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Are there endogenous estrone fatty acyl esters in human plasma or ovarian follicular fluid?

Veera Vihma[a,b,*], Anja Koskela[a], Ursula Turpeinen[c], Esa Hämäläinen[c], Aila Tiitinen[d], Kristiina Wähälä[e], Matti J. Tikkanen[a,b], Herman Adlercreutz[a]

[a] Folkhälsan Research Center, Biomedicum Helsinki, P.O.B. 63, FIN-00014 University of Helsinki, Finland
[b] Institute of Clinical Medicine, Department of Medicine, University of Helsinki, and Division of Cardiology, Helsinki University Central Hospital, 00290 Helsinki, Finland
[c] Department of Clinical Chemistry, Helsinki University Central Hospital, 00290 Helsinki, Finland
[d] Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, 00290 Helsinki, Finland
[e] Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, 00140 Helsinki, Finland

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ABSTRACT

Background: Estrone and its sulfated esters are the most abundant estrogens in blood in men and in women after the menopause. However, previous studies on the esterification of estrone with fatty acids have yielded conflicting results, some studies reporting high nanomolar concentrations of estrone fatty acyl esters in plasma.
Methods: We developed an estrone radioimmunoassay (RIA) method to determine endogenous concentrations of estrone and after saponification, applied it to male and female plasma. In addition, the concentration of estrone fatty acyl esters in ovarian follicular fluid was analyzed by gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS).
Results: By estrone RIA, we did not find measurable amounts of estrone fatty acyl esters in male or female plasma, except for one premenopausal woman who had the highest plasma concentration of nonesterified estrone. The concentration of hydrolyzed estrone fatty acyl esters determined by LC–MS/MS in follicular fluid obtained from women undergoing ovarian stimulation was below the limit of quantification of <10 pmol/l (<2.7 ng/l).
Conclusions: In contrast to previous data by others, our study suggests that estrone fatty acyl esters are in most cases not detectable in plasma of healthy men or healthy nonpregnant women.

1. Introduction

Estrone is one of the major estrogens secreted by the ovaries. In plasma, estrone-3-sulfate is the most abundant estrogen, derived from the peripheral conversion of estrone and 17β-estradiol. Estrone is also the major estrogen in plasma after menopause, produced in the peripheral tissues by aromatization of steroid precursors [1]. One of the metabolic pathways of steroids is esterification with long chain fatty acids [2,3]. A significant proportion of steroid hormone precursors pregnenolone and dehydroepiandrosterone circulate as lipophilic fatty acid esters associated with lipoproteins in human blood [4–6]. The concentration of 17β-estradiol fatty acid esters in female plasma is substantially lower than the esters of pregnenolone or dehydroepiandrosterone but is increased by oral estrogen therapy and during pregnancy [7–9]. The possibility that estrone would occur naturally in the fatty acid esterified form in plasma has been less studied. However, studies by M. Alemany's group have suggested that estrone fatty acyl esters would circulate in human blood in high nanomolar concentrations, far exceeding those of unconjugated and sulfated estrone [10–12]. In this communication, we developed an indirect estrone radioimmunoassay (RIA) method to analyze concentrations of hydrolyzed estrone fatty acyl esters in human male and female plasma. As ovarian follicular fluid obtained from women undergoing ovarian stimulation is known to have high concentrations of estrogens [13], we also studied whether it contained estrone in the form of fatty acyl esters.

Abbreviations: CV, coefficient of variation; GC–MS, gas chromatography–mass spectrometry; HDL, high density lipoprotein; HPLC, high pressure liquid chromatography; IS, internal standard; LCAT, lecithin:cholesterol acyltransferase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ND, not detectable; RIA, radioimmunoassay.

* Corresponding author at: Folkhälsan Research Center, Biomedicum Helsinki C415, Haartmaninkatu 8, 00290 Helsinki, Finland. Tel.: +358 9 4717 1881; fax: +358 9 4717 4013.
E-mail address: veera.vihma@hus.fi (V. Vihma).

