Acute Recapitulation of the Hyperinsulinemia and Hyperlipidemia Characteristic of Metabolic Syndrome Suppresses Gonadotropins

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Objective: To determine the effect of lipid/heparin versus saline infusion, with or without concurrent euglycemic hyperinsulinemia, on serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Obesity is associated with hyperlipidemia, insulin resistance, and relative hypogonadotropic hypogonadism. It was hypothesized that acutely elevated fatty acids and insulin would impair gonadotropin secretion.

Methods: Regularly cycling women and men without obesity underwent a crossover 6-hour infusion study over four visits. Participants received infusions of saline-control, lipid/heparin, insulin, and lipid/heparin plus insulin. Serum FSH and LH were measured by immunoassay.

Results: In women (n = 10), infusion of lipid plus insulin significantly reduced LH, from 4.6 IU/L (3.7-5.4) (mean [95% confidence interval]) to 3.3 IU/L (2.3-4.4); P = 0.03, and FSH, from 3.9 IU/L (3.2-4.6) to 3.1 IU/L (2.3-3.8); P = 0.03, compared to saline-control. Similarly, in men (n = 10), LH, 3.3 IU/L (2.4-4.1), and FSH, 2.1 IU/L (1.4-2.8), were significantly reduced after the combined infusion (2.2 [1.3-3.1] IU/L and 1.5 [0.8-2.1] IU/L; P = 0.03, P = 0.02, respectively). Neither lipid nor insulin alone significantly impacted gonadotropin levels compared to saline-control.

Conclusions: Hyperinsulinemia combined with elevated lipids acutely suppresses LH and FSH, providing a possible mechanism underlying the relative hypogonadotropic hypogonadism of obesity. Effects of insulin on the hypothalamic-pituitary-gonadal axis may be dependent on the concomitant metabolic environment.

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Introduction

Obesity is associated with high circulating free fatty acids and triglycerides (TGs), insulin resistance, and impaired secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Obesity can lead to metabolic syndrome, in which an individual has a clustering of three or more of the following: hypertension, low high-density lipoprotein, hypertriglyceridemia, high fasting glucose, and abdominal obesity. Collectively, obesity has been implicated in reproductive dysfunction and adverse pregnancy outcomes (1-4). Thus, it is important to understand the mechanisms by which obesity impacts the reproductive axis.

We have previously shown that pituitary LH and FSH secretion is impaired in women with obesity (5). Subjects with obesity had a reduction in mean serum LH and FSH and in LH pulse amplitude compared to women of normal weight included as study controls, and they had a blunted pituitary response to exogenous gonadotropin releasing hormone (6). These data imply that metabolic changes due to obesity result in a functional impairment of the hypothalamic-pituitary-ovarian axis at the level of the pituitary, impacting LH and FSH synthesis and/or secretion (6-11).

Numerous studies have implicated insulin and free fatty acids in the regulation of LH and FSH synthesis and secretion. However, results have often been contradictory, and interpretations are frequently made in the context of polycystic ovarian syndrome (PCOS) and/or frank type 2 diabetes. Elevated insulin levels have been shown to suppress LH in sheep (12) but appeared to have no effect in rhesus monkeys (13). It is, however, difficult to separate effects of insulin from those of hypoglycemia in animal models in which insulin is...
infused. In hyperinsulinemic euglycemic clamp (HEC) studies of women, circulating LH levels were slightly reduced in women undergoing insulin infusion, as well as those with worsening insulin resistance (14). In vitro studies are no more consistent. Insulin was found to stimulate LH and FSH release from cultured pituitary cells (15), while free fatty acid administration induced LHβ mRNA but suppressed that of FSHβ (16). The latter study did not examine hormone release. To date, studies have not addressed the combinatorial effects of both insulin and TGs/free fatty acids on gonadotropins under euglycemic conditions in metabolically healthy human subjects.

