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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

N_2, and dissolved in hexane–chloroform (1:1, v/v) and subjected to LH-20 chromatography. Estradiol in either the ester or free fractions were quantitated by time-resolved fluoroimmunoassay (TR-FIA). Instruments and related reagents were obtained from Wallac Oy (PerkinElmer), Turku, Finland. Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) technique was performed using the samples, controls (estradiol-17β-sterate in serum or distilled water), and calibrators (non-radioactive 17β-estradiol (Steraloids, Inc.)). The results were adjusted to reflect the recovery based on the internal standard and dilution factors used.

2.6. [^{3}H]estradiol cellular uptake

Purified HDL containing [^{3}H]estradiol or the intracellular and extracellular fractions after the cell incubations with HDL-[^{3}H]estradiol were applied to Sephadex LH-20 hydrophobic chromatography columns to separate the [^{3}H]estradiol ester from the free [^{3}H]estradiol based on an existing protocol [16,26]. The method for LH-20 chromatography is outlined in Section 2.5.

2.7. Cholesterol and triglyceride determination

Serum total cholesterol (TC) and triglycerides (TG) were determined with an automated Cobas Mira analyser (Hoffman-La Roche, Basel, Switzerland) by enzymatic methods (Hoffman-La Roche kits #0722138 and #0715166, respectively). Serum HDL-C was quantified by phosphotungstic acid/magnesium chloride precipitation procedures (Hoffman-La Roche kit #0720674). Serum low-density lipoprotein cholesterol (LDL-C) was calculated from the Friedewald formula [LDL cholesterol = TC-(HDL-C)-TG/2.2] [27].

2.8. Apolipoprotein A-I and E analysis

ApoA-I and E were quantified by ELISA assays as described [28,29].

2.9. Thin-layer chromatography

Analysis of BSA-associated estradiol oleate or BSA was performed by TLC method as described [30].

2.10. Other methods

Protein from the isolated lipoprotein fractions was quantified by the Lowry method [31].

2.11. Statistics

Paired student's *t*-tests were used to assess the statistically significant differences. Means and standard errors of the mean are presented.

3. Results

To determine the role that HDL-associated estradiol esters play in mediating macrophage cholesterol efflux potential, male HDL, enriched with estradiol oleate, was incubated with differentiated THP-1 macrophage cells (loaded with [^{3}H]cholesterol oleate labeled acLDL) for 16 h and the cholesterol efflux potential, expressed as percentage media [^{3}H]cholesterol, was measured. Results assessing the media [^{3}H]cholesterol c.p.m. per milligram of cell protein were similar to the percentage values (unpublished data).

Fig. 1 shows the media cholesterol efflux percentage after 16 h of incubation with HDL-associated estradiol oleate, male HDL, BSA-associated estradiol oleate, BSA, and RPMI media with no acceptor. Maximum stimulation of cholesterol efflux was caused by HDL-associated estradiol oleate (Fig. 1; male HDL + estradiol-oleate, 30.3 ± 1.6%; male HDL, 22.7 ± 0.7%; BSA + E2-oleate, 16.9 ± 0.6%; BSA, 13.9 ± 0.6%). BSA-associated estradiol oleate had a significantly greater percentage of cholesterol efflux compared to BSA lacking estradiol oleate. BSA-associated estradiol oleate was included in these experiments to emphasize the importance of HDL as a carrier of estradiol esters and as a facilitator for estradiol-mediated macrophage cholesterol efflux.

