Sex Steroids and Plasma Lipoprotein Levels in Healthy Women: The Importance of Androgens in the Estrogen-Deficient State

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The role of endogenous estrogens and androgens and their potential interaction in atherosclerosis is not well understood. Therefore, we investigated the effects of natural menopause and endogenous sex steroids on triglycerides (TG), a major risk factor for cardiovascular disease in women. Fasting lipid and lipoprotein concentrations, postheparin lipase activities, kinetic indicators of triglyceride lipolysis, and various hormone levels, including dehydroepiandrostenedione-sulfate (DHEA-S), (bioavailable) testosterone, and androstenedione, were determined in 18 premenopausal and 18 postmenopausal women, matched for age and body composition. Fasting plasma TG were 0.69 ± 0.29 mmol/L in postmenopausal women and 0.73 ± 0.33 mmol/L in premenopausal women (difference not significant [NS]). Approximately 30% of all plasma TG were present in the very-low-density lipoproteins (VLDLs) in both groups. No differences were found between groups in plasma lipolytic potential of TG-rich lipoproteins. Univariate analysis revealed that VLDL-TG concentrations were strongly related to insulin ($r = 0.84$, $P = .0001$) and androstenedione ($r = 0.65$, $P = .004$) in postmenopausal women. Multivariate analysis of potential determinants of VLDL-TG showed that insulin, androstenedione, and bioavailable testosterone were independent variables, explaining 87% of the variability ($r = 0.93$, $P = .0001$) in postmenopausal women. In contrast, in premenopausal women, the only identified predictor of fasting VLDL-TG in univariate and multivariate analysis was insulin ($r = 0.72$, $P = .001$). Our results show that the association of androgens with TG varied depending on androgen concentrations, the relative androgenic potential, and most importantly on hormonal milieu. Endogenous androgens were only related to plasma VLDL-TG in the estrogen-deficient state.

ENDOGENOUS estrogen deficiency by natural menopause has been associated with reduced protection from coronary heart disease (CHD).[1,2] At present, there is debate on the cause of this increased risk in postmenopausal women. A generally accepted view is that endogenous estrogen deficiency results in an atherogenic lipid profile. The transition to menopause is known to be accompanied by a raise in low-density lipoprotein (LDL) cholesterol,[3] and the results of the statin studies emphasize the importance of LDL as risk factor. However, LDL-cholesterol is a poor predictor of future cardiovascular disease in women.[4,5] In contrast, levels of plasma triglycerides (TG) are better predictors in epidemiological studies,[4] and some have even argued that their predictive power is considerably underestimated.[6] Both heterogeneity of the hypertriglyceridemic state and its complex relationship with coronary artery disease have been put forward as explanations. TG are increased in postmenopausal women in most studies,[7,8] but not all.[9,10] It remains unclear how levels of plasma TG are regulated. This is interesting for potential therapeutic interventions or risk factor modification. Few studies have addressed the subject of endogenous hormones and endogenous lipid parameters. In fact, many attempts to solve this question were unsuccessful because pharmacological manipulations have hampered physiological inference in this field. Excellent investigations with stable isotopes have been performed on the effects of exogenous estrogens on lipoprotein metabolism in postmenopausal women.[11] However, these studies did not provide insight into the effects of endogenous estrogen deficiency on TG metabolism, nor did they identify potential determinants by which effects on lipoprotein metabolism were mediated. Similarly, studies on treatment with low doses of androgens in postmenopausal women do not allow any interpretation on the interaction between estrogens and androgens.[12,13]

For this purpose, extensive sex steroid analysis were performed in pre- and postmenopausal women independent of possible confounding factors as age, body composition, smoking, or use of medication. Our studies clearly show, for the first time, the importance of estrogen status on the relationships between endogenous androgens and TG.