We hypothesized that the reproductive milieu of women with obesity includes both insulin resistance and dyslipidemia, and that both factors together might be responsible for the pituitary impairment we have observed in such women. We further hypothesized that if our notion were correct, we should be able to reproduce a similar reproductive defect in individuals characterized as healthy and nonobese by induction of insulin resistance and hyperlipidemia. To test this hypothesis, we investigated serum LH and FSH profiles in women and men without obesity, during a lipid or saline infusion in the presence or absence of hyperinsulinemia with euglycemia using a HEC. We measured LH and FSH across the infusions to determine whether a short-term mimicking of the metabolic changes of interest (elevated lipids and hyperinsulinemia) impacted the hypothalamic-pituitary-gonadal (HPG) axis, inducing the impaired gonadotropin secretion that is characteristically observed in obesity.

Methods
Participants
This study was performed as a secondary analysis of an ongoing study. Parent study recruitment consisted of men and women without obesity (BMI 18-28 kg/m²), in good health, aged 18-40 years old, and reporting sedentary to moderately active lifestyles (vigorous exercise no more than three times per week). History, physical exam, and laboratory testing confirmed good health status, defined as absence of exclusionary comorbid conditions including hypertension, pregnancy, impaired glucose tolerance (75 g oral glucose tolerance test with 2-hour glucose ≥140 mg/dl), impaired fasting glucose (≥100 mg/dl), overt diabetes, TG >250 mg/dl, liver or kidney disease, pulmonary disease, chronic inflammatory conditions, coagulopathy, anemia, abnormal cardiac function, or evidence of ischemic heart disease. Additional exclusions included use of medications known to impact insulin production or sensitivity, the presence of soy or egg allergies (due to possible reaction to the lipid infusate), any type of tobacco use, and use of any medications or supplements that would impact reproductive hormones, including systemic hormonal contraception. All women were premenopausal, with a history of regular menstrual cycles, and underwent study only during the follicular phase of the menstrual cycle. Male participants were without reproductive complaints and not taking reproductive medications. Detailed sexual function information and genital examinations were not performed. All subjects refrained from any exercise for 3 days prior to study visits.

Study design
This was a secondary analysis of a subset of samples from a parent study designed to examine the impact of acute fatty acid elevation and the resulting insulin resistance on exercise performance parameters in adults characterized as healthy and nonobese (17,18). Participants underwent a maximum of five study visits: a screening visit with a dual-energy x-ray absorptiometry (DXA) scan to determine body composition and a series of four visits, in random order, for infusion of saline-control or lipid/heparin, each in the presence or absence of a HEC (Figure 1). Heparin was coadministered with lipid infusion to enhance liberation of free fatty acids. Visits were at least 1 week apart and, for women, restricted to days 5 through 10 of the follicular phase of the menstrual cycle. Male participants were without reproductive complaints and not taking reproductive medications. Detailed sexual function information and genital examinations were not performed. All subjects refrained from any exercise for 3 days prior to study visits.
required to abstain from moderate to vigorous exercise. Subjects were provided with breakfast at the time of infusion start. Breakfast composition was adjusted for the saline and lipid infusion visits such that cumulative morning caloric intake was constant across all visits. Blood samples, for measurement of hormones, were obtained at regular intervals, beginning immediately prior to the start of each 6-hour infusion (Figure 1). Not all participants completed the full protocol. The study was approved by the University of Colorado Institutional Review Board, and informed consent was obtained from all participants.

**Lipid/heparin infusion**
Participants underwent saline-control or lipid/heparin infusions (Liposyn® II or Intralipid®; 20% lipid emulsion at 45 cc/h, heparin at 0.4 U/kg/min), in the presence or absence of a HEC, conducted at four separate visits, for 6 hours commencing at approximately 6 AM. The study was initiated with Liposyn II (Abbott Laboratories, North Chicago, IL; 10% safflower oil, 10% soybean oil, 1.2% egg phosphatides, and 2.5% glycercin; major component fatty acids: approximately 65.8% linoleic, 17.7% oleic, 8.8% palmitic, 3.4% stearic, and 4.2% linolenic acid), but due to a product recall, some participants received Intralipid (Baxter Healthcare Corporation, Deerfield, IL; 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycercin; major component fatty acids: linoleic [44%-62%], oleic [19%-30%], palmitic [7%-14%], linolenic [4%-11%], and stearic [1.4%-5.5%]) instead. Liposyn and Intralipid are reported to induce similar degrees of insulin resistance (20,21), and this was confirmed within this study (see Results).

