Insulin Resistance and Inflammation in Hypogonadotropic Hypogonadism and Their Reduction After Testosterone Replacement in Men With Type 2 Diabetes

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OBJECTIVE

One-third of men with type 2 diabetes have hypogonadotropic hypogonadism (HH). We conducted a randomized placebo-controlled trial to evaluate the effect of testosterone replacement on insulin resistance in men with type 2 diabetes and HH.

RESEARCH DESIGN AND METHODS

A total of 94 men with type 2 diabetes were recruited into the study; 50 men were eugonadal, while 44 men had HH. Insulin sensitivity was calculated from the glucose infusion rate (GIR) during hyperinsulinemic-euglycemic clamp. Lean body mass and fat mass were measured by DEXA and MRI. Subcutaneous fat samples were taken to assess insulin signaling genes. Men with HH were randomized to receive intramuscular testosterone (250 mg) or placebo (1 mL saline) every 2 weeks for 24 weeks.

RESULTS

Men with HH had higher subcutaneous and visceral fat mass than eugonadal men. GIR was 36% lower in men with HH. GIR increased by 32% after 24 weeks of testosterone therapy but did not change after placebo ($P = 0.03$ for comparison). There was a decrease in subcutaneous fat mass (−3.3 kg) and increase in lean mass (3.4 kg) after testosterone treatment ($P < 0.01$) compared with placebo. Visceral and hepatic fat did not change. The expression of insulin signaling genes (IR-β, IRS-1, AKT-2, and GLUT4) in adipose tissue was significantly lower in men with HH and was upregulated after testosterone treatment. Testosterone treatment also caused a significant fall in circulating concentrations of free fatty acids, C-reactive protein, interleukin-1β, tumor necrosis factor-α, and leptin ($P < 0.05$ for all).

CONCLUSIONS

Testosterone treatment in men with type 2 diabetes and HH increases insulin sensitivity, increases lean mass, and decreases subcutaneous fat.

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The first report of frequent occurrence of subnormal free testosterone concentrations in males with type 2 diabetes demonstrated that while the duration of diabetes or the quality of its control had no relationship with plasma testosterone concentrations, the latter were inversely related to BMI (1,2). The subnormal free testosterone concentrations were associated with inappropriately low leutinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations, which responded with normal increases to gonadotropin-releasing hormone stimulation. These patients had a normal MRI of the brain and the pituitary. It was also demonstrated later that these patients with hypogonadotropic hypogonadism (HH) had significantly greater plasma concentrations of C-reactive protein (CRP), consistent with an increase in systemic inflammation (3). This is suggestive of an increased potential of atherogenicity and insulin resistance. Indeed, several studies have shown that low testosterone concentrations constitute a risk for future cardiovascular events (4). In addition, some studies have shown that subjects with low testosterone concentrations, irrespective of diabetes, have an increase in insulin resistance as measured by HOMA of insulin resistance (HOMA-IR) (5,6). Thus, it follows that patients with HH may have insulin resistance, and this may be mediated through an increase in inflammatory mediators that have been shown to interfere with insulin signaling. However, no study has delineated the impact of HH on insulin sensitivity in men with type 2 diabetes. Studies evaluating changes in insulin resistance (measured by HOMA-IR) after testosterone replacement in hypogonadal men with type 2 diabetes have shown inconsistent results (7–10). In any case, HOMA-IR is inadequate as an index of insulin resistance, especially in patients with type 2 diabetes, since β-cell loss and inadequate insulin secretion can lead to inappropriately low insulin concentrations and HOMA-IR. The best way to assess insulin resistance is through hyperinsulinemic-euglycemic (HE) clamps.

On the basis of the above, we hypothesized that patients with HH have an increase in insulin resistance and in inflammatory mediators, which may interfere with insulin signal transduction. In addition, we hypothesized that the replacement of testosterone suppresses inflammatory mediators, enhances the expression of elements of insulin signal transduction, and, thus, decreases insulin resistance. Finally, we also hypothesized that the anti-inflammatory and insulin-sensitizing effects of testosterone replacement occur in parallel with the replacement of adipose tissue with lean body mass (muscle).

RESEARCH DESIGN AND METHODS

This was a randomized, parallel, placebo-controlled, double-blind, prospective, single-center trial to assess *1*) the impact of HH on insulin resistance, inflammation, and body composition in men with type 2 diabetes and *2*) the effects of intramuscular testosterone replacement on insulin sensitivity, inflammation, and body composition. The trial was conducted at the research center of the Division of Endocrinology, Diabetes and Metabolism, State University of New York at Buffalo (Buffalo, NY). The protocol was approved by the Human Research Board of the State University of New York at Buffalo. An informed consent was signed by all subjects.

Study Population

Male subjects with type 2 diabetes between the ages of 30 and 65 years, HbA_{1c} ≤8% (64 mmol/mol), and stable diabetes regimen for 3 months were recruited between December 2010 and January 2014 by advertisements. Subjects on androgens, glucocorticoids, or opiates in the last 6 months or with panhypopituitarism, congenital HH, prolactinoma, head trauma, severe hepatic or kidney disease (glomerular filtration rate <30 mL/min/1.73 m^2), HIV, prostate-specific antigen (PSA) >4 ng/mL, or contraindications to testosterone replacement therapy (11) were excluded from the study.

At the screening visit, all subjects had the following laboratory tests performed in a fasting state in the morning: hemoglobin/hematocrit, serum electrolytes, creatinine, liver enzymes, testosterone, sex hormone–binding globulin (SHBG), and LH. Subjects then returned after 2 weeks for a repeat testosterone measurement. HH was defined as calculated free testosterone (cFT) concentration <6.5 ng/dL on both occasions along with low or normal LH concentrations. Men with normal cFT concentrations on both occasions were considered eugonadal. As per study design, symptoms were not considered for the diagnosis of hypogonadism.

Out of 186 people screened, 94 men qualified for and agreed to the study (50 men with normal cFT concentrations and 44 men with HH). Men with HH were randomized to testosterone (22 men) or placebo (22 men). Two men in testosterone group and eight men in placebo group dropped out. There were no differences in baseline characteristics of study completers and dropouts (Supplementary Table 1).

