

# The effect of 17 $\beta$ -estradiol on cholesterol content in human macrophages is influenced by the lipoprotein milieu

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## Abstract

Estrogen and testosterone are thought to modulate coronary heart disease (CHD) risk. To examine how these hormones affect human macrophage cholesterol transport, a key factor in atherogenesis, we obtained monocytes from healthy male and postmenopausal female donors (age 50–70 years). Cells were allowed to differentiate in autologous serum. Human monocyte-derived macrophages (HMDMs) were exposed to estrogen, testosterone, or vehicle, during differentiation. Cells were cholesterol enriched with oxidized low-density lipoprotein (oxLDL) in the presence of treatment. Cell cholesterol mass, efflux, and the expression of proteins involved in HMDM cholesterol transport were examined. Estrogen significantly reduced cholesteryl ester (CE) content in both female and male HMDMs while having no measurable effect on cholesterol efflux. Testosterone did not affect cholesterol content or efflux. Both hormones significantly but modestly affected the gene expression of several proteins involved in HMDM transport, yet these effects did not translate into significant changes in protein expression. In THP-1 macrophages, the effect of estrogen on CE content was more potent in unloaded macrophages and was estrogen receptor dependent. A trend for a reduction in non-oxLDL uptake by estrogen was observed and was also found to be dependent upon estrogen receptor activation. Our data indicate that estrogen, but not testosterone, reduces CE accumulation in HMDMs obtained from a CHD age relevant population, independent of changes in the expression of proteins important to macrophage cholesterol transport. In THP-1 cells, this effect is reduced in the presence of oxLDL, indicating that a pro-atherogenic lipoprotein milieu is an important variable in sex hormone modulation of CHD.

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## Introduction

Macrophage cholesterol transport is an important variable in the initiation and progression of atherosclerosis. Cholesterol influx of modified lipoproteins via the scavenger receptor (SR) pathway results in cholesterol enrichment and subsequent foam cell formation, an early hallmark of atheroma lesions (Kunjathoor *et al.* 2002). Transporters important to influx include CD36 and the macrophage SR type A (SR-A; Kunjathoor *et al.* 2002). Acyl coenzyme A:cholesterol acyltransferase 1 (ACAT1) esterifies free cholesterol for storage in lipid droplets, while cholesteryl ester hydrolase (CEH) de-esterifies this cholesterol, presumably leading to cholesterol efflux (Buhman *et al.* 2001). Proteins important to cholesterol efflux include ABCA1, ABCG1, and apolipoprotein E (apoE; Cullen *et al.* 1998, Wang *et al.* 2007, Out *et al.* 2008). The SR-B1 may also play a role in cholesterol accumulation as it is a bidirectional cholesterol transporter, the function of which, however, is complicated and poorly understood, with evidence supporting roles for efflux, efflux

inhibition, and influx (Chen *et al.* 2000, Langer *et al.* 2002, Yancey *et al.* 2003, Wang *et al.* 2007).

The steroid hormones 17 $\beta$ -estradiol (E<sub>2</sub>) and testosterone are thought to play a role in modulating atherogenesis. E<sub>2</sub> and testosterone treatments have been shown to alter cholesterol metabolism in human monocyte-derived macrophage cells (HMDMs) obtained mainly from younger men and premenopausal women (Tomita *et al.* 1996, McCrohon *et al.* 1999, 2000, Napolitano *et al.* 2001, Ng *et al.* 2003). E<sub>2</sub> has been shown to reduce CE content in female, but not in male, HMDMs (McCrohon *et al.* 1999), an effect possibly caused by reduced cholesterol esterification and uptake of modified lipoproteins (Tomita *et al.* 1996, Sulistiyani & St Clair 1997, McCrohon *et al.* 1999, 2000, Napolitano *et al.* 2001). Enhanced cholesterol efflux in both male and female HMDMs by E<sub>2</sub> has also been observed, but at super-physiological concentrations (Napolitano *et al.* 2001). Conversely, dihydrotestosterone, the more potent, non-aromatizable form of testosterone, has been shown to increase CE content in male but not in female-derived HMDMs (McCrohon *et al.* 1999, 2000, Ng *et al.* 2003).

These studies have predominantly utilized HMDMs derived from younger male and premenopausal female donors. It is not known how HMDMs obtained from older individuals will respond to physiological concentrations of E<sub>2</sub> or testosterone. Because the prevalence and severity of atherosclerosis increases with age (Lloyd-Jones *et al.* 2010), we aimed to investigate the effect of E<sub>2</sub> and testosterone on cholesterol accumulation in HMDMs obtained from older male and postmenopausal female donors. Additionally, we studied how oxidized lipoproteins modulate the effects of E<sub>2</sub> and testosterone on human macrophage cholesterol accumulation.

