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Review

Fatty acid esters of steroids: Synthesis and metabolism in lipoproteins and adipose tissue

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ABSTRACT

At the end of the last century ideas concerning the physiological role of the steroid fatty acid ester family were emerging. Estrogens, fatty acylated at C-17 hydroxyl group and incorporated in lipoproteins were proposed to provide antioxidative protection to these particles. A large number of studies involving non-estrogenic adrenal steroids, and their fatty acylated forms, demonstrated their lipoprotein-mediated transport into cells and subsequent intracellular activation, suggesting a novel transport mechanism for lipophilic steroid derivatives. After these important advances the main focus of interest has shifted away from C-19 and C-21 steroids to fatty acylated estrogens. However, interest in their lipoprotein-mediated transport has decreased because only minute amounts of these derivatives were detected in circulating lipoproteins, and their antioxidative activity remained unconfirmed under physiological circumstances. It now appears that the overwhelming majority of estradiol in postmenopausal women resides in adipose tissue, most of it in esterified form. This is poorly reflected in plasma levels which are very low. Recent data suggest that estrogen fatty acid esters probably represent a storage form. The future focus of investigation is likely to be on firstly, the enzymatic mechanisms regulating the esterification and de-esterification of estradiol and other steroids residing in adipose tissue and secondly, on the role of insulin and other hormones in the regulation of these enzymatic mechanisms. Thirdly, as a large proportion of fatty acid esterified C-19 and C-21 non-estrogenic steroids is transported in lipoproteins and as they are important precursors of androgens and estrogens, this field should be investigated further.
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Contents

1. Introduction 66
2. Human studies: fatty acid esterification of steroids 66
3. Studies in experimental animals 68
   3.1. Fatty acid esterification of steroids 68
   3.2. Studies in adipose tissue and the mammary gland 68
4. Antioxidative effects of fatty acylated estrogens in lipoproteins 69
5. Metabolism of lipoprotein-associated steroid fatty acid esters 69
6. Endogenous steroid fatty acid esters in humans 71
   6.1. Circulating steroid fatty acid esters 71
   6.2. Adipose tissue 73
   6.3. Ovarian follicular fluid 73

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; ACAT, acyl coenzyme A:cholesterol acyltransferase; apoAI, apolipoprotein AI; BLT-1, blocker of lipid transport 1; CETP, cholesterol ester transfer protein; DHEA, dehydroepiandrosterone; DTNB, dithionitrobenzoic acid; eNOS, endothelial nitric oxide synthase; GC–MS, gas chromatography–mass spectrometry; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; IVF, *in vitro* fertilization; LAL, lysosomal acid lipase; LCAT, lecithin:cholesterol acyltransferase; LC–MS, liquid chromatography–mass spectrometry; LDL, low density lipoprotein; PPARα, peroxisome proliferator-activated receptor alpha; RIA, radioimmunoassay; SHBG, sex hormone-binding globulin; SR-B1, scavenger receptor class B, type 1; TLC, thin layer chromatography.

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7. Fatty acid esters of sex steroids in invertebrates 74
8. Conclusions 74
Disclosure statement 74
Acknowledgments 74
References 74

1. Introduction

A landmark review was published in 1998 by Richard B. Hochberg [1]. It summarized everything known up to that time of a unique hormone family consisting of lipophilic derivatives of steroids which were transported in the circulation incorporated in lipoprotein particles. These substances were characterized as steroids bound to long-chain fatty acids by an ester bond. They are formed by lecithin:cholesterol acyltransferase (LCAT) in plasma but by different acyltransferases in other tissues. In plasma, cholesterol is the most avidly esterified steroid by LCAT, followed by adrenal steroids pregnenolone and dehydroepiandrosterone. 17β-Estradiol (estradiol), the principal and most potent estrogen in women, is also esterified with fatty acids in plasma and other tissues. Estradiol fatty acid esters do not bind to estrogen receptors and therefore require to be hydrolyzed to estradiol to exert hormonal effects. As indicated by metabolic studies, this hydrolysis of the ester bond is relatively slow. Accordingly, fatty acid esterified estradiol administered to experimental animals has shown stronger and more sustained estrogenic effects as compared with estradiol.

By 1998 major theories concerning the physiological role of steroid fatty acid esters were emerging [1]. Estrogens containing one or more hydroxyl groups in the aromatic A ring of the molecule exhibited antioxidative activity in lipid–aqueous systems *in vitro* and were proposed to provide antioxidative protection for lipoprotein particles. This was plausible as the estrogens were fatty acylated at C-17 OH group leaving the proposed antioxidative aromatic hydroxyl group at C-3 free (Fig. 1) [1]. In addition, some studies involving non-estrogenic C-19 and C-21 steroids demonstrated that esterified steroids carried in low density lipoprotein (LDL) particles could be internalized into cells *via* lipoprotein receptors, and converted to other biologically active metabolites [1]. The data suggested that esterified steroids had a dual role in lipoproteins. Some of them acted as antioxidants and had therefore antiatherogenic potential. They were also being transported in lipoprotein particles to various tissues in which they could enter the cells and become activated and metabolized further. Also, the first quantitative methods for steroid fatty acid ester determination in blood, ovarian follicular fluid and adipose tissue were developed and initial results raised the possibility that hydrophobic derivatives of steroid hormones might be storage forms.

2. Human studies: fatty acid esterification of steroids

The enzyme responsible for the esterification of steroid hormones in humans has only been characterized in plasma and ovarian follicular fluid [1]. In these tissues, the esterifying enzyme is LCAT, the same enzyme that esterifies cholesterol. LCAT is known to catalyze the esterification of pregnenolone, dehydroepiandrosterone, 5-androstene-3β,17β-diol (androstenediol) and estradiol [2]. The biosynthesis of fatty acid esters of estriol, a less lipophilic estrogen, was characterized for the first time by Kanji et al. in human plasma, adipose tissue and placenta [3]. The rate of estriol esterification in plasma was slow, less than 10% of that of estradiol. The LCAT inhibitor, dithionitrobenzoic acid (DTNB), markedly inhibited the esterification of both estriol and estradiol in plasma. Characterized by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), almost equal amounts of estriol 16α- and 17β-esters were formed in adipose tissue and placenta while in plasma, the ratio of 17β-esters to 16α-esters was approximately three. This predominant esterification of estriol at the 17β-hydroxyl was considered unexpected because this site is sterically hindered by the adjacent C-18 methyl group [3]. The study also indicated that there exist several enzymes which catalyze fatty acylation of sex steroids in various human tissues. Androstenediol became esterified in plasma only at the 3β-hydroxyl but in adipose tissue mainly 17β-esters were formed. In placenta, approximately equal amounts of 3- and 17-esters were formed. Despite the similar D-ring structure of testosterone and estradiol, no testosterone fatty acid esters were synthesized in plasma confirming that testosterone is not a substrate for LCAT. However, in other tissues, testosterone was esterified at the 17β-hydroxyl. In summary, only estrogens, not structurally similar C-19 steroids, were esterified by LCAT at the D ring of the molecule, and predominantly at the C-17 hydroxyl.

