Endocr (2010) 37:329–335
DOI 10.1007/s12020-010-9307-7

ORIGINAL ARTICLE

Endogenous estrogen levels are associated with endothelial function in males independently of lipid levels

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Received: 23 September 2009 / Accepted: 23 December 2009 / Published online: 16 January 2010

Abstract Estrogens and androgens may play an important role in vascular health in both sexes. The aim of this study was to examine the relation of endogenous sex hormone levels with early markers of atherosclerosis in a cohort of apparently healthy males. 124 males (age 46.25 ± 9.56) attending a preventive medicine program were examined for unrecognised features of the metabolic syndrome. Flow-mediated dilatation (FMD) and intima-media thickness (IMT) of the common carotid artery were evaluated. Obesity parameters were recorded; estradiol, testosterone, SHBG, free testosterone, insulin, as well as glucose and lipid levels were measured. FMD was positively correlated with estradiol ($r = 0.201$, $P = 0.041$) and negatively with total cholesterol ($r = -0.205$, $P = 0.022$), low density lipoproteins ($r = -0.232$, $P = 0.009$), and triglyceride levels ($r = -0.179$, $P = 0.046$). In multivariate analysis, the association of FMD with estrogen was independent of BMI and lipid levels. No significant association between FMD and testosterone levels was found. Subjects with an increased mean IMT (>0.73 mm, i.e., >3rd tertile) had lower levels of free ($P = 0.021$) and bioavailable ($P = 0.016$) testosterone. In multivariate logistic regression analysis, this association was no longer significant when age or cholesterol levels were considered. Endogenous estrogen levels are associated with FMD, independently of age and lipid levels, showing a protective effect in middle-age male subjects. Circulating androgens are associated, although not independently, with structural changes such as the IMT of carotid artery; this effect is possibly influenced by lipid levels and age.

Keywords Flow-mediated dilatation · Intima-media thickness · Males · Estrogen · Androgen · Cardiovascular

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Introduction

Several studies have shown that endogenous sex hormones may play an important role in cardiovascular health in both sexes [1–4]. It has been shown that estrogens may modulate several vasoactive, pro-inflammatory, and metabolic factors which influence the vasculature [5]. Moreover, it has been suggested that estrogen may play an important role in the regulation of endothelium-dependent vasodilatation [4, 6, 7].

In women, estrogen appears to act protectively: cardiovascular disease is less common in premenopausal women when compared to men and to postmenopausal women [8]. Moreover, in postmenopausal women, estrogen administration has been shown to improve endothelial function [9].

Circulating estrogen is lower, but measurable in men, mostly originating from peripheral aromatization of androgen precursors. Important information about the significance of estrogen for cardiovascular health in males came from the study of a young man carrying a disruptive mutation in the estrogen receptor alpha gene, which caused

insensitivity to estrogens; he was found to have endothelial dysfunction and premature cardiovascular disease [10, 11]. It is also known that estrogen receptors are expressed in male vascular endothelial cells, as in females [12].

As concerns normal males, the effect of estrogens on the vasculature is not well-established. There are few reports, if any, concerning direct associations of endogenous estrogen levels with markers of endothelial function in healthy middle-age males under normal conditions. Most of the reports in the literature have examined the effect of exogenous estrogen administration on endothelial function parameters and showed rapid vasodilatory effect [13] as well as improvement in endothelial function [14]. Long term estrogen administration in male-to-female transsexuals or in hypogonadal men may also improve vascular function [15–17]; however, other studies have shown conflicting results [18, 19]. The possible beneficial effect of exogenous estrogen on endothelial function in males [3] may be mediated through rapid stimulation of nitric oxide release [20].

Concerning androgen, a beneficial effect on vascular function parameters and cardiovascular risk factors has been described in several reports especially in older male populations [21–23]. Associations of early markers of atherosclerosis with endogenous testosterone levels in healthy male populations have been studied, but have not always led to similar conclusions [23–25].

The aim of our study was to examine associations of endogenous estradiol and testosterone with markers of early atherosclerosis in a cohort of apparently healthy middle-age male individuals.

Subjects and methods

We studied 124 males, who attended a preventive medicine program for free examination for unrecognised features of the metabolic syndrome in the outpatients' clinic of our hospital over a period of 12 months. Mean age was 46.25 ± 9.56, while mean BMI range was 20.4–43.3 (median 27.14 kg/m^2).