MATERIALS AND METHODS

Subjects

Participants were recruited by means of advertisements in local newspapers. A questionnaire on health and menopausal status was sent to all responders. Women were defined as postmenopausal when they reported their last menstrual period to have been at least 12 months ago and had elevated follicle-stimulating hormone (FSH $>$ 40 IU/L) levels at both screening and test days. Women were considered premenopausal when they had an unchanged and regular menstrual pattern during the previous 5 years, without typical climacteric complaints, and had compatible low FSH levels (20 IU/L). All healthy women, ranging in age from 47 to 52 years, were eligible for enrollment in the study when they had a uterus and 2 ovaries in situ, used no medication, currently as well as in the preceding year, and did not smoke. During a screening visit, the participant's medical history was taken with special attention to the menstrual cycle, and a physical examination was performed. At this visit, plasma lipids, FSH, and estradiol levels were determined. All women who were normolipidemic at screening (cholesterol $<$ 6.5 mmol/L and TG $<$ 2.0 mmol/L, representing approximately the 75th percentile for Dutch women in this age group and with body mass index [BMI] values between 19 and 30 kg/m^2) participated

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in the study. This careful selection of healthy women was performed because ill-defined menopausal status, age, smoking, body composition, disease, and use of various medications are known confounders in studies of risk factors on lipoprotein metabolism. Premenopausal women were tested in the follicular phase of their menstrual cycle. The study was part of a larger study to the effects of estrogens on postprandial lipid metabolism. All participants gave their written informed consent prior to the study. The study was approved by the ethical committee of the University Medical Center Utrecht.

Study Protocol

Participants had been fasting for 12 hours after a standardized oral fat-loading test, which was performed at 8 AM.[10] Fasting blood samples were then drawn at 8 PM for lipoprotein determination. Immediatialy thereafter, heparin (Heparine Leo, Leo Pharmaceutical Products BV, Weesp, The Netherlands) was given as a bolus injection in a dose of 50 U/kg body weight via an indwelling catheter in an antecubital vein for determination of plasma lipase activities. Twenty minutes after heparin administration, blood was drawn from the same antecubital vein in pre-cooled tubes and these were put instantly into ice water. To evaluate the effects of heparin-induced lipolysis on lipoprotein constituents in distinct density fractions, blood was collected in EDTA-containing tubes (Venoject, Terums, Leuven, Belgium; 0.047 mmol EDTA/10 mL tube) at baseline and 20 minutes postheparin. To determine plasma lipase activities, blood was obtained 20 minutes postheparin in heparinized tubes (Vacutainer, BD, Erebodegem, Belgium; lithium heparine 143 U/10 mL tube). No lipase inhibitor was used. Plasma was prepared by centrifugation at 4°C (2,500 relative centrifugal force [RCF] for 15 minutes) and maintained at this temperature throughout the preparation procedures.

Laboratory Assays and Measurements

Lipoprotein fractionation was done by density gradient ultracentrifugation according to Redgrave et al.[14] Preparation of lipoprotein fractions was carried out on the test day. Lipoprotein lipase (LPL) and hepatic lipase (HL) activities were measured as described.[15] Results are expressed as nmol of released free fatty acids min^{-1} (mU) per mL plasma. Whole plasma and lipoprotein fractions were assayed for cholesterol and TG with commercial enzymatic reagents. Average recoveries of TG and cholesterol in all lipoprotein fractions compared to plasma were 91.6% and 98.4%, respectively. Plasma apolipoprotein (apo)B, apoA-I, and apoE genotype were determined as reported previously.[16]

