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Increasing Insulin Resistance Is Associated with a Decrease in Leydig Cell Testosterone Secretion in Men

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Insulin resistance is associated with low testosterone (T) levels in men, the mechanism of which is unclear. Thus, the aim of this study was to evaluate the hypothalamic-pituitary-gonadal axis in men with a spectrum of insulin sensitivity. Twenty-one men (aged 25–65 yr) had a glucose tolerance test and assessment of insulin sensitivity using a hyperinsulinemic-euglycemic clamp. Insulin sensitivity, expressed as the M value (milligrams per kilograms^{-1} per minute^{-1}), was calculated from the glucose disposal rate during the final 30 min of the clamp. Eighteen subjects had blood sampling every 10 min for 12 h to assess LH pulsatility. Hypogonadism was then induced with a GnRH antagonist, followed by sequential stimulation testing with GnRH (750 ng/kg, iv) and human chorionic gonadotropin (hCG; 1000 IU, im) to assess pituitary and testicular responsiveness, respectively. Nine subjects had normal glucose tolerance, nine had impaired glucose tolerance, and three had diabetes mellitus. There was a positive relationship between M and T levels (r = 0.46; $P < 0.05$). No relationship was seen between M and parameters of LH secretion, including mean LH levels, LH pulse amplitude, LH pulse frequency, and LH response to exogenous GnRH administration. In contrast, a strong correlation was observed between M and the T response to hCG (r = 0.73; $P < 0.005$). Baseline T levels correlated with the increase in T after hCG administration (r = 0.47; $P < 0.05$). During the clamp, T levels increased from a baseline level of 367 ± 30 to 419 ± 38 ng/dl during the last 30 min ($P < 0.05$). From these data we conclude that insulin resistance is associated with a decrease in Leydig cell T secretion in men. Additional studies are required to determine the mechanism of this effect. (*J Clin Endocrinol Metab* 90: 2636–2641, 2005)

CROSS-SECTIONAL STUDIES have shown an inverse correlation between serum testosterone (T) and fasting insulin levels in men (1–5). Furthermore, men with insulin resistance states such as obesity (2, 5, 6) and type 2 diabetes mellitus (DM2) (7, 8) have significantly lower T levels than age-matched normal weight and nondiabetic controls. To date, the mechanism underlying the low T levels associated with insulin resistance in men has not been elucidated.

It has been suggested that the inverse relationship between T and insulin is due to obesity (9, 10), given that the latter is associated with both insulin resistance and low SHBG levels. If this hypothesis is correct, the reduction in T levels seen with increasing obesity in men should be accounted for by low levels of SHBG alone, and the free T fraction should be normal. However, a number of studies have shown that total and free T levels decline in parallel in proportion to the degree of obesity (5, 11). Similarly, we have demonstrated a positive relationship between total T levels and insulin sensitivity in men independent of SHBG (12), whereas others have demonstrated an inverse correlation between free T and fasting insulin levels independent of body fat (4). In contrast, two recent studies support the hypothesis that the relationship between T and insulin sensitivity is mediated by obesity and SHBG (9, 10). Discrepancies between studies may reflect the different methodologies used to assess insulin sensitivity and free T levels. None of the studies used equilibrium dialysis, the method regarded as the gold standard for measurement of free T levels (13–15).

An alternative explanation for the inverse relationship between T and insulin is that they are directly linked independent of SHBG levels. The available evidence suggests that this relationship may, in fact, be bidirectional. Insulin signaling in the brain plays an important role in regulating reproductive function (16). Insulin promotes GnRH secretion in a hypothalamic GnRH neuronal cell line (17) and stimulates gonadotropin secretion from pituitary cell cultures (18), and T secretion from cultured Leydig cells (19, 20). In animal studies, lowering plasma insulin levels decreases pituitary LH content and plasma LH levels (21). In obese men, acute hyperinsulinemia causes a modest increase in T levels (22), whereas lowering insulin levels with diazoxide reduces serum T levels (23). This stimulatory effect of insulin on the hypothalamic-pituitary-gonadal (HPG) axis appears to contradict the inverse relationship between T and insulin levels noted in epidemiological studies (1–5). However, this apparent paradox could be explained by the decreased sensitivity of the HPG axis to insulin action in insulin-resistant states.

