Independent Effects of Testosterone on Lipid Oxidation and VLDL-TG Production

A Randomized, Double-Blind, Placebo-Controlled, Crossover Study

Christian Høst,[1] Lars C. Gormsen,[1] Britt Christensen,[1] Niels Jessen,[1,2] David M. Hougaard,[3] Jens S. Christiansen,[1] Steen B. Pedersen,[1] Michael D. Jensen,[4] Søren Nielsen,[1] and Claus H. Gravholt[1]

Low testosterone (T) levels in men have been shown to predict development of the metabolic syndrome, but the effects of T on lipid metabolism are incompletely understood. In a randomized, double-blind, placebo-controlled, crossover study, 12 healthy, young males received gonadotropin-releasing hormone agonist treatment 1 month prior to 3 of 4 trial days to induce castrate levels of T. On trial days, T gel was applied to the body containing either high or low physiological T dose or placebo. On the 4th trial day, participants constituted their own eugonadal controls. Each study comprised a 5-h basal period and a 3-h hyperinsulinemic-euglycemic clamp. Short-term hypogonadism did not affect VLDL triglyceride (TG) secretion, nor did it affect VLDL-TG concentrations. It was, however, characterized by lower total lipid oxidation. In addition, acute rescue with high physiological T increased VLDL-TG secretion during both basal and clamp conditions. These data show that T can act through fast nongenomic pathways in the liver. In addition, the early hypogonadal state is characterized by decreased total lipid oxidation, but whether these changes represent early hypogonadal metabolic dysfunction warrants further investigations. T is not a major determinant of resting VLDL-TG kinetics in men. *Diabetes* 62:1409–1416, 2013

I
n recent years, interest in testosterone (T) has been increasing due to the accumulating evidence associating low T levels in men with augmented endocrine and cardiovascular morbidity and mortality (1–3). Fundamentally, T is regarded as an anabolic and lipolytic hormone, and in hypogonadal patients, T therapy increases fat-free mass and muscle mass (4) and decreases fat mass (FM) (5). Despite favorable body composition changes, the effects of T on lipid metabolism, including levels of lipids and triglycerides (TGs), are contradictory (6–11) and, unfortunately, much of what is known has been inferred by examining results derived from rodent studies or studies comparing men and women.

Hypogonadal men are prone to develop a metabolic profile characterized by insulin resistance and hypertriglyceridemia (2,12). Although diabetic patients have greater VLDL-TG secretion and preserved suppression after acute insulin exposure (13), very little data demonstrate T's direct effects on VLDL-TG kinetics. Recently, however, overweight hypogonadal patients were shown to store more meal-derived free fatty acids (FFAs) in fat depots and have lower 6-h postabsorptive fat oxidation than BMI-matched controls (14). This underscores the importance of considering both fasting and postabsorptive conditions when investigating T effects on whole-body lipid turnover.

Compared with women, men have greater fasting plasma concentrations of total TG (15) and VLDL-TG (16), whereas similar (17) or lower VLDL-TG secretion and clearance rates (18,19) have been reported. If T was an important determining factor for such sex differences, it would imply a greater secretion and clearance of VLDL-TG particles in the hypogonadal state, whereas T substitution would have the opposite effect. This hypothesis is supported by rodent studies, in which androgen receptor knockout mice show increased hepatic lipogenesis and decreased lipid oxidation (20), and castrated male rats show increased TG uptake in intra-abdominal fat depots (21). Conversely, T increases lipolysis and reduces adipose tissue lipoprotein lipase (LPL) activity, decreasing TG uptake in abdominal fat (22,23). It also stimulates palmitate oxidation in myotubes from male donors (24). Any T treatment, however, will inevitably lead to significant body composition changes and, as a result, changes in resting energy expenditure (REE), substrate oxidation, and aerobic capacity.

Therefore, to test if T exerts direct effects on hepatic and tissue lipid metabolism, it is necessary to measure these parameters before body composition changes. To overcome this problem, we chose a model of acute sex steroid withdrawal of healthy young men and timed our measurements to occur before significant changes in body composition could take place. VLDL-TG kinetics and oxidation were investigated using ex vivo–labeled [1-14C]triolein and the primed-constant isotope dilution technique (25). In addition, we investigated the regulation of T on key enzymes involved in lipolysis during basal and hyperinsulinemic-euglycemic circumstances.

RESEARCH DESIGN AND METHODS

Subjects. Twelve healthy, nonsmoking male volunteers participated in this study. All volunteers displayed normal primary and secondary sex characteristics

and none of them used medication or had a positive family history of diabetes. The exclusion criteria included known heart disease, vascular disease, present or former cancer, and use of androgenic steroids. Men who were planning to participate in competitive sport events during the subsequent year were not included. At baseline, all volunteers had normal fasting plasma glucose (5.2 [4.7–5.7] mmol/L), insulin (33.4 [15.8–54.6] pmol/L), erythrocyte sedimentation rate, complete blood count, lipid profile, and renal and hepatic blood tests, and all had normal levels of T (18.6 [8.3–32.9] nmol/L) as well as luteinizing hormone (4.8 [1.7–8.1] IU/L) and follicle-stimulating hormone (3.2 [1.2–6.6] IU/L). All volunteers received oral and written information concerning the study prior to giving written, informed consent. The protocol was approved by the Local Ethical Scientific Committee (M-20070046), registered at clinicaltrials.gov (NCT-00613288), and performed in accordance with the Helsinki Declaration II.

Research design. This study was a randomized, double-blinded, placebo-controlled, crossover study with a washout period of at least 4 weeks. The 12 eligible men were randomly assigned to different treatment series consisting of four arms with two coupled days: a day 1 or "metabolic day" and a day 2, allocated for dual-energy X-ray absorptiometry estimation of body composition and VO_2max, respectively. For the latter, a 6-min submaximal exercise test with continuous monitoring of heart rate was performed on a bicycle ergometer (Monark Ergometric 829 E; Monark Exercise, Varberg, Sweden) using a workload of 300–1,200 kpm/min, depending on age and reported physical activity by the subject, and maximal aerobic capacity (VO_2max) was calculated (26). Hypogonadism was achieved by subcutaneous injection of gonadotropin-releasing hormone (GnRH) agonist (7.5 mg leuprorelide, Eligard; Astellas Pharma, Wallisellen, Switzerland) 1 month before trial session in three out of four trial sessions. Initially, GnRH agonist treatment results in a burst of T followed by gradual T decline. Therefore, gonadotropins and T levels were measured 1 week before trial days, and volunteers that did not present with castrate levels of T at this point were reinjected with GnRH agonist and the session was postponed. Thus, hypogonadal trial days were preceded by at least 7–10 days of castrate levels of T, designed to achieve stable changes in their metabolic state.

