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# Human macrophage cholesterol efflux potential is enhanced by HDL-associated 17β-estradiol fatty acyl esters

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ABSTRACT

High-density lipoprotein (HDL) and 17β-estradiol independently provide protection against atherosclerosis. Estradiol fatty acyl esters incorporate into HDL and whether this association enhances the atheroprotective properties of HDL is unclear. The study objective was to clarify the role that HDL-associated estradiol fatty acyl esters play in mediating the initial steps of reverse cholesterol transport. Cholesterol efflux potential from cholesterol loaded macrophage cells to HDL-associated estradiol ester or between HDL from premenopausal women and age-matched males and the cellular receptors involved were examined. Human THP-1 macrophages, loaded with [$^3$H]cholesterol oleate, acetylated low-density lipoprotein, were pretreated with or without SR-BI inhibitors or an estrogen receptor antagonist and incubated with either HDL-associated estradiol oleate, HDL lacking estradiol oleate, or isolated HDL from females and males, and cholesterol efflux was measured. Cellular internalization and hydrolysis of HDL-associated [$^3$H]estradiol ester were determined. HDL-associated estradiol oleate and premenopausal female HDL demonstrated significantly higher cholesterol efflux capacity to media than male HDL. SR-BI and estrogen receptor inhibition significantly reduced this effect. Cells internalized and subsequently hydrolyzed HDL-associated [$^3$H]estradiol ester to [$^3$H]estradiol and again SR-BI inhibition reduced this internalization. These results demonstrate that HDL-mediated macrophage cholesterol efflux potential is enhanced by HDL-associated estradiol esters.



## 1. Introduction

Compared to premenopausal women, risk factors for cardiovascular disease (CVD) are increased in males and in postmenopausal women. This conveys an important role for endogenous estrogens, present in premenopausal women, to protect against CVD [1,2]. This is supported by a large body of experimental and epidemiological evidence [2–4]. Additionally, in men, there exists an inverse relation between serum 17β-estradiol, the most biologically potent estrogen, and CVD risk, which further reinforces the atheroprotective function of endogenous estrogens [5].

High-density lipoproteins (HDLs) as well as estradiol both defend against the progression of atherosclerosis. HDL has many atheroprotective properties, one of which is reverse cholesterol transport, i.e. HDLs ability to remove excess cholesterol from macrophage foam cells and deliver it to the liver for excretion [6]. An initial step in atherosclerosis is the deposition of oxidized apoB-containing lipoproteins especially oxidized low-density lipoproteins (oxLDL) in the arterial intima, which triggers the release of inflammatory markers [7]. Macrophages are targeted to these sites and take up oxLDL in an unregulated manner, becoming cholesterol ester-enriched, atherogenic, macrophage foam cells [7]. HDL interacts with macrophage membrane receptors, such as the adenosine triphosphate-binding cassette transporter A1 (ABCA1), ABCG1, and the scavenger receptor class B, type I receptor (SR-BI) to promote active cholesterol removal (efflux) [8]. Estrogen treatment of macrophage foam cells promotes cholesterol efflux and decreases oxidized low-density lipoprotein (LDL) influx [9,10]. Mechanisms for these estrogenic effects are presently not fully understood.

Gender-specific differences in HDL function exist. HDL from premenopausal women enhances hepatic cellular cholesterol efflux rates and eNOS-mediated endothelial cell vasodilation compared to age-matched males [11,12]. Association of estrogen with HDL in these women may boost HDLs cardioprotective properties.

Naturally, estrogen is partitioned into lipoprotein particles, both LDL and HDL, as estrogen fatty acyl esters, and free (unesterified) estrogen makes up a minority [13–16]. It appears that 17β-estradiol is transported by lipoproteins as esters to cellular targets [16,17].

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Studies by our group have determined that lecithin-cholesterol acyltransferase (LCAT) and cholesterol ester transfer protein (CETP) mediate *in vitro* generation and transfer of estradiol esters between HDL and LDL [14,18]. In addition, hepatic cells internalize HDL-derived estradiol esters via the LDL receptor and SR-BI-facilitated mechanisms. These estradiol esters are then hydrolyzed into free estradiol in the intracellular compartment [16]. Related studies have focused on free estradiol associated with HDL rather than the estradiol esters and have determined that HDL-associated estradiol influences cellular processes via estrogen receptors and SR-BI [12,19]. Whether HDL-associated estradiol esters interact with macrophage lipoprotein receptors to modulate cholesterol efflux is uncertain.

Since estradiol associates almost exclusively as estradiol fatty acyl esters in lipoproteins in circulation [13,15], the metabolism and function of these still remain unclear. Since estradiol fatty esters follow the HDL metabolic pathway [14,16,18], the question arises: do HDL-associated estradiol esters enhance the function of HDL in reverse cholesterol transport compared to HDL that is lacking estradiol? In this report, we addressed whether: (i) HDL-mediated macrophage cholesterol efflux potential is increased by HDL-associated estradiol fatty acyl esters; (ii) HDL and estrogen receptors are critical for this process; (iii) HDL-associated estradiol esters are internalized through these macrophage receptors; and (iv) a gender-specific effect exists for this efflux.

## 2. Materials and methods

### 2.1. Human plasma lipoproteins

Total HDL (1.063–1.21 g ml$^{-1}$) was isolated from pooled plasma from either four normolipidemic premenopausal, or four age-matched male volunteers, respectively, by sequential gradient ultracentrifugation using KBr for density adjustments [20]. Informed oral consent was granted by the volunteers. All women were premenopausal, non-pregnant, reproductively mature individuals with no reported use of contraceptive drugs. The phase of their menstrual cycle was unknown to the investigators. LDL (1.019–1.063 g ml$^{-1}$) was obtained from pooled plasma [20], from males and females, received from the Finnish Red Cross.