Hormonal Analysis

Measurements of hormones were performed in fasting blood samples. Serum was obtained by centrifugation within 1 hour after venipuncture, distributed in aliquots in small plastic cups, and stored at −80°C until analysis (on average 6 months later) was performed. Insulin was analyzed using the enzyme-linked immunosorbent assay method (Boehringer Mannheim, Almere, The Netherlands). Levels of growth hormone, insulin-like growth factor-1 (IGF-1), sex hormone–binding globulin (SHBG), FSH, and luteinizing hormone (LH) were determined as described.[16] Estrone (E1) and 17β-estradiol (E2) were extracted with diethyl-ether, purified, and separated by chromatography on Sephadex LH-20 columns using toluene-methanol (92:8, vol/vol) as eluent and quantitated by radioimmunoassay (RIA). Interassay variations for E1 were 11.0%, 7.0%, and 7.5% at 124, 425, and 1,180 pmol/L, respectively. Those for E2 were 23.6%, 10.0%, and 14.8% at levels of 88, 411, and 1,170 pmol/L, respectively. The sensitivity of the estradiol method is 40 pmol/L.Total testosterone was measured by RIA after extraction with diethyl ether. The interassay variations were 7.6% at 0.76 nmol/L, 5.1% at 2.3 nmol/L, and 8.1% at 13.2 nmol/L. Androstenedione (A) was also determined by RIA after extracion with hexane-toluene mixture (80:20, vol/vol) with an interassay variation of 7.6% at 1.6 nmol/L, 5.2% at 6.3 nmol/L, and 5.3% at 15.4 nmol/L. Determinations of of dehydroepiandrostenedione-sulfate (DHEA-S) were made by RIA directly in serum using the commercially available reagents from Diagnostic Products Corp (Coat-a-Count; Los Angeles, CA). The interassay variations were approximately 9% at concentrations ranging from 1 to 10 μmol/L. Bioavailable testosterone was calculated indirectly by the use of equations, as described by Nanjee and Wheeler.[17]

Statistical and Kinetic Analysis

Data are given as mean ± SD. Unpaired *t* test was used to determine the significance of mean differences between post- and premenopausal women. The focus of the analysis reported here was to assess the extent to which fasting levels of TG-rich lipoproteins in postmenopausal and premenopausal women could be attributed directly to body composition and fat distribution, postheparin lipase activities, kinetic indices of TG catabolism, and endogenous hormone (-binding) levels. The primary analytic approach, therefore, was to regress fasting very-low-density lipoprotein (VLDL) measures on the levels of these potential determinants. Pearson's correlation coefficients were used to assess the strength of univariate associations. Spurious correlations due to outliers were excluded after visual confirmation of homogeneous distribution of all variables. To compensate for multiple testing, statistical significance was reached below the .01 level. Because of strong colinearity among some of the variables and the interest in controlling potential confounders, stepwise regression analysis was employed to determine the best subset of potential determinants. First, potential confounders were added in a stepwise fashion to the regression model. Hormones, SHBG, and glucose levels were then added, again in a stepwise fashion, to a model that included the subset of the variables that contributed significantly to the model at the first step. Because of skewed distributions, estradiol concentrations were log-normally transformed. All other variables had normal distributions.

Kinetic indices of TG lipolysis, ie, VLDL-TG lipolysis rates ($V_{e,VLDL\text{-}TG}$) and apparent first-order constants ($k_{e,\ VLDL\text{-}TG}$), were calculated according to standard formulas of pharmacokinetics. It was assumed that heparin injection resulted in the instantaneous release of LPL or HL and in their immediate and homogeneous distribution throughout the plasma compartment. Statistical analysis was done with SPSS 11.5 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Clinical and metabolic characteristics of subjects in this report are shown in Table 1. Both groups were of similar age, BMI, and waist-to-hip ratio. Postmenopausal women had, by definition, high FSH and LH concentrations and low concentrations of estrogens when compared to premenopausal women. Total testosterone levels, but not bioavailable testosterone concentrations, were significantly higher in premenopausal women. In postmenopausal women, plasma cholesterol and LDL-cholesterol were significantly higher and apoB tended to be raised. Fasting plasma TG were identical in both groups (0.69 ± 0.29 mmol/L *v* 0.73 ± 0.33 mmol/L). Approximately 30% of all plasma TG were present in the VLDL in both groups. No differences were seen in the intermediate-density lipoproteins (IDL) and in high-density lipoprotein (HDL)-cholesterol. ApoE genotypes in both groups were comparable: E2/E3 (postmenopausal, 0; premenopausal, 2), E2/E4 (1, 0), E3/E3 (13, 12), E3/E4 (3, 3), and E4/E4 (1, 1). Dietary intakes,