On 3 of 4 trial days (day 1), gel containing physiological or supraphysiological T gel (Testogel, 50 or 150 mg; Bayer Schering Pharma AG, Berlin, Germany) or placebo gel (produced by the local hospital pharmacy) was applied to the upper body of the healthy volunteer and allowed to dry. T gel has previously been shown to result in normophysiological plasma T levels within 2–3 h (27). On one of four trial sessions, the healthy volunteers served as their own eugonadal controls. In order to minimize carryover and period effects, each of the four treatment modalities were equally distributed throughout the 12 treatment series, in random order, and with at least 1-month intervals between sessions. One volunteer completed only two of four sessions due to an unrelated event (prolapse of an intervertebral disc).

Three days before the study, volunteers were instructed not to participate in heavy physical exercise or to drink alcoholic beverages. After an overnight fast (>10 h), subjects were admitted to the clinical research unit and confined to bed. At 07:00 A.M. ($t = -120$ min), a catheter was placed into an antecubital vein for infusion of saline to maintain catheter patency. Another catheter was inserted into a contralateral hand vein and kept in a thermo-regulated heating box (60°C) for sampling of arterialized venous blood. After collection of baseline blood samples, T or placebo gel was applied ($t = -120$ min) as described above. At 08:00 A.M. ($t = -60$ min), 20% of the [1-14C]VLDL-TG tracer was infused as a bolus, and a constant infusion of the remaining 80% was started. At $t = 180$ min, a 3-h infusion of human insulin (Actrapid; Novo Nordisk A/S, Bagsværd, Denmark) commenced (0.6 mU · kg total body mass^{-1} · min^{-1}). Plasma glucose was measured every 10 min and clamped at 5 mmol/L by a variable infusion of 20% glucose. The glucose infusion rate during the last hour of the clamp (M value) was used as an index of insulin sensitivity. Blood samples were drawn to determine VLDL-TG specific activity (SA) at $t = 0$, 120, 150, and 180 min (basal period) and 300, 330, and 360 min (clamp period). Insulin and metabolite concentrations were determined every 60 min. Breath samples to determine $^{14}CO_2$ SA were obtained at $t = 0$, 120, 150, 180, 300, 330, and 360 min. Indirect calorimetry was performed from $t = 60–80$ min and from $t = 360–380$ min. At $t = 120$ and 210 min, fat and muscle biopsies were obtained. At 360 min, all catheters were removed, plasma glucose was stabilized, and the participants had lunch and were discharged.

VLDL-TG kinetics. VLDL-TG kinetics were assessed by a previously validated method from our laboratory, in which the TG moiety of the VLDL particle is ex vivo labeled by [1-14C]triolein and subsequently reinfused as a primed-continuous infusion. This approach enables a short-term experiment circumventing possible recirculation of the tracer and enables calculation of VLDL-TG kinetics without the use of complicated compartmental models. It assumes isotopic and metabolic steady-state conditions but is otherwise conceptually straightforward (25).

VLDL-TG Ra (Rate of appearance): primed-constant infusion. Isotopic and metabolic steady state was reached within the first 2 h of the infusion (slope

of regression not significantly different from zero), and we therefore calculated VLDL-TG Ra (μmol/min) by standard isotopic dilution principles:

$$VLDL\text{-}TG\ Ra\ (\mu mol/min) = \frac{F}{SA}$$

where F (dpm/min) denotes infusion rate and SA denotes plateau SA (dpm/μmol). We used approximately one-fifth of total tracer available as priming bolus.

Clearance was calculated as

$$VLDL\text{-}TG\ clearance\ (mL/min) = \frac{VLDL\text{-}TG\ Ra}{VLDL\text{-}TG\ conc}$$

VLDL-TG fatty acid oxidation: primed-constant infusion. Fractional oxidation (%) of the infused [1-14C]VLDL-TG was calculated as follows:

$$fractional\ VLDL\text{-}TG\ oxidation = \frac{^{14}CO_2\ SA \times VCO_2}{k \times Ar \times F}$$

Here, $^{14}CO_2$ SA is SA of $^{14}CO_2$ in expired air (dpm/μmol CO_2), VCO_2 is CO_2 flow rate (mL/min), k is the volume of CO_2 at 20°C and 1 atm pressure (22.4 L/mol), Ar is the fractional acetate carbon recovery factor in breath CO_2, and F is the tracer infusion rate. Sidossis et al. (28) has previously calculated Ar to be 0.56 for resting conditions.

The total VLDL-TG oxidation rate (μmol/min) was calculated as

$$VLDL\text{-}TG\ oxidation\ (\mu mol/min) = fractional\ VLDL\text{-}TG\ oxidation \\ \times VLDL\text{-}TG\ Ra$$

VLDL-TG tracer preparation. The procedure used to label the TG moiety of the VLDL particle ex vivo has been described previously in detail (29). In brief, 80 mL of blood was drawn from each participant 1 week before trial sessions, plasma was separated by a short spin, 30 μCi [1-14C]triolein (PerkinElmer, Inc., Turkku, Finland) dissolved in 300 mL ethanol (Merck & Co., Inc., Whitehouse Station, NJ) was added, and the solution was then sonicated at 37.8°C for 6 h. Subsequently, labeled VLDL particles were separated from other particles in plasma by 18 h of ultracentrifugation at 98,350g and 4°C.

Body composition. Body weight was measured to the nearest 0.1 kg, height was measured to the nearest 0.5 cm at inclusion, and BMI was calculated. We measured total and regional FM (g) and lean body mass (LBM) (g) by dual-energy X-ray absorptiometry using a Hologic 2000/w osteodensitometer (Hologic, Waltham, MA). Visceral FM was determined manually after placement of a region of interest from L2 to L4 and laterally extending to the outer rim of the rib cage (30), an area shown to contain predominantly visceral adipose tissue (31). Additionally, this region was corrected for area (Table 1).

Indirect calorimetry. REE and substrate oxidation rates were measured by indirect calorimetry (Deltatrac monitor; Datex Instruments, Helsinki, Finland).

Tissue biopsies. At $t = 120$ and 210 min (30 min into the clamp), muscle biopsies were obtained from the vastus lateralis muscle with a Bergström biopsy needle under local anesthesia. A total amount of ~200 mg muscle was aspirated, and biopsies were cleaned for blood (within 15 s) and snap frozen in liquid nitrogen. Muscle biopsies were stored at –80°C until analyzed. After the muscle biopsy, a subcutaneous fat biopsy of ~1–2 g from the periumbilical region was obtained by liposuction, cleaned for blood, and snap frozen in liquid nitrogen.