Another important finding by Kanji et al. [3] was that the rate of estradiol esterification in human adipose tissue was high. This was different from early studies in the rat which reported a very low estradiol esterification activity in the rat adipose tissue [4,5]. In human adipose tissue, the acyltransferase activity for estradiol was approximately twenty times higher than in placenta and also several-fold higher than in plasma [3]. This is in line with the high

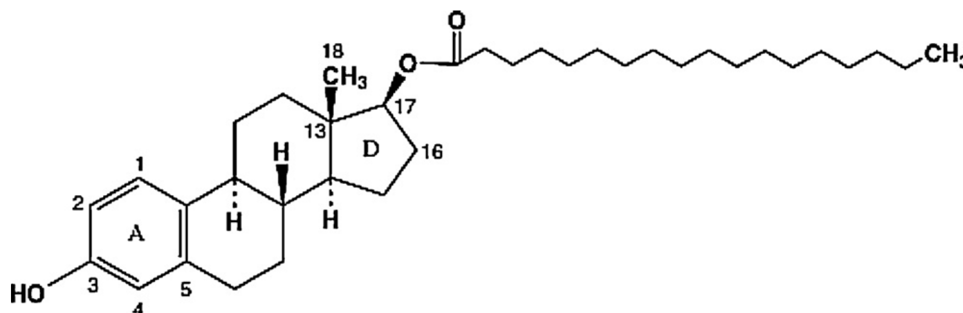

Fig. 1. Structure of 17β-estradiol 17-stearate. The 17β-hydroxyl in the D ring is esterified with a long-chain fatty acid.

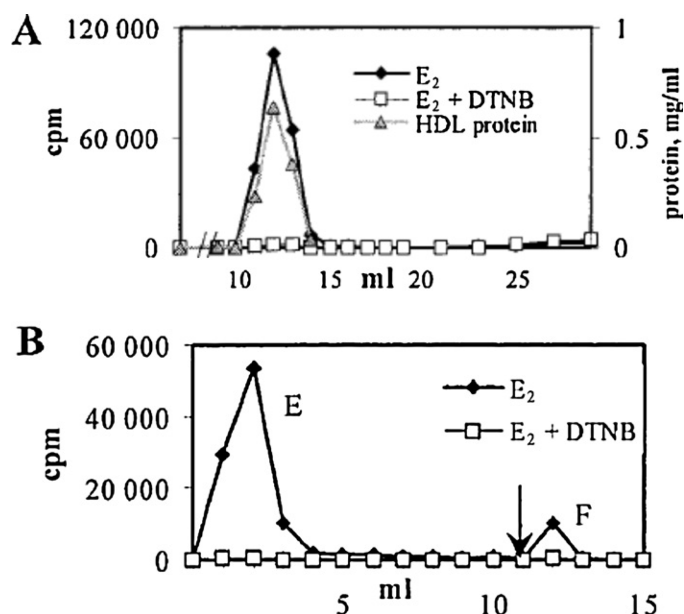

Fig. 2. The radioactivity elution pattern of follicular fluid HDL after chromatography on Sephadex G-25 and Sephadex LH-20 columns. Ovarian follicular fluid was incubated in the presence of [^{3}H]estradiol with and without the LCAT inhibitor dithionitrobenzoic acid (DTNB), followed by the isolation of HDL by ultracentrifugation. (A) The [^{3}H]radioactivity coeluted with the protein peak in the void volume on Sephadex G-25. When follicular fluid was incubated in the presence of DTNB, almost no radioactivity was recovered in the elution position of HDL, suggesting that esterification was a prerequisite for the incorporation of [^{3}H]estradiol into HDL. (B) After extraction with organic solvents, HDL was subjected to chromatography on Sephadex LH-20. A major part of the radioactivity in HDL (83%) was eluted in fractions 1–3 (the estradiol ester fraction) and only 9% was recovered in fraction 12 (nonesterified estradiol fraction). No radioactivity was detected in the ester fraction of the sample that was incubated in the presence of DTNB.
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concentrations of estradiol fatty acid esters detected in early studies in human adipose tissue [6].

Recent investigations have focused on the association and fatty acylation of estrogens in lipoproteins. Pioneering studies reported that follicular fluid in stimulated ovaries contained far greater amounts of esterified estradiol than any other tissue [7]. Based on these findings, we set out to explore the incorporation of [^{3}H]estradiol in human lipoproteins in follicular fluid (HDL) (Fig. 2A) and in plasma (LDL and HDL) [8]. Incubation of [^{3}H]estradiol with human ovarian follicular fluid resulted in increased radioactivity in the lipophilic fatty acid ester fraction obtained from isolated HDL, as demonstrated by column chromatography (Fig. 2B) and TLC. After saponification of the ester fraction, the radioactivity was recovered in the unesterified estradiol fraction [8]. The association of estradiol with HDL in follicular fluid was inhibited by DTNB, the inhibitor of the estradiol esterifying LCAT enzyme. Next, the possible exchange of esterified estradiol between lipoprotein fractions was investigated. To obtain radiolabeled HDL, [^{3}H]estradiol was incubated with human plasma followed by isolation and purification of HDL. In the co-incubation of [^{3}H]estradiol-HDL and non-labeled LDL in the presence of increasing amounts of exogenous cholesterol ester transfer protein (CETP), the transfer of labeled estrogen from HDL to LDL moderately increased, suggesting that CETP contributed to this [8]. This was different from the fatty acid esters of pregnenolone and dehydroepiandrosterone, which reportedly are transferred from HDL to LDL by a CETP-independent mechanism [9].

Estrone is the other major estrogen secreted by the ovaries. After menopause, estrone is the principal cycling estrogen in women,

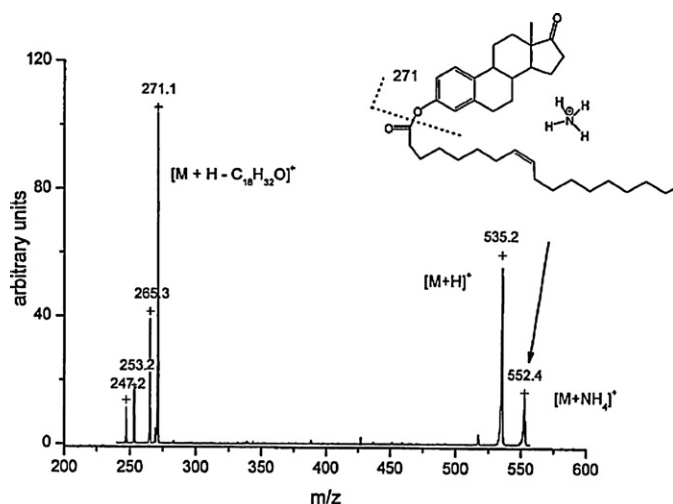

Fig. 3. Daughter ion spectrum of estrone 3-oleate. The ion produced from estrone 3-oleate, the NH_4^+ adduct $[M+NH_4]^+$ (552.4 *m*/*z*), was isolated and subjected to fragmentation. The main fragmentations are loss of NH_3 (535.2 *m*/*z*) and loss of NH_3 and fatty acid as ketene (271.1 *m*/*z*). The loss of fatty acid yields the same product ion for all estrone esters therefore the ion at 271.1 *m*/*z* can be used as an indicator of this substance class. The small fragments at 265.3, 253.2 and 247.2 have not been identified.
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derived from aromatization of the androgens in the peripheral tissues. Several studies have shown that underivatized estrone as a lipophilic estrogen also associates with lipoproteins during incubation with plasma [10–12]. However, no study has reported of biosynthesis of estrone fatty acid esters in plasma [2,10] or in tissues [4,13]. In a preliminary experiment, we incubated [^{3}H]estrone with follicular fluid which unexpectedly resulted in the formation of labeled non-polar estrone derivatives bound to HDL [14]. This led to further studies demonstrating the formation of lipophilic estrone derivatives during incubation of human plasma with [^{3}H]-labeled estrone [14]. During incubation, radioactivity became associated with both LDL and HDL, but this association was smaller when DTNB was added to the incubation medium. Subsequently, the radioactivity associated with LDL and HDL was fractionated to estrone ester and underivatized estrone fractions by Sephadex LH-20 column chromatography. There was slightly more of unesterified estrone than esterified estrone associated with HDL, whereas equal amounts of label were detected in the unesterified and ester fractions in LDL. In contrast, when [^{3}H]estrone was incubated in the presence of follicular fluid, most of the radioactivity (59%) in the ester fraction of the isolated HDL was found to be lipophilic derivatives of estradiol. This suggested conversion of estrone to estradiol by 17β-hydroxysteroid dehydrogenase enzymes present in follicular fluid HDL [14]. The biosynthetic derivatives of estrone associated with plasma HDL were characterized by liquid chromatography–mass spectrometry (LC–MS) demonstrating ions of the intact esters following incubation of non-labeled estrone with plasma [14]. A fragmentation spectrum of estrone 3-oleate obtained from a pure standard is shown in Fig. 3. The main biosynthetic fatty acid esters of estrone were estrone linoleate, oleate, palmitate, arachidonate and stearate. Thus, the study indicates that human plasma is capable of esterifying estrone with fatty acids at C-3 hydroxyl, a reaction which was shown to be partly inhibited by DTNB. However, we hypothesized that the enzyme esterifying estrone could be another acyltransferase and not LCAT which esterifies hydroxyl groups only at C-17 or C-16 of estrogens but not the phenolic hydroxyl group at C-3. DTNB blocks thiols in the active site of LCAT and might block thiol groups of other acyltransferase enzymes as well.