In addition to the impact of insulin on T secretion, there

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Abbreviations: BMI, Body mass index; CV, coefficient of variation; DM2, type 2 diabetes mellitus; E_2, estradiol; hCG, human chorionic gonadotropin; HOMA-IR, homeostatic model assessment for insulin resistance; HPG, hypothalamic-pituitary-gonadal; IGT, impaired glucose tolerance; M, glucose disposal rate; NGT, normal glucose tolerance; T, testosterone; WHR, waist to hip ratio.

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is evidence to support an effect of T on insulin sensitivity. In male rats, acute castration induces significant insulin resistance (24). In men, low T levels predispose to central obesity (25, 26) and predict the development of both the metabolic syndrome (27) and DM2 (28–32). The impact of T administration on insulin sensitivity in men is still unclear, with some (33, 34), but not all (35), studies showing an improvement.

The few studies that have attempted to identify the mechanism of the low T levels seen in obese men have methodological limitations (6, 11, 36). Pharmacological doses of GnRH (6) and human chorionic gonadotropin (hCG) (6, 11, 36) were employed to assess pituitary and testicular reserve. In addition, interpretation of sex steroid responses was confounded by the presence of both positive and negative feedback, thus limiting the ability to localize defects at individual levels of the HPG axis.

The aim of the present study was to determine the mechanism of the low T levels associated with insulin resistance in men by systematically evaluating each level of the HPG axis. To circumvent the limitations of previous studies, we used a GnRH antagonist to first remove the confounding variable of endogenous reproductive hormones, followed by sequential stimulation with physiological doses of GnRH and hCG to assess target cell responsiveness of the pituitary and testes, respectively.

Subjects and Methods

Study population

Twenty-one men (aged 25–65 yr; mean, 47.9 ± 2.0 yr) participated in this study. All subjects had normal hematocrit, TSH, and prolactin levels as well as normal liver function tests. Subjects were excluded if they had a history of a reproductive disorder or use of medications known to interfere with androgen synthesis/action or glucose homeostasis. The study was approved by the Human Research Committee at Massachusetts General Hospital, and all subjects provided written informed consent.

Anthropometric assessment

Height and weight were measured by standard procedures to calculate body mass index (BMI) as weight (kilograms) divided by the square of the height (meters), which was used to provide an index of generalized adiposity. The waist to hip ratio (WHR) was calculated in the erect position by measuring waist circumference at the level of the umbilicus and hip circumference at the level of the greatest hip girth to provide a marker of central adiposity.

Glucose tolerance

A 2-h oral glucose tolerance test using a 75-g glucose load was performed according to World Health Organization criteria (37).

Insulin sensitivity

Insulin sensitivity was assessed using the hyperinsulinemic-euglycemic clamp study (38) and the homeostatic model assessment for insulin resistance (HOMA-IR) (39).

