Sex hormone modulation of proinflammatory cytokine and C-reactive protein expression in macrophages from older men and postmenopausal women

Michael P Corcoran¹, Mohsen Meydani², Alice H Lichtenstein³, Ernst J Schaefer¹, Alice Dillard³ and Stefania Lamon-Fava¹

¹Lipid Metabolism Laboratory, ²Vascular Biology Laboratory and ³Cardiovascular Nutrition Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, 711 Washington Street, Boston, Massachusetts 02111, USA

(Correspondence should be addressed to S Lamon-Fava; Email: stefania.lamon-fava@tufts.edu)

Abstract

Inflammation plays a central role in the development and progression of coronary heart disease (CHD). The sex hormones estrogen and testosterone have been shown to modify the inflammatory response by influencing cytokine expression in human macrophages obtained from younger individuals. The effect of these hormones on the expression of proinflammatory markers in macrophages obtained from a CHD age-relevant population has not been studied. Human monocyte-derived macrophages (HMDMs) were obtained from healthy normolipidemic men and postmenopausal women (age 50–70 years), and cultured in autologous serum along with both physiological and supraphysiological concentrations of estrogen or testosterone. HMDMs were stimulated with oxidized low-density lipoproteins, and the expression of the cytokines tumor necrosis factor α (TNF- α or TNF), interleukin (IL)6, and IL-1 β (IL1B) and of the

acute-phase protein C-reactive protein (CRP) was measured. Both physiological and supraphysiological concentrations of testosterone reduced the expression and secretion of TNF- α and reduced the expression of IL-1 β , but did not affect the expression of IL6 or CRP. Estrogen did not modify the expression of TNF- α , IL6, and IL-1 β . Estrogen caused a variable response in CRP expression that was positively associated with the plasma small dense LDL-cholesterol concentration of the donors. There were no gender differences in any of the observed effects. Our results indicate that testosterone may exert anti-inflammatory effects by reducing macrophage TNF- α expression, while the effects of estrogen on macrophage CRP expression may depend upon the extracellular lipid environment.

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Introduction

The pathology of coronary heart disease (CHD) is complex and multifaceted, with inflammation playing a central part (Libby 2002). Peripheral blood monocytes recruited into the intima-media layer of an artery play a pivotal role in the local inflammation (Brown & Goldstein 1983). Recruited monocytes differentiate into macrophages and begin to take up oxidized lipoproteins, leading to the formation of foam cells. Foam cells are a primary component of early atheroma lesion formation (Lloyd-Jones *et al.* 2009), and are a significant source of proinflammatory cytokines (Tipping & Hancock 1993), which include tumor necrosis factor α (TNF- α or TNF), interleukin (IL)6, and IL-1 β (IL1B). These cytokines actively participate in atherogenesis by promoting endothelial dysfunction, further monocyte recruitment, and smooth muscle cell apoptosis (Valente *et al.* 1992).

C-reactive protein (CRP) is an acute-phase protein that serves as a marker of systemic inflammation, and has been shown to be an independent predictor of CHD risk (Torres &

Ridker 2003). Most circulating CRP is secreted by the liver, yet a small amount of CRP is produced by macrophages present in atherosclerotic plaques (Yasojima *et al.* 2001). Levels of CRP mRNA and protein have been found to be up to tenfold higher in arterial plaque tissue than in the normal artery, suggesting that at least a portion of atheroma CRP content is locally produced (Kobayashi *et al.* 2003). *In vitro* and animal studies indicate that CRP may actively participate in plaque development by promoting endothelial dysfunction (Bisoendial *et al.* 2007), monocyte adhesion to the endothelium (Li *et al.* 2004), macrophage cholesterol accumulation (Singh *et al.* 2008), and fibrin breakdown (reduced plaque stability; Williams *et al.* 2004).