## Materials and methods

### Materials

Estrogen, testosterone, DNaseI, BSA, apoA-I, formaldehyde, malondialdehyde, acrylamide, phorbol 12-myristate 13-acetate (PMA), and protease inhibitor cocktail were purchased from Sigma. ICI 182 780 was purchased from Tocris Biosciences (Ellsville, MO, USA). THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Charcoal/dextran-treated fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Ficoll-Paque was obtained from GE Healthcare (Piscataway, NJ, USA). Amplex Red Cholesterol Assay Kit was purchased from Molecular Probes (Eugene, OR, USA). RNeasy Mini Kit was purchased from Qiagen. Phenol-free RPMI1640 medium and Superscript III Reverse Transcriptase Kit were obtained from Invitrogen. Power SYBR Green Master Mix was purchased from Applied Biosystems (Carlsbad, CA, USA). Bicinchoninic Acid Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). DiI-low-density lipoprotein (LDL) was purchased from Biomedical Technologies (Stoughton, MA, USA). 3[H]-Cholesterol was obtained from Perkin-Elmer (Waltham, MA, USA). CD36, LDL receptor (LDLR), and ACAT1 antibodies were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The remaining antibodies were purchased from Novus Biologicals (Littleton, CO, USA). ECL detection assay Kit was obtained from Amersham Biosciences.

### Subjects

Healthy normolipidemic male ( $n=10$ ) and postmenopausal female ( $n=10$ ) volunteers between 50 and 70 years of age were recruited for this study. Subjects were included if they had no history of coronary heart disease (CHD), cancer, diabetes, or renal, liver, or thyroid diseases. Inclusion criteria were LDL cholesterol (LDL-C) <160 mg/dl, high-density lipoprotein cholesterol (HDL-C) >40 mg/dl, triglycerides (TG)

**Table 1** Characteristics and fasting metabolic and lipid profiles of study volunteers. Values are expressed as mean (+s.d.)

	Women ( $n=10$ )	Men ( $n=10$ )	<i>P</i> value
Age (years)	58.9 (4.4)	60.8 (5.9)	0.4236
BMI (kg/m <sup>2</sup> )	27.6 (6.2)	26.0 (2.9)	0.4808
Lipids			
TC (mg/dl)	205.5 (30.5)	177.8 (33.6)	0.0698
LDL-C (mg/dl)	111.1 (32.5)	87.24 (35.8)	0.1366
HDL-C (mg/dl)	66.0 (15.7)	46.1 (15.5)	0.0107*
TG (mg/dl)	78.2 (27.8)	99.3 (45.8)	0.2292
VLDL (mg/dl)	14.3 (6.8)	10.4 (5.8)	0.2381
Glucose (mg/dl)	90.9 (6.7)	91 (8.0)	0.9762
ERα IVS1	T/C=5, T/T=5	T/C=8, T/T=2	0.3498

\**P* value <0.05 for gender difference. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; VLDL, very low-density lipoprotein.

<150 mg/dl, and glucose <100 mg/dl. Subjects who smoked or had hypertension were excluded from the study. Volunteers were also not taking any medications to control blood lipid or glucose levels. Women were considered postmenopausal if irregular menstrual periods exceeded 1 year. Most women ( $n=8$ ) in this study had been postmenopausal for >5 years. None of the women were on hormone replacement therapy. The study was approved by the Institutional Review Board of Tufts Medical Center, and all subjects provided informed consent. Characteristics of the subjects are shown in Table 1.

### Lipid measurements, and LDL isolation and oxidation

Plasma lipid measurements including total cholesterol (TC), TG, LDL-C, and HDL-C were determined by enzymatic assays (Roche diagnostics). LDL was isolated from pooled donor plasma by rapid single-step ultracentrifugation using a Beckman NVT90 rotor (Beckman Coulter, Miami, FL, USA) as described previously (Vieira *et al.* 1996). LDL was oxidized at 37 °C by the addition of 100 μM CuSO<sub>4</sub>/100 μg protein. Oxidation extent was monitored by absorbance increases at 234 nm. When absorbance began to increase exponentially ( $\approx 1.5$  h), LDL was placed on ice and immediately desalted to stop further oxidation. This typically produced a thiobarbituric acid-reactive substance (TBARS) value of between 6 and 8 nM malondialdehyde/μg protein, which is considered mildly oxidized LDL (oxLDL; Weidtmann *et al.* 1995), a form likely present *in vivo* (Yla-Herttuala *et al.* 1989). TBARS measurements were performed as described previously (Cathcart *et al.* 1991). oxLDL was stored at -80 °C in the dark for up to 2 months, as TBARS values and 234 nm readings were found to remain stable for this duration. Protein aggregation of

the LDL particles was also found to be minimal for this period of time. The same batch of oxLDL was used for all experiments.