Table 1. Characteristics of Postmenopausal and Premenopausal Women

	Postmenopausal Women (n = 18)	Premenopausal Women (n = 18)
Age (yr)†	50 (47-52)	49 (47-52)
Amenorrhoea (yr)*	3.0 (1-7.5)	Regular menses
BMI (kg/m^2)	24.6 ± 2.9	24.0 ± 2.6
Waist-to-hip ratio	0.76 ± 0.04	0.78 ± 0.08
FSH (IU/L)	93.3 ± 20.2	8.0 ± 5.7§
LH (IU/L)	33.6 ± 8.6	7.8 ± 6.9§
Estradiol (pmol/L)	56.7 ± 42.5	660.3 ± 473.3§
Estrone (pmol/L)	113.1 ± 34.3	390.8 ± 179.8§
Androstenedione (nmol/L)	3.14 ± 1.01	3.66 ± 0.83
Testosterone (nmol/L)	0.89 ± 0.23	1.05 ± 0.21†
Bioavailable testosterone (nmol/L)	0.15 ± 0.05	0.17 ± 0.03
DHEA-S (μmol/L)	2.76 ± 1.12	2.68 ± 1.25
SHBG (nmol/L)	45.7 ± 20.5	50.2 ± 17.9
Insulin (mE/L)	7.7 ± 4.6	7.4 ± 4.0
Glucose (mmol/L)	5.1 ± 0.3	5.2 ± 0.7
Plasma		
apoB (g/L)	1.00 ± 0.22	0.89 ± 0.20
apoA-1 (g/L)	1.46 ± 0.25	1.43 ± 0.19
TG (mmol/L)	0.69 ± 0.29	0.73 ± 0.33
Cholesterol (mmol/L)	5.43 ± 0.69	4.81 ± 0.73†
VLDL ($d < 1.006$ g/mL)		
TG (mmol/L)	0.26 ± 0.22	0.24 ± 0.28
Cholesterol (mmol/L)	0.10 ± 0.11	0.09 ± 0.13
IDL ($1.019 < d < 1.063$ g/mL)		
TG (mmol/L)	0.06 ± 0.03	0.06 ± 0.03
Cholesterol (mmol/L)	0.31 ± 0.29	0.24 ± 0.24
LDL ($1.063 < d < 1.21$ g/mL)		
TG (mmol/L)	0.14 ± 0.03	0.17 ± 0.05
Cholesterol (mmol/L)	3.23 ± 0.48	2.77 ± 0.68‡
HDL ($1.063 < d < 1.21$ g/mL)		
TG (mmol/L)	0.10 ± 0.03	0.13 ± 0.03‡
Cholesterol (mmol/L)	1.51 ± 0.38	1.39 ± 0.29

NOTE. Data are mean ± SD.

*Median and ranges. †$P < .05$, ‡$P < .01$, §$P < .001$ for differences in mean concentrations by unpaired t test.

including ethanol, were similar for both groups (data not shown).

Heparin administration released equal amounts of LPL activities in postmenopausal women and premenopausal women (205.9 ± 58.6 *v* 178.9 ± 59.9 mU/mL) after 20 minutes. By contrast, postheparin HL activities were significantly higher in postmenopausal women (418.1 ± 50.2 *v* 336.9 ± 69.0, $P = .0001$). As a result of heparin administration, VLDL-TG levels decreased from 0.26 ± 0.22 to 0.07 ± 0.11 mmol/L in postmenopausal women and from 0.24 ± 0.28 to 0.06 ± 0.13 mmol/L in premenopausal women. Kinetic analysis of the TG disappearance in $d < 1.006$ g/mL fractions revealed that both groups responded in a similar fashion to the heparin injections. Both VLDL-TG half-life times ($T_{½,\ VLDL\text{-}TG}$) (8.8 ± 10.1 min *v* 10.1 ± 7.1 min, NS) and VLDL-TG lipolysis rate ($V_{e,VLDL\text{-}TG}$) (9.4 ± 6.15 *v* 6.8 ± 7.8 $\mu mol \cdot L^{-1} \cdot min^{-1}$) were the same. Lipolysis rates in both groups were highly related to fasting TG (substrate) concentrations ($r = 0.97$, $P = .0001$) but not to LPL or HL activities. These substrate-dependent (first-order) reaction velocities were highly similar for postmenopausal and premenopausal women. Postheparin lipase acitivities were not related to hormones or any fasting lipoprotein concentrations in both groups.

