CLINICAL STUDY

Acute estrogen exposure does not affect basal very low-density lipoprotein–triglyceride production or oxidation in postmenopausal women

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Abstract

Context: Long-term hormone replacement therapy (HRT) with estradiol (E₂) is associated with an altered lipid profile including unfavorable increases in triglyceride (TG) concentrations and augmented hepatic very low-density lipoprotein (VLDL)–TG production. There are indications that this effect of estrogens may be immediate.

Objective: To study the *in vivo* effect of a single dose of E₂ on VLDL–TG kinetics and oxidation in humans.

Methods: Eight healthy, postmenopausal women were given a single dose of either placebo or E₂ (4 mg) orally. VLDL–TG kinetics was assessed by a 240-min primed-continuous infusion of *ex vivo* labeled [1-¹⁴C]triolein-labeled VLDL. Fractional and absolute VLDL–TG oxidation was determined by hyamin trapping of exhaled ¹⁴C label. Indirect calorimetry provided measurements of lipid oxidation.

Results: Administration of 4 mg of E₂ orally rapidly increased plasma E₂ concentrations from below detection threshold to premenopausal levels. Free fatty acids (FFA) and TG concentrations were unaltered. No immediate effect was observed on either VLDL–TG production (placebo versus E₂): 20.0 ± 12.4 vs 24.1 ± 10.7 μmol/min, *P* = 0.33; VLDL–TG oxidation: 12.3 ± 10.9 vs 12.6 ± 5.6 μmol/min, *P* = 0.93; or VLDL–TG clearance rates: 51.4 ± 16.8 vs 64.9 ± 28.8 ml/min, *P* = 0.34).

Conclusions: Short-term E₂ elevation does not affect VLDL–TG production, oxidation, or clearance in humans. We therefore propose that HRT-associated dyslipidemia has a gradual rather than immediate onset.

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Introduction

Postmenopausal hormone replacement therapy (HRT) is associated with favorable changes in lipid profile including lowered levels of low-density lipoprotein (LDL) and increased high-density lipoprotein (HDL) (1). Administration of estrogens, however, may cause hypertriglyceridemia, a non-favorable effect (2) on the individual's risk of developing cardiovascular disease. Increased concentrations of circulating triglycerides (TGs) may stem from either: i) increased hepatic production and secretion of TG rich very low-density lipoproteins (VLDLs), or ii) reduced peripheral clearance by lipoprotein lipase (LPL), hepatic lipase, direct catabolism, and/or LPL receptors (3). Whereas LPL activity is modified by estradiol (E₂) according to whether the enzyme is located in muscle or adipose tissue, there is now general agreement that long-term estrogen administration augments hepatic VLDL production (1) and leads to elevated TG levels.

Although it is certainly possible that the TG increasing effect of estrogens may develop gradually over weeks and months, there are indications that the effect may be more immediate. Animal studies indicate that the hypertriglyceridemic effect of estrogen may have an acute onset. Thus, cultured chick hepatocytes stimulated by estrogen for 3 h secrete significantly more VLDL particles than control hepatocytes (4). Moreover, both APOB mRNA and secretion of VLDL increase following a single injection of either diethylstilbestrol (5) or E₂ (6), an effect possibly mediated by the activation of membrane-bound estrogen receptors (ERs), enabling rapid cellular signaling (7). Thus, the effect of E₂ on lipid metabolism and hence on body composition appears to be direct and mediated via ERs. These observations have led to renewed interest in adipocyte receptor subgroups, and recently, Dos Santos *et al.* (8) demonstrated the existence of a membrane-bound ERα. In that study, the authors demonstrated that physiological concentrations of E₂ rapidly lead to activated p42/p44

MAPK, and that this effect could be blocked by an estrogen antagonist. Furthermore, infusion of conjugated estrogens increases insulin action within hours, and decreases plasma glycerol concentrations during a hyperinsulinemic euglycemic clamp (9), rendering it plausible that a range of signaling cascades is activated acutely by E₂.

No previous study has assessed whether E₂ has an immediate effect on hepatic VLDL-TG production in humans; thus, we studied the effect of oral administration of 4 mg E₂ on VLDL-TG kinetics and oxidation in postmenopausal women using *ex vivo* labeled [1-¹⁴C]triolein and the primed-constant isotope dilution technique (10).