Höckerstedt et al. further studied the regulation of estradiol esterification in HDL isolated from plasma, in particular the two subfractions HDL_2 and HDL_3 [15]. When increasing amounts of purified LCAT enzyme were added, a dose–response was observed in the esterification of [^{3}H]estradiol in HDL. Analysis by TLC showed that these esters were 17-monoesters of estradiol. Similar experiments carried out with HDL subfractions showed that estradiol fatty acid esters were synthesized almost exclusively in the small, less mature HDL_3 particles, in agreement with previous work by Leszczynski and Schafer [11]. The larger HDL_2 particles had very little esterifying activity, and the addition of purified LCAT enzyme did not increase the association of labeled estradiol with this lipoprotein fraction to any significant extent [15,16]. Thus, it appears that the surface configuration of HDL particles plays a role in the activity of the estradiol esterifying reaction by LCAT, in the same way as in cholesterol esterification.

Subsequent studies were carried out to investigate the effect of the lipid composition of HDL_3 on the estradiol esterification rate. HDL_3 was obtained from hypertriglyceridemic men (serum triglycerides 2.6–11.2 mmol/l) and normolipidemic control men and incubated with ^{3}H-labeled estradiol and exogenous LCAT [16]. The total LCAT activity was measured in HDL_3 and it was positively related to the amount of labeled estradiol associated with HDL_3 in both hypertriglyceridemic ($P < 0.05$) and normolipidemic subjects ($P < 0.005$). Interestingly, there was a statistically significant positive correlation between the HDL_3 triglyceride content and the amount of labeled estradiol associated with HDL in all subjects (strength of association $r = 0.739$), suggesting that the esterification of estradiol by LCAT might be enhanced in triglyceride-rich HDL_3 particles. In agreement with this, another study reported higher plasma LCAT activities in hypertriglyceridemic than in normolipidemic men, and LCAT activity was positively related to plasma triglyceride concentration [17]. It was proposed that structural alterations in HDL particles, such as more efficient unfolding of apoAI on the surface of triglyceride-rich HDL, could enhance the esterification of estradiol by LCAT [16].

3. Studies in experimental animals

3.1. Fatty acid esterification of steroids

Early studies carried out in the rats and other experimental animals have shown that a large number of steroids become fatty acylated in various tissues such as the liver, brain and placenta [1]. More recently, Xu et al. studied the esterification of estradiol and other steroids in female rats [18]. In the rat liver, the highest specific estradiol acyltransferase activity was detected in the microsomal fraction, in accordance with previous studies [1], and K_m was 4–6 μmol/l for estradiol at the optimal pH of 5, similarly to a previous study in bovine placental microsomes [19]. The lysosomal, nuclear and mitochondrial fractions contained about one third of the acyltransferase activity detected in the microsomal fraction, while no activity was detected in the cytosolic fraction [18]. The liver acyltransferase activity was the highest for estradiol, followed by testosterone, dehydroepiandrosterone, pregnenolone and corticosterone [13], in accordance with previous work by Pahuja and Hochberg [20]. Although estradiol 17-stearate has been previously shown to be the most abundant biosynthetic estradiol ester in rat liver microsomes [20], arachidonoyl-CoA and oleyl-CoA exhibited the highest affinities for estradiol [18]. This supports the concept that the concentration rather than the affinity of the specific fatty acyl-CoA determines the abundance of various estradiol fatty acid esters in the tissue [1].

Interestingly, Xu et al. showed that the biosynthesis of steroid fatty acid esters in rat liver microsomes may be increased in a dose-dependent manner by the pretreatment of rats with peritoneal or oral clofibrate or gemfibrozil, the peroxisome proliferator-activated receptor alpha (PPARα) agonists [13,18]. This is in contrast to the hepatic cholesterol esterifying enzyme, acyl coenzyme A:cholesterol acyltransferase (ACAT), the activity of which is reduced by clofibrate [21]. Fatty acylation of estradiol, testosterone, dehydroepiandrosterone, pregnenolone and corticosterone in rat liver was stimulated by 14–22-fold by oral clofibrate [13]. In PPARα null (−/−) mice, however, a potent PPARα agonist Wy-14,643 did not increase fatty acylation of estradiol or testosterone, suggesting that PPARα was involved in the stimulatory effect of clofibrate on steroid esterification [22]. Accordingly, clofibrate did not increase the esterification of estradiol in tissues with less PPARα expression compared to liver, such as in adipose tissue, uterus or brain [13]. Thus, these studies indicate that clofibrate administration enhances the fatty acid esterification of steroid hormones specifically in the rat liver.

In different tissues of the rat, studies on the esterification of estradiol and other steroids have yielded conflicting results. Xu et al. recently described the highest rate for estradiol fatty acid ester biosynthesis in the brain, followed by lung, uterus and liver tissues, and the lowest rate for estradiol esterification was detected in the kidney [13], similarly to the 3β-hydroxy-Δ^5 steroids pregnenolone and dehydroepiandrosterone according to an earlier report [23]. On the contrary, previous studies by Hochberg's group indicated that rat brain tissue had a relatively low activity of estradiol fatty acylation compared to the lung, uterine and kidney tissues [4,5]. In the rat brain, dehydroepiandrosterone fatty acid esters were described to increase the fluidity of cell membranes and thus possibly influence membrane function [24]. Recently, however, Liere et al. have questioned the existence of sulfate and fatty acid esters of pregnenolone and dehydroepiandrosterone in the rodent brain [25]. They show that cholesterol may contaminate the steroid fraction and become auto-oxidized to pregnenolone or dehydroepiandrosterone during the experimental procedures, resulting in falsely elevated concentrations of these analytes. According to the authors, the existing data on rat brain 3β-hydroxy-Δ^5 steroid esters, also those obtained by LC–MS of intact steroid fatty acid esters [26], need to be re-evaluated [25].

3.2. Studies in adipose tissue and the mammary gland

Recent studies indicate that rat breast and adipose tissues might be active sites of steroid fatty acid ester hydrolysis and metabolism. Xu et al. studied the hydrolysis of estradiol fatty acid esters in different tissues of the rat and described higher esterase activities in adipose and mammary tissues compared to the liver [13]. Interestingly, Mills et al. found the highest estradiol esterase activity in the cytosolic fraction of rat mammary tumor tissue, followed by normal mammary and abdominal adipose tissues while the liver, pituitary gland and uterine tissues had much lower esterase activities [27].

