Estrogen and Progesterone Reduce Lipid Accumulation in Human Monocyte-Derived Macrophages
A Sex-Specific Effect

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Background—Males have an earlier onset and greater prevalence of clinical atherosclerosis than age-matched females, which is consistent with an atheroprotective effect of the female sex steroids, estrogen and progesterone. We therefore examined the effects of estrogen and progesterone on human foam cell formation, a key early event in atherogenesis.

Methods and Results—Monocytes from healthy female and male donors were obtained from white cell concentrates and allowed to differentiate into macrophages over 10 days. These human monocyte-derived macrophages (MDMs) were exposed to either control (0.1% vol/vol ethanol) or estrogen or progesterone treatment on days 3 through 10. Lipid loading was achieved on days 8 through 10 by incubation with acetylated LDL. Lipid from the MDMs was then extracted for analysis of cholesteryl ester (CE) content. 17β-Estradiol at both physiological (2 nmol/L) and supraphysiological (20 and 200 nmol/L) concentrations produced a significant reduction in macrophage CE content (88 ± 3%, 88 ± 2%, and 85 ± 4%, respectively; P < 0.02 compared with control). Physiological and supraphysiological levels of progesterone (2, 10, and 200 nmol/L) produced an even more dramatic reduction in CE content (74 ± 9%, 56 ± 10%, and 65 ± 8%, respectively; P < 0.002 compared with control). This effect could be abrogated by coincubation with the progesterone receptor antagonist RU486. Neither estrogen nor progesterone produced a reduction in lipid loading in male-donor–derived MDMs. Detailed lipid trafficking studies demonstrated that both estrogen and progesterone altered macrophage uptake and/or processing of modified LDL.

Conclusions—Physiological levels of estrogen and progesterone are associated with a female-sex–specific reduction in human macrophage lipid loading, which is consistent with an atheroprotective effect. (Circulation. 1999;100:2319-2325.)

Key Words: cells • atherosclerosis • lipoproteins • lipids
of deoxygenated PBS (calcium and magnesium free; Flow Laboratories) containing 0.1 mg/mL chloramphenicol (Boehringer Mannheim) and 1.0 mg/mL EDTA. The LDL was stored in the dark at 4°C and used within 2 weeks.

Acetylation of LDL
LDL was acetylated at 4°C by a modification of a previous method\textsuperscript{20} with 6 μL of acetic anhydride per 1 mg of LDL protein. After acetylation, LDL was again dialyzed 4 times in 1 L of PBS containing chloramphenicol (0.1 g/L) and EDTA (1 mg/mL) over 16 hours to remove excess saturated sodium acetate and acetic anhydride, filtered (0.45 μm filter), and stored in the dark at 4°C to be used within 1 week. Adequate acetylation of LDL was confirmed before use by observation of a relative electrophoretic mobility of ≥2.5 for AcLDL compared with native LDL on 1% Universal agarose gels (Ciba-Corning) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 minutes.

Iodination of AcLDL
AcLDL was iodinated with \textsuperscript{125}I (carrier-free sodium iodide, activity 200 MBq) by the iodine monochloride method.\textsuperscript{21} The \textsuperscript{125}I-labeled AcLDL was passed through a PD-10 column (Sephadex G-25M, Pharmacia), to remove unreacted iodide and then dialyzed 4 times against 1 L of PBS/EDTA/chloramphenicol, filtered (0.45 μm filter), and stored at 4°C to be used within 1 week. Specific activity was assessed before use and ranged between 250 and 750 counts/min per 1 mg of LDL protein.

Preparation of Macrophage Cell Extracts
After the human MDMs were washed 3 times with ice-cold PBS, cells were lysed with 0.6 mL of cold 0.2 mol/L NaOH at 4°C for 15 minutes. From the lysate, 0.2 mL was used for cell protein estimation, and the remaining 0.4 mL was added to 0.6 mL of ice-cold PBS and immediately extracted into methanol (2.5 mL) and hexane (5 mL) in the presence of 20 μmol/L butylated hydroxytoluene (Sigma) and 2 mmol/L EDTA. Samples were stored after extraction at −80°C until analysis for free cholesterol (FC) and cholesteryl ester (CE) was performed, usually within 7 days.

Analysis of Cholesterol and CE's
Cholesterol and CE's were separated by reverse-phase high-performance liquid chromatography at room temperature on a C-18 column (Supelco) as described previously.\textsuperscript{22} CE's were analyzed with an eluent of acetoni triol/isopropanol (30:70 vol/vol), whereas FC values were assessed with acetoni triol/isopropanol/water (44:54:2 vol/vol), with detection at 210 nm absorbance for both parameters (Activeon UV-200 absorbance detector).