Hyperinsulinemic-euglycemic clamp

Subjects consumed a fixed 300-g carbohydrate diet daily for 3 d before the study. After a 12-h overnight fast, an iv catheter was inserted into an antecubital vein for infusions of insulin and glucose. A second catheter was inserted retrogradely in a hand vein for blood sampling, and the hand was kept heated in a warming chamber to arterialize venous samples. After obtaining three basal samples at 10-min intervals, the hyperinsulinemic-euglycemic clamp was initiated with an infusion of regular insulin (Humulin, Eli Lilly & Co., Indianapolis, IN) at a dose of 80 $mU/m^2 \cdot min^{-1}$. For subjects with fasting glucose levels in the normal range, the plasma glucose level was clamped at the prevailing basal level of that individual (*i.e.* the mean of the three basal samples) with an infusion of 20% glucose for 2 h. In subjects with fasting hyperglycemia, plasma glucose was allowed to fall to 95 mg/dl and was then clamped at that level. Plasma samples were obtained every 5 min for determination of glucose using an on-site glucose analyzer (Beckman Instruments, Fullerton, CA) and every 10 min for determination of insulin. Insulin sensitivity, expressed as the glucose disposal rate (M), was determined from the mean rate of glucose infusion during the last 30 min of the clamp, corrected for glucose space and assumed total suppression of hepatic glucose output as previously described (38). Baseline T, estradiol (E_2), SHBG, and leptin levels were measured from a pooled aliquot of the −30, −20, and −10 min blood samples. The effect of hyperinsulinemia on serum T levels was measured from a pooled aliquot of the 100, 110, and 120 min blood samples.

HOMA-IR

HOMA-IR was calculated as $[(IB_f \times GB_f)]/22.5$, where IB_f is the fasting insulin level (microunits per milliliter), and GB_f is the glucose level (millimoles per liter) (39).

HPG axis evaluation

Eighteen subjects underwent the following detailed neuroendocrine evaluation (Fig. 1). On d 1, subjects were admitted to the General Clinical Research Center and, starting at 2000 h had blood sampling every 10 min for 12 h to assess the endogenous LH secretion pattern. Pulsatility was analyzed using the modified Santen and Bardin method previously validated by the authors (40). After completing blood sampling at 0800 h on d 2, hypogonadotropic hypogonadism was induced using the GnRH antagonist, acyline (41). Acyline was originally synthesized by Jean Rivier at The Salk Institute and is being distributed by the National Institute of Child Health and Human Development. Acyline (75 μg/kg) was administered sc at 0800 h on d 2 and 4 to suppress the HPG axis for the remainder of the study. Pituitary sensitivity was assessed on the morning of d 3 by measuring the LH response to 750 ng/kg GnRH, a dose demonstrated to overcome competitive antagonist inhibition (data not shown). Samples were drawn 15 min before and 0, 15, 30, 45, and 60 min after the GnRH bolus injection, with the peak LH response used as a measure of pituitary sensitivity. On completing the GnRH test, 1000 IU hCG were administered im, and serum T levels were measured 24 and 48 h later (0800 h on d 4 and 5).

Hormone assays

Serum LH concentrations were determined by microparticle enzyme immunoassay using the automated Abbott AxSYM system (Abbott Lab-

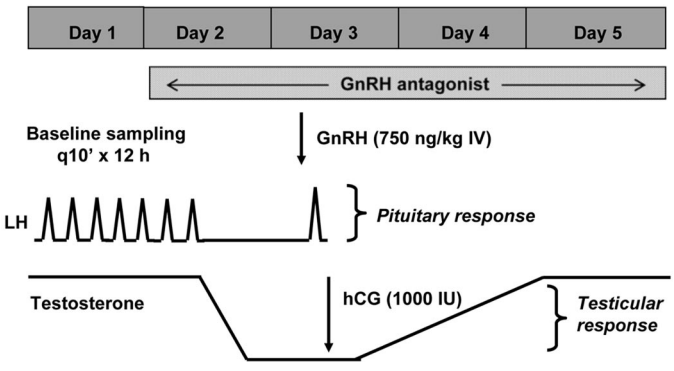

FIG. 1. Experimental paradigm to evaluate each level of the HPG axis, including a 12-h frequent blood-sampling study for assessment of LH pulsatility (d 1), followed by suppression of the HPG axis with the GnRH antagonist, acyline, for 4 d (d 2–5), during which the responses to sequential stimulation testing with physiological doses of exogenous GnRH and hCG are assessed.

