
ESTRADIOL FATTY ACID ESTERIFICATION IN HIGH DENSITY LIPOPROTEIN

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

- I Helisten H*, Höckerstedt A*, Wähälä K, Tiitinen A, Adlercreutz H, Jauhiainen M, Tikkanen MJ. Accumulation of high-density lipoprotein-derived estradiol 17 β -fatty acid esters in low-density lipoprotein particles. *J Clin Endocrinol Metab.* 86(3):1294-300. 2001
- II Höckerstedt A, Tikkanen MJ, Jauhiainen M. LCAT facilitates transacylation of 17 beta-estradiol in the presence of HDL3 subfraction. *J Lipid Res.* 43(3): 392-7, 2002
- III Höckerstedt A, Jauhiainen M, Tikkanen MJ: Lecithin:cholesterol acyl transferase induces estradiol esterification in high density lipoprotein increasing its antioxidant capacity. *J Clin Endocrinol Metab.* 89(10):5088-93, 2004
- IV Höckerstedt A, Jauhiainen M, Tikkanen MJ: Estradiol fatty acid esterification is increased in high density lipoprotein subclass 3 isolated from hypertriglyceridemic subjects. *Submitted.*

*) equal contribution

ABBREVIATIONS

ApoA1	apolipoprotein A1
ApoB	apolipoprotein B
ABCA1	ATP-binding cassette transporter A1
d	density
DTNB	dithionitrobenzoic acid
chol	cholesterol
CE	cholesterol ester
CETP	cholesterol ester transfer protein
E ₂	17 β -estradiol
eNOS	endothelial nitric oxide synthase
HDL	high density lipoproteins
HDL ₂	high density lipoprotein subfraction 2
HDL ₃	high density lipoprotein subfraction 3
HTG	hypertriglyceridemia
HL	hepatic lipase
HRT	hormone replacement therapy
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoproteins
LPL	lipoprotein lipase
OxLDL	oxidized low density lipoprotein
NO	nitric oxide
NTG	normotriglyceridemia
PBS	phosphate buffered saline
PLTP	phospholipid transfer protein
r	correlation coefficient
rHDL	reconstituted HDL
SD	standard deviation
SEM	standard error of mean
SR-B1	scavenger receptor class B type 1
TG	triglyceride
TLC	thin-layer chromatography
v	volume
VLDL	very low density lipoprotein

ABSTRACT

Background. Endogenous estrogens are considered protective against atherosclerosis, but the exact mechanism remains unclear. Estrogens exert favorable effects on some of the major risk factors of atherosclerosis, including beneficial changes in levels of low density lipoprotein (LDL) and high density lipoprotein (HDL) and improved endothelial function. A widely investigated feature is their role as antioxidants in lipoproteins. Estrogen fatty acid esters, a unique class of hormones, are water-insoluble and they are carried lipoprotein-bound in circulation. In contrast to unesterified estrogen, they have been shown to inhibit lipoprotein oxidation at physiological concentrations *in vitro*. Indirect evidence suggests that they are formed in HDL by lecithin:cholesterol acyltransferase (LCAT), but they have also been shown to be present in LDL.

Aims of the study. The present thesis is focused on the mechanism of estradiol fatty acid esterification and incorporation in lipoproteins. Experimental methods were set up to investigate the formation, incorporation and transport of estradiol esters in different lipoprotein fractions, to provide direct evidence that the estradiol esters are generated by the function of LCAT and to demonstrate that, once incorporated in HDL, the antioxidant potential of this lipoprotein is increased, and finally, to investigate the effect of increased triglyceride content in HDL on estradiol esterification in this class of lipoproteins.

Methods. Incubations of labeled estradiol were carried out first with plasma and with ovarian follicular fluid in the presence and absence of an LCAT inhibitor, followed by ultracentrifugal isolation of lipoproteins and analysis of the radioactive material associated with lipoproteins by chromatographic methods. Next, incubation experiments were made with isolated LDL, HDL₂ and HDL₃ with labeled estradiol (E₂) with and without purified, exogenous LCAT. The effect of the triglyceride content of HDL₃ on the E₂ esterification reaction was investigated by comparing esterification of E₂ in hypertriglyceridemic HDL₃ with that in normotriglyceridemic HDL₃.

Coincubations with native LDL and labeled E₂-ester –containing HDL in the absence and presence of cholesterol ester transfer protein (CETP) were made followed by separation of these lipoproteins and analysis of attached radioactivity. To investigate whether the resistance of HDL to oxidation is increased when it contains E₂ esters, an *in vitro* model system was established with supraphysiological concentrations of E₂ and purified LCAT to produce E₂ ester -containing HDL particles. Copper-induced oxidation of purified HDL was measured after incubations of i) HDL alone, ii) HDL in the presence of

exogenous E₂, iii) HDL in the presence of exogenous LCAT, iv) HDL in the presence of both E₂ and LCAT and v) HDL in the presence of E₂, LCAT and the LCAT inhibitor.

Results. The results demonstrated that LCAT-mediated synthesis of esterified 17 β -estradiol occurred mainly in HDL₃, whereas HDL₂ and LDL contained only trace amounts of estradiol ester. Chromatographic analysis confirmed that the radioactivity migrated in a position corresponding to that of 17 β -E₂ 17-monoester standard. The amount of radioactivity associated with HDL₃ representing esterified E₂ was significantly increased by addition of purified LCAT but only limited increases of radioactivity were observed in HDL₂ and LDL with purified LCAT. After coincubations with E₂ ester containing HDL, also LDL contained E₂ esters and this LDL-associated radioactivity increased as a function of time in the presence of CETP. Increased triglyceride content caused an accelerated E₂ esterification in HDL. The LCAT-facilitated esterification and incorporation of E₂ esters increased oxidation resistance of HDL and there was a significant correlation between LCAT activity and oxidation resistance.

Conclusions. HDL subfractions differ in their potential to regulate estradiol esterification by LCAT. E₂ 17-ester formation occurred almost exclusively in the HDL₃ particles both in the presence of endogenous HDL-associated LCAT as well as after addition of exogenous LCAT. The experiments provide direct evidence that E₂ is esterified by LCAT in a dose-dependent manner, but also other factors such as HDL composition and particle size modify these effects. E₂ esters present in HDL can be transported in plasma to LDL particles in a process facilitated by CETP.

In summary, LCAT facilitates the formation of hydrophobic E₂ fatty acid esters that become incorporated in HDL particles where they protect the lipoprotein from oxidation. Based on experiments *in vitro*, E₂ esters formed in HDL could be transported to LDL in plasma via a partly CETP-dependent mechanism. This may result, in theory, in effective antioxidant protection of LDL even after the particles have penetrated the vascular wall. This would ultimately increase the subendothelial antioxidant potential and hinder the foam cell formation.

Elucidation of the possible *in vivo* role of HDL-associated estrogen esters requires further critical studies including experiments with physiological hormone concentrations.

1. INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death in the Western world, and although premenopausal women are at lower risk than men of same age, the incidence of CVD in women rises markedly after natural or surgical menopause (McGill et al. 1979; Wenger et al. 1993). The protective role of hormone replacement therapy (HRT) against CVD is controversial as many observational studies have shown beneficial effects (Grady et al. 1992; Barrett-Connor et al. 1998), but clinical trials have failed to show any cardiovascular risk reduction (Hulley et al. 1998; Herrington et al. 2000). However, endogenous estrogens are considered cardioprotective but the underlying mechanisms are still unclear. Cardiovascular disease is the clinical manifestation of the complicated process of atherosclerosis, and estrogens exert favorable effects on some of the major risk factors of atherosclerosis, including beneficial changes in levels of LDL and HDL (Walsh et al. 1991) and improved endothelial function (Mendelsohn 2002).

In the present thesis we have focused i) on estradiol fatty acid esters which are members of a unique steroid family transported in the circulation exclusively associated with lipoprotein particles, and ii) on one of the possible atheroprotective mechanisms of endogenous estrogens: protection of lipoproteins from oxidation. We have investigated the formation and transfer of estradiol esters between lipoproteins, a process which is considered even a prerequisite for the antioxidant effect of estrogens (Shwaery et al. 1997).

2. REVIEW OF THE LITERATURE

This thesis concentrates on lipoprotein-associated estradiol and on the underlying mechanisms by which endogenous estrogens may be atheroprotective. Accordingly, the vast literature concerning HRT is beyond the scope of this review. In addition, since the investigations have demonstrated a central role for HDL, the major emphasis is on this lipoprotein.

2. 1. Overview of lipoprotein metabolism in atherosclerosis

Atherosclerosis is a complex, multifactorial process which takes decades to develop. The process begins with vessel wall inflammation, and the earliest signs are increased endothelial permeability, migration of circulating monocytes into the arterial intima followed by accumulation of cholesterol in monocyte-derived macrophages (Ross 1999).

2. 1. 1. Low density lipoprotein

Cholesterol is transported in plasma lipoproteins, predominantly in low density lipoprotein (LDL). LDL is thought to be formed in two-step process by lipoprotein lipase -mediated lipolysis of liver-derived very low density lipoprotein (VLDL) and subsequent lipolysis of intermediate density lipoprotein (IDL) via the action of hepatic lipase (HL). The fatty acids released in this process are taken up by the involved tissues and by nascent HDL. Part of triglycerides can also be transported to high density lipoprotein (HDL) in exchange for cholesterol esters by cholesterol ester transfer protein (CETP). LDL can be removed from the circulation via receptor-mediated endocytosis into the liver by hepatic LDL receptors (Brown et al. 1981) or by LDL-receptor-related protein (LRP) which together explain 75% of LDL clearance (Herz et al. 1988) or taken up by peripheral cells through LDL receptors located on cell surfaces (Brown et al. 1986). Oxidatively modified LDL can be taken up by macrophages via scavenger receptors resulting in cholesterol accumulation in the vessel wall (Steinberg et al. 1989).

2. 1. 2. High density lipoprotein

Nascent HDL particles originate from apolipoprotein A-I (apoAI) –containing particles secreted by the liver and intestine or from excess surface components produced during lipolysis of chylomicrons or VLDL (Eisenberg 1984). Lipid-poor, nascent HDLs (also called pre β 1-HDL particles) acquire cholesterol from peripheral cells via the function of ATP-binding cassette transporter A1 (ABCA1) (Lawn et al. 1999) with apoAI serving as a ligand in the binding to ABCA1 on macrophages. Phospholipid transfer protein (PLTP) continues phospholipidation of nascent HDL in the circulation (Huuskonen et al. 2001). Free cholesterol of the discoidal HDL is esterified by lecithin:cholesterol acyltransferase (LCAT) and the esters form a lipophilic core changing the particle shape to spherical high density lipoprotein subclass 3 (HDL₃) particles (Rye et al. 1999) and subsequently to larger, more mature high density lipoprotein subclass 2 (HDL₂) particles. The esterified cholesterol in HDL particles may be transferred to LDL and VLDL by CETP (Tall 1993), or alternatively selectively taken up by scavenger receptor B1 (SR-B1), a HDL receptor located mainly in the liver. This cholesterol can be converted to bile acids and eliminated via the bile (Barter et al. 1996). SR-B1 receptors have also been found in adrenal gland, ovaries, testes (Krieger 1999) and the vessel wall (Yeh et al. 2002).

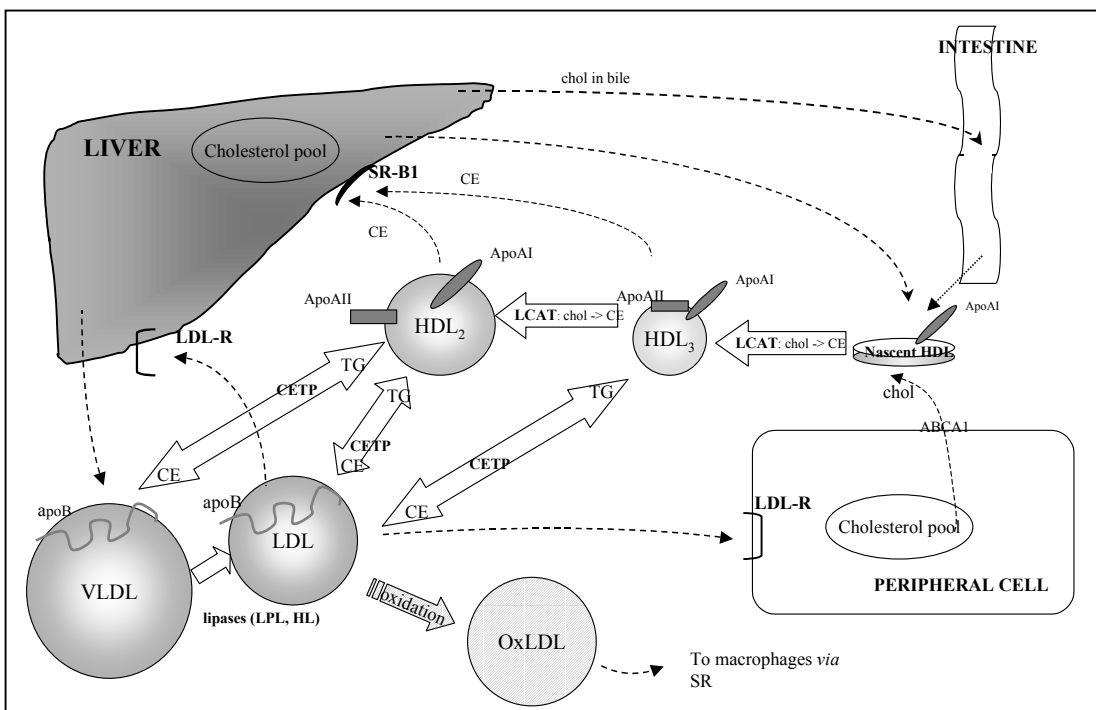


Figure 1. A schematic overview of lipoprotein metabolism.

