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Supraphysiological concentrations of estradiol in menopausal women given repeated implant therapy do not adversely affect lipid profiles

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BACKGROUND: The effects of oral estrogen therapy (ERT) on lipids and metabolic parameters are well known, in contrast to the effects of subcutaneously administered estrogen, particularly high concentrations of estrogen. We examined metabolic parameters in cohorts of women with and without subcutaneous estrogen therapy with concomitant supra-normal concentrations of estradiol (SE). METHODS: Lipids and lipoprotein concentrations, low density lipid (LDL) subfractions, and activity of hepatic lipase (HL) were assessed in 30 menopausal women with SE and 19 control subjects not using ERT, matched for body mass index and age. RESULTS: Waist–hip ratio (WHR) and fasting insulin (FI) concentrations were lower in the SE group compared with the women not on ERT ($P < 0.05$). The concentrations of triglyceride and high density lipid (HDL) cholesterol were similar ($P > 0.1$), whereas total cholesterol ($P < 0.05$), LDL cholesterol ($P < 0.05$), and HL activity ($P < 0.01$) were lower in the SE group. Concentrations of the large, buoyant LDL I subfraction were significantly lower in the SE group ($P < 0.05$), but there was no difference in LDL III concentrations. CONCLUSIONS: Women with SE have similar triglyceride and HDL cholesterol levels but lower LDL cholesterol concentrations compared with post-menopausal women not taking ERT. The observations that the SE group showed reduced fasting insulin and WHR suggest that supra-normal circulating concentrations of estradiol, delivered subcutaneously, may beneficially influence insulin metabolism.

Key words: hormone replacement therapy/insulin resistance/LDL subfractions/menopause/tachyphylaxis

Introduction

Loss of ovarian function after menopause results in an increase in cardiovascular disease (CVD) risk factors, such as adverse changes in glucose and insulin metabolism, body fat distribution, coagulation and fibrinolysis, and vascular endothelial function (Mendelsohn and Karas, 1999). Derangements of lipoprotein profiles, particularly the elevation in total cholesterol concentrations, are believed to contribute to the increased risk of CVD after the menopause in women that may be independent of the effects of ageing (Stevenson *et al.*, 1993; Spencer *et al.*, 1997). This is largely thought to be due to an increase in circulating low density lipid (LDL) cholesterol, but additionally, there is a reduction in LDL particle size after the menopause that may confer an increased atherogenic potential (Campos *et al.*, 1988).

Smaller LDL particles (LDL-III) are considered more atherogenic than larger buoyant species, because of their increased susceptibility to oxidation (McNamara *et al.*, 1992) and their increased residence time in the plasma (Dejager, 1993). Indeed, their preponderance in the circulation (even in the presence of normal total LDL concentrations) is strongly associated with an increased incidence of CVD and type 2 diabetes (Crouse *et al.*, 1985; Austin, 1992).

Plasma triglyceride concentrations have a determinative influence on the concentration of small, dense LDL in the normal population (McNamara *et al.*, 1992; Rainwater, 2000). Studies in men demonstrate that when plasma triglyceride concentrations are above 1.3–1.7 mmol/l, larger LDL particles become triglyceride enriched and thus suitable for conversion to smaller species by the action of the hepatic lipase (HL) enzyme. Increased HL activity, in turn, is associated with insulin resistance (Baynes *et al.*, 1991), and exhibits strong sexual dimorphism with exogenous androgens up-regulating, and estrogens down-regulating its activity (Hazzard *et al.*, 1984).

Most studies of ERT in post-menopausal women have examined the effects of oral administration. For example, oral unopposed estradiol (E_2) replacement therapy has a favourable

effect by reducing total and LDL, and increasing high density lipid (HDL) cholesterol concentrations (Godsland, 2001). The LDL cholesterol-lowering effect following ERT is thought to be directly related to changes in estrogen level, through enhanced LDL receptor activity, which is manifested as an increase in fractional catabolic rate for LDL apolipoprotein B (Karjalainen *et al.*, 2000). Paradoxically, however, concentrations of plasma triglyceride have been shown to increase (and the LDL particle diameter decrease) following oral estrogen replacement therapy (Campos *et al.*, 1988; Griffin *et al.*, 1993; Rajman *et al.*, 1996). The magnitude of change in triglyceride concentrations appears to be related to the type of estrogen (being more marked with oral conjugated equine estrogen than with 17β-estradiol), and also appears to be related to the mode of administration.