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2. Experimental

2.1. Subjects and samples

Blood was drawn from ten healthy men aged 43–68 yrs and six healthy nonpregnant women (aged 23–56 yrs) to EDTA-containing vacuum tubes. Plasma was prepared by centrifugation within 1 h ($2500 \times g$, 15 min, +4 °C) and stored at −20 °C until analyzed. In addition, pooled sera from 50 to 60 male donors were purchased from The Finnish Red Cross, Helsinki, Finland. Ovarian follicular fluid was obtained from women undergoing ovarian stimulation for *in vitro* fertilization at the Helsinki University Central Hospital as described in [7]. Follicular fluid was centrifuged twice ($2300 \times g$, 15 min, +10 °C) to remove blood cells and cell debris, and stored at −80 °C. The study was approved by the Ethics Committee of Helsinki University Central Hospital, and written informed consent was obtained from subjects.

2.2. Estrogens

Estrone was purchased from Makor Chemicals (Jerusalem, Israel). Estrone-3-oleate was synthesized as described in [14]. [2,4,6,7-^{3}H(N)]Estrone (specific activity 74 Ci/mmol) and [6,7-^{3}H(N)]estrone sulfate, ammonium salt (specific activity 53 Ci/mmol) were purchased from NEN, Boston, MA. 4-[^{14}C]Estrone-3-oleate was synthesized from 4-[^{14}C]estrone (PerkinElmer Life Sciences Inc., Boston, MA; specific activity 51.3 mCi/mmol) as described in [15]. [^{14}C]Estrone-3-oleate was purified by Sephadex LH-20 column chromatography in hexane:chloroform (1:1, v/v) prior to use.

2.3. Extraction, separation of esterified from unesterified estrone, saponification and chromatographic purification of the ester fraction for RIA

The outline of the method is shown in Fig. 1. Plasma (1 ml) or ovarian follicular fluid (0.1 ml or 0.2 ml) was pipetted into disposable extraction tubes. [^{14}C]Estrone-3-oleate [~20,000 cpm (180 pmol) in 10 μl of ethyl acetate] was added to three tubes and used as an internal standard to measure recovery. The samples were extracted four times with two volumes of diethyl ether–ethyl acetate (1:1 by volume), as described in [7]. The combined organic phases were evaporated to dryness under N_2. To separate fatty acid esterified estrone from nonesterified estrone, we used hydrophobic chromatography on Sephadex LH-20 columns (5 mm × 50 mm in disposable Pasteur pipettes; GE Healthcare Bio-Sciences AB, Uppsala, Sweden), modified from Vihma et al. and Miilunpohja et al. [7,16]. In short, the samples were applied to the columns in two 0.2-ml aliquots of hexane–chloroform (2:1 by volume). The estrone ester fraction was eluted with 4 ml of the same solvent. The nonesterified estrone fraction was then eluted with 3 ml of methanol. The estrone fatty acyl ester fraction was saponified at +60 °C for 2 h in 0.5 ml of methanolic KOH (1 mol/l). After incubation, 0.5 ml of water was added and the sample was neutralized with 125 μl of 4 mol/l HCl. After evaporation under N_2 until ~0.5 ml of the water phase was left in the tubes, the samples were extracted twice with 1.5 ml of diethyl ether, the ether phases were combined and evaporated.

To remove lipophilic substances that might interfere with the immunoassay, the samples containing hydrolyzed estrone esters were subjected to a second Sephadex LH-20 column chromatography in hexane–chloroform (2:1 by volume; Fig. 1). The sample was applied to a 3-cm column in two 0.2-ml aliquots of the same solvent. After eluting lipophilic impurities with 3 ml of the same solvent, the hydrolyzed estrone ester fraction was eluted with 3 ml of methanol. After evaporation, the samples were subjected to a third Sephadex LH-20 column chromatography carried out using 9% methanol in toluene to separate estrone from estradiol and estriol [17], and analyzed by RIA as described below.