### Isolation and culture of HMDMs

Blood was drawn for the isolation of monocytes, plasma (for HDL/LDL isolation), and serum following a 12 h fast. Buffy coats were obtained by layering blood cells diluted 1:2 with phenol-free RPMI1640 over Ficoll-Paque and centrifuged (37 min, 394 000 *g* at room temperature). White blood cells were collected and washed twice in RPMI, then plated in serum-free RPMI. After 3–4 h of incubation, non-adherent cells were washed off, and the remaining monocytes were cultured in RPMI containing 10% autologous serum. Cells were allowed to differentiate for 10 days in the presence of vehicle (0.1% ethanol), physiological concentrations of E<sub>2</sub> or testosterone (2 and 10 nM respectively), or superphysiological concentrations of E<sub>2</sub> or testosterone (20 and 100 nM respectively).

HMDMs were treated with 50  $\mu$ g/ml oxLDL from days 10 to 12 in the presence of 10% autologous serum and hormone treatment. Cells were then harvested for protein, RNA, or cholesterol mass measurement. Cells were collected in lysis buffer (0.1 M KH<sub>2</sub>PO<sub>4</sub>, 0.05 M NaCl, 5 mM cholic acid, and 0.1% Triton X-100), and cell protein was quantified using the bicinchoninic acid method with BSA as a standard.

### Cholesterol efflux

For cholesterol efflux, cells were treated with 2.5  $\mu$ Ci/ml <sup>3</sup>[H]-cholesterol in addition to 50  $\mu$ g/ml oxLDL during days 10–12. After oxLDL/<sup>3</sup>[H]-cholesterol exposure, cells were equilibrated in RPMI supplemented with 2 mg/ml fatty acid-free BSA for 6–8 h, then incubated with 25  $\mu$ g/ml HDL + 10  $\mu$ g/ml apoA-1+ treatments for 24 h in serum-free medium. Exposure of HMDM to vehicle, E<sub>2</sub>, or testosterone was constant throughout these experiments. HDL used in the cholesterol efflux assay was isolated from pooled donor plasma by sequential ultracentrifugation as described previously (Havel *et al.* 1955). Fraction of cholesterol efflux was calculated by dividing the medium disintegrations per minute (DPM) counts by the total (medium + cell) DPM counts. These values were then adjusted for cell protein concentration.

### THP-1 cells

THP-1 monocytic leukemia cells were differentiated with 100 ng/ml PMA in phenol-free RPMI medium + 10% charcoal/dextrin-treated FBS for 72 h. Cells were then washed twice in PBS and treated for 48 h with

vehicle, E<sub>2</sub> 20 nM, vehicle + ICI 182 780 (an ER antagonist) 200 nM, or E<sub>2</sub> 20 nM + ICI 182 780 200 nM in 10% postmenopausal female serum (PFS). Cells were then cultured with treatments in the following conditions for 48 h: PFS, PFS + oxLDL, and for cholesterol efflux measurement: PFS + <sup>3</sup>[H]-cholesterol or PFS + oxLDL + <sup>3</sup>[H]-cholesterol.

### Measurement of cellular cholesterol content

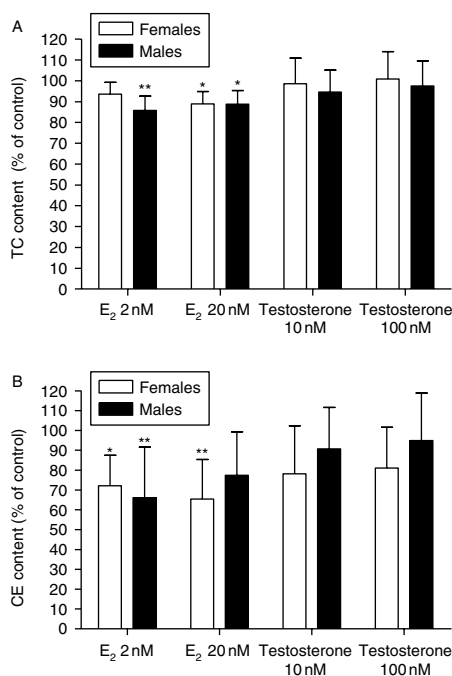
Cellular cholesterol content was measured fluorometrically using the Amplex Red Cholesterol Assay Kit as described by the manufacturer. Cell monolayers were washed twice and then lysed in 1 $\times$  reaction buffer supplemented with 10 U/ml DNaseI. Because this lysate was also used for protein expression analysis, lysates were also supplemented with a protease inhibitor cocktail. Plates were rocked at 4  $^{\circ}$ C for 15 min, and cells were scraped into 1.5 ml centrifuge tubes and incubated on ice for 1 h. An aliquot of this lysate was used for cell cholesterol mass measurement, while the remaining lysate was used for protein analysis. The aliquot for cholesterol measurement was vortexed for 30 min at room temperature to ensure complete homogenization of the cells. Samples were heated at 60  $^{\circ}$ C for 30 min to inactivate enzymes that could compete with the assay. Because this assay included cholesterol esterase, both TC and CE could be quantified. Cholesterol content was adjusted for cell protein quantity.