Few studies have examined the effects of non-oral ERT on lipoprotein parameters, and none have examined the effects of subcutaneous administration on LDL size and density. Transdermally delivered estrogen appears to have minimal effects on lipoprotein profile, and it has a less marked effect on plasma triglyceride concentrations than oral preparations, probably because they avoid the hepatic first pass effect (Seed, 1994; Lahdenpera *et al.*, 1996). However, it has been suggested that high concentrations of estrogen delivered transdermally may adversely effect the LDL subfraction profile (Lobo, 1991), however, conclusive data are lacking.

Tachyphylaxis is a recognized complication of implanted estrogen replacement therapy that describes the return of menopausal women to the clinic requesting implant renewal at more frequent intervals than recommended, because of the premature return of symptoms (Garnett *et al.*, 1990). This results in a cumulative effect of increasingly high concentrations of serum E_2 (Cardozo *et al.*, 1984). This provides a good model to study the effects of unopposed, high concentrations of circulating estradiol on lipoprotein profile, HL activity, and LDL subfraction distribution, in the absence of first pass liver interactions.

The aim of this study was to use a cross-sectional population design to examine, for the first time, the effect of supranormal circulating E_2 upon lipid and lipoprotein subfraction concentrations. We hypothesized that despite very high concentrations of systemic estrogen, plasma lipids (in particular triglyceride) would be only minimally altered in women with tachyphylaxis due to avoidance of hepatic first-pass metabolism. Since there is evidence that sex hormones influence insulin metabolism and body fat distribution, we also examined anthropometric parameters and fasting insulin concentrations in this model.

Materials and methods

Patient recruitment

Thirty post-menopausal patients receiving E_2 implants who had demonstrated tachyphylaxis were identified from the menopause clinic at Glasgow Royal Infirmary, and were invited to participate in the study after confirmation of their excessive circulating E_2 concentrations. All patients in the SE group had undergone surgical menopause at least 2 years prior to the study. These patients were studied for at least 6 weeks following implant administration. Supraphysiological concentration of E_2 was diagnosed when the circulating $E_2 > 272$ pg/ml (conversion to SI units×3.68), or >1000 pmol/l, at least 4 weeks after implant administration. Nineteen post-menopausal patients who were not on ERT comprised the control group. They were selected from the local general practitioner's register, were residing in the same postal code region as the study subjects, and did not have any concurrent disease. None of the subjects or controls was taking any medication known to affect lipoprotein metabolism in the 2 months preceding the study. All patients had normal thyroid function.

Study protocol

The study was approved by the local hospital ethics committee, and written informed consent was obtained from all study subjects. All subjects were studied after an overnight fast of 12 h between 0800 and 1030 h.

Anthropometric measurements were made by the same-trained observer using standard techniques (World Health Organization, 1989). Body weight was measured using digital scales (Seca®, Hamburg, Germany) to within 100 g in light clothes; height was measured barefoot using stadiometer to within 0.5 cm. Body mass index (BMI) was calculated as: weight (kg)/height $(m)^2$. Circumferences were measured to within 1 mm using flexible tape in the standing position. For calculation of waist–hip ratio (WHR), waist circumference was measured mid-way between the lowest rib margin and the iliac crest at the end of gentle expiration, and hip circumference at the widest level of the greater trochanters.

The antecubital vein was cannulated with a butterfly needle (21 gauge) and 50 ml blood was withdrawn for hormone, lipoprotein and LDL subfraction estimations. Post-heparin plasma samples were obtained for hepatic lipase estimation 10 min following an i.v. injection of heparin at 70 IU/kg body weight. Samples for endocrine assays were harvested at 4°C by low speed centrifugation and aliquots of serum and plasma for hormonal analyses were stored at –20°C until analyses. Samples for lipid, and lipoprotein, and lipoprotein subfractions were placed on ice and were centrifuged within 2 h of venesection at 1000 *g* for 10 min. Aliquots of separated serum or plasma were either frozen immediately at –70°C for estimation of hepatic lipase activity, or stored temporarily at 4°C.