ABCA1, ATP-binding cassette transporter; apoB, apolipoprotein B; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; chol, cholesterol; CE, cholesterol ester; CETP, cholesterol ester transfer protein; HDL₂, high density lipoprotein subclass 2; HDL₃, high density lipoprotein subclass 3; HL, hepatic lipase LDL, low density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL-R, LDL-receptor; LPL lipoprotein lipase; SR, scavenger receptor, SR-B1, scavenger receptor B class 1; TG triglyceride; VLDL, very low density lipoprotein

HDL metabolism in hypertriglyceridemia

Hypertriglyceridemia results in an increased mass transfer of triglyceride from triglyceride (TG)-rich lipoproteins to HDL through the action of CETP leading to TG enrichment of HDL and reduction of the cholesterol content of each HDL particle (Lewis et al. 1996). TG enrichment of HDL may have a significant impact on the metabolism of HDL particles. These particles are more prone to lipolysis by HL, resulting in a formation of smaller particles (Lamarche et al. 1999). ApoA-I is in a more dissociable form on TG-enriched HDL and may be shed from the particle (Liang et al. 1994). These mechanisms may all contribute to a significant extent for the increased fractional catabolic rate (FCR) of apoA-I observed in hypertriglyceridemic states and provide an explanation for the inverse relationship between plasma triglyceride and HDL levels (Lamarche et al. 1999).

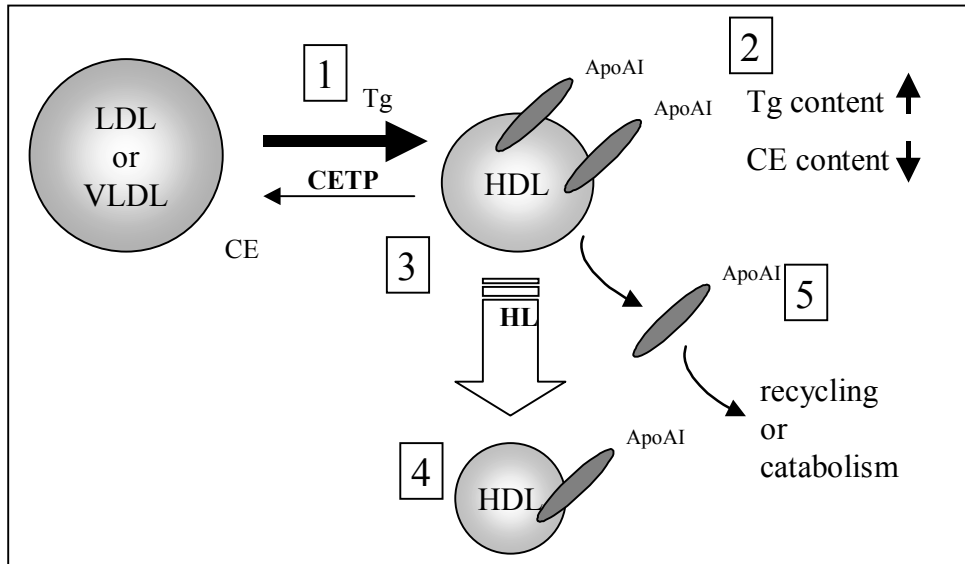


Figure 2. A model of HDL metabolism in hypertriglyceridemia

In hypertriglyceridemia, (1) an increased mass transfer of triglycerides (TG) occurs from TG-rich lipoproteins to HDL particles through the action of cholesteryl ester transfer protein (CETP) leading to TG enrichment of HDL. TG-enriched HDL (2) has been shown to a better substrate for lipolysis by hepatic lipase (HL) (3), which results in formation of small, lipolytically modified HDL particles (4). Apolipoprotein A-I (apoAI) may be shed from the particle in this process (5).

High density lipoprotein subpopulations

HDL particles are multi-shaped structures with varying density, fluidity, size, charge and antigenicity, a result of qualitative and quantitative differences in lipids, apolipoproteins, enzymes and lipid transfer proteins (Skinner 1994). Mature HDL have a globular shape with a hydrophobic core and a hydrophilic surface layer. The HDL core consists of cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipids and unesterified cholesterol in which various proteins are embedded. The major proteins of HDL are apolipoproteins (apo) AI and AII. In addition, HDL contain a large number of less abundant proteins including apo AIV, apoC's, apoE, and apo J (Barter et al. 2003).

The heterogeneity of plasma HDL particles results from continuous remodeling of HDLs by different proteins CETP, LCAT and PLTP associated with HDL, and

by the lipolytic activities of HL and LPL (Skinner 1994). HDL particles can be classified according to particle size, protein composition or density in ultracentrifugation, where the two main fractions are HDL₂ and HDL₃. HDL₂ particles are less dense and contain a relatively higher amount of cholesterol and phospholipids than HDL₃ (Gotto et al. 1986). Small, dense HDL₃ displays a higher capacity to accept cholesterol from peripheral tissues and they are better substrates for LCAT. They have also been reported to be more effective in protection of LDL from oxidative stress (Yoshikawa et al. 1997) as compared with large, light, lipid-rich HDL₂.

HDL particles can be artificially reconstituted. Discoidal reconstituted HDL (rHDL) contains defined numbers of apolipoprotein molecules (usually one or two molecules of apoA1) and a high amount of phospholipid molecules (usually phosphatidylcholine)(Jonas 1986). These discoidal particles –in addition to reconstituted spherical particles- are frequently used in *in vitro* studies to delineate the biological effects of distinct proteins and lipids which are present in native HDL.

Lecithin:cholesterol acyltransferase (LCAT)

Lecithin:cholesterol acyltransferase is a soluble plasma enzyme that converts cholesterol and phosphatidylcholines (lecithins) to cholesteryl esters and lyso-phosphatidylcholines on the surface of HDL (Glomset 1968) and participates in cholesterol movement from tissues into HDL (Fielding et al. 1995). Most of this glycoprotein is synthesized by the liver and circulates in blood reversibly bound to lipoproteins. LCAT preferentially binds to HDL, where apoA1, the major apolipoprotein component of HDL functions as the main activator of LCAT (Yokoyama et al. 1980).

Antiatherogenic functions of HDL

The inverse correlation between HDL cholesterol concentration and atherosclerosis is well established (Nikkilä 1953; Gordon et al. 1977; Watkins et al. 1986) and the mechanisms are under intense investigation. Reverse cholesterol transport –a process of excess cholesterol flux from peripheral cells to the liver (Fielding et al. 2000)- is at present considered the most important antiatherosclerotic action of HDL. Also other antiatherogenic mechanisms have been postulated, like inhibition of endothelial cell adhesion molecule expression and protection of LDL against oxidation (Nofer et al. 2002).

2. 2. Lipoprotein oxidation

2. 2. 1. Measurement of lipoprotein oxidation

Oxidation of lipoprotein lipids generates conjugated dienes (rearrangements of double bonds in polyunsaturated acyl chains) that absorb light at 234 nm (Esterbauer et al. 1989). The kinetics of lipoprotein oxidation can be monitored by measuring the formation of conjugated dienes by spectrophotometry according to the method of Esterbauer et al (1989). A number of other methods to measure lipoprotein oxidation have been described including thiobarbituric acid reactive substances (TBARS) assay, which detects aldehyde products of lipid peroxidation, as well as number of other assays for phospholipid and cholesterylester hydroperoxides and aldehydic lipid peroxidation products (Esterbauer 1996).

When oxidation of LDL *in vitro* has been initiated by copper and monitored by measuring the formation of conjugated dienes, the oxidation resistance of LDL can be expressed as the length of time during which no significant lipid peroxidation occurs. This period of inhibited oxidation, termed the 'lag time', is thought to be partially due to the radical scavenging reactions of the antioxidants contained in the LDL particle (Giesege et al. 1994). Increase in the antioxidative capacity of LDL causes prolongation of lag time and a shift to the right of the oxidation curves (**Fig 3**).

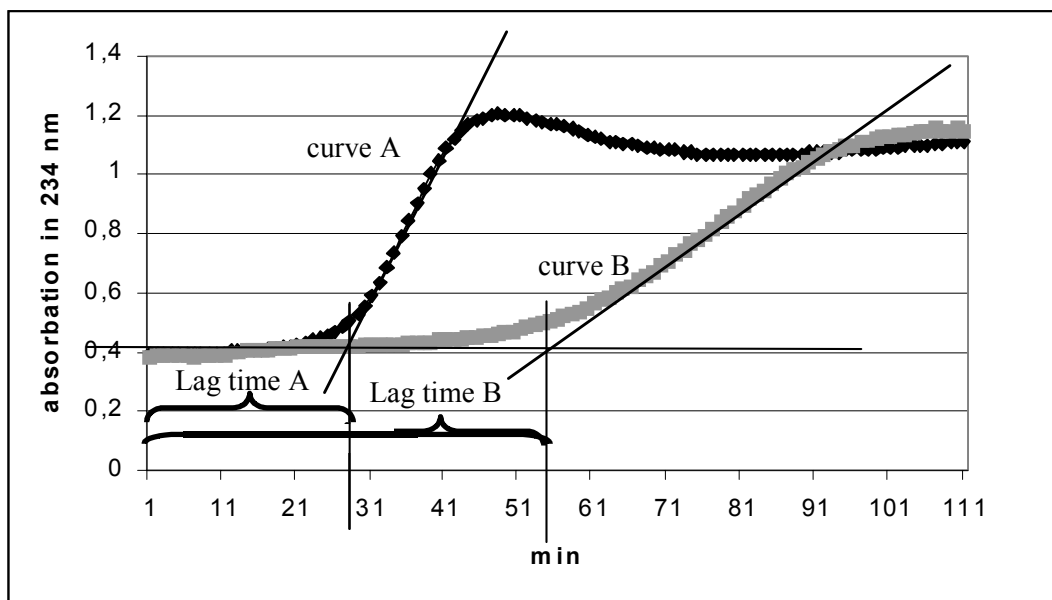


Figure 3. An example of lipoprotein oxidation measured by determination of conjugated diene formation as a function of time. The oxidation resistance of the two samples A and B is compared as a difference in lag times, the lag time of curve A being 29 min and the lag time of curve B 53 min (prolongation indicating increased antioxidant capacity).

2. 2. 2. Metabolic aspects of lipoprotein oxidation

The fact that human plasma is endowed with a number of antioxidant defense mechanisms involving many free radical scavengers, reducing agents and antioxidant enzymes suggests that the oxidation of lipoproteins occurs preferentially in subendothelial space rather than in plasma (Frei et al. 1988). The term “oxidation” is used here to include the process of lipid peroxidation and the protein modifications that accompany or result from lipid peroxidation. Lipoproteins have been reported to be oxidized by various vascular cells including macrophages (Parthasarathy et al. 1986), lymphocytes (Lamb et al. 1992) endothelial cells (Steinbrecher et al. 1984) and smooth muscle cells (Heinecke et al. 1987). *In vitro*, iron and copper are used to modify LDL and copper oxidized LDL resembles to some extent endothelial cell –modified LDL in its chemical and biological properties (Esterbauer et al. 1992).

Susceptibility of LDL to oxidation is among other factors increased by reduced particle size and high content of polyunsaturated fatty acid (Korpela et al. 1999), and decreased by high vitamin E content in the particle (Tsimikas et al. 1998). It is presumed that lipid peroxidation in LDL begins in the acyl chains of the phospholipids and propagates to the neutral lipids in the core of the particle, which is later accompanied by fragmentation of apolipoprotein B and antigenic changes in the protein (Steinbrecher et al. 1987).

Oxidation of LDL and its intimal accumulation are considered crucial steps in the development and progression of atherosclerosis, i. e. LDL must be oxidatively modified to trigger the pathological events of atherosclerosis (Steinberg et al. 1989; Witztum et al. 1991; Ross 1993; Heinecke 1998; Ehara et al. 2001). This hypothesis is supported by a number of *in vitro* and *in vivo* studies demonstrating the proatherogenic properties of oxidized LDL and the occurrence of oxidatively modified LDL in atherosclerotic (Klatt et al. 1996). The uptake of normal, unoxidized LDL is regulated by LDL receptors as increased intracellular cholesterol concentration downregulates the cholesterol synthesis and LDL-receptor expression (Brown et al. 1986). Oxidized LDL (oxLDL) is taken up by a different receptor, a scavenger receptor of macrophages in vessel wall, which does not recognize normal LDL. The scavenger receptor is not downregulated by the inflowing cholesterol, which leads to accumulation of cholesterol in intimal macrophages and subsequent development to foam cells,

and, finally to formation of fatty streaks, the first visible signs of an atherosclerotic arterial wall (Goldstein et al. 1979).

HDL inhibits LDL oxidation and reverses the stimulatory effect of oxidized LDL on monocyte infiltration (Parthasarathy et al. 1990; Berliner et al. 1995; Mackness et al. 1995; Banka 1996). The principal lipid and protein constituents of HDL may have antioxidant potential, but minor components of HDL have also been implicated, including transferrin which can associate with apoA1 (Kunitake et al. 1992), and enzymes such as paraoxonase (Mackness et al. 1993; Watson et al. 1995), platelet activating factor (PAF)–acetylhydrolase (Watson et al. 1995) and LCAT (Vohl et al. 1999; Mackness et al. 2000). In particular, paraoxonase can hydrolyze oxidized phospholipids in minimally modified LDL (mmLDL) (Watson et al. 1995), and reduce the accumulation of lipid peroxides in copper-oxidized LDL (Mackness et al. 1993).

However, also HDL moieties may become oxidized and the degree of oxidation depends in part on the type, concentration, and duration of oxidant exposure. Tyrosyl radical and hypochlorite both preferentially attack apoproteins rather than lipids of HDL, with tyrosyl radical generating low levels of lipid oxidation products (Francis et al. 1996). In contrast, the initial site of attack by metal ions (copper, iron, and manganese), hydroxyl radical, and lipoxygenase appears to be HDL lipids, with modification of apoproteins occurring secondarily (Garner et al. 1998).

