that is characteristic of leptin, which suggests there are factors independent of adiposity which chronically influence leptin secretion in amenorrheic women, and also supports the hypothesis that leptin mediates the regulation of reproductive function. Although adiposity remains the primary regulator of leptin concentration in amenorrheic women (23), other modulators of leptin must be present to explain the reduced leptin concentrations which is sometimes observed in amenorrheic women in comparison to their cycling peers, even after adjusting for adiposity (20-22).
Elevated sympathetic activation of adipose tissue in amenorrheic women may be one possible mechanism explaining the low circulating leptin concentrations (20-24) observed in these women (Figure 1). Sympathetic activation of adipose tissue has been shown to cause an acute inhibition of leptin expression (61). Trayhurn and coworkers (62) first demonstrated the role of the sympathetic system in the cold-induced suppression of leptin expression in mice. Leptin gene expression decreased in mice after an overnight (18h) exposure to cold (4 °C). To test whether this decrease was mediated
by sympathetic activity, mice maintained in the warm were given injections of either norepinephrine or the beta-adrenoreceptor agonist, isoprenaline, which were reported to decrease leptin mRNA to 16% and < 1% of control values, respectively (62). Investigators have confirmed the direct inhibitory role of sympathetic activity and beta-adrenergic stimulation in WAT on leptin expression using both animal and human models (63-66). Further evidence supporting the inhibitory role of sympathetic activity was provided by Rayner and coworkers (64), who observed an increase in circulating leptin concentrations in mice after inhibiting norepinephrine production from adipose via blockade of tyrosine hydroxylase. Jeon and colleagues (67) also demonstrated that sympathetic stimulation facilitates the acute reduction in leptin production in response to fasting. They observed that sympathectomized men failed to exhibit the immediate decrease in leptin production in response to a 12-hour fast that was evident in the intact subjects (67).

The inhibitory impact of SNA on leptin production in humans has been corroborated by many research studies (65). Pinkney et al (68) showed that administration of isoprenaline, a sympathetic agonist, increased lipolysis and decreased leptin concentrations to 81.3% of baseline values in healthy men and women. Donahoo and colleagues (69) found similar results after isoproterenol infusion. Ricci, Fried, and Mittleman (70) reported a decrease in leptin concentrations to 86% of baseline values in healthy women after 90 minutes of cold exposure (6.3 degrees Celsius). They also reported that plasma glycerol increased by 134% after 90 minutes of cold exposure, indicating elevated levels of fatty acid mobilization due to increased sympathetic activation of WAT. Lastly, Carulli et al (71) showed that direct epinephrine infusion
caused leptin levels to decrease by 47% of basal values. It has been consistently demonstrated that sympathetic activation of adipose tissue inhibits leptin gene expression (72), which demonstrates that sympathetic activation of WAT may act to suppress circulating leptin concentration.

During times of low energy availability, such as in response to an acute fast, an increase in sympathetic nervous activity to white adipose tissue (WAT) (73), yet a general decrease in sympathetic activation of other tissues, such as the heart (74), has been observed in rodents. Low energy availability is a metabolic stress, and increased sympathetic activity to adipose tissue is a stress response that stimulates the mobilization of fat in order to meet energy demands and thus reduce the threat to homeostasis (75, 76). Direct sympathetic activation of adipose tissue causes norepinephrine to be released from the activated nerve terminals innervating adipose tissue (72). An increase in epinephrine release from the adrenal medulla has also been observed in response to stress when blood glucose falls (77), such as due to exercise or in response to a fast, and can likewise stimulate the mobilization of fuels to counter the threat to homeostasis. Both norepinephrine and epinephrine can activate beta adrenergic receptors, which stimulate lipolysis, thereby causing an increase in free fatty acid (FFA) and glycerol release into the blood (78). Although it is well established that sympathetic activation of WAT in rats is elevated in response to metabolic stress (73) and other stressors (79), whether or not women with hypothalamic amenorrhea exhibit increased sympathetic activity in adipose tissue has not been measured. However, it has been reported that anorexic women exhibit elevated sympathetic activity in adipose tissue at rest and during exercise (80). Assuming the underlying problem in women with exercise-associated amenorrhea is also related to
chronic energy deficiency, one would expect to see evidence of elevated sympathetic activation of WAT in amenorrheic women.

Other studies also support the hypothesis that women with exercise-associated amenorrhea may exhibit low leptin concentrations due to elevated sympathetic activation of adipose tissue secondary to a hypo-metabolic state (72, 80). For example, fasting-induced sympathetic activation of adipose tissue acutely inhibits leptin synthesis (67), as previously discussed, independent of any change in adiposity (53, 81, 82). By contrast, food intake transiently promotes \textit{ob} gene expression and thus increases leptin production (83). Jenkins and colleagues (84) reported that carbohydrate availability, and presumably oxidizable fuel availability, increases leptin production. These findings corroborate those of Hilton and Loucks (55), who demonstrated that low energy availability suppresses 24-hour mean leptin levels and disrupts its diurnal secretion pattern. Hilton and Loucks (55) also demonstrated that the inhibitory impact of low energy availability on circulating leptin is attenuated in the presence of increased carbohydrate availability. Taken together, these findings support the notion that leptin functions as an acute regulator of energy balance, and suggest that women with exercise-associated amenorrhea may exhibit reduced leptin concentrations secondary to chronic low energy availability, independent of adiposity (20-22). This hypothesis is supported by the findings that women with hypothalamic amenorrhea exhibit elevated levels of ghrelin (85, 86), a hunger signal, as well as evidence of disordered eating (20, 25) and of a hypo-metabolic state (87).

Although it has not been directly demonstrated in women with exercise-associated amenorrhea, it nevertheless seems likely that low energy availability inhibits leptin secretion (55) via elevated sympathetic activation of adipose tissue.
As mentioned previously, metabolic stress increases direct sympathetic activity to adipose tissue (73). Other stressors have likewise been shown to increase sympathetic activity and may also contribute to sympathetic inhibition of leptin production (62, 79, 88, 89). For example, sympathetic activity is reportedly higher in response to physical stress (i.e. exercise) and mental stress (88), Type A behavior patterns (89), as well as in response to cold exposure, as previously mentioned (62, 79). Since sympathetic stimulation of adipose tissue acutely inhibits leptin expression (72), it is possible that chronic hyperactivity of the sympathetic system, due to any stressor that elevates adrenergic stimulation in adipose tissue, could cause a reduction in leptin synthesis. Since leptin facilitates reproductive function, stress-induced sympathetic stimulation and subsequent suppression of leptin may explain why reproductive disturbances are often reported in response to stress (8, 43, 44).

Leptin is a hormone needed for reproductive competency (90). It is therefore logical for leptin production to be inhibited at times when energy is scarce and during times of stress, i.e. when conditions are unfavorable for reproduction (91). It appears that a primary mechanism whereby leptin is down-regulated is via sympathetic activation of adipose tissue (72). It is not surprising that the activation of the stress response is coupled to the inhibition of leptin synthesis (92). Similarly, it makes sense that the stress of low fuel availability stimulates fuel mobilization (93), i.e. lipolysis, as well as a decrease in leptin expression. Women with exercise-associated amenorrhea may exhibit low circulating leptin due to increased sympathetic inhibition of leptin production. The increased sympathetic activation of adipose tissue in these women would likely be due to metabolic stress as a result of high exercise energy expenditure coupled with inadequate
caloric intake, but may likewise be due to elevated psychosocial (94) and/or exercise stress (95).

**Measuring elevated sympathetic activation of adipose tissue**

Sympathetic activity is up-regulated to adipose tissue in response to fasting (73), but as previously mentioned is decreased to most other tissues (74). It is therefore difficult to assess sympathetic activation of adipose tissue using conventional measures of sympathetic activity, such as elevated blood pressure, increased heart rate, and plasma epinephrine or norepinephrine concentrations, which are better indicators of total sympathetic activity. Sympathetic activation of WAT stimulates lipolysis, and markers of lipolysis can therefore be used as proxy indicators of sympathetic nervous activity in WAT (78, 96). Serum glycerol concentration has been shown to be increased by both norepinephrine and epinephrine (70, 78), which suggests it could be used as a surrogate indicator of sympathetic activation of adipose tissue. Similarly, increased fat utilization, due to elevated rates of lipolysis, results in a lower respiratory quotient (97). Matsuo and Suzuki (97) observed a negative correlation between respiratory quotient and norepinephrine concentration in healthy young women, which suggests RER measurements can likewise be used as indirect measures of lipolysis, and thus as proxy indicators of sympathetic activation of adipose tissue. Training volume and diet have also been shown to correlate with resting RER (98), and must therefore be controlled for when using RER to assess sympathetic nervous activation of adipose tissue.

There are many factors to consider when using fasting serum glycerol concentration and resting RER as indirect measures of sympathetic activation of adipose
tissue. Dietary fat and carbohydrates serve as substrates for energy metabolism during rest and exercise, and the relative contribution of each substrate to power ATP production during exercise depends on the pre-exercise diet (99, 100) and on the intensity of exercise (101). Similarly, the relative contribution of fat and carbohydrates to energy production at rest depends on dietary fat intake and serum FFA concentrations (98). Higher fat intakes and FFA concentrations are negatively correlated with RER, indicating the proportion of energy production from fat is increased (98). Training status, the proportion of type I muscle fibers and resting muscle glycogen content are also significantly related to resting RER (98), yet are positively correlated. The proportion of type I muscles fibers (102) and muscle glycogen content (103) are significantly related to exercise training status. It is therefore important to control for diet and training status when using RER as a proxy indicator of sympathetic activation of adipose tissue.

**Other potential modulators of leptin synthesis**

**Training Status**

Although the impact of exercise training on leptin production is not well understood, Hickey and colleagues (104) reported that long-term exercise training had an inhibitory impact on serum leptin concentrations in women, independent of any change in adiposity. Exercise was also shown to decrease peak and average 24-hour leptin concentrations in healthy, lean men (105). These observations support the finding that both amenorrheic and cycling athletes exhibit leptin concentrations lower than their sedentary counterparts (22), yet does not explain the further reduction in leptin concentrations observed in the amenorrheic athletes, nor does it explain the low leptin
levels observed in non-athlete amenorrheic women. Exercise acutely elevates sympathetic nervous activity (95), which we now know inhibits leptin gene expression (19), yet the low leptin levels observed in amenorrheic women appear to represent a chronic adaptation because they persist throughout the day even in the absence of exercise (22). Regardless of its acute impact on sympathetic activity, long-term exercise training can theoretically potentiate the attenuation of leptin production due to the consequent increase in energy expenditure, which may reduce adiposity and fuel availability.

*Carbohydrate Intake and Insulin Status*

Decreased carbohydrate availability, independent of changes in body mass, is associated with reduced leptin production (84), suggesting carbohydrate availability plays a permissive role in the regulation of leptin synthesis. Insulin is secreted in response to elevated blood glucose, i.e. adequate carbohydrate availability, and has been shown to potently increase leptin synthesis and secretion from adipose cells (106-109). Serum insulin concentrations are reduced in women with hypothalamic amenorrhea (22, 87); suggesting insulin-stimulated leptin production is attenuated in these women.