Hormone-sensitive lipase is a triglyceride esterase with broad substrate specificity, and its activity may be increased by clofibrate in cultured rat preadipocytes [28]. Hormone-sensitive lipase can also catalyze the hydrolysis of steroid fatty acid esters [29]. Xu et al. showed that clofibrate treatment enhanced the hydrolysis of ^{3}H-labeled estradiol 17-oleate by 70% in rat liver and by 40% in mammary tissue [13]. In adipose tissue, clofibrate increased the hydrolysis of estradiol 17-oleate even more, by 125% and 90% by microsomal and cytosolic esterases, respectively [30]. In summary, these data suggest that the effects of clofibrate on the steroid fatty acid ester metabolism in the rat adipose tissue are principally hydrolytic. Next, Xu et al. investigated whether clofibrate would influence the estrogenic effects of estradiol treatment in ovariectomized rats [30]. Co-treatment with clofibrate and subcutaneous estradiol significantly increased the number of lobules and lob-

ular cell proliferation in the rat mammary gland compared with estradiol treatment alone. In contrast, the uterotrophic effect of estradiol was not further increased by cotreatment with clofibrate. The authors speculated that clofibrate might have potentiated the estrogenic effect of estradiol on the mammary gland by enhancing the hydrolysis of estradiol fatty acid esters in the surrounding fatty tissues [30].

Early studies have indicated that, when injected parenterally, estradiol 17-esters are metabolized more slowly than nonesterified estradiol leading to an increased duration of the estrogenic signal [31–33]. More recently, Paris et al. observed that also orally administered estradiol 17-stearate was more slowly metabolized and induced a stronger uterotrophic effect than unesterified estradiol in juvenile female rats [34]. Mills et al. studied the growth stimulatory effect of continuous estradiol 17-stearate administration on the adult rat mammary and uterine cells by measuring 5-bromo-2′-deoxyuridine labeling index [27,35]. Interestingly, subcutaneous estradiol 17-stearate treatment had a stronger stimulating effect on mammary cell proliferation than estradiol at equimolar doses [35]. In contrast, estradiol 17-stearate enhanced the uterine endometrial cell proliferation less than estradiol [27,35]. The authors concluded that esterified estradiol appeared to have a stronger mitogenic effect on mammary cells as compared to endometrial cells. They speculated that the lipophilic estradiol fatty acid ester might accumulate in the adipose tissue surrounding mammary glandular cells [35]. In line with this hypothesis, several studies have indicated that estrogen administration leads to increased concentrations of estradiol fatty acid esters in adipose tissue as reported in the rats and cattle [36–38]. Esterified estradiol might then enhance mammary cell proliferation after hydrolysis to biologically active estradiol. The differential effects of estradiol ester on the breast and uterus might also be explained by different esterase activities in these tissues. As mentioned above, the same group reported a higher estradiol fatty acid ester hydrolyzing activity in the breast compared to the uterine tissue [27].

4. Antioxidative effects of fatty acylated estrogens in lipoproteins

All estrogens have a hydroxyl group in the aromatic A-ring, a structural feature which is suggestive of antioxidative activity. Based on that, several laboratories have carried out studies to clarify this issue. Shwaery et al. studied the association of radiolabeled estrogens estradiol, estriol and estrone with LDL in plasma and their effect on the oxidation of LDL *in vitro* [10]. When human plasma was incubated in the presence of 10–100 nmol/l of estradiol, corresponding to estrogen concentrations in human pregnancy serum, estradiol became esterified and incorporated in LDL leading to increased resistance of LDL against Cu^{2+}-mediated oxidation. Estriol, a more polar estrogen, became markedly less associated with LDL, but still showed some hydrophobic modification during incubation with plasma. Unesterified estrone became incorporated in LDL but did not act as antioxidant in these experiments. The concentration of esterified estradiol that inhibited the oxidation of LDL *in vitro* was many times lower in these experiments than previously described for the antioxidative effect of nonesterified estradiol [39]. This led to a hypothesis that fatty acylation could markedly increase the antioxidative potential of estradiol. However, the mechanism of the antioxidative action of estradiol fatty acid esters was not clear. The authors speculated that it was not likely to be free radical scavenging because no protection from oxidation by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), a source of peroxyl radicals, was detected [39]. Moreover, after plasma incubation in the presence of high concentration (100 nmol/l) of radiolabeled estradiol, the LDL estrogen content averaged 0.86 pmol/mg LDL protein [10], which corresponds to less than one estradiol molecule per one thousand LDL particles.

The results obtained by Shwaery et al. [10,39] have, however, not been confirmed elsewhere. We incubated increasing concentrations (10–5000 nmol/l) of estradiol, estradiol 17-oleate and estradiol 17-stearate with plasma. The concentration of esterified estradiol in LDL, as measured by gas chromatography–mass spectrometry (GC–MS) after saponification of the esters, increased to 15 pmol/mg LDL protein after plasma incubation with 5000 nmol/l of estradiol, and to 140 pmol/mg LDL protein after plasma incubation with 5000 nmol/l of estradiol 17-stearate [40]. However, these LDL fractions did not show any increased resistance to copper-mediated oxidation. Similar results were obtained in experiments with HDL [40]. When a Celite dispersion system was used in the incubation of LDL with estradiol 17-stearate or 17-oleate to further increase the incorporation of esterified estradiol in LDL, it was shown that only concentrations exceeding 500 pmol estradiol equivalents/mg LDL protein significantly increased the LDL oxidation resistance. Thus, approximately one estradiol equivalent per three LDL particles was a level required for increasing the oxidation resistance of LDL *in vitro*.

Höckerstedt et al. set out to provide direct proof that estradiol esters are formed in HDL in a reaction catalyzed by LCAT and that these fatty acid esters of estradiol increase the resistance of HDL particles against copper-mediated oxidation *in vitro* [41]. Compared with native HDL, the oxidation resistance of HDL increased statistically significantly when either supraphysiological concentrations of estradiol or purified LCAT enzyme were incubated with HDL, suggesting that also LCAT alone had some antioxidative activity. However, the oxidation resistance of HDL increased markedly more when both exogenous LCAT and estradiol were present in the incubation. Furthermore, the oxidation resistance of HDL was positively related to the activity of LCAT when both exogenous estradiol and LCAT were incubated with HDL prior to oxidation, but not when only exogenous LCAT with no added estradiol was present. When DTNB, the inhibitor of LCAT enzyme, was added to the HDL incubation along with estradiol and LCAT, the concentration of estradiol fatty acid esters in HDL was only 1% of that detected in the absence of the inhibitor. Moreover, the lag time of HDL oxidation was similar to that of native HDL [41]. These results clearly indicated that the formation of estradiol esters by active LCAT in HDL is associated with increased resistance of HDL to oxidation *in vitro*. It was, however, estimated that these *in vitro* experiments produced one thousand times higher concentrations of HDL-associated fatty acylated estradiol compared to the concentrations previously reported in pregnant women [42], not to mention women taking oral or transdermal estrogen therapy who have even lower levels [43]. In summary, the studies mentioned above [40,41] suggest that incorporation of esterified estradiol in lipoproteins can enhance the oxidation resistance of these particles *in vitro* but this can be achieved only at very high supraphysiological concentrations. This is in line with other recent experimental studies [44,45]. Clinical studies on the effect of estrogen therapy on the LDL oxidation resistance *in vitro* have yielded conflicting results [46–52]. Current experimental evidence therefore suggests that any physiological estradiol fatty acid ester levels, or those achieved during oral or transdermal estradiol substitution therapy, are probably not sufficient to influence lipoprotein oxidation resistance [53].