Real-time RT-PCR for mRNA analysis. Total RNA was isolated from adipose tissue biopsies using standard techniques. cDNA was synthesized with the Verso cDNA Kit AB 1453 (Thermo Fisher Scientific) using random hexamers. Real-time PCR for target genes was performed with mRNA levels of β2-microglobulin as internal control. The primers listed in Table 2 were used. The PCR reactions were performed in duplicate using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Inc., Woburn, MA) in an ICycler from Bio-Rad (Hercules, CA) using the following protocol: one step at 95°C for 10 min and then 95°C for 3 s, 57°C for 20 s, and 72°C for 1 s. The threshold cycle was calculated, and the relative gene expression was calculated essentially as described in User Bulletin no. 2, 1997 (PerkinElmer Cetus, Norwalk, CT).

Cell signaling analysis. Fat and muscle biopsies were homogenized as previously described (32). Western blot analyses were used to assess expression and phosphorylation levels of various proteins. The phosphospecific (Ser^{79})-ACC antibody was from Millipore (Billerica, MA), total ACC expression was assessed using horseradish peroxidase–conjugated streptavidin (Pierce Chemical, Rockford, IL), and CD36 and anti–β-actin were measured with antibodies from Abcam (Cambridge, U.K.). Goat anti-rabbit IgG horseradish peroxidase (GE Healthcare, Buckinghamshire, U.K.) was used as secondary antibody. Blots were developed using enhanced chemiluminescence reagents (Amersham) and quantified using the UVP BioImaging System (UVP, Upland, CA).

Analytical techniques. Plasma glucose was analyzed in duplicate using the glucose oxidase method (Beckman Coulter, Palo Alto, CA). Insulin and

TABLE 1
Characteristics of healthy subjects at each of four treatment modalities

	Control	H+P	H+T50	H+T150	P value*
n	12				
Age (years)	23.1 ± 2.5				
BMI (kg/m^2)	23.7 ± 1.8	23.7 ± 2.0	23.5 ± 1.9	23.5 ± 1.5	0.99
Total FM (kg)	13.7 ± 4.8	13.6 ± 4.4	13.0 ± 3.6	13.6 ± 4.6	0.98
Visceral FM (g)	1,004 ± 382.2	1,022.7 ± 321.1	977.5 ± 328.2	973.2 ± 338.3	0.99
Visceral FMcorr (g/cm^2)	18.2 ± 7.7	18.8 ± 6.3	18.0 ± 6.8	18.3 ± 6.6	0.99
Total LBM (kg)	60.4 ± 8.0	59.9 ± 7.6	59.8 ± 7.9	59.5 ± 7.3	0.99
VO$_{2max}$ (mL O$_2$/kg · min)	47.2 ± 2.9	46.9 ± 2.9	47.3 ± 2.9	47.3 ± 4.0	1
REE (kcal/day) BASAL	1,823.1 ± 60.6	1,815.3 ± 52.5	1,800.7 ± 59.3	1,768.5 ± 76.6	0.93
REE (kcal/day) CLAMP	1,833 ± 55.0	1,837.3 ± 44.7	1,801.6 ± 49.2	1,829.3 ± 50.6	0.99

Data are mean ± SD. FMcorr, fat mass corrected for area; VO$_{2max}$, maximal oxygen uptake. *ANOVA, four-arm model.

C-peptide were measured with an immunoassay (DAKO, Glostrup, Denmark). Serum FFA was determined using a commercial kit (Wako Chemicals, Neuss, Germany). T was measured by liquid chromatography tandem mass spectrometry. The limit of detection was 0.1 nmol/L, and the working range was 0.2–100 nmol/L, with a coefficient of variation of <10%. Sex hormone–binding globulin (SHBG), follicle-stimulating hormone, and luteinizing hormone were analyzed on the Architect i2000 platform (Abbott, IL). The working ranges were 0.1–250 nmol/L, 0.05–150 IE/L, and 0.07–250 IE/L, respectively.
Breath $^{14}CO_2$ SA. Subjects expired into breath bags (IRIS breath bags; Wagner Analysen Technik, Bremen, Germany), and exhaled air passed through a solution containing benzethonium hydroxide (Sigma-Aldrich, Inc.) with thymolphthalein (Sigma-Aldrich, Inc.) in a scintillation vial. A color change (blue to clear) occurredwhen exactly 0.25 mmol CO_2 was trapped in the solution. Scintillation fluid was added to the vial, and $^{14}CO_2$ activity was measured by liquid scintillation counting to <2% counting error.
Plasma VLDL-TG concentration and SA. VLDL was isolated from ~3 mL of each plasma sample by ultracentrifugation. The supernatant containing the VLDL fraction was obtained by tube slicing (Beckman Instruments) and transferred to a scintillation vial. A sample was analyzed for TG concentration, and the plasma concentration of VLDL-TG was calculated. Scintillation fluid was added, and ^{14}C activity was counted.
Statistics. Statistical comparisons for groups over time were analyzed by repeated-measures ANOVA, with Geisser-Greenhouse adjustments for nonsphericity and Bonferroni post hoc, where appropriate. One-way ANOVA was used to analyze baseline data and data obtained only once per period (basal and clamp periods, respectively). All statistical calculations were performed using SPSS for Windows, version 18.01 (SPSS Inc., Chicago, IL). Nonnormally distributed data were transformed to better approximate Gaussian distribution.

We considered three statistical models of relevance: the full four-arm model, the three-arm model consisting of the hypogonadal arms only, thereby assessing the acute intervention with the placebo arm as the functional "control" arm, and lastly the two-arm model comparing sustained hypogonadism to the eugonadal state. Data are shown as mean ± SD or median (range). P values <0.05 were regarded as statistically significant.
Sample size was determined as follows. Based on our volunteer's body composition, presumed VLDL-TG levels, and previous studies performed in our laboratory, we presumed basal VLDL-TG production rates of 60 mmol/min with an SD of Δ values of 10 mmol/min. Power was set at 0.80 and α at 5%. As no prior studies have investigated the effects of T on VLDL-TG production rates, we considered differences between study days to be ~20% or 12.5 mmol/min. Sample size was then calculated to be 12 subjects.

RESULTS

Subject characteristics. Body composition, fat distribution, VO$_2$max, and REE were similar in all four situations (Table 1).
Testosterone. At baseline ($t = -120$), all hypogonadal states had significantly lower T concentrations than the control state. T concentrations were significantly different during the basal period ($t = 180$), clamp period ($t = 360$), and trial day, with placebo arm levels significantly lower at all time points (all $P < 0.001$) (Fig. 1).