The oxidation of HDL leads to impairment of its antioxidative actions (Mertens et al. 2003) and also to decline in other atheroprotective properties like reduced reverse cholesterol transport (Morel 1994; Francis 2000) and decreased LCAT activity (Maziere et al. 1993)

2. 3. Endogenous estrogens and atherosclerosis

2. 3. 1. Overview of metabolism of endogenous estrogens

The principal estrogen in fertile women is 17 β -estradiol (E2), and together with other naturally occurring estrogens such as estrone (E1) and estriol (E3), it belongs to the C18 steroid family derived from cholesterol. It is synthesized primarily in steroidogenic tissues, which include the ovaries, the placenta (during pregnancy) and adrenal glands. Estrogens are also produced in fat tissue by aromatization of androgenic precursors originating from the testes and adrenal glands (MacDonald et al. 1979). Unconjugated estradiol is transported in blood mainly by sex hormone binding globulin (SHBG) and albumin (Anderson 1974), but a small fraction is circulating free and can be taken up by estrogen target tissues. Estrogens are metabolized by sulfation or glucuronidation, and these water-soluble conjugates are excreted into urine and bile. The biliary conjugates can re-enter blood following hydrolysis and intestinal reabsorption (Adlercreutz et al. 1976). Other metabolic pathways are hydroxylation and methylation of hydroxyl groups, which yield catechol and methoxylated estrogens (Osawa et al. 1993) as well as fatty acid esterification (Hochberg 1998). A small part of serum estradiol is associated in lipoproteins in the form of estradiol fatty acid esters (Vihma et al. 2001), which are also the focus of the current studies.

2. 3. 2. Antiatherogenic effects of endogenous estrogens

Estrogens have beneficial effect on lipids: they increase plasma HDL and decrease plasma LDL levels (Tikkanen et al. 1978; Wakatsuki et al. 1995; Tikkanen 1999) and they have, in addition, been reported to possess antioxidative properties and reduce lipoprotein oxidation (Maziere et al. 1991; Rifici et al. 1992; Sack et al. 1994; Ayres et al. 1996; Ayres et al. 1998; Wakatsuki et al. 1998; Meng et al. 1999).

Estrogens exert also several direct effects on vessel wall, e.g. inducing vasodilatation by relaxing smooth muscle tone (White et al. 1995). Notably, they increase the formation of nitric oxide (NO), a vasodilator that is also capable of preventing platelet aggregation and leukocyte chemotaxis (Kim et al. 1999; Saito et al. 1999; Rubanyi et al. 2002). Other suggested cardioprotective mechanisms of estrogens include inhibition of smooth cell proliferation (Suzuki

et al. 1996) and facilitation of angiogenesis and collateral vessel formation in ischemic tissues (Rubanyi et al. 2002).

2. 3. 3. Estradiol esterification and estradiol esters in lipoproteins

E₂ fatty acid esters are formed by the esterification of the hydroxyl at position C-17 β in the D-ring with a (predominantly) long chain fatty acid (Kanji et al. 1999) (Figure 4). Estradiol esters are synthesized *in vivo* in various tissues including spleen, lung, uterus, liver and adipose tissue (Schatz et al. 1981) by direct esterification of E₂ (Larner et al. 1992) in a reaction catalyzed by acyl coenzyme A : cholesterol acyltransferase (ACAT) (Martyn et al. 1987), but these E₂ esters are not secreted from the tissues into the circulation (Schatz et al. 1981). Previous studies have provided indirect evidence that the esterification reaction in blood and ovarian follicular fluid occurs in HDL particles and that the reaction is catalyzed by a different enzyme, LCAT (Shwaery et al. 1997; Hochberg 1998; Kanji et al. 1999; Lamarche et al. 1999; Lewis et al. 2001; Tikkanen et al. 2002) Pahuja et al. 1995) (see figure 4), which mainly esterifies cholesterol (Pahuja et al. 1995; Pahuja et al. 1995).

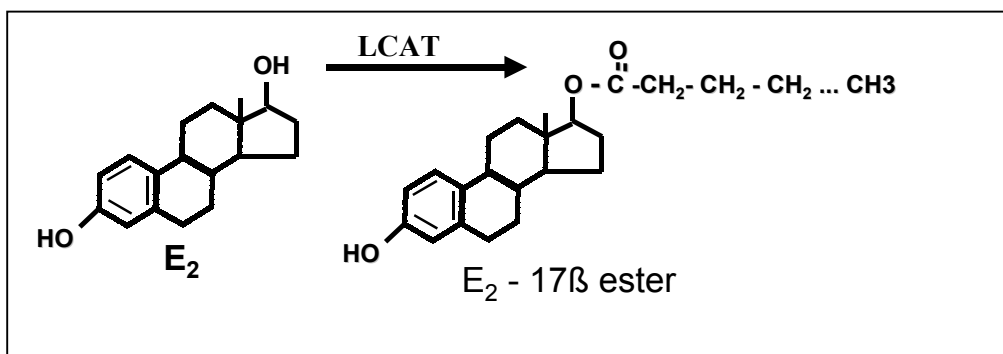


Figure 4. Formation of estradiol fatty acyl 17 β ester in plasma. The 17 β -hydroxyl in the D ring is esterified with a long chain fatty acid in a reaction that can be catalyzed by LCAT. The aromatic A ring has an intact hydroxyl group at carbon 3.

The estradiol esters formed are transported in blood where they are exclusively bound to lipoproteins (Janocko et al. 1983; Larner et al. 1992).

Unesterified E₂ displays only a weak association with HDL particle (Tang et al. 1997), but E₂ fatty acid derivatives are able to incorporate into lipoprotein particles. Thus, these fatty acid ester derivatives of E₂ are preferential structural forms that associate with HDL.

Studies in both rats and humans have indicated that the plasma half-life of E₂ fatty acid esters is significantly longer compared with unesterified E₂ (Larner et al. 1985) and their effects in various tissues (uterus, liver, brain, pituitary gland) has been found to be more prolonged than that of unesterified E₂ (Larner et al. 1985; MacLusky et al. 1989). This suggests that estradiol fatty acid esters could function as a storage form of estradiol and be hydrolyzed to free hormone when needed (Hochberg et al. 1990).

There is evidence that the esterification reaction is a prerequisite for estradiol to function as an antioxidant in LDL particles at physiological levels, a conclusion based on *in vitro* experiments with plasma in which physiological E₂ concentrations were used (Shwaery et al. 1997). However, the antioxidant effect of E₂ added directly to LDL, but not to HDL, has reportedly been lost after dialysis. This suggests that the E₂ esters are formed in HDL and subsequently transported to LDL (Abplanalp et al. 2000).

3. AIMS OF THE STUDY

Estrogen fatty acid esters, a unique class of hormone derivatives, are water-insoluble and they are carried in the circulation in lipoprotein particles. In contrast to unesterified estrogen, they have been shown to inhibit lipoprotein oxidation at physiological concentrations *in vitro*.

The aims of the present study were:

1. To study the formation, incorporation and transport of estradiol esters in different lipoprotein fractions (I and II)
2. To provide direct evidence that estradiol is esterified by the function of LCAT and then incorporated into HDL in the form of estradiol esters, which subsequently increases the antioxidant potential of HDL (III)
3. To investigate the effect of HDL composition, in particular of increased triglyceride content, on estradiol esterification (IV)

4. SUBJECTS, MATERIALS AND METHODS

4. 1. Subjects

4. 1. 1. Samples of normolipidemic plasma (Studies I-IV)

Blood was drawn from healthy, normolipidemic volunteers (aged, 21–35) who were taking no medication, into EDTA-containing tubes. Plasma was prepared by immediate centrifugation at 2500 x g, 15 min, at +4°C or 2 300 x g for 10 min at +10°C and was used for experiments during the same day.

4. 1. 2. Samples of follicular fluid (Study I)

Fresh follicular fluid was obtained from aspirates of women undergoing oocyte retrieval for *in vitro* fertilization at the Helsinki University Central Hospital. Ovarian stimulation was induced by combining gonadotrophin releasing hormone (GnRH) analog with gonadotrophins. The only lipoprotein that follicular fluid contains is HDL. Follicular fluid was centrifuged twice (2300 x g, 15 min, at +10°C) to remove blood cells and granulosa cells as well as nonspecific cell debris.

4. 1. 3. Samples of hypertriglyceridemic plasma (Study IV)

Blood was collected from 8 hypertriglyceridemic men (plasma TG 2.6-11.2 mmol/l) after an overnight fast into EDTA-containing tubes and centrifuged at 2300 g for 10 min at +10°C to obtain plasma.

The study protocols have been approved by the Ethics Committee of the Department of Obstetrics and Gynecology and by the Ethics committee of Department of Medicine, Helsinki University Central Hospital.

4. 2. Materials and Methods

4. 2. 1. Sequential ultracentrifugation and purification of serum lipoproteins

The lipoproteins were isolated by sequential ultracentrifugation (Havel et al. 1955) at the following cut-off densities: VLDL ($d < 1.006$ g/ml), LDL ($1.006 < d < 1.063$ g/ml), total HDL ($1.063 < d < 1.21$ g/ml) HDL₂ ($1.063 < d < 1.125$ g/ml) and HDL₃ ($1.125 < d < 1.21$ g/ml) using a Beckman Optima LE-80K ultracentrifuge and a Ti 50.4 rotor. Prior to the incubation with E₂ (see incubation of estrogen with lipoproteins), ultracentrifugally isolated lipoprotein fractions were gel filtrated on Sephadex G-25 (column dimensions 1 x 20 cm, Pharmacia Biotech, Uppsala, Sweden) to remove EDTA and other small molecules. Lipoproteins were eluted with 2.5-3 ml of phosphate buffered saline (PBS) pH, 7.4.

4. 2. 2. Separation of esterified estradiol from non-esterified estradiol

The lipoprotein fractions obtained by gel filtration on Sephadex G25 were pooled and extracted four times with 2.5 volumes of diethyl ether:ethyl acetate (1:1, v/v) by mixing them vigorously for 3 min. The water phase was frozen in a dry ice-ethanol mixture and the ether phase was decanted into a glass tube. The extraction was repeated four times and the combined ether phases were evaporated to dryness under nitrogen.

In order to separate esterified E₂ from the free E₂, samples were chromatographed on a Sephadex LH-20 hydrophobic matrix (column dimensions 0.5 x 5cm, Pharmacia Biotech, Uppsala, Sweden) using hexane:chloroform, (1:1, v/v) as the eluting solvent at room temperature. In short, disposable Pasteur pipettes (145 mm, Volac) plugged with cotton were packed to a height of 5 cm with a suspension of Sephadex LH-20 in hexane:chloroform (1:1, v/v). After washing the columns with fresh solvent, the dry samples were dissolved in 0.3 ml of hexane:chloroform (1:1, v/v) and applied to the columns twice in 0.3 ml of the same solvent. The estradiol ester fraction was eluted with 6 ml of hexane:chloroform (1:1, v/v). The non-esterified estradiol fraction was then eluted with 5 ml of methanol. Both fractions were evaporated to dryness under nitrogen and stored in the appropriate solvent (ester fraction in hexane:chloroform, free fraction in methanol).

4. 2. 3. Analysis of 17 β -estradiol derivatives by thin-layer chromatography (TLC).

Samples (approx. 4000 dpm or all radioactivity available) obtained from the 17 β -estradiol "ester" fractions after chromatography on Sephadex LH-20 were applied to TLC plates (20 x 20 cm, Silica gel 60, Merck, Germany) and developed in a hexane/ethyl ether (1:7, vol/vol) solvent system. The following non-radioactive standards were used: 17 β -E₂ (Steraloids, Newport, USA), 17 β -E₂ 17-stearate (Steraloids, Newport, USA), 17 β -E₂ 3-oleate and 17 β -E₂ 3,17-dioleate. The last two esters were prepared as described previously (Meng et al. 1999). TLC plates were dried in air and the location of the standards was determined by visualization under ultraviolet (UV) light after rhodamine staining. All bands were scraped from the TLC plates and counted directly for [³H] radioactivity in a liquid scintillation counter (Rack-beta, Wallac, Turku, Finland). 17 β -estradiol and 17 β -estradiol 17-ester fractions of the samples were identified by comparing their R_F-values to those of the standards (Studies I, II and IV).

4. 2. 4. Incubation experiments with estradiol

Plasma (Study I)

Labelled [2,4,6,7-³H(N)] 17 β -E₂ (specific activity of 72 Ci/mmol, New England Nuclear, Boston, USA) in 0.5 mol/l N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid sodium salt (HEPES) was added to plasma to reach a final concentration of 2 x 10⁶ cpm/ml (corresponding to 52 nmol/l E₂) in a total volume of 4 ml. The mixture was incubated for 24 h at +37°C in the absence and presence of 1.5 mmol/l dithionitrobenzoic acid (DTNB) (Sigma, St. Louis, MO).

Follicular fluid (Study I)

Incubations with follicular fluid were performed identically to the plasma incubations during the same experimental set.

