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

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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.

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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).

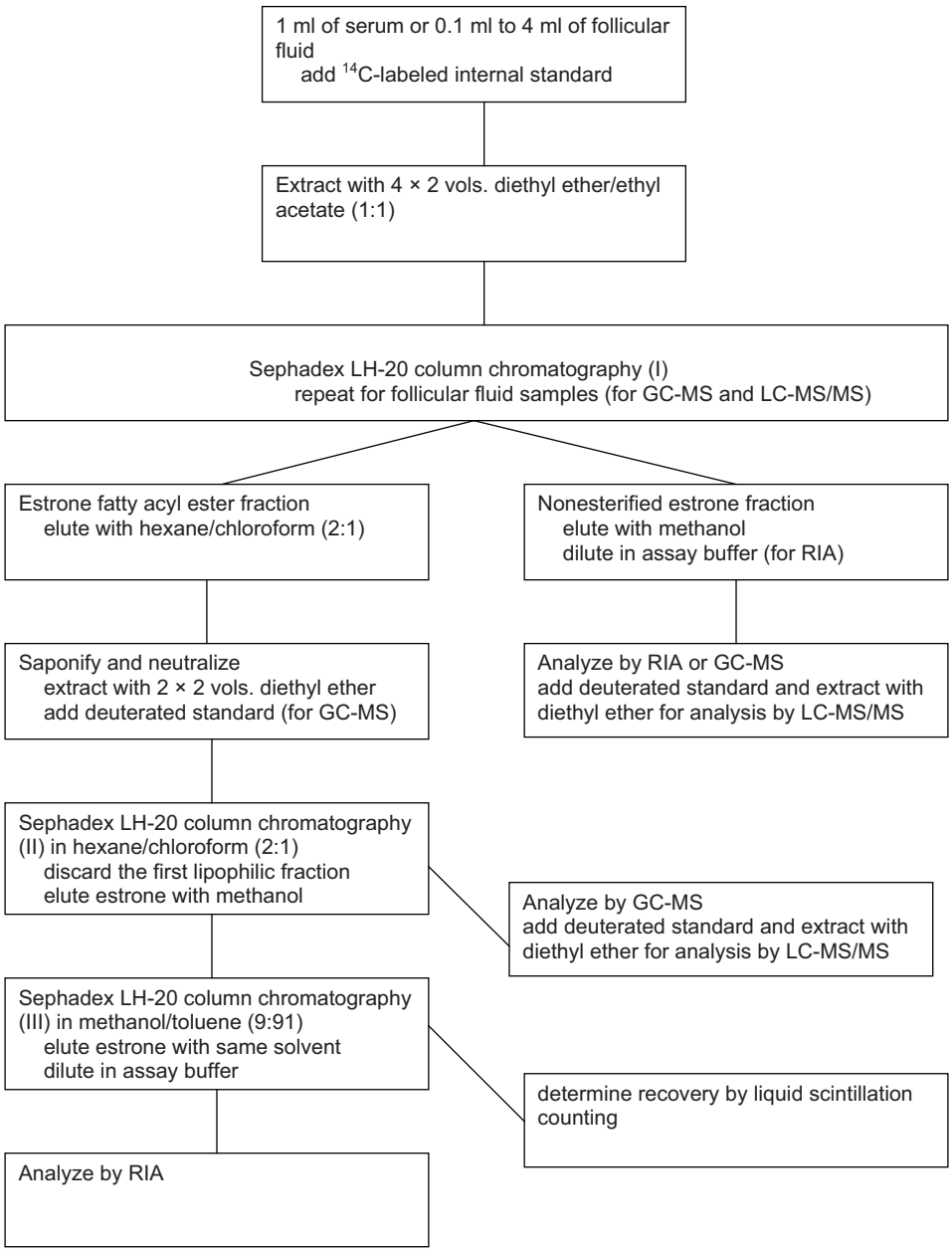

Fig. 1. Outline of the method for determination of estrone fatty acyl esters and estrone in plasma and ovarian follicular fluid.