### Real-time PCR

Total cellular RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. RNA was reverse transcribed using a Superscript III Reverse Transcription Kit and amplified on an Applied Biosystems 7300 real-time PCR using specific primers validated for efficiency and specificity by standard curve dilution and melting point analysis. Real-time PCR using power SYBR Green Master Mix was carried out for 40 cycles of 95  $^{\circ}$ C (15 s) + 60  $^{\circ}$ C (1 min). Changes in gene expression were assessed by  $\Delta\Delta C_t$  analysis with  $\beta$ -actin as the control/housekeeping gene, as the expression of this gene was unchanged with treatment (data not shown). Changes were expressed as percent of control (vehicle).

### Western immunoblot assay

For HMDM, 20  $\mu$ g of total cell protein were separated on a 10% acrylamide gel overnight and transferred onto a nitrocellulose membrane. After blocking the membranes with 5% non-fat dry milk in TBS-0.5% Tween 20 (blocking buffer) for 1–2 h, the membranes were incubated with primary antibody in blocking buffer overnight at 4  $^{\circ}$ C. Following washing, the



**Figure 1** Effect of 17 $\beta$ -estradiol (E<sub>2</sub>) or testosterone on HMDM cholesterol content. Macrophage cells were cultured for 10 days in autologous serum along with control (ethanol), E<sub>2</sub> 2 nM, E<sub>2</sub> 20 nM, testosterone 10 nM, or testosterone 100 nM, and then treated with oxLDL (with hormones). (A) Effect of E<sub>2</sub> or testosterone treatment on HMDM total cholesterol (TC) content. (B) Effect of E<sub>2</sub> or testosterone treatment on HMDM cholesteryl ester (CE) content. Data are shown as percent of control. \* $P < 0.05$ , \*\* $P < 0.01$ .

membranes were incubated with secondary antibody in blocking buffer for 1 h at room temperature. Bands were visualized by Amersham ECL detection assay. Changes in protein expression were assessed by band optical density analysis with  $\beta$ -actin as the control. Changes were expressed as percent of control (vehicle).

### DiI-LDL uptake

THP-1 cells were treated with E<sub>2</sub> 20 nM + 5  $\mu$ g/ml DiI-LDL in RPMI medium containing 10% PFS for 48 h following differentiation with PMA. Cells were then washed several times with PBS containing 3 mg/ml fatty acid-free BSA, trypsinized (0.25%), and fixed with 4% formaldehyde prior to FACSCalibur analysis. Cells (10 000) were counted for fluorescence with an emission spectra set at 571 nm and an excitation spectra set at 554 nm.

### Statistical analysis

Statistical analyses were performed using SAS Software (v9.1, Cary, NC, USA) while graphs were created using GraphPad Prism Software (v4, La Jolla, CA, USA). Results are expressed as mean percent of control

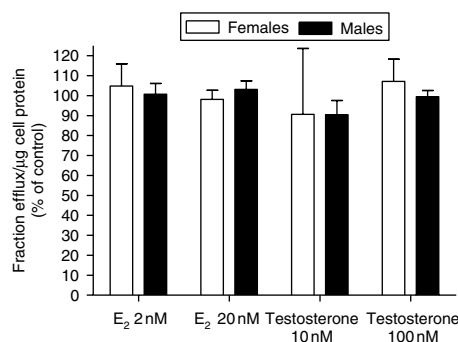
(+s.d.), except where noted. Means and s.d. are representative of the treatment response in macrophage cultures obtained from ten females or ten males. For THP-1 experiments, results are from three to four independent experiments where each treatment was performed in triplicate. Statistical differences were determined by two-way ANOVA for both treatment effect and sex effect using Tukey's Student Range test or by one-way ANOVA for treatment effect alone where appropriate. Significance was considered at  $P < 0.05$ .

## Results

Table 1 describes the characteristics of participating subjects. Women had significantly higher plasma HDL-C levels than men. Plasma levels of HDL-C, LDL-C, and TG did not significantly correlate with HMDM cholesterol content in control cells nor did these levels influence the treatment effect (data not shown).