Study Design

Eugonadal subjects who qualified after the screening visit were asked to fill out a questionnaire regarding their mood and sexual symptoms for 7 days. They then underwent DEXA and MRI of abdomen. They returned in 7 days to undergo HE clamp studies. Subcutaneous fat biopsies were performed before the clamp. Men in HH group underwent the same procedures as eugonadal men at baseline. Upon completion of the clamp, they were given 250 mg testosterone cypionate (200 mg/mL; Watson Pharmaceuticals, Parsippany, NJ) or placebo (1.25 cc saline) intramuscularly in the buttock. Subjects returned to the study center every 2 weeks to receive study drug injection. Dose of testosterone was adjusted to keep cFT concentrations in normal range (6.5–25 ng/dL) (12) (Supplementary Fig. 1).

Subjects underwent HE clamp at week 3 to assess change in insulin sensitivity prior to any changes in body composition that were expected with testosterone therapy.

Subjects underwent end-of-study HE clamp and subcutaneous fat biopsy 1 week after the final study drug injection (23 weeks after the first study drug injection). Subjects also had DEXA and MRI measurements within 7 days of HE clamp. Thus, the total study duration was 24 weeks.

Study Procedures

HE Clamp

Insulin clamp was started with a priming dose of short-acting human insulin (Humulin R; Eli Lilly & Co., Indianapolis, IN) given over 10 min and then an infusion at the rate of 80 mU/m^2/min (13).

Glucose infusion (20%) was titrated to maintain arterialized blood glucose concentration at 100 mg/dL. Insulin sensitivity was calculated from the glucose infusion rates (GIRs) during the last 30 min of 4-h HE clamp (steady state) and expressed as glucose uptake per lean body mass. The mean coefficient of variation of glucose during the clamps was 5.8%. The mean glucose concentrations during the last 30 min were 99.4 ± 4.9 mg/dL and 99.7 ± 3.4 mg/dL at 0 weeks in men with and without HH. The mean glucose concentration during steady state was 103.9 ± 15.8 mg/dL at 23 weeks in men with HH (P = 0.20 compared with 0 weeks). Mean insulin concentrations achieved during the clamps were 89 μU/mL (range 75–110) and were not different between baseline and end-of-study clamps.

Fat Aspiration Procedure

Subcutaneous fat tissue aspiration was performed, before the start of clamp, on abdomen at a 10-cm distance from umbilicus under sterile conditions and local anesthesia; 0.5–3 g was aspirated and cleared from blood and fluids contaminants by centrifugation. The upper adipose tissue was collected into a separate sterile tube, washed twice with cold sterile PBS, and centrifuged to remove the PBS. Total RNA, nuclear extracts, and total cell lysates were prepared from the adipose tissue.

Imaging

Lean body mass and fat mass, total and regional (appendicular and trunk), were measured by DEXA at baseline and week 23. Hepatic fat fraction was assessed by MRI using a modification of the Dixon 3-point technique. Visceral adipose tissue volumes were measured by abdominal multislice imaging on a General Electric 1.5T MRI scanner as previously described (14,15). Images were analyzed independently with commercial image analysis software by the Image Reading Center, Inc. (New York, NY).

Clinical Laboratory Assays

All measurements were carried out by Quest Diagnostics. Total testosterone and estradiol concentrations were measured by liquid chromatography–tandem mass spectrometry (16). Tracer equilibrium dialysis is considered the gold standard for measuring free steroid hormone concentrations, and this methodology was used to separate the free testosterone and free estradiol (Nichols Institute, Chantilly, VA, and San Juan Capistrano, CA) (16,17). SHBG, LH, and FSH concentrations were measured by a solid-phase, chemiluminescent immunometric assay (IMMULITE 2500; Siemens). Free testosterone concentrations were also calculated from the concentrations of total testosterone, SHBG, and albumin by the formulas of Söderġard et al. (18) and Vermeulen et al. (19).

Mononuclear Cell Isolation

Blood samples were collected in Na-EDTA and carefully layered on Lympholyte medium (Cedarlane Labs, Hornby, ON). Samples were centrifuged and two bands separate out at the top of the red blood cell pellet. The mononuclear cell (MNC) band was harvested and washed twice with Hanks' balanced salt solution. This method provides yields >95% MNC preparation.

Quantification of mRNA Expression by RT-PCR

Expression of inflammatory mediators involved in insulin resistance and those that mediate insulin signaling was tested by RT-PCR in mRNA isolated from MNC and adipose tissue. Total RNA was isolated from MNC and adipose tissue using a commercially available RNAqueous-4PCR kit and adipose tissue RNA Isolation kit (Ambion, Austin, TX). Real-time RT-PCR was performed using the Stratagene Mx3000P qPCR system (La Jolla, CA), SYBR Green Master Mix (Qiagen, CA), and gene-specific primers for IKK-β, SOCS-3, PTEN, PTP-1B, JNK-1, TLR-4, IL-1β, IR, IRS-1, AKT-2, and GLUT4 (Life Technologies, Frederick, MD). All values were normalized to the expression of a group of housekeeping genes including actin, ubiquitin C, and cyclophilin A. The normalization factor used is calculated by GeneNorm software and is based on the values of all housekeeping genes used.

Western Blotting

MNCs and adipose tissue total cell lysates were prepared and electrophoresis and immunoblotting were carried out as described before (20). Polyclonal antibodies against IR (Abcam, Cambridge, MA), AKT-2, SOCS-3, IKK-β, and actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used, and all values were corrected for loading to actin.

Plasma Measurements

Glucose concentrations were measured in plasma by a YSI 2300 STAT Plus glucose analyzer (Yellow Springs, OH). Free fatty acid (FFA) levels were measured by a colorimetric assay (Wako Chemicals, Richmond, VA). ELISA was used to measure plasma concentrations of insulin (EMD Millipore, Billerica, MA), and CRP was measured using ELISA assay (American Diagnostica, Inc.). Leptin, adiponectin, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β were measured by ELISA (R&D Systems, Minneapolis, MN).

Sexual Function and Mood Questionnaire

The subjects were asked to complete a questionnaire daily for seven consecutive days (21,22). The subjects assessed their level of sexual desire, sexual enjoyment, and satisfaction of erection using a 7-point Likert-type scale (0–7) and assessed the percentage of full erection from 0 to 100%. The subjects rated their mood using a 0–7 score. The parameters assessed included positive mood responses (alert, full of pep, friendly, well/good) and negative mood responses (angry, irritable, sad or blue, tired, nervous). Weekly average scores were calculated.

Safety Tests

Hemoglobin, hematocrit, and PSA were measured at week 15. Digital rectal exams were done at baseline and at week 15.