*Gonadal Hormone Status*

Likewise, the ovarian hormones—estrogen and progesterone—have been shown to increase serum leptin concentrations (110, 111). By definition, amenorrheic women exhibit hypogonadism, which may further contribute to their attenuated levels of leptin production because estrogen- and progesterone-stimulated leptin production (110, 111) is presumably absent. Conversely, androgens been shown to decrease leptin production
(112-114), and are thought to contribute to the lower leptin concentrations observed in men (115).
CHAPTER III

STUDY 1: A Wide Range of Circulating Leptin Concentration is Observed in Association with Exercise-Associated Amenorrhea

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**Key Words:** Leptin, Amenorrhea

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INTRODUCTION

The mechanism whereby reproductive function becomes disrupted in women with functional hypothalamic amenorrhea (FHA) remains unclear, yet evidence suggests it occurs when a relative energy deficiency or other stressor disrupts the secretion of gonadotropin-releasing hormone (GnRH) and other neuroendocrine axes (1-7). Leptin, the hormone product of the obesity (ob) gene, has been implicated as the signal whereby nutritional status is relayed from the periphery to the hypothalamus (8), where it acts to regulate appetite (9), energy balance (10, 11), and reproduction (12, 13). Leptin is secreted by adipocytes and circulates at concentrations highly correlated to adiposity (14), which explains why women with very low body fat, and presumably low leptin concentrations, are at risk for the development of FHA (15). Leptin may also be a key signal whereby stress down-regulates reproductive function. Leptin production is inhibited by sympathetic nervous activity (16)—one of two primary physiological systems activated by stress—suggesting leptin concentration may be lower in some women with amenorrhea secondary to stress. Both psychosocial and metabolic stressors have been shown to activate the sympathetic nervous system (17-20).

In support of the hypothesis that leptin serves as a key regulator of reproductive function, women with FHA have been shown to exhibit low leptin concentrations (2, 21-26). In some cases low leptin was observed secondary to low body fatness, such that after correcting for adiposity there was no difference in leptin concentration in amenorrheic women compared to their cycling peers (2, 24, 26), which is consistent with the hypothesis that leptin serves as a key signal whereby a relative energy deficit is relayed to the hypothalamus. However, amenorrheic women who are of normal weight and
adiposity also exhibit low leptin concentrations (23). These women exhibit hypoleptinemia, such that their leptin concentrations are lower than can be explained by adiposity alone (21-23, 25), which suggests there are factors other than adiposity that are modulating leptin production in these women. Thus, it is possible that these additional modulators play a role in the suppression of reproductive function. In all cases (21-25), leptin is significantly lower in women with hypothalamic amenorrhea than in regularly cycling women, which supports its role in the etiology of reproductive disturbances. However, what is not clear from the literature is whether low leptin, *per se*, is the signal whereby reproductive function is disrupted, or whether some other modulator of leptin that acts to reduce its concentration independent of body fat is the signal. Currently there exist only a few studies focusing on leptin concentrations in carefully defined FHA subjects and data from these studies have yielded conflicting results (23-25).

The purpose of this study was to examine the range of leptin concentrations observed in women with FHA in comparison to their ovulatory peers and determine whether there appears to be a threshold concentration of leptin below which reproductive function is compromised, or whether reproductive suppression associated with FHA exists in association with a wide range of leptin concentrations. If the latter were found to be the case, a secondary purpose of this study was to determine whether FHA was associated with leptin concentrations that were low relative to adiposity, such that a state of “hypoleptinemia” was associated with FHA. If the latter were found to be true, it is plausible that additional modulators of leptin are associated with FHA.
SUBJECTS AND METHODS

Experimental Design

This investigation was part of a larger, prospective study at the University of Toronto and Penn State University. It 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) and amenorrhea (the absence of menses for greater than 90 days). Three groups of exercising women have been studied, including an ovulatory control group, a group exhibiting severe EAMD that was randomized to have their calories increased, i.e., EAMD+Calories, and a group exhibiting severe EAMD that was randomized to maintain their normal caloric intake, i.e., EAMD Controls. The current investigation includes only baseline data from two experimental groups, including an exercising amenorrheic group (Amen) (derived from the larger group of EAMD subjects) and an exercising ovulatory control group (OV). Data collected from both study sites was used. The exercising Amen group included subjects that were deemed to exhibit FHA, and that went on to be randomized to either the EAMD+Calories or EAMD Control group. 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 EAMD. The primary outcome variables of this cross-sectional investigation were menstrual status, i.e., ovulatory or amenorrheic, fasting serum leptin concentration and percentage body fat.
Recruitment

Volunteers were recruited by posters targeting physically active women for a study on the impact of increased caloric intake on bone health and menstrual cyclicity in energy deficient exercising women. Inclusion criteria for this study were: 1) no history of any serious medical conditions; 2) no current clinical diagnosis of an eating or psychiatric disorder; 3) age 18-30 years; 4) BMI 16-25 kg/m²; 5) weight stable (± 2kg) for the past 6 months; 6) non-smoking; 7) no medication use that would alter metabolic or reproductive hormone concentrations; 8) ≥ 3 hrs/wk aerobic exercise for EAMD women, and ≥ 2 hrs/wk aerobic exercise for eumenorrheic women; 9) no menses within the past 3 months or 6 or fewer menses within the past year, or regular menses within the past 6 months; 10) 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. There were 176 volunteers who met initial inclusion criteria and who started screening. Of those, 84 volunteers entered the study and completed the baseline assessment period. 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.

Subject Grouping

All subjects collected first morning urine samples throughout the baseline monitoring period. Day one of baseline was the first day of menses for eumenorrheic women and an arbitrary day for women with EAMD. Classification of menstrual status was based on urinary estrone-1-glucoronide (E1G), pregnanediol-3-glucuronide (PdG) and luteinizing hormone (LH) profiles, and on self-reported menstrual histories. Subjects
who reported no menses within the last 3 months or 6 or fewer menses within the past year, and whose baseline menstrual cycle confirmed diagnosis of amenorrhea were assigned to the amenorrheic group (Amen). These subjects were also deemed to not have any clinical or biochemical signs of PCOS, and exhibited low concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Subjects who reported regular menses within the last 6 months, confirmed by a normal (26-35 days) ovulatory baseline cycle, were assigned to the ovulatory group (OV). Of the 84 women who completed baseline, 24 women met the criteria for Amen, and 26 women met the criteria for OV. The other women were classified as having oligomenorrheic cycles, anovulatory cycles, short cycles, or PCOS. Baseline comparisons were made between the exercising Amen and exercising OV women.

Classification of Menstrual Status

The first morning urine samples collected during baseline were assayed for LH, E1G and PdG to characterize menstrual cycle status. Ovulatory status was determined by the presence or absence of the urinary LH surge, identified as the LH peak on the day of or the day after the E1G peak (27). Menstrual cycle length was defined as the number of days from the first day of menses to the next menses. Volunteers were placed in the OV group if the following criteria were met: menses occurred at regular intervals of 26-35 days and ovulation was detected. Amenorrhea was confirmed by chronically suppressed E1G and PdG profiles. To determine estrogen exposure, E1G urinary metabolites were compared between the groups using a modified trapezoidal integrated area under the curve (AUC) technique. To determine progesterone exposure, PdG urinary metabolites were compared between the groups using a modified trapezoidal integrated area under the
curve (AUC) technique. All the women exhibited an FAI < 3, which was calculated according to the following equation: FAI = [total testosterone (nmol/L) / serum hormone binding globulin (nmol/L)]*100 (28). An FAI of 3 was used as the cutoff because women diagnosed with PCOS (29), often exhibit calculated FAIs ≥3 (30, 31), while women without PCOS exhibit calculated FAIs below 3 (30, 31).

**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 two samples were pooled for all baseline hormone analyses. Blood was always collected before 1000 following an overnight fast and after subjects refrained from exercise and caffeine for at least 12 hours. All samples were allowed to clot for 30 minutes and were then centrifuged at 4 °C for 15 minutes at 3000 rpm. The serum was then aliquotted into 2-ml polyethylene storage tubes and stored frozen at -80 °C until analysis.

**Serum Hormone Analysis**

Plasma leptin concentration was measured using a solid-phase sandwich enzyme-linked immunoassay (ELISA) for total leptin (Milipore, St. Charles, MI). All samples were measured in duplicate, in the same laboratory and by the same technician, and the concentration 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 for the low control were 6.2% and 4.6%, respectively. This assay is sensitive to leptin concentrations ranging from 0.5 to100 ng/ml. Total testosterone was measured using a radioimmunoassay kit (Siemens, Los Angeles, CA) through competitive immunoassay. Analytical sensitivity for the testosterone assay was 0.14 nmol/L (4 ng/dL). The
intraassay and interassay coefficients of variation were 6.4% and 7.5%, respectively. Serum hormone binding globulin (SHBG) was analyzed using a chemiluminescence analyzer (Immule, Euro Diagnostic Products Corporation, Lianberis, UK) through competitive immunoassay. Analytical sensitivity for the SHBG was 0.2nmol/L. The intraassay and interassay coefficients of variation were 6.4% and 8.7%, respectively. FSH and LH were sent out for analysis (Quest Diagnostics, Pittsburgh, PA).

**Urinary hormone measurements**

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (32) which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (32). Microtiter plate competitive enzyme immunoassays were used to measure the urinary metabolites E1G and PdG. The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by Coralie Munro University of California (Davis, CA). The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 12.2% and 14.0%, respectively. The PdG intra- and inter-assay variability was determined in house as 13.6% and 18.7%, respectively. The validity of the urine technique as representative of the 24 hour pattern of E1G and PdG excretion has been reported by other investigators (33, 34). Urinary LH was determined by coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The sensitivity of the LH assay is 0.15mIU/ml. The intra- and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

**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, and were provided with a food scale and measuring cup to weigh and measure food if needed. Diet analysis of the 3-day food logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN).

**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 on five occasions (at screening and four times during baseline), and the mean of these measurements is presented for Baseline weight (Seca, Model 770, Hamburg, Germany). Height was measured to the nearest 1.0 cm during screening. Baseline body mass index (BMI) was calculated as the baseline weight divided by screening height squared (kg/m²).

**Body Composition**

Body composition was assessed using dual-energy x-ray absorptiometry (DXA). Forty-six subjects were scanned on one of two scanners at different centers, either a GE Lunar Prodigy (n=29, enCORE 2002 software version 6.50.069) or a GE Lunar iDXA (n=17, 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, fourteen participants were scanned in triplicate on both machines. The majority (n=8) were scanned on both machines within 5 days; however, there was approximately one month between scans for some subjects (n=6). The values for body composition measured on
the different scanners were found to be highly correlated with no significant difference between the population mean values. Equations were derived using simple linear regression to remove these biases and report the Prodigy values calibrated to the iDXA. The remaining subjects (n=4) were scanned on a Hologic QDR 4500W. A cross-calibration study between the Hologic and iDXA is in process to generate agreement between the data collected on the two machines in the case the measurements significantly differ.

*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 (35, 36). Subjects recorded daily exercise activities and heart rates (by radial palpation) throughout study participation on weekly exercise logs which were used to quantify purposeful exercise in minutes per week.

*Statistical Analysis*

All demographic and descriptive data were analyzed using independent t-tests to determine whether the groups differed. Regression equations for serum leptin vs. body fat percentage were analyzed separately for the Amen and OV groups and compared by analysis of covariance (ANCOVA) to determine whether there was a difference in slopes. For the regression equations, serum leptin concentration was log transformed to normalize its distribution. 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 differences in leptin concentration using
an independent t-test between ovulatory women and women with severe menstrual
disturbances, i.e., amenorrhea. Sample size was based on the detection of a meaningful
difference in leptin concentration of 2.8 ng/ml and an S.D. of 6.0 ng/ml for both group
means, which was based off of preliminary data from this study. In order to achieve 80%
power using a 0.05 level of significance, a sample size of 73 cycles for each cycle
category (OV and Amen) would be required. The calculation of this sample size was very
conservative. Using data reported by Miller and colleagues (21), a sample size of 23
cycles for each category would be required to detect a difference of 3.5ng/ml and an S.D.
of 4.9ng/ml and 3.0ng/ml for the mean fasting leptin concentrations in cycling women
and amenorrheic women, respectively. For all analyses, $P < 0.05$ was considered
statistically significant. Statistical analyses were performed using SPSS for Windows
(Version 18.0; Chicago, IL) statistical software. Data are reported as mean ± SEM.
RESULTS

Descriptive Characteristics

Table 1: Baseline descriptive data for exercising ovulatory women (OV) and exercising amenorrheic women (Amen).