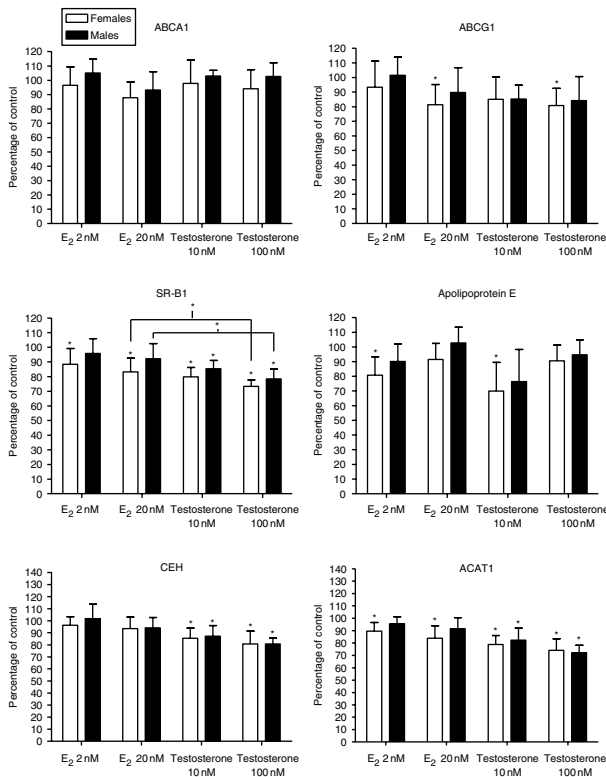
E<sub>2</sub> 2 nM significantly reduced TC content in males only, while this reduction was significant in both genders at E<sub>2</sub> 20 nM (Fig. 1A). Conversely, E<sub>2</sub> significantly reduced CE content in women at both concentrations. In men, the reduction in CE was significant at the 2 nM concentration, with a non-significant trend ( $P = 0.054$ ) at the 20 nM concentration (Fig. 1B). No dose-dependent effect was observed. In contrast, testosterone treatment had no significant effect on TC or CE content in male or female donors (Fig. 1A and B). Free cholesterol levels did not change with either hormone treatment (data not shown). Neither hormone had any significant effect on HMDM cholesterol efflux (Fig. 2).

The effect of E<sub>2</sub> or testosterone treatment on the expression of genes important for cholesterol transport was also studied. E<sub>2</sub> induced modest reductions



**Figure 2** Effect of E<sub>2</sub> or testosterone on HMDM cholesterol efflux. HMDMs were exposed to hormones + oxLDL (described in Fig. 1) along with <sup>3</sup>H-cholesterol. Following this, cells were exposed to HDL-C, apoA-1, and hormone treatment for 24 h, and then fraction of cholesterol efflux (<sup>3</sup>H medium counts/<sup>3</sup>H cell counts) was measured.





**Figure 3** Effect of E<sub>2</sub> or testosterone on the expression of select genes involved in HMDM macrophage cholesterol transport. Following oxLDL treatment of HMDMs (described in Fig. 1), RNA was isolated and reverse transcribed, and the expression of genes involved in cholesterol efflux was measured by real-time PCR. Data are expressed as percent of control. \**P* < 0.05.

(10–20%) in *ABCG1*, *SR-B1*, *apoE*, and *ACAT1* gene expression in female but not in male HMDMs (Fig. 3), yet these effects were not observed at the protein level (Fig. 4). Testosterone reduced the gene expression of *ABCG1*, *SR-B1*, *apoE*, *ACAT1*, and *CEH* (Fig. 3) by 10–30% but did not elicit any measurable change at the protein level (Fig. 4). No effect of E<sub>2</sub> or testosterone was observed on the gene or protein expression of *ABCA1*, *CD36*, or *MSR* (data not shown).

To understand how E<sub>2</sub> reduced cellular CE content in the absence of an increase in cholesterol efflux or any significant changes in the major proteins involved in cholesterol transport, we used THP-1 cells as a model. E<sub>2</sub> treatment significantly reduced CE content in cells not exposed to oxLDL (Fig. 5B). This effect was abrogated by ICI 182 780 treatment (Fig. 5B). After oxLDL exposure, the effect of E<sub>2</sub> on CE content was smaller than that observed prior to oxLDL exposure (–20 vs –40% respectively) and did not reach statistical significance despite a clear trend (Fig. 5D). There was no significant difference in TC (Fig. 5A and C) or free cholesterol (data not shown) with E<sub>2</sub> treatment. However, we did observe a significant increase in TC