Cholesterol and CE's were quantified by the derivation of standard curves by use of commercially available standards (Sigma). The curves expressed a linear relation between the chromatographic peak areas and the mass of the standard, which enabled us to quantify individual cholesterol compounds in nanomoles per milligram of cell protein.

Protein Estimation
All protein estimations were performed by the bicinchoninic acid method (Sigma) with BSA used as a standard. Samples were incubated for 60 minutes at 60°C before measurement of absorbance at 562 nm.

\textsuperscript{125}I-Labeled AcLDL Trafficking Experiments
For all of the following lipoprotein trafficking experiments, MDMs were treated as described previously. Instead of AcLDL loading on day 8, cells were treated with \textsuperscript{125}I-labeled AcLDL to assess lipid-handling mechanisms.

Cell-Surface Lipoprotein Binding at 4°C
After 8 days of propagation, cells were washed twice with PBS and cooled to 4°C for 20 minutes, and cell-surface–bound AcLDL was determined after a 4-hour incubation at 4°C with \textsuperscript{125}I-AcLDL (1, 2,
5, 8, 10, 20, 30, and 40 μg/mL) with and without a 30-fold excess of unlabeled AcLDL to assess specific and nonspecific binding. Each condition was performed in triplicate cultures. After incubation, macrophages were washed 5 times with ice-cold PBS containing 2 mg/mL BSA, then washed 3 times with PBS as described by Goldstein et al.23 Cells were lysed with 0.2 mol/L NaOH, and lysates were assessed for protein content and cell-associated (cell surface) radioactivity. Binding parameters were analyzed by the LIGAND computer program.24

**Incubation at 37°C Followed by Surface Binding at 4°C**
Macrophages were incubated at 37°C for 4 hours with 20 μg/mL unlabeled AcLDL and washed as described previously at 4°C, and the media were replaced with RPMI (containing 2 mg/mL BSA and 20 μg/mL 125I-AcLDL) for an additional 4 hours at 4°C to reassess surface binding–site availability after metabolism of AcLDL at 37°C.

**Retroendocytosis Studies**
Previous studies in the THP-1 cell line have shown that estrogen action in reducing macrophage lipoprotein metabolism is independent of lysosomal degradation of AcLDL.25 We therefore explored the possibility that estrogen and progesterone might affect the membrane traffic of AcLDL in human macrophages. A role for sex steroid hormone modulation of cellular lipoprotein-receptor pathways has been demonstrated in the estrogen-mediated increase in hepatocyte LDL transcytosis via the asialoglycoprotein receptor.26 In macrophages, internalized lipoprotein-scavenger receptor complex dissociates in the acidic environment of the endosome, with the receptor recycling to the cell surface and the majority of the AcLDL proceeding to lysosomal degradation.27 A shift in the balance between degradation and retroendocytosis of intact AcLDL would contribute to a reduction in net uptake of modified lipoprotein. We examined rates of retroendocytosis by internalizing labeled AcLDL 4 times between 15 minutes and 2 hours and then measuring the release of intact AcLDL to the extracellular media over 60 minutes. At 8 days, cells were incubated with 20 μg/mL 125I-AcLDL at 37°C for either 15, 30, or 120 minutes. At each time point, the respective wells were rapidly cooled to 4°C to inhibit additional intracellular transport and washed as described previously28 with the exception of an acid wash (0.2 mol/L acetic acid, 0.5 mol/L NaCl, pH 2.4) to remove surface-bound 125I-AcLDL, followed by the final 3 PBS washes. By removing ≥80% of the specific surface-bound AcLDL during acid washing29 before the chase incubation, these studies ensured that the intact AcLDL in the TCA-treated media was comprised of lysosomal degradation. At each time point, the chase incubation involved the addition of fresh RPMI (containing 2 mg/mL BSA and 100 μg/mL of unlabeled AcLDL) at 37°C for 1 hour. The media were then collected and precipitated with TCA, and the radioactivity of the pellet was measured (intact AcLDL released into the media from inside cells). In addition, a separate chase of only 15 minutes was performed on the cells that received a 2-hour pulse, to preferentially measure retroendocytosis from organelles close to the surface (e.g., early endosomes). Viability after each trafficking experiment was assessed by the trypan blue exclusion method and confirmed to be ≥90%.