<table>
<thead>
<tr>
<th></th>
<th>OV (n = 26)</th>
<th>Amen (n = 24)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</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 characteristics</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>Fat Free Mass (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 (yr)</td>
<td>12.5 ± 0.2</td>
<td>13.4 ± 0.3</td>
<td>0.017</td>
</tr>
<tr>
<td>Gynecological Age (yr)</td>
<td>10.7 ± 0.7</td>
<td>8.1 ± 0.7</td>
<td>0.012</td>
</tr>
<tr>
<td>E1G AUC (days (ng/ml))</td>
<td>1010.6 ± 86.1</td>
<td>468.5 ± 46.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PdG AUC (days (ng/ml))</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>-</td>
<td>n/a</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/wk)</td>
<td>468 ± 71</td>
<td>662 ± 85</td>
<td>0.084</td>
</tr>
<tr>
<td>Exercise Activity (min/wk)</td>
<td>236 ± 26</td>
<td>388 ± 53</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Metabolic characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary Intake (kcal/day)</td>
<td>1897 ± 109</td>
<td>2163 ± 243</td>
<td>0.310</td>
</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>
</tbody>
</table>

Values are mean ± S.E.M.
*n = 20 for Ovulatory Women and n= 21 for Amenorrheic Women

Baseline descriptive characteristics for Amen and OV are shown in Table 1. Amen and OV did not differ in terms of age, height, and body weight. However, a significantly reduced BMI and percentage body fat, elevated peak aerobic capacity, and younger gynecological age and age of menarche were observed in Amen.
Menstrual Characteristics and Urinary Ovarian Steroid Data

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

Menstrual characteristics for OV and Amen women are shown in table 1. Amen women had a significantly older age of menarche, younger gynecological age, and lower
estrogen and progesterone exposure. Composite graphs of the daily concentrations of E1G and PdG are presented in figure 1.

**Leptin Measures**

![Graph showing leptin measures for Amen and OV women](image)

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

Fasting serum leptin concentration was significantly lower in Amen compared with OV exercising women (Table 1). However, as seen in Figure 2, the range in serum leptin concentration for exercising Amen and for exercising OV women were similar (Range for exercising Amen women: 0.30-16.98ng/ml; Range for exercising OV women: 2.57-18.28ng/ml).
Figure 3: Log leptin vs. body fat relationship in exercising women with amenorrhea (Amen: closed circles) and exercising women with ovulatory menstrual cycles (OV: open circles).

Table 2: Regression models to predict log leptin concentration in all exercising women (Amen + OV), exercising women with amenorrhea (Amen), and exercising women with ovulatory menstrual cycles (OV).

<table>
<thead>
<tr>
<th>Subjects included in each model</th>
<th>$R^2$</th>
<th>$\beta$ body fat (%)</th>
<th>$\beta$ intercept</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amen + OV</td>
<td>0.733</td>
<td>0.065</td>
<td>-0.815</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amen</td>
<td>0.657</td>
<td>0.064</td>
<td>-0.813</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OV</td>
<td>0.547</td>
<td>0.056</td>
<td>-0.577</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Regression analyses demonstrated a positive logarithmic correlation between leptin and percent body fat ($r^2 = 0.733$, $P < 0.001$), regardless of menstrual status (Figure 3). Using regression analyses performed on each group separately (Table 2), the slopes of the regression equation were not significantly different ($P = 0.744$), as assessed by ANCOVA, which demonstrates that the percentage body fat vs. leptin concentration
relationship is similar for both groups. The difference in serum leptin concentration between groups was no longer significant after adjusting for adiposity (Figure 4).

**Figure 4:** Actual mean serum leptin concentrations ± S.E.M and mean serum leptin concentrations after adjusting for percentage body fat ± S.E.M in exercising amenorrheic (solid bars) and exercising ovulatory (open bars) women.

**DISCUSSION**

Although strong evidence supports the view that leptin mediates the dependence of reproductive function on energy availability (12), no studies to date confirm the precise mechanism whereby leptin is sensed in the hypothalamus to regulate reproductive function. We assessed reproductive status and measured fasting serum leptin concentration and body composition in 52 premenopausal women to explore whether low leptin, per se, mediates reproductive suppression, or whether hypoleptinemia, i.e., having lower leptin than can be accounted for by adiposity, is the signal that suppresses the reproductive axis. Corroborating the findings of others (24, 25), we found that functional hypothalamic amenorrhea was associated with low percentage body fat and low fasting serum leptin concentration in comparison to cycling women. However, the findings from
this study indicate that it is not low leptin, \textit{per se}, that is suppressing reproductive function in all exercising amenorrheic women. Although many of the exercising amenorrheic women exhibited 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 that a threshold concentration of leptin, below which reproductive function is suppressed, is the primary mechanism underlying reproductive suppression, as proposed by others (37).

Many of the exercising amenorrheic women in our study exhibited circulating leptin concentrations that were no different from those observed in the ovulatory women. However, the lowest leptin concentration observed in the group of exercising ovulatory women was 2.57ng/ml, and several of the exercising amenorrheic subjects exhibited leptin concentrations that were lower than 2.57ng/ml. One could therefore argue that there is a critical concentration of serum leptin, and that the critical value is about 2.5ng/ml, which is in close agreement with the plasma leptin threshold identified by others (24, 25). Nevertheless, many women with FHA exhibited leptin concentrations that were above this “threshold”, which suggests that relative or individual changes in leptin concentrations are also sensed by and influence the reproductive system.

The primary regulator of leptin (\textit{ob}) gene expression is adiposity (14), which led us to question whether hypoleptinemia—having a lower leptin concentration than can be accounted for by adiposity alone—suppresses the reproductive system. However, our data did not support the hypothesis that low leptin concentrations relative to body fatness mediate reproductive suppression. After adjusting for adiposity there was no difference in mean leptin concentration between the exercising women with and without amenorrhea.
Others (2, 24) have reported similar findings in women with amenorrhea, regardless of exercise status, and have concluded that significantly lower body fat can account for the difference in leptin levels between amenorrheic and cycling women. These findings support the view that leptin acts as a peripheral signal to relay long-term energy status to the hypothalamus, which explains why women with low adiposity are at risk for amenorrhea (15), but fails to explain the mechanism whereby normal-weight women experience impaired reproductive function.

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 (38), and may explain why impaired reproductive function is not always associated with low body fatness. It is possible that we were unable to detect hypoleptinemia in our women due to our sampling method, because we only measured fasting serum leptin concentration in our women, and were thus unable to detect the presence of the nocturnal rise in leptin. Similarly, even though we consistently measured serum leptin concentration after an overnight fast when leptin levels reach their lowest (22), it is possible that some of the variability we observed in leptin concentration at a given value of adiposity is due to the fact that we probably were not always capturing the nadir in leptin concentration. In other words, it is possible that some of the variability we saw in leptin concentration was due to the timing of the sample, because it cannot be determined whether or not the blood samples were collected at the time leptin was at its lowest. Therefore, a single measurement of circulating leptin concentration does not appear to be a good indicator of hypoleptinemia due to its diurnal secretion pattern (39). Laughlin and Yen (22) found that
the relationship between leptin levels and the integrity of the reproductive system is related to the absence of the diurnal rhythm that is characteristic of leptin, rather than to low leptin per se, which supports our interpretation of our data.

In further support of this interpretation, Welt et al (40) demonstrated that administration of recombinant human leptin to women with hypothalamic amenorrhea in doses designed to mimic the normal diurnal variation in leptin levels stimulated folliculogenesis, improved LH pulsatility, and restored normal ovulatory status, independent of any lifestyle changes. These findings also demonstrate that changes in leptin concentration independent of lifestyle modifications and/or changes in energy status can influence the reproductive system. Although leptin production is primarily regulated by adiposity (14), it is also under the influence of other modulators, including, but not limited to, sympathetic nervous activity (41), carbohydrate availability (42), and training status (43). Therefore, circulating leptin concentration reflects the integrated stimulatory and inhibitory effects of these and other modulators on its production.

Perhaps, then, identifying the factors that contribute to the variability in circulating leptin concentration that is not accounted for by adiposity may shed some light on whether these factors modulate leptin concentration in a way that can impair reproductive function.

Our data indicate that women with exercise-associated FHA exhibit circulating leptin concentrations that are, on average, lower than those observed in exercising ovulating women. However, the range of leptin concentrations associated with exercise-associated amenorrhea is wide, which suggests that the reproductive system is sensitive to relative changes in leptin concentration, and not just a critical concentration of leptin.
REFERENCES


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CHAPTER IV

STUDY 2: The Resumption of Menses in Amenorrheic Women is Associated with an Increase in Fasting Serum Leptin Concentration

Noll Laboratory, Women’s Health and Exercise Laboratory, and the Department of Kinesiology, Pennsylvania State University, University Park, PA 16801

Key Words: Amenorrhea, Leptin, Resumption of Menses

United States Department of Defense CDMRP
Peer Reviewed Medical Research Program PR054531
INTRODUCTION

Leptin, the hormone product of the obesity (*ob*) gene, plays a key role as the signal whereby nutritional status is relayed from the periphery to the brain, allowing for the integrated control of energy balance and reproduction (1, 2). In *ob/ob* mice and humans, the lack of leptin production is associated with obesity, weight gain, and infertility, and the administration of recombinant leptin reverses these abnormalities (3-5). Leptin administration has also been shown to advance the age of reproductive maturity in weanling mice (6, 7), prevent the fasting-induced suppression of gonadotropins in *ob/ob* and normal mice (8, 9), and stimulate folliculogenesis in women with hypothalamic amenorrhea (10) who are consistently shown to exhibit low circulating leptin concentrations (11-16). These findings strongly support the current view that leptin serves as a key regulator of reproductive function. Although the precise mechanism whereby leptin regulates reproductive function remains unknown, evidence suggests it mediates the neuroendocrine response to a relative energy deficit or other stressor, resulting in reduced gonadotropin-releasing hormone and subsequent reproductive dysfunction (17-20).

Although women with exercise-associated functional hypothalamic amenorrhea (FHA) exhibit leptin concentrations that are, on average, lower than those observed in their regularly cycling peers (12-14), low leptin *per se* does not appear to be the cause of reproductive dysfunction in most of these women (Study 1). Hypoleptinemia, defined by leptin concentrations that are lower than can be explained by adiposity alone, likewise does not seem to fully explain the presence of reproductive dysfunction in women with exercise-associated hypothalamic amenorrhea (Study 1). Nevertheless, Welt *et al* (10)
performed a key study that confirmed the importance of leptin in the etiology of hypotalamic amenorrhea by demonstrating that the administration of recombinant human leptin restored normal ovulation and LH pulsatility in amenorrheic women. Leptin administration was likewise shown to increase markers of bone formation and improve thyroid and growth hormone axes in these women (10). These findings suggest that increasing leptin concentrations can reverse the suppression of reproductive function and restore normal neuroendocrine function.

However, the above mentioned study was pharmacological and the women who were treated all started with very low leptin (mean 3.9 ng/ml, range 1.4-6.9 ng/ml) concentrations. We have previously shown that exercising women with exercise-associated amenorrhea exhibit a wide range of circulating leptin concentrations, reaching upwards of 15ng/ml (Study 1). Higher mean leptin concentrations have also been observed by others in normal-weight women with FHA (12, 16). We were therefore interested in monitoring women with FHA who exhibit leptin concentrations across a wide range to determine whether a relative increase in leptin is evident in exercising women who resume menses without pharmacologic treatment. A secondary purpose of this study, presuming an increase in leptin is detected with resumption of menses, was to examine the magnitude of increase in leptin concentration observed. To this end, changes in circulating leptin concentration were measured in exercising women with hypothalamic amenorrhea during a 6-month period and compared between women who resumed menses and those who did not.
SUBJECTS AND METHODS

Recruitment

Volunteers were recruited by posters targeting physically active women for a study on the impact of increased caloric intake on bone health and menstrual cyclicity in energy deficient exercising women. Eligible women for this study experienced no menses within the past 3 months or 6 or fewer menses within the past year. Additional inclusion criteria for this study were: 1) no history of any serious medical conditions; 2) no current clinical diagnosis of an eating or psychiatric disorder; 3) age 18-30 years; 4) BMI 16-25 kg/m²; 5) weight stable (± 2kg) for the past 6 months; 6) non-smoking; 7) no medication use that would alter metabolic or reproductive hormone concentrations; 8) ≥ 3 hrs/wk aerobic exercise; 9) 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. Forty-three women met initial screening criteria and completed a 28-day baseline observational period. 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 Institutional Review Board.