TABLE 2
Primers for PCR analysis

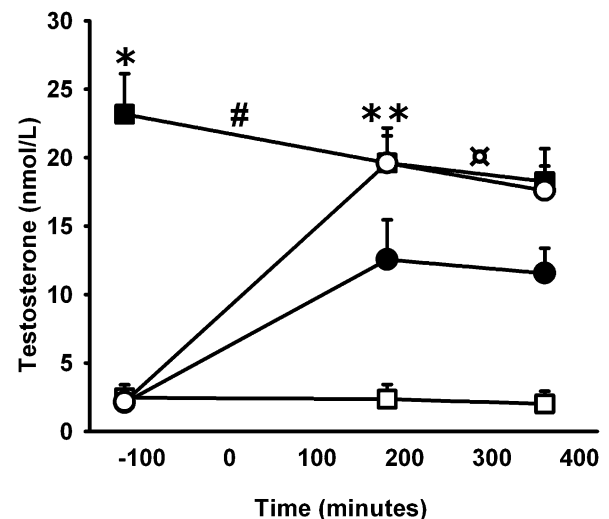| mRNA	Primer pairs
HSL	5′-GAAGGCGGCACGGACGCC-3′ 5′-GCTGGTGCGGCGGGACAC-3′
LPL	5′-ATGGAGAGCAAAGCCCTGCTC-3′ 5′-CACCCAACTCTCATACATTCC-3′
Perilipin	5′-GGAGCGAGGATGGCATGCAAC-3′ 5′-TCTGGAAGATTCGCAGGT-3′
Androgen receptor	5′-CTCCAACGCCTCCACACCCAG-3′ 5′-CCCCGAGAGAGGTTGCGTCCC-3′
ERα	5′-CAGAGAAAGATTGGCCAGTACC-3′ 5′-CTTGTGTTTCAACATTCTCCC-3′
ADR-α2	5′-GGCCTGCTCATGCTGCTCACC-3′ 5′-TCGCACCAAGTCTTGCCGAAGT-3′
ADR-β1	5′-GTGGCCCTACGCGAGCAGAAG-3′ 5′-GCGTAGCCCAGCCAGTTGAAGA-3′
ADR-β2	5′-ACAGCCATTGCCAAGTTCGAC-3′ 5′-ATGATCACCCGGGCCTTATTCTT-3′
β2-microglobulin	5′-AATGTCGGATGGATGAAACC-3′ 5′-TCTCTCTTTCTGGCCTGGAG-3′

FIG. 1. Testosterone levels for T150 (hypogonadal + 150 mg T substitution, ○), T50 (hypogonadal + 50 mg T substitution, ●), P (placebo, □), and C (control, ■) during trial days. Data are means, and error bars are SD. *, baseline (hypogonadal arms vs. control, arm); #, basal period (T value at $t = 180$); ¤, clamp period (T value at $t = 360$); **, trial day ($t = -120$ to 360); all $P < 0.001$. Data are mean ± SEM.

Circulating metabolites, insulin, and FFA. Concentrations of TG, VLDL-TG, FFA (Fig. 2*A* and *B*), insulin, glucose, and glucose infusion rates (Supplementary Fig. 1) were comparable during both basal and clamp periods in all statistical models (data not shown). TG, VLDL-TG, and FFA showed a similar course over time.

VLDL-TG secretion and clearance. VLDL-TG SA steady state was effectively reached in the last hour of both the basal and the clamp period. Basal and clamp VLDL-TG secretion were comparable in both the two- and four-arm models ("2" and "4"). Acute T treatment of the hypogonadal state, however, resulted in greater secretion at the T150 arm (three-arm model ["3"]: basal, $P < 0.05$; clamp, $P = 0.003$) (Fig. 2*C*). The suppression of VLDL-TG secretion during clamp was highly significant in all arms (all <0.03 [paired Student t test]; Δcontrol = 20.2% vs. Δplacebo = 22.2% vs. ΔT50 = 23.6% vs. ΔT150 = 31.5%), but there was no difference between the treatment arms. There were no differences in VLDL-TG clearance between groups during either period (Fig. 2*D*).

VLDL-TG fatty acid oxidation. Breath $^{14}CO_2$ SA steady state was reached in the clamp period, but not in the basal period, allowing calculation of VLDL-TG FA oxidation only during clamp (Fig. 3*A*). The fraction of VLDL-TG secretion that was oxidized tended to be higher in controls (two-arm model, $P = 0.07$), but there was no effect of T intervention (three-arm model, $P = NS$). Likewise, eugonadal VLDL-TG FA oxidation was comparable with placebo, and no effect of acute T was detected (Fig. 3*B* and *C*, respectively).

REE. The respiratory quotient (RQ) during clamp was significantly greater in all hypogonadal arms as compared with control (four-arm model, $P = 0.03$; two-arm model, $P = 0.002$) (Fig. 4*A*), and ΔRQ increased significantly more in the placebo arm as compared with the control arm (two-arm model, $P = 0.008$) (Fig. 4*B*). Accordingly, basal lipid oxidation was comparable between study arms but significantly lower during clamp in all hypogonadal arms as compared with the control state (four- and two-arm models, both $P = 0.002$) (Fig. 4*C*). However, it was unaffected by acute T (three-arm model, $P = NS$). No overall effect on insulin-mediated glucose disposal corrected for LBM was seen (Glucose [Rd]; four-arm model, $P = NS$), but a significantly greater oxidative glucose disposal rate was seen with sustained hypogonadism, but only a trend for total glucose disposal was detected (two-arm model, $P < 0.05$ and $P = 0.07$, respectively) (Fig. 4*D*).