**Statistical Analysis**
Results are expressed as mean±SEM of ≥3 wells per condition per donor, with n=11 for female-donor MDMs and n=8 for male-donor MDMs. Because of individual donor variability, results for each donor have been expressed as a percentage of control values, and individual experimental results (in percent of control values) have been pooled to give the final results for male and female-donor cells under control and hormone-treated conditions. Statistical significance was analyzed by a 1-way ANOVA and post hoc pairwise testing between conditions. Statistical significance was inferred at a 2-sided P value <0.05.

**Results**

**Effects of Estrogen and Progesterone on Macrophage Lipid Loading**
17β-Estradiol produced a significant reduction in macrophage CE accumulation in premenopausal female MDMs (n=11) (Figure 1A). This occurred at both physiological concentrations and supraphysiological concentrations of 17β-estradiol (88±3%, 88±2%, and 85±4% for 2, 20, and 200 nmol/L estrogen, respectively; P<0.02 for each concentration compared with control but no significant differences...
between concentrations). This effect could not be reversed by coincubation with the estrogen receptor antagonist ICI 182780 (shown in Figure 1A). Furthermore, MDMs exposed to the potent nonsteroidal estrogen receptor agonist diethylstilbestrol did not show a significant reduction in CE content (95±4%; \( P = 0.1 \) compared with controls), which further suggests that the effect of \( 17\beta \)-estradiol occurred independently of the classic estrogen receptor known to be present in these cells.

Physiological levels of progesterone produced an even more dramatic reduction in CE accumulation (74±9%, 56±10%, and 65±8% for 2, 10, and 200 nmol/L progesterone, respectively; \( P < 0.002 \) for each concentration compared with control but no significant difference between concentrations) (Figure 1A). This reduction in cellular CE content with progesterone could be abrogated by coincubation with the progesterone receptor antagonist RU 486 (112±8% and 91±13% for 2 nmol/L progesterone per 100 nmol/L RU 486 and 10 nmol/L progesterone per 100 nmol/L RU 486, respectively; \( P > 0.5 \) compared with controls) (Figure 1). RU 486 treatment alone was not significantly different from controls (93±8%, \( P = 0.1 \)).

Intracellular FC levels were also reduced in the estrogen-treated female-donor MDMs (86±4%, 88±3%, and 85±6% for 2, 20, and 200 nmol/L estrogen, respectively; \( P < 0.02 \) for each concentration compared with controls) but not in progesterone-treated MDMs (108±8%, 105±8%, and 117±11% for 2, 10, and 200 nmol/L progesterone, respectively; \( P > 0.1 \) compared with controls).

By contrast, CE accumulation in MDMs from male donors was not decreased by either \( 17\beta \)-estradiol or progesterone (for example, 105±9% and 115±8% for estrogen 200 nmol/L and progesterone 10 nmol/L, respectively; \( P > 0.05 \) compared with controls). Similarly, FC levels in male-donor MDMs were not significantly influenced by either female hormone (data not shown).

These sex-specific effects of estrogen and progesterone were also observed when the male- and female-donor MDMs were propagated in phenol red–free RPMI supplemented with 10% male serum (as opposed to 10% postmenopausal female serum). There was no significant effect of \( 17\beta \)-estradiol or progesterone on lipid loading of male-donor MDMs compared with controls (data not shown), but there was a persistent reduction in lipid loading in female-donor MDMs exposed to female hormones in the presence of male or postmenopausal female serum in the growth media. For example, cellular CE and FC values (expressed as nmol/mg cell protein) for MDMs from the same female donor, maintained in RPMI containing 10% postmenopausal female serum compared with 10% male serum, were not significantly different from each other for each of the treatment conditions (control 277±12 versus 301±3, \( P = 0.1 \); estrogen 200 nmol/L 233±9 versus 264±12, \( P = 0.1 \); progesterone 10 nmol/L 221±18 versus 245±15, \( P = 0.4 \)).

**Estrogen and Progesterone Action on Macrophage Uptake and Processing of Modified LDL**

To understand the effects of estrogen and progesterone on AcLDL loading, we conducted binding and intracellular processing studies of radiolabeled AcLDL in premenopausal female-donor macrophages, in which there were significant hormonal effects on intracellular lipid loading.