Statistical Analysis

Group comparisons were performed by two-sided t tests, Mann-Whitney rank sum tests, and χ^2 tests as appropriate. Adjustment for variables in group comparisons was done with ANCOVA and generalized linear model analysis. Data that were not normally distributed (Kolmogorov-Smirnov test) were log transformed to perform the parametric statistical tests. Pearson correlation and multiple linear regression analyses between variables were done using SPSS software (SPSS, Chicago, IL). Data are presented as means ± SD (or means ± SE where indicated) for normally distributed data and median [25th, 75th percentile] for nonnormal data. $P < 0.05$ was considered significant. Results for posttreatment group comparisons are presented as mean difference (95% CI). Correction for multiple testing was not considered because there was only one primary end point (insulin sensitivity).

The exploratory secondary end points (body composition and inflammation) were designed to study the mechanisms underlying change in insulin sensitivity.

Sample Size Calculation

The primary end point of the study was to detect a difference in insulin sensitivity as measured by whole-body glucose uptake during HE clamp in subjects treated with testosterone for 24 weeks compared with placebo. The change from baseline in primary end point was compared by generalized linear model analysis between the testosterone and placebo arms. We conservatively estimated a change in whole-body glucose uptake of 20% in the testosterone arm compared with placebo. Assuming a dropout rate of 25%, we estimated that 22 patients per treatment arm should provide adequate power ($\beta = 0.2$) to detect a significant difference ($\alpha = 0.05$) between the treatment arms provided the SD of the residuals is not equal to or greater than the mean difference.

RESULTS

Comparison of Eugonadal and Hypogonadal Men

All measures of fat mass were greater in men with HH with the notable exception of hepatic fat. Total lean mass was higher in men with HH, while lean mass when expressed as a percentage of total body weight was lower in men with HH compared with eugonadal men ($58 \pm 5\%$ vs. $62 \pm 7\%$, respectively, $P = 0.003$), consistent with previous studies (23). Men with HH scored lower on measures of sexual function compared with eugonadal men.

Men with HH were more insulin resistant than eugonadal men as evidenced by lower GIR (by 36%) during HE clamps (Table 1). GIR was inversely related to visceral fat ($r = -0.63$, $P < 0.001$), hepatic fat ($r = -0.30$, $P = 0.02$), total body subcutaneous fat ($r = -0.48$, $P < 0.001$), and BMI ($r = -0.54$, $P < 0.001$). GIR was positively related to total testosterone ($r = 0.50$, $P < 0.001$), free testosterone ($r = 0.41$, $P < 0.001$), cFT ($r = 0.45$, $P < 0.001$), and SHBG ($r = 0.35$, $P = 0.002$) concentrations. GIR was not related to age ($r = 0.12$, $P = 0.32$). In a multivariate regression analysis model that included free testosterone, visceral fat, hepatic fat, and subcutaneous fat as independent variables and GIR as the dependent variable, visceral fat was the only determinant of GIR in both eugonadal men ($\beta = -0.48$, $P = 0.03$) and in men with HH ($\beta = -0.74$, $P = 0.02$).

We compared the GIR among men with and without HH after adjustment for visceral fat, hepatic fat, and total body subcutaneous fat difference between the two groups. After this adjustment, the GIR between the two groups was not different (Table 1).

Basal Insulin Signaling Mediators

The expression of genes that mediate insulin signaling, IR-β, IRS-1, AKT-2, and GLUT4, was significantly lower in the adipose tissue from HH men compared with eugonadal men (Fig. 1), while there was no significant difference in the expression of these genes in the MNC between the groups (data not shown).

Basal Inflammatory Mediators

There was no significant difference in the serum concentrations of FFA, leptin, adiponectin, and inflammatory mediators (CRP, IL-1β, and TNF-α) of men with or without HH (Table 1). There was also no difference between HH and eugonadal men in basal mRNA expression of proinflammatory mediators known to interfere with insulin signaling including JNK-1, IKK-β, SOCS-3, PTP-1B, and TLR-4 in MNC and in adipose tissue (data not shown).

Results of Testosterone Treatment

The men randomized to testosterone or placebo arms were similar in age, duration of diabetes, or use of antidiabetes medications (Supplementary Table 2). There was no significant difference in any of the baseline measures of body composition, insulin sensitivity, or inflammation of men randomized to testosterone or placebo arms (Table 2). There was a significant increase in testosterone and estradiol concentrations after testosterone treatment (Table 2). The changes in free testosterone and free estradiol were positively related ($r = 0.61$, $P = 0.04$).

Three Weeks

Subjects returned in 3 weeks after the study drug for an HE clamp. There was no significant change in body weight in the testosterone group compared with placebo (mean difference 2.6 kg [95% CI −1.9, 7.0], $P = 0.25$). The GIR also did not change (mean difference −0.25 mg/kg/min [−1.6, 1.1], $P = 0.70$).

Study End Points at Completion (23 Weeks)

The results are presented in Table 2.

Glucose, Insulin, and Lipid Concentrations

There was no change in HbA_{1c}, but there was a significant reduction in the mean fasting glucose concentration in the testosterone-treated group compared with placebo group. Plasma insulin concentrations fell significantly in the testosterone-treated group. Only two subjects, both in placebo group, reported a change in their diabetes drugs by their physicians. In one subject, glargine dose was increased, and in the other, sulfonylurea dose was increased. There was no change in serum lipid concentrations.

Body Composition

Subjects receiving testosterone therapy had an increase in lean body mass and a decrease in subcutaneous, but not visceral or hepatic, fat mass (Table 2). The changes in lean mass and subcutaneous fat mass in testosterone group were inversely related ($r = -0.47$, $P = 0.05$).

Insulin Sensitivity

Treatment with testosterone for 6 months increased glucose uptake during HE clamp by 32% (Table 2). The change in insulin sensitivity in the testosterone group was not related to the change in lean mass ($r = 0.34$, $P = 0.16$), subcutaneous fat ($r = -0.27$, $P = 0.91$), visceral fat ($r = -0.32$, $P = 0.91$), hepatic fat ($r = -0.14$, $P = 0.63$), or baseline GIR ($r = -0.26$, $P = 0.18$). The change in GIR in testosterone group was significantly higher than that in placebo group even after adjustment for baseline GIR, visceral fat, hepatic fat, and subcutaneous fat (mean difference 2.07 mg/kg fat-free mass/min [95% CI 0.06, 4.08], $P = 0.04$). The change in insulin sensitivity in testosterone group was not related to the change in free testosterone ($r = -0.05$, $P = 0.85$) or free estradiol concentrations ($r = -0.04$, $P = 0.9$).