Experimental Design

This investigation was conducted on a subset of subjects from a larger, prospective study that began at the University of Toronto and was moved to Penn State University. It was originally 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 irregular menstrual cycles)
and amenorrhea (the absence of menses for greater than 90 days). This investigation includes data from fourteen women with the most severe EAMD (i.e. amenorrhea) who completed 6 months of the intervention. These women were randomly assigned to a treatment group (EAMD + Calories; n = 7) or a control group (EAMD controls, n = 7) at the beginning of the intervention. At the end of 6-months of intervention, for the purposes of this investigation, the exercising women with amenorrhea (Amen) were then categorized into one of two groups, regardless of intervention group: 1) those who resumed menses between months one and six of intervention (Amen-R; n = 5), and 2) those who did not resume menses within 6 months of intervention (Amen-NR; n = 7). Two women resumed menses within the first month of the intervention, and were thus excluded because the concentration of leptin coincident with the resumption of menses could not be determined. Of the 5 women assigned to the Amen-R group, 3 were originally in the Amen + Cal group and 2 were in the Amen control group. Of the 7 women categorized as Amen-NR, 4 were originally in the EAMD + Cal group and 3 were in the EAMD control group. All intervention comparisons were made between baseline (Pre) and either month six of intervention or at the time of resumption (Post) in Amen-NR and Amen-R, respectively. The time of resumption was defined as the day the subject resumed menses.

Repeated measures of weight, body composition, resting energy expenditure, weekly training volume, dietary intake, serum hormones, and urinary metabolites were collected during the study. The primary outcome variables pertaining to this study include measures of body composition, serum leptin concentration, and menstrual status.
Classification of Menstrual Status

All subjects collected first morning urine samples throughout the 28-day baseline monitoring period. Day one of baseline was an arbitrary day because the women were not cycling. Classification of menstrual status was based on urinary estrone-1-glucuronide (E1G), pregnanediol-3-glucuronide (PdG) and luteinizing hormone (LH) profiles, as previously reported (21), and on self-reported menstrual histories. Subjects who reported no menses within the last 3 months and whose baseline monitoring cycle confirmed diagnosis of amenorrhea were included in this study. Women who exhibited oligomenorrheic cycles (between 35 and 90 days long) or an FAI > 3 were excluded. The FAI was calculated according to the following equation: FAI = [total testosterone (nmol/L) / serum hormone binding globulin (nmol/L)]*100 (22). Twenty-four of the forty-three women who completed baseline were identified as having FHA, and only fourteen of those women entered and completed the 6-month intervention. The other ten women discontinued their participation in the study.

Blood Sampling

<table>
<thead>
<tr>
<th>Subject</th>
<th>Resumption Day</th>
<th>Resumption Week</th>
<th>Blood Sample(s) Used</th>
<th>Blood Sample Day Relative to Day of Resumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>11</td>
<td>Week 9</td>
<td>-16</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>6</td>
<td>Week 5</td>
<td>-1</td>
</tr>
<tr>
<td>3*</td>
<td>89</td>
<td>13</td>
<td>Week 9</td>
<td>-19</td>
</tr>
<tr>
<td>4*</td>
<td>114</td>
<td>17</td>
<td>Week 9 and 21</td>
<td>-57, +27</td>
</tr>
<tr>
<td>5</td>
<td>118</td>
<td>17</td>
<td>Week 17</td>
<td>-7</td>
</tr>
</tbody>
</table>

*Subjects did not have blood drawn during weeks 13 and 17
For the current study, fasting morning venous blood samples were collected once during week 3 of baseline and once at the end of baseline for all subjects. The two samples were pooled for all baseline hormone analyses. Additional blood samples were collected once monthly in the Amen women who entered the intervention, during weeks 5, 9, 13, 17 and 21. The week 21 blood sample was used for Post measurements in Amen-NR women, and the blood sample(s) nearest to and prior to the time of resumption were used for Post measurements in Amen-R women (Table 1). In the case where more than one sample was used for the Post measurement, the reported value is the average of the two samples. Blood was always collected before 1000 hr following an overnight fast and after subjects refrained from exercise and caffeine for at least 12 hours. All samples were allowed to clot for 30 minutes and were then centrifuged at 4 °C for 15 minutes at 3000 rpm. The serum was then aliquotted into 2-ml polyethylene storage tubes and stored frozen at -80 °C until analysis.

Serum Hormone Analysis

Plasma leptin concentration was measured using a solid-phase sandwich enzyme-linked immunoassay (ELISA) for total leptin (Millipore, St. Charles, MI). All samples were measured in duplicate, in the same laboratory and by the same technician, 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 for the low control were 6.2% and 4.6%, respectively. This assay is sensitive to leptin concentrations ranging from 0.5 to 100 ng/ml. Total testosterone was measured using a radioimmunoassay kit (Siemens, Los Angeles, CA) through competitive immunoassay. Analytical sensitivity for the testosterone assay was 0.14 nmol/L (4ng/dL). The intraassay
and interassay coefficients of variation were 6.4% and 7.5%, respectively. Serum hormone binding globulin (SHBG) was analyzed using a chemiluminescence analyzer (ImmunoLite, Euro Diagnostic Products Corporation, Lianberis, UK) through competitive immunoassay. Analytical sensitivity for the SHBG was 0.2nmol/L. The intraassay and interassay coefficients of variation were 6.4% and 8.7%, respectively.

Urinary hormone measurements

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (23) which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (23). Microtiter plate competitive enzyme immunoassays were used to measure the urinary metabolites E1G and PdG. The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by Coralie Munro University of California (Davis, CA). The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 12.2% and 14.0%, respectively. The PdG intra- and inter-assay variability was determined in house as 13.6% and 18.7%, respectively. The validity of the urine technique as representative of the 24 hour pattern of E1G and PdG excretion has been reported by other investigators (24, 25). Urinary LH was determined by coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The sensitivity of the LH assay is 0.15mIU/ml. The intra- and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

Dietary energy intake

Dietary energy intake and macronutrient composition was estimated using a 3-day diet log (2 weekdays and 1 weekend day) once during baseline in all subjects, as well as
once monthly in all Amen subjects who completed the intervention. Subjects were provided with detailed instructions on how to record types and quantities of foods eaten, and were provided with a food scale and measuring cup to weigh and measure food if needed. Diet analysis of the 3-day food logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN).

*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 on five occasions for subjects who completed Baseline (at screening and four times during baseline), and the mean of these measurements is presented for Baseline weight (Seca, Model 770, Hamburg, Germany). Additional weights were recorded biweekly during the intervention. Height was measured to the nearest 1.0 cm during screening. Baseline body mass index (BMI) was calculated as the baseline weight divided by screening height squared (kg/m²). Post BMI was calculated as the Post weight divided by screening height squared (kg/m²).

*Body Composition*

Body composition was assessed during screening and at weeks one, five, nine and twenty-one during intervention using dual-energy x-ray absorptiometry (DXA). Ten subjects were scanned on one of two scanners at different study centers, either a GE Lunar Prodigy (n=5, enCORE 2002 software version 6.50.069) or a GE Lunar iDXA (n=5, 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, fourteen participants were scanned in triplicate on both machines. The majority (n=8) were scanned on both machines within 5 days; however, there was approximately one month between scans for some subjects (n=6). The values for body composition obtained on each scanner were found to be highly correlated with no significant difference between the population mean values. Equations were derived using simple linear regression to remove these biases and report the Prodigy values calibrated to the iDXA. The remaining subjects in the current study (n=2) were scanned on a Hologic QDR 4500W. A cross-calibration study between the Hologic and iDXA is in process to generate agreement between the data collected on the two machines in the case the measurements significantly differ.

Training Status

Peak aerobic capacity (VO2 peak) was measured once during week 3 of Baseline during a progressive treadmill test to volitional exhaustion using open-circuit spirometry, using methods that have been previously reported (26, 27). Subjects recorded daily exercise activities and heart rates (by radial palpation) throughout study participation on weekly exercise logs, which were used to quantify purposeful exercise in minutes per week.

Statistical Analysis

All demographic and descriptive data were analyzed using independent t-tests to determine whether the groups differed at Pre-intervention. Changes in variables from Pre- to Post-intervention were compared between groups using paired t-tests on change scores. A repeated-measures ANOVA was used to determine whether changes in serum leptin
concentration from Pre- to Post-intervention were significantly different between groups. Regression equations for serum leptin concentration vs. percentage body fat were analyzed separately for Amen-R and Amen-NR in order to test for differences in slope, as assessed by analysis of covariance. To control for variables showing significant differences in the independent t-tests at baseline, baseline serum leptin concentration was also assessed by analysis of covariance. A specific power calculator was used to determine samples sizes required to detect differences in the change in leptin concentration using an independent t-test between exercising Amen women who resumed menses and those who did not. Sample size was based on a meaningful difference of 6.3 ng/ml, and S.D.s of 1.5 ng/ml and 4.1 ng/ml, which was based off of data reported by Welt et al. (10) in amenorrheic women before and after one month of receiving recombinant human leptin. In order to achieve 80% power using a 0.05 level of significance, a sample size of 6 women for each group would be required. In all analyses, $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS for Windows (Version 18.0; Chicago, IL) statistical software. Data are reported as mean ± SEM.
RESULTS

**Pre-study characteristics**

Table 2: Descriptive data for exercising amenorrheic women who resumed menses (Amen-R) and those who did not resume menses (Amen-NR) during the 6-month study.

<table>
<thead>
<tr>
<th></th>
<th>Amen-R (n=5)</th>
<th>Amen-NR (n=7)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>24.6±1.9</td>
<td>-</td>
<td>21.4±1.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.7±1.2</td>
<td>-</td>
<td>168.6±2.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>55.5±1.3</td>
<td>57.0±1.1</td>
<td>54.8±3.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.2±0.3</td>
<td>21.8±0.3</td>
<td>19.3±0.9</td>
</tr>
<tr>
<td><strong>Body Composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>24.0±1.9</td>
<td>24.7±2.0</td>
<td>17.3±1.8</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>13.4±2.6</td>
<td>14.0±1.2</td>
<td>9.4±1.2</td>
</tr>
<tr>
<td>Fat Free Mass (kg)</td>
<td>42.7±1.6</td>
<td>43.1±1.7</td>
<td>44.9±2.4</td>
</tr>
<tr>
<td><strong>Menstrual History</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of Menarche (yr)</td>
<td>13.8±0.6</td>
<td>-</td>
<td>13.9±0.7</td>
</tr>
<tr>
<td>Gynecological Age (yr)</td>
<td>10.8±2.4</td>
<td>-</td>
<td>7.4±1.3</td>
</tr>
<tr>
<td><strong>Training Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise Activity (min/wk)</td>
<td>400±182</td>
<td>390±149</td>
<td>380±98</td>
</tr>
<tr>
<td>VO₂ peak (ml/kg/min)*</td>
<td>55.1±3.5</td>
<td>-</td>
<td>50.9±3.0</td>
</tr>
<tr>
<td>Exercise History (min/wk)</td>
<td>658±224</td>
<td>-</td>
<td>466±82</td>
</tr>
<tr>
<td><strong>Metabolic Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>6.5±2.8</td>
<td>11.0±2.9</td>
<td>2.3±0.7</td>
</tr>
<tr>
<td>Dietary Intake (kcal/day)</td>
<td>2530±774</td>
<td>2585±49</td>
<td>2046±157</td>
</tr>
<tr>
<td>CHO Intake (g/day)</td>
<td>379±126</td>
<td>375±95</td>
<td>299±18</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

**Time** = main effect of time, **Group** = main effect of group, **X** = interaction effect

BMI – body mass index

CHO – carbohydrate

*n = 6 for Amen-NR

Descriptive characteristics are shown in Table 2. Amen-R and Amen-NR did not differ at baseline with respect to age, weight, BMI, gynecological age, or maximal aerobic capacity, yet significant differences in height (P = 0.049), percentage body fat (P = 0.033), and fat mass (P = 0.043) were observed. Although mean serum leptin concentration was greater in Amen-R vs. Amen-NR at baseline, the difference was not statistically significant (P = 0.207)
Figure 1: Pre and Post study fasting serum leptin concentrations for individual subjects (open circles, solid lines) and group means (filled circles, dashed lines) for exercising amenorrheic women who resumed menses (top panel) and those who did not (bottom panel).
**Figure 2**: Serum leptin vs. body fat relationship between exercising amenorrheic women who resumed menses (Amen-R; open markers) and those who did not (Amen-NR; filled markers) at Pre (squares) and Post (triangles) study.