Lipoproteins (Studies I-IV)

Study I: Transfer of [³H] estradiol-17 β between lipoproteins

Purified, non-radioactive LDL (described under isolation and purification of lipoproteins) was incubated with HDL isolated from the same subject, which contained [³H]-E₂ esters (prepared by incubation with plasma as described

above). Incubations were carried out in 3 ml PBS, each incubation contained HDL corresponding to 12,500 cpm (HDL protein between 0.3 and 0.9 mg). The amount of LDL (defined as LDL protein) used in each incubation was half of the HDL protein used (*i.e.* 0.15–0.45 mg). The incubations were carried out in the absence as well as in the presence of various amounts of CETP at +37°C for 3, 6, 12 and 24 h. After incubations, lipoproteins were reisolated by 24h ultracentrifugation as described under *Isolation of lipoproteins*. The transfer of [³H]-E₂-17β esters from HDL to LDL was measured by determining the radioactivity in both donor (HDL) and acceptor (LDL) lipoproteins (Study I)

Study II: Incubation of LDL, HDL₂, HDL₃ and rHDL with [³H]-E₂

[³H]-E₂ in 0.5 mol/l HEPES buffer was added to ultracentrifugally isolated and purified HDL as well as to the major HDL subfractions HDL₂ and HDL₃ (1 mg or 2 mg protein in 3 ml TBS (Tris-HCl buffer, pH 7.4) to give a total radioactivity of 200 000 dpm (corresponding to 1.3 nmoles of E₂). For comparison, incubations were carried out using LDL, and reconstituted discoidal HDL (rHDL, apoA1/cholesterol/egg PC in a molar ratio of 1:8:140). The mixture was incubated at +37°C for 24 h in the absence or presence of LCAT (activity 10-74 nmol/h/ml) as well as in the absence and presence of DTNB, final concentration of 3 mmol/l.

Study III: Incubation of HDL with non-radioactive and radioactive estradiol

Estradiol-17β (concentration of E₂ prior to incubation step was 300 μmol/l in the final experimental solution) was added to HDL (1 or 2 mg total protein) in total sample volume of 1.5-3 ml and the mixture was incubated at +37°C for 24 h in the absence or presence of exogenous purified LCAT (activity 16.9 nmol/h/ml ± 1.0 (SEM), range 1.6 - 44.7 nmol/h/ml, n=10) as well as in the absence or presence of the LCAT inhibitor, DTNB, final concentration 3 mmol/l. Simultaneous experiments were made with labeled 17β-estradiol in 0.5 mol/l HEPES buffer, added to HDL to give a total radioactivity of 2x10⁵-2x10⁶ dpm.

Study IV: Incubation of HDL₂ and HDL₃ isolated from normo- (NTG) and hypertriglyceridemic (HTG) males with radioactive and non-radioactive estradiol

[³H]-E₂ in 0.5 mol/l HEPES buffer was added to the HDL₂ (200 μg protein in 2.5 ml) or HDL₃ (600 μg protein in 2 ml) solution to give a total radioactivity of

2×10^6 dpm. The mixture was incubated at $+37^\circ\text{C}$ for 24 h in the absence or presence of exogenous purified LCAT. The same amount of exogenous LCAT was added to HTG and NTG samples in each experiment, taking in the consideration that the activities of different LCAT preparations varied between 3.7 and 37.2 nmol/h/ml (median 15.6 nmol/h/ml) in the 7 different experiments. Parallel incubation experiments with HDL subfractions were made with non-radioactive estradiol- 17β in the presence and absence of exogenous LCAT, as well as control incubations without addition of estradiol. Altogether 7 different experiments were carried out using simultaneously both HTG and NTG samples with the exception of one incubation with two HTG samples. E₂-ester enriched HDL₃ was used in oxidation experiments.

Following incubations in all experiments, the lipoproteins were purified by size-exclusion chromatography on Sephadex G-25 to remove small molecular weight substances not associated with HDL particles. Protein concentration was determined by the method of Lowry et al (1951). Radioactivity in the eluted fractions was determined by liquid scintillation counting (Rack-beta, Wallac, Turku, Finland). The proportion of generated, lipoprotein-associated E₂ esters was measured by a recently developed quantitative method by Vihma et al (Vihma et al. 2003).

4. 2. 3. Preparation of reconstituted discoidal HDL (rHDL)

The sodium cholate dialysis method was used essentially as described (Jauhiainen et al. 1986) in the preparation of rHDL discs from human apolipoprotein A-I, egg phosphatidyl choline (PC) and cholesterol. Purified human plasma apoA-I was kindly provided by Dr Peter Lerch, Swiss Red Cross, Bern, Switzerland. Briefly, reaction mixture contained apoA-I/cholesterol/egg PC/sodium cholate in a molar ratio of 1:8:140:140.

ApoA-I was added into detergent-containing lipid dispersion and all the incubations were performed in 10 mmol/l TBS, pH 7.4 containing 1 mmol/l EDTA. First the reaction mixture was incubated at $+25^\circ\text{C}$ for 20 min with slight shaking. Exhaustive dialysis to remove sodium cholate was carried out at $+4^\circ\text{C}$ against 5 l of 10 mmol/l TBS – 1 mmol/l EDTA buffer, pH 7.4 (changing fresh buffer 4 times during dialysis period). After dialysis the discoidal rHDL preparation was analyzed for the composition, purged with nitrogen and stored at $+4^\circ\text{C}$ for a maximum period of 4 weeks.

4. 2. 4. Purification and assay of human plasma lecithin-cholesterol acyltransferase (LCAT)

Human plasma LCAT was purified from fresh lipoprotein deficient plasma $d > 1.21$ g/ml by combining Phenyl-Sepharose CL-4B, ion-exchange and hydroxyl-apatite chromatographies essentially as described previously (Jauhiainen et al. 1986; Zhou et al. 1991), and LCAT activity was analyzed by a radiometric assay (Jauhiainen et al. 1986). Enzyme preparations did not express any CETP, PLTP, LPL or HL activity. Endogenous LCAT activity in isolated lipoprotein fractions was determined prior to adding exogenous LCAT (studies II-IV).

4. 2. 5. Purification and assay of cholesterol ester transfer protein (CETP)

Purification of CETP from human plasma was performed essentially as described (Ohnishi et al. 1990; Rye et al. 1998) from the bottom fraction ($d > 1.21$ g/ml) of ultracentrifuged plasmapheresis plasma. CETP activity was determined with a radiometric method in which CETP transfers radiolabeled cholesteryl esters from LDL to HDL (Groener et al. 1986). The CETP-active fractions were stored at -70°C until use. The activity of CETP in different purified batches varied between 15 and 25 nmol /ml /h. Purified CETP did not contain PLTP, LCAT, LPL, or HL activity (Study I).

4. 2. 6. Other analytical methods

HDL-associated estradiol and its fatty acyl derivatives in the samples were measured in a single experiment (Study IV) by the recently published method (Vihma et al. 2003). The concentrations of apoA1 and apoB were measured with commercial kits by an immunoturbidometric assay (Orion Diagnostica, Finland), and concentrations of cholesterol (Boehringer Mannheim, Mannheim, Germany), triglycerides (Roche, Switzerland), and phospholipids (Wako Chemicals, Germany) by enzymatic tests. Concentration of unesterified E_2 was measured by RIA (Sorin Diagnostics, Italy).

4. 2. 9. Statistical analyses

Statistical analyses were carried out with SPSS 9.0-program for Windows. Data are expressed as the mean \pm standard error of the mean (SEM) or when the data were not normally distributed, as median and range. Between-group differences were assessed by unpaired *t*-test, variables with skewed distribution were \log_{10} transformed before the analyses. Continuous variables were compared between the groups by univariate analysis of variance (ANOVA). Correlation coefficients (*r*) for the associations between characteristics were examined either by Pearson's correlation analysis or Spearman's non-parametric correlation analysis, when appropriate.

5. RESULTS

5. 1. Formation of 17 β -estradiol 17-monoesters in HDL 3 subclass by LCAT (Study I and II)

Incubation of [³H]E₂-17 β with follicular fluid and plasma

Following incubation of [³H]E₂-17 β with follicular fluid as well as normolipidemic male plasma with and without DTNB, LDL and HDL were isolated by a combination of ultracentrifugation and gel filtration on Sephadex G25. **Figure 4** shows that there was a significant amount of radioactivity associated with the protein fractions (fractions 7-9) of HDL (**Fig 4 A**) and LDL (**Fig 4 C**) after plasma incubations with radioactive E₂. Results with HDL derived from follicular fluid were identical. When incubations were carried out in the presence of the LCAT inhibitor DTNB, almost no radioactivity could be detected in the elution position of lipoproteins (**Fig 4 A-D**) suggesting that esterification was a prerequisite for incorporation of [³H]-E₂-17 β into lipoproteins.

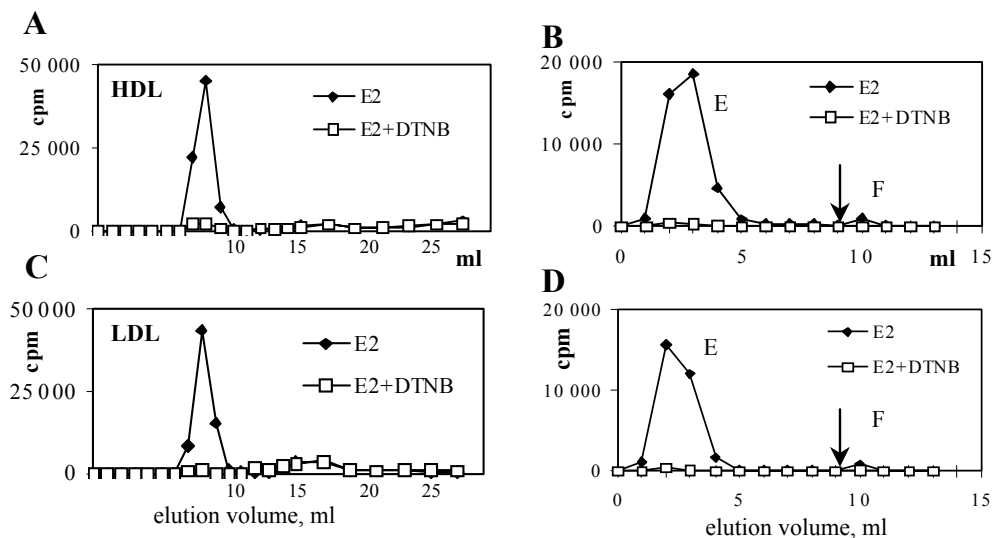


Figure 4. The radioactivity elution patterns of plasma HDL after Sephadex G25 (A) and Sephadex LH-20 (B) columns and of plasma LDL after Sephadex G25 (C) and Sephadex LH-20 (D) columns.

Plasma was incubated with [³H]E₂-17 β with and without DTNB for 24 h. After incubation, LDL and HDL were isolated by sequential ultracentrifugation. HDL and LDL were purified by gel filtration on Sephadex G25 (A, C) to remove unattached [³H]-E₂ and protein and radioactivity levels were analyzed. After extraction with ethylacetate/diethylether, samples were chromatographed on Sephadex LH-20 (B, D) first in hexane-chloroform, to elute the [³H]-E₂ esters (E) and the solvent was changed to methanol to elute free [³H]-E₂ (F) (arrow indicates start of methanol elution).

Analysis of radioactivity attached to lipoproteins

Following extraction of the lipoprotein-containing fractions with hexane/chloroform and subsequent hydrophobic chromatography on Sephadex LH-20, a major part of the radioactivity was eluted in the "ester fraction" (indicated by "E") and only a trace amount was detected in the "free fraction" (indicated by "F") in both HDL (**Fig 4B**) and LDL samples (**Fig 4D**). There was no detectable radioactivity in the "ester" fraction of the sample that was incubated in the presence of DTNB (**Fig 4B, Fig 4D**). The "ester fractions" were further analyzed by TLC that offered further evidence that most of the radioactivity migrated in the same position as the ester standard (E_2 - 17β -stearate, R_f -value 0.27).

Some samples from the saponified ester fraction were chromatographed on Sephadex LH-20 (9% toluene in methanol), which separates estrone, 17β - E_2 , and estriol from each other showing that estrone or estriol was not present (data not shown). This excluded the possibility of esterification and incorporation into HDL of estrone or estriol formed from 17β - E_2 .

Effect of exogenous, purified LCAT on 17β -estradiol esterification in HDL.

To find direct evidence of the role of LCAT in the estradiol esterification, isolated HDL was incubated with labeled E_2 in the presence and absence of exogenous LCAT. The radioactivity peaks coincided with the protein peak after gel filtration on Sephadex G25. Also, addition of exogenous LCAT significantly increased the esterification and incorporation of [3 H]- E_2 into HDL ($p < 0.05$) whereas addition of DTNB significantly decreased this effect ($p < 0.005$). When the incubations of HDL with [3 H]- E_2 were carried out by adding increasing amounts of exogenous LCAT (0, 36, 73 and 110 nmol/h/ml), a clear dose-response in the esterification of 17β -estradiol (**Fig. 5**) was observed. The esterification activity present in the absence of exogenous LCAT was probably due to endogenous enzyme activity remaining in HDL after ultracentrifugation, a finding observed also previously (Meng et al. 1999). Endogenous LCAT activity ranged between 3-107 nmol/h/ml among the different HDL preparations used.

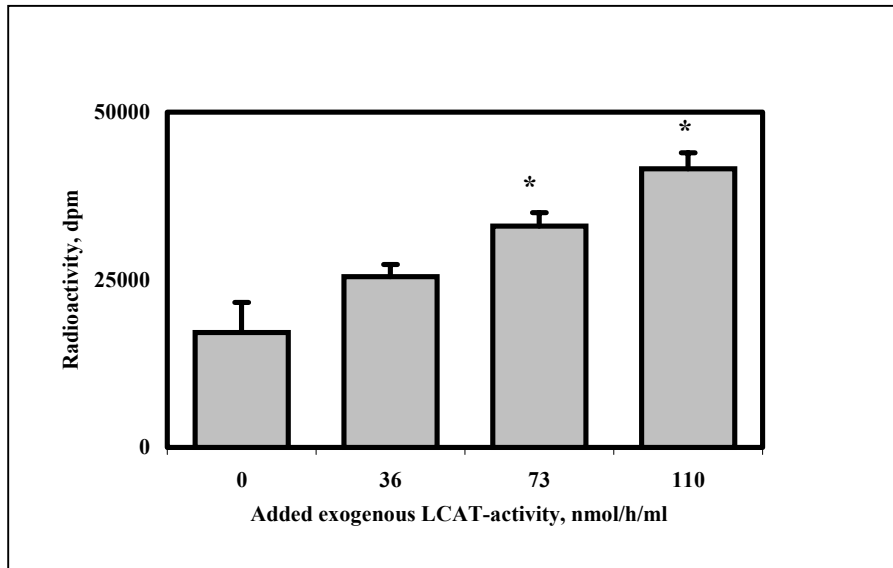


Fig 5 Effect of exogenous, purified LCAT on E₂ esterification in HDL.