**HEC studies**
HECs were performed during the final 3 hours of a 6-hour saline or lipid/heparin infusion in the late morning. Clamp visits included a single-stage (40 mU/m²/min) insulin infusion performed as previously reported (22,23). Insulin sensitivity is reported as glucose infusion rate (average space-corrected glucose infusion rate in mg glucose/kg lean body mass/min over the last 30 minutes).

**Caloric estimates**
Daily caloric needs for the prescribed diet were determined from fat free mass (FFM; determined by DXA, as described previously (24)) and activity using the equation Total Energy = (FFM × 23.9) + 372 × activity factor, where FFM = lean mass + bone mineral content in kilograms. An activity factor of 1.4-1.6 was used for this largely sedentary population (25).

**Hormone assays**
Serum FSH and LH were measured using specific, solid-phase immunofluorometric assays (DELFIA®, Perkin Elmer, Turku, Finland) as previously described (24,26). Inter-assay and intra-assay coefficients of variation (CVs) were 6.3% and 4.2%, respectively, for FSH and 4.7% and 5.4%, respectively, for LH. Estradiol and sex hormone binding globulin (SHBG) were measured by immunoassay (ADVIA Centaur® XP, Siemens, Malvern, PA). Inter-assay and intra-assay CVs were 10.6% and 10.6% for estradiol. SHBG was measured using a single kit; therefore, there is no inter-assay CV to report. Intra-assay CV for SHBG was 3%. Testosterone was measured by Access testosterone assay (Beckman Coulter, Brea, CA); intra- and inter-assay CVs were 2.1% and 5.1%, respectively.

**Metabolic assays**
Serum insulin, hemoglobin A1c (HbA1c), glucose, nonesterified fatty acids (NEFA), and TGs were determined by the University of Colorado CTRC laboratory, as described previously (27).

**Statistical analysis**
The last two observations per lipid-only, insulin-only, and the combined infusion were assumed to represent steady state for each condition. These were averaged to yield one observation per condition per person; for the saline condition, all samples were averaged for one observation per condition as steady state would be reached immediately. A second analysis using identical timing of end point assessments revealed the same findings (not shown). For each outcome (TGs, estradiol, testosterone, SHBG, NEFA, FSH, LH), a linear mixed-effects regression model was estimated, parameterized with gender, condition, and the interaction between gender and condition included as fixed effects, a random intercept, and an unstructured covariance. For estradiol and testosterone, models included only condition as a fixed effect, as only males contributed to the model of testosterone and females to the model of estradiol. The use of mixed-effects regression allows for incorporating repeated observations from all conditions per participant into one model for pairwise testing, while adjusting the variance for repeated measures and incorporating available data from participants who did not complete the protocol. Values are expressed in text and plotted as means (95% confidence intervals). TGs, estradiol, and NEFA were analyzed on the log scale and presented as geometric means and 95% confidence intervals. P values ≤ 0.05 were considered statistically significant. No adjustments were made for multiple comparisons (28).

**Results**

**Participation in each arm of the study**
Ten males and ten females participated in these studies and were used for the analysis. Among the women, four completed all four visits, three completed three visits, two completed two visits, and one woman completed just one visit. Among the men, six completed all four visits, two completed three visits, one completed two visits, and one completed only one visit. Because of the random visit order, this resulted in a per-condition sample size of saline-control (nine men and eight women), lipid alone (seven men and nine women), insulin alone (nine men and eight women), or lipid plus insulin (eight men and five women).

As shown in Table 1, male and female participants were of a similar age (women 32.5 [28.2-36.8] years and men 31.4 [24.3-33.3] years) and of nonobese mean BMI. There was no evidence of insulin resistance based on mean fasting insulin levels, fasting glucose, or glucose intolerance. HbA1c and body fat were also within normal ranges.