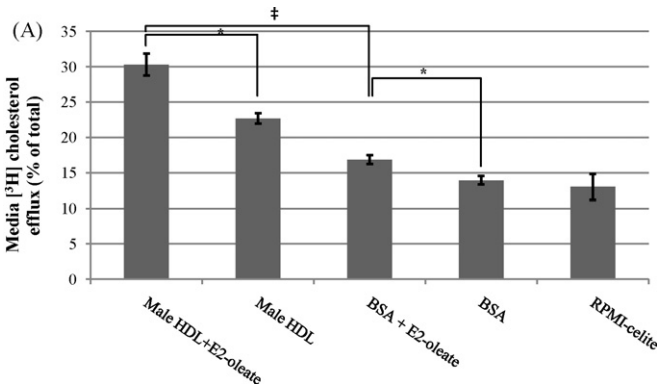

Fig. 1. 17β-Estradiol oleate enriched HDL- or BSA-mediated cholesterol efflux from THP-1 macrophage foam cells. Estradiol oleate (3 μmol) was incorporated into either 1 mg (as protein) of male HDL or BSA by using a Celite 545 AW transfer system, as described in Section 2. Differentiated THP-1 macrophages loaded with [^{3}H]cholesterol oleate labeled acetylated LDL were incubated in the presence of 25 μg (as HDL-protein) of HDL- or BSA-containing estradiol oleate, HDL or BSA without hormone. After incubation cholesterol efflux was evaluated as the percentage [^{3}H]cholesterol effluxed to media after 16 h. Triplicate wells were used in incubations. Data is expressed as the mean ± S.E. of 3 independent experiments using different preparations of HDL-associated estradiol oleate Celite preparations. $^*P \leq 0.05$ and $^‡P \leq 0.001$.

Table 1 represents the quantification of free and esterified estradiol in serum and male HDL after Celite incubations. The levels of serum free and esterified estradiol displayed similar pattern as reported previously for females and males [32]. BSA-associated estradiol oleate was confirmed by TLC analysis (unpublished data).

Since HDL-associated estradiol oleate promoted an enhancement in macrophage cholesterol efflux compared to male HDL, SR-BI, a major cellular HDL receptor, and estrogen receptors were tested for their involvement in this process (Fig. 2A and B). Inhibition of SR-BI, by BLT1 (Fig. 2A) or a specific SR-BI blocking antibody (Fig. 2B), attenuated the enhanced effect on cholesterol efflux, from [^{3}H]acLDL loaded macrophages, by HDL-associated estradiol oleate, and yielded similar values to male HDL. Cholesterol efflux enhancement by HDL-associated estradiol oleate was also impeded by ICI, an anti-estrogen. When BLT1 or SR-BI blocking antibody and ICI were coincubated with HDL-associated estradiol oleate, HDL-associated estradiol oleate was less effective to stimulate cholesterol efflux potential than male HDL. Male HDL did not show any significant differences in cholesterol efflux potential upon inhibitor treatment (Fig. 2A: male HDL + E2 oleate, 30.1 ± 1.3%; male HDL + E2 oleate + BLT1, 22.7 ± 1.5%; male HDL + E2 oleate + ICI, 23.3 ± 1.6%; male HDL + E2 oleate + BLT1 + ICI, 21.6 ± 0.8%; Fig. 2B: male HDL + E2 oleate, 28.6 ± 1.9%; HDL + E2 oleate + SR-BI blocking antibody, 24.1 ± 1.4%; male HDL + E2 oleate + ICI, 23.3 ± 1.6%; male HDL + E2 oleate + SR-BI blocking antibody + ICI, 22.5 ± 0.9%). In addition, we did not find any significant differences employing either BLT1 or SR-BI blocking antibody on the attenuation in HDL-associated estradiol oleate mediated cholesterol efflux potential.

Fig. 3 illustrates macrophage uptake of HDL-associated [^{3}H]estradiol esters and their hydrolysis after the incubation time period of 16 h. After LH-20 chromatography, HDL-associated [^{3}H]estradiol was presented as 91% [^{3}H]estradiol esters and 9%

Table 1
Estradiol (expressed as free and fatty acyl esters) quantification in serum and HDL.

	Male		Female	
	Ester	Free	Ester	Free
Serum (pmol ml^{-1} E2)	0.064 (0.057–0.071)	0.069 (0.063–0.072)	0.097 (0.069–0.129)	0.173 (0.069–0.274)
	Celite	Incubations		
Male HDL + E2-oleate (pmol E2 mg^{-1} HDL prot.)	20.74 (11.54–29.93)	ND		

Estradiol (E2) median and range values (in parentheses) are presented for serum and HDL. ND = not detectable. Values represent 3 independent isolations/preparations of samples.

free [^{3}H]estradiol. After the 16 h time of incubating HDL-associated [^{3}H]estradiol esters with differentiated, non-tritiated acetylated cholesterol ester loaded macrophages, the intracellular distribution of HDL-associated [^{3}H]estradiol was 70.1% esterified and 29.9% free. SR-BI and estrogen receptor inhibition resulted in a decrease in overall [^{3}H]estradiol counts but did not change the percent distribution of intracellular ester to free estradiol.