Post-study measurements as well as changes from pre- to post-study are presented in Table 2. Amen-R exhibited a significant increase in weight ($P = 0.013$) and BMI ($P = 0.015$) during the study, yet did not exhibit significant increases in adiposity ($P = 0.257$). The increase in fasting serum leptin concentration from pre- to post-study was significantly greater in Amen-R in comparison to Amen-NR (Interaction effect: $P = 0.002$, Time Effect: $P = 0.001$, Group Effect: $P = 0.031$; Figure 1), as assessed by repeated-measures ANOVA. Figure 2 shows the serum leptin vs. percentage body fat relationship in the two groups. The increase in fasting serum leptin concentration observed in the Amen-R group was not paralleled by an increase in adiposity.

**DISCUSSION**

Leptin is increasingly recognized as the hormonal mediator allowing for the coordinated regulation of energy status and reproductive function, yet this is the first study to demonstrate that an increase in fasting serum leptin concentration is associated
with the resumption of menses in women with hypothalamic amenorrhea. Welt and colleagues (10) have demonstrated that administration of recombinant human leptin to women with hypothalamic amenorrhea stimulates follicle growth, improves LH pulsatility and restores ovulation, yet it was a pharmacological study and therefore the results did not represent normal physiological changes. Hypothalamic amenorrhea is associated with chronic energy deficiency (28), yet the women who received recombinant human leptin treatment and who displayed improved reproductive status exhibited significant weight loss and percentage body fat loss (10). This is likely because leptin acts as a satiety signal to reduce feeding (29, 30). In order to reverse amenorrhea in these women without pharmacological treatment, a more physiological treatment would be to increase caloric intake (31).

We observed an increase in serum leptin concentration in all of the women who resumed menses, yet the magnitude of increase observed was varied, ranging from 18% to 230%. Similarly, the leptin concentrations measured at the time of resumption were varied, ranging from 2.8ng/ml to 20.0ng.ml. These observations support the findings from Study 1, which demonstrated that a leptin concentration below 2.5ng/ml may signal reproductive suppression, yet that low leptin per se is not always associated with reproductive suppression, and in such cases perhaps it is relative changes in leptin concentration that are sensed by the reproductive system.

The increases in leptin observed in association with the resumption of menses in exercising women with amenorrhea were not paralleled by significant increases in adiposity, which demonstrates that factors independent of body fatness might have been suppressing leptin levels in the women prior to resumption. This suggests that other
modulators of leptin production can influence the reproductive axis via their impact on leptin levels, and may explain why stress is often found in association with compromised reproductive function (32, 33). In animal and human models, sympathetic nervous activity has been shown to inhibit leptin production (34, 35). Activation of the sympathoadrenal axis is observed in response to many different types of stressors, including exercise stress (36), cold stress (19), psychosocial stress (37) and metabolic stress (38). It is therefore possible that stress may contribute to reproductive dysfunction due to its inhibitory impact on leptin production. Increased hypothalamic-pituitary-adrenal (HPA) axis activity, another stress response that results in the secretion of cortisol, has also been observed in women with amenorrhea (39, 40), and hypercortisolemia was found in association with hypoleptinemia in exercising women with amenorrhea (13). These findings support the theory that stress can impair reproductive function by inhibiting leptin production.

Other factors have also been shown to modulate leptin production, and may therefore contribute to the increases in leptin concentration observed in the women who resumed menses. Carbohydrate availability (i.e. glucose availability) and carbohydrate intake, as well as insulin have been shown to stimulate leptin production (34, 41), for example, while an acute state of low energy availability has been shown to suppress leptin production (17, 42). It is therefore possible that an increase in leptin concentration was observed in the women who resumed menses secondary to improved nutritional status. However, we did not observe significant changes in dietary measures. Similarly, exercise training has been shown to suppress leptin production in women (43, 44). We did not observe a significant decrease in training volume to account for the increase in
leptin concentration, but the inhibitory impact of exercise training on leptin may contribute to the greater prevalence of menstrual disturbances observed in exercising women (45).

Our data support the hypothesis that a relative increase in leptin concentration can restore menses in women with exercise-associated hypothalamic amenorrhea who exhibit a wide range of leptin concentrations. We did not assess changes in the diurnal secretion pattern of leptin, and are therefore unable to determine whether the resumption of menses observed in our group of women with amenorrhea was associated with changes in the diurnal secretion pattern of leptin. Nevertheless, our data indicate that relative changes in leptin concentration, rather than a critical concentration of leptin, are sensed by and influence the reproductive axis.
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CHAPTER V

STUDY 3: Determinants of the Variability in Fasting Serum Leptin in Exercising Women with Ovulatory Menstrual Cycles and in Women with Exercise-Associated Hypothalamic Amenorrhea Before and After Resumption of Menses

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Key Words: Amenorrhea, Leptin, Adiposity, Insulin, Glycerol, Exercise, Diet, Respiratory Exchange Ratio

United States Department of Defense CDMRP Peer Reviewed Medical Research Program PR054531
INTRODUCTION

Leptin, the hormone product of the obesity \textit{(ob)} gene, has been implicated as the signal whereby energy status, both acute and long-term, is relayed from the periphery to the brain, providing a negative-feedback regulation of weight homeostasis (1-3). It has also been suggested that leptin is the signal allowing for the integrated control of nutrition and reproduction (4), such that a relative energy deficit can cause the suppression of reproductive function. Compelling evidence to support this theory has been reported in women with hypothalamic amenorrhea, who often experience reproductive suppression secondary to chronic low energy availability due to high levels of energy expenditure and insufficient nutritional intake, or both (5-7), and who likewise exhibit low circulating leptin concentrations (8-14).

However, not all women with hypothalamic amenorrhea exhibit signs of a relative energy deficit due primarily to training and dietary regimens. These women are thought to experience reproductive dysfunction secondary to psychosocial stress (15, 16). Although leptin is primarily regulated by long term energy status, and thus lower in people with reduced adiposity, leptin production is also under the regulation of other factors, such as sympathetic nervous activity, exercise status, gonadal steroids, and diet (17-22). Consequently, leptin secretion may not always reflect adiposity (i.e. long-term energy status). It seems likely that modulators of leptin production could theoretically influence reproductive function via their impact on circulating leptin concentration. This could explain how psychosocial stress, in combination with or independent of an energy deficiency, contributes to reproductive dysfunction (23, 24).
Although there is strong evidence to support the involvement of leptin in the etiology of hypothalamic amenorrhea (8-14), the precise mechanism whereby leptin regulates reproductive function remains unknown. There does not appear to be a critical threshold of leptin concentration, below which reproductive function is suppressed because a wide range of leptin has been observed in women with hypothalamic amenorrhea (Study 1). Similarly, mixed findings have been reported regarding whether hypoleptinemia—leptin concentrations that are lower than can be explained by adiposity alone—is associated with hypothalamic amenorrhea (Study 1)(10, 11). We have previously observed that increases in circulating leptin concentration are associated with the resumption of menses in women with hypothalamic amenorrhea independent of increases in adiposity (Study 2). This supports the hypothesis that hypoleptinemia may contribute to reproductive suppression and suggests that leptin production in women with FHA is being suppressed by factors independent of long-term energy status. We were therefore interested in determining whether modulators of leptin production contributing to the variance in leptin concentration not accounted for by adiposity differ depending on menstrual status. Insulin (25), carbohydrate intake (19), glucose availability (26), sympathetic nervous activity (27), and other factors (28-30) have been shown to influence leptin production. To this end, we tested the hypothesis that modulators of leptin concentration, after adjusting for adiposity, are present in exercising women with hypothalamic amenorrhea but not in exercising ovulatory women. A second purpose of this study was to explore whether changes in modulators of leptin production are found in association with the increase in leptin that is observed in women with hypothalamic amenorrhea who resume menses independent of any changes in adiposity.
SUBJECTS AND METHODS

Experimental Design

This investigation is a subset of a larger, prospective study that started at the University of Toronto and moved to Penn State University. It was originally designed to determine whether a 12-month intervention of increased caloric intake would improve indices of bone health and menstrual cyclicity in premenopausal women who suffer from severe exercise-associated menstrual disturbances (EAMD), including oligomenorrhea (long and irregular menstrual cycles) and amenorrhea (the absence of menses for greater than 90 days). This investigation includes baseline data from 24 women with the most severe EAMD, i.e. amenorrhea, and 26 women with ovulatory menstrual cycles, as well as data from 14 exercising amenorrheic women who completed 6 months of the intervention. The primary outcome variables of this investigation were menstrual status, fasting serum leptin concentration, and known modulators of leptin production.

Inclusion Criteria

Subject inclusion criteria were: 1) no history of any serious medical conditions; 2) no current clinical diagnosis of an eating disorder; 3) age 18-35 years; 4) BMI 16-26 kg/m²; 5) weight stable (± 2kg) for the past 6 months; 6) gynecological age ≥ 5 years; 7) non-smoking; 8) no medication use that would alter metabolic hormone concentrations; 9) not taking hormonal contraceptives for the past 6 months; 10) no menses within the last 3 months or 6 or fewer menses within the last 12 months (EAMD) or regular menses within the last 6 months (OV); 11) ≥ 3 hours/week aerobic exercise for women with EAMD and ≥ 2 hours/week aerobic exercise for OV women; 12) no history of a clinical diagnosis of PCOS or a free androgen index (FAI) ≥ 3 with other corroborative
symptoms of PCOS. 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.

**Subject Groupings**

All subjects were studied for a baseline period equivalent to either one menstrual cycle for eumenorrheic women or one 28-day monitoring period for women not regularly cycling. Subjects who met the inclusion criteria for OV controls and whose baseline menstrual cycle confirmed normal ovulatory cycles (24-35 days) were assigned to a control group (OV Controls). Subjects who met inclusion criteria for EAMD and whose baseline menstrual cycle confirmed diagnosis of amenorrhea (Amen) were randomly assigned to one of two intervention groups: 1) an increased calorie intake treatment group (Amen + Calories), or 2) an Amen control group (Amen Controls). Regardless of intervention group, Amen subjects were also retrospectively grouped based on whether or not they resumed menses during 6 months of observation. Amen women who resumed menses between months one and six of the study were classified as Amen-R, while those who did not resume menses by month six of the study were classified as Amen-NR.