**Hyperlipidemia and insulin resistance**
In response to lipid infusion, NEFAs were increased, in both males and females, to the high physiologic levels frequently observed in postprandial metabolic syndrome patients (29). This was in sharp contrast to the NEFA levels seen with saline infusion (Figure 2). As expected, insulin reduced NEFA levels. However, NEFA levels in
the infusion of lipid plus insulin remained significantly higher than those during saline-control. The circulating TG response to the various infusions was similar for men and women. During saline-control infusion, mean TGs in men (130 [92-183] mg/dl) and women (121 [85-174] mg/dl) decreased to 57.4 mg/dl (41-80) and 99 mg/dl (69-143), respectively, with concomitant insulin infusion. During lipid infusion, TG levels increased to 227 mg/dl (161-321) and 105 mg/dl (74-149) and were 239 mg/dl (167-343) and 87 mg/dl (53-141) mg/dl with concomitant insulin infusion (to convert mg/dl to mmol/L, multiply by 0.01129).

As shown in Figure 3, both sexes exhibited a significant decrease in their glucose infusion rate during the euglycemic clamp with lipid infusion, indicating development of acute insulin resistance (23,27). Taken together, these results indicate that the elevated NEFA levels, elevated insulin levels, and insulin resistance characteristic of metabolic syndrome were achieved in both male and female subjects.

To evaluate whether the use of Liposyn or Intralipid had different effects on insulin resistance, the value of delta-glucose infusion rate, a measure of the change in insulin sensitivity, was calculated for each treatment. For Liposyn, delta-glucose infusion rate was 2.73 mg/kg/min (1.79-3.67), and for Intralipid it was 2.83 mg/kg/min (1.06-4.60), confirming no significant difference (P = 0.9) between these two treatments in the overall cohort.

Gonadotropin response to insulin and lipid infusion

Figure 4 illustrates the effect of each infusion on serum LH levels. In women, compared to saline-control LH (4.57 [3.69-5.45] IU/L), the infusion of lipid (4.55 [3.70-5.40] IU/L) or insulin alone (3.94 [3.06-4.82] IU/L) had no effect on LH levels. However, the combination of lipid plus insulin (3.33 [2.28-4.38] IU/L) significantly decreased LH levels compared to both lipid infusion (P = 0.028) and saline-control (P = 0.031) (Figure 4A). Among males, LH did not differ from saline-control (3.27 [2.43-4.12] IU/L) in response to either lipid infusion (2.52 [1.60-3.44] IU/L) or insulin infusion (3.00 [2.16-3.84] IU/L). In contrast, as seen in the women, the combination of lipid plus insulin significantly decreased LH (2.18 [1.30-3.06] IU/L, P = 0.02) compared to saline-control (Figure 4B).

A similar pattern was observed for FSH (Figure 5), where mean saline-control FSH levels (3.88 [3.20-4.56] IU/L) in women did not differ with infusion of lipid (4.22 [3.56-4.88] IU/L) or insulin (4.48 [3.80-5.16] IU/L) (Figure 5A). However, infusion of lipid plus insulin significantly lowered FSH (3.06 [2.29-3.83] IU/L) compared to saline-control (P = 0.025), lipid alone (P = 0.002), or insulin alone (P < 0.001). In men (Figure 5B), serum FSH did not differ in response to infusion of lipid (1.69 [0.99-2.40] IU/L) or insulin (1.94 [1.28-2.60] IU/L) compared to saline-control (2.10 [1.44-2.76] IU/L). As in the women, the combination of lipid plus insulin significantly decreased FSH (1.47 [0.79-2.15] IU/L, P = 0.03) in males compared to saline-control.