The steroid hormones 17 β -estradiol (E₂) and testosterone are thought to play a role in modulating inflammation and thereby influencing atherogenesis. In postmenopausal women, E₂ replacement therapy does not affect circulating TNF- α and IL6 concentrations (Pradhan *et al.* 2002, Zegura *et al.* 2003). However, hormone treatment may cause changes in cytokine levels in the arterial lesions that may not be

entirely predicted by plasma cytokine levels. Very little work has been conducted examining the effect of E₂ on cytokine expression in human monocyte-derived macrophages (HMDMs), with one study showing that E₂ withdrawal results in greater proinflammatory cytokine expression in female premenopausal HMDMs after 24 h of treatment than in HMDMs continually exposed to the hormone for 48 h (Kramer *et al.* 2004). Using testosterone, the expression of TNF- α , IL6, and IL-1 β was shown to be reduced both in rodent macrophage cell models and in human monocytes obtained from younger individuals (Chao *et al.* 1995, Kanda *et al.* 1996, 1997, D'Agostino *et al.* 1999); yet, no studies have been done in HMDMs obtained from older individuals. The effect of E₂ and testosterone on CRP expression in HMDMs has not been studied. The purpose of this study was to assess the effect of E₂ and testosterone treatment on the expression of proinflammatory cytokines and CRP by macrophages obtained from a CHD age-relevant population.

Materials and Methods

Materials

E₂ and testosterone were purchased from Sigma–Aldrich. Phenol-free RPMI1640 medium was purchased from Gibco. Ficoll-Paque was obtained from GE Healthcare (Piscataway, NJ, USA). RNeasy mini kit was purchased from Qiagen. Penicillin, streptomycin, 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).

Subjects

Male (*n*=10) and postmenopausal female (*n*=10) volunteers between 50 and 70 years of age were recruited. Subjects were included if they reported no history of CHD; cancer; diabetes; or renal, liver, or thyroid disease. Subjects who smoked or had hypertension were excluded from the study. Volunteers were not taking any medications to control plasma lipid or glucose levels. Inclusion criteria were low-density lipoprotein cholesterol (LDL-C) <160 mg/dl, high-density lipoprotein cholesterol (HDL-C) $\geq$ 40 mg/dl, triglycerides (TG) <150 mg/dl, and glucose $\leq$ 100 mg/dl. Women were considered postmenopausal if absence of menstrual periods exceeded 1 year. Most women (*n*=8) in this study had been postmenopausal for >5 years. Lastly, because it had been reported that the estrogen receptor α (*ER α* or *ESR1*) gene polymorphism IVS1-401 T/C, located within the first intron, can affect the plasma lipid response to E₂ (Herrington *et al.* 2002), volunteers were genotyped for this mutation, and subjects with the IVS1 C/C genotype were excluded. Characteristics of the subjects are given in Table 1.

Table 1 Characteristics and fasting metabolic and lipid profile of the study volunteers. Values are expressed as means (s.d.)

	Women (<i>n</i> =10)	Men (<i>n</i> =10)	<i>P</i> value*
Age (years)	59 (4)	61 (6)	0.423
BMI (kg/m ²)	27.6 (6.2)	26.0 (2.9)	0.481
Lipids			
TC (mg/dl)	206 (31)	178 (34)	0.069
LDL-C (mg/dl)	111 (33)	87 (36)	0.136
sdLDL-C (mg/dl)	26 (8)	29 (13)	0.555
HDL-C (mg/dl)	66 (16)	46 (16)	0.011*
TG (mg/dl)	78 (28)	99 (46)	0.229
Glucose (mg/dl)	91 (7)	91 (8)	0.976
Plasma CRP (μ g/ml)	3.9 (3.6)	1.4 (0.91)	0.053
ER α IVS1	T/C=5, T/T=5	T/C=8, T/T=2	0.349

**P* value for gender difference.

Plasma lipid measurements and LDL isolation and oxidation

Plasma lipid levels were determined using enzymatic assays (Roche Diagnostics). Plasma levels of small dense LDL-C (sdLDL-C) were assessed using an enzymatic assay (Denka Seiken Corporation, Tokyo, Japan) as described previously (Ai *et al.* 2008). Plasma CRP levels were measured using a high-sensitivity immunoturbidimetric assay (Roche Diagnostics).