**Surface Binding Studies**

Surface binding studies performed at 4°C over a range of concentrations for radiolabeled AcLDL with or without a 30-fold excess of unlabeled ligand showed no significant difference in binding between control cells and cells treated with estrogen (Figure 2A) or progesterone (Figure 2B). When specific and nonspecific binding kinetics were analyzed by the LIGAND computer program,24 scavenger receptor affinity and binding-site numbers were not significantly different between treatment groups (\( K_d \) values 1.0×10⁻⁸, 9.7×10⁻⁹, and 9.6×10⁻⁹ mol/L for control, estrogen 2 nmol/L, and progesterone 10 nmol/L, respectively, \( P > 0.9 \); receptor site concentrations 8.8×10⁻¹¹, 7.1×10⁻¹¹, and 7.1×10⁻¹¹ mol/L for control, estrogen 2 nmol/L, and progesterone 10 nmol/L, respectively, \( P > 0.4 \)).

Because of the previously reported temperature-sensitive nature of the scavenger receptor ligand-binding domain,30 binding at 4°C may not be representative of in vivo binding. We therefore incubated the MDMs with unlabeled AcLDL for 4 hours at 37°C and then rapidly cooled the cells to 4°C before challenging them with a single concentration of
radioactive ligand and excess AcLDL. In this protocol, binding represents the steady state, with unoccupied receptor sites remaining at the surface during uptake at 37°C. Compared with controls, estrogen- and progesterone-treated macrophages were able to bind a significantly greater proportion of labeled AcLDL (114 ± 6% for estrogen 2 nmol/L and 161 ± 15% for progesterone 10 nmol/L versus 100 ± 4% control, **P < 0.001 and 0.001, respectively) (Figure 3). This suggests that at physiological temperatures, estrogen and progesterone reduce scavenger receptor occupancy and therefore subsequent internalization of ligand-receptor complexes.

Retroendocytosis Studies

Progesterone significantly increased the amount of intact AcLDL that had undergone retroendocytosis under all conditions tested (P < 0.008) (Figure 4). Furthermore, the fractions of intact ligand that had undergone retroendocytosis after 15 minutes of labeling (Figure 4, A), 30 minutes of labeling (B), and a 2-hour labeling period (C) were similar. Because early endosomes would be filled by the 15-minute time point, this suggests that retroendocytosis predominantly occurs from the early endosomal compartment. Release of AcLDL from the cells was also rapid, with the majority occurring during the first 15 minutes of the 60-minute chase (Figure 4, D). This observation is also consistent with the recycling time of lipids from an early endosomal compartment. Retroendocytosis in estrogen-treated macrophages was not significantly different from control cells.

Discussion

In this study, we found that physiological concentrations of both 17β-estradiol and progesterone decreased CE formation in human MDMs. This effect was sex specific, being observed in female- but not male-donor cells. The estrogen effect was not mediated via the classic estrogen receptor and was due predominantly to an effect on scavenger receptor occupancy and therefore lipoprotein uptake. The progesterone effect, in contrast, was receptor mediated and due to changes in both lipoprotein uptake and intracellular processing, in particular to increased retroendocytosis of modified LDL. Both estrogen and progesterone receptors are known to exist in macrophages.

Previous studies have suggested other potentially antiatherogenic effects of estrogen, including beneficial effects on the lipid profile,6,7 coagulation factors,12 cell adhesion molecule expression,31,32 arterial plaque size, and cellular proliferation.33 In an immortalized cell line that does not express estrogen receptors, supraphysiological doses of 17β-estradiol have been shown to decrease lipid uptake by macrophages25; however, the effects of physiological levels of estrogen and its antagonists on foam cell formation and the mechanism of lipoprotein uptake in female and male primary human macrophages have not been studied previously. Our observation of an uptake-mediated, receptor-independent reduction in macrophage lipid loading in female cells only is consistent with other recently published work33,34 that has also noted sex-specific effects of sex steroid hormones on atherosclerotic processes.

Few previous studies have examined the effect of progesterone on basic atherogenic processes. The mechanism for the observed benefit of progesterone on MDM lipid loading in the present study most likely relates to both reduced modified LDL internalization at 37°C and greater retroendocytosis of undegraded lipoprotein to the surface. Time-course experiments (Figure 4) suggested that retroendocytosis must occur from early endosomal compartments of the cell, because it is close to its maximal value after 15 minutes, which is approximately twice the t1/2 (8 minutes) of passage through this compartment in Chinese hamster ovary cells.27 The precise effects on the endosome remain uncertain. Altered endosomal acidification affecting receptor-ligand dissociation is one plausible explanation, although previously reported effects with estrogen in hepatocytes were small.35
Modulation of receptor affinity and sorting are other possible methods of sex steroid action.