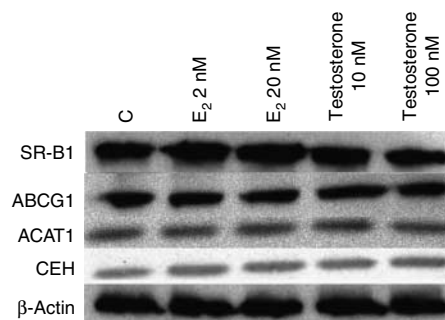
and CE content by ICI treatment in THP-1 cells exposed to oxLDL (Fig. 5C and D). Reductions in THP-1 gene expression (*ABCG1*, *SR-B1*, *apoE*, and *ACAT1*) by E<sub>2</sub> were also similar to those in the HMDMs, and there was no change in cholesterol efflux by E<sub>2</sub> (Fig. 6A).

THP-1 cells exposed to E<sub>2</sub> 20 nM and treated with non-oxidized DiI-LDL exhibited a non-significant trend toward a reduced LDL uptake, compared with control cells, that was ablated by ICI 182 780 treatment (Fig. 6B). There were no measurable differences in the protein expression of *CD36*, *MSR*, and *LDLR* (data not shown).

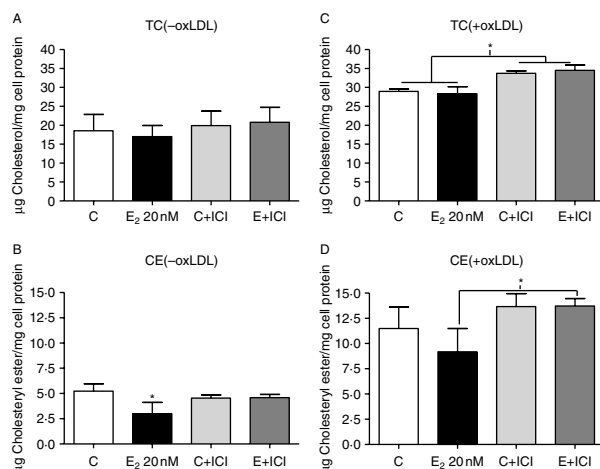
## Discussion

In this study, we found that E<sub>2</sub>, but not testosterone, reduces CE content without altering cholesterol efflux in macrophage cells obtained from 50- to 70-year-old men and postmenopausal women. This effect was not dependent upon changes in the expression of proteins important to cholesterol transport. In the human macrophage cell line THP-1, the E<sub>2</sub>-associated reduction in CE was dependent upon ER activation. More importantly, the reduction in CE content was more pronounced in cells not exposed to oxLDL indicating that a pro-atherogenic lipoprotein milieu is an influential factor in estrogen modulation of CHD.

The reduction in macrophage CE content by E<sub>2</sub> should supposedly lead to the inhibition of foam cell formation. By mitigating foam cell formation, E<sub>2</sub> may inhibit the progression of CHD. A reduction in CE content has also been reported previously in HMDM from younger donors (McCrohon *et al.* 1999), with E<sub>2</sub> reducing CE content in premenopausal female HMDM donors, but not in male donors. We did not observe any sex-specific effects in our study, possibly due to the use



**Figure 4** Effect of E<sub>2</sub> or testosterone treatment on the expression of select proteins involved in HMDM macrophage cholesterol transport. Following oxLDL treatment (described in Fig. 1), cell protein was collected, and the quantity of proteins involved in HMDM cholesterol transport that exhibited hormone-induced changes in gene expression was measured by western blotting. Blot shown is a representative.

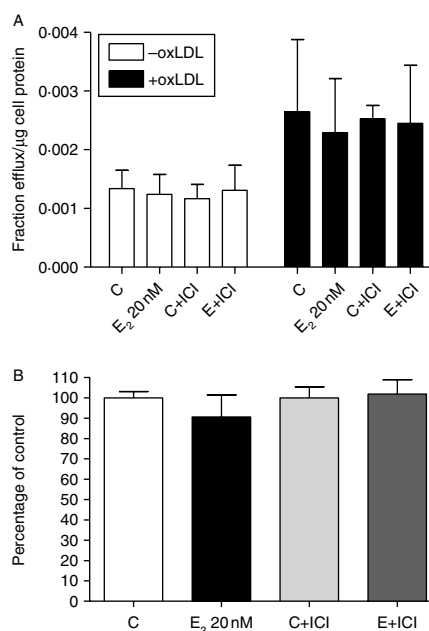


**Figure 5** Effect of E<sub>2</sub> treatment on THP-1 macrophage cholesterol content in cells with or without oxLDL exposure. THP-1 monocytes were differentiated for 3 days and then exposed to vehicle (C), E<sub>2</sub> 20 nM, C+ICI 1 82 780 200 nM, or E<sub>2</sub>+ICI 1 82 780 for 2 days. TC, free cholesterol, and CE content were measured in one group and another group was treated with hormones+oxLDL for additional 48 h, followed by cholesterol measurement. Results are expressed as mean + s.d. from three to four independent experiments, where each treatment was performed in triplicate. \**P*<0.05.