Radioactivity associated with HDL after incubation of HDL, [³H]-E₂ and increasing amounts of LCAT. Asterisks indicate significant differences vs. baseline (0 = no addition of exogenous LCAT; p<0.05), using ANOVA followed by pairwise comparison by t-test with Bonferroni correction. Vertical bars indicate mean ± SEM.

Effect of exogenous, purified LCAT on 17β-estradiol esterification in HDL subfractions and LDL

Experiments identical with those carried out with total HDL were performed using HDL subfractions, HDL₂ and HDL₃, as well as rHDL and LDL. A significant peak of HDL₃-associated radioactivity (**Fig. 6C**) as well as rHDL-associated radioactivity (data not shown) was recovered in the void volume after gel filtration on Sephadex G25. Only a trace amount of radioactivity, however, was attached to HDL₂ subfraction (**Fig 6B**) and LDL (**Fig 6A**).

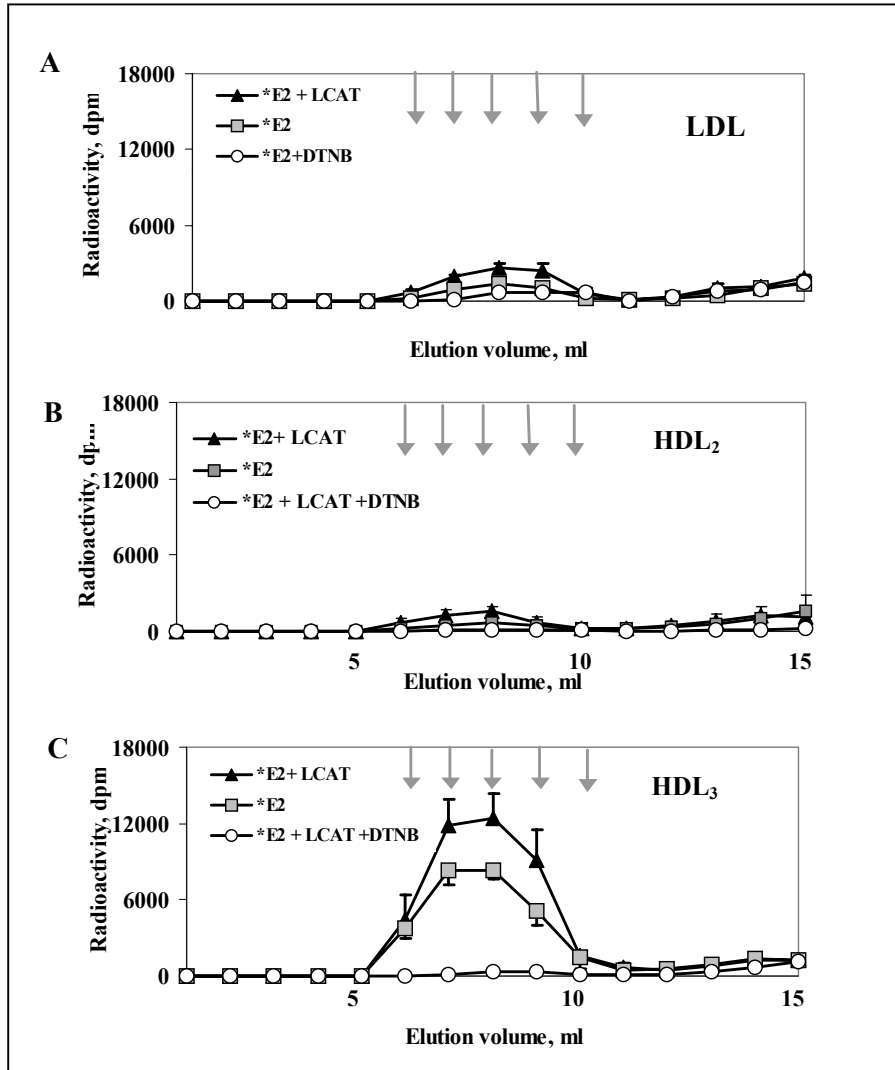


Figure 6. Elution patterns of LDL, HDL₂ and HDL₃-associated radioactivity after gel filtration on Sephadex G25.

LDL, HDL₂ and HDL₃ were incubated with [³H]-E₂ in the presence (▲) or in the absence (■) of exogenous LCAT. Incubations with DTNB (○) are also shown. After incubation, lipoproteins were purified by gel filtration on a Sephadex G25 column. **Panel A:** incubation with LDL ($p < 0.0005$, LDL vs. HDL₃ in the absence of exogenous LCAT; $p < 0.003$, LDL vs. HDL₃ in the presence of exogenous LCAT); **Panel B:** incubation with HDL₂ ($p < 0.00001$, HDL₂ vs. HDL₃ in the absence of exogenous LCAT; $p < 0.002$, HDL₂ vs. HDL₃ in the presence of exogenous LCAT); **Panel C:** incubation with HDL₃ ($p = 0.07$, HDL₃ + LCAT vs. HDL₃ - LCAT). Wilcoxon signed-rank test was used for statistical analysis. Arrows indicate the fractions containing proteins.

Presence of label in the HDL₃-fraction without addition of exogenous LCAT was due to endogenous HDL₃-associated LCAT-activity, which ranged between 22 and 50 nmol/h/ml. Endogenous LCAT activities in LDL and HDL₂ were very low, ranging between 0.1 and 1.2 nmol/h/ml for LDL and between 0 and 4.2 nmol/h/ml for HDL₂. Addition of purified LCAT to the incubations enhanced the formation and incorporation of 17 β -E₂ 17-esters in the presence of HDL₃ (p=0.07) and rHDL (p<0.05). Addition of LCAT to HDL₂ or LDL caused only a small increase in the radioactivity associated with these lipoproteins (p<0.00001, HDL₂ vs. HDL₃ in the absence of exogenous LCAT; p<0.002; HDL₂ vs. HDL₃ in the presence of exogenous LCAT; p<0.0005, LDL vs. HDL₃ in the absence of exogenous LCAT; p<0.003, LDL vs. HDL₃ in the presence of exogenous LCAT).

Extraction with ethylacetate/diethylether (1:1) followed by Sephadex LH-20 chromatography indicated that the radioactivity attached to total HDL, HDL₃ and rHDL represented esterified [³H] 17 β -E₂ (data not shown). Again, further analysis of the ester fraction by TLC confirmed that the radioactivity comigrated with the 17-E₂ 17-stearate standard suggesting that they represent 17 β -E₂ fatty acid 17-monoesters.

Thus, in line with previous results (Abplanalp et al. 2000), E₂ esterification occurred in HDL, and not in LDL (even in the presence of exogenous LCAT). Separate incubations with HDL subfractions indicated that E₂ 17-ester formation occurred almost exclusively in the small HDL₃ subfraction. In addition, these experiments provide direct evidence that E₂ is esterified by LCAT in a dose – response manner. The esterification by both endogenous and exogenous LCAT occurred mainly in HDL₃ subfraction.

5. 2. The role of cholesterol ester transfer protein (CETP) in the transfer of 17 estradiol esters (Study I)

Since E₂ esterification occurs only in HDL, not in LDL, but after plasma incubations or co-incubations with HDL, E₂ esters are also present in LDL (Meng et al. 1999; Abplanalp et al. 2000), we explored the possibility that [³H]-E₂-17 β -esters formed in HDL could be transferred to LDL by means of a CETP-facilitated process.

Non-radioactive (native) LDL was incubated with [³H]-E₂ ester -containing HDL (12,500 cpm, obtained from plasma incubations with [³H]E₂-17 β) in the absence and presence of CETP. As shown in Figure 7, during incubation without adding CETP, the radioactivity in LDL increased proportionally with time, while

subsequently the radioactivity in HDL decreased. In the presence of purified CETP (exogenous cholesteryl ester transfer activity per incubation: 8 nmol cholesteryl ester transferred/h) this shift in radioactivity from HDL to LDL was significantly accelerated (**Fig. 7**) The transfer of label from HDL (donor) to LDL (acceptor) in multiple incubations was quantitated by determining the LDL/HDL (cpm/cpm) ratio at various time points. The significance of the difference between the mean LDL/HDL count ratios at each time point was analyzed by paired *t* test (mean and SD): 3 h (0.23 ± 0.05 vs. 0.34 ± 0.06 , $n = 4$), $P = 0.028$ without and with CETP. The corresponding values for the other time points were: 6 h (0.40 ± 0.17 vs. 0.55 ± 0.15 , $n = 5$), $P = 0.032$; 12 h (0.46 ± 0.09 vs. 0.76 ± 0.19 , $n = 3$), $P = 0.035$; 24 h (0.82 ± 0.21 vs. 0.90 ± 0.26 , $n = 5$), $P = 0.388$.

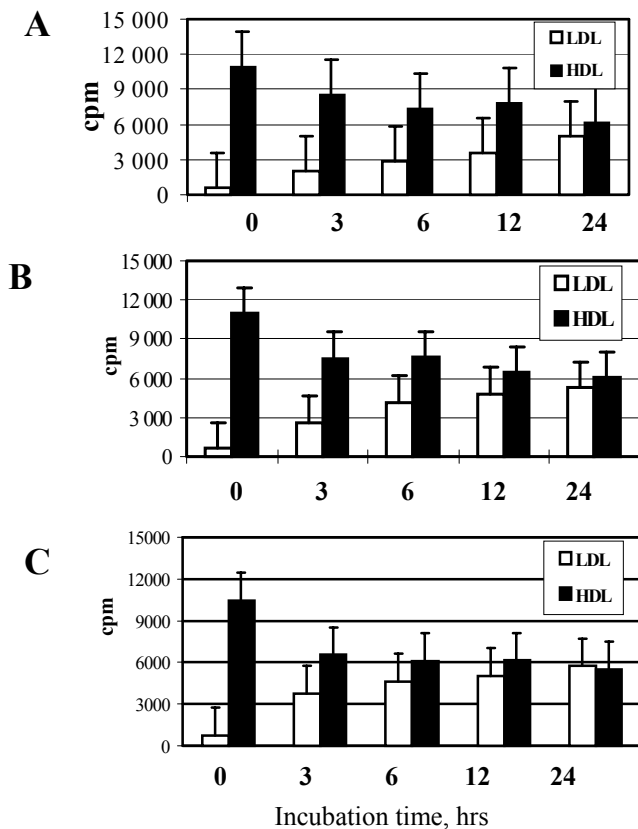


Figure 7. Incubation of native LDL with [³H]-E₂ labelled HDL. [³H]-E₂ labelled HDL was incubated with non-labelled LDL, the lipoproteins reisolated by ultracentrifugation after 3, 6, 12 and 24 hours, and their radioactivity determined. Incubations were carried out in the absence (**Panel A**) and presence (**Panel B**) of added purified CETP. The transfer of label from HDL (donor) to LDL (acceptor) was assessed in multiple incubations by determining the LDL/HDL (cpm/cpm) ratio at various time points. The significance of the difference between the mean LDL/HDL count ratios at each time point was analyzed by paired t-test (mean and S.D.): **3h** p=0.028 without and with CETP. The corresponding values for the other time points were: **6h** p=0.032; **12h** p=0,035; **24h** p=0.388. In other incubations additional CETP (final cholesteryl ester transfer activity / incubation 20 nmol /h) was used (**Panel C**) which indicated further enhancement of transfer.

To further investigate the role of endogenous CETP present in HDL, the transfer activities of HDL ultracentrifuged once or twice were compared, the second spin being used to remove residual endogenous CETP. The results indicated that the amount of spontaneously transferred radioactivity was significantly diminished, but not totally eliminated by this "washing" procedure (data not shown). Analysis of the twice ultracentrifuged HDL samples by Western blotting demonstrated that there still remained immunodetectable CETP in HDL in the washed HDL. Thus, [³H]-E₂ esters present in HDL could be transported to LDL particles in a process that is facilitated by CETP.

5. 3. The effect of elevated triglyceride content of HDL on E₂ esterification (Study IV)

Experiments were made with both HDL subclasses 2 and 3 isolated from HTG and NTG subjects, but in line with our results (Study II) accumulation of label in HDL₂ subclass was minimal (median radioactivity associated with HDL₂ was only 7% compared with that detected in HDL₃). Thus, only results concerning HDL₃ are reported.

5. 3. 1. Characteristics of HDL₃ isolated from HTG and NTG subjects

To explore the difference in the ability of HTG vs NTG HDL₃ to function as a substrate in LCAT-facilitated estradiol esterification, HDL₃ fractions were isolated from the normotriglyceridemic (NTG) and hypertriglyceridemic (HTG) subjects and their mass composition was determined (**Fig. 8**). The amount of triglycerides was significantly higher in HTG HDL₃ than in NTG HDL₃ (mean ± SEM, 7.9 ± 0.9 mass % in HTG vs 1.7 ± 0.2 mass % in NTG; p<0.001). Other lipid parameters as well as apolipoprotein levels did not differ between the HDL₃ species (p>0.1 for all).