oratories, Inc., Chicago, IL). The Second International Reference Preparation was used as the reference standard. The assay sensitivity for LH is 1.6 IU/liter. The intraassay coefficient of variation (CV) for LH was less than 7%, with an interassay CV less than 7.4%. Serum T levels were measured using the Coat-A-Count RIA kit (Diagnostics Products Corp., Los Angeles, CA), which had intra- and interassay CVs less than 10%. E_2 was measured by RIA, using hexane ethylacetate extraction and LH-20 chromatography (Esoterix, Calabasas Hills, CA). The E_2 assay has a sensitivity of 5 pg/ml (18 pmol/liter), an intraassay CV of 4.9%, and an interassay CV of 15%. Immunoreactive insulin was determined by an insulin-specific, double-antibody system using human insulin standards and tracer (Linco Research, Inc., St. Charles, MO). The antiserum was raised against highly purified human insulin and does not cross-react with human proinsulin (<0.1%). SHBG was measured by a chemiluminescent enzyme immunometric assay (Immulite, Diagnostics Products Corp.), which has an intraassay CV less than 7% and an interassay CV less than 8%. Leptin was measured using a commercially available RIA kit (Linco Research, Inc.).

Statistical methods

The data are expressed as the mean ± SE, except for data that were not normally distributed, in which case median values and ranges are reported. Correlations were assessed using the Pearson's correlation coefficient or Spearman rank order depending on whether the data were normally distributed. $P < 0.05$ was considered statistically significant.

Results

Anthropometric assessment

The BMI of the subjects ranged from 23.7–46.3 kg/m^2 (median, 30.9). WHR ranged from 0.89–1.15 (median, 0.97). A negative correlation was observed between T and BMI ($r = -0.49$; $P < 0.05$) and between T and WHR ($r = -0.46$; $P = 0.06$).

Glucose tolerance and insulin sensitivity

Nine subjects had normal glucose tolerance (NGT), nine had impaired glucose tolerance (IGT), and three had DM2. Fasting plasma glucose and insulin levels ranged from 77–204 mg/dl (median, 96) and from 3.5–46.8 μU/ml (median, 8.5), respectively. Plasma insulin levels during the clamp were stable and averaged 1189 μU/ml. The glucose disposal rate (M) during the final 30 min of the clamp ranged from 1.6–12.8 mg/kg^{-1}·min^{-1} (mean, 6.2 ± 0.7). HOMA-IR ranged from 0.8–23.5 (median, 2.8). A significant inverse correlation was observed between HOMA-IR and M ($r = -0.7$; $P < 0.005$).

Neuroendocrine assessment

Neuroendocrine profiling was obtained for nine men with NGT and nine men with IGT. There was a range of T levels from 170–619 ng/dl (mean, 359 ± 28), E_2 levels from 16–55 pg/ml (mean, 26 ± 3), SHBG levels from 4–55 nmol/liter (mean, 24 ± 3.0), and leptin levels from 2–37 ng/ml (median, 8.4). Mean LH levels during the 12-h overnight frequent blood sampling study were 8.9 ± 0.7 IU/liter, with an LH pulse amplitude of 5.3 ± 0.5 IU/liter and an LH pulse frequency of 5.4 ± 0.4 pulses/12 h. There was no difference in mean LH levels, LH amplitude, or LH pulse frequency between those with NGT and those with IGT.

Acyline caused the desired degree of suppression of the HPG axis, as evidenced by low levels of gonadotropins (mean, LH 1.7 ± 0.2 IU/liter) and hypogonadal T levels (42 ± 3 ng/dl). After GnRH administration, there was a wide range of responses, with the peak increase in LH levels ranging from 3.1–30.1 IU/liter (mean, 11.9 ± 1.5; Fig. 2A). Men with NGT tended to have a smaller increase in LH after GnRH treatment those with IGT (9.0 ± 0.5 *vs.* 14.8 ± 2.7 IU/liter; $P = 0.06$). After the administration of hCG, a similarly wide range of responses was seen, with an increase in serum T levels ranging from 115 to 677 ng/dl at 24 h (mean, 393 ± 38) and from 74 to 1230 ng/dl at 48 h (mean, 535 ± 59; Fig. 2B). Subjects with NGT had a greater increase in T after hCG treatment than men with IGT at both 24 h (466 ± 50 *vs.* 319 ± 40; $P < 0.05$) and 48 h (639 ± 89 *vs.* 431 ± 67; $P < 0.05$).