To gain insight in potential regulatory mechanisms of fasting TG-rich lipoprotein concentrations, the strength of their associations with anthropometric measures, postheparin lipase activities, and various hormones were calculated. Insulin was the single most important variable identified for both postmenopausal and premenopausal women (Fig 1A and B). In addition, androstenedione was positively associated with TG in postmenopausal women (Fig 1D), but not in premenopausal women (Fig 1C). Stepwise multiple regression analysis of potential determinants of VLDL-TG concentrations showed that insulin, LH, androstenedione, and DHEA-S were independent variables, explaining 94% of the variability ($r = 0.97$, $P < .0001$) in postmenopausal women (Table 2, model A). DHEA-S was of borderline significance ($P = .013$). Note that in the regression equation both LH and DHEAS were inversely related to VLDL-TG concentrations. When the gonadotrophic LH was excluded from analysis, because its effects were likely to be mediated by other hormones, bioavailable testosterone became a significant contributor to the regression equation and the association with androstenedione became stronger (Table 2, model B). Thus, 87% of the variability in fasting VLDL-TG concentration in postmenopausal women was predicted by insulin, bioavailable testosterone, and androstenedione. Again, note the opposite direction of the associations of bioavailable testosterone and androstenedione with VLDL-TG. Similar but weaker relationships were found when plasma TG were introduced as the dependent variable in stead of VLDL-TG. In premenopausal women, the only identified predictor of VLDL-TG in multiple regression analysis was insulin. Weak univariate associations ($P < .05$) were found between some nonhormonal variables (eg, BMI and waist circumference) and fasting VLDL-TG in both post- and premenopausal women, but none reached significance ($P < .01$). These potential confounders did not contribute significantly to the final regression analysis. No significant relationships were found between HDL-cholesterol and hormones or lipase activities.

DISCUSSION

The present study showed that androgens are strongly associated with fasting VLDL-concentrations only in postmenopausal women. Effects of androgens were independent of insulin and were not related to kinetic indices of catabolism of VLDL particles. Clinical states of androgen excess in women are known to be associated with increased TG concentrations, often in combination with hyperinsulinemia,[18] but not always.[19] However, this is the first study that reports on the importance of physiological levels of androgens and insulin on triglyceride metabolism in the estrogen-deficient state.

Insulin was the main determinant identified in both groups. Note that before and after the menopause, the relation of insulin with fasting VLDL-TG was identical in both groups and that estrogen deficiency or androgen predominance did not alter this relation. Associations of elevated insulin levels with a cluster of metabolic alterations including increased TG have been observed in various study populations and several reviews have addressed the nature of this association.[20,21]