Gene expression. Muscle hormone-sensitive lipase (HSL) mRNA expression was similar during basal and clamp periods, except for the two-arm model during clamp ($P = 0.03$). This may represent a type 1 statistical error, because
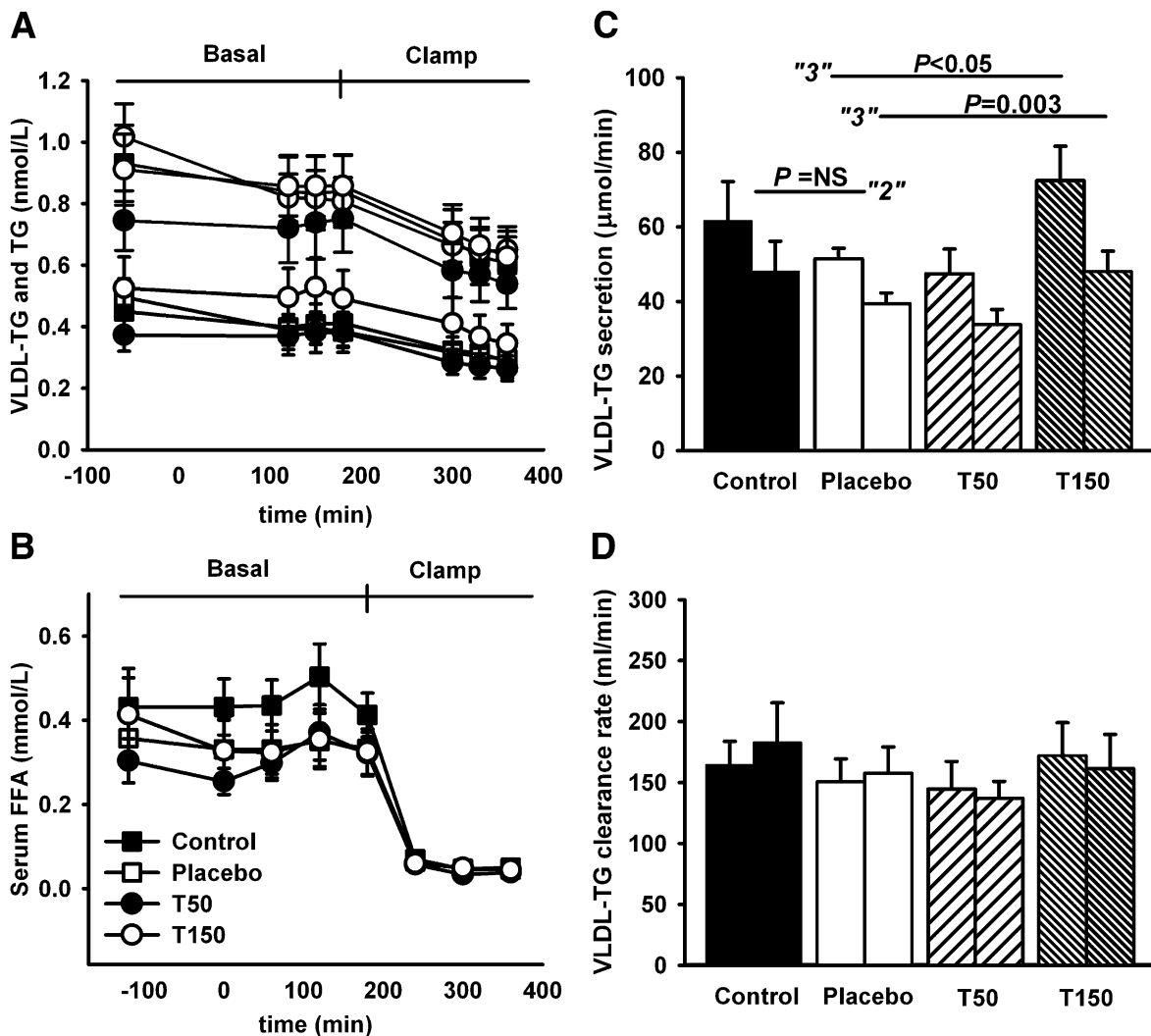

FIG. 2. TG and VLDL-TG (*A*) and FFA (*B*) levels during basal and clamp period. Both were equally suppressed during hyperinsulinemia. VLDL-TG secretion (*C*) and clearance rates (*D*) during basal and clamp periods. Data are mean ± SEM.
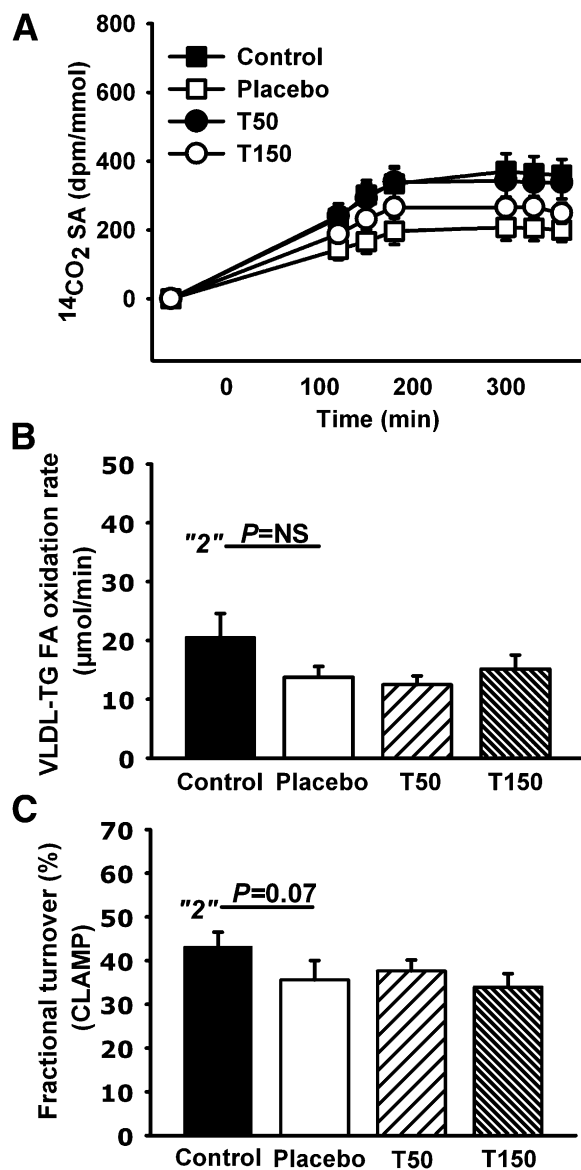
FIG. 3. Breath $^{14}CO_2$ SA steady state was reached in the clamp period, but not in the basal period (*A*). Therefore, only VLDL-TG FA oxidation data from the clamp period are illustrated and analyzed statistically. VLDL-TG FA oxidation rates (*B*) and as a fraction of secretion (*C*). Data are mean ± SEM.

we found no obvious physiological explanation for suppression of HSL only in controls during clamp. In support, no change in adipose tissue HSL expression was detected. Muscle LPL expression was similar during both conditions, but basal fat tissue LPL mRNA abundance was significantly reduced in the eugonadal condition compared with placebo (two-arm model, $P = 0.03$). Perilipin mRNA expression was comparable in muscle and fat tissue at all instances (Supplementary Fig. 2). In addition, basal expression of the androgen receptor, estrogen receptors (ERα and ERβ), and adrenergic receptors (ADR-α2, -β1, and -β2) in both muscle and adipose tissue was not different between conditions (data not shown).

DISCUSSION

This study is the first to investigate direct and temporal effects of T on VLDL-TG kinetics and energy expenditure

in men. We report that short-term hypogonadism and acute T rescue have differential effects on VLDL-TG kinetics. Although VLDL kinetics and levels of VLDL-TG are unaffected by short-term chronic T fluctuations, acute high-normal T increases VLDL-TG secretion in male hypogonadism within hours, independently of FFA concentrations and body fat distribution. In addition, the early hypogonadal state is characterized by intact VLDL-TG oxidation but impaired total lipid oxidation, which we speculate may be an early feature of hypogonad metabolic dysfunction.

Currently, our understanding of T's metabolic effects in both acute and longer-term settings is incomplete, and we have no definite understanding of genomic versus nongenomic effects. Many studies have been performed on different hypogonadal populations with regard to age, body composition, and degree of hypogonadism, which makes firm conclusions difficult. For instance, recent epidemiological studies have demonstrated a higher cardiovascular disease risk in men (33), both among T-sufficient and untreated hypogonadal men (3,34,35). Likewise, the effects of T therapy on glucose control (36) and risk factors such as cholesterol, C-reactive protein (37), and TG concentrations are contradictory (6–11), which calls for studies assessing direct effects of T.