Correlation of insulin sensitivity with neuroendocrine parameters

A significant relationship was observed between baseline T levels and insulin sensitivity assessed by M ($r = 0.46$; $P < 0.05$) and HOMA-IR ($r = -0.5$; $P < 0.05$). A negative correlation was observed between T and fasting insulin levels ($r = -0.5$; $P < 0.05$); there was a trend for T levels to correlate with fasting glucose levels ($r = -0.39$; $P = 0.08$). For SHBG, the correlation was stronger with M ($r = 0.47$; $P < 0.05$) than with HOMA-IR ($r = -0.4$; $P = 0.06$). There was no relationship between E_2 levels and either M ($r = -0.1$; $P = 0.7$) or HOMA-IR ($r = 0.09$; $P = 0.7$). Similarly, no correlation was observed between mean LH levels and M ($r = 0.06$; $P = 0.8$), HOMA-IR ($r = -0.3$; $P = 0.2$), or leptin ($r = -0.3$; $P = 0.3$); between LH pulse amplitude and M ($r = 0.3$; $P = 0.3$), HOMA-IR ($r = -0.1$; $P = 0.8$), or leptin ($r = -0.3$, $P = 0.3$); between LH pulse frequency and M ($r = -0.1$; $P = 0.8$), HOMA-IR ($r = 0.1$; $P = 0.6$), or leptin ($r = 0.1$; $P = 0.8$); or between the LH response to exogenous GnRH administration and M ($r = -0.3$; $P = 0.3$), HOMA-IR ($r = 0.3$; $P = 0.3$), or leptin ($r = 0.03$; $P = 0.9$).

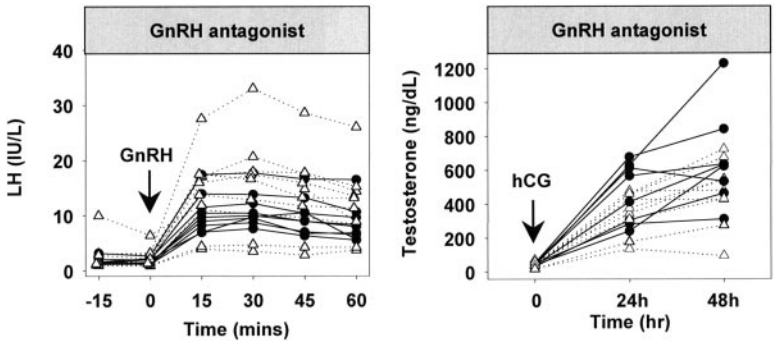

FIG. 2. The LH response to exogenous GnRH stimulation (750 ng/kg, iv; *left*) and the T response to hCG (1000 IU, im; *right*) in the presence of GnRH antagonist-induced hypogonadism in a cohort of 18 men, nine with NGT (●) and nine with IGT (△).

Correlation of insulin sensitivity with Leydig cell function

hCG-stimulated T levels at 24 h showed a positive correlation with M ($r = 0.7$; $P < 0.005$) and a negative correlation with HOMA-IR ($r = -0.7$; $P < 0.005$), fasting insulin levels ($r = -0.7$; $P < 0.005$), and leptin ($r = -0.6$; $P < 0.05$). A similar relationship was seen with T levels 48 h after hCG treatment and M ($r = 0.73$; $P < 0.005$; Fig. 3), HOMA-IR ($r = -0.6$; $P < 0.05$), fasting insulin levels ($r = -0.6$; $P < 0.05$), and leptin ($r = -0.55$; $P < 0.05$). The increase in T levels 48 h after hCG administration correlated positively with baseline T levels ($r = 0.47$; $P < 0.05$; Fig. 3). No relationship was observed between fasting glucose levels and hCG-stimulated T levels at 24 h ($r = -0.2$; $P = 0.4$) or 48 h ($r = -0.2$; $P = 0.4$).