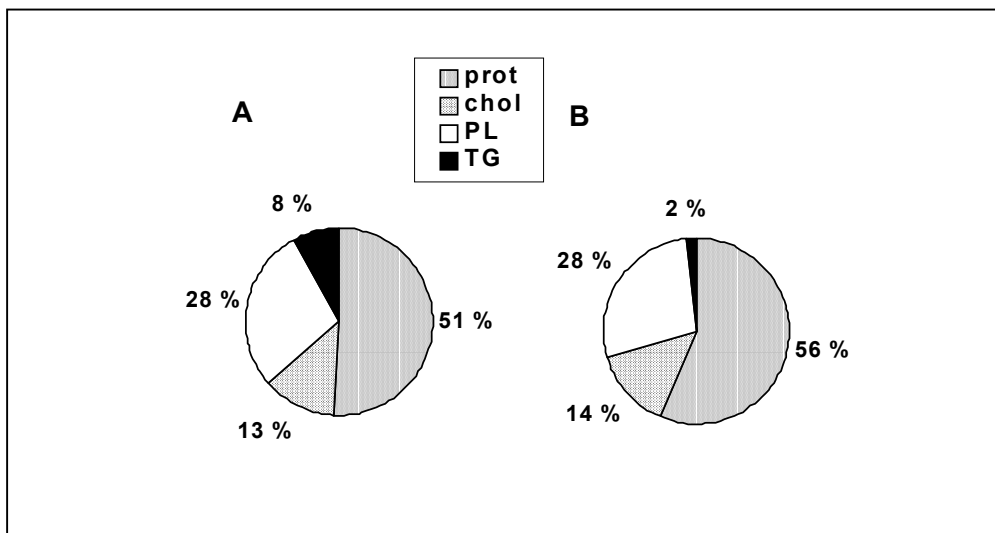


Figure 8. Composition of HDL₃ isolated form HTG (panel A) and NTG (panel B) plasma. The data present mass % values of individual components of HDL₃.

5. 3. 2. Detection of endogenous LCAT-activity in HTG and NTG HDL₃

Since LCAT-free HDL is difficult to obtain with conventional ultracentrifugal methods, endogenous LCAT activity was measured prior to E₂ esterification experiments. At baseline, endogenous LCAT activity ranged between 2.3 and 54 nmol/h/ml (median 11.4 nmol/h/ml, n=8) in hypertriglyceridemic HDL₃ and between 3.5 and 23 nmol/h/ml (median 6.7 nmol/h/ml, n=6) in normotriglyceridemic HDL₃ incubations (**Table 1**). These data indicate that the HDL₃ particles have their own endogenous potential to launch estradiol esterification due to tightly-associated LCAT activity.

Table 1. Incubation of HDL₃ obtained from HTG- and NTG- individuals with radioactive estradiol in the absence and presence of purified LCAT.

	HTG		NTG	
	LCAT -	LCAT +	LCAT -	LCAT +
N	8	8	6	6
Tg (mmol/l)	5.7	5.7	1.0	1.0
Tg range (mmol/l)	2.6-11.7	2.6-11.7	0.45-1.76	0.45-1.76
[³ H]-E ₂ ester (dpm)	49 600 a)	76 300 b)	18 000 a)	39 600 b)
[³ H]-E ₂ ester range (dpm)	20 900 - 66300	56 000 - 105 000	13 700 - 26 200	27900-46 300
LCAT act (nmol/h/ml)	11.4	28.8	6.7	20.6
LCATact range (nmol/h/ml)	2.3 - 53.6	22.7 – 74.4	3.5 – 22.9	13.9 – 60.1

Data are expressed as median. Statistical calculations were made using log-transformed data. ^{a)} P<0.001, ^{b)} P<0.00005

5. 3. 3. Comparison of the LCAT-mediated esterification of E₂ between hypertriglyceridemic (HTG) and normotriglyceridemic (NTG) HDL₃ as substrates

In order to compare the function of HTG- and NTG-HDL₃ as substrates for LCAT in E₂ esterification, HDL₃ was incubated with [³H]-E₂ in the presence and absence of added, exogenous LCAT. The same amount of exogenous LCAT was added to HTG and NTG samples in each experiment. After incubation, the radioactive compound(s) associated with HDL₃ particles as well as the final LCAT activity were analysed. In 7 separate HDL₃ incubations, final median LCAT activity was 28.8 nmol/h/ml (range 22.7 - 74.4 nmol/h/ml) in HTG samples while the final median LCAT activity was 20.6 nmol/h/ml (range 13.9 - 60.1 nmol/h/ml) in NTG samples (**Table1**). The radioactivity associated with HDL₃ following each incubation is demonstrated in **Fig. 10**. Significantly more

radioactivity representing esterified E_2 was associated with HTG HDL₃ compared with NTG HDL₃, both in the absence ($p < 0.001$) and in the presence ($p < 0.00005$) of exogenous LCAT. The addition of exogenous LCAT (mean added activity 15.6 nmol/h/ml) to the incubation mixture caused a significant increase in HDL₃-associated radioactivity in both HTG HDL₃ (mean increase 29 000 dpm) and NTG HDL₃ (mean increase 20 000 dpm) ($p < 0.005$ and $p < 0.0005$, respectively).

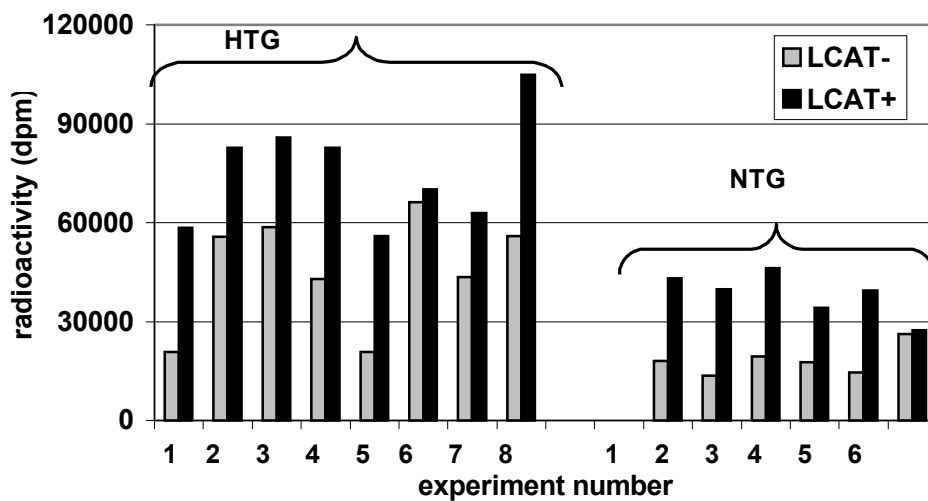


Figure 10. Radioactivity associated with HTG- and NTG- HDL₃ representing esterified E_2 . Labeled 17β -estradiol was added to the HDL₃ in the absence (■) and presence (■) of added, exogenous LCAT. Following incubation, the lipoprotein was purified by Sephadex G-25 and radioactivity in the eluted fractions was determined by liquid scintillation counting. Significantly more E_2 esters were formed in HTG HDL₃ than NTG HDL₃ without the addition of exogenous LCAT ($p < 0.001$), addition of exogenous LCAT to the incubation mixture resulted in significant increased E_2 esterification in both hyper- and normotriglyceridemic samples: median increase was from 49 600 to 76 300 dpm in HTG HDL₃ and from 18 000 to 39 600 dpm in NTG HDL₃ ($p < 0.005$ and $p < 0.0005$, respectively). Each experiment represents HDL₃ from different individuals.

The median endogenous LCAT-activity was non-significantly greater in the HTG group than in the NTG group, and this activity did not correlate with the higher amount of label in HDL₃. There was a highly significant correlation between the HDL₃ triglyceride content and radioactivity associated with HDL₃ both in the absence ($r=0.744$; $p=0.01$) and in the presence ($r=0.739$; $p=0.002$) of exogenous LCAT in the incubation mixture as demonstrated in **Figure 11**. The triglyceride content (mass%) of HDL₃ did not correlate with endogenous LCAT-activity. These results strongly suggest that increase in HDL₃ triglyceride content improves its substrate properties in the LCAT reaction and leads to increased esterification of E₂.

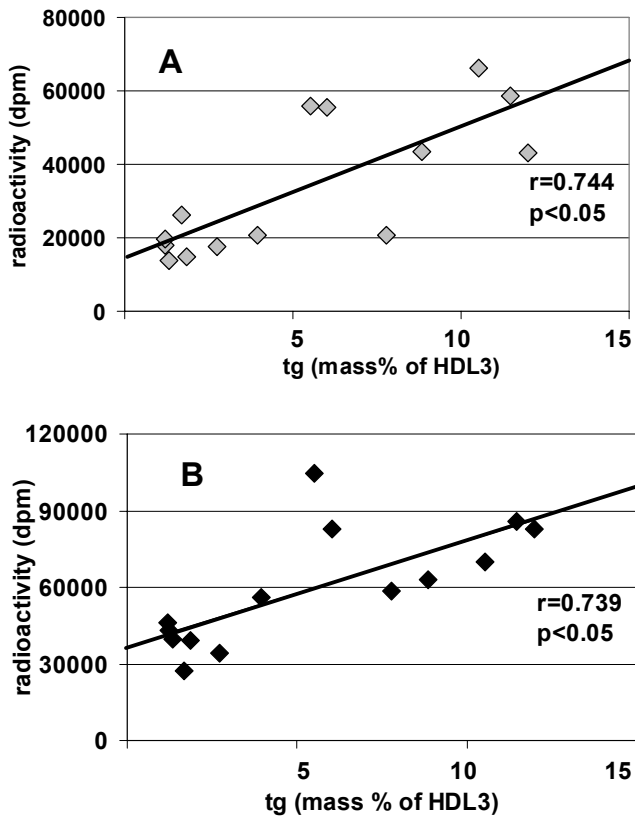


Figure 11. Correlation between triglyceride mass% of HDL₃ and radioactivity associated in HDL₃ after incubation with [³H]-E₂ in the absence (panel A) and presence (panel B) of exogenous LCAT. Labeled 17β-estradiol was added to the HDL₃ solution in the absence and presence of added, exogenous LCAT. Following incubation, the lipoprotein was purified by Sephadex G-25 and radioactivity in the eluted fractions was determined by liquid scintillation counting. Spearman's correlation analysis was used in statistical analysis

As demonstrated in Study II, E₂ was present in HDL₃ mainly in fatty acid ester form. Analysis of the radioactivity contained in HDL₃ by a combination of hydrophobic gel chromatography Sephadex LH-20 and TLC demonstrated that it co-eluted and comigrated completely with the 17β-E₂ ester standard. This data confirms that the estradiol derivative bound to HDL₃ is a fatty acid ester and not unesterified E₂.

5. 4. The effect of estradiol ester formation and incorporation into lipoproteins on the antioxidant potential of lipoproteins (Study III)

In order to investigate the antioxidative effects of fatty acyl derivatives of estradiol incorporated in HDL, E₂ was incubated with HDL in the presence or absence of purified LCAT and the kinetics of copper-facilitated HDL oxidation was monitored.

Figure 12 represents the copper-induced oxidation of HDL as a function of time measured by Esterbauer method (Esterbauer et al. 1989). Incubation of HDL in the presence of both exogenous estradiol and LCAT caused a marked shift of the oxidation curve to the right. However, when only E₂ or only exogenous LCAT had been incubated with HDL, the corresponding HDL oxidation curves moved halfway to the right between the oxidation curves of native HDL and the curve resulting from the incubation of HDL with both E₂ and LCAT (**Fig. 12**).

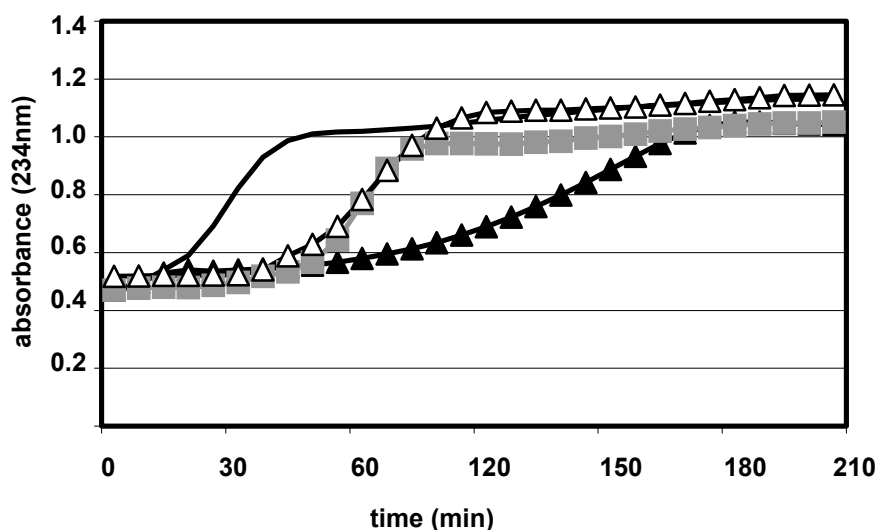


Figure 12. Effects of E₂ and LCAT-generated E₂ fatty acyl esters on copper-mediated HDL oxidation.

HDL was incubated with E₂ in the presence (▲, n=9) or absence (■, n=7) of exogenous LCAT. Incubations were also carried out without addition of E₂ in the presence (Δ, n=9) and absence of LCAT (— n=10). After incubation, HDL was isolated and purified and oxidation was started with CuSO₄ and diene formation was monitored.