Materials and methods

Subjects

The participants were eight healthy, postmenopausal women (56 ± 4 years, 65 ± 8 kg body weight, 24.2 ± 1.2 kg/m² body mass index, 40 ± 4 kg lean body mass (LBM), 22 ± 5 kg fat mass). None used HRT, and all had E₂ levels below 0.07 nM. Informed consent as well as ethics committee approval was obtained from all participants prior to the study, which was conducted in accordance with the Declaration of Helsinki II. Volunteers were recruited from an ongoing study of the impact of E₂ on lipolysis. The study is in preparation for submission.

Protocol

The subjects were examined on 2 days separated by 1 month. On all examination days, the subjects received either placebo or E₂ (4 mg) at 0600 h (*t* = −120 min) after a 10-h overnight fast. Two i.v. catheters were inserted – one in an antecubital vein for infusion of the VLDL tracer and saline, and the other one in a dorsal vein on the contralateral hand. The latter was used for blood sampling. The hand was placed in a heated box in order to obtain arterialized blood. At *t* = 0 min, a bolus followed by a constant infusion of VLDL tracer was administered. The VLDL infusion was maintained for 240 min, and blood samples for determination of VLDL-TG concentration, specific activity (SA), and metabolites were drawn at *t* = 180, 210, and 240 min. Indirect calorimetry was performed for 30 min from *t* = 210 min.

VLDL tracer preparation The procedure used to label the TG moiety of the VLDL particle *ex vivo* has been described previously in detail (11). In brief, 80-ml blood was drawn from each participant, plasma was separated by a short spin, 30 µCi [1-¹⁴C]triolein (PerkinElmer, Inc., Turku, Finland) dissolved in 300 µl ethanol (Merck & Co., Inc.) was added, and the solution was then sonicated at 37 °C for 6 h. Subsequently, labeled VLDL particles were separated from other particles in plasma

by 18 h of ultracentrifugation at 98 350 *g* and 4 °C. Samples were tested for bacterial growth to ensure sterility, and the solutions were stored at 4 °C until use (<1 week).

VLDL-TG concentration and SA VLDL particles were isolated from ~3 ml of each plasma sample by ultracentrifugation as described above. The supernatant containing the VLDL fraction was obtained by tube slicing (Beckman Instruments, Inc., Palo Alto, CA, USA). Then, 300 µl were transferred to a scintillation vial, and ¹⁴C activity was measured by dual-channel liquid scintillation counting to <2% counting error (11).

Breath ¹⁴CO₂-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) occurred when exactly 0.25 mmol CO₂ was trapped in the solution. Scintillation fluid was added to the vial, and ¹⁴CO₂ activity was measured by liquid scintillation counting to <2% counting error.

Indirect calorimetry CO₂ flow and substrate oxidation rates (12) were measured by indirect calorimetry (Deltatrac monitor, Datex Instrumentarium, Helsinki, Finland). After 5 min, adaptation to the hood VCO₂ measurements was averaged from *t* = 185 to 210 min.

Calculations VLDL-TG production – primed-constant infusion:

VLDL production and clearance rates were calculated as described in detail previously (10):

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

VLDL-TG Clearance (ml/min)

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

VLDL-TG fatty acid oxidation – primed-constant infusion:

Fractional oxidation of the infused [1-¹⁴C]VLDL-TG was calculated as follows:

$$\text{Fractional VLDL-TG oxidation} = \frac{{}^{14}\text{CO}_2 \text{ SA} \times \text{VCO}_2}{k \times \text{Ar} \times F}$$

Here, *k* is the volume of CO₂ at 20 °C and 1 atm. pressure (22.4 l/mol), and Ar is the fractional acetate carbon recovery factor in breath CO₂, and *F* is the tracer infusion rate. Sidossis *et al.* (13) has previously calculated Ar to be 0.56 for resting conditions. The total VLDL-TG oxidation rate (µmol/min) was

calculated as follows:

VLDL-TG oxidation rate

$$= \text{fractional VLDL-TG oxidation} \times \text{VLDL-TG Ra}$$

Statistical analysis

Results were expressed as mean $\pm$ s.d. (parametric data) unless otherwise specified. Statistical comparisons between study days were assessed by paired *t*-test or Wilcoxon's test. $P < 0.05$ was considered significant.