Current drug therapy, clinical history, as well as cardiovascular risk factors, such as arterial hypertension and dyslipidemia, were also recorded. Exclusion criteria in our study were a history of known coronary heart disease or a previous history of stroke, as well as a history of diabetes mellitus (previously diagnosed according to the American Diabetes Association criteria). The presence of hypertension was defined as systolic and/or diastolic blood pressure higher than 139 mmHg and/or 89 mmHg, respectively, and/or current use of antihypertensive drugs.

Of the studied individuals included in the analysis 21% had hypertension and 44.4% had dyslipidemia. The study was approved by the institutional Ethics Committee, and all subjects gave their informed consent.

In the clinical examination, height and weight were measured with subjects wearing indoor clothes without shoes. Body mass index (BMI) was calculated according to the formula weight (kg)/height (m^2). Waist and hip perimeter (cm) measurements were performed and waist to hip ratio (W/H R) was used to evaluate fat distribution. Systolic and diastolic arterial pressure was obtained after 5 min of rest using a standard mercury sphygmomanometer, while all subjects were in a sitting position.

Early markers of atherosclerosis such as endothelium-dependent vasodilatation (flow-mediated dilatation, FMD) and intima-media thickness (IMT) of the common carotid artery were recorded.

Brachial artery reactivity was assessed by measuring endothelium-dependent vasodilatation as previously described [26]. Briefly, in each visit, scans of the brachial artery were recorded at rest, during reactive hyperemia, and after administration of nitrate, using B-mode ultrasound imaging with a 7.0-MHz linear array transducer (Acuson 128XP, Mountain View, Calif). FMD was induced in response to reactive hyperemia after deflation of a cuff placed distally to the site of measurement. It is expressed as the percentage change of internal diameter of the brachial artery from baseline. FMD has been previously shown to be nitric oxide-dependent in humans, and it is considered as a marker of endothelial function [27]. Ultrasound analysis was performed in each case by two independent observers. The inter-and intra-observer variability for brachial artery diameter measurements in our laboratory is 0.1 ± 0.12 and 0.08 ± 0.19 mm, respectively, while the FMD variability measured in the same patient on two different days varied by 1.1 ± 1% (mean ± SD).

Carotid intima-media thickness, a significant predictor of coronary and cerebrovascular events [28], was measured using again B-mode ultrasound imaging in three paired segments of both right and left carotid arteries: common carotid artery (defined as the segment 1 cm proximal to carotid dilatation), carotid bulb (defined as the segment between the carotid dilatation and carotid flow divider), and internal carotid artery (defined as 1 cm long arterial segment distal to the flow divider). In each segment, three measurements of the maximal carotid IMT in the far wall were averaged. Subsequently, the average maximal carotid IMT of all six segments was calculated [29].

Early morning fasting blood samples were obtained by venipuncture between 08:00 and 09:00 h for the measurement of estradiol, testosterone, SHBG, insulin, as well as glucose and lipid levels. Glucose, total cholesterol, high density (HDL), low density (LDL) cholesterol, triglycerides, and uric acid levels were measured immediately using an automated analyzer Integra 400, Roche). Specimens were kept frozen at −20°C until the determination of the levels of 17β-estradiol, SHBG, testosterone, and insulin using the same

or consecutive batches of assays. Estradiol was measured using the method Spectria E2 sensitive (Biomedica). The reference range for men was 9–61 pg/ml, while the intra- and inter-assay variability were 2.8% (for 23.7 pg/ml) and 5.8% (for 25.6 pg/ml), respectively. Total testosterone was measured by chemiluminescence (DPC-immulite 2000, Los Angeles, USA). The reference range was 2.12–15.1 ng/ml, while the intra- and inter-assay variability were 7.2 and 8.2% (for 4.14 ng/ml), respectively. Finally, serum insulin and SHBG were determined by IRMA (Biosource Europa SA, Nivelles, Belgium and Radim S.p.A, Via del Mare, Domecia Roma Italia, respectively). The HOMA basal insulin resistance index (HOMA), was calculated according to the formula FI × G/22.5 where FI = fasting insulin (mU/l) and G = fasting glucose (mmol/l). Free and bioavailable testosterone were estimated using a computer program to solve the equation published and validated by Vermeulen et al. [30].