E_2 (SI: pg/ml×3.67 = pmol/l) and progesterone (SI: ng/ml×3.18 = nmol/l) were measured using a competitive fluoroimmunoassay (Wallac Ltd, Turku, Finland). Testosterone (SI: ng/ml×3.46 = nmol/l) was measured using competitive radioimmunoassay (Coat-A-Count® T; DPC, Los Angeles, CA, USA). LH, FSH and sex hormone binding globulin were assayed using specific non-competitive sandwich fluoroimmunoassay (Delfia hLH, Delfia hFSH, Delfia SHBG; Wallac Ltd, Turku, Finland). The free androgen index (FAI) was calculated as testosterone concentration (nmol/l) ×100, divided by SHBG concentration (nmol/l). Plasma glucose was measured using the glucose oxidase method (Glucose Reagent Kit, Olympus AU5200®; Olympus Optical Co Ltd), while insulin was measured using a competitive radioimmunoassay (Coat-A-Count® I; DPC).

Plasma total cholesterol, triglyceride, HDL-C, very low density lipoprotein cholesterol (VLDL-C) and LDL-C measurements were performed by a modification of the standard Lipid Research Clinics protocol (1975).

The LDL subfractions were isolated by upward elution though six-step density gradient ultracentrifugation. The method was developed in our laboratory (Griffin *et al.*, 1990). Briefly, major LDL subfractions were identified by peak maxima that occurred between hydrated density intervals of 1.025–1.034 g/ml (LDL I), 1.034–1.044 g/ml (LDL II) or 1.044–1.060 g/ml (LDL III). The individual subfraction

areas beneath the LDL profile were quantified using Beckman data graphics software (Beckman, High Wycombe, Bucks, UK). The detection system measured LDL concentration as absorbance at 280 nm and this was corrected to lipoprotein mass equivalence by applying previously calculated extinction coefficients. LDL-I 1 optical density unit (OD) = 2.63 mg lipoprotein/ml, LDL-II 1OD = 2.94 mg lipoprotein/ml and LDL-III 1 OD = 1.92 mg lipoprotein/ml. The integrated areas were corrected for differences in extinction coefficient and expressed as percentage of total LDL concentrations in mg of lipoprotein/dl plasma. The concentrations of the individual lipoprotein subfractions were determined by proportioning the mass (i.e. the sum of protein, cholesterol, cholesterol ester, triglyceride and phospholipid content) of total LDL (density 1.019–1.063) prepared by sequential centrifugation according to the areas under the density gradient absorbance profile.

Hepatic lipase activity was estimated by the method described (Belfrage and Vaughan, 1969). It was assayed in post-heparin plasma (PHP). Briefly, PHP was incubated with 14C-labelled triglyceride/gum arabic emulsion; the free fatty acid (FFA) released by lipase activity was captured by albumin and extracted into a solvent. The ratio of radioactivity in the extracted fraction to the total present in blank incubations provided the basis of calculating the activity of the enzyme. Enzyme activities were expressed in mmol of fatty acids released per hour per ml of plasma (mmolFFA/ml/h).

Statistics

Data distributions were examined by drawing normality plots of all variables, and by performing Shapiro–Wilks test. A P value of < 0.05 was considered significant deviation from normality. Variables (E_2, LH, FSH, testosterone, androstenedione, SHBG, triglyceride, HDL cholesterol, VLDL cholesterol, HL, and LDL subfractions mass and percentage) not normally distributed were log ($\log_e$) transformed. Results for normally distributed variables are shown as mean [95% confidence interval (CI)], while geometric mean (95% CI) is quoted for log-transformed variables.

Results in the two groups were compared using independent sample t-tests on normal and log normal variables. χ^2-tests were used to determine the difference in proportions in the study groups. Pearson's product moment correlation coefficients were calculated in the study subjects and controls (pooled data).

Results

Mean duration of treatment with subcutaneous estrogen in the SE group was 96 months (95% CI 77.3–115.0 months). At the time of the study, one patient had shown a decay of E_2 below the diagnostic concentration. Table I shows the characteristics of the 30 subjects with supraphysiological E_2 concentrations (SE), and the 19 control subjects. There was no significant difference in age between the two groups, and time since menopause was not significantly shorter in the control group [P = not significant (0.082)]. There was no significant difference in the proportion of subjects who were smokers in each group [P = not significant (0.703)]. BMI was not statistically different but WHR was significantly lower in the SE group ($P < 0.05$). The SE group had significantly higher concentrations of E_2, and SHBG, and significantly lower serum concentrations of gonadotrophins and testosterone compared with the control group ($P < 0.001$).