Sample size was determined as follows: based on our volunteers body composition, presumed VLDL-TG levels, and previous studies done in our lab, we considered average basal VLDL-TG production rates of 50 $\mu\text{mol/min}$ with s.d. 10 $\mu\text{mol/min}$ realistic. Risk of type I and II errors (α and β) was set at 5% giving us a power of 95%. Earlier studies have indicated that VLDL-TG production rates may be augmented by 32–40% by month-long estrogen administration (14, 15). We therefore estimated differences between study days to be $\sim 25\%$ or 12.5 $\mu\text{mol/min}$. Sample size can then be calculated as $n = (C2\alpha + C\beta)^2 \times \text{s.d.}^2 / \Delta^2$, where $C2\alpha$ = constant for type I error (two-sided), $C\beta$ = constant for type II error (power 95%), and Δ = estimated difference. In our case, this gives $n = (1.96 + 1.645)^2 \times (10)^2 / 12.5^2$ – eight subjects.

Results

Circulating hormones and metabolites

By design, all participants had E_2 concentrations below detection threshold on the placebo study day, while administration of 4 mg E_2 resulted in an increase to premenopausal levels ((placebo versus E_2) E_2 (nM): 0.07 ± 0.01 vs 0.25 ± 0.04 , $P < 0.001$) by the end of the study period. E_2 treatment tended to lower circulating FFA, although not statistically significant (FFA (mM): 0.61 ± 0.13 vs 0.52 ± 0.14 , $P = 0.25$). Total TG concentrations were comparable (TG (mM): 1.22 ± 0.59 vs 1.10 ± 0.33 , $P = 0.48$).

Lipid and glucose oxidation

Lipid oxidation tended to decrease during E_2 treatment (lipid oxidation (mg/kg per min): 0.66 ± 0.32 vs 0.47 ± 0.08 , $P = 0.12$); in contrast, glucose oxidation increased significantly (glucose oxidation (mg/kg per min): 1.02 ± 0.20 vs 1.40 ± 0.36 , $P = 0.03$).

VLDL-TG kinetics

VLDL-TG SA and VLDL-TG concentrations are depicted in Fig. 1A and B. As seen, SA increased tenfold during the primed-continuous infusion, and isotopic steady state was present throughout the sampling period.

Figure 1 Effects of a single dose of oral estradiol 4 mg. (A) Total triglyceride and VLDL-triglyceride concentrations (open square, placebo; solid circle, estradiol). (B) VLDL-triglyceride-specific activity during primed-constant infusion of [$1\text{-}^{14}\text{C}$]VLDL-triglyceride. Isotopic steady state was obtained during the sampling period ($t = 180\text{--}240$ min). (C) VLDL-TG production rate. (D) VLDL-triglyceride clearance rate. Values are mean $\pm$ S.E.M.

VLDL-TG production (VLDL-TG production ($\mu\text{mol}/\text{min}$): 20.0 ± 12.4 vs 24.1 ± 10.7 , $P=0.33$) and clearance rates (VLDL-TG clearance rates (ml/min): 51.4 ± 16.8 vs 64.9 ± 28.8 , $P=0.34$) are presented in Fig. 1C and D. No significant difference in VLDL-TG production or clearance rate was observed.

VLDL-TG fractional oxidation

Fractional VLDL-TG oxidation (Fig. 2B) was not affected by E_2 treatment (Fractional VLDL-TG oxidation: 0.57 ± 0.17 vs 0.53 ± 0.08 , $P=0.53$). Likewise, absolute VLDL-TG oxidation (VLDL-TG oxidation ($\mu\text{mol}/\text{min}$): 12.3 ± 10.9 vs 12.6 ± 5.6 , $P=0.93$) did not differ significantly between study days.

Discussion

Although long-term estrogen treatment is known to increase plasma TG (2), little is known about the immediate effect on hepatic VLDL-TG production and peripheral clearance. The present study is the first to provide data demonstrating that a single dose of E_2 (4 mg) does not result in measurable changes in hepatic VLDL-TG production or oxidation in humans.

The high density of ERs on hepatocytes (16) has lead authors to speculate that E_2 impacts on hepatic VLDL particle formation and secretion. Indeed, animal studies have shown that E_2 stimulates hepatocyte VLDL-TG production and secretion (5, 6). Moreover, a few human tracer studies have demonstrated that long-term E_2 treatment augments hepatic VLDL-TG (14) secretion as well as VLDL apoB secretion (i.e. particle number) (17).