Statistical analysis

Descriptive data are shown as range as well as mean ± SD. Categorical data are presented as percentages. The SPSS (version 11) statistical package was used to perform all the statistical analysis. Linear regression analysis was used to investigate correlations between continuous variables. Because the number of participants in relation to the total number of parameters included in the univariate regression analysis was relatively small, in the stepwise multivariate regression analysis, we included as possible confounders age and all the variables for which there was a correlation with the dependent variable with a P value of at least <0.2 in the univariate analysis. This cutoff was chosen [29] to filter out parameters without significant contribution to the statistical model and to simultaneously include marginally correlated parameters. This strategy allows a more parsimonious model which yields better power to the study. Because mean carotid IMT did not follow normal distribution, this variable was transformed into ordinal, and was divided based on those subjects in the highest tertile with IMT > 0.73 mm and the rest of the population. Student's t-test was used to compare mean values between groups where the distribution was normal. Multivariate binary logistic regression analysis was performed to assess the effect of possible confounders when significant differences in hormone levels were detected between IMT subgroups. A P value of <0.05 was taken as statistically significant.

Results

The clinical and biochemical characteristics of the studied population are presented in Table 1. The range of testosterone levels in our population was 0.49–12.6 ng/ml. One subject had total testosterone levels lower than the normal range of our laboratory.

Table 1 Anthropometric, clinical and biochemical parameters of the studied population

Parameters	%	Mean	Range
Age (years)		46.25 ± 9.56	34–72
Weight (Kg)		86.76 ± 15	60–142
BMI (Kg/m^2)		27.75 ± 4.2	20.3–43.3
Waist (cm)		97.75 ± 13.77	53–130
W/H R		0.95 ± 0.09	0.52–1.36
Dyslipidemia	44.4		
Arterial hypertension	21		
Smoking	52		
E2 (pg/ml)		19.7 ± 7.7	8.7–50.3
Testosterone (ng/ml)		5.9 ± 2.1	0.49–12.6
SHBG (nmol/l)		31.9 ± 12.8	12.2–83.4
Glucose (mg/dl)		91.6 ± 10.5	68–121
Insulin (μU/ml)		9.5 ± 6.4	1.1–41.0
HOMA-IR		2.21 ± 1.6	0.04–11.0
Total Cholesterol (mg/dl)		211.8 ± 39.9	133–336
HDL (mg/dl)		51.6 ± 13.8	29–109
LDL (mg/dl)		152.4 ± 39.0	78–233
Triglycerides (ng/dl)		116.0 ± 50.0	39–314
Uric Acid (mg/dl)		6.1 ± 1.2	3.9–11.0

Flow-mediated dilatation (FMD) was positively correlated with estradiol levels ($r = 0.201$, $P = 0.041$, Fig. 1). FMD was negatively associated with total cholesterol ($r = -0.205$, $P = 0.022$), low density lipoproteins ($r = -0.232$, $P = 0.009$) and triglyceride levels ($r = -0.179$, $P = 0.046$). No significant associations between FMD and HOMA-IR ($P = 0.816$), BMI ($P = 0.162$), or waist to hip

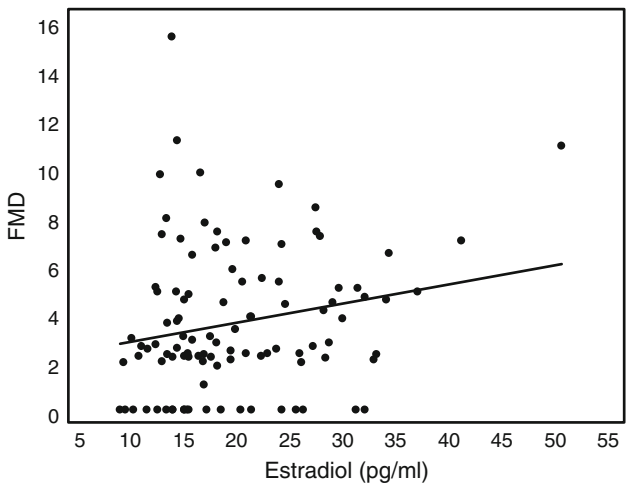

Fig. 1 Association of flow-mediated dilatation with estradiol levels in apparently healthy males ($r = 0.20$, $P = 0.041$)