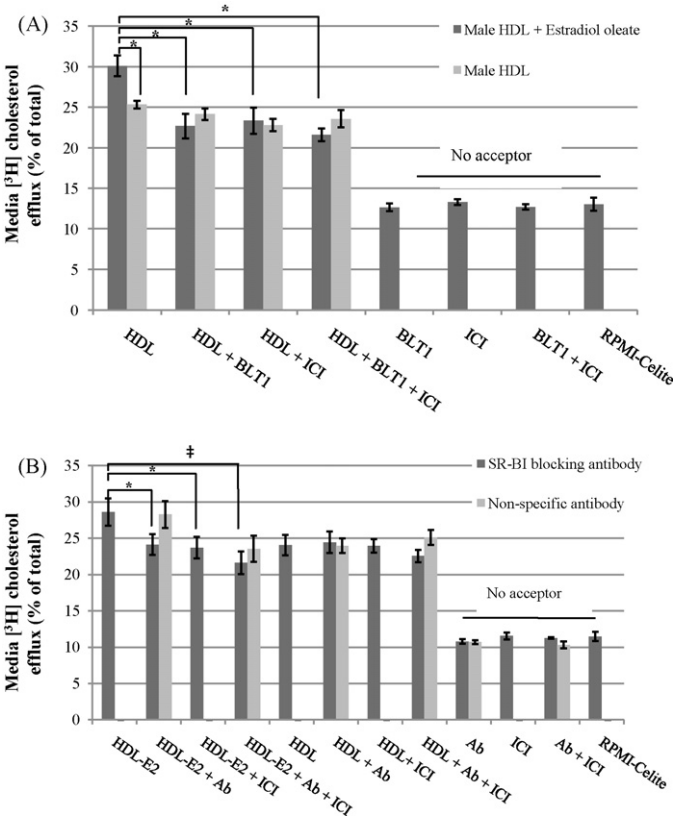

Fig. 2. Effect of SR-BI and estrogen receptor antagonist treatment on 17β-estradiol oleate enriched HDL-mediated cholesterol efflux from THP-1 macrophage foam cells. (A) Macrophages loaded with [^{3}H]cholesterol oleate-acetylated LDL were pretreated for 90 min with either 10 μM of BLT1 (a SR-BI chemical inhibitor), 1 μM of ICI (estrogen receptor antagonist), BLT1 and ICI together, or vehicle. 17β-Estradiol oleate enriched male HDL (25 μg HDL protein; male HDL + estradiol oleate) or male HDL lacking estradiol oleate (25 μg HDL protein; male HDL) was then added to the media. Cells were also treated with media containing only the respective inhibitors but lacking HDL (no acceptor; BLT1, ICI, or BLT1 + ICI) and [^{3}H]cholesterol efflux from macrophages was measured as indicated in Fig. 1 and in Section 2. (B) [^{3}H]cholesterol oleate acetyl LDL loaded macrophages were pretreated for 60 min with either 200 μg ml^{-1} of SR-BI blocking antibody or non-specific antibody. Next, male HDL with or without Celite facilitated estradiol oleate enrichment (25 μg HDL protein; HDL-E2, HDL, respectively) was added to the incubation media and treated exactly as in panel A (above). Cells were cultured in triplicate wells. Data is expressed as the mean ± S.E. of 3 independent experiments using different preparations of HDL-E2-oleate-Celite preparations.
$^*P \leq 0.05$ and $^‡P \leq 0.001$.