However, there are several points favoring the notion that E_2 could augment VLDL-TG production acutely: i) estrogens exert their effects not only via nuclear but also through membrane-bound receptors (7) enabling a rapid intracellular signaling response in the hepatocyte to changes in E_2 levels. ii) TG levels fluctuate during the day in healthy humans, and are modified within hours through changes in circulating hormones (e.g. insulin and GH) or metabolites (e.g. glucose and FFAs). iii) The increase in E_2 levels observed during the end of the follicular phase is in some studies (18) mirrored by a concomitant increase in TG levels. The impact of menstrual phase on VLDL-TG kinetics was therefore further explored by Magkos *et al.* (19), who, contrary to the author's initial hypotheses, found no effect of menstrual phase on VLDL-TG kinetics. However, that particular study was not designed to delineate the independent effects of E_2 perturbations on VLDL-TG kinetics but rather the combined effects of hormonal changes (E_2 and progesterone) during the menstrual cycle. Indeed, Wolfe *et al.* (20) demonstrated that estrogen combined with micronized progesterone administered for 7 weeks does not result in changes in VLDL particle turnover or VLDL-TG concentrations.

Figure 2 (A) Breath $[^{14}\text{CO}_2]$ production rate. Values are derived from measurement of $[^{14}\text{CO}_2]$ -specific activity in expired air and VCO_2 (indirect calorimetry). Isotopic steady state was reached during the sampling period ($t=180\text{--}240$ min) (open square, placebo; solid circle, estradiol). (B) Fractional oxidation (%) of infused VLDL-triglyceride fatty acid tracer. (C) VLDL-TG fatty acid oxidation rate ($\mu\text{mol}/\text{min}$). Values are mean $\pm$ S.E.M.

The present finding of no significant effect of short-term E_2 exposure on VLDL-TG production therefore indicates either i) that key enzymes involved in VLDL particle assembly (e.g. microsomal triacylglycerol transfer protein or diacylglycerol transferase) are only up-regulated after long-term exposure, or ii) that substrate availability (circulating FFAs or intrahepatic TG pools) increases after weeks of E_2 treatment. However, E_2 has been demonstrated to inhibit lipolysis (21) resulting in

decreased circulating FFAs (as also observed – albeit not significantly – in this study). It therefore seems plausible that E₂ treatment alters hepatic VLDL-TG formation via slow genomic effects on enzymes involved in both VLDL-TG synthesis and VLDL particle formation.

Oral estrogen treatment has been demonstrated to decrease lipid oxidation in a dose-dependent manner in humans (22), and has been shown to reduce ketogenesis (a by-product of hepatic lipid oxidation) in cell culture studies (23). E₂ action in the liver therefore likely involves channeling FFAs from oxidative to nonoxidative pathways (primarily VLDL-TG production). We therefore hypothesized that peripheral VLDL-TGs would be diverted in a similar manner away from oxidation and toward storage, i.e. that VLDL-TG oxidation in skeletal muscle would decrease following E₂ exposure. Again, our finding of no significant change in VLDL-TG oxidation supports the notion that E₂ affects lipid metabolism via slow pathways, both in the liver and in peripheral tissues. We chose a dose of 4 mg of E₂ in order to make sure that we would achieve premenopausal circulating levels of serum E₂ in the pre-menopausal range, which is often not achieved with lower doses (1–2 mg) of E₂ often used in the typical postmenopausal setting (24, 25). A limitation to the study is the possible type 2 statistical error due to the low number of subjects. Although our power calculation shows that eight subjects in a cross over design is sufficient to detect a statistical difference, our results need to be confirmed in larger studies. Our results cannot be extended to describe the effects of other routes of delivery, e.g. transdermal estrogen. While oral estrogen results in greater concentrations and VLDL-TG particle production (Apo-B100), transdermal estrogen administration results in unchanged or reduced VLDL-TG concentrations (26) and unchanged VLDL-TG particle production. The latter finding is, to our knowledge, only reported in one study by Walsh *et al.* (1). Moreover, we cannot conclude whether short-term treatment (e.g. 1 week) is sufficient to elicit statistical differences in VLDL-kinetics or whether treatment withdrawal results in rapid reversal of such effects. Thus, future studies should address the effect of short-term treatment and as well as the impact of treatment withdrawal in order to gain a more complete knowledge of the time-dependent changes in VLDL-kinetics. In summary, short-term E₂ exposure does not alter VLDL-TG production, clearance, or oxidation in healthy postmenopausal women. These data therefore indicate that HRT-associated dyslipidemia has a gradual rather than immediate onset.

Declaration of interest

L C Gormsen, C Høst, B E Hjerrild, and S Nielsen have no disclosures. C Gravholt has received lecture fees from Novo Nordisk and Genentech. S Nielsen has received lecture fees from Sanofi-Aventis and MSD.

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