At first glance, the preserved VLDL-TG secretion with short-term hypogonadism and increment after acute T150 substitution seems puzzling. It may, however, be explained by the capability of T to activate both genomic and nongenomic pathways, in which the effects can be biphasic depending on the actual hormone concentration (38,39) and temporal exposure. VLDL-TG secretion is a complicated and not fully understood process, in which the activity of numerous enzymes stimulates the two-step process characterized by the formation of the ApoB-containing VLDL precursor in the endoplasmic reticulum (first step) and the later fusion with a larger triacylglyceride droplet to form the mature VLDL particle (second step) (40). Although there is no available data supporting a role for T governing these enzymes, longer-term T treatment may well influence hepatic lipid homeostasis through different pathways. For instance, isolated hepatocytes from castrated male rats show increased TG synthesis comparable to that of female rats, whereas 1 month of low-dose T substitution suppresses synthesis to eugonadal levels (38). This is in line with the increased lipogenesis and hepatic steatosis found in androgen receptor knockout mice fed a high-fat diet (20). Conversely, VLDL secretion in male rats is unaffected by castration (41), which accords with unchanged VLDL Apo-B kinetics during 6 months of T and/or growth hormone treatment in healthy, elderly, eugonadal men (9) and both VLDL-TG and VLDL Apo-B-100 kinetics in obese, premenopausal women after 3 weeks of T treatment (42). Thus, T may affect hepatic TG synthesis and VLDL-TG secretion differently, and our data support the concept that T is not a major determinant of the VLDL-TG fatty acid secretion pattern in men.

The increased VLDL-TG secretion during acute T treatment expands our current knowledge on the ability of androgens to activate fast nongenomic pathways (20). However, no data exist describing the molecular regulation of secretion pattern by T, but we speculate that T can elicit cAMP and PKA activation through the SHBG receptor in the plasma membrane (43). In liver cells, physiological levels of T have been shown to increase cAMP levels within 15 min after exposure, but when used at higher, nonphysiological concentrations, only increased inhibition of
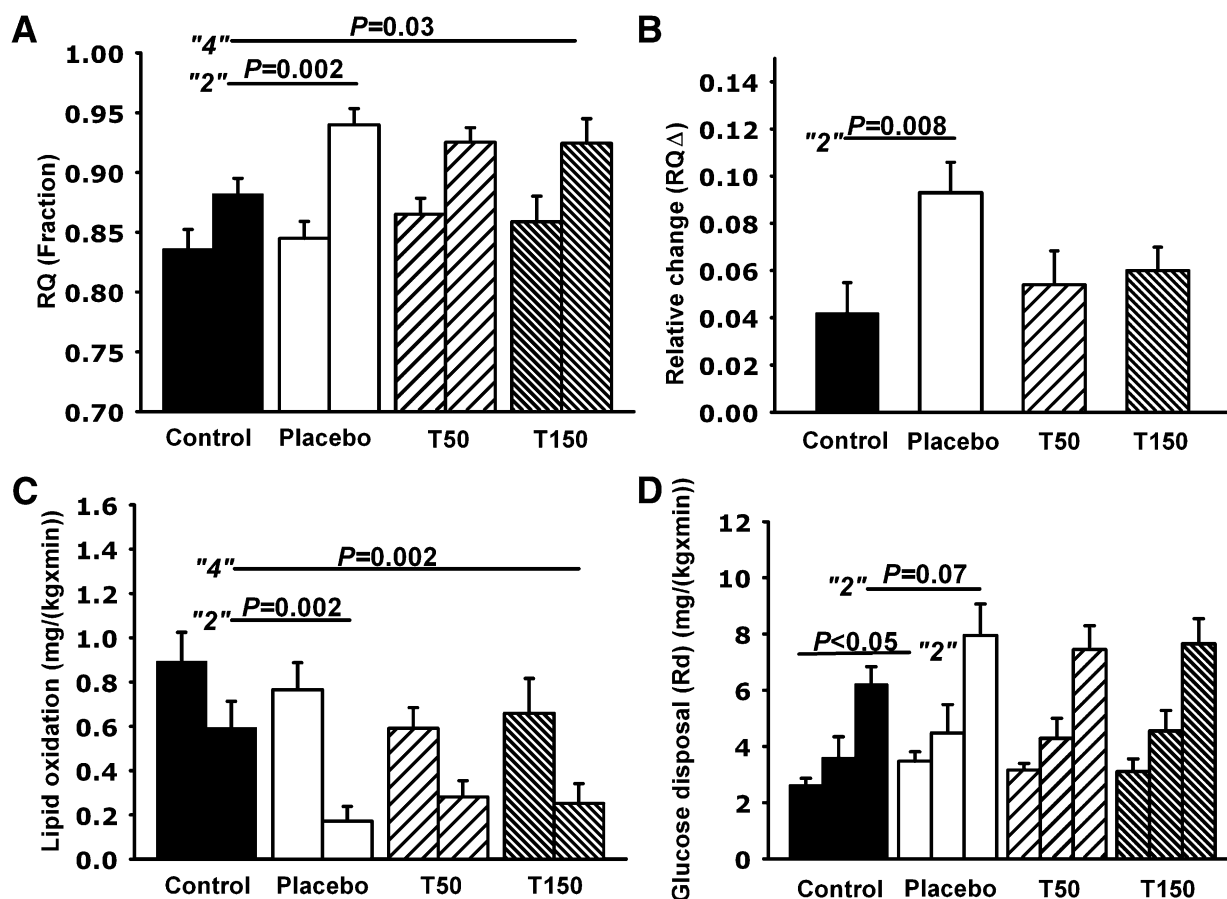
FIG. 4. RQ (*A*), change in RQ (ΔRQ) (*B*), lipid oxidation rates (*C*), and insulin-stimulated glucose disposal (columns represent oxidative glucose disposal, nonoxidative glucose disposal, and total glucose disposal) (*D*). *A* and *C*: Columns represent basal and clamp period, respectively. Data are mean ± SEM.

the SHBG-steroid complex binding to membranes occurred (39). Other studies have shown that activation of AMP-activated protein kinase–dependent mechanisms within minutes facilitates exocytosis of lipoproteins and TGs (44), and yet others have demonstrated that fluctuations in hepatocellular Ca^{2+} concentrations can contribute either directly or indirectly to microsomal TG transfer protein activation, and thereby to the assembly and secretion of mature VLDL-TG particles (45). We therefore speculate that the T150 substitution, by reaching a certain threshold, could have activated such pathways and facilitated assembly and exocytosis of mature VLDL particles. Another mechanistic explanation includes the activation of a recently described lipase, arylacetamide deacetylase, which catalyzes the lipolytic mobilization of the cytosolic triacylglyceride, and which has considerable identity with HSL (46). Since androgens can upregulate lipolytic β ADRs (47) and increase catecholamine-stimulated HSL activity (22), an increased arylacetamide deacetylase activity with T substitution, although speculative, could also increase VLDL secretion acutely.