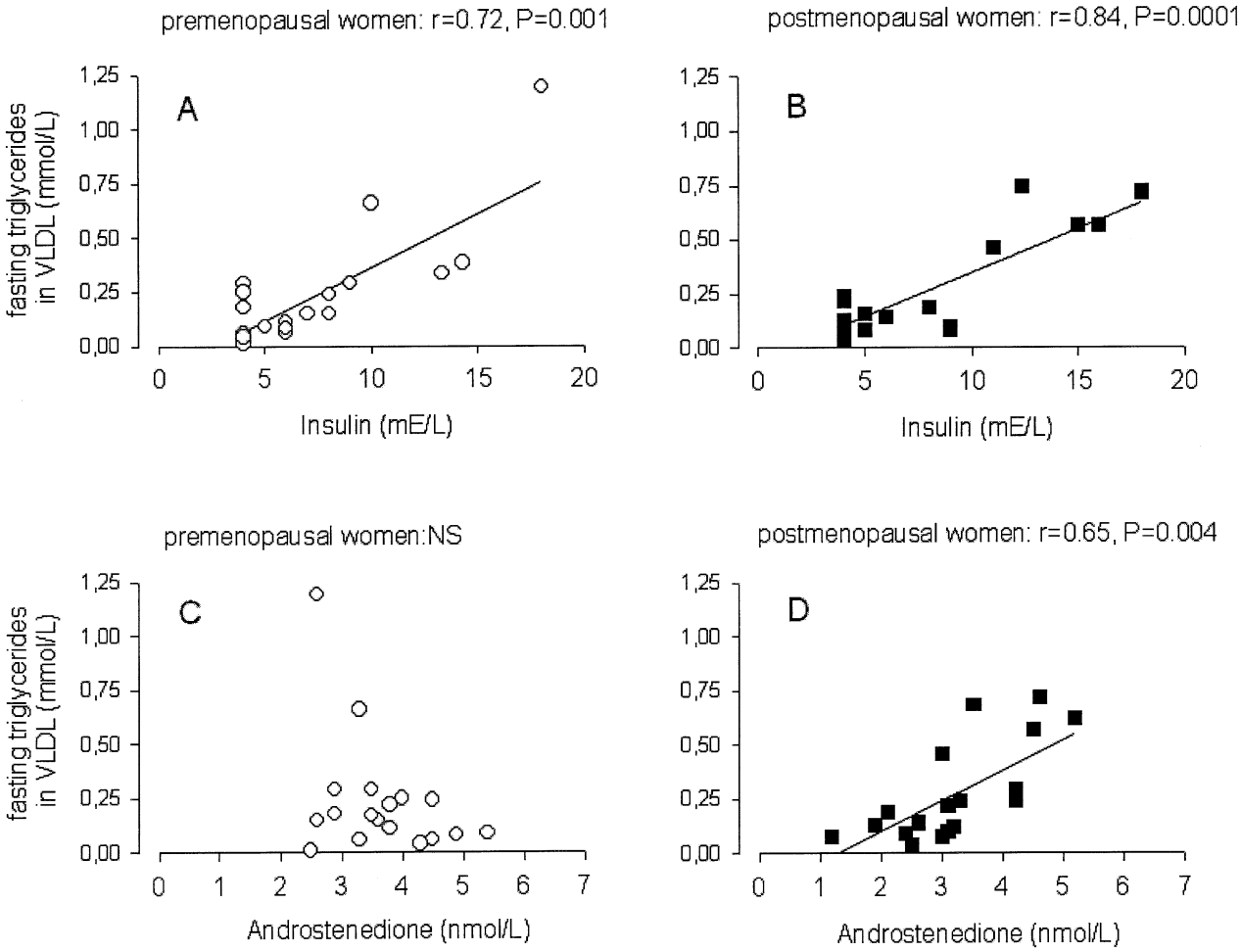

Fig 1. Associations of insulin (A and B) and androstenedione (C and D) with fasting VLDL-TG concentrations in premenopausal women (A and C, ○) and postmenopausal women (B and D, ■).

Androgens were associated with TG only in postmenopausal women. The various androgens in our study had opposite effects on VLDL-TG. Thus, there may be a partial agonistic and antagonistic effect. Testosterone, the principal circulating androgen in normal women, was inversely related to TG. Although results of clinical studies on the effects of androgen therapy are conflicting, due to diversity in treatment modalities and study populations, studies on treatment with physiological

Table 2. Multiple Regression Analysis of the Relation of Fasting VLDL-TG to Various Hormones

	Independent Variables					Model	
Postmenopausal Women	Insulin	LH	Bioavailable Testosterone	Androstenedione	DHEA-S	R^2*	*P*
Dependent variable: VLDL-TG							
Model A	0.66 (<.0001)	−0.35 (0.0001)	NS	0.38 (0.0001)	−0.22 (0.013)	0.94	<.0001
Model B	0.74 (<.0001)	Excluded	−0.38 (0.006)	0.55 (<0.0001)	NS	0.87	<.0001

	Dependent Variables					Model	
Premenopausal Women	Insulin	LH	Bioavailable Testosterone	Androstenedione	DHEA-S	R^2*	*P*
Dependent variable: VLDL-TG	0.72 (.001)	NS	NS	NS	NS	0.51	.0001

NOTE. Stepwise multiple regression analysis was performed by including growth hormone, IGF-1, TSH, FSH, LH, estrone, estradiol, androstenedione, total testosterone, bioavailable testosterone, DHEA-S, SHBG, insulin, and glucose into the model. Data are partial correlation coefficients and *P* value of variable in regression equation.