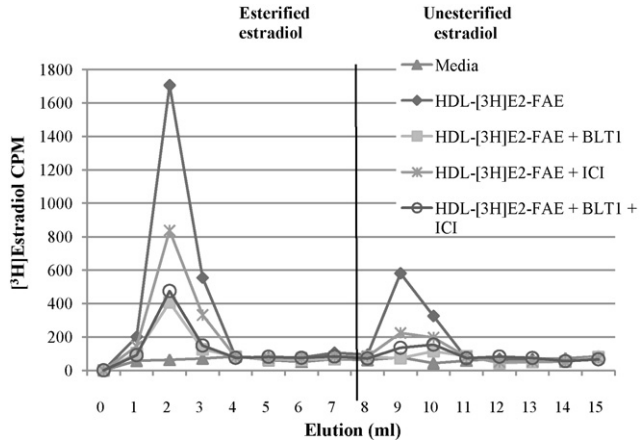

Fig. 3. Internalization of HDL-derived estradiol ester in THP-1 cells. Male serum and [^{3}H]17β-estradiol was incubated for 24 h at 37 °C. Total HDL containing [^{3}H]estradiol (360 μg HDL protein) was isolated and incubated with acetylated LDL containing non-labeled cholesterol oleate loaded THP-1 cells in the absence (HDL-[^{3}H]E2-FAE) or presence of 10 μM of BLT-1, 1 μM ICI, or BLT-1 and ICI together. Cellular lipids were extracted and [^{3}H]estradiol and [^{3}H]estradiol esters were separated by LH-20 chromatography, as described in Section 2. Vertical bar divides the ester fraction (hexane–chloroform extraction) to the unesterified fraction (methanol extraction). Prior to the incubation, HDL-containing [^{3}H]estradiol partitioned as 91% [^{3}H]estradiol fatty acyl esters and 9% as free [^{3}H]estradiol. Intracellular [^{3}H]estradiol (c.p.m.) as a function of the elution volume is depicted and the distribution of esterified and free estradiol (unesterified) is marked, respectively. Cells were cultured in 15-cm plates. Figure data is a representative of two replicate experiments.

To assess the difference in premenopausal female and male serum or isolated total HDL stimulated macrophage cholesterol efflux, each were incubated with [^{3}H]acLDL loaded macrophages and then cholesterol efflux potential was determined. Premenopausal female serum (0.5%, v/v) and HDL stimulated greater cholesterol efflux percentages compared to the respective male samples (Fig. 4A; female serum, 40.2 ± 2.2%; male serum, 28.7 ± 1.0%; female total HDL-fraction, 32.8 ± 2.3%; male total HDL-fraction, 25.9 ± 2.6%). BLT1 and ICI treatment eliminated the difference in macrophage cholesterol efflux between female and male HDL.

Table 2 outlines the lipid and apolipoprotein profiles in pooled serum from either four premenopausal or four age-matched males. Three different pools were collected and these data represent the mean values. No significant differences were detected.

4. Discussion

The presence of estradiol esters in female HDL prompts the question of their function and the likely protective nature against atherosclerosis. Causes of the gender-specific differences in foam cell cholesterol efflux potential remain unclear. These results provide a novel and preliminary mechanism by which HDL-associated