LDLs were isolated from the pooled plasma of the donors by rapid single-step ultracentrifugation using a Beckman NVT90 rotor as described previously (Vieira *et al.* 1996). After desalting, LDLs were oxidized by the addition of 100 μ M CuSO₄/100 μ g protein. Oxidation extent was monitored by the formation of conjugated dienes at 234 nm. When absorbance began to increase exponentially ($\approx$ 1.5 h), LDLs were placed on ice and immediately desalted using chromatography columns (Bio-Rad Laboratories) to stop further oxidation. This typically produced a thiobarbituric acid-reactive substance (TBARS) value of 6–8 nM malondialdehyde/ μ g protein. TBARS measurements were performed as described previously (Cathcart *et al.* 1991). The moderately oxidized LDLs (oxLDL) were 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. The same batch of oxLDLs was used for all the experiments.

Isolation and culture of HMDMs

Blood was drawn in tubes containing 0.1% EDTA and centrifuged at 250 *g* for 30 min (25 °C) to remove plasma. Buffy coats were obtained by layering blood diluted 1:2 with RPMI culture medium over Ficoll-Paque followed by centrifugation (37 min, 500 *g*, 25 °C). White blood cells were collected and washed twice with RPMI medium. Cells were plated in RPMI medium containing 100 U/ml penicillin and 100 μ g/ml streptomycin. After 3–5 h of incubation at 37 °C in 5% CO₂, nonadherent cells were washed off, and the remaining monocytes were cultured in

RPMI medium containing 10% autologous serum. Cells were allowed to differentiate for 10 days in the presence of vehicle (ethanol), E_2 (2 or 20 nM), or testosterone (10 or 100 nM), with the medium being changed every 3–4 days. E_2 and testosterone solutions were prepared fresh under sterile conditions every 2 weeks by dissolving in 100% ethanol, and were stored in the dark at -80°C . HMDMs were treated with 50 $\mu\text{g}/\text{ml}$ oxLDL for 48 h (days 11–12) in the presence of 10% autologous serum and hormone, and were then exposed to hormones in addition to the medium without serum for an additional 24 h. The serum-free medium was collected, centrifuged to remove cell debris, and stored at -80°C . Cells were collected in a lysis buffer (0.1 M KH_2PO_4 , 0.05 M NaCl, 5 mM cholic acid, and 0.1% Triton X-100), and the cell protein was quantified using the bicinchoninic acid method with BSA as a standard.

Real-time PCR

Total cellular RNA was isolated using the RNeasy mini kit according to the manufacturer's instructions. Hundred nanograms of RNA were reverse transcribed using the SuperScript III Reverse Transcription kit, and were amplified on a real-time PCR system 7300 using specific primers. The primer sequences were as follows: β -actin (F' -TGA-AGTGTGACGTGGACATCC and R' -CTCAGGAGGAG-CAATGATCTTG), *TNF- α* (F' -TGGAGAAGGGTG-ACCGACTC and R' -TCCTCACAGGGCAATGATCC),

IL6 (F' -GTGGCTGCAGGACATGACAA and R' -TGAG-GTGCCCATGCTACATTT), *IL-1 β* (F' -TTATTACAGTG-GCAATGAGGATGAC and R' -CGCCATCCAGAGGGCAG), CRP (F' -ATTCAAGGCCCTTGT-ATCACTGG and R' -AACAGCTTCTCCATGGTCACG), androgen receptor (AR) (F' -GACTCCGTGCAGCCTATTGC and R' -TCTGCC-ATCATTTCCGGAA), *ER α* or *ESR1* (F' -CGGCATTC-TACAGGCCAAA and R' -GCGAGTCTCCTTGGCAGATTTC), and *ER β* or *ESR2* (F' -TACAATCGATAAAAA-ACCGGCG and R' -GGGAGCCACACTTCACCATT). Primers were designed using Primer Express software (Foster City, CA, USA), included intron/exon boundaries, and were validated for efficiency and specificity using the standard curve dilution and melting point analyses. Real-time PCR was carried out for 40 cycles at 95°C (15 s) + 60°C (1 min) using the Power SYBR Green Master Mix. Changes in gene expression were assessed by $\Delta\Delta C_t$ analysis with β -actin as the control/housekeeping gene. Changes were expressed as the percent of control (vehicle only).