5. Metabolism of lipoprotein-associated steroid fatty acid esters

Esterified steroids incorporated in HDL or LDL can be taken up into cells *via* lipoprotein receptors and act as substrates for

steroid synthesis. Pioneering studies by Bélanger's group in the 1990s showed that lipoprotein-bound esterified steroids acted as a source of inactive hormones that could be hydrolytically activated following lipoprotein receptor-mediated intake into cells, as summarized in Hochberg's review [1]: LDL and HDL particles containing ^{3}H-labeled pregnenolone fatty acid ester were incubated with porcine granulosa cells which were shown to synthesize ^{3}H-labeled progesterone. Addition of excess LDL or HDL inhibited the formation of [^{3}H]progesterone with both lipoprotein fractions indicating that [^{3}H]pregnenolone fatty acid ester was entering the cells *via* receptor-mediate intake. The findings also suggested the intracellular presence of steroid fatty acid esterase activity for the cleavage of the fatty acid residue, followed by a Δ^5-dehydrogenase-isomerase for oxidation to progesterone [54].

Two recent studies demonstrated that also lipoprotein-associated estradiol may be internalized into target cells *via* lipoprotein receptors. Gong et al. showed that HDL-associated estradiol can be delivered to endothelial cells *via* HDL binding to scavenger receptor class B, type 1 (SR-B1), leading to an increase in endothelial nitric oxide synthase (eNOS)-mediated vasorelaxation [55]. Brodeur et al. [56] demonstrated that osteoblastic cells express SR-B receptors and that these receptors are involved in the uptake of radiolabeled estradiol associated with LDL and HDL_3. This selective uptake of HDL_3-associated estradiol could be partly inhibited by competitive SR-B ligands: LDL or oxidized LDL. However, the nature of the lipoprotein-associated estradiol was not determined in either of the studies. Neither did the authors discuss the possibility that the lipoprotein-bound estradiol would be in the form of fatty acid esters [57]. R. Badeau et al. set out to investigate the mechanisms of cellular uptake of HDL-incorporated estradiol fatty acid esters and their possible conversion to biologically active estradiol in Fu5AH rat hepatoma cells. This study showed for the first time that liver-originated cells can internalize HDL-incorporated radiolabeled estradiol fatty acid esters [58]. Interestingly, this uptake was initially rapid compared to the selective uptake of cholesterol esters by the same cells. During a 24-h period of cellular incubation, intracellular radioactivity in the esterified estradiol fraction decreased by approximately one half with a corresponding increase in the unesterified estradiol fraction indicating esterase activity in these cells. The cellular uptake of labeled estradiol esters was partly inhibited by chemically blocking the SR-B1-mediated lipid transport. Furthermore, by adding increasing amounts of LDL to compete with apolipoprotein E-containing HDL binding to LDL receptor, cellular uptake of estradiol esters decreased even more (Fig. 4). This suggested that both SR-B1- and LDL receptors were involved in the cellular uptake of HDL-associated estradiol fatty acid esters. However, as this uptake was not completely blocked by SR-B1 inhibition and LDL receptor competition, also other lipoprotein receptors might be involved.

Dehydroepiandrosterone functions as a precursor of sex steroids in peripheral tissues [59] and its fatty acid esters are transported by lipoproteins in the circulation [60]. The ratio of fatty acid esterified to nonesterified dehydroepiandrosterone in human male plasma is approximately 0.5 [61]. Wang et al. studied the metabolism of LDL-associated dehydroepiandrosterone fatty acid esters in human cultured HeLa cells [62]. The study indicated that the cellular uptake of LDL-associated dehydroepiandrosterone esters was mediated by LDL receptors or related receptors, as excess of LDL competitively inhibited the internalization of radiolabeled dehydroepiandrosterone esters. Moreover, the results also suggested that lysosomal acid lipase (LAL) which hydrolyzes LDL-associated cholesteryl esters in the cells is also involved in the hydrolysis of dehydroepiandrosterone esters. Further experiments in LAL-deficient human fibroblasts showed, however, that this hydrolysis was dependent on LAL activity only to limited extent, suggesting the possibility of hormone-sensitive lipase being involved (see Section 3.2). Interestingly, after 48-h incubation of the cells with LDL-associated labeled dehydroepiandrosterone fatty acid esters, the fraction containing esterified dehydroepiandrosterone was recovered in the cellular fraction, while biologically active, unesterified dehydroepiandrosterone and its metabolites, androstenediol and androstenedione were secreted into the cell culture medium [62]. Thus, the findings supported the concept that in this cell line, in addition to esterase activity, other enzyme activities needed for the conversion of dehydroepiandrosterone to other metabolites are present.

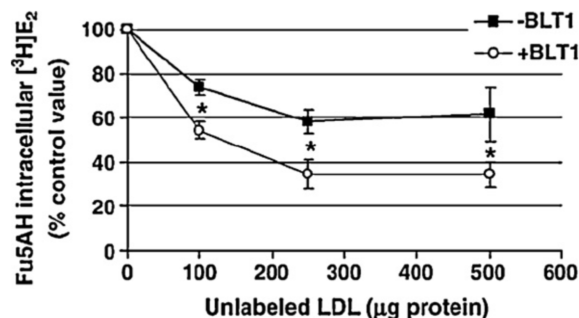

Fig. 4. Effects of LDL addition and scavenger receptor class B, type 1 inhibition on HDL-[^{3}H]estradiol uptake by the Fu5AH cells. Incubations for 1 h of HDL-containing [^{3}H]estradiol (83% esterified and 17% unesterified) in the presence of increasing concentrations of LDL reduced [^{3}H]estradiol uptake by Fu5AH cells in a dose-dependent manner (−BLT-1). Pretreatment of the cells with scavenger receptor class B, type 1 (SR-B1) inhibitor further decreased [^{3}H]estradiol uptake (+BLT-1). Cells incubated with 80 μg of HDL-[^{3}H]estradiol but devoid of LDL and BLT-1 treatment was the 100% control value. Data are expressed as mean ± S.E. (n = 3). *Statistically significant difference from the control value (P < 0.05). E_2, estradiol; BLT-1, blocker of lipid transport 1 (SR-B1 inhibitor).
Reprinted from R. Badeau et al. [58]. Copyright (2007), with permission from Elsevier.

Paatela et al. [63] explored the possibility that esterified dehydroepiandrosterone contained in HDL particles could enhance their vasodilatory effect. This hypothesis was based on previous reports indicating that HDL improves endothelial vasodilatory function by a mechanism mediated by eNOS activity [64,65] and other studies showing that dehydroepiandrosterone also enhances endothelium-dependent vasodilatation [66]. First, they demonstrated that incubation of [^{3}H]dehydroepiandrosterone with plasma resulted in the incorporation of fatty acylated dehydroepiandrosterone in HDL particles while addition of LCAT inhibitor reduced label incorporation to trace amounts. Second, they enriched HDL particles with non-labeled dehydroepiandrosterone in a similar plasma incubation and demonstrated that HDL containing fatty acylated dehydroepiandrosterone caused increased vasodilatation in an *ex vivo* experimental animal model (Fig. 5). Since Roy and Bélanger [61] as well as we have previously reported that lipoprotein-associated steroid fatty acid esters can enter cells by lipoprotein receptor-mediated pathways and intracellularly become hydrolyzed into biologically active steroid hormones [58,62], one possibility is that HDL containing esterified dehydroepiandrosterone could enter endothelial cells followed by the hydrolysis of the ester producing biologically active dehydroepiandrosterone with vasodilatory properties [66].

Accordingly, there is increasing evidence that lipoprotein particles may act as carriers of inactive fatty acylated steroids to various tissues, in which they can be taken up by cells *via* lipoprotein receptors and activated by intracellular esterases. Lipoprotein-mediated transport may be of significance especially for dehydroepiandrosterone and pregnenolone, a great proportion of which circulate in human blood as fatty acid esters [67].