Our kinetic data suggest that fluctuations in circulating T levels, both in the acute and short-term chronic setting, have no effects on VLDL-TG clearance rates. Since clearance is a composite parameter, these data do not provide detailed information on tissue-specific clearance. It does, however, seem likely that the transient increase in secreted VLDL-TG during T150 substitution must have been met by a matched increase in peripheral TG removal, explaining the comparable levels of total TG and VLDL-TG

found across study arms. Our findings on TG levels are supported by most other studies (6–9), but T therapy has also been found to suppress (10) or even increase TG levels (11). Our data, however, contrast with the general perception that hypogonadism is associated with higher TG levels (10,48), but since VLDL-TG synthesis is partly a substrate-driven process (40,49), such elevated levels may well be the result of a higher FFA release. Therefore, our finding of comparable concentrations of FFA and of gene expression levels of perilipin, HSL, and ADRs in both muscle and fat tissue could indicate that in vivo, T does not have immediate effects on tissue lipolysis. On the other hand, eugonadal FFAs were in fact higher at single time points during the basal period along with suppressed adipose tissue LPL expression. This could indicate an increased channeling of TG away from storage in fat tissue toward oxidative pathways in muscle. Although no differences in muscle LPL expression were detected, this hypothesis is supported by the trend toward higher eugonadal VLDL-TG oxidation rates and the significantly lower total lipid oxidation found in all hypogonadal arms regardless of intervention.

Impaired lipid oxidation, even before any signs of insulin resistance or changes in REE, may therefore be an early metabolic feature of male hypogonadism, in which noninhibited peripheral LPL activity may lead to increased TG uptake and storage. This, in turn, provides one potential mechanism by which hypogonadal insulin resistance may ensue over time. We do, however, acknowledge that the

current study was not designed to explore such later metabolic perturbations, and current knowledge supports the concept that multiple factors are involved in the development of hypogonadal metabolic dysfunction (50). Whereas T primarily stimulates extrahepatic and not hepatic lipid oxidation in humans (51), limited data are available on other tissues such as heart, kidney, and brain, warranting further research. This is further complicated by the fact that T treatment of hypogonadal populations of different age and etiology seems to affect metabolism and body composition differently (52).

In conclusion, short-term chronic fluctuations in T levels affect whole-body lipid oxidation, but have no effect on REE, VLDL-TG secretion, or the clearance or oxidation pattern in healthy young men. Although acute high T does not affect total lipid oxidation, it does increase VLDL-TG secretion, indicating that androgens can affect hepatic lipid metabolism through fast nongenomic pathways. In addition, we speculate that impaired lipid oxidation during hyperinsulinemia may be one contributor in the development of the obese, insulin-resistant, hypogonadal phenotype. Future studies combining tracer techniques and standardized meals should expose both healthy subjects and hypogonadal patients to graded intervals of longer-term T treatment to fully understand androgenic effects on lipid metabolism.

ACKNOWLEDGMENTS

REFERENCES

1. Jones TH. Testosterone deficiency: a risk factor for cardiovascular disease? Trends Endocrinol Metab 2010;21:496–503
2. Stanworth RD, Jones TH. Testosterone in obesity, metabolic syndrome and type 2 diabetes. Front Horm Res 2009;37:74–90
3. Bojesen A, Juul S, Birkebaek N, Gravholt CH. Increased mortality in Klinefelter syndrome. J Clin Endocrinol Metab 2004;89:3830–3834
4. Bhasin S, Storer TW, Berman N, et al. Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. J Clin Endocrinol Metab 1997;82:407–413
5. Bhasin S, Woodhouse L, Casaburi R, et al. Testosterone dose-response relationships in healthy young men. Am J Physiol Endocrinol Metab 2001; 281:E1172–E1181
6. Berg G, Schreier L, Geloso G, Otero P, Nagelberg A, Levalle O. Impact on lipoprotein profile after long-term testosterone replacement in hypogonadal men. Horm Metab Res 2002;34:87–92
7. Whitsel EA, Boyko EJ, Matsumoto AM, Anawalt BD, Siscovick DS. Intramuscular testosterone esters and plasma lipids in hypogonadal men: a meta-analysis. Am J Med 2001;111:261–269
8. Snyder PJ, Peachey H, Berlin JA, et al. Effects of testosterone replacement in hypogonadal men. J Clin Endocrinol Metab 2000;85:2670–2677
9. Giannoulis MG, Jackson N, Shojaee-Moradie F, Sonksen PH, Martin FC, Umpleby AM. Effects of growth hormone and/or testosterone on very low density lipoprotein apolipoprotein B100 kinetics and plasma lipids in healthy elderly men: a randomised controlled trial. Growth Horm IGF Res 2006;16:308–317
10. Lanfranco F, Zitzmann M, Simoni M, Nieschlag E. Serum adiponectin levels in hypogonadal males: influence of testosterone replacement therapy. Clin Endocrinol (Oxf) 2004;60:500–507
11. Jockenhövel F, Bullmann C, Schubert M, et al. Influence of various modes of androgen substitution on serum lipids and lipoproteins in hypogonadal men. Metabolism 1999;48:590–596
12. Bojesen A, Kristensen K, Birkebaek NH, et al. The metabolic syndrome is frequent in Klinefelter's syndrome and is associated with abdominal obesity and hypogonadism. Diabetes Care 2006;29:1591–1598
13. Sørensen LP, Andersen IR, Søndergaard E, et al. Basal and insulin mediated VLDL-triglyceride kinetics in type 2 diabetic men. Diabetes 2011;60: 88–96
14. Santosa S, Jensen MD. Effects of male hypogonadism on regional adipose tissue fatty acid storage and lipogenic proteins. PLoS ONE 2012;7:e31473
15. Freedman DS, Otvos JD, Jeyarajah EJ, et al. Sex and age differences in lipoprotein subclasses measured by nuclear magnetic resonance spectroscopy: the Framingham Study. Clin Chem 2004;50:1189–1200
16. Magkos F, Mohammed BS, Mittendorfer B. Plasma lipid transfer enzymes in non-diabetic lean and obese men and women. Lipids 2009;44:459–464
17. Gormsen LC, Jensen MD, Schmitz O, Møller N, Christiansen JS, Nielsen S. Energy expenditure, insulin, and VLDL-triglyceride production in humans. J Lipid Res 2006;47:2325–2332
18. Magkos F, Patterson BW, Mohammed BS, Klein S, Mittendorfer B. Women produce fewer but triglyceride-richer very low-density lipoproteins than men. J Clin Endocrinol Metab 2007;92:1311–1318
19. Mittendorfer B, Patterson BW, Klein S. Effect of sex and obesity on basal VLDL-triacylglycerol kinetics. Am J Clin Nutr 2003;77:573–579
20. Lin HY, Yu IC, Wang RS, et al. Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor. Hepatology 2008;47: 1924–1935
21. Li M, Björntorp P. Effects of testosterone on triglyceride uptake and mobilization in different adipose tissues in male rats in vivo. Obes Res 1995;3: 113–119
22. Rebuffé-Scrive M, Mårin P, Björntorp P. Effect of testosterone on abdominal adipose tissue in men. Int J Obes 1991;15:791–795
23. Mårin P, Odén B, Björntorp P. Assimilation and mobilization of triglycerides in subcutaneous abdominal and femoral adipose tissue in vivo in men: effects of androgens. J Clin Endocrinol Metab 1995;80:239–243
24. Salehzadeh F, Rune A, Osler M, Al-Khalili L. Testosterone or 17beta-estradiol exposure reveals sex-specific effects on glucose and lipid metabolism in human myotubes. J Endocrinol 2011;210:219–229
25. Sørensen LP, Gormsen LC, Nielsen S. VLDL-TG kinetics: a dual isotope study for quantifying VLDL-TG pool size, production rates, and fractional oxidation in humans. Am J Physiol Endocrinol Metab 2009;297:E1324–E1330
26. ASTRAND I. Aerobic work capacity in men and women with special reference to age. Acta Physiol Scand Suppl 1960;49:1–92
27. Fabbri A, Giannetta E, Lenzi A, Isidori AM. Testosterone treatment to mimic hormone physiology in androgen replacement therapy. A view on testosterone gel and other preparations available. Expert Opin Biol Ther 2007;7:1093–1106
28. Sidossis LS, Magkos F, Mittendorfer B, Wolfe RR. Stable isotope tracer dilution for quantifying very low-density lipoprotein-triacylglycerol kinetics in man. Clin Nutr 2004;23:457–466
29. Gormsen LC, Jensen MD, Nielsen S. Measuring VLDL-triglyceride turnover in humans using ex vivo-prepared VLDL tracer. J Lipid Res 2006;47:99–106
30. Carey DG, Jenkins AB, Campbell LV, Freund J, Chisholm DJ. Abdominal fat and insulin resistance in normal and overweight women: direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM. Diabetes 1996;45:633–638
31. Ross R, Shaw KD, Martel Y, de Guise J, Avruch L. Adipose tissue distribution measured by magnetic resonance imaging in obese women. Am J Clin Nutr 1993;57:470–475
32. Witczak CA, Jessen N, Warro DM, et al. CaMKII regulates contraction- but not insulin-induced glucose uptake in mouse skeletal muscle. Am J Physiol Endocrinol Metab 2010;298:E1150–E1160
33. Kapoor D, Malkin CJ, Channer KS, Jones TH. Androgens, insulin resistance and vascular disease in men. Clin Endocrinol (Oxf) 2005;63:239–250
34. Basaria S, Coviello AD, Travison TG, et al. Adverse events associated with testosterone administration. N Engl J Med 2010;363:109–122