2.6. LC–MS/MS

The estrone ester and nonesterified estrone fractions of follicular fluid were processed as described in Section 2.5 for GC–MS (Fig. 1). After the last chromatography on Sephadex LH-20, the samples were evaporated and dissolved in 1 ml of methanol–water (1:1 by volume). The follicular fluid nonesterified estrone fractions were diluted 1000-fold prior to analysis by LC–MS/MS. Deuterated estrone (d_2-E_1; 30 μl) was added as an internal standard to an aliquot of 250 μl of the sample. After extraction with 4 ml of diethyl ether and evaporation, the samples were dissolved in 125 μl of methanol–water (1:1 by volume) and a 25-μl aliquot was injected to LC–MS/MS (API 4000 triple quadrupole mass spectrometer; MSD Sciex, Concord, CA). Peripherals included an Agilent series 1200 HPLC system with a binary pump (Waldbronn, Germany) and a SunFire C_{18} column (2.1 mm × 100 mm; 3.5 μm; Waters, Milford, MA). A six-point calibration curve was included with each assay, using calibrator concentrations from 25 to 1000 pmol/l in 4% bovine albumin. The limit of quantification was 10 pmol/l (S/N = 10). The total coefficient of variation (CV) was 11% at 144 pmol/l. Data were acquired and processed with Analyst Software (MSD Sciex).

3. Results

3.1. Assay characteristics (RIA) and estrone fatty acyl esters in human plasma and ovarian follicular fluid

The precision profile of estrone RIA was calculated from nine replicates of each calibrator. The working range of the estrone RIA was 43–7400 pmol/l (12–2000 ng/l) when a CV of 15% was taken as the discrimination limit. The lower limit of detection of the estrone fatty acyl ester method was 69 pmol/l (19 ng/l), defined as the mean + 3 SD concentration [18] calculated from 47 determinations of the reagent blank in 16 consecutive assays. The mean (SD) reagent blank in these assays was 27 (14) pmol/l [7.3 (3.9) ng/l]. The mean analytical recovery of three different concentrations

Table 1
Analytical recovery by RIA of estrone-3-oleate and estrone added to pooled female plasma.

Sample	*n*	Estrone-3-oleate (pmol/l)[a]		Recovery[c] (%)
		Added	Observed[b]	
1	4	169	151	89
2	5	507	506	100
3	5	845	730	86
		Estrone (pmol/l)		
		Added[d]	Observed[b]	
4	5	128	136	106

[a] Concentration of estrone-3-oleate is expressed as pmol/l estrone.
[b] Expressed as median from multiple determinations in the same assay. The value for pooled female plasma without added estrogen was subtracted from the samples.
[c] Calculated as (observed/added) × 100.
[d] Nonesterified estrone was added to the ester fraction obtained from the first Sephadex LH-20 column chromatography (Fig. 1), saponified and analyzed similarly to estrone esters.

of estrone-3-oleate added to pooled female plasma is shown in Table 1. The recovery of nonesterified estrone added to estrone ester fraction obtained from the first Sephadex LH-20 column chromatography, and then saponified and analyzed similarly to estrone esters, is also shown in Table 1. The mean (SD) recovery of the radioactive internal standard, [^{14}C]estrone-3-oleate, added to plasma or ovarian follicular fluid samples was 64% (3.8) in 11 assays, number of determinations $n = 32$. Regarding the specificity, the reported cross-reactivity of estrone antiserum was less than 0.1% for estradiol, estriol and other steroids. As studied with [^{3}H]estrone sulfate, most of it remained in the water phase in the extraction from plasma with organic solvents and less than 0.2% of the [^{3}H]radioactivity was recovered in the organic solvent fraction. Estradiol and estriol were removed from the samples in the end of the method by the last Sephadex LH-20 column chromatography in methanol–toluene (Fig. 1). When varying amounts of nonradioactive estrone [5.1 nmol/l to 5.1 μmol/l (1.4 μg/l to 1.4 mg/l)] were added to plasma in several experiments, less than 0.05% of added estrone was recovered in the estrone ester fraction as analyzed by RIA.

Plasma from men ($n = 10$) was pooled into four pools. The male plasma pools as well as plasma obtained from women were analyzed for estrone esters as single determinations in seven to eight consecutive assays by RIA (Table 2). Also, pooled female plasma was analyzed in duplicate in five consecutive assays. The concentration of nonesterified estrone in plasma was determined in one single assay (Table 2). The estrone ester concentration in all male plasma pools was below the limit of detection (Table 2). In women, the plasma estrone ester concentrations were below the detection limit and in most cases similar to the value of the reagent blank in all but one 50-yr old female. In this subject, the measured concentration of hydrolyzed estrone esters was above the detection limit in only four out of seven assays. Thus, it is possible that the estrone ester found is artifactual due to a possible contamination or matrix effect interfering with the immunoassay method. The plasma concentration of unconjugated estrone in this subject was the highest among the women studied (Table 2).