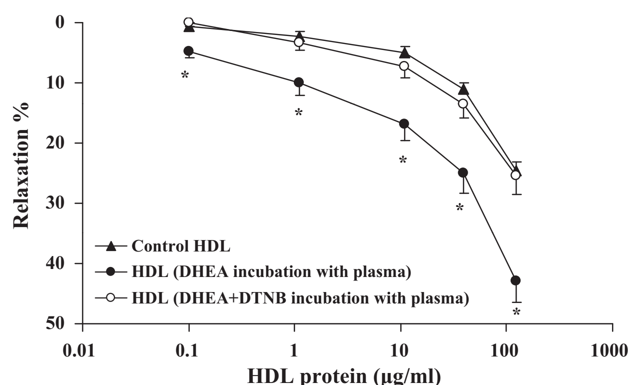

Fig. 5. Relaxation responses to HDL in isolated endothelium-intact rat mesenteric arterial rings after precontraction with noradrenaline. Relaxations to cumulative doses of HDL isolated and purified after plasma incubation in the presence of 10 μmol/l DHEA (●, $n = 22$), 10 μmol/l DHEA + DTNB (○, $n = 16$), or the vehicle control (▲, $n = 24$). The maximal relaxation response to HDL isolated after plasma incubation in the presence of DHEA and DTNB was significantly weaker than the response to HDL from a plasma incubation in the presence of DHEA without DTNB ($P < 0.001$), and did not differ from the response to the control HDL. Symbols indicate mean ± S.E.M. *$P < 0.01$ *vs.* control HDL (ANOVA and Sheffe's post hoc test). DHEA, dehydroepiandrosterone; DTNB, dithionitrobenzoic acid, lecithin:cholesterol acyltransferase (LCAT) inhibitor.
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6. Endogenous steroid fatty acid esters in humans

6.1. Circulating steroid fatty acid esters

The presence of lipophilic estradiol derivatives in human blood was originally described by Janocko and Hochberg [68,69]. The same group was the first to quantify hydrolyzed estradiol fatty acid esters in human tissues by a GC–MS method [6]. In this early study, low concentrations of esterified estradiol were detected in blood in four premenopausal women, ranging between 2 and 22% of serum nonesterified estradiol, but higher serum estradiol ester concentrations were reported in three women receiving gonadotrophin treatment for infertility. However, in postmenopausal women and men, serum estradiol ester levels were considered to be under the detection limit, and the value of the male serum was subtracted as a blank from the other serum values [6]. By estradiol time-resolved fluoroimmunoassay [42], Vihma et al. more recently measured serum concentrations of estradiol fatty acid esters in different groups of women as well as in pooled male serum. The concentration of serum estradiol esters was in the same range in postmenopausal women [70] and in men but slightly higher in premenopausal women [43] (Table 1). In men, the greater proportion of serum esterified estradiol compared to premenopausal women could be attributed to their greater proportion of free, nonprotein-bound estradiol in serum [71]. In women, the median serum estradiol ester to estradiol ratio was the highest in postmenopausal, intermediate in premenopausal women and the lowest in pregnancy (Table 1) [72]. This suggests that the proportion of esterified estradiol in the circulation might increase with decreasing serum estradiol levels. Interestingly, studies of non-estrogenic steroids *in vivo* [67,73] and estrogens *in vitro* [74] have suggested that ageing might be associated with increased esterification of steroids. In theory, fatty acylation might provide a storage mechanism for steroids which could be more pronounced during estrogen depletion.

In early pregnancy, at 7–10 weeks of gestation, serum estradiol ester concentrations were in the same range as in nonpregnant women (Table 1) [42,75], and consistent with a previous report by Larner et al. as studied in one pregnant subject [6]. From early to late pregnancy, the concentration of serum estradiol esters increased several-fold (Table 1) [42,75] and was positively related to serum estradiol concentration [72]. The proportion of esterified estradiol of serum nonesterified estradiol, however, decreased from 1.2% in early to 0.6% in late pregnancy (Table 1), indicating that fatty acylation is not a major metabolic pathway of circulating estradiol in pregnancy [72], unlike glucuronidation or sulfation.

The elevated concentration of estradiol esters in late pregnancy allowed Vihma et al., for the first time, to quantify endogenous estradiol fatty acid esters in all major human lipoprotein fractions [75]. Lipoproteins were isolated from plasma by sequential ultracentrifugation and purified by gel filtration prior to the analysis of estrogens. The study showed that lipoproteins are the major carriers of esterified estradiol in human blood (Fig. 6), in agreement with previous experimental data [76]. Most of the endogenous estradiol esters in human serum were recovered in HDL (54%) and LDL (28%) (Fig. 7) [75]. Estradiol esters quantified in both HDL_3 and LDL fractions were positively related to serum estradiol levels in late pregnancy. Also, the amount of endogenous estradiol esters measured in the LDL fraction correlated statistically significantly

Table 1
Plasma estradiol (E_2) and estradiol fatty acid ester concentrations in postmenopausal women before and after transdermal or oral estrogen therapy, in premenopausal and pregnant women and in pooled human male serum; and adipose tissue estradiol and estradiol fatty acid ester concentrations in postmenopausal, premenopausal and pregnant women.

	n	E_2 (pmol/l or pmol/kg)	E_2 fatty acid ester (pmol/l or pmol/kg)	E_2 ester/E_2 ratio (%)
Serum[a]				
Postmenopausal women	20	32 (20–120)	57 (24–91)	170(47–450)
Transdermal estrogen	20	220 (28–560)	53 (27–130)	22 (9.4–95)
Oral estrogen	20	330 (140–720)	73 (35–120)	23 (9.5–52)
Men[c]		72 (61–81)	52 (45–66)	75(62–100)
Premenopausal women	7	250 (149–704)	75 (62–107)	29 (9.2–55)
Early pregnancy[d]	8	6480 (2940–10,500)	70 (56–97)	1.2 (0.7–2.4)
Late pregnancy	20	92,100 (31,000–240,000)	480 (208–1170)	0.6 (0.2–1.5)
Adipose tissue, visceral[b]				
Postmenopausal women	6	250 (100–360)	740 (420–1190)	390(140–840)
Premenopausal women	7	650 (320–1550)	820 (470–1710)	150(30–362)
Late pregnancy	12	38,900 (23,300–74,900)	4240 (1860–6520)	9.6 (5.8–16.5)
Adipose tissue, subcutaneous[b]				
Postmenopausal women	5	180 (100–470)	740 (520–1570)	490(110–890)
Premenopausal women	7	410 (270–1670)	1140 (560–2580)	260(37–540)
Late pregnancy	13	46,700 (23,600–68,900)	3870 (1590–12,600)	11 (6.2–19)

Data are shown as median (range).
[a] Expressed as pmol/l estradiol [42,43,70,72,75].
[b] Expressed as pmol/kg (fmol/g) estradiol. [Reproduced with permission from M. Badeau et al. [90]. Copyright (2007) The Endocrine Society.]
[c] Pooled human male serum obtained from 50 to 60 donors. The results are calculated from six consecutive assays.
[d] Blood samples were collected at 7–10 weeks of gestation from women who had received treatment for infertility by ovulation induction.