35. Hyde Z, Norman PE, Flicker L, et al. Elevated LH predicts ischaemic heart disease events in older men: the Health in Men Study. Eur J Endocrinol 2011;164:569–577
36. Grossmann M. Low testosterone in men with type 2 diabetes: significance and treatment. J Clin Endocrinol Metab 2011;96:2341–2353
37. Dandona P, Dhindsa S. Update: hypogonadotropic hypogonadism in type 2 diabetes and obesity. J Clin Endocrinol Metab 2011;96:2643–2651
38. Elam MB, Umstot ES, Andersen RN, Solomon SS, Heimberg M. Deprivation and repletion of androgen in vivo modifies triacylglycerol synthesis by rat hepatocytes. Biochim Biophys Acta 1987;921:531–540
39. Frairia R, Fortunati N, Berta L, Fazzari A, Fissore F, Gaidano G. Sex steroid binding protein (SBP) receptors in estrogen sensitive tissues. J Steroid Biochem Mol Biol 1991;40:805–812
40. Gibbons GF, Wiggins D, Brown AM, Hebbachi AM. Synthesis and function of hepatic very-low-density lipoprotein. Biochem Soc Trans 2004;32:59–64
41. Weinstein I, Seltzer M, Belitsky R. The interrelation of glucagon and gonadectomy upon hepatic triglyceride metabolism in rats. Biochim Biophys Acta 1974;348:14–22
42. Wang X, Smith GI, Patterson BW, et al. Testosterone increases the muscle protein synthesis rate but does not affect very-low-density lipoprotein metabolism in obese premenopausal women. Am J Physiol Endocrinol Metab 2012;302:E740–E746
43. Rosner W, Hryb DJ, Kahn SM, Nakhla AM, Romas NA. Interactions of sex hormone-binding globulin with target cells. Mol Cell Endocrinol 2010;316:79–85
44. Puljak L, Parameswara V, Dolovcak S, et al. Evidence for AMPK-dependent regulation of exocytosis of lipoproteins in a model liver cell line. Exp Cell Res 2008;314:2100–2109
45. Cho HJ, Kang HC, Choi SA, Ju YC, Lee HS, Park HJ. The possible role of Ca2+ on the activation of microsomal triglyceride transfer protein in rat hepatocytes. Biol Pharm Bull 2005;28:1418–1423
46. Trickett JI, Patel DD, Knight BL, Saggerson ED, Gibbons GF, Pease RJ. Characterization of the rodent genes for arylacetamide deacetylase, a putative microsomal lipase, and evidence for transcriptional regulation. J Biol Chem 2001;276:39522–39532
47. Xu XF, De Pergola G, Björntorp P. Testosterone increases lipolysis and the number of beta-adrenoceptors in male rat adipocytes. Endocrinology 1991;128:379–382
48. Isidori AM, Giannetta E, Greco EA, et al. Effects of testosterone on body composition, bone metabolism and serum lipid profile in middle-aged men: a meta-analysis. Clin Endocrinol (Oxf) 2005;63:280–293
49. Lewis GF. Fatty acid regulation of very low density lipoprotein production. Curr Opin Lipidol 1997;8:146–153
50. Traish AM, Miner MM, Morgentaler A, Zitzmann M. Testosterone deficiency. Am J Med 2011;124:578–587
51. Birzniece V, Meinhardt UJ, Handelsman DJ, Ho KK. Testosterone stimulates extra-hepatic but not hepatic fat oxidation (Fox): comparison of oral and transdermal testosterone administration in hypopituitary men. Clin Endocrinol (Oxf) 2009;71:715–721
52. Zitzmann M. Testosterone deficiency, insulin resistance and the metabolic syndrome. Nat Rev Endocrinol 2009;5:673–681