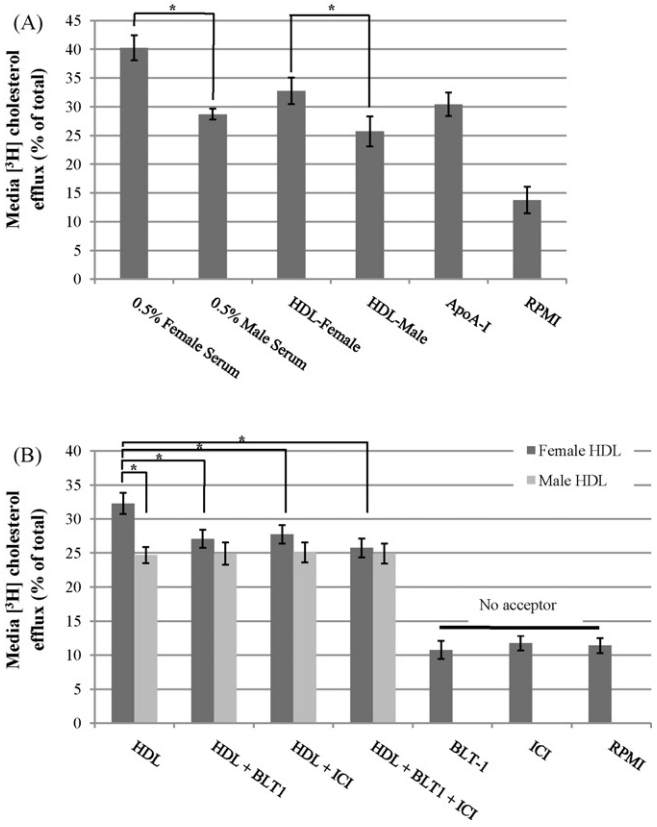

Fig. 4. Gender-specific serum and total HDL-mediated cholesterol efflux from [^{3}H]acetyl LDL cholesterol oleate loaded THP-1 macrophage foam cells. Combined serum from either four non-pregnant, premenopausal women or from four aged-matched males was collected and total HDL was isolated from the respective preparations. (A) Premenopausal female serum (0.5%, v/v), female total HDL (25 μg HDL protein), male serum (0.5%, v/v), male total HDL (25 μg HDL protein), or apoA-I (15 μg), was added to wells and incubated for 16 h at 37 °C with differentiated THP-1 macrophages loaded with [^{3}H]cholesterol oleate-acetylated LDL. Data is expressed as the [^{3}H]cholesterol efflux percentage, as described in Section 2. (B) Cells were pretreated for 90 min with 10 μM BLT1, 1 μM ICI, or BLT1 and ICI together. Female or male total HDL (25 μg HDL protein, respectively) was added to the wells and incubated for 16 h. Cells were also treated with media containing only the respective inhibitors but lacking HDL (no acceptor; BLT1, ICI, or BLT1 + ICI) and macrophage [^{3}H]cholesterol efflux was measured as indicated in Fig. 1 and in Section 2. Cells were treated in triplicate wells and data is expressed as the mean ± S.E. of three experiments using different preparations of serum and HDL from human volunteers. $^*P \leq 0.05$.

17β-estradiol fatty acyl ester and also natural, premenopausal women's HDL favorably affect cholesterol efflux from macrophage foam cells. We demonstrated that this occurs by a process involving SR-BI and estrogen receptors. Since macrophage foam cell development is a critical stage in atherosclerosis and there is a clear difference in the atherosclerosis risk between the genders, the effect of estradiol esters, transported by HDL to macrophages, may be an important regulatory mechanism in atherosclerosis development.

Table 2
Serum lipid levels, apoA-I, and apoE levels in premenopausal female and male subjects.

	Female	Male	*P*
Age (range)	38 (29–45)	37 (31–55)	
n	3	3	
Cholesterol (mmol l^{-1})	4.78 ± 0.9	4.53 ± 1.1	NS
LDL-C (mmol l^{-1})	2.71 ± 0.5	2.59 ± 0.5	NS
HDL-C (mmol l^{-1})	1.56 ± 0.2	1.47 ± 0.3	NS
ApoA-I (g l^{-1})	1.47 ± 0.9	1.32 ± 0.4	NS
ApoE (mg l^{-1})	35.6 ± 0.3	31.0 ± 0.2	NS
Triglycerides (mmol l^{-1})	1.05 ± 0.3	1.14 ± 0.1	NS

Data is expressed as mean ± S.D. and are from data collected from 3 independent pooled samples taken from human volunteers. NS = $P > 0.05$.