Table II shows that circulating concentrations of fasting insulin (FI), total cholesterol and LDL cholesterol were significantly lower in the study subjects relative to controls ($P < 0.05$). Although concentrations of HDL cholesterol, VLDL cholesterol, and plasma triglyceride were similar in the two groups, HL activity and the concentrations of LDL I were significantly lower in the SE group compared to controls (Table III). Due to the large proportion of smokers in each group, we examined whether smoking was a potential confounder in our study. Similar trends were observed when data

Table I. Patient characteristics, anthropometric data and endocrine profile in subjects with supraphysiological estradiol (E_2) concentrations (SE) and controls

	SE (n = 30)	Controls (n = 19)
Age (years)	51.5 (48.6–54.4)	53.2 (49.6–56.8)
Smokers (n)	11	8
Time since menopause (months)	117 (89–146)	80 (50–110)
BMI (kg/m^2)	26.0 (24.5–27.5)	27.0 (25.1–28.9)
Waist (cm)	78.9 (75.2–82.5)	83.6 (78.2–88.9)
WHR	0.78 (0.75–0.80)	0.82 (0.79–0.85)[b]
E_2 (pg/ml)[a]	398.0 (360.0–440.0)	28.66 (24.7–33.2)[c]
LH (IU/l)[a]	1.1 (0.84–1.48)	24.9 (17.50–30.0)[c]
FSH (U/l)[a]	0.73 (0.51–1.03)	40.6 (28.0–58.8)[c]
Testosterone (ng/ml)[a]	0.18 (0.14–0.24)	0.34 (0.28–0.42)[c]
SHBG (nmol/l)[a]	140 (122–161)	54 (42–69)[c]

Mean and [a]geometric mean (95% CI); [b]$P < 0.05$; [c]$P < 0.0001$.
SHBG = sex hormone binding globulin; BMI = body mass index; WHR = waist–hip ratio.

Table II. Insulin, lipoprotein concentrations and hepatic lipase activity in subjects with supraphysiological E_2 concentrations (SE) and controls

	SE (n = 30)	Controls (n = 19)
Insulin (IU/l)	8.2 (6.6–9.8)	11.6 (8.4–14.7)[b]
Cholesterol (mmol/l)	5.5 (5.1–5.9)	6.2 (5.4–6.9)[b]
Triglyceride (mmol/l)[a]	1.40 (1.14–1.70)	1.38 (1.05–1.83)
HDL cholesterol (mmol/l)[a]	1.47 (1.32–1.63)	1.35 (1.21–1.50)
LDL cholesterol (mmol/l)	3.2 (2.9–3.5)	4.0 (3.4–4.5)[b]
VLDL cholesterol (mmol/l)[a]	0.53 (0.45–0.64)	0.65 (0.48-0.88)
Hepatic lipase (mmol FFA/ml/h)[a]	7.97 (6.55–9.68)	13.34 (11.10–16.04)[c]

Mean and [a]geometric mean (95% CI); [b]$P < 0.05$; [c]$P < 0.0001$.
HDL = high density lipid; LDL = low density lipid; VLDL = very low density lipoprotein; FFA = free fatty acids.

Table III. LDL subfraction distribution in subjects with supraphysiological E_2 concentrations (SE) and controls

	SE (n = 30)	Controls (n = 19)
LDL I (mg/dl)	56 (47.5–66.6)	80 (59.0–107.0)[a]
LDL II (mg/dl)	172 (145.0–204.0)	209 (169.0–260.0)
LDL III (mg/dl)	40 (29.0–55.0)	42 (28.0–61.0)
%LDL I	19.0 (16.0–22.6)	22.3 (17.5–28.6)
%LDL II	58.0 (50.3–66.8)	59.0 (52.6–66.0)
%LDL III	13.4 (10.0–18.0)	11.7 (8.2 16.6)

[a]$P < 0.05$.
Data presented as geometric mean (95% CI); note therefore that percentage abundance of LDL subfractions do not necessarily add up to 100%.

in smokers or non-smokers were examined separately (data not shown).