**Menstrual Cycle Characteristics**

Subjects collected first morning urine samples every morning throughout baseline. These samples were assayed for luteinizing hormone (LH), estrone-1-glucuronide (E1G) and pregnanediol-3-glucuronide (PdG) to characterize menstrual cycle status. Ovulatory status was determined by the presence or absence of the urinary LH surge, identified as the LH peak on the day of or the day after the E1G peak (31). Menstrual cycle length was defined as the number of days from the first day of menses to
the next menses. Volunteers were considered eumenorrheic if menses occurred at regular intervals of 26-35 days. Amenorrhea was confirmed by chronically suppressed E1G and PdG profiles. To determine estrogen exposure, E1G urinary metabolites were compared between the groups using a modified trapezoidal integrated area under the curve (AUC) technique. To determine progesterone exposure, PdG urinary metabolites were compared between the groups using a modified trapezoidal integrated area under the curve (AUC) technique. All women exhibited an FAI < 3, which was calculated according to the following equation: FAI = [total testosterone (nmol/L) / serum hormone binding globulin (nmol/L)]*100 (32). An FAI of 3 was used as the cutoff because women diagnosed with PCOS (33), often exhibit calculated FAIs ≥3 (34, 35), while women without PCOS exhibit calculated FAIs below 3 (34, 35).

Urinary hormone measurements

All urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells) to account for hydration status (36) which has been reported to perform as well as creatinine correction for adjusting urinary hormone concentrations (36). Microtiter plate competitive enzyme immunoassays were used to measure the urinary metabolites E1G and PdG. The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by Coralie Munro University of California (Davis, CA). The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 12.2% and 14.0%, respectively. The PdG intra- and inter-assay variability was determined in house as 13.6% and 18.7%, respectively. The validity of the urine technique as representative of the 24 hour pattern of E1G and PdG excretion has been reported by other investigators (37, 38). Urinary LH was determined by coat-a-count
immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The sensitivity of the LH assay is 0.15mIU/ml. The intra- and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

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 two samples were pooled for all baseline hormone analyses. Additional blood samples were collected once monthly in the Amen women who entered the intervention, during weeks 5, 9, 13, 17 and 21. The week 21 blood sample was used for Post measurements in Amen-NR women, and the blood sample(s) nearest to the time of resumption were used for Post measurements in Amen-R women (Table 1). In the case where more than one sample was used for the Post measurement, the reported value is the average of the two samples. Blood was always collected before 10:00am following an overnight fast and after subjects refrained from exercise for at least 12 hours. Blood was always collected before 10:00am following an overnight fast and after subjects refrained from exercise and caffeine for at least 12 hours. All samples were allowed to clot for 30 minutes and were then centrifuged at 4 °C for 15 minutes at 3000 rpm. The serum was then aliquotted into 2-ml polyethylene storage tubes and stored frozen at -80 °C until analysis.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Resumption Day</th>
<th>Resumption Week</th>
<th>Blood Sample(s) Used</th>
<th>Blood Sample Day Relative to Day of Resumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>11</td>
<td>Week 9</td>
<td>-16</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>6</td>
<td>Week 5</td>
<td>-1</td>
</tr>
<tr>
<td>3*</td>
<td>89</td>
<td>13</td>
<td>Week 9</td>
<td>-19</td>
</tr>
<tr>
<td>4*</td>
<td>114</td>
<td>17</td>
<td>Week 9 and 21</td>
<td>-57, +27</td>
</tr>
<tr>
<td>5</td>
<td>118</td>
<td>17</td>
<td>Week 17</td>
<td>-7</td>
</tr>
</tbody>
</table>

*Subjects did not have blood drawn during weeks 13 and 17
**Serum hormone analysis**

Serum leptin concentration was measured using a solid-phase sandwich enzyme-linked immunoassay (ELISA) for total leptin (Millipore, St. Charles, MI). All samples were measured in duplicate, in the same lab and by the same person, 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 0.5ng/ml. Serum glycerol concentration was measured using a glycerol assay kit (Cayman Chemical Company, Ann Arbor, MI). The intra-assay and inter-assay coefficients of variance are 7.9% and 8.0%, respectively. This assay is sensitive to glycerol concentrations of 0-20 mg/dl. Serum insulin concentration and sex hormone binding globulin (SHBG) concentration were analyzed using a chemiluminescence analyzer (Immulite, Euro Diagnostic Products Corporation, Lianberis, UK) through competitive immunoassays (Siemens Medical Solutions Diagnostics). The assay for insulin is sensitive to insulin concentrations of 2μIU/ml (13.89μmol/L), and the assay for SHBG is sensitive to SHBG concentrations of 0.2 nmol/L. Total testosterone was measured using a radioimmunoassay kit (Siemens, Los Angeles, CA) through competitive immunoassay. Analytical sensitivity for the testosterone assay was 0.14 nmol/L (4 ng/dL). The intraassay and interassay coefficients of variation were 6.4% and 7.5%, respectively.

**Energy Status Measurements**

Resting energy expenditure (REE) and resting respiratory exchange ratio (RER) were determined using a ventilated hood (Sensormedics Vmax Series). Subjects were
tested in the morning, between 06:00 and 10:00 in a fasted state, having refrained from exercise and caffeine for 24 hours. 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 arriving at the laboratory, the subject lay in a supine position for 30-45 minutes to establish resting conditions and acclimate to room temperature. The ventilated hood was then placed over the subject, and REE measurements were collected for 45 minutes or until the subject reached steady state. Steady state was defined as VO₂ and VCO₂ varying by <10% and RER varying by <5% from minute to minute. The first 5 minutes were automatically excluded. Oxygen consumption (VO₂) and carbon dioxide production (VCO₂) were collected every 30 seconds. Steady state data for VO₂ and VCO₂ were averaged and REE was calculated using the Weir equation (39). Resting RER was calculated as the average ratio of VCO₂ to VO₂ during steady state.

**Dietary energy intake**

Dietary energy intake and macronutrient composition was estimated using a 3-day diet log (2 weekdays and 1 weekend day). Subjects were provided with detailed instructions on how to record types and quantities of foods eaten, and were provided with a food scale and measuring cup to weigh and measure food if needed. Diet analysis of the 3-day food logs were performed using Nutrition Data System for Research (NDS-R 2008, Minneapolis, MN).

**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 on five occasions
for subjects who completed Baseline (at screening and four times during baseline), and
the mean of these measurements is presented for Baseline weight (Seca, Model 770,
Hamburg, Germany). Additional weights were recorded biweekly during the
intervention. Height was measured to the nearest 1.0 cm during screening. Baseline body
mass index (BMI) was calculated as the baseline weight divided by screening height
squared (kg/m²). Post BMI was calculated as the Post weight divided by screening height
squared (kg/m²).

*Body Composition*

Percentage body fat, fat mass, and fat free mass (FFM) were assessed using dual-
energy x-ray absorptiometry (DXA) once during screening in all subjects, as well as
during weeks one, five, nine and twenty-one in the subjects who completed the 6-month
intervention. Forty-six subjects were scanned on one of two scanners at different centers,
either a GE Lunar Prodigy (n=29, enCORE 2002 software version 6.50.069) or a GE
Lunar iDXA (n=17, 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. Fourteen participants were
scanned in triplicate on both machines. The majority (n=8) were scanned on both
machines within 5 days; however, there was approximately one month between scans for
some subjects (n=6). The values for body composition that were obtained on each
scanner were found to be highly correlated with no significant difference between the
population mean values. Equations were derived using simple linear regression to
remove these biases and report the Prodigy values calibrated to the iDXA. The
remaining subjects in the current investigation (n=4) were scanned on a Hologic QDR
4500W. A cross-calibration study between the Hologic and iDXA is in process to generate agreement between the data collected on the two machines if the measurements significantly differ.

**Training Status**

Peak aerobic capacity (VO\textsubscript{2} peak) was measured during a progressive treadmill test to volitional exhaustion during week 3 of baseline using open-circuit spirometry. The VO\textsubscript{2} peak test was initiated by the volunteer selecting a comfortable running speed at 0.0% grade. The grade of the treadmill was increased 2.0% after every 2 minutes for the first 8 minutes of the test, after which the grade was then increased 1.0% for each subsequent minute. VO\textsubscript{2} and VCO\textsubscript{2} were measured using an on-line Moxus Modular VO\textsubscript{2} System (Applied Electrochemistry Inc., Pittsburg, PA). Subjects also recorded daily exercise activities and heart rates (by radial palpation) 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: 1) percentage body fat (18), 2) serum insulin concentration (40), 3) weekly exercise minutes and peak VO\textsubscript{2}, as indicators of training status (20), 4) dietary carbohydrate intake and RER, as indicators of carbohydrate availability (19), 5) RER per kg FFM and total
kilocalories consumed per day, as indicators of acute energy status (22), 6) E1G AUC and PdG AUC as well as the E1G and PdG concentrations coinciding with the day of the leptin sample, as indicators of gonadal steroid milieu (17, 30), and 7) glycerol, as an indicator of lipolysis to reflect sympathetic activation of adipose tissue (21). These variables were then used in multivariate analysis, by use of stepwise regression, to determine the model which best predicted leptin in each group. Repeated-measures ANOVA were used to investigate whether variables changed differently in amenorrheic women who resumed menses and those who did not resume menses during the 6-month study. A specific power calculator was used to determine sample sizes required to detect differences in RER and fasting serum glycerol concentrations using an independent t-test between exercising women with Amen who resumed menses and those who did not. Sample size was based on the detection of a meaningful difference in RER of 0.038 and a S.D. of 0.05 for both means. In order to achieve 80% power using a 0.05 level of significance, a sample size of 44 women for each group would be required. The calculation of this sample size was very conservative. To detect a meaningful difference in fasting serum glycerol concentration of 42μmol/l (0.39 mg/dl), with a S.D. of 17μmol/l (0.16 mg/dl), which was based off a study comparing glycerol concentrations after a 13-hour fast in healthy non-obese men (41), a sample size of 10 people for each group would be required. Statistical analyses were performed using SPSS for Windows (Version 18.0; Chicago, IL) statistical software. All results are presented as mean ± S.E.M, and an alpha level of $P < 0.05$ was considered to be statistically significant.
RESULTS

Descriptive Characteristics

Table 2: Baseline descriptive data for exercising ovulatory women (OV) and exercising amenorrheic women (Amen).

<table>
<thead>
<tr>
<th></th>
<th>OV (n = 26)</th>
<th>Amen (n = 24)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</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>Body composition</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>Fat Free Mass (kg)</td>
<td>43.1 ± 0.8</td>
<td>44.3 ± 1.0</td>
<td>0.349</td>
</tr>
<tr>
<td>Menstrual characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of Menarche (yr)</td>
<td>12.5 ± 0.2</td>
<td>13.4 ± 0.3</td>
<td>0.017</td>
</tr>
<tr>
<td>Gynecological Age (yr)</td>
<td>10.7 ± 0.7</td>
<td>8.1 ± 0.7</td>
<td>0.012</td>
</tr>
<tr>
<td>E1G AUC (days (ng/ml))</td>
<td>1010.6 ± 86.1</td>
<td>468.5 ± 46.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PdG AUC (days (ng/ml))</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>-</td>
<td>n/a</td>
</tr>
<tr>
<td>Training characteristics</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/wk)</td>
<td>468 ± 71</td>
<td>662 ± 85</td>
<td>0.084</td>
</tr>
<tr>
<td>Exercise Activity (min/wk)</td>
<td>236 ± 26</td>
<td>388 ± 53</td>
<td>0.012</td>
</tr>
<tr>
<td>Serum hormones</td>
<td></td>
<td></td>
<td></td>
</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 (mg/dl)</td>
<td>3.5 ± 0.2</td>
<td>4.2 ± 0.4</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>Metabolic characteristics</td>
<td></td>
<td></td>
<td></td>
</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 (kcal/day)</td>
<td>1271 ± 26</td>
<td>1233 ± 39</td>
<td>0.430</td>
</tr>
<tr>
<td>REE (kcal/kgFFM/day)</td>
<td>29.6 ± 0.5</td>
<td>27.9 ± 0.7</td>
<td>0.053</td>
</tr>
<tr>
<td>Nutritional characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary Intake (kcal/day)</td>
<td>1897 ± 109</td>
<td>2163 ± 243</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>
<tr>
<td>Fat Intake (g/day)</td>
<td>63 ± 4</td>
<td>66 ± 8</td>
<td>0.731</td>
</tr>
<tr>
<td>Fat Intake (%)</td>
<td>30.0 ± 1.3</td>
<td>26.7 ± 1.5</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

*n = 20 for Ovulatory Women and n = 21 for Amenorrheic Women
Baseline descriptive characteristics for the exercising OV women and the exercising Amen women are presented in Table 2. The exercising groups of women were similar with regards to age, height and weight. However, the exercising OV group had a significantly greater BMI and percentage body fat and fat mass than the exercising Amen group. The exercising Amen group had a greater peak VO₂ and current weekly training volume than the exercising OV group. There were no differences between the exercising groups of women with respect to dietary characteristics or total REE, however the exercising Amen women exhibited a lower REE relative to FFM and a significantly greater resting RER than the exercising OV women.