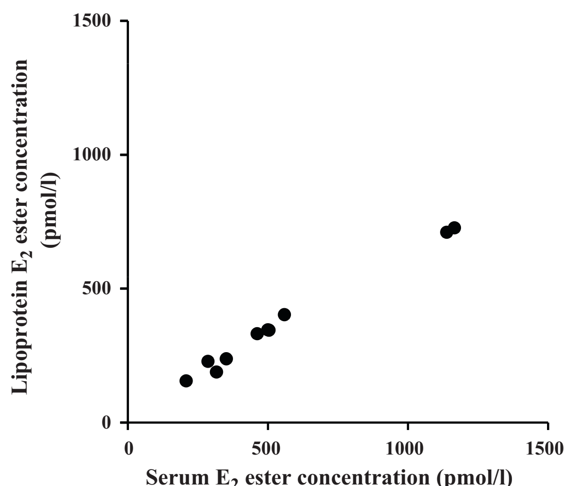

Fig. 6. Correlation between concentrations of estradiol fatty acid esters measured in serum and in isolated total lipoprotein fraction in pregnant women. The concentration of estradiol esters determined in the lipoprotein fraction was strongly positively related to the concentration of estradiol esters measured in whole serum (n = 10, r = 0.98, P < 0.001). Total lipoprotein fraction was isolated by single ultracentrifugation at density less than 1.21 g/ml, followed by gel filtration by Sephadex G-25. E_2 ester, estradiol fatty acid ester (expressed as pmol/l estradiol).
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with the estradiol ester content in HDL_3. Thus, the data support the concept of synthesis of estradiol fatty acid esters in HDL_3 by LCAT using circulating estradiol as the substrate and further transfer of estradiol esters from HDL_3 to LDL, as indicated by experimental studies [12,15]. In all of the HDL_3 and the majority of the LDL and VLDL fractions, estradiol was detected only in the form of fatty acid esters, and the concentration of nonesterified estradiol was below the limit of detection in 29 out of the 40 individual late pregnancy lipoprotein fractions analyzed [75]. This is consistent with experimental data indicating that the association of nonesterified estradiol with lipoproteins is nonspecific and weak [11,12]. Thus, the data obtained from pregnant women indicate that endogenous estradiol is incorporated into lipoproteins almost entirely in the fatty acid esterified form.

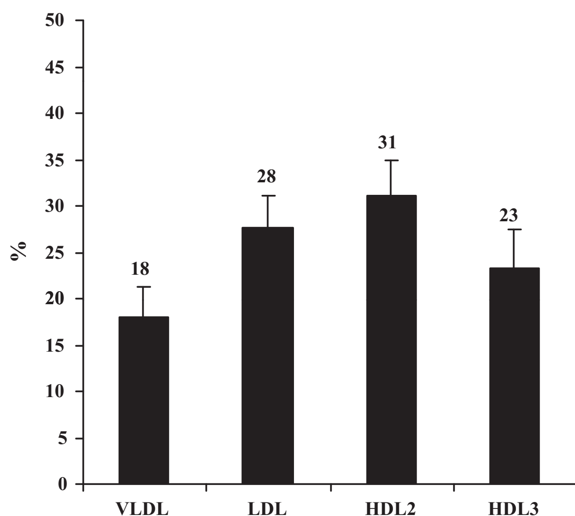

Fig. 7. The distribution of estradiol fatty acid esters in isolated and purified lipoprotein fractions in human late pregnancy. Most of the estradiol fatty acid esters in lipoproteins were recovered in HDL and LDL fractions. The results are expressed as the mean (SD) percentage of estradiol ester concentration in lipoproteins.
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We hypothesized that serum estradiol and thereby also estradiol fatty acid ester concentrations might increase during estrogen therapy. The effect of 12 weeks of oral micronized estradiol (2 mg/d) and transdermal estradiol (patch delivering 50 μg/d) and placebo treatment on serum estradiol ester concentrations was investigated in 26 postmenopausal women [43]. In the oral estradiol group, the median concentration of estradiol esters in serum increased by 27% (P = 0.028), in association with serum concentration of estradiol which increased approximately nine-fold. In contrast, in the transdermal estradiol and placebo groups, serum estradiol fatty acid ester concentrations remained unchanged despite the five-fold increase in serum estradiol during transdermal estrogen therapy. The increases in the calculated concentration of serum free, nonprotein-bound estradiol were similar in the oral and transdermal groups. Next, the effects of increasing doses of oral (2–4 mg/d of estradiol valerate) and transdermal (patch delivering 50–100 μg/d of estradiol) estrogen therapy were compared in a cross-over study in 20 postmenopausal women of which 10 women had been diagnosed having intrahepatic cholestasis of pregnancy approximately 31 years previously [70]. A history of intrahepatic cholestasis of pregnancy appeared to have no effect on the esterification of serum estradiol. Similarly to the first study on estrogen therapy [43], four weeks of oral estrogen therapy increased the median serum estradiol fatty acid ester concentration by 29% (P = 0.002) (Table 1), which correlated positively with increases in serum estradiol, estrone and sex hormone-binding globulin (SHBG) concentrations. The median serum estradiol ester concentration returned to pretreatment levels during the four-week wash-out period. During transdermal estradiol treatment in the same subjects, concentration of estradiol esters in serum did not change despite a seven-fold increase in the median serum estradiol concentration (Table 1). These differential effects of orally and transdermally administered estrogen may be, at least partly, explained by the greater total estrogen dose in oral intake. Orally administered estrogen is subjected to hepatic first pass metabolism, in contrast to transdermal administration [70]. Moreover, the presumably higher peak estradiol concentrations in serum after oral estrogen administration may have enhanced the esterification of estradiol.

Interestingly, there was a positive correlation between the change in serum estradiol ester concentration and the change in forearm blood flow responses during postmenopausal estrogen therapy [43]. Both the acetylcholine-induced endothelium-dependent vasodilatation (r = 0.43, P = 0.028) and sodium-nitroprusside-induced endothelium-independent vasodilatation (r = 0.441, P = 0.024) were positively related to the change in serum estradiol ester concentration in postmenopausal women. No such correlation, in contrast, was observed between blood flow responses and concentrations of nonesterified estradiol [43,77]. This raised the possibility that serum estradiol esters might contribute to the increased vasodilatation during oral estrogen therapy. However, other possible causes for improved endothelial function observed during oral estradiol therapy include increased HDL cholesterol and serum estrone concentrations as well as decreased LDL cholesterol, lipoprotein(a) and serum free testosterone levels [43].

Couillard et al. [78] studied the association of ageing with plasma steroid concentrations in healthy men. The concentration of dehydroepiandrosterone fatty acid esters in plasma, as determined by radioimmunoassay (RIA) after saponification of the esters, decreased significantly with age from approximately 12 to 5.9 nmol/l, along with nonesterified dehydroepiandrosterone levels. However, the proportion of esterified dehydroepiandrosterone in plasma increased during ageing, in line with previous studies [67,73]. In his review Hochberg summarized the work

of Bélanger and colleagues who described the biosynthesis and metabolism of fatty acylated non-estrogenic C-21 and C-19 steroids such as pregnenolone and dehydroepiandrosterone and its androgenic metabolites [1]. After this important work, the group has focused on other aspects of steroid conjugation, such as water-soluble metabolites. Based on a variety of studies, including trials of dehydroepiandrosterone administration to women, these investigators have emphasized that dehydroepiandrosterone is converted into active androgens and to a smaller degree, into estrogens in various tissues [59,79]. However, these hormones exert their effects at the site of their synthesis with no or only a small release of active steroids in the circulation. They are metabolized in the same cells in which they were synthesized to inactive glucuronidated and sulfated conjugates which are released into the circulation. Thus, peripheral androgen synthesis in women is reflected in the plasma levels of water-soluble steroid glucuronides which permits the estimation of the androgen pool [80].

