The effect of 17β-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 cholesteryl efflux (Buhman et al. 2001). Proteins important to cholesteryl 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 17β-estradiol (E2) and testosterone are thought to play a role in modulating atherogenesis. E2 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). E2 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 E2 has also been observed, but at superphysiological 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 E2 or testosterone. Because the prevalence and severity of atherosclerosis increases with age (Lloyd-Jones et al. 2010), we aimed to investigate the effect of E2 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 E2 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 1 82 780 was purchased from Tocris Biosciences (Ellisville, 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 and RT-PCR reagent kit were purchased from Applied Biosystems (Carlsbad, CA, USA). Bicinchoninic Acid Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). DiH2-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) <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 lipids 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.

| 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)      | P value |
| Age (years)     | 58.9 (4.4)      | 60.8 (5.9)      | 0.4236  |
| BMI (kg/m²)     | 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)     | 93.9 (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 (6.0)      | 0.9762  |
| ERα IVS1        | T/C=5.          | T/C=8,          | 0.3498  |
| T/T=5           |                 | T/T=2           |

*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.

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 CuSO4/100 μg protein. Oxidation extent was monitored by absorbance increases at 234 nm. When absorbance began to increase exponentially (~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 E2 or testosterone (2 and 10 nM respectively), or superphysiological concentrations of E2 or testosterone (20 and 100 nM respectively).

HMDMs were treated with 50 μg/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 (0.1M K H2PO4, 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 μCi/ml 3[H]-cholesterol in addition to 50 μg/ml oxLDL during days 10–12. After oxLDL/3[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 μg/ml HDL + 10 μg/ml apoA-I + treatments for 24 h in serum-free medium. Exposure of HMDM to vehicle, E2, 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, E2 20 nM, vehicle + ICI 182 780 (an ER antagonist) 200 nM, or E2 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 + 3[H]-cholesterol or PFS + oxLDL + 3[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 1X reaction buffer supplemented with 10 U/ml DNaseI. Because this lystate was also used for protein expression analysis, lysates were also supplemented with a protease inhibitor cocktail. Plates were rocked at 4°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 lystate was used for cell cholesterol mass measurement, while the remaining lystate 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°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°C (15 s) + 60°C (1 min). Changes in gene expression were assessed by ΔΔCt analysis with β-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 μg 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°C. Following washing, the
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 β-actin as the control. Changes were expressed as percent of control (vehicle).

DiI-LDL uptake

THP-1 cells were treated with E2 20 nM + 5 μg/ml Dil-LDL in RPMI medium containing 10% FBS 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 (10000) 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).

E2 2 nM significantly reduced TC content in males only, while this reduction was significant in both genders at E2 20 nM (Fig. 1A). Conversely, E2 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 E2 or testosterone treatment on the expression of genes important for cholesterol transport was also studied. E2 induced modest reductions...
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 E2 were also similar to those in the HMDMs, and there was no change in cholesterol efflux by E2 (Fig. 6A).

THP-1 cells exposed to E2 20 nM and treated with non-oxidized Dil-LDL exhibited a non-significant trend toward a reduced LDL uptake, compared with control cells, that was ablated by ICI 1 82 780 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 E2, 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 E2-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 E2 should supposedly lead to the inhibition of foam cell formation. By mitigating foam cell formation, E2 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 E2 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
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 E2 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 E2 on cholesterol efflux may also be lost with age, as previous work indicated that E2 may enhance cholesterol removal from HMDM at least in male donors (Napolitano et al. 2001). In this report, however, very high concentrations of E2 (1.5 µM) were used, HMDM were exposed to acetylated LDL, and the differences between control and E2-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 E2 to promote macrophage cholesterol efflux needs to be investigated. Increased activity of CEH, but not protein mass, by E2 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 E2 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). E2–HDL-C may represent a more efficient mode of cellular delivery than simply the addition of E2 dissolved in ethanol as we have used. Most of E2 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 E2 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 E2-induced heat shock protein 27 (Rayner et al. 2010). We have not studied this mechanism of action of E2 in our cell experiments.

In our study, the most plausible explanation for the observed reduction in HMDM CE content by E2 in the absence of increased efflux is reduced cholesterol uptake. We chose THP-1 cells to study the effect of E2 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.
Cells were exposed to either human serum alone, or human serum supplemented with oxLDL. The percent reduction in CE content by E₂ 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 E₂ occurs on macrophages before they are lipid loaded. The magnitude of the CE reduction by E₂ 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 E₂ 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 (Sulistiyani & 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 E₂ 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 through a ‘time’ effect. This needs to be further investigated. If the effect of E₂ 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 E₂ 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 E₂. 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 E₂ 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 E₂, but not testosterone, reduces CE content in HMDM obtained from 50- to 70-year-old donors. The reduction in macrophage CE content by E₂ was more pronounced in cells that were not exposed to oxLDL, indicating that oxLDL may mitigate the estrogen response. Clinically, the protective effects of E₂ 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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