In ovarian follicular fluid ($n = 4$), the median concentration of estrone fatty acyl esters analyzed by RIA was 46.5 nmol/l and that of nonesterified estrone was 286 nmol/l. Based on these determinations, human ovarian follicular fluid was thought to contain rather high concentrations of endogenous fatty acid esters of estrone. Subsequently, intra- and interassay imprecision of the RIA method was assessed by analyzing estrone esters in samples of ovarian follicular fluid diluted with different amounts of pooled human male sera. Although the method showed adequate within-run reproducibility

Table 2
Determination of hydrolyzed estrone fatty acyl esters and nonesterified estrone in male and female plasma by RIA.

	Assays (*n*)	Estrone fatty acyl ester (pmol/l)[a]	Estrone (pmol/l)[b]
Men			
Plasma pool 1[d]	8	ND	<43
Plasma pool 2[e]	8	ND	79
Plasma pool 3[f]	8	ND	103
Plasma pool 4[g]	7	ND	96
Women			
23 yrs	8	ND	124
50 yrs (premenop.)	7	87 ($n = 4$)[c]	1420
Plasma pool[h]	5	ND	148
54 yrs (postm.)	8	ND	<43
55 yrs (postm.)	8	ND	<43
56 yrs (postm.)	7	ND	<43

ND, not detectable; premenop., premenopausal; postm., postmenopausal.
[a] Single determinations in consecutive assays. The detection limit of the estrone ester method was 69 pmol/l.
[b] Single determination in one assay.
[c] The median of the detectable concentrations in four assays. Data are expressed as pmol/l estrone.
[d] Pooled plasma from a 43-yr-old male (time interval between the two blood samples, 2 weeks).
[e] Pooled plasma from four men (aged 50–61 yrs, mean age 55 yrs).
[f] Pooled plasma from a 67-yr-old male (time interval between the first and third blood sample, 6 months).
[g] Pooled plasma from four men (aged 67 yrs).
[h] Pooled plasma from three women (aged 27, 53 and 54 yrs). Duplicate determinations in 5 assays.

(mean CV of 8%, 10% and 13% for three different dilutions, number of determinations $n = 10$ or 12), the interassay variation of the same samples was 18% or higher, (number of assays $n = 7$). Moreover, in contrast to the good dilution linearity of the nonesterified estrone fraction (data not shown), the dilution linearity of the hydrolyzed estrone ester fraction of follicular fluid, diluted either with male plasma or RIA buffer, was not satisfactory. This led us to suspect that something was interfering with the analysis of estrone esters in follicular fluid by RIA. Some kind of matrix effect was one possibility. Cross-reaction with other estrogens present in high concentrations in follicular fluid was another possibility, despite the many chromatographic purification steps as well as the specific antiserum used.

3.2. Estrone fatty acyl esters in ovarian follicular fluid analyzed by GC–MS and LC–MS/MS

Further analysis of estrone fatty acyl esters in ovarian follicular fluid by GC–MS gave much lower concentrations compared to those originally obtained by RIA. The concentration of esterified estrone ranged between 0.5 nmol/l and 2.5 nmol/l in three individual follicular fluid samples (duplicate determinations in two separate assays), and that of nonesterified estrone between 30 nmol/l and 89 nmol/l (number of determinations $n = 4$ in a single assay). To investigate the possibility that nonesterified estrone was leaking into the estrone ester fraction thus falsely giving elevated values for estrone esters, follicular fluid from the same subjects were subjected to two subsequent chromatographies on Sephadex LH-20 instead of one (see Section 2.5), to separate estrone esters from nonesterified estrone. When these samples were analyzed by GC–MS, no estrone in the fatty acid ester fraction was detectable. This indicated a previous contamination of the estrone ester fraction by the abundant follicular fluid nonesterified estrone. Analysis of the same samples by LC–MS/MS confirmed that human ovarian follicular fluid contained no quantifiable amounts (<10 pmol/l) of estrone fatty acyl esters. The concentration of nonesterified estrone

in follicular fluid ranged between 40 nmol/l and 53 nmol/l, and correlated with the results obtained by GC–MS.