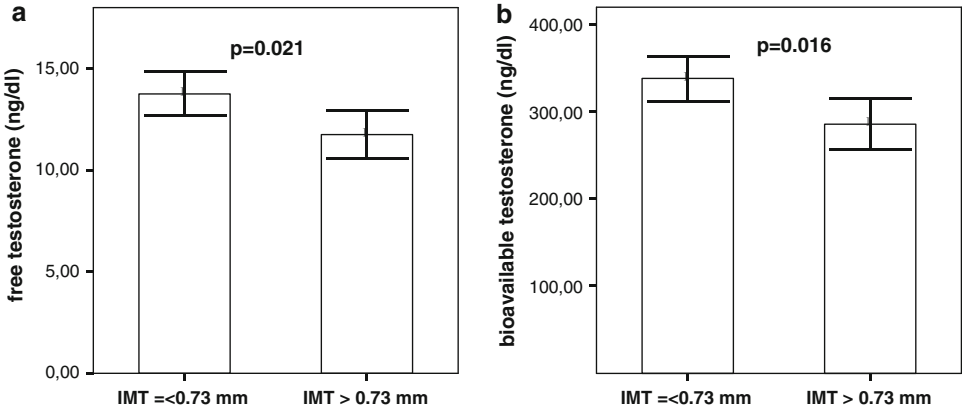

Fig. 2 Differences in (**a**) free testosterone and (**b**) bioavailable testosterone levels between apparently healthy males with low (≤0.73 mm, $n = 82$) and increased (>0.73 mm, $n = 42$) mean carotid IMT. *Error bars* represent 95% confidence intervals

ratio ($P = 0.597$) were found. FMD did not differ between smokers and non-smokers ($P = 0.909$).

Higher estrogen levels were associated with lower cholesterol levels ($r = -0.196$, $P = 0.047$) and higher BMI ($r = 0.279$, $P = 0.004$). No significant association of estradiol levels with HOMA-IR was found.

Multivariate analysis showed that the association of FMD with estradiol was independent of age and lipid levels ($r = 0.292$, $P = 0.041$). In a different model, when BMI was also taken into account, again estradiol remained a significant predictor of FMD (Table 2). No association of FMD with total or the free fractions of testosterone was found.

Subjects with an increased mean carotid artery IMT (>0.73 mm, i.e., >3rd tertile) had lower levels of free and bioavailable testosterone as compared to the rest of the population (11.72 ± 3.46 vs. 13.74 ± 4.43 ng/dl, $P = 0.021$ and 337.74 ± 107.61 vs. 286.18 ± 84.73 ng/dl, $P = 0.016$, respectively, Fig. 2); no difference was observed in total testosterone (5.51 ± 1.85 vs. 6.11 ± 2.25 ng/ml, $P = 0.169$) and estradiol levels (19.8 ± 7.7 vs. 19.6 ± 7.7 pg/ml, $P = 0.898$).

Free testosterone was negatively associated with HOMA-IR ($r = -0.210$, $P = 0.040$, Spearman's correlation) and total cholesterol levels ($r = -0.2064$, $P = 0.038$). Total testosterone was inversely associated with HOMA-IR ($r = -0.257$, $P = 0.009$). Using multivariate logistic regression analysis differences in free and bioavailable testosterone between high and low IMT were lost, when total cholesterol or age were taken into account. In the final analysis, age remained the only independent predictor of an increased mean carotid IMT. The results were not affected when the individual with low testosterone was excluded from the analysis.

Table 2 Multivariate analysis (step) of the association of flow-mediated dilatation (FMD) with biochemical parameters in apparently healthy males

Variable	Parameter	Beta	t	P	Overall r^2
FMD	Estradiol	0.202	2.068	0.041	$r^2 = 0.041$
	Total cholesterol		−1.15	0.25	
	Age		−0.06	0.948	
	Triglycerides		−1.18	0.24	
	BMI		−1.64	0.103	

Discussion

The findings of the present study suggest a possible protective effect of endogenous estrogen on endothelial function in healthy mostly middle-age male individuals. FMD is an early marker of endothelial dysfunction, and has been associated with a higher risk for cardiovascular disease [31]. The association of FMD with estradiol levels in our study was independent of lipid levels and BMI. In absolute terms, the effect of endogenous estrogen may be weak and thus the clinical significance may not seem very strong. It should be noted that, in most published studies, the reported relevant associations of FMD with classical predisposing risk factors for cardiovascular disease are also weak [29, 32]. The interesting finding of our study was that estrogen levels remained the only independent determinant of FMD when classical predisposing factors were taken into account. As far as we know, this is the first study which shows a possible beneficial effect of endogenous estrogen on the vasculature in healthy middle-age men under normal conditions. Our findings imply that estrogens may indeed play a role in endothelial function. It should be noted that in men endogenous estrogen levels are measurable and higher than in postmenopausal women [33]. Thus, one might suggest that their measurement could be included in epidemiological studies examining early cardiovascular risk factors in males.