T levels during the hyperinsulinemic-euglycemic clamp

During the clamp, T levels increased from a baseline level of 376 ± 30 to 419 ± 38 ng/dl during the last 30 min ($P < 0.05$). When stratified by BMI greater or less than 30 kg/m^2, the increase in T levels was significant in the nine obese subjects (349 ± 58 to 424 ± 67 ng/dl; $P < 0.005$) and approached statistical significance in the nine normal weight subjects (403 ± 24 to 433 ± 30 ng/dl; $P = 0.08$).

Discussion

We recently reported a positive correlation between serum T levels and insulin sensitivity, independent of SHBG, in a large cohort of men with a wide spectrum of BMI (12). The present study was designed to establish the mechanism of the low T levels associated with insulin resistance in men. Using a novel experimental paradigm to systematically isolate each level of the HPG axis, we show a strong correlation between insulin sensitivity and Leydig cell function assessed by the T response to physiological stimulation with hCG.

Serum T levels reflect not only the integrity of the HPG axis, but also the concentration of SHBG. Our previous demonstration that T levels correlate with insulin sensitivity independently of SHBG (12) implies that the low T levels seen in insulin-resistant men cannot be explained by low levels of SHBG alone, but, rather, indicate a functional defect at one or more levels of the HPG axis. In the present study we saw no correlation between insulin sensitivity and parameters of either endogenous LH secretion or the LH response to exogenous GnRH, implying that the low T levels associated with insulin resistance do not result from a defect in the hypothalamus or pituitary. Previous studies, which examined the HPG axis in obese men with low T levels, gave conflicting results. The few neuroendocrine studies published report normal mean LH levels and LH amplitude in men with moderate obesity (42), whereas men with severe obesity have lower LH levels and attenuated LH pulse amplitude than normal weight controls (42, 43). Other studies reported that obese men have similar LH responses to GnRH and clomiphene citrate as normal weight men (36). The limitations of these studies include use of pharmacological doses of GnRH to stimulate the pituitary, thus eliminating the possibility of detecting anything other than gross differences in response. Although the results of the present study do not support an involvement of the neuroendocrine axis in causing the low T levels in our cohort of men with mild to moderate obesity, they do not exclude the possibility that such an association may exist in a morbidly obese population.

This is the first study to demonstrate a strong positive correlation between hCG-stimulated T secretion and insulin sensitivity in men. A previous study reported a diminished T response to hCG in obese men, which correlated with baseline leptin, but not fasting insulin levels (11). Other studies have shown no differences in the T response to hCG stimulation in obese men *vs.* normal weight controls (6, 36). Our contrasting results are likely to be explained by methodological differences, given our use of physiological doses of hCG in the presence of experimentally induced hypogonadism, thus permitting the detection of more subtle differences in response.

Although it is well established that T biosynthesis is regulated primarily by pulsatile secretion of LH, there is compelling evidence that Leydig cell steroidogenesis is also modulated by circulating and/or locally produced hormones, growth factors, and cytokines (44). Insulin receptors are present on Leydig cells (20), and insulin stimulates T production in Leydig cell cultures (19, 20). These *in vitro* data seem to be at variance with the demonstration in our study that high baseline insulin levels are inversely correlated with baseline serum T levels and the T response to hCG. However, we hypothesize that in insulin-resistant states such as obesity, Leydig cell steroidogenesis is impaired because of target organ resistance to insulin action and/or production of cytokines/hormones by adipose tissue.