Menstrual Characteristics and Urinary Ovarian Steroid Data

Menstrual characteristics for OV and Amen exercising women are shown in table 1. Exercising amen women had a significantly older age of menarche, younger gynecological age, and lower estrogen and progesterone exposure in comparison to the exercising ovulatory women. Composite graphs of the daily concentrations of E1G and PdG are presented in figure 1.
Figure 1. Composite graphs of daily estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) in exercising women with ovulatory menstrual cycles (top panel) and exercising women with amenorrhea (bottom panel). The E1G and PdG data for ovulatory women are aligned by day of the luteinizing hormone (LH) peak defined as day 0. The amenorrheic women’s E1G and PdG data are aligned by chronological day of daily urinary hormone collections. Values are mean ± SEM.
Correlations with Log Leptin

Variables that were positively correlated with log leptin when both groups were combined included percentage body fat \( (r = 0.856, \ P < 0.001) \), REE per kg FFM \( (r = 0.370, \ P = 0.006) \), and log insulin \( (r = 0.363, \ P = 0.012) \). Weekly exercise minutes \( (r = -0.303, \ P = 0.029) \), VO2 peak \( (r = -0.385, \ P = 0.010) \) and RER \( (r = -0.285, \ P = 0.035) \) were negatively correlated with log leptin when both groups were combined. Dietary carbohydrate intake was not significantly correlated with log leptin.

Determinants of Leptin

**Table 3: Multivariate analysis for Log Leptin**

<table>
<thead>
<tr>
<th></th>
<th>( R^2 )</th>
<th>( R^2 ) Change</th>
<th>( B )</th>
<th>( p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>0.522</td>
<td>0.522</td>
<td>0.722</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Amen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>0.657</td>
<td>0.657</td>
<td>0.826</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Log Insulin</td>
<td>0.713</td>
<td>0.056</td>
<td>0.308</td>
<td>0.012</td>
</tr>
<tr>
<td>Log Glycerol</td>
<td>0.775</td>
<td>0.061</td>
<td>0.258</td>
<td>0.030</td>
</tr>
</tbody>
</table>

The statistical models that best predicted log leptin in the OV and Amen group, respectively, are presented in Table 2. Log leptin was best predicted by percentage body fat in both groups. For the Amen group, percentage body fat explained 65.7% of the total variability in leptin concentration. Log insulin concentration explained an additional 5.6%, and log glycerol concentration an additional 6.1% of the total variability observed in circulating leptin concentration. In contrast to the Amen group, percentage body fat was the only variable that significantly predicted log leptin concentration in the OV group, accounting for 52.2% of the total variability.
Possible Confounding Variables

There are a number of variables that were associated with, and may have confounded, the interpretation of the relationships with log leptin concentration as described above. For example, percentage body fat was significantly correlated with RER ($r = -0.353, P = 0.007$), peak VO$_2$ ($r = -0.404, P = 0.007$), and REE per kg FFM ($r = 0.362, P = 0.006$). Weekly exercise minutes were significantly correlated to dietary carbohydrate intake (grams) ($r = 0.528, P < 0.001$) and log insulin ($r = -0.372, P = 0.013$). Lastly, peak VO$_2$ was significantly correlated to weekly exercise minutes ($r = 0.514, P < 0.001$).
Resumption of Menses

Table 4: Descriptive data for exercising amenorrheic women who resumed menses (Amen-R) and those who did not resume menses (Amen-NR) during the 6-month study.

<table>
<thead>
<tr>
<th></th>
<th>Amen-R (n=5)</th>
<th>Amen-NR (n=7)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>24.6±1.9</td>
<td>-</td>
<td>21.4±1.2</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.7±1.2</td>
<td>-</td>
<td>168.6±2.4</td>
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<tr>
<td>Weight (kg)</td>
<td>55.5±1.3</td>
<td>57.0±1.1</td>
<td>54.8±3.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.2±0.3</td>
<td>21.8±0.3</td>
<td>19.3±0.9</td>
</tr>
<tr>
<td>Body Composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>24.0±1.9</td>
<td>24.7±2.0</td>
<td>17.3±1.8</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>13.4±2.6</td>
<td>14.0±1.2</td>
<td>9.4±1.2</td>
</tr>
<tr>
<td>Fat Free Mass (kg)</td>
<td>42.7±1.6</td>
<td>43.1±1.7</td>
<td>44.9±2.4</td>
</tr>
<tr>
<td>Menstrual History</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Age of Menarche (yr)</td>
<td>13.8±0.6</td>
<td>-</td>
<td>13.9±0.7</td>
</tr>
<tr>
<td>Gynecological Age (yr)</td>
<td>10.8±2.4</td>
<td>-</td>
<td>7.4±1.3</td>
</tr>
<tr>
<td>Training Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise Activity (min/wk)</td>
<td>400±182</td>
<td>390±149</td>
<td>380±98</td>
</tr>
<tr>
<td>VO₂ peak (ml/kg/min)*</td>
<td>55.1±3.5</td>
<td>-</td>
<td>50.9±3.0</td>
</tr>
<tr>
<td>Exercise History (min/wk)</td>
<td>658±224</td>
<td>-</td>
<td>466±82</td>
</tr>
<tr>
<td>Serum Hormones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>6.5±2.8</td>
<td>11.0±2.9</td>
<td>2.3±0.7</td>
</tr>
<tr>
<td>Glycerol (mg/dl)</td>
<td>3.7±1.0</td>
<td>3.2±0.5</td>
<td>4.4±0.7</td>
</tr>
<tr>
<td>Metabolic Variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RER</td>
<td>0.84±0.01</td>
<td>0.90±0.03</td>
<td>0.87±0.02</td>
</tr>
<tr>
<td>REE (kcal/kgFFM/day)</td>
<td>29.5±1.4</td>
<td>31.3±1.0</td>
<td>26.2±0.9</td>
</tr>
<tr>
<td>Nutritional Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary Intake (kcal/day)</td>
<td>2530±774</td>
<td>2585±49</td>
<td>2046±157</td>
</tr>
<tr>
<td>CHO Intake (g/day)</td>
<td>379±126</td>
<td>375±95</td>
<td>299±18</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

Time = main effect of time, Group = main effect of group, X = interaction effect

BMI – body mass index

VO₂ peak – peak oxygen consumption

RER – respiratory exchange ratio

REE – resting energy expenditure

CHO – carbohydrate
Figure 2. Change in fasting serum leptin concentration vs. change in RER from pre- to post-study for exercising women with amenorrhea who resumed menses during the study (Amen-R, filled circles) and those who did not resume menses (Amen-NR, unfilled circles). \( r = 0.584, P = 0.046 \).

Descriptive variables between Amen-R and Amen-NR can be seen in Table 4. As assessed by repeated-measures ANOVA, the increase in fasting serum leptin concentration observed in the Amen-R group from pre- to post-study was significantly greater than in the Amen-NR group (Interaction effect: \( P = 0.002 \), Time effect: \( P = 0.001 \), Group effect: \( P = 0.032 \)), demonstrating that an increase in serum leptin concentration was associated with the resumption of menses in amenorrheic women (Study 2).

Similarly, a significant increase in RER was observed in the Amen-R group from pre- to post-study, but not in the Amen-NR group (Interaction effect: \( P = 0.006 \), Time effect: \( P = 0.534 \), Group effect: \( P = 0.506 \)). As seen in Figure 2, the change in RER was significantly correlated with the change in leptin concentration during the study when Amen-R and
Amen-NR were considered together ($r=0.584, P=0.046$). This demonstrates that an increase in carbohydrate utilization was observed in association with the resumption of menses. A significant increase in percentage body fat was observed over time ($P = 0.036$), and a significant group effect was likewise observed ($P = 0.049$), yet the Amen-R group did not exhibit a greater increase in body fatness than the Amen-NR group ($P = 0.406$). This demonstrates that an increase in adiposity was not associated with the resumption of menses, and therefore did not account for the increase in serum leptin concentration. Changes in resting energy expenditure were not observed from pre- to post-study ($P = 0.102$), and were likewise not associated with the resumption of menses ($P = 0.489$). However, a significant group effect was observed ($P = 0.010$), such that the Amen-R women exhibited an elevated REE in comparison to the Amen-NR women. Glycerol concentration, weekly exercise minutes, dietary energy intake and diet composition did not significantly differ between groups or significantly change over the 6-month study.

**DISCUSSION**

The first major finding of this study was the differential regulation of leptin production in women with and without hypothalamic amenorrhea. It has consistently been demonstrated that women with hypothalamic amenorrhea exhibit leptin concentrations that are lower than those observed in regularly cycling women (9-13), yet the difference is largely attributed to differences in adiposity (9, 13). Although percentage body fat was the primary predictor of log leptin concentration in both groups of women, and the only significant predictor in regularly cycling women, log insulin concentration and log glycerol concentration were also significant predictors of log leptin concentration.
in women with amenorrhea. This demonstrates that factors other than adiposity are influencing leptin production and may play a role in the etiology of FHA.

Our findings suggest that increases in insulin concentration are associated with increases in leptin production in amenorrheic women. Insulin stimulates leptin synthesis (40, 42), which supports our observations. Similarly, circulating insulin concentrations are often lower in exercising women with FHA (43), which may contribute to the lower leptin concentrations observed in these women. Our data supports the above mentioned theory and demonstrate that amenorrheic women who exhibit low 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 (10, 11). Laughlin and Yen (8) observed hypoleptinemia in amenorrheic women in association with hypoinsulinemia, which further corroborates our findings.

Log glycerol concentration was found to be positively associated with log leptin concentration in women with hypothalamic amenorrhea. Circulating glycerol is a marker of lipolysis. Lipolysis is primarily stimulated by sympathetic stimulation of adipose tissue (44), which has been shown to inhibit leptin synthesis via activation of beta-adrenergic receptors (27, 45, 46). 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, elevated lipolysis and presumably increased fat utilization could spare glucose and thus preserve carbohydrate availability. Carbohydrate availability is positively correlated with leptin production (19, 26), and may therefore explain why we observed increased leptin concentrations in association with elevated
glycerol levels. We also measured serum glycerol concentration, which reflects total body lipolysis rather than adipose-specific lipolysis (47).

Diet and training status also influence fuel utilization at rest, and may therefore influence circulating glycerol concentration (48). A decrease in fat utilization, and presumably a decrease in serum glycerol concentration, has been observed in association with endurance training (48). Since exercise training is often reported to reduce circulating leptin concentrations in women (20, 49), it is possible that we are observing increases in leptin concentration in association with elevated glycerol concentration secondary to a lower training status. Although we tried to control for training status by including weekly exercise minutes in our model, the data were self-reported. Therefore, it seems possible that we observed the inhibitory effect of training on circulating leptin concentration via the effect of training on fat utilization.