of autologous serum and oxLDL (as opposed to acetylated LDL, which acutely increases HMDM CE content, yet is not present *in vivo* (Brown & Goldstein 1983)). It is, however, possible that the sex-specific effect of E<sub>2</sub> on macrophage CE content may be lost with age. This needs to be further investigated by direct comparison with younger donors under similar conditions. The effect of E<sub>2</sub> on cholesterol efflux may also be lost with age, as previous work indicated that E<sub>2</sub> may enhance cholesterol removal from HMDM at least in male donors (Napolitano *et al.* 2001). In this report, however, very high concentrations of E<sub>2</sub> (1.5  $\mu$ M) were used, HMDM were exposed to acetylated LDL, and the differences between control and E<sub>2</sub>-treated HMDMs had dissipated by 24 h (Napolitano *et al.* 2001). Another report showed that THP-1 cells treated with HDL isolated from older individuals exhibited reduced efflux capacity compared with cells treated with HDL isolated from younger donors (Berrougui *et al.* 2007). This effect was linked with reduced ABCA1-mediated efflux (Berrougui *et al.* 2007), possibly indicating a reduced prevalence of lipid-poor apoA-I and nascent HDL particles in older individuals (Wang *et al.* 2007). How age-related HDL particle distribution influences the ability of E<sub>2</sub> to promote macrophage cholesterol efflux needs to be investigated. Increased activity of CEH, but not protein mass, by E<sub>2</sub> has been reported (Tomita *et al.* 1996, Napolitano *et al.* 2001) and should theoretically lead to increased cholesterol efflux. Because we did not observe an increase in cholesterol

efflux, it is unlikely that this occurred in our study to a significant extent. A recent study has shown that when E<sub>2</sub> is incorporated into HDL-C particles, the efflux capability of HDL-C is enhanced in THP-1 cells (Badeau *et al.* 2009). This effect was both ER and SR-B1 dependent (Badeau *et al.* 2009). E<sub>2</sub>-HDL-C may represent a more efficient mode of cellular delivery than simply the addition of E<sub>2</sub> dissolved in ethanol as we have used. Most of E<sub>2</sub> *in vivo* is bound to plasma proteins such as sex hormone-binding globulin or lipoproteins (Vihma *et al.* 2003). Therefore, the extracellular lipid environment may play an important role with respect to the effect that E<sub>2</sub> has on macrophage cholesterol efflux. Recently, it has also been suggested that estrogen may regulate cholesterol transport and uptake through the inhibition of the macrophage SR-A by the E<sub>2</sub>-induced heat shock protein 27 (Rayner *et al.* 2010). We have not studied this mechanism of action of E<sub>2</sub> in our cell experiments.

In our study, the most plausible explanation for the observed reduction in HMDM CE content by E<sub>2</sub> in the absence of increased efflux is reduced cholesterol uptake. We chose THP-1 cells to study the effect of E<sub>2</sub> on cholesterol accumulation because preliminary experiments by our group as well as by others (Cutolo *et al.* 2001) have shown that THP-1 cells express ERs.



**Figure 6** Effect of E<sub>2</sub> treatment on cholesterol efflux and nascent DiI-LDL uptake in THP-1 macrophages. (A) THP-1 macrophage cells were treated as described in Fig. 2, with or without pretreatment with oxLDL. (B) Cells were treated with control (C), E<sub>2</sub> 20 nM (E<sub>2</sub>), C+ICI 1 82 780, or E<sub>2</sub>+ICI 1 82 780 in the presence of 10% postmenopausal human serum + 5  $\mu$ g/ml nascent DiI-LDL. Data shown for A and B are representative of four independent experiments, where each treatment was performed in triplicate.

Cells were exposed to either human serum alone, or human serum supplemented with oxLDL. The percent reduction in CE content by E<sub>2</sub> was greater in cells exposed to human serum without oxLDL than in cells exposed to oxLDL, but the absolute reduction was similar, suggesting that the majority of the effect of E<sub>2</sub> occurs on macrophages before they are lipid loaded. The magnitude of the CE reduction by E<sub>2</sub> 20 nM in THP-1 cells exposed to oxLDL was identical to the degree of CE reduction observed in the HMDMs (20–30%) treated with either E<sub>2</sub> 2 or 20 nM. Based on these results, it is possible that a significant portion of the HMDM reduction reported in this study and in previous studies (Sulistyani & St Clair 1997, McCrohon *et al.* 1999) may have occurred prior to exposure to modified lipoproteins.