The precise etiology of the low serum levels of T and SHBG

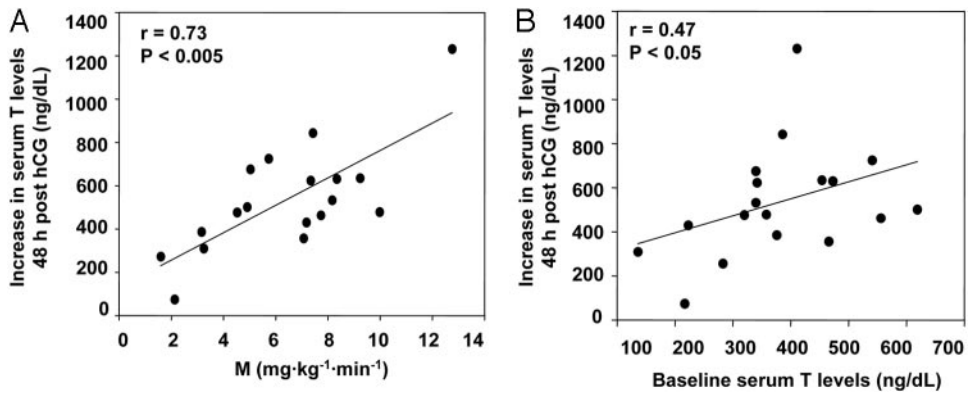

FIG. 3. The relationship between insulin sensitivity (M) and plasma T levels 48 h after the administration of hCG (A) and the correlation between baseline T levels and the T response to hCG at 48 h (B) in 18 healthy men.

reported in men with obesity (2, 5, 6) and DM2 (7, 8) is unclear. However, the demonstration that they are reversed by weight loss suggests a link to adipocyte function (45). It is thus tempting to propose that signals produced by adipose tissue, which are known to modulate insulin action, may also play a direct role in regulating gonadal function. Leptin is one such regulator of reproductive function, which is tightly linked with insulin resistance (46–48). Several studies have demonstrated that leptin is inversely correlated with serum T levels (49–51), and an excess of circulating leptin has been implicated in the pathogenesis of low T levels in obese men (11). Leptin receptors are present in Leydig cells, and leptin inhibits hCG-stimulated T secretion from rat Leydig cells at concentrations similar to those seen in obese men (52). The demonstration in the present study of a negative correlation between leptin and the T response to hCG is consistent with these findings. TNF-α, a cytokine elevated in adipose tissue of obese subjects (53, 54), has also been implicated in the pathogenesis of insulin resistance (55–59). From the reproductive standpoint, intratesticular delivery of TNF-α has been shown to reduce both basal and hCG-stimulated steroidogenic acute regulatory protein expression and T biosynthesis in the rat (60). Additional studies are required to determine the interaction between TNF-α and T in the human.

Regardless of the mechanism by which insulin resistance is associated with decreased T production, it has important clinical implications. In men with low T levels, consideration should be given to screening for insulin resistance using fasting insulin levels or HOMA-IR. Indeed, some investigators believe that an alteration in the sex hormone milieu is such a key component to the metabolic syndrome that the more appropriate name should be the glucose-insulin-lipid-hypertension-T-estrogen, or GILHT-E, syndrome (61).

During the hyperinsulinemic milieu of the glucose clamp, we demonstrated an increase in serum T levels, consistent with previous human studies (22) and *in vitro* data (17–20), suggesting that high levels of insulin can overcome insulin resistance in the testis. Although the increase in T levels during the clamp was greatest in the obese men, T levels also tended to increase in the lean subjects; the failure of these changes to reach statistical significance is probably the result of the small sample size and limited statistical power. Plasma glucose levels were maintained in the normal range during the clamp, thus excluding a role for changes in glucose in mediating the increase in T levels (62).

In summary, this study demonstrates that the low T levels associated with insulin resistance result in part from an alteration in Leydig cell function, the molecular mechanism for which is still unclear. Interventional studies are clearly needed to assess the potential role of insulin-sensitizing agents in increasing T production in insulin-resistant men.

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