Insulin Signaling Mediators

The expression of insulin signaling genes, IR-β, IRS-1, AKT-2, and GLUT4, was significantly upregulated in adipose tissue after testosterone treatment (Fig. 2). There was no change in the

Table 1—Demographics, laboratory measures, sexual questionnaire scores, body composition, insulin sensitivity, and inflammatory mediators in men with and without HH

	Hypogonadal	Eugonadal	*P*
Number of subjects	44	50	
Age (years)	54.6 ± 7.9	51.5 ± 8.9	0.075
Duration of diabetes (years)	9.4 ± 8.6	10.9 ± 9.8	0.50
BMI (kg/m^2)	39.8 ± 7.8	34.0 ± 6.4	<0.001
Testicular size (mL)	17.4 ± 4.7	19.1 ± 5.3	0.19
Laboratory measurements			
Total testosterone (ng/dL)	252 ± 82	485 ± 183	<0.001
Free testosterone (ng/dL)	4.4 ± 1.2	7.6 ± 2.2	<0.001
cFT (ng/dL)	5.4 ± 1.1	9.5 ± 1.8	<0.001
SHBG (nmol/L)	26.3 ± 12.8	36.0 ± 23.5	0.01
LH (IU/L)	3.9 [2.4, 5.8]	5.0 [3.6, 6.1]	0.05
FSH (IU/L)	5.3 [3.5, 9.4]	6.9 (4.2, 9.3]	0.34
PSA (ng/mL)	0.6 [0.4, 0.8]	0.6 [0.4, 1.0]	0.64
HbA_{1c}, % (mmol/mol)	7.0 ± 1.1 (53 ± 12)	7.1 ± 1.1 (54 ± 12)	0.66
Total estradiol (pg/mL)	29.6 ± 13.2	25.0 ± 9.9	0.11
Free estradiol (pg/mL)	0.65 ± 0.32	0.60 ± 0.23	0.48
Total cholesterol (mg/dL)	154 ± 42	173 ± 50	0.07
HDL cholesterol (mg/dL)	39 ± 9	44 ± 14	0.06
LDL cholesterol (mg/dL)	89 ± 29	100 ± 36	0.13
Triglycerides (mg/dL)	128 [103, 211]	113 [80, 177]	0.44
Hemoglobin (g/dL)	13.8 ± 1.3	14.6 ± 1.2	0.002
Hematocrit (%)	41.2 ± 3.8	43.8 ± 3.2	0.001
Medications (%)			
Metformin	89	68	0.05
Sulfonylureas	41	34	0.29
Insulin	50	46	0.82
Glucagon-like peptide 1 agonists	16	16	0.92
Dipeptidyl peptidase-4 inhibitors	14	16	0.64
Thiazolidinediones	14	12	0.89
Diet only	0	6	0.28
Ethnicity (%)			
Caucasian	68	68	1.0
African American	23	24	1.0
Hispanic	7	8	1.0
Sexual questionnaire scores			
Sexual desire (0–7)	2.5 ± 1.6	3.6 ± 1.5	0.009
Sexual enjoyment (0–7)	1.7 ± 1.4	2.6 ± 1.9	0.04
Satisfaction with erection (0–7)	2.6 ± 2.1	4.0 ± 1.9	0.02
Percentage of full erection (0–100%)	37 ± 30	60 ± 29	0.007
Positive mood (0–7)	4.5 ± 1.2	5.0 ± 1.3	0.15
Negative mood (0–7)	1.4 ± 1.1	1.4 ± 1.4	0.93
Body composition			
Waist circumference (cm)	125 ± 21	116 ± 18	0.06
Hip circumference (cm)	119 ± 20	113 ± 12	0.17
Waist-to-hip ratio	1.06 ± 0.07	1.01 ± 0.09	0.03
Trunk fat mass by DEXA (kg)	28 ± 7	22 ± 8	<0.001
Leg fat mass (kg)	12.3 ± 5.1	8.5 ± 3.7	<0.001
Arm fat mass (kg)	4.6 ± 1.9	3.2 ± 1.5	<0.001
Total body subcutaneous fat mass (kg)	46 ± 14	34 ± 12	<0.001
Visceral fat (L)	8.22 ± 3.24	5.96 ± 2.53	0.001
Hepatic fat (%)	5.77 ± 6.93	7.10 ± 7.81	0.43
Leg lean mass (kg)	21.8 ± 3.8	19.8 ± 3.0	0.01
Arm lean mass (kg)	8.0 ± 1.5	8.0 ± 1.4	0.99
Total body lean mass (kg)	71 ± 11	64 ± 9	0.002
Insulin sensitivity			
GIR (mg/kg fat-free mass/min)	6.53 ± 4.01	10.29 ± 5.55	0.002
GIR adjusted for total subcutaneous, visceral, and hepatic fat	7.67 ± 4.09	9.40 ± 5.72	0.15
HOMA-IR	3.92 ± 0.66	3.44 ± 0.59	0.53

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Table 1—Continued

	Hypogonadal	Eugonadal	*P*
Inflammatory mediators			
FFAs (mmol/L)	0.60 ± 0.19	0.57 ± 0.19	0.54
CRP (mg/L)	4.5 ± 3.9	2.9 ± 3.1	0.10
Leptin (ng/mL)	20.0 ± 2.1	18.1 ± 3.6	0.13
Adiponectin (μg/mL)	6.6 ± 0.9	5.6 ± 0.6	0.81
IL-1β (pg/mL)	1.41 ± 0.20	1.13 ± 0.19	0.14
TNF-α (pg/mL)	2.51 ± 0.28	2.28 ± 0.38	0.18

Data are means ± SD, except for LH, FSH, PSA, and triglycerides, which were not normally distributed and are reported as median [25th, 75th percentile]. All patients had testosterone concentrations measured twice at baseline. The concentrations for total, free, and calculated free testosterone are an average of the two measurements.

expression of these genes in the MNC (data not shown).

Inflammatory Mediators

After testosterone treatment in HH men, there was a significant fall in circulating levels of FFA, CRP, IL-1β, TNF-α, and leptin (Table 2), while there was no significant change in adiponectin concentrations. Except for IL-1β, the changes after testosterone treatment were significantly different compared with placebo by week 15 (Supplementary Fig. 2). In the MNC, testosterone therapy suppressed the mRNA expression of the proinflammatory and insulin resistance mediators SOCS-3, IKK-β, and PTEN as well as protein levels of SOCS-3 compared with placebo treatment in HH men ($P < 0.05$) (Supplementary Fig. 3). These changes were apparent at 15 weeks after the start of the treatment (data not shown). There was no change in TLR-4, PTP-1B, or JNK-1 mRNA or protein after testosterone or placebo treatments (data not shown).