### 2.2. Labeling of lipoproteins

LDL was acetylated using acetic anhydride and radiolabeled by incubations with [1α,2α(*n*)-$^3$H]-cholesterol oleate (Amersham Biosciences, Buckinghamshire, UK) [21,22]. [$^3$H]cholesterol oleate acetyl LDL ([$^3$H]acLDL) preparations had specific activities ranging from 2 to 3 × 10$^4$ c.p.m. per μg LDL protein. For experiments using total HDL with [$^3$H]estradiol, 10 ml of male serum was incubated for 24 h at 37 °C with 2 × 10$^6$ c.p.m. per ml of [2,4,6,7-$^3$H-N]17β-estradiol (Perkin-Elmer Life Sciences, Boston, MA; specific activity 51 Ci per mmol) and total HDL, containing both [$^3$H]estradiol and [$^3$H]estradiol fatty acyl esters, was isolated as described above. Isolated HDL-associated [$^3$H]estradiol had a radioactivity of approximately 100 c.p.m. per μg total HDL protein. All HDL preparations were purified by size-exclusion chromatography (Sephadex G-25, column dimensions, 2 cm × 20 cm or 1 cm × 20 cm: GE Healthcare, Uppsala, Sweden) [16]. [$^3$H]acLDL and LDL were purified by extensive dialysis against LDL buffer containing 150 mM NaCl and 1 mM EDTA, pH 7.4 [22].

### 2.3. Cell culture

THP-1 cells (human acute monocytic leukemia cells; American Type Culture Collection, Monassas, VA, catalog no. TIB-202) were maintained in phenol-red free RPMI 1640 medium supplemented with 100 U ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin, 2 mM L-glutamine, and 10% (v/v) fetal bovine serum. To differentiate cells into macrophages, cells were plated onto 24-well plates at a density of 1.5 × 10$^6$ cells per well and 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, St. Louis, MO) was added to the cell media at day 0. At day 3, the cells were washed with phosphate-buffered saline, pH 7.4 (PBS) twice and then loaded with 25 μg of [$^3$H]acLDL total protein in media containing lipoprotein deficient bovine serum (5%, v/v) [21,22]. At day 5, the cells were washed with PBS twice and incubated with the experimental samples for 16 h. For experiments using the estrogen receptor antagonist, fulvestrant (Faslodex$^{TM}$; ICI 182,780; AstraZeneca, Cheshire, UK) and/or the SR-BI inhibitors, BLT1 (blocking lipid transporter-1; ChemBridge, San Diego, CA), SR-BI blocking antibody, raised against amino acid residues 230–280 in the extracellular domain of scavenger receptor class B (Scavenger receptor class I + II antibody, number ab36970, Abcam, Cambridge, UK) or a non-specific antibody, isolated from immunized rabbits, cells were pretreated for 90 min with 1 μM of ICI 182,780 or 10 μM of BLT1 or 60 min with 200 μg ml$^{-1}$ blocking antibodies in serum-free media at 37 °C and then the experimental samples were incubated with the cells without removing the inhibitors [16,23,24]. For experiments using HDL-associated [$^3$H]estradiol, 360 μg of total HDL was added to differentiated, non-radiolabeled acLDL loaded, macrophages and incubated as outlined above. At the end of the incubation period, the cellular media was removed and put into a tube. Cells were washed twice with PBS and then 0.2 M NaOH was added to the cell monolayer. Radioactivity was measured in the media and cellular fractions by scintillation counting. Percentage cholesterol efflux was expressed as the percentage of media [$^3$H]cholesterol from the total amount of [$^3$H]cholesterol (intracellular and media [$^3$H]cholesterol). Total macrophage protein was then determined from the 0.2 M NaOH cell extracts.

### 2.4. Preparation of estradiol fatty acyl ester containing HDL particles

Estradiol 17β-monooleate (kindly provided by Dr. K. Wähälä) was loaded into male HDL or bovine serum albumin (BSA) by using a Celite 545 AW (Sigma–Aldrich, St. Louis, MO) transfer system [17,25]. Celite 545 (33 mg) and 3 μmol ml$^{-1}$ estradiol oleate was added to 1 ml of chloroform, mixed, and the chloroform was evaporated under $N_2$. HDL (1 mg HDL protein) or 1 mg BSA was added to the Celite dispersion, mixed, and incubated at 37 °C for 24 h. Next, Celite beads were pelleted with low-speed centrifugation (at 3000 rpm for 30 min). The supernatant was removed, filtered through a Millipore filter (0.22 μm) and applied to a Sephadex G-25 chromatographic column to remove unbound estradiol oleate and EDTA. As a control, HDL, BSA, or RPMI cell media lacking estradiol were processed in the same way. The estradiol oleate was tested prior to the Celite incubations by TLC for purity.

### 2.5. Quantification of estradiol in lipoprotein and serum fractions

Free (non-esterified) and esterified estradiol in different samples were determined by an established method [26]. Briefly, an internal standard, [$^3$H]estradiol-3,17β-dioleate was added to each sample and then lipids were extracted four times with 2.5 volumes of diethyl ether-ethyl acetate (1:1, v/v) and evaporated to dryness under $N_2$. Sephadex LH-20 chromatography (Pharmacia Biotech, Uppsala, Sweden) was used to isolate estradiol fractions. Samples, dissolved in hexane–chloroform (1:1, v/v), were applied to a 5-cm column containing a Sephadex LH-20 slurry. Estradiol esters were eluted using hexane–chloroform and the free estradiol was then eluted with methanol. Estradiol ester fractions were hydrolyzed, neutralized, and then the organic phase was removed and the lipids were again extracted with diethyl ether, dried under