To highlight the importance of estradiol esters in mediating macrophage cholesterol efflux potential, we loaded male HDL with estradiol oleate by a hydrophobic exchange reaction using the Celite approach. The amount of estradiol ester present was 300-fold greater than the amount detected in female HDL (Table 1). Incubating HDL-associated estradiol oleate with macrophage foam cells promoted a significantly greater cholesterol efflux percentage after 16 h, a time course commonly used in cholesterol efflux experiments, compared to non-loaded HDL or BSA with or without estradiol oleate (Fig. 1). Firstly, this demonstrates that estradiol oleate has an effect on macrophages and secondly, that HDL is a potent carrier of estradiol oleate to stimulate these effects. Since HDL is required for the observed enhanced cholesterol efflux and SR-BI participates in estradiol uptake in hepatocytes [16], we considered that HDL receptors may play a role also in macrophages. Inhibiting SR-BI with BLT1 resulted previously [16] in a marked decrease in HDL-estradiol's effect (Fig. 2A). A related investigation into HDL-associated estradiol 's effect on cells employed the ICI antiestrogenic compound [12]. Therefore, we used ICI to test the influence of estrogen receptors on macrophage cholesterol efflux. ICI treatment also resulted in a significant decrease in efflux and combined use of ICI and BLT1 did not add further to the reduction of cholesterol efflux potential (Fig. 2A). In order to confirm that the results were not inaccurate due to a non-selective reaction of BLT1 to the cells rather than inhibiting SR-BI exclusively, we used a blocking antibody to SR-BI and found similar results to BLT1 treated cells (Fig. 2B). These results imply that SR-BI and estrogen receptors manage the enhanced macrophage cholesterol efflux potential stimulated by HDL-associated estradiol oleate.

To determine if estradiol is internalized by macrophage cells, we radiolabeled HDL with [^{3}H]estradiol. Incubations of [^{3}H]estradiol with serum results in esterified estradiol stemming from the LCAT reaction [16,18]. After 16 h, the cells internalized and hydrolyzed estradiol esters into their free state (Fig. 3). SR-BI inhibition with BLT1 resulted in a 75% reduction in the total amount of intracellular [^{3}H]estradiol counts, which strengthens SR-BI's importance in this mechanism. ICI treatment showed approximately a 50% reduction in internalization of [^{3}H]estradiol counts (Fig. 3). Therefore, functional estrogen receptors and SR-BI significantly contribute to HDL-associated estradiol ester internalization.

We next tested the effect of premenopausal female or male serum and HDL on macrophage cholesterol efflux. Premenopausal female serum and HDL showed significantly greater cholesterol efflux potential compared to males (Fig. 4A and B). Premenopausal female serum and HDL showed significantly greater cholesterol efflux potential compared to males (Fig. 4A and B). Also, premenopausal female serum had higher median estradiol content (Table 1) and correspondingly, Gong et al. [12] showed that HDL from premenopausal women have greater estradiol quantities compared to HDL from males, which have non-detectable amounts. Interestingly, there was no significant difference between female HDL (female HDL, Fig. 4) and supraphysiologic HDL (male HDL + estradiol oleate, Fig. 1) on macrophage cholesterol efflux. We think that an explanation for this is that the Celite incubations with estradiol oleate and male HDL may have modified the HDL particle to be less efficient for cellular estradiol uptake and the orientation of estradiol ester in the HDL particle, after these incubations, may not have been positioned properly for maximum effect as compared to the estradiol in the native HDL from premenopausal women. It is also possible that there is a limit to the amount of estradiol ester that is needed to cause these effects on cholesterol efflux. The fact that male HDL, loaded with estradiol ester, can trigger a

significant increase in cholesterol efflux compared to male HDL lacking it, demonstrates that estradiol esters do modulate these effects.

This investigation contains certain limitations. First, the specificity of ICI for estrogen receptors is somewhat unknown and the possible cooperativity between estrogen receptors and SR-BI needs to be further investigated. Secondly, since we conducted a pilot experiment to test whether a difference in macrophage cholesterol efflux between premenopausal women and males exists, the small sample population used here makes it difficult to draw conclusions about the results obtained and an investigation with a larger population is warranted.

In summary, we have demonstrated here that HDL targets estradiol fatty acyl esters to macrophage cells and estradiol ester-stimulated cholesterol efflux is dependent on SR-BI and estrogen receptors. These esters undergo cellular internalization and get liberated into free estradiol over time. This investigation lends novel evidence that macrophage cholesterol efflux by HDL is enhanced by HDL-associated estradiol fatty acyl esters.

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