Previous studies\(^6\) in the mouse cell line J774 have suggested that progesterone inhibits FC translocation from the plasma membrane, leading to decreased CE accumulation in foam cell macrophages. This mechanism was not observed in the present study, in which decreased intracellular CE levels in progesterone-treated MDMs were not accompanied by significant increases in FC concentration. A reduction in acyl-CoA:cholesterol acyltransferase (ACAT) activity may also lead to reduced cellular CE content; however, progesterone has been shown to be only a weak inhibitor of ACAT activity in previous studies in mouse peritoneal macrophages.\(^37\)

Competitive ligand-receptor–binding experiments at 4°C did not reveal altered scavenger receptor binding kinetics between treatment groups; however, our data showing less ligand-receptor occupancy at 37°C with estrogen and progesterone treatment could be attributed to a change in ligand-receptor affinity at 37°C. Hence, 4°C binding data may not be representative of in vivo hormone effects, consistent with the previously described temperature-sensitive nature of the scavenger receptor ligand-binding domain.\(^30,38\) The progesterone- and estrogen-related increase in radioiodinated AcLDL surface binding at 4°C after a 37°C incubation could be due to (1) reduced cell-surface scavenger receptor occupancy with modified LDL at 37°C or (2) an increase in cell-surface scavenger receptor numbers. The latter explanation is unlikely, because our data showed no treatment differences in cell-surface binding site number in MDMs exposed to hormones for 6 days at 37°C until just before the 4°C binding experiments. A plausible explanation is that estrogen and progesterone decrease receptor affinity for AcLDL at 37°C, leading to an overall decrease in ligand binding and internalization at this temperature. We attempted to examine receptor-ligand affinity at 37°C indirectly using receptor occupancy as a marker because of the inadequacy of Scatchard binding kinetics at this temperature and the limitations imposed by inhibitors of internalization.

The present study, in which decreased intracellular CE levels in progesterone-treated MDMs were not accompanied by significant increases in FC concentration, is unlikely, because our data showed no treatment differences in cell-surface binding site number in MDMs exposed to hormones for 6 days at 37°C until just before the 4°C binding experiments. A plausible explanation is that estrogen and progesterone decrease receptor affinity for AcLDL at 37°C, leading to an overall decrease in ligand binding and internalization at this temperature. We attempted to examine receptor-ligand affinity at 37°C indirectly using receptor occupancy as a marker because of the inadequacy of Scatchard binding kinetics at this temperature and the limitations imposed by inhibitors of internalization such as N-ethylmaleimide, whose broad-specificity alkylating potential could also modify sulfhydryl groups in the scavenger receptor, potentially confounding interpretation of binding data.

Sex-specific reduction in lipid loading under the influence of the female sex steroids, estrogen and progesterone, occurs in the presence of either male or postmenopausal female serum. This suggests that the sex differences are unlikely to be related to nonspecific perturbations in the cell environment but rather to specific cellular characteristics. One plausible explanation would be a difference in sex steroid hormone receptor levels or hormonally responsive cellular pathways between the sexes; this possibility requires additional study.

Foam cell formation is intimately involved in early atherogenesis\(^19,40\) and has been studied extensively in mouse and more recently in immortalized human cell lines.\(^41,42\) The methods used to purify primary monocytes from healthy donors have enabled the study of macrophage lipoprotein uptake with primary human cells from both males and females. This might be expected to reflect the in vivo situation more closely than could previously be accomplished. The presence of sex steroid receptors in these primary cells\(^33–45\) has been demonstrated recently and is important in an examination of sex steroid modulation of lipoprotein metabolism. The use of AcLDL is well established as a standard form of modified lipoprotein in foam cell experiments, because it is specific for the scavenger receptor pathway for lipoprotein uptake.\(^46\) The probable importance of this pathway in contributing to atherosclerosis has been confirmed in several animal studies.\(^47\)

In summary, the present study has shown that physiological levels of estrogen and progesterone are associated with reduced foam cell formation in a primary human cell culture system. Our data demonstrate that sex steroid hormones might influence macrophage lipoprotein metabolism by changing ligand-receptor interactions and membrane traffic. These results are consistent with a role for both estrogen and progesterone in the atheroprotection conferred by female premenopausal status and the observed sex difference in atherosclerosis development.

Acknowledgments

Dr McCrohon is supported by Sydney University and the Department of Cardiology, Royal Prince Alfred Hospital. Dr Jessup is supported by the NHMRC. Dr Celermajer is supported by the Medical Foundation, University of Sydney. We thank Dr David Handelsman and Dr Paul Williams from the Department of Endocrinology, University of Sydney, for their technical advice and assistance.

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27. McCrohon et al. Female Hormones Reduce Foam Cell Formation.
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_Circulation_. 1999;100:2319-2325
doi: 10.1161/01.CIR.100.23.2319

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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