TNF- α ELISA

TNF- α concentration in the cell culture medium was measured using an ultrasensitive human *TNF- α* ELISA kit according to the manufacturer's instructions (Alpco Diagnostics, Salem, NH, USA). The final *TNF- α* concentrations were adjusted for cell protein.

Figure 1 Effect of E_2 or testosterone treatment on proinflammatory cytokine expression in HMDMs. HMDMs were cultured in 10% autologous serum and differentiated over 10 days in the presence of vehicle (control), 2 nM E_2 , 20 nM E_2 , 10 nM testosterone, or 100 nM testosterone. HMDMs were then exposed for 48 h to oxLDLs with the hormone. RNA was isolated, and the expression of proinflammatory cytokines was measured. Data are expressed as the percent of control; * $P < 0.05$.

Statistical analyses

Statistical analyses were performed using SAS software (version 9.1; SAS Institute, Cary, NC, USA), while correlations were determined using GraphPad Prism software (version 4; La Jolla, CA, USA). Gene expression results are expressed as the percent of control (vehicle treatment only). Means and s.d. are representative of the treatment response in macrophage cultures donated by ten females or ten males. Statistical differences were determined by two-way ANOVA for both treatment effect and sex effect using Tukey's Student Range test.

Results

Both hormone receptors *AR* and *ER α were expressed in HMDMs (average C_t of 33 and 32 respectively, on real-time PCR, compared with an average C_t of 24 for β -actin). There was no difference in the expression of *AR* between female and male HMDMs, while the expression of *ER α was threefold higher in female HMDMs than in male HMDMs. The expression of *ER β was too low to be accurately quantified ($C_t > 36$).***

E_2 treatment did not significantly affect the expression of *TNF* α , *IL6*, and *IL-1* β in HMDMs (Fig. 1). Relative to control, both physiological and pharmacological concentrations of testosterone significantly reduced *TNF* α expression in HMDMs obtained from both males and females (Fig. 1). These reductions averaged 20–25% at 10 nM and 25–30% at 100 nM (Fig. 1). *TNF* α concentration in the medium was measured in the vehicle-, 2 nM E_2 -, and 10 nM testosterone-treated cells (Fig. 2). Similar to the effect on gene expression, testosterone treatment, but not E_2 treatment,

Figure 2 Effect of E_2 or testosterone treatment on *TNF* α secretion by HMDMs. As described in Fig. 1, HMDMs were differentiated for 10 days in 10% autologous serum and the corresponding hormone, and were then exposed to oxLDLs for 48 h, followed by exposure to the medium without serum for 24 h. Indicated hormone was given for the entire duration of the experiment. Medium was collected, and *TNF* α concentration was measured and adjusted for cell protein. Data are expressed as the percent of control; * $P < 0.05$.

Figure 3 Effect of E_2 or testosterone treatment on HMDM CRP expression. HMDMs were cultured as described in Fig. 1, and the expression of CRP was measured. Data are expressed as the percent of control.

significantly reduced HMDM *TNF* α secretion, compared with the control. There was no statistical difference between the genders. Testosterone treatment significantly reduced *IL-1* β expression at 10 nM in males and at 100 nM in females, but it did not affect *IL6* expression (Fig. 1).

The effect of E_2 on CRP expression was variable in both women and men (Fig. 3). The change in CRP expression by HMDMs in response to E_2 treatment, but not in response to testosterone treatment, was positively associated with the concentration of sdLDL-C in the plasma of the donors (Fig. 4A and B). Donors with plasma sdLDL-C > 30 mg/dl showed a significant increase in HMDM CRP expression with estrogen treatment, whereas there was no effect in HMDMs obtained from donors with sdLDL-C < 30 mg/dl (Fig. 4C). This effect was gender independent. The change in CRP expression with E_2 was not associated with other parameters (age, body mass index, other plasma lipid, or CRP levels).