Table 2 shows the mean lag times after incubation of i) HDL alone, ii) HDL in the presence of exogenous E₂, iii) HDL in the presence of exogenous LCAT iv) HDL in the presence of both exogenous E₂ and LCAT and v) HDL in the presence of exogenous E₂, LCAT and the LCAT inhibitor DTNB. There was a significant increase in the lag time of HDL when both E₂ and LCAT were present in the incubation (p<0.0001). In contrast, when the incubations were made in the presence of DTNB, no prolongation of lag time was detected when compared with incubations of native HDL. Also incubations in the presence of either E₂ alone (p=0.013) or LCAT alone (p=0.002) resulted in a prolongation of lag time compared with native HDL. This data emphasize the antioxidant role of esterified estradiol associated with HDL, but also demonstrates some antioxidant activity for LCAT alone.

Table 2. Lag times of HDL oxidation.

<u>Sample</u>	<u>n</u>	<u>Lag time (min)</u>	<u>P</u>
HDL (native)	10	27.8 ± 4.2	
HDL + E ₂	7	55.2 ± 8.6	0.013
HDL + LCAT	9	59.7 ± 4.2	0.002
HDL + E ₂ + LCAT	9	105.6 ± 10.7	<0.00001
HDL + E ₂ + LCAT + DTNB	3	25.1 ± 2.8	ns

Data presented as mean ± SEM, comparisons calculated vs. native HDL. HDL oxidations were induced by addition of Cu⁺⁺ and measured by monitoring diene formation at A₂₃₄.

Prior to addition of exogenous LCAT, endogenous LCAT activity in isolated HDL ranged between 1.6 and 20.8 nmol/h/ml (mean ± SEM: 10.0 ± 1.2 nmol/h/ml). The amount of added exogenous LCAT activity varied between 8.7 and 26.9 nmol/h/ml (mean ± SEM: 14 ± 3.0 nmol/h/ml) resulting in a final activity of 16.9 ± 2.3 nmol/h/ml (range 1.6 - 44.5 nmol/h/ml) during all incubations. There was a significant correlation between LCAT activity in the incubation mixture and the lag time of HDL oxidation (Fig. 13). These results provide further evidence that HDL-associated catalytically active LCAT provides antioxidant protection in our *in vitro* system, thus representing one of the mechanisms that catalyze E₂ esterification.

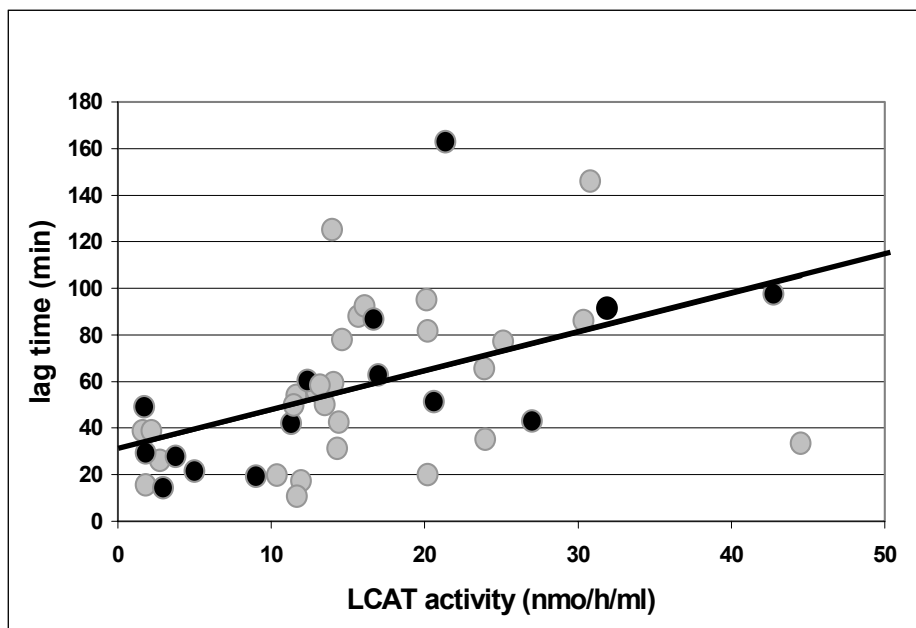


Figure 13. Correlation between LCAT activity and lag time in HDL oxidation

HDL was incubated with LCAT in the absence (O) and presence (●) of exogenous E₂. After incubation, the oxidation of purified HDL was started with CuSO₄ and followed by monitoring diene formation.

Free (nonesterified) E₂ and esterified E₂ were quantitated in the HDL fraction by a recently developed method (Vihma et al. 2003) in one of our experiments. The addition of E₂ and exogenous LCAT (final activity 17 nmol/h/ml) into the HDL incubation mixture induced the formation of 380 pmoles E₂ esters per 1mg HDL-protein. Addition of DTNB to this incubation mixture caused a significant reduction in both LCAT activity by 80% and in the concentration of E₂ esters by 99%. When no exogenous E₂ was added to the HDL incubation mixture, the E₂ ester concentrations were 0.33 pmoles per 1 mg HDL-protein with added LCAT and 0.17 pmoles per 1 mg HDL-protein without LCAT.

6. DISCUSSION

6. 1. The role of LCAT in 17 β -E₂ esterification

Incubation of [³H]-labeled 17 β -E₂ with human plasma and follicular fluid produced a lipoprotein-bound [³H]-labeled product, most of which coeluted with 17 β -E₂ ester fatty acid standards on a hydrophobic chromatography as well as comigrated with E₂-17 β monoester during TLC analysis. The addition of the LCAT inhibitor DTNB to the incubations almost completely abolished the lipoprotein-bound radioactivity, suggesting indirectly that the reaction was catalyzed by LCAT, which is in line with previous studies (Pahuja et al. 1995; Kanji et al. 1999). It has been presumed a role for LCAT in E₂ esterification based on the following observations: i) E₂ esterification did not occur in incubations with isolated LDL but it occurred in incubations with plasma (Shwaery et al. 1997; Shwaery et al. 1998) and during coincubation of LDL and HDL (Abplanalp et al. 2000), ii) E₂ esterification was inhibited by an enzyme-inhibitor, a well-known sulfhydryl reagent DTNB (Shwaery et al. 1997; Shwaery et al. 1998), indirectly suggesting a role for LCAT.

The experimental system was designed to provide direct evidence for the role of LCAT and to clarify the specific roles of the HDL₂ and HDL₃ subfractions in the formation of E₂ 17-fatty acid esters. Experiments using total HDL demonstrated a clear-cut dose-response for LCAT in E₂ esterification *i.e.* stepwise increases in E₂ 17-fatty acid ester formations were observed when increasing amounts of LCAT were added to the incubations. Separate incubations with HDL subfractions indicated that 17 β -E₂ ester formation occurred almost exclusively in the small HDL₃ subfraction.

It can be concluded that LCAT facilitates the esterification of 17 β -E₂ and the magnitude/rate of esterification is dependent on the LCAT substrate used. From a mechanistic point of view it is interesting that LCAT, which normally esterifies the 3 β -OH group of cholesterol and several hydroxysteroids is able to facilitate the fatty acylation of the 17 β -OH group of estradiol. The specificity of the LCAT enzyme toward various acyl acceptors has not been intensively investigated, but sterol molecules such as β -sitosterol, cholestanol and desmosterol have been shown to be esterified in human plasma, indicating that sterol molecules other than cholesterol can also function as the fatty acyl group acceptors (Skrede et al. 1974). Several steroids that function as substrates for LCAT lack the side chain (Piran et al. 1979; Leszczynski et al. 1989). Similarly, 17 β -E₂ also lacks the carbon side chain which suggests that the hydrophobic side chain of sterols at carbon-17 is not a prerequisite for the acylation of the 3 β -hydroxyl

group. In the case of 17β -estradiol the A-ring is aromatic, and the hydroxyl group at position C-3 is phenolic. This group is not fatty acylated because LCAT requires that the sterol acyl acceptor has a 3β -configuration for the hydroxyl group (Jonas et al. 1984). TLC-analysis in the present study clearly demonstrated that LCAT catalyzed acylation of the 17β -OH group.

Szedlacsek and co-workers (Szedlacsek et al. 1995) have investigated the role of LCAT in forming fatty acid esters of oxysterols in lipoprotein particles. They discovered that following esterification of the preferred site (3β -hydroxyl group), the 27-hydroxyl group was esterified by LCAT *in vitro*, which suggested that the esterification at this less common site was dependent on particle size, *i.e.*, the physical characteristics of the lipoprotein particle surface appeared to influence the LCAT reaction. Our data using 17β -E₂ suggests that although the 17β -OH group of E₂ is located at the other end of the molecule opposite to the 3-OH group it is well oriented at the enzyme active site and the fatty acyl-group can be transferred from the acyl-enzyme intermediate. This is in line with the findings of Kanji et al (Kanji et al. 1999).

Although the results provide evidence that E₂ 17-esters are preferably formed in HDL₃ subfraction, direct conclusions can not be made concerning the underlying mechanism. However, substrate specificity for LCAT-facilitated cholesterol esterification has been widely investigated, and analogies to estradiol esterification can be presumed. Activation of LCAT by apoA1 has been demonstrated to depend directly upon the binding of apoA1 to a lipid surface (Chung et al. 1979) and the physicochemical characteristics of the particle surface where the interaction of LCAT with substrate takes place appear to be important (Jonas 1986). Small HDL particles (HDL_{3bc}) as well as discoidal apoA1-phospholipid-cholesterol particles provide the most active surface configuration for the LCAT reaction (Barter et al. 1985; Dobiasova et al. 1994) whereas large HDL (HDL_{2b}) particles may inhibit the enzymatic reaction (Barter et al. 1984; Karpe et al. 1990). It is possible that the rate of estradiol esterification by LCAT is influenced by HDL surface configuration in the same way as cholesterol esterification.

6. 2. The transfer of E₂ esters from HDL to LDL

The fact that 17β -E₂ esters have been detected in LDL (Study I, (Shwaery et al. 1998; Kanji et al. 1999; Abplanalp et al. 2000)), but incubations of isolated LDL with E₂ did not result in E₂ esterification and incorporation (Study II), raised the question how these lipophilic estrogen esters produced in HDL were transported to LDL particles. We hypothesized that CETP could be responsible for this transport of 17β -E₂ esters between lipoproteins. This was considered possible since cholesterol and 17β -E₂ share a somewhat similar ring structure. It was

demonstrated that coincubation of native LDL with HDL containing ^3H -labeled $17\beta\text{-E}_2$ ester in a buffer solution resulted in transfer of the label to LDL and that this transfer was significantly accelerated by addition of purified CETP to the incubation mixture (Study I). Conversely, "washing" of the HDL fraction by a second ultracentrifugation reduced the spontaneous transfer, presumably due to reduction of the CETP adhered to HDL particles. The study provides for the first time *in vitro* evidence that CETP participate in the transfer of E_2 esters (Study I).

The current results are compatible with the concept that this transfer may *in vivo* occur directly from HDL_3 to LDL. Another possibility is that the $17\beta\text{-E}_2$ 17-esters are retained *in vivo* in the HDL particle during the LCAT-facilitated maturation of HDL_3 to HDL_2 particles, and are then transferred from HDL_2 to LDL. Either way, LDL would receive powerful lipophilic antioxidant molecules which could, in theory, increase the oxidation resistance of LDL particles. This is supported by the reports of Shwaery et al (Shwaery et al. 1997) indicating that incubation of physiologically relevant concentrations of $17\beta\text{-E}_2$ with male plasma caused a significant prolongation of lag times in an *in vitro* LDL oxidation system.

CETP has specificity for both neutral lipids (cholesterol esters and triglycerides) and phospholipids (Tall 1993). Our observation of the CETP facilitated acceleration of the transfer of $17\beta\text{-E}_2$ esters from HDL to LDL is topical, since recent data have proposed a new model of the transfer reaction mechanism of CETP. The model suggested that i) a hydrophobic pocket in the amino-terminal domain of CETP may have a role in neutral lipid binding and transfer (Tall 1993; Beamer et al. 1997) and ii) the N-terminal active site may be involved in determining the substrate species specificity for the transfer (Kotake et al. 1997). Accordingly, the transfer of E_2 esters might be mediated via this site. The role of CETP in the transfer of two other neutral steroids, dehydroepiandrosterone (DHEA) and pregnenolone, has also been investigated (Provost et al. 1997), and the results indicated that the fatty acid esters of these compounds were transferred from HDL to VLDL and LDL by a mechanism not dependent on CETP. However, this observation does not contradict the possibility that CETP mediates estradiol ester transfer, but suggests that DHEA and pregnenolone esters have very weak or null affinity to the lipid-binding pocket of CETP. Additional studies are needed to elucidate in detail the CETP-mediated transfer of E_2 esters. Nevertheless, our data show that CETP is able, at least *in vitro*, to transfer esterified $17\beta\text{-E}_2$ from HDL to LDL particles.

6. 3. Effect of increased triglyceride content of HDL on E_2 esterification

Our data indicated that fatty acid esterification rate of $[^3\text{H}]\text{-E}_2$ in the HDL_3 subclass increased with increasing mass % of triglyceride in HDL_3 *i.e.* as HTG- HDL_3 had a significantly higher triglyceride content than that in NTG- HDL_3 ,

these TG-enriched HDL₃ species also exhibited a greater accumulation of [³H]-E₂ fatty acid ester (Study IV). Previous studies have shown that E₂ is esterified with fatty acids in a LCAT-mediated reaction at position C-17 (Kanji et al. 1999). LCAT is tightly bound to HDL particles, mainly to those in the HDL₃ subfraction and nascent discoidal HDL, and is only incompletely removed by sequential ultracentrifugation (Dolphin 1992). This finding was confirmed in our studies which showed significant but greatly variable endogenous LCAT activities in all isolated HDL samples. There was a highly significant correlation between the label associated with HDL₃ and total LCAT activity in both HTG- and NTG-HDL₃ incubations following addition of exogenous LCAT in the presence of [³H]-E₂ (Study IV). One possible explanation is that since the triglyceride-rich HDL₃ particles become more susceptible to lipolysis by HL resulting in smaller particle size (Lamarche et al. 1999), the high degree of curvature at the lipid/water interface is a better setting for LCAT to function (Fielding et al. 1971; Pinon et al. 1980). This results in enhanced rate of E₂ fatty acid esterification, which is in line with the central role of LCAT in E₂ esterification.