*Cumulative R^2. In model B, the gonadotrophic LH was excluded from analysis since its effects were likely to be mediated by other hormones.

doses of androgens in postmenopausal women report a decrease in plasma TG.[12,13] Further, increased levels of TG by estrogen replacement therapy may be blunted by addition of androgens or androgenic progestins.[22,23] Accordingly, in vitro studies on the treatment of human hepatoma (HepG2) cells with various sex hormones showed that testosterone alone had no effects on apolipoprotein A and CII secretion, but it antagonized the effects of low-dose estrogen on these lipoproteins.[24] Thus, testosterone at physiological concentrations may have a beneficial effect on TG, an established risk factor for the development of CHD in women.[4] Studies on harder end points in postmenopausal women show favorable effects of testosterone on carotid intima media thickness,[25] or the absence of the alleged association of cardiovascular mortality risk and androgens.[26] However, these results should be interpreted with some caution because there is also evidence in literature for adverse effects of androgens on TG metabolism in postmenopausal women.[27,28]

Is it possible that the observed effects of androgens are spurious, for example mediated by estrogens, as testosterone is the direct precursor of estradiol? We believe that this is not the case for several reasons. First, we did not observe any relations between estrogens and lipoprotein concentrations, although it must be noted that the estradiol method may not be sensitive enough at low (postmenopausal) concentrations. Second, multivariate analysis emphasized the importance of the ovaries as main androgen-producing glands in postmenopausal women.[29] The effects of the gonadotrophic LH appeared to be mediated directly by testosterone and androstenedione. Further, the complex partial agonist and antagonistic relations of testosterone, androstenedione, and DHEA-S with TG showed that all androgens are involved. In men, androstenedione was inversely related to plasma TG.[30] Third, estrogenic effects on VLDL would enhance production, rather than result in lower VLDL concentrations.

Few studies are available on the mechanisms of action, especially those conducted as controlled trials. Wolfe and Huff found that levonorgestrel, a potent androgenic progestin, reduces plasma concentrations of VLDL by increasing catabolism and decreasing production rates.[31,32] Further, there is strong evidence that anabolic steroids increase postheparin HL activity.[3] However, in our study we found that HL, with significantly different activities in post- and premenopausal women, had no influence on fasting TG levels, nor was it in any way associated with androgens. Studies by Tikkanen et al. showed that HL activity is not an major regulator of VLDL catabolism under physiological circumstances.[33] It has been argued that TG lipolysis by the action of LPL is a major determinant of plasma levels of TG-rich lipoproteins.[34] Not only the amount of LPL, but also the efficacy of VLDL as substrate for the lipase, and its contact time with lipase are factors that may account for lipoprotein concentrations. We found no differences in LPL activity, nor in the lipolytic degradation of fasting TG-rich lipoproteins in postmenopausal and premenopausal women. This suggests that potential compositional changes in VLDL between both groups do not influence its lipolytic degradation. However, our recent findings may be limited by the fact that we performed in vivo lipolysis experiments in the fasting state, when very low concentrations of TG in the VLDL lipoproteins were present. Saturation of the lipolytic pathway is predicted to occur at much higher TG concentrations.[35] Few data exist on androgens and their receptors with regard to the expression of lipoprotein receptors. Estradiol activates the LDL receptor (LDLR) promoter in transiently transfected HepG2 cells, with normal regulation of LDLR gene transcription.[36] Compounds with androgen receptor agonist activity (eg, testosterone and androgenic progestins) attenuate the estrogen-induced upregulation of LDLR in this in vitro system. In vivo expression of hepatic LDLR in middle-aged and elderly women showed positive relations with plasma concentrations of free estrone, but not with testosterone.[37] To our knowledge, no studies have been performed on the influence of androgens on the expression of other lipoprotein receptors. Additional studies are needed to elucidate the role of androgens on these receptors.

In conclusion, insulin and androgens may be important independent determinants of fasting VLDL-TG in postmenopausal women. In our study, testosterone showed potential beneficial effects on TG, but further controlled studies are needed to elucidate the precise role of physiological concentrations of androgens on TG metabolism.

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