Discussion

The effect of sex hormones on macrophage inflammation is an important area of investigation because of the contribution of inflammation to CHD. The hormone receptors AR and ER, which are expressed in both male and female HMDMs, are the likely mediators of the effects of sex hormones on macrophage function (Cutolo *et al.* 1996). Low serum testosterone levels in men have been associated with increased atheroma formation (Hak *et al.* 2002). In addition, hypogonadal men have been found to have higher serum cytokine levels than healthy men, and androgen supplementation was found to reduce these levels (Yesilova *et al.* 2000). Testosterone treatment has been shown to suppress cytokine expression in rodent macrophages and in human

Figure 4 Association between fasting plasma sdLDL-C concentration of the donors and HMDM *CRP* gene expression in response to E₂. (A) Association between the change in *CRP* expression during 2 nM E₂ treatment, relative to the control (vehicle), and the sdLDL-C levels of the donors. (B) Association between the change in *CRP* expression during 20 nM E₂ treatment, relative to control (vehicle), and the sdLDL-C levels of the donors. Both the correlations were significant ($P < 0.01$) and were gender independent. (C) Effect of E₂ on *CRP* expression as a function of the plasma sdLDL-C level of the donors. The number of subjects with sdLDL-C < 30 mg/dl is 12, and the number of subjects with sdLDL-C > 30 mg/dl is 8. Data are expressed as the percent of control; * $P < 0.05$.

monocytes obtained from younger individuals (Chao *et al.* 1995, Kanda *et al.* 1996, 1997, D'Agostino Milano *et al.* 1999). Therefore, we hypothesized that similar changes would occur in HMDMs obtained from older individuals. In cells that were exposed to moderately oxidized LDLs, testosterone significantly reduced proinflammatory cytokine expression, specifically *TNF- α* and *IL-1 β* expression, in agreement with the previous studies (Chao *et al.* 1995, Kanda *et al.* 1996, 1997, D'Agostino Milano *et al.* 1999). The effects of testosterone on *TNF- α* and *IL-1 β* were observed at physiological and supraphysiological concentrations. Macrophages express the enzyme aromatase, and are therefore able to convert testosterone to E₂ (Mor *et al.* 2001). Since reductions in *TNF- α* and *IL-1 β* were only observed with testosterone, it is reasonable to assume that the effect observed with testosterone treatment was not due to aromatization to E₂, but due to an androgen-specific action of testosterone.

NF- κ B (NFKB), a transcription factor that plays a direct role in the expression of numerous proinflammatory cytokines including *TNF- α* and *IL-1 β* (Li & Verma 2002), may be the mediator of the repression of cytokine expression by testosterone. Several studies have shown that AR activation suppresses *NF- κ B* activity (Hatakeyama *et al.* 2002, Libby 2002, Itoh *et al.* 2007), presumably by increasing the expression of *I κ B- α* (NFKB1A; Death *et al.* 2004). *NF- κ B* inhibition by testosterone is also associated with enhanced macrophage apoptosis (Cutolo *et al.* 2005), suggesting that the reduction in *TNF- α* expression is accompanied by an immunosuppressive effect.

The effect of E₂ on cytokine expression is less clear. Short-term exposure to E₂ (<1 h) *in vitro* has been reported to decrease the production of proinflammatory cytokines in lipopolysaccharide (LPS)-activated rodent macrophages (Ghisletti *et al.* 2005). In contrast, several studies have reported that long-term exposure to E₂ *in vivo* enhances the proinflammatory cytokine production in LPS-activated rodent macrophages (Soucy *et al.* 2005, Calippe *et al.* 2008). In our study, in which HMDMs obtained from 50- to 70-year-old male and postmenopausal female donors were cultured in the continual presence of E₂, we observed no effect on proinflammatory cytokine expression compared with the vehicle-treated cells.