Observational and randomized intervention studies (Miller *et al.* 1995, Hulley *et al.* 1998, Grodstein *et al.* 2000, 2006, Herrington *et al.* 2000, Rossouw *et al.* 2002, 2007) reveal that the actions of E<sub>2</sub> within the vasculature are complex and appear to depend upon numerous factors including age, number of years postmenopause, and the presence of CHD or associated risk factors (Masood *et al.* 2010). Analysis of the Nurse's Health and WHI studies indicated that women who initiated estrogen replacement therapy closer to menopause had a reduced CHD risk, while women who initiated this therapy farther from menopause had an increased CHD risk (Grodstein *et al.* 2006, Rossouw *et al.* 2007). Several hypotheses have been proposed with the predominant 'timing' hypothesis suggesting that an environment without estrogen for a prolonged period of time can lead to an altered vascular state whereby the hormone acts in a different vasotoxic manner compared with a premenopausal/early postmenopausal vascular environment (Xing *et al.* 2009). Our findings support this hypothesis, specifically in that the effects of E<sub>2</sub> on macrophage CE content are influenced by the extracellular lipoprotein milieu. In a diseased artery, more likely present in the older postmenopausal individual, the presence of oxidized lipoproteins is likely to be significant. E<sub>2</sub> may not be as efficacious in a postmenopausal artery that already has a substantial accumulation of cholesterol-laden foam cells, whereas in a healthy artery, E<sub>2</sub> may reduce macrophage cholesterol uptake, thereby preventing foam cell formation early on (Salpeter *et al.* 2009). Two trials, the Kronos Early Estrogen Prevention Study (KEEPS) and the Early versus Late Intervention Trial with Estradiol (ELITE), will test this timing hypothesis (Harman *et al.* 2005, Taylor & Manson 2011).

LDL uptake by E<sub>2</sub> was reduced, although this effect did not reach statistical significance. This effect may partially be contributing toward the reduction in CE content by E<sub>2</sub> as we observed no change in cholesterol efflux, regardless of whether cells were treated with

oxLDL or not. LDL naturally present in human serum is mainly taken up by the LDLR, unlike oxLDL or acetylated LDL (Brown & Goldstein 1983). Our data indicate that E<sub>2</sub> does not affect LDLR protein expression and therefore, the mechanism may involve altered LDL surface binding to the LDLR and subsequent reduction in lipoprotein internalization. This needs to be further investigated. If the effect of E<sub>2</sub> on reducing LDL uptake is in part mediated by the LDLR, then this effect would presumably be lost with the oxLDL-induced downregulation of the LDLR from elevated CE accumulation (Linton *et al.* 1999). Accounting for the remaining reduction in CE content may include enhanced oxysterol conversion and reductions in *de novo* cholesterol synthesis.

The reduction in macrophage CE content by E<sub>2</sub> was found to be ER dependent as evidenced by ICI 1 82 780 treatment. This finding is not in agreement with McCrohon *et al.* (1999), who found that ICI treatment did not prevent the reduction in female HMDM CE accumulation by E<sub>2</sub>. We used THP-1 cells to test the effect of ICI, and therefore it is possible that HMDMs may respond differently. Interestingly, we observed a significant increase in TC and CE content with ICI in cells that were treated with oxLDL. The mechanism for this effect is unknown. Several studies suggest that ICI may act as an agonist for GPR30, a non-classical estrogen receptor, highlighting the complexities of the estrogen signaling network (Thomas *et al.* 2005, Filardo *et al.* 2007). It is unclear whether GPR30 activation may influence macrophage cholesterol metabolism. Alternatively, complete antagonism of the ER in the presence of oxLDL may slightly exacerbate cholesterol accumulation. Since THP-1 cells were cultured in postmenopausal serum, it is possible that the low levels of E<sub>2</sub> present in the serum of the non ICI-treated control group may have resulted in enough ER activation to reduce cholesterol accumulation.

In summary, we show that E<sub>2</sub>, but not testosterone, reduces CE content in HMDM obtained from 50- to 70-year-old donors. The reduction in macrophage CE content by E<sub>2</sub> was more pronounced in cells that were not exposed to oxLDL, indicating that oxLDL may mitigate the estrogen response. Clinically, the protective effects of E<sub>2</sub> replacement in early postmenopausal women without CHD may in part be due to the inhibition of arterial macrophage cholesterol uptake, an effect that is not as potent in the presence of oxLDL such as in advanced atherosclerotic lesions.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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