Glycerol concentration and percentage body fat did not significantly change in the amenorrheic women who resumed menses during the 6-month monitoring period, suggesting other factors were contributing to the increases in leptin concentration observed in these women. The only factor that significantly changed over time in the amenorrheic women who resumed menses was resting RER. An increase in resting RER, which indicates an increase in carbohydrate-derived energy production, was likewise the only factor that increased in association with leptin. The increase in carbohydrate utilization could reflect an increase in carbohydrate availability or a decrease in fatty acid availability. As mentioned earlier, increased carbohydrate availability has a stimulatory effect on leptin production (19, 22). If the increase in RER reflects increased carbohydrate availability, it would explain why it was observed in association with
increases in leptin concentration. However, we did not observe an increase in carbohydrate intake or changes in exercise volume to account for an increase in carbohydrate availability. It is likewise possible that the increase in RER reflects a decrease in sympathetic activation of adipose tissue, and a subsequent decrease in lipolysis and fatty acid availability (50). Since sympathetic stimulation of adipose tissue inhibits leptin synthesis (51), it is possible that the increases in leptin observed in the exercising amenorrheic women who resumed menses may be due to a decrease in the sympathetic inhibition of leptin synthesis (52). The decrease in sympathetic activity could reflect an improved metabolic state (53) and/or a decrease in other stressors known to activate the sympathetic axis, such as exercise stress (47), mental stress (54) or emotional stress (55).

The interpretation of our data concerning sympathetic nervous activity is constrained by the indirectness of our measurements. Unfortunately, we cannot better determine whether sympathetic nervous activation of adipose tissue was present using data from the current study. A future study that directly assesses sympathetic activation of adipose tissue and its effect on leptin concentration is needed in women with FHA. The findings reported by Bartak et al (47), who used microdialysis to demonstrate an increase in sympathetic stimulation of adipose tissue in anorexic women at rest and during exercise, are encouraging that similar findings may be observed in exercising women with amenorrhea.

Our data demonstrate that leptin production is differentially regulated in exercising women with and without hypothalamic amenorrhea, and supports the theory that modulators of leptin production may play a role in the etiology of hypothalamic
amenorrhea. Although percentage body fat remains the primary regulator of leptin production, it appears that other modulators known to acutely influence leptin production, such as insulin concentration and sympathetic nervous activity, may be exerting a chronic impact on leptin production in amenorrheic women.
REFERENCES


34. Barber, T.M., et al., Metabolic characteristics of women with polycystic ovaries and oligo-amenorrhoea but normal androgen levels: implications for the


CHAPTER VI

CONCLUSIONS

Leptin, the hormone product of the obesity (ob) gene, is primarily regulated by adiposity and acute energy status (16, 116), and is directly involved with the regulation of energy homeostasis (54). Leptin has been implicated as a key signal allowing for the integrated control of nutritional status and reproductive function, explaining why abnormal hypothalamic-pituitary-gonadal (HPG) function is observed in association with low adiposity or a relative energy deficiency (15, 23). However, leptin synthesis is likewise influenced by factors other than adiposity and acute energy status (65, 70, 105, 110). To date, most of the studies examining the role of leptin in the etiology of functional hypothalamic amenorrhea (FHA) have only considered how it functions to relay nutritional status from the periphery to the hypothalamus (20, 21, 24, 55). It is therefore difficult to determine whether factors other than adiposity that suppress leptin production, such as sympathetic nervous activity, contribute to reproductive suppression in women with FHA. The primary goals of these papers were to determine the role of leptin in the regulation of reproductive function in women with FHA who exhibit a wide range of body fatness, and to identify whether factors independent of adiposity are acting to suppress leptin production in women with FHA.

The first study was designed to identify the mechanism whereby leptin is sensed in the hypothalamus to suppress the reproductive system in women with FHA. Although mean fasting serum leptin concentration was lower in women with FHA than in women with ovulatory menstrual cycles, there does not appear to be a critical concentration of leptin accounting for the suppression of the HPG axis in these women because they
exhibited a wide range of leptin concentrations. However, the lowest leptin concentration observed in exercising women with ovulatory cycles was 2.57ng/ml, and many exercising amenorrheic women exhibited leptin concentrations that were lower than 2.57ng/ml. Even though many exercising amenorrheic women exhibited leptin concentrations that fell within the range observed in exercising ovulating women, it is nevertheless possible that 2.57ng/ml represents a critical concentration of leptin needed for reproductive competency. For the exercising amenorrheic women who exhibited serum leptin concentrations that were above 2.57ng/ml, low leptin per se does not appear to be the cause for the reproductive suppression observed in these women. It also does not appear that hypoleptinemia, defined as having a leptin concentration that is lower than can be accounted for by adiposity alone, is suppressing the reproductive system in exercising women with amenorrhea. After adjusting for adiposity, the difference in leptin concentration observed between exercising women with and without amenorrhea was no longer present. Therefore, it appears that relative changes in leptin concentration are also important.

The next study was designed to explore whether the resumption of menses in exercising women with amenorrhea is associated with increases in serum leptin concentration. Although the women exhibited a wide range of leptin concentrations before and after they resumed menses, they all experienced a relative increase in leptin concentration. The increase in leptin, however, was not paralleled by an increase in adiposity, which demonstrates that other modulators of leptin production are contributing to the increase in leptin concentration observed in exercising amenorrheic women with the resumption of menses. The findings from this study support our interpretations from
Study 1 that relative changes in serum leptin concentration are important. However, at the time of resumption, all of the exercising amenorrheic women who resumed menses exhibited leptin concentrations that fell within the range observed in exercising ovulatory women. The lowest leptin concentration observed in exercising ovulatory women, as mentioned above, was 2.57ng/ml. All of the exercising amenorrheic women who resumed menses exhibited leptin concentrations that were greater than 2.57ng/ml at the time they resumed menses. One exercising amenorrheic subject who resumed menses had a baseline leptin concentration of 2.04ng/ml, which was lower than the proposed threshold concentration of 2.57ng/ml, and a leptin concentration of 2.81ng/ml at the time of resumption. Although there was not a large increase in leptin concentration from baseline to the time of resumption of menses observed in this subject, the increase was sufficient in the sense that serum leptin concentration went from being below the proposed threshold concentration of leptin needed for reproductive competency to above it. Therefore, this study demonstrated the importance of relative changes in serum leptin concentration, while also supporting the finding from Study 1 that a leptin concentration of 2.57ng/ml may represent a threshold of leptin, below which reproductive function becomes suppressed.

The last study was designed to identify whether other modulators of leptin production, such as sympathetic nervous activity, might be contributing to the variability observed in circulating leptin concentration that is not accounted for by adiposity in exercising women with amenorrhea and those with ovulatory menstrual cycles. In exercising ovulatory women, percentage body fat was the only significant predictor of leptin concentration. However, in exercising women with amenorrhea, serum insulin and
glycerol concentrations were positively associated with leptin concentration. Hypoinsulinemia is characteristic of metabolic stress, and has been observed in exercising women with FHA secondary to a relative energy deficiency (87). Insulin stimulates leptin synthesis (106, 107), explaining why lower leptin concentrations were found in association with lower insulin concentrations in exercising women with amenorrhea. Elevated glycerol concentration is a good indicator of elevated lipolysis (80), which in turn is often found in association with elevated sympathetic nervous activation of adipose tissue (68, 73, 78, 96). Therefore, since leptin synthesis is inhibited by sympathetic activity (72), one would reason that elevated glycerol concentrations should be found in association with lower leptin concentrations. A possible explanation for the positive association we observed between glycerol and leptin concentrations in exercising women with amenorrhea could be that elevated lipolysis, and presumably elevated fatty acid utilization, would spare blood glucose. Increased glucose availability and carbohydrate intake are positively associated with leptin production (84, 116), which would explain why elevated lipolysis may be associated with increases in leptin concentration. However, resting RER, which reflects total body substrate utilization at rest, was not a significant predictor of log leptin concentration in exercising women with amenorrhea. Regardless, the findings from this study demonstrate that leptin production is differentially regulated in exercising women with and without FHA, and suggests that factors other than adiposity can influence the reproductive axis through their impact on leptin production.

The increase in leptin concentration observed in exercising women with FHA in association with the resumption of menses was not associated with changes in adiposity,
but rather with an increase in resting RER. The exercising women with amenorrhea who resumed menses during the study exhibited a significantly greater increase in both leptin and RER from baseline to the time they resumed menses in comparison to the exercising amenorrheic women who did not resume menses during the study. The change in leptin concentration was correlated to the change in resting RER in both the exercising amenorrheic women who resumed and those who did not resume from pre- to post-study. An increase in resting RER reflects increased carbohydrate-derived energy production, which in turn may reflect increased carbohydrate availability and/or decreased fatty acid availability. Carbohydrate availability is positively associated with leptin production (55, 84), as mentioned earlier, which supports our findings. Similarly, decreased fatty acid availability could be due to decreased sympathetic-stimulated lipolysis and subsequent fatty acid release (96), which would similarly support our findings because sympathetic activation of adipose tissue inhibits leptin synthesis (19, 106). Although serum glycerol concentration also reflects lipolysis and sympathetic activation of adipose tissue (70, 78), we did not observe a significant change in glycerol concentration in association with the changes observed in leptin concentration. Serum glycerol concentration reflects total body lipolysis, rather than lipolysis stimulated by sympathetic activation of adipose tissue (80), which may explain why we did not observe a significant association between serum glycerol and leptin concentration. However, modulators of leptin production, such as sympathetic nervous activation of adipose tissue, may contribute to the increase in leptin concentration observed in association with the resumption of menses in exercising women with amenorrhea.
Our findings and interpretations are limited due to the study design and because many of our measurements were indirect or self-reported. For example, we only measured fasting serum leptin concentration and were thus unable to observe the diurnal secretion pattern of leptin. Similarly, due to the long intervals between blood sampling, we were not able to capture the serum leptin concentrations that coincided with the exact day of menses in our FHA subjects who resumed. We were also limited by subject compliance for many of our self-reported outcome variables, such as dietary measures and weekly exercise minutes, as well as by adherence of the EAMD+Calories group to the increased calorie diet. However, we had good compliance with urine collection and with REE and blood draw appointments, and with other study visits. Lastly, we were limited by sample size when addressing the association between changes in leptin and other variables with the resumption of menses.

Taken together, it appears that the reproductive system senses and responds to relative changes in leptin concentration rather than to a critical concentration of leptin. Changes in leptin concentration may not always reflect changes in adiposity, and in such cases may be due to the influence of other modulators of leptin synthesis. Elevated sympathetic nervous activity, for example, due to psychosocial stress (89), metabolic stress (67), or exercise stress (80) may contribute to the suppression of leptin production in these women, and may therefore play a role in the development of FHA.
FUTURE STUDIES

Our previous findings support the theory that leptin production is modulated by factors other than adiposity in women with FHA, and suggest that these factors may contribute to the etiology of FHA. However, our interpretations of the precise mechanisms whereby these factors regulate leptin production are speculative because we were not able to directly measure their impacts on leptin production. Thus, future studies directly assessing whether these modulators of leptin production play a role in FHA would have practical implications and would perhaps shed light on the mechanism whereby stress down-regulates the HPG axis. It would interesting to corroborate the findings of Bartak et al (80), for example, who demonstrated elevated sympathetic activation of adipose tissue at rest and during exercise in anorexic women using microdialysis. If sympathetic activation of adipose tissue is also present in women with FHA, it would likely be contributing to the suppression of leptin production, which could be determined by administration of a beta blocker. If sympathetic inhibition of leptin production is present in women with FHA, then there would be strong support for the hypothesis that factors independent from energy status can influence the HPG axis.
REFERENCES


APPENDIX

MAP OF R-DRIVE DATA FILES

R-Drive > “Williams” Folder > “Maggie” Folder > “Thesis” Folder

Thesis SPSS Database

Workbooks of Statistical Analyses
  Study 1 Workbook
  Study 2 Workbook
  Study 3 Workbook

Excel Data File (Pre, Post and Change)
  Subject IDs
  All Figures
  Leptin Data
  Glycerol Data
  RER Data
  Percentage Body Fat Data
  E1G and PdG Data

Compiled Thesis
  Word Document
  PDF

Final Thesis Manuscripts
  Study 1
  Study 2
  Study 3

Thesis Endnote Library

Thesis Defense Presentation