Randomized, placebo-controlled trials in postmenopausal women have shown that orally delivered, but not transdermally delivered, estrogen therapy increases plasma CRP concentrations (Cushman *et al.* 1999, Hodis *et al.* 2008). It has been speculated that hepatic metabolism of the orally delivered therapy is responsible for this rise in plasma CRP levels (Zegura *et al.* 2003). However, since CRP is also produced by macrophages in the aortic lesions, plasma CRP level changes in response to E₂ treatment may not predict CRP changes in the arterial wall. Because CRP has been shown to elicit proatherogenic effects such as the promotion of macrophage cholesterol accumulation (Singh *et al.* 2008), CRP secreted by macrophages may play a role in lesion development. The CRP expression in response to E₂ treatment was quite variable in both female and male

HMDM donors. It has been shown previously that the lipoprotein composition of culture serum may influence the cellular response (de la Llera Moya *et al.* 1994). Since HMDMs were cultured in 10% autologous serum, we tested the hypothesis that changes in *CRP* expression by E_2 may be dependent on the plasma levels of lipoproteins of the donors. The *CRP* gene response to E_2 was significantly correlated with plasma sdLDL-C levels, with a greater increase in *CRP* expression in subjects with higher sdLDL-C levels. Individuals with a high concentration of sdLDL-C often display some degree of dyslipidemia and a greater degree of chronic inflammation and CHD (Vakkilainen *et al.* 2003, Krauss & Siri 2004). This indicates that in a proatherogenic lipid environment, E_2 may promote arterial disease, an effect that is not present under a healthy lipid environment. This concept is supported by clinical evidence suggesting that E_2 therapy is beneficial in younger premenopausal women, but harmful in older postmenopausal women (Rossouw *et al.* 2007). In support of our findings, a recent study done by Norata *et al.* (2009) has shown a greater expression of several proinflammatory molecules in HMDMs obtained from individuals with high sdLDL-C levels than in those obtained from individuals with lower sdLDL-C levels. Furthermore, in postmenopausal women, plasma sdLDL-C levels have been found to be significantly associated with plasma CRP concentration, further indicating the link between sdLDL-C and inflammation (Muzzio *et al.* 2007). We did not observe a significant correlation between these two factors in our study (data not shown). However, this may be due to the small sample size.

To our knowledge, our study is the first to report a modulation of *CRP* expression by E_2 in HMDMs. The mechanism by which high sdLDL-C levels may alter the effect of E_2 on *CRP* expression is not currently known. *IL6* and *IL-1 β* are the known stimulators of *CRP* expression (Calabro *et al.* 2003). In the current study and in the study done by Norata *et al.* (2009), sdLDL-C concentrations were not associated with HMDM *IL6* expression. Similarly, the expression of *IL6* and *IL-1 β* did not change with E_2 treatment. Therefore, the observed up-regulation of macrophage *CRP* expression by E_2 is not driven by greater macrophage *IL6* or *IL-1 β* expression. An *IL6*-independent stimulation of *CRP* by hormone replacement has also been suggested by observational and intervention studies (Lakoski & Herrington 2005). The expression of *Stat3*, a transcription factor that regulates the expression of *Crp*, has been shown to be increased by estrogen in the ob/ob mouse model of obesity, possibly through *E α* binding to the promoter region of *Stat3* (Gao *et al.* 2006). How sdLDLs may modulate the effect of E_2 on *CRP* expression is currently not known. sdLDLs are known to be in circulation longer than LDLs, and therefore are more likely to undergo oxidation (Millar & Packard 1998).

Overall, these data suggest that testosterone may protect against the progression of atherosclerosis by inhibiting the expression of select proinflammatory cytokines in human macrophages, while E_2 may not be as potent in this regard.

Furthermore, E_2 may actually encourage macrophage *CRP* production under conditions of high sdLDL-C, thereby potentially exacerbating atherosclerosis in individuals at risk of the disease. Understanding the mechanism of sdLDL-C-induced macrophage inflammation and how E_2 modulates this is an important step in defining the role of both sdLDL-C and E_2 in the progression of CHD.

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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