In the adipose tissue, testosterone treatment reduced the mRNA expression of PTP-1B and TLR-4 but not of other proinflammatory genes that interfere with insulin signaling (SOCS-3, IKK-β, JNK-1, and PTEN). Testosterone treatment also suppressed the protein levels of PTP-1B compared with placebo (Supplementary Fig. 4).

Sexual Function

Subjects randomized to testosterone had improvement in some measures of sexual function (Table 2). There was a modest reduction in testicular size (3 mL) with testosterone therapy.

Safety End Points

PSA, hemoglobin, and hematocrit concentrations were measured at week 15. PSA concentrations did not change during the study (mean difference between groups 0.11 ng/mL [95% CI −0.11, 0.32], $P = 0.82$). Hemoglobin and hematocrit concentrations increased by mean between-group difference (95% CI) of 0.54 g/dL (−0.19, 1.27) and 2.3% (0.2, 4.8), respectively ($P = 0.10$ and 0.03). No subject developed hemoglobin >18 g/dL, hematocrit >55%, supranormal PSA concentrations (>4 ng/mL), or a prostate nodule during the trial.

CONCLUSIONS

Our results show clearly that the state of HH in patients with type 2 diabetes is associated with a significant increase in insulin resistance as reflected in decreased GIR by 36%. Consistent with this, there was a marked reduction in the expression of IR-β, IRS-1, AKT-2, and GLUT4, the major genes mediating insulin signaling responsible for glucose transport. Since the difference in GIR between the two groups was not significant after correction for adiposity (especially visceral fat), the lower insulin sensitivity of men with HH is probably due to increased adiposity.

After testosterone administration, there was a significant reduction in total and truncal fat mass (by ~3 kg overall) and an increase in lean body mass by ~3 kg without a net change in body weight, consistent with other testosterone replacement studies (22,24). In parallel with this, there was an increase in insulin sensitivity as reflected in 32% increase in GIR, but the increase in insulin sensitivity was related neither to changes in lean body mass nor to fat mass. However, there was a significant increase in the expression of IR-β, IRS-1, AKT-2, and GLUT4 in adipose tissue, which provides a mechanistic explanation for the increase in insulin sensitivity. Testosterone administration also induced a reduction in FFA concentrations, probably through the suppression of lipolysis. FFAs are known to induce oxidative and inflammatory stress and to interfere with insulin signal transduction (25,26). The improvement in insulin sensitivity after testosterone treatment was also associated with suppression of other inflammatory mediators, which interfere with insulin signaling. These included IKK-β, SOCS-3, and PTEN in MNC and TLR-4 and PTP-1B in adipose tissue. While SOCS-3 interferes with insulin signal transduction by causing the ubiquitination and proteasomal degradation of IRS-1 (27), IKK-β induces serine phosphorylation of IRS-1 and thus prevents insulin signal transduction though IRS-1 (28). PTP-1B dephosphorylates the insulin receptor after the autophosphorylation by tyrosine kinase and thus limits insulin signaling (29). The expression of PTEN, another protein that interferes with insulin signaling (30), was also suppressed in MNC after testosterone treatment. While many studies have documented a decrease in CRP with testosterone replacement (31–33), the suppression of these mediators of insulin resistance in the adipose tissue and MNC of obese humans with testosterone treatment was not demonstrated before. However, we cannot state whether they are the direct actions of testosterone or are indirectly mediated by changes in adiposity.

Prior studies in men with type 2 diabetes have investigated the effect of testosterone therapy on insulin sensitivity by calculating HOMA-IR (7,8). The results, however, have not been consistent (10). This study is the first to demonstrate the insulin-sensitizing effect of testosterone therapy through the use of HE clamp in hypogonadal men with type 2 diabetes, consistent with

studies in obese men without diabetes (34–37). In contrast, studies in nonobese men do not show a change in insulin sensitivity after testosterone replacement (24,38,39). It is possible that testosterone-induced increase in insulin sensitivity occurs only in obese and insulin-resistant men.

We did not observe a change in HbA_{1c} despite a marked decrease in insulin resistance. However, we observed a significant decrease in fasting glucose concentrations after testosterone therapy. It is possible that the duration of the study was too short to induce a change in HbA_{1c}, especially in the context of relatively low baseline HbA_{1c} concentrations (mean 7.0 ± 1.1% [53 ± 12 mmol/mol]). Previous trials of testosterone therapy on glycemic control have been mixed (8,10); some studies have shown a benefit (9,40), while others have not (7,41). Although our data provide evidence that testosterone increases insulin sensitivity and fasting glucose concentrations, longer-term studies will be required to establish whether it can improve overall diabetes control.

While there were no significant differences in inflammation or fat mass–adjusted insulin sensitivity at baseline among hypogonadal and eugonadal men, testosterone therapy clearly induced an improvement in both these parameters. This could be because the difference in cFT concentrations between hypogonadal and eugonadal men (4.4 ng/dL) was much lower than the increase in cFT concentrations after testosterone therapy (12.2 ng/dL).

The major limitation of our study was a high rate (36%) of dropouts in the placebo arm compared with testosterone arm (9%). The predominant reason was lack of time to follow the study protocol in entirety. The overall dropout rate in the study (23%) was lower than