As the quantitative analysis of steroid fatty acid esters is methodologically complicated the studies on the endogenous concentrations of these metabolites in human tissues are not many. Firstly, esterified steroids constitute a heterogeneous family of fatty acid esters which does not allow direct immunological assays for their quantification. Secondly, several steps are needed to separate the analyte from the abundant other steroid conjugates and lipid substances that might interfere with the assay. For example, cholesterol and cholesteryl esters circulate in human blood at approximately 10^6 times higher concentrations than pregnenolone and dehydroepiandrosterone and their fatty acid esters. In most of the studies, steroid fatty acid esters have been quantified as unconjugated steroids after hydrolysis of the ester bond. One group has described unexpectedly high levels of circulating estrone fatty acid esters, approximately 200-fold higher than nonesterified estrone, in both men and premenopausal women [81,82]. The estrone fatty acid ester concentration was determined as total estrone after extraction from plasma, followed by saponification, but without any chromatographic separation of the unconjugated and conjugated steroids. We have not been able to confirm these findings (unpublished). Recently, Jung et al. [83] analyzed intact saturated fatty acid esters of dehydroepiandrosterone and pregnenolone in five women and men by GC–MS. In contrast to previous work, the reported male plasma concentration of a representative pregnenolone fatty acid ester, pregnenolone 3-stearate, was over 100 times higher (99–1800 nmol/l) than the total plasma pregnenolone fatty acid ester concentration as previously analyzed by indirect methods (1–6.5 nmol/l) [2,54,84]. Unfortunately, the plasma concentration of unesterified pregnenolone was not reported [83]. Definitely, the development of more specific and sensitive analytical methods is needed for the quantification of steroid fatty acid esters in human tissues.

6.2. *Adipose tissue*

Adipose tissue constitutes an important site of steroid hormone synthesis and metabolism in humans as demonstrated by several-fold higher concentrations of dehydroepiandrosterone, androstenedione, estrone, estradiol and their metabolites in adipose tissue compared to plasma [85,86]. Adipose tissue has all the necessary enzymes for the production of estrogens from dehydroepiandrosterone providing the possibility of continuous *de novo* synthesis of estrogen [87]. Especially in postmenopausal women, steroid hormones are synthesized primarily in the adipose tissue from adrenal and gonadal precursors [88]. Early studies indicate that adipose tissue may serve as a reservoir of esterified estradiol [6]. Hormone-sensitive lipase can catalyze hydrolysis of steroid fatty acid esters [29,89]. The activity of this enzyme is regulated by insulin and several other endocrine factors and probably has a role in modifying the metabolism of steroids in adipose tissue.

To study the relationship of serum and tissue estradiol levels at different hormonal states in women, M. Badeau et al. quantified serum and adipose tissue concentrations of estradiol and estradiol fatty acid esters in pre- and postmenopausal as well as in pregnant women (Table 1) [90]. In nonpregnant premenopausal and postmenopausal women, the median estradiol concentrations in adipose tissue were several-fold higher than in serum, comparable to previous studies [85,86,91]. Most of the estradiol in adipose tissue was in the fatty acid esterified form (Table 1), consistent with previous report by Larner et al. [6]. In postmenopausal adipose tissue, the median concentration of estradiol esters was approximately five times higher than that of nonesterified estradiol. This indicates a high estradiol fatty acylating capacity of human adipose tissue, in line with the experimental work of Kanji et al. [3]. Moreover, in postmenopausal women, the median concentration of estradiol esters was more than 10-fold higher in adipose tissue than in serum (740 pmol/kg in subcutaneous and 750 pmol/kg in visceral adipose tissue *vs.* 57 pmol/l in serum), strongly suggesting that estradiol fatty acid esters were formed and stored locally in the adipose tissue [90]. In late pregnancy, different from nonpregnant women, less of the estradiol in visceral and subcutaneous adipose tissue (10%) was in the fatty acid esterified form (Table 1). We speculate that at least in theory, stimulation of enzymatic hydrolysis of estrogen fatty acid esters could produce biologically active endogenous estrogen in the postmenopause. Studies on the regulation of hormone sensitive lipase are of interest in this respect.

In pre- and postmenopausal breast cancer patients, Blankenstein et al. have previously detected higher levels of nonesterified estradiol than esterified estradiol in breast adipose tissue [92]. Interestingly, they reported of endogenous lipophilic derivatives of estrone in breast adipose tissue obtained from breast cancer patients, the mean concentration ranging from approximately 100 pmol/kg in postmenopausal to 200 pmol/kg in premenopausal breast adipose tissue [92]. In all subjects, the mean concentration of unconjugated estrone in breast adipose tissue was approximately ten times higher than that of lipophilic estrone conjugates. However, the analytical method for estrogens and their derivatives was not fully described. In contrast to the situation in the postmenopause, inactivation to estrogens by the inhibition of enzymatic hydrolysis of estrogen fatty acid esters might be beneficial for the female breast. Keeping a major proportion of estrogens in fatty acid ester form could reduce the risk of breast cancer.

6.3. *Ovarian follicular fluid*

Early studies showed that in ovarian follicular fluid obtained from women undergoing ovarian stimulation for *in vitro* fertilization (IVF), a high proportion of estradiol is in the fatty acid esterified form [7]. Recent studies have confirmed the high concentration of estradiol fatty acid esters and nonesterified estradiol in follicular fluid from both women undergoing ovarian stimulation and regularly menstruating women [42,93,94]. In women undergoing ovarian stimulation for IVF, we analyzed estradiol fatty acid esters by fluoroimmunoassay after chromatographic separation and saponification of the ester fraction [42]. The estradiol fatty acid ester-to-estradiol ratio in the pooled follicular fluid samples ranged between 0.03 and 0.1 which was lower than previously determined in individual follicles by GC–MS or RIA, 0.2–1.65 [7]. This difference can be partly explained by the high variation in concentrations of both estradiol and estradiol esters between individual follicles and by the different methodology used [7]. Cigliano et al. measured estradiol and estradiol 17-linoleate concentrations by HPLC in follicular fluid from stimulated follicles during the preovulatory period in 19 women [93]. Based on experiments *in vitro*,

they suggested that estradiol esterification rate may depend on the quantity of cholesterol present in follicular fluid, since addition of free cholesterol appeared to competitively inhibit LCAT-mediated esterification of estradiol.

7. Fatty acid esters of sex steroids in invertebrates

Animals from several invertebrate groups have vertebrate-type sex hormones. However, their biosynthetic pathways have not been fully characterized [95]. When different tissues have been incubated with estradiol, dehydroepiandrosterone or testosterone, the formation of lipophilic steroid conjugates has been reported in several invertebrate groups including molluscs, echinoderms and crustaceans [96–99]. Some mollusc species appear to synthesize lipophilic steroid conjugates upon exposure to environmental estradiol [100–102] or testosterone [103]. Upon laboratory exposure of the mussel *Mytilus edulis* to estradiol, Labadie et al. identified these conjugates by GC–MS/MS as three D-ring fatty acid esters of estradiol [104]. Several studies indicate that tributyltin, a marine pollutant, might decrease the fatty acylation of steroids in aquatic invertebrates [98,99,105], leading to a relative increase in the amount of unconjugated, biologically active steroid and possibly, to endocrine disruption. Thus, although the biological role of steroid fatty acid esters in invertebrates is not known, fatty acylation might be associated with the homeostatic regulation of bioactive steroid levels in these animals [106,107].

8. Conclusions

During the past decade, investigations on the physiological role of steroid fatty acid esters have concentrated on lipophilic estrogen derivatives in man and experimental animals. In contrast, the number of new reports on fatty acid esterified C-19 and C-21 non-estrogenic steroids has become smaller following the successful experimental demonstration in the 1990s of the LDL-mediated transport of these hormones into cells and subsequent intracellular activation. As a large proportion of these steroids are transported in lipoproteins and as they are important precursors of both androgens and estrogens, this field should be investigated further. In the estrogen field, the role of the fatty acylated estradiol providing antioxidative protection for lipoprotein particles under physiological conditions has not been confirmed. Neither does it appear likely that lipoprotein-mediated transport of estradiol has a major role in providing this steroid to various tissues, as only minute amounts of fatty acylated estradiol are transported in the circulation while the overwhelming majority of estradiol resides in adipose tissue, most of it in esterified form. The future focus of investigation is likely to be on firstly, the enzymatic mechanisms regulating the esterification and de-esterification of estradiol and other steroids residing in adipose tissue and secondly, on the role of insulin and other hormones in the regulation of these enzymatic mechanisms.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

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