4. Discussion

We studied male and female plasma by an indirect estrone RIA method and, in addition, ovarian follicular fluid also by GC–MS and LC–MS/MS and found no reliably quantifiable amounts of estrone fatty acyl esters in either human body fluid. In most of the RIA analyzes, the plasma concentrations of hydrolyzed estrone fatty acyl esters in men and women were similar to the value of the reagent blank. In the case of follicular fluid, something was interfering with the analysis of hydrolyzed estrone esters by RIA, but further analyzes by mass spectrometry clearly demonstrated the absence of quantifiable amounts of estrone in the isolated estrone fatty acyl ester fraction. The concentrations of nonesterified estrone in plasma as determined by RIA, and in follicular fluid by GC–MS and LC–MS/MS, were in agreement with previous studies which used LC–MS/MS as the analytical method [13,19,20].

Our data are in contrast to previous studies by M. Alemany's group which has reported of high estrone fatty acyl ester concentrations (>100 nmol/l) in male and female plasma [10,11]. Recently, they reported limitations of their previous estrone RIA method [21] and that the actual concentration of fatty acyl esterified estrone in human plasma was lower than they had previously reported, ranging from <5 nmol/l to 50 nmol/l, as analyzed by a different method [12,22]. However, based on our present study in 16 subjects by a validated RIA method, we conclude that estrone fatty acyl esters could not be detected in human blood in men and the majority of the women (Table 2). Moreover, if estrone would naturally become esterified with fatty acids in the human body, we hypothesized that ovarian follicular fluid from stimulated ovaries with high concentrations of various steroids and also their fatty acylated forms [7,13,23,24] would most likely contain these metabolites. However, we detected no quantifiable amounts of estrone in the fatty acyl ester fraction of follicular fluid by either GC–MS or a highly sensitive estrone LC–MS/MS method.

Several experimental studies have previously indicated that estrone is not esterified in human plasma [25,26]. In addition, studies in experimental animals have shown that estrone is not converted to fatty acid esters in tissues with high steroid esterifying activity like in rat mammary tumors [27] or hepatic microsomes [28]. In experiments using microsomal fraction from the rat brain, Vourc'h et al. observed slight conversion of estrone to lipophilic conjugates but this was attributed to conversion of estrone to estradiol and subsequently to estradiol-17-esters [29]. However, in our previous work, we were able to identify intact fatty acid esters of estrone in the isolated high density lipoprotein (HDL) fraction by LC–MS after having incubated supraphysiological concentrations of estrone in the presence of human female plasma [16]. This was surprising and in contrast to the previous findings by other investigators who did not detect lipophilic estrone conjugates upon incubating plasma in the presence of exogenous estrone [25,26]. In the case of ovarian follicular fluid, incubation of [^{3}H]estrone in the presence of follicular fluid resulted mostly in the formation of lipophilic derivatives of estradiol associated with the HDL fraction, but we also detected lipophilic derivatives of [^{3}H]estrone in the follicular fluid HDL [16]. The enzyme responsible for the esterification of estrone in these experiments was not identified. Estrone is a very nonpolar compound, and as shown by experimental studies, becomes associated with lipoproteins *in vitro* [26,30,31]. However, HDL-associated lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible of the esterification of estradiol and many other steroids in plasma, is not supposed to have esterifying activity towards estrone which has a ketone group at C-17 and a phenolic hydroxyl group at C-3. Thus, although fatty acid esters of estrone may be formed experimentally by incubating very high concentrations of estrone in the presence of human plasma [16], we did not, with the exception of one female, find measurable amounts of naturally occurring estrone esters in plasma obtained from healthy men or healthy nonpregnant women.

In conclusion, the possibility that estrone fatty acyl esters might occur in low concentrations in human plasma cannot be excluded, although most of our study subjects had no detectable amounts of estrone esters in plasma. In ovarian follicular fluid, esterification with fatty acids does not appear to be a physiologically relevant reaction for estrone. However, the question whether estrone fatty acid esters would have a role in other tissues, for example in human adipose or breast tissue [32], was not assessed in this study.

Disclosure

The authors have nothing to disclose.

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