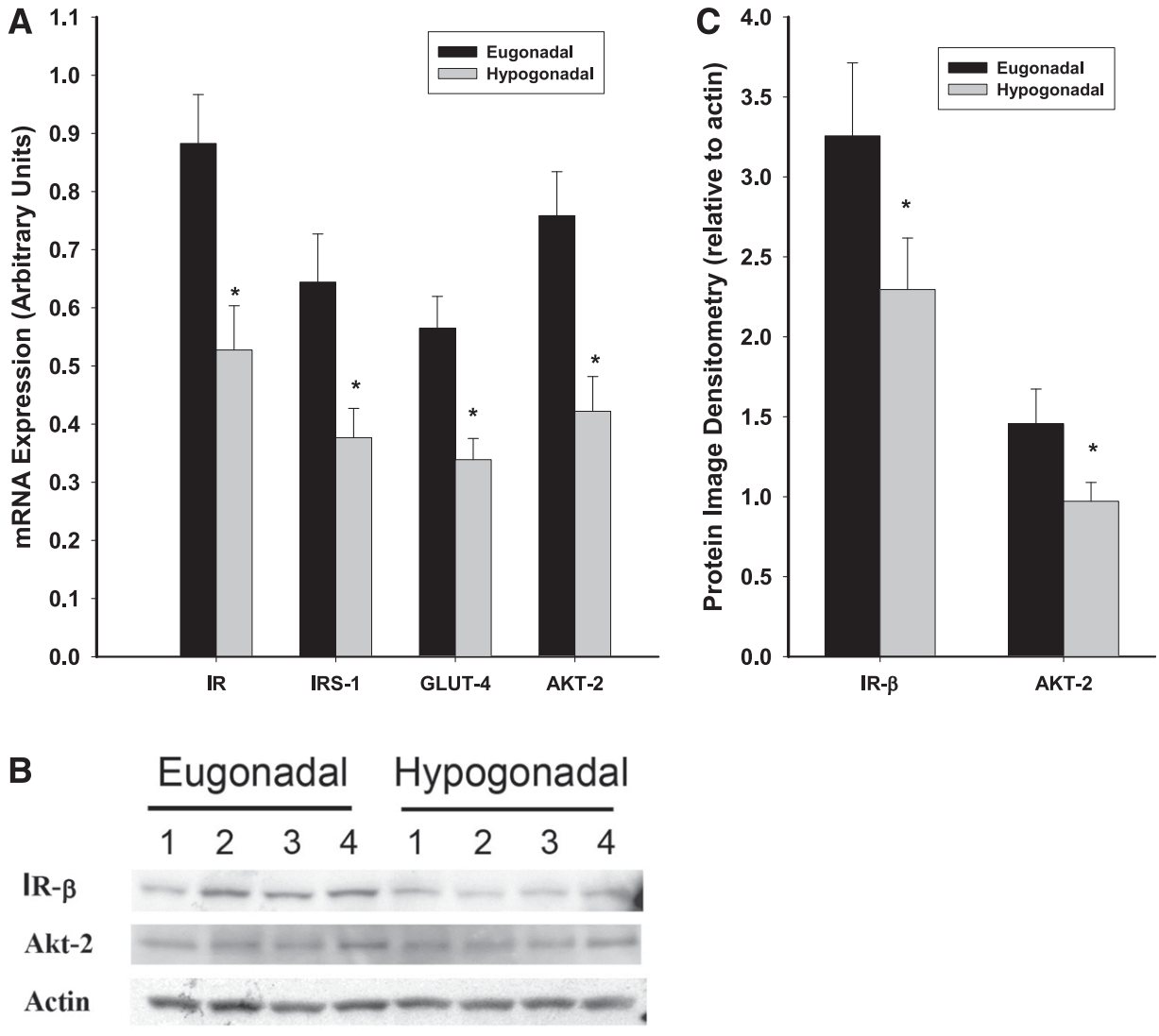

Figure 1—*A*: Basal mRNA expression of insulin signaling mediators (IR-β, IRS-1, GLUT4, and AKT-2) in adipose tissue from HH and eugonadal men. *N* = 26 vs. 27 (HH vs. eugonadal). The mRNA expression of IR-β, IRS-1, AKT-2, and GLUT4 was significantly lower by 39%, 37%, 41%, and 33%, respectively, in men with HH ($P < 0.01$ for all). *B*: Representative Western blot showing basal protein levels of IR-β subunit and AKT-2 in adipose tissue from 4 HH and 4 eugonadal men with type 2 diabetes. *C*: Basal protein levels of IR- β subunit and AKT-2 in adipose tissue from HH and eugonadal men. *N* = 26 vs. 27 (HH vs. eugonadal). The protein levels of IR-β subunit and AKT-2 were lower by 24% and 21%, respectively, in men with HH ($P < 0.05$). We were unable to detect an evaluable protein signal for IRS-1 and GLUT4, possibly due to quality of antibodies used. Data are represented as mean ± SE. Densitometry is corrected for loading using actin protein levels. *$P < 0.05$ by *t* test.

Table 2—Laboratory parameters, sexual questionnaire score, body composition, insulin sensitivity, and inflammatory mediators after testosterone and placebo treatment