HL activity correlated with fasting insulin concentration ($r = 0.34$; $P = 0.018$), with measures of body fat distribution (waist circumference: $r = 0.48$; $P = 0.001$, and WHR: $r = 0.40$; $P = 0.008$), and negatively with sex hormone binding globulin (SHBG) ($r = –0.62$; $P < 0.0001$). In turn, FI correlated significantly with waist circumference ($r = 0.58$; $P < 0.0001$), WHR ($r = 0.40$; $P = 0.004$), and with triglyceride concentrations ($r = 0.42$; $P = 0.002$). Weaker, but significant correlation was observed between plasma triglyceride concentrations and the concentrations of LDL III ($r = 0.33$; $P = 0.031$).

Discussion

The present study details, for the first time, lipid and lipoprotein subfractions concentrations in women with supraphysiological estrogen concentrations associated with tachyphylaxis syndrome. Women with supraphysiological E_2 concentrations showed lower total and LDL cholesterol concentrations relative to a control group not on ERT, and interestingly, triglyceride concentrations were not raised in the SE group. We did not observe a difference in the concentrations of HDL cholesterol or LDL III, even though the hepatic lipase activity was lower in the SE group.

Reduction in LDL cholesterol observed in this study could be due to an estrogen-mediated increased LDL catabolism secondary to enhanced LDL receptor expression (Griffin *et al*., 1993). These receptors have greater affinity for larger LDL particles resulting in selective clearance of LDL subfractions. Consistent with this, we noted that LDL-I concentrations were 30% lower in the SE group (56 versus 80 mg/dl; $P < 0.05$). By contrast, LDL-III was similar in the two groups.

Triglyceride concentrations were not higher in the SE group relative to controls. These data support the concept that the dose dependent increase in triglyceride concentration following oral estrogen therapy is likely a first pass pharmacological effect, since transdermal estrogen administration has little effect on plasma triglyceride concentrations (Walsh *et al.*, 1991; Lahdenpera *et al*., 1996). Given the absence of an effect of SE on triglyceride concentrations, and the established influence of triglyceride on LDL III concentrations (McNamara *et al*., 1992; Coresh *et al.*, 1993; Rainwater, 2000), it is not surprising that the concentration of small, dense LDL-III was not different in the SE group. This observation indicates that the perturbations in LDL subfraction distribution following oral, conjugated estrogen replacement therapy are explained by the increased plasma triglyceride concentrations that result from this form of therapy (Campos *et al.*, 1993; Griffin *et al*., 1993; Rajman *et al*., 1996). Absolute LDL-III concentrations only increase once plasma triglyceride reaches concentrations above 1.5–1.7 mmol/l (Tan *et al.*, 1995; Sattar *et al.*, 1997; Pirwany *et al*., 2001).

HL plays a key role in the remodelling of LDL, and is thus a key determinant of LDL-III concentration (Griffin *et al*., 1993). HL activity was 40% lower in the SE group, which is in keeping with the proposed effects of estrogen on this enzyme (Tikkanen and Nikkila, 1987).

Although we cannot exclude the effects of selection bias (e.g. differences in diet) in our study, it is noteworthy that mean WHR and fasting insulin were significantly lower in the SE group than in the controls, suggesting a difference in insulin sensitivity in the two groups. Our results appear to contrast with those observed in oral contraceptive users in whom the estrogen component (ethinyl estradiol) is associated with a reduction in insulin sensitivity (Godsland *et al.*, 1992); however, such effects were not observed with transdermally administered 17β-estradiol (Spencer *et al.*, 2000). These combined data indicate that the effects of estrogen on insulin sensitivity are dependent both on the type of estrogen used, balance of estradiol and estrone in the circulation, and the mode of administration.

In our population, there was a significant proportion of smokers in the two groups (37% and 42% in the SE and control groups respectively). Although we did not detect any differences in the pattern of results when smoking was examined, larger prospective studies are needed to establish if smoking influences the metabolic benefits of estrogen treatment.

In conclusion, our results show that women with SE had similar circulating triglyceride and HDL cholesterol concentrations but lower LDL cholesterol concentrations compared to age, smoking and BMI matched post-menopausal women not taking ERT. The data concerning correlates of insulin sensitivity (fasting insulin, and WHR) suggest that supra-normal circulating concentrations of E_2, delivered subcutaneously, may beneficially influence insulin metabolism. Future prospective studies are needed to confirm these cross-sectional observations.

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