2.4. Radioimmunoassay

After evaporation, the samples were dissolved in 1.1 ml of phosphate buffer [NaH_2PO_4, Na_2HPO_4, NaCl, thiomersal, gelatin (Merck), H_2O; pH = 7]. The recovery of the internal standard, hydrolyzed [^{14}C]estrone-3-oleate, was determined by liquid scintillation counting (Rack-beta, Wallac Oy, Turku). Two 0.5-ml aliquots were taken for RIA. The ovarian follicular fluid samples were diluted 2.5 to 5-fold for the analysis of hydrolyzed estrone ester fraction and 25-fold for the analysis of estrone fraction. The calibrators for RIA [29 pmol/l to 7400 pmol/l (7.8 ng/l–2000 ng/l)] were made from an estrone stock solution in methanol by serial dilutions with the assay buffer. Estrone antiserum (Medicorp Inc., Montreal, Canada) was diluted 1:150 with the assay buffer, according to the protocol of the manufacturer. Mixture of dextran (0.625 g; Dextran T70, Pharmacia) and charcoal (0.625 g, NoritA, Pharmacia) in 100 ml of assay buffer was prepared by mixing for 30 min and stored overnight at +4 °C. Antiserum (100 μl) and [^{3}H]estrone (50 μl) were added to the samples which were then incubated for 20 h at +4 °C. To the ice cold samples, 200 μl of dextran–charcoal mixture was added. After mixing for 45 s, the samples were incubated in an ice bath for 10 min and then centrifuged for 2.5 min ($8000 \times g$). 500 μl of the supernatant was taken for determination of radioactivity by liquid scintillation counting.

2.5. GC–MS

For follicular fluid (1–4 ml), the extraction and the first Sephadex LH-20 chromatography to separate esterified and nonesterified estrone were performed as explained in Section 2.3 (Fig. 1). In part of the experiments, the ester fraction was evaporated to dryness and re-chromatographed on Sephadex LH-20. The ester fraction was collected, evaporated to dryness and subjected to saponification (see Section 2.3 and Fig. 1). After saponification and neutralization, the deuterated internal standard (IS) d4-estrone (74.8 ng in 20 μl of methanol) was added to the samples. Thereafter the samples were extracted and the lipophilic substances removed by chromatography as explained in Section 2.3. The hydrolyzed estrone ester fraction was evaporated to dryness. An equal amount of deuterated estrone internal standard was added to the calibrators, ranging from 0.67 ng to 66.7 ng of estrone (corresponding to 40 pg to 4002 pg injected on-column). Derivatization was performed by incubating the samples and calibrators in the presence of 50 μl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide:ammonium iodide:dithioerythritol (1000:2:4; v/w/w) for 30 min at +60 °C. A 3-μl aliquot of the samples and calibrators was injected into the GC–MS using splitless injection mode. The analytes were separated by gas chromatography using a BP-1 column (length 15 m, I.D. 0.22 mm, film thickness 0.25 μm; SGE International Pty Ltd., Ringwood, Australia) with helium as carrier gas (flow 1 ml/min) and detected by GC–MS-SIM method utilizing 70 eV EI+ ionization. The ions monitored were *m/z* 414.2 and 399.2 for estrone-diTMSi, and 417.2 and 402.2 for d4-estrone-diTMSi (IS). The instruments included a Fisons Instrument (Milan, Italy) MD 1000 quadrupole mass spectrometer and a Fisons GC 8000 gas chromatography system. Data were processed using XcaliburConfig MFC Application (version 1.0.0.1). The calibration curve was linear with a mean correlation coefficient of 0.999 ($n = 3$). The limit of quantification of GC–MS was 10 pg of estrone standard on-column (a signal to noise ratio, S/N = 5).