	Testosterone (n = 20)			Placebo (n = 14)			Mean difference	
	Baseline	24 weeks	P	Baseline	24 weeks	P	(95% CI)	P
Weight (kg)	123 ± 23	123 ± 24	0.69	124 ± 30	128 ± 31	0.22	−3.5 (−8.7, 1.7)	0.13
BMI (kg/m^2)	39.0 ± 7.6	38.9 ± 8.1	0.73	39.4 ± 7.9	40.6 ± 8.4	0.22	−1.0 (−2.6, 0.6)	0.14
Testicular size (mL)	18 ± 4	14 ± 4	0.006	17 ± 5	16 ± 5	0.31	−3 (−6, 0)	0.04
Laboratory measurements								
Total testosterone (ng/dL)	259 ± 85	561 ± 183	<0.001	239 ± 81	280 ± 132	0.08	246 (106, 385)	<0.001
Free testosterone (ng/dL)	4.5 ± 1.3	13.8 ± 4.1	<0.001	4.2 ± 1.2	5.1 ± 1.7	0.07	7.3 (4.0, 10.6)	<0.001
cFT (ng/dL)	5.5 ± 1.1	17.6 ± 5.1	<0.001	5.2 ± 1.0	5.7 ± 1.8	0.36	8.9 (4.6, 13.2)	<0.001
Total estradiol (pg/mL)	30.1 ± 17.2	62.6 ± 42.7	0.01	26.1 ± 8.3	26.4 ± 10.6	0.92	30 (9, 51)	0.008
Free estradiol (pg/mL)	0.66 ± 0.43	1.55 ± 1.10	0.02	0.57 ± 0.20	0.59 ± 0.22	0.96	0.74 (0.16, 1.31)	0.014
SHBG (nmol/L)	27 ± 14	24 ± 10	0.06	26 ± 13	27 ± 13	0.82	−3 (−8, 1)	0.13
HbA_{1c}, % (mmol/mol)	6.8 ± 0.9 (51 ± 9.8)	7.2 ± 0.9 (55 ± 9.8)	0.27	7.0 ± 1.4 (53 ± 15.3)	7.1 ± 1.5 (54 ± 16.4)	0.72	0.15 (−0.59, 0.90)	0.40
Insulin (μU/mL)	13.6 ± 3.0	9.9 ± 1.8	0.04	11.8 ± 2.2	13.9 ± 4.0	0.54	−6.1 (−7.4, 0.87)	0.07
Glucose (mg/dL)	126 ± 8	115 ± 7	0.03	119 ± 10	132 ± 13	0.31	−26.4 (−48.1, −4.6)	0.02
Total cholesterol (mg/dL)	157 ± 38	145 ± 27	0.26	156 ± 37	156 ± 31	0.92	−7 (−32, 17)	0.39
HDL cholesterol (mg/dL)	34 ± 7	33 ± 8	0.36	39 ± 10	40 ± 12	0.79	−2 (−8, 4)	0.46
LDL cholesterol (mg/dL)	87 ± 37	77 ± 23	0.20	83 ± 23	82 ± 28	0.89	−8 (−35, 19)	0.23
Triglycerides (mg/dL)	222 ± 197	192 ± 142	0.73	167 ± 96	172 ± 100	0.73	−10 (−112, 92)	0.36
Sexual questionnaire scores								
Sexual desire (0–7)	2.7 ± 1.8	3.9 ± 1.2	0.03	2.2 ± 1.5	2.5 ± 2.2	0.57	0.7 (−0.8, 2.2)	0.25
Sexual enjoyment (0–7)	1.6 ± 1.3	3.0 ± 1.6	0.03	1.4 ± 1.3	1.0 ± 1.1	0.59	0.9 (0.4, 2.1)	0.03
Satisfaction with erection (0–7)	2.2 ± 2.0	3.9 ± 1.8	0.03	3.4 ± 2.2	3.5 ± 2.1	0.94	1.5 (−0.3, 3.4)	0.11
Percentage of full erection (0–100%)	35 ± 35	57 ± 31	0.07	44 ± 25	51 ± 26	0.12	14 (−14, 41)	0.27
Positive mood (0–7)	4.4 ± 1.3	4.9 ± 0.8	0.25	4.3 ± 0.9	4.4 ± 1.1	0.89	−0.02 (−1.46, 1.42)	0.19
Negative mood (0–7)	1.2 ± 0.9	0.9 ± 1.1	0.35	1.7 ± 1.7	1.8 ± 1.4	1.0	−0.4 (−2.1, 1.3)	0.73
Body composition								
Waist circumference (cm)	128 ± 20	126 ± 18	0.73	124 ± 30	124 ± 19	0.22	−2 (−7, 3)	0.29
Waist-to-hip ratio	1.06 ± 0.08	1.04 ± 0.07	0.34	1.06 ± 0.04	1.01 ± 0.05	0.02	0.03 (−0.02, 0.08)	0.20
Trunk subcutaneous fat mass (kg)	27.2 ± 8.1	25.3 ± 7.3	0.03	26.7 ± 6.9	27.2 ± 7.9	0.36	−2.5 (−4.8, −0.2)	0.03
Arm fat mass (kg)	4.5 ± 2.1	4.4 ± 1.9	0.48	4.4 ± 2.0	5.1 ± 1.9	0.04	−0.9 (−1.7, −0.11)	0.03
Leg fat mass(kg)	11.8 ± 4.5	11.2 ± 4.5	0.28	12.3 ± 6.7	11.9 ± 5.2	0.55	−0.2 (−1.8, 1.4)	0.81
Total body subcutaneous fat mass (kg)	44.5 ± 13.7	42.1 ± 12.5	0.02	44.5 ± 15.0	45.4 ± 14.4	0.11	−3.3 (−5.8, −0.8)	0.01
Visceral fat (L)	7.81 ± 3.03	7.25 ± 2.86	0.40	7.33 ± 2.73	7.44 ± 3.18	0.61	−1.09 (−3.00, 0.82)	0.25
Hepatic fat %	6.17 ± 6.45	4.41 ± 3.80	0.22	3.93 ± 6.06	3.39 ± 4.06	0.55	−1.49 (−4.86, 1.89)	0.37
Arm lean mass (kg)	8.1 ± 1.3	8.8 ± 1.3	0.01	7.9 ± 1.5	8.4 ± 1.8	0.08	0.2 (−0.5, 0.9)	0.61
Leg lean mass (kg)	21.6 ± 3.2	22.5 ± 3.2	0.07	21.2 ± 4.3	20.9 ± 3.1	0.74	1.2 (−0.6, 3.1)	0.18
Total body lean mass (kg)	70.6 ± 9.2	73.2 ± 10.7	0.001	69.1 ± 13.4	68.3 ± 13.0	0.41	3.4 (1.2, 5.6)	0.003
Hip bone density (g/cm^2)	1.15 ± 0.19	1.14 ± 0.19	0.61	1.15 ± 0.17	1.14 ± 0.15	0.20	0.01 (−0.01, 0.04)	0.29
Spine bone density (g/cm^2)	1.27 ± 0.17	1.28 ± 0.15	0.57	1.25 ± 0.19	1.31 ± 0.21	0.04	−0.04 (−0.11, 0.02)	0.09
Total body bone density (g/cm^2)	1.36 ± 0.13	1.36 ± 0.13	0.85	1.35 ± 0.12	1.33 ± 0.13	0.45	0.01 (−0.01, 0.04)	0.29
Insulin sensitivity								
GIR (mg/kg fat-free mass/min)	6.66 ± 4.36	8.73 ± 4.27	0.004	5.12 ± 2.79	5.06 ± 3.62	0.76	2.16 (0.39, 3.92)	0.03
HOMA-IR	4.1 ± 0.9	2.7 ± 0.5	0.03	3.5 ± 0.6	3.8 ± 0.9	0.85	−1.72 (−3.88, −0.65)	0.03
Inflammatory mediators								
FFAs (mmol/L)	0.62 ± 0.20	0.40 ± 0.13	<0.001	0.58 ± 0.20	0.56 ± 0.20	0.52	−0.21 (−0.37, −0.5)	0.04
CRP (mg/L)	4.3 ± 4.5	3.5 ± 3.8	0.003	4.7 ± 4.1	4.6 ± 3.9	0.71	−0.7 (−1.7, −0.2)	0.02
IL-1β (pg/mL)	1.69 ± 0.23	1.22 ± 0.17	0.001	0.99 ± 0.31	0.93 ± 0.27	0.65	−0.40 (−0.82, −0.09)	0.03
TNF-α (pg/mL)	2.53 ± 0.41	2.13 ± 0.32	0.001	2.08 ± 0.22	2.03 ± 0.2	0.85	−0.403 (−0.022, −0.783)	0.04
Leptin (ng/mL)	19.0 ± 2.7	15.4 ± 2.4	0.007	22.9 ± 3.1	24.1 ± 2.6	0.41	−4.8 (−7.9, −2.7)	0.01
Adiponectin (μg/mL)	6.8 ± 0.1	6.4 ± 0.1	0.45	6.3 ± 0.2	6.1 ± 0.2	0.68	−0.2 (−0.8, 0.4)	0.61

Data are mean ± SD unless otherwise indicated.

the rate accounted for in the study design (25%) and was similar to that in other studies (8). The clinical characteristics of the patients who dropped out were similar to those who completed the study. Since the changes in body composition, insulin sensitivity, and inflammation occurred simultaneously, we are unable to determine which of these factors was the primary driver of these changes. Lastly, our study was 6 months in duration, and, therefore, longer-term studies will be required to establish the durability of these effects.