Associations between endogenous estrogen levels and FMD in apparently healthy mostly middle-aged male individuals are very rare in the literature. Our results agree with

the report by Lew et al., who performed a placebo-controlled study in healthy young men. In this study, the administration of anastrozole, an aromatase inhibitor which results in decreased endogenous estrogen levels, resulted in an impairment of flow-mediated dilatation compared to the placebo-control group [7]. Similar results have been reported by Komesaroff et al. [34]. Furthermore, better endothelial function has been reported in men with decreased sensitivity of the androgen receptor [24], who are expected to have higher circulating testosterone and thus higher estrogen levels [35]. Higher estradiol levels, associated with "resistant" androgen receptor, were also reported to be associated with less severe coronary artery disease in men [36]. Finally, observational studies have shown that higher levels of endogenous estrogen are associated with decreased cardiovascular mortality and morbidity [37].

This protective effect of estrogen could also be mediated through effects on lipids. Indeed, estrogen levels were inversely associated with total cholesterol, LDL cholesterol, and triglycerides. Similar findings, indicating the favorable influence of estrogen on lipidemic profile, have been previously reported [37]. Similarly, an improvement in the lipidemic profile has been observed in men after estrogen administration [38]. However, in our study, this effect was independent of lipid levels. The estradiol effect was not mediated through associations with BMI either [38, 39].

In our study, we did not find any significant association between IMT and the levels of endogenous estrogen. This is in accordance with previous reports [22]. However, Tivesten et al. have reported that total and free estradiol were independent predictors of the progression of carotid IMT in 58 years old males during a 3-year follow up. It should be noted that this was a population-based, prospective cohort study, and this could account for the differences from our study which was a cross-sectional study. Moreover, our population included younger subjects. It is possible that such structural changes take longer to emerge and may thus be more readily apparent in an older population [40].

The second finding in our study concerns the association of IMT of the common carotid artery with testosterone levels in this group of healthy male individuals. Subjects with an increased mean carotid artery IMT (>3rd tertile) had lower levels of free and bioavailable testosterone, although in multiple regression analysis this was not independent. There are reports in the literature concerning similar associations of IMT with androgen levels mostly in older populations [21, 22, 25]. Moreover, low levels of androgen may be associated with increased risk for atherosclerosis [41]. We thus confirm that endogenous testosterone may have a protective role for the cardiovascular system [42, 43].

We did not find any association between FMD and testosterone levels, as has been previously reported [24]. The influence of testosterone on FMD in the literature is controversial possibly because several factors, such as age, cardiovascular health status, and the sex hormone exposure may alter the vascular response to testosterone [44–46].

The local conversion of androgen to estrogen at the tissue level may be implicated in the androgen effect on the cardiovascular system as previously described [47, 48]. It is interesting that the administration of dihydrotestosterone that cannot be aromatized to estrogen has no effect on endothelial function in men [49]. Similarly an impaired FMD in the aorta has been described in an aromatase knock out mouse model [50]. Therefore, circulating estrogen levels may not reflect the tissue estrogen production [33]. Thus, this association between IMT and free or bioavailable testosterone points to the same direction with the main finding of this study concerning the beneficial effect of estrogen on endothelial function in males.

Our study has several limitations. The first one is that this is a cross-sectional study and included a relatively small number of subjects. However, it is a homogenous population of mostly middle-age subjects attending a preventive medicine program of our hospital and the vascular study performed was quite detailed. Furthermore, our data do not provide proof for a cause-effect association.

In conclusion, endogenous estrogen levels are associated with FMD, showing a protective effect, in apparently healthy, middle-aged male subjects. This appears to be an effect of estrogen on cardiovascular health which does not depend on age and lipid levels. We further confirmed that circulating androgen may be favorable for structural changes, such as the IMT thickness of carotid artery. One cannot exclude the possibility that the androgen effects could also be mediated through estrogen.

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