The underlying mechanisms explaining increased substrate properties of TG-enriched HDL particles have been explored previously in functional PLTP studies (Rye et al. 1998; Settasatian et al. 2001) and are applicable here. It was demonstrated that the apoAI in TG-enriched reconstituted HDL can unfold more readily in comparison to apoAI on the surface of TG-poor HDL. This may prevent some α -helices of apoAI to interact with surface phospholipid fatty acyl chains (Frank et al. 1997) and may enable it to interact in a more effective way with LCAT and therefore function as a better substrate. Recently it was reported that three arginine residues of apoAI located at the hydrophobic/hydrophilic boundary of the amphipathic helix are critical for activation of LCAT (Roosbeek et al. 2001). This region could be more available for LCAT in TG-enriched HDL and interact better with LCAT, which could explain the strong correlation between LCAT and accumulation of [³H]-E₂ esters in HDL₃.

6. 4. Antioxidative effect of lipoprotein-bound E₂ fatty acid esters

Estrogens have been suggested to possess strong antioxidant effects *in vitro* (Maziere et al. 1991; Rifichi et al. 1992; Sack et al. 1994; Ayres et al. 1996; Ayres et al. 1998; Wakatsuki et al. 1998; Meng et al. 1999), but very little is known about the factors that influence the expression of the antioxidant effects of estrogens *in vivo*. However, studies by Shwaery et al (Shwaery et al. 1997; Shwaery et al. 1998) have suggested an important role for conversion of estrogens to lipophilic derivatives, which occur in plasma. Fatty acyl esterification of estrogens is considered a prerequisite for their incorporation into lipoproteins (Hochberg 1998; Abplanalp et al. 2000) and even a key event for their antioxidant actions (Shwaery et al. 1997). However, previous studies on the antioxidant efficacy of E₂ esters have provided only indirect evidence for the

following chain of events: LCAT mediates esterification of estradiol, which incorporates into lipoproteins, which in turn increases the oxidation resistance of the lipoprotein particles. (Shwaery et al. 1997; Shwaery et al. 1998). Although these studies have provided valuable information, they have not documented direct proof that E₂ esters are formed in HDL in a reaction catalyzed by LCAT and that these E₂ esters provide protection of HDL particles against oxidation.

We used an *in vitro* model system with supraphysiological concentrations of E₂ and purified LCAT to produce E₂ ester -containing HDL particles for studies of oxidation resistance. The lag time of HDL oxidation significantly increased with increasing contents of HDL-associated E₂ esters (Study III). We estimated that the amount of HDL-associated E₂ esters observed in our experiments were approximately 760 pmol/ml in plasma. Thus, the E₂-ester concentrations presented here are more than a 1000-fold excess of the E₂-ester levels in plasma *in vivo*, which have been measured recently in pregnant women 200-750 pmol/l (Vihma et al. 2003). To our knowledge, the highest concentrations of E₂ esters *in vivo* have been determined in human ovarian follicular fluid (mean 106 nmol/l, range 60-260 nmol/l) (Vihma et al. 2001). However, in our study (Study III) we used supraphysiological levels of E₂ to study the basic mechanism underlying the formation and function of HDL-associated E₂ fatty acid esters. Esterbauer's method (Esterbauer et al. 1989) used in our experiments is very applicable for this kind of first step analysis, but it is relatively insensitive and requires sufficient amounts of antioxidants present necessitating the use of high concentrations of E₂. However, E₂, although the principal human estrogen, is only one among many estrogens like estrone and estriol, which may contribute to the antioxidant protection of HDL, and to possible differences between female and male HDL.

Functional endogenous LCAT provided antioxidant efficacy by enhancing exogenous E₂ esterification to some extent in the absence of added LCAT (Study III). Incorporation of estradiol fatty acyl esters in HDL was confirmed by quantitating the free and esterified estradiol in HDL with a recently developed sensitive method (Vihma et al. 2003). In some experiments in which pharmacological E₂ amounts were incubated with HDL under conditions of low LCAT activity, also free E₂ adhered to some extent to the HDL particles. On the other hand, it has been previously demonstrated that under physiological conditions nonesterified estradiol has low binding affinity to serum lipoproteins (Vihma et al. 2003). In Study III, greater estradiol ester contents in HDL were associated with prolonged oxidation lag times. The fact that also addition of LCAT alone gave rise to increased antioxidant capacity is in line with results of Vohl et al (Vohl et al. 1999) who proposed that scavenging free radicals was the underlying mechanism for LCAT -induced antioxidation.

Thus, two different mechanisms are suggested to explain the increased resistance to oxidation of HDL, one being the incorporation of E₂ esters and the other the inherent antioxidant activity of LCAT . We hypothesize that E₂ esterification and LCAT activity could have physiological roles together with the

antioxidative enzymes carried by HDL, the platelet-activating factor acetylhydrolase and paraoxonase (Tselepis et al. 1995; Watson et al. 1995). These mechanisms may contribute to blocking the build-up of oxidized lipids in HDL thus preserving its ability to inhibit LDL oxidation (Bowry et al. 1992; Hahn et al. 1994). Appropriate in this context is also the observation demonstrating that E_2 esters formed in HDL are transported to LDL by a mechanism that is at least partly dependent on CETP (Study I). Thus, E_2 esters formed in HDL might be important in protection of both HDL and LDL against oxidation and the activity of both LCAT and CETP might have an important role in the build-up of antioxidative capacity of LDL.

There is currently methodologically no easy way to clarify in what position the 17β - E_2 esters are located in the lipoprotein structure, but for instance modern NMR-technology might be a promising novel way to track the E_2 ester location in lipoproteins. One possibility is that they are aligned on the lipoprotein surface in the same way as phospholipids, the fatty acid chains directed toward the core and the ring nucleus with the hydrophilic A-ring hydroxyl group on the surface of the lipoprotein, thus providing functionally essential conformation in the antioxidant process, see **Figure 14**.

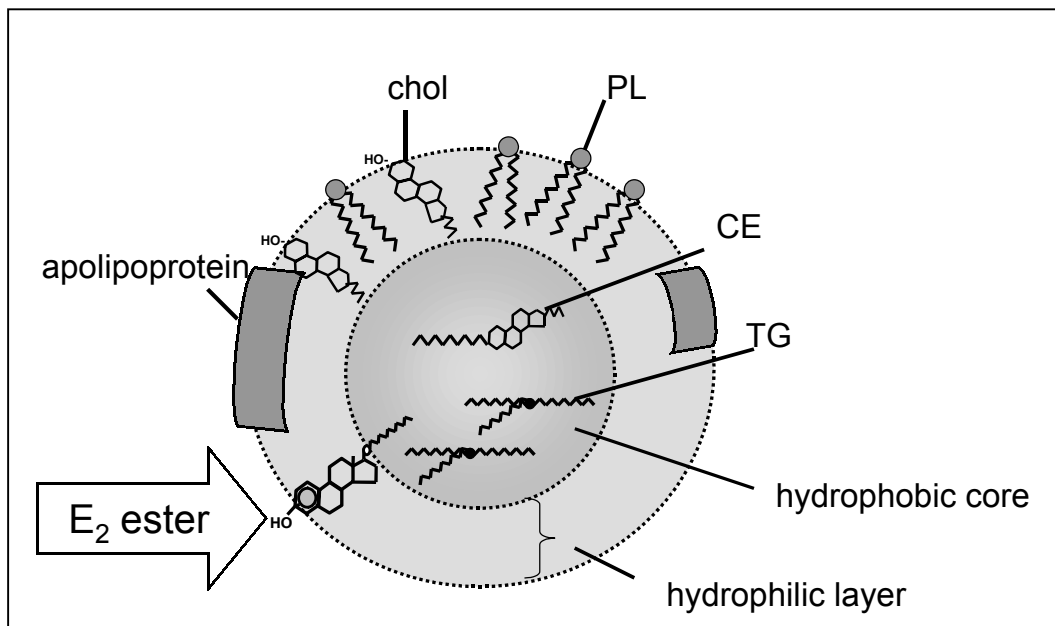


Figure 14. hypothesized location of E_2 esters in lipoproteins.
 chol, cholesterol; CE, cholesterol ester; PL, phospholipid; TG, triglyceride

6. 5. Other possible antiatherogenic actions of lipoprotein-bound E₂ fatty acid esters

A recent study has proposed that HDL binds to the scavenger receptor B class I (SR-BI) which is also expressed in vascular endothelial cells and delivers estrogen to endothelial nitric oxide synthase (eNOS), thereby stimulating the enzyme (Gong et al. 2003). The authors, however, did not measure esterified estradiol and it is not clear how the estradiol activated eNOS. Recent observations by Nofer et al. (Nofer et al. 2004) have presented critical aspects stating that native estradiol concentrations in HDL are by far too low to explain eNOS activation *via* SR-BI route. On the other hand, it is quite possible that certain membrane domains of the endothelium may cluster molecules like estradiol esters from native HDL and form high local concentrations thus being able to facilitate/initiate relevant physiological responses. Interestingly, earlier data on lipoprotein-bound non-estrogenic steroid esters have suggested that they can be taken up into cells *via* lipoprotein receptors and further hydrolyzed into free steroid forms (Provencher et al. 1992; Roy et al. 1992; Roy et al. 1993). In any case, it is quite evident that the possible physiological role of HDL-receptor facilitated endothelial entry of HDL associated estrogen esters must be assessed critically in future studies.

The experiments in this thesis were designed using supraphysiological concentrations of E₂ and were aimed at providing basic information concerning the role of HDL composition changes in determining the esterification rate of estrogens *via* the function of LCAT. Our results indicate that LCAT facilitates the formation of hydrophobic estradiol fatty acid esters that become incorporated in HDL particles where they significantly increase its antioxidant potential. These E₂ esters can be transported to LDL *via* a mechanism which is at least partly CETP-dependent. In physiological terms, estrogen esters contained in HDL could enter the arterial subendothelial compartment. Alternatively, E₂ esters formed in HDL could be transported to LDL in plasma resulting, in theory, in effective antioxidant protection of LDL even after the particles have penetrated the vascular endothelium and become sequestered from the water-soluble antioxidants in plasma. This would ultimately increase the subendothelial antioxidant potential and decrease the foam cell formation. **Figure 15** summarises the hypothesized mechanism.

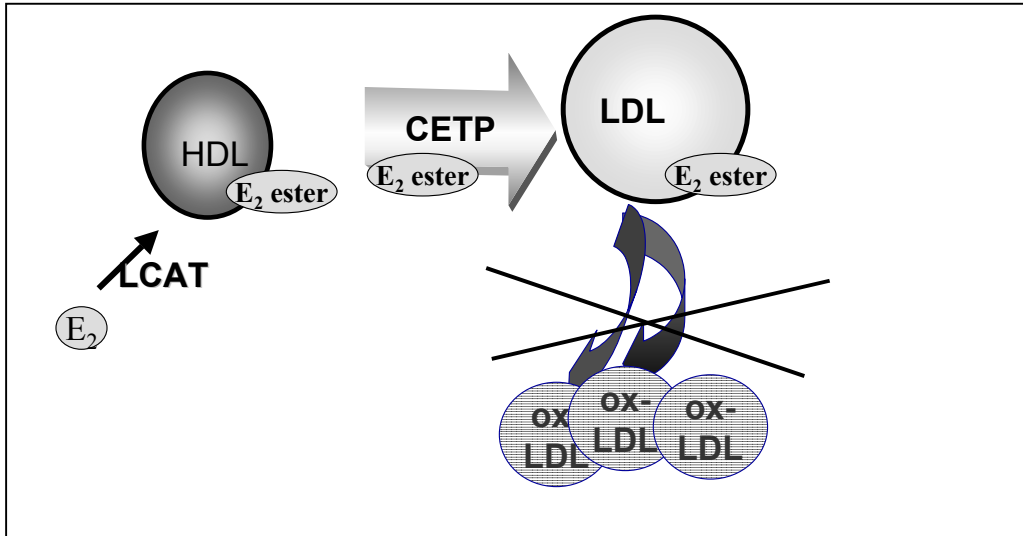


Figure 15. Suggested mechanisms of formation and transport of E₂ esters as well as possible atheroprotective mechanisms

E₂ esters formed in HDL by LCAT could be transported to LDL in plasma partly *via* a CETP-dependent mechanism resulting, in theory, in effective antioxidant protection of LDL even after the particles have penetrated the vascular endothelium.

7. SUMMARY AND CONCLUSIONS

The present series of studies aimed at elucidating the mechanism and role of estrogen fatty acid esterification in high density lipoprotein *in vitro* and can be summarized as follows:

I HDL subfractions differ in their potential to regulate estradiol esterification by LCAT. E₂ 17-ester formation occurred almost exclusively in the small less mature HDL₃ particles both in the presence of endogenous HDL-associated LCAT as well as after addition of exogenous LCAT.

II The data demonstrate that E₂ is esterified by LCAT in a dose –dependent manner, but also other factors such as HDL composition and particle size have an effect in the process.

III The results provide *in vitro* evidence that E₂ esters present in HDL can be transported in plasma to LDL particles in a process that is facilitated by CETP.

IV The results prove directly that HDL-associated catalytically active LCAT provide antioxidant protection by E₂ esterification.

Elucidation of the possible *in vivo* role of HDL-associated estrogen esters requires further critical studies including experiments with physiological hormone concentrations.

8. ACKNOWLEDGEMENTS

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Helsinki, December 2004

A handwritten signature in black ink, appearing to be 'V. O.', with a long horizontal flourish extending to the right.

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