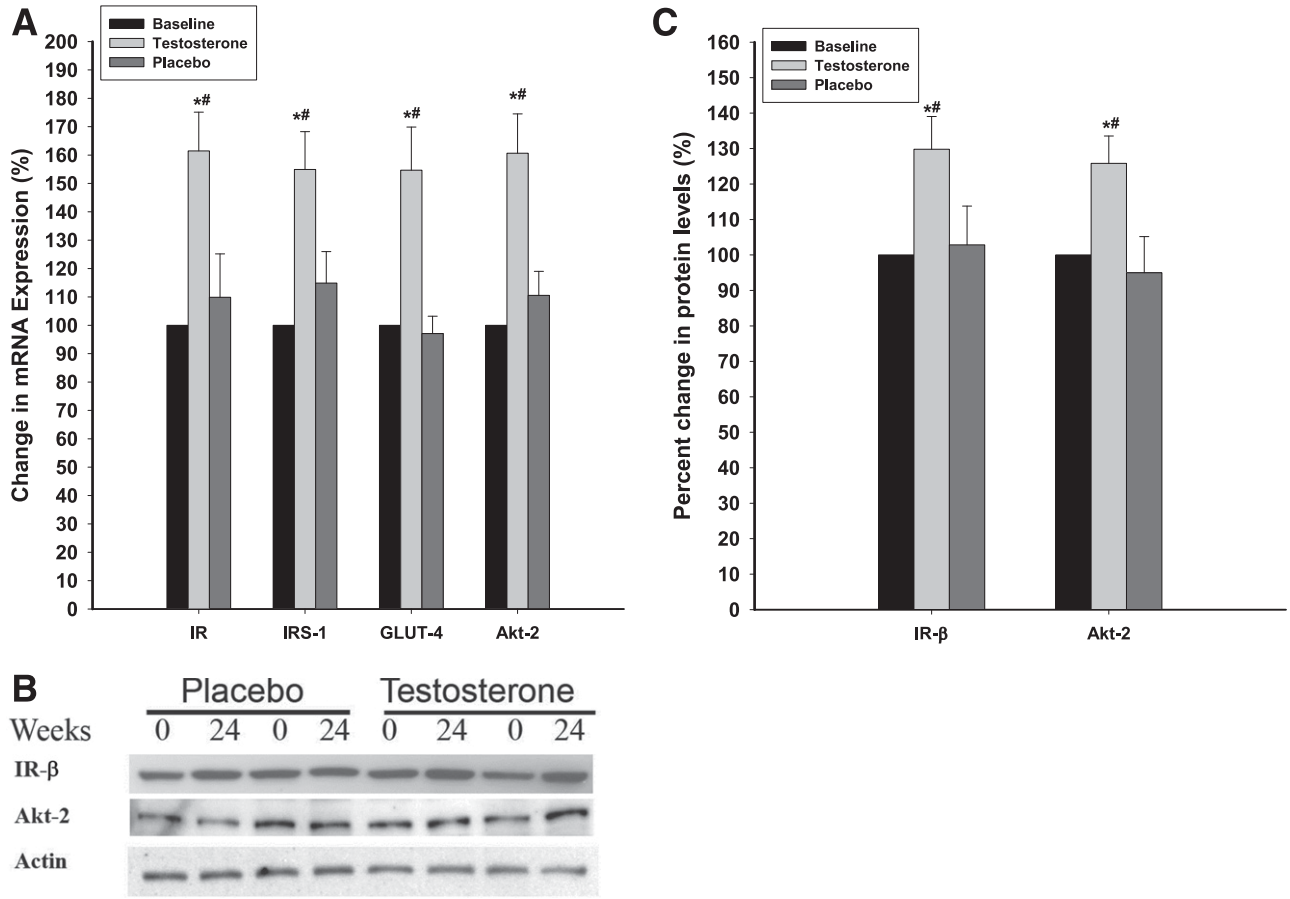

Figure 2—*A*: Percent change in mRNA expression of insulin signaling mediators (IR-β, IRS-1, GLUT4, and AKT-2) in adipose tissue after 24 weeks of testosterone or placebo treatment. $N = 16$ vs. 10 (testosterone vs. placebo). IR, IRS-1, AKT-2, and GLUT4 mRNA expression increased significantly by 60 ± 11%, 54 ± 10%, 54 ± 12%, and 59 ± 10%, respectively, after testosterone treatment compared with placebo ($P < 0.01$ for all). *B*: Representative Western blot showing basal protein levels of IR-β subunit and AKT-2 in adipose tissue after 24 weeks of testosterone or placebo treatment. *C*: Percent change in protein levels of IR-β subunit and AKT-2 in adipose tissue after 24 weeks of testosterone or placebo treatment. $N = 16$ vs. 10 (testosterone vs. placebo). Protein density was measured and corrected for loading with actin. Protein levels of IR-β subunit and AKT-2 increased by 29 ± 10% and 25 ± 11%, respectively ($P < 0.05$), after testosterone treatment compared with placebo. Data are represented as mean ± SE. Baselines normalized to 100% and changes from baselines calculated; therefore, the first bar in each group is the baseline for both testosterone and placebo treatment. Change in testosterone group was compared by *t* test with baseline (*$P < 0.05$) and with placebo (#$P < 0.05$).

We conclude that testosterone treatment in hypogonadal men with type 2 diabetes has insulin-sensitizing and anti-inflammatory effects in addition to a reduction in adiposity and an increase in the lean body mass. The increase in the expression of genes related to insulin signal transduction and the suppression of genes interfering with the action of insulin probably account for this insulin-sensitizing effect. There is also an improvement in sexual function. Future long-term studies will determine whether these effects are sustained in the long term and whether they translate into an improvement in clinical outcomes.

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Author Contributions. S.D. planned and executed the study, wrote the manuscript, and performed the statistical analysis. H.G. analyzed samples, performed statistical analyses, and wrote the manuscript. M.B., N.D.K., S.S., A.M., and A.C. executed the study and reviewed the manuscript. S.A. and K.G. analyzed samples. J.H. executed the study. M.P. analyzed MRI images. P.D. put forth the hypothesis, planned and interpreted the study, and wrote the manuscript. S.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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