Thus, while neither agent alone significantly affected serum gonadotropins, the combination of elevated insulin and lipids, in the context of acute insulin resistance, significantly reduced LH and FSH levels in both men and women.
Sex steroids and SHBG response to insulin and lipid infusion

We further examined sex steroid and SHBG responses to these short-term infusions to see whether they explained any of our findings with respect to LH and FSH. As shown in Figure 6, for women, estradiol was not found to differ between the three conditions. SHBG also did not differ across all infusions, suggesting unchanged bioavailability of estradiol (Figure 6). All males studied had a testosterone level within the normal range (177-548 ng/dl) in saline-control (manufacturer’s 95% reference interval: 175-781 ng/dl) and 151-722 ng/dl across all groups. In males, mean testosterone was significantly and equally increased, after lipid or lipid plus insulin infusion, compared to saline-control or insulin alone (Figure 6). Similar to the women, SHBG levels in the men were not changed by any of the infusions, implying that the observed differences in testosterone were not directly attributable to differential SHBG binding or bioavailability (Figure 6).

Discussion

We demonstrate herein that the combination of acutely increased circulating lipid plus insulin, under euglycemic conditions, causes a reduction in serum LH and FSH in healthy men and women without obesity. The combinatorial effect of the infusion of lipid plus insulin induced a 20% or greater reduction in LH secretion in both women and men, relative to saline-control. A similar pattern was observed with the administration of lipid plus insulin on FSH levels. Thus, the acute manipulation of the metabolic milieu created by lipid plus insulin significantly impaired net pituitary gonadotropin secretion in both sexes. Alternatively, gonadotropin clearance could have been increased by the intervention. However, we have previously shown that endogenous LH clearance does not differ by body size among normally cycling women, and others have reported a prolonged half-life of endogenous LH in women of obese BMI with PCOS (26,30). These findings provide support for the notion that the metabolic challenges of obesity...
translate into circulating factors that adversely affect pituitary function, and they support our previous findings that obesity-related reproductive function is, at least in part, linked to pituitary dysfunction (6).

Effects of hyperinsulinemia on the pituitary gland are unclear, in part because it is not known whether or not the pituitary gland is capable of acquiring the characteristic insulin resistance observed in the liver and muscle of individuals with obesity. Studies using the...
pituitary insulin receptor knockout mouse have indicated that the pituitary likely remains insulin sensitive in the face of obesity and its metabolic challenges (31). It is tempting to speculate that excess insulin action in the pituitary, when exposed to increased free fatty acids, causes their rapid incorporation into the cell, thereby inducing a lipotoxic endoplasmic reticulum stress and induction of the unfolded protein response (32) and pausing gonadotropin gene translation.

Although our sample size is too small to draw firm conclusions, these findings suggest a possible sexual dimorphism in HPG axis function between women and men when exposed to a high-fat environment. Lipid infusion was associated with a short-term and small increase in testosterone in men and a trend for a small decrease in estradiol in women. It is possible that the reduction in gonadotropins in males undergoing lipid plus insulin infusion may have been a result of negative feedback of this small increase in testosterone. However, all testosterone levels remained within the normal range. Nonetheless, the short-term effect of this infusion did not recapitulate hypogonadism in men or women, as neither testosterone nor estradiol was acutely lowered. An effect of lipid or insulin on testosterone would not be expected to be observed in women, because of the overriding effect of estradiol on the female hypothalamic-pituitary axis and the much lower circulating testosterone levels. However, reduction of androgen activity in women with flutamide has been shown to improve the lipid profile in women with PCOS (33). Regardless, the high, supraphysiologic concentrations of insulin and lipid provided in this study are far more likely than the modest elevation of testosterone to have accounted for the negative feedback we observed in men. Unsurprisingly, SHBG, which has a long serum half-life, was unaltered in concentration over the course of these short-term infusions.

Strengths of our study include the carefully controlled conditions of each infusion, with excellent achievement and stability of elevated insulin and NEFA levels during each infusion. The repeated nature of our study design, with the same subjects participating in up to four treatment arms, is also a strength. Weaknesses of our study include its relatively small sample size, multiple comparisons, a lack of very frequent blood sampling (q 10 minutes), required to allow assessment of pulsatile gonadotropin secretion, and the fact that not all participants were able to complete all infusion types.

In conclusion, the combination of acute exposure to elevated fatty acids and hyperinsulinemia acutely downregulates pituitary gonadotropin levels in both women and men. Thus, mimicking metabolic syndrome in individuals characterized as healthy and nonobese can induce the reproductive phenotype characteristic of obesity.

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