Estradiol Inhibits Leukocyte Adhesion and Transendothelial Migration in Rabbits In Vivo
Possible Mechanisms for Gender Differences in Atherosclerosis

Lauren Nathan, Shehla Pervin, Rajan Singh, Michael Rosenfeld, Gautam Chaudhuri

Abstract—The mechanism by which estrogens protect against atherosclerosis is not known. We evaluated in vivo whether there is a gender difference in monocyte adhesion and subendothelial migration in hypercholesterolemic rabbits and whether any gender differences observed are due to estradiol. Monocyte adhesion and subendothelial migration were assessed in a blinded fashion by analyzing a standardized segment of aorta using a scanning electron microscope. We also assessed whether estradiol modulates induction of vascular cell adhesion molecule-1 (VCAM-1) protein using Western blot and flow cytometric analyses. We observed that male rabbits develop more monocyte adhesion and subendothelial migration than do female rabbits during hypercholesterolemia. We also observed that oophorectomized rabbits given physiological estradiol supplementation demonstrate fewer adherent and subendothelial monocytes than do oophorectomized rabbits given placebo. VCAM-1 protein expression was increased in aortae obtained from hypercholesterolemic, oophorectomized animals supplemented with placebo, and this increase was attenuated by estradiol. Finally, in cultured rabbit aortic endothelial cells stimulated with lysophosphatidylcholine, we observed an increase in VCAM-1 protein that was inhibited in a concentration-dependent fashion by estradiol. We have demonstrated in vivo that there is a gender difference in monocyte adhesion to endothelial cells and transendothelial migration after hypercholesterolemia and that this gender difference is due in part to estradiol. Our results also suggest that estradiol inhibits monocyte adhesion by inhibiting expression of VCAM-1.

Key Words: estrogen ■ estradiol ■ atherosclerosis ■ vascular cell adhesion molecule-1 ■ monocyte adhesion

Women in the reproductive age group are at lower risk for developing cardiovascular disease (CVD) as compared with men of similar age.1,2 This gender difference has been well documented in both human and animal studies.2–7 Hamm et al5 demonstrated that in nonhuman primates there was significantly more coronary artery stenosis in males than in females in response to an atherogenic diet. Furthermore, they found that differences in lipid levels did not account for this influence of gender in the development of CVD. Similar observations have been made in male and female rabbits after administration of a cholesterol (2%)-enriched diet.6 The protection against CVD observed in females has been ascribed to estrogen. Estrogen replacement therapy in postmenopausal women reduces the risk of CVD relative to the risk in postmenopausal women who do not receive estrogen replacement.7,8 In support of these clinical observations, Adams et al9 observed that ovariectomy in nonhuman primates resulted in a doubling of the extent of atherosclerosis in animals fed an atherogenic diet compared with a similar group of animals receiving estrogen replacement. Adams et al10 also demonstrated that in animals fed an atherogenic diet, administration of 17β-estradiol (E2) resulted in the formation of a plaque area that was 50% smaller than that seen in animals given no hormone replacement. Studies in oophorectomized rabbits also showed that estrogen administration attenuated aortic accumulation of cholesterol11 and reduced the degree of atherosclerosis after an atherogenic diet.12 However, the mechanisms by which estrogens protect against atherosclerosis are not known.

The adhesion of monocytes to endothelial cells coupled with transendothelial migration are essential components of an inflammatory response and occur continuously throughout the entire atherogenic process.13 We therefore decided to assess whether there was a gender difference in the number of monocytes that both adhere to and migrate across the endothelial lining of the aorta in vivo in rabbits fed a cholesterol-enriched (0.5%) diet and whether E2 was responsible for this difference. To further elucidate possible mechanisms by which E2 may prevent monocyte adhesion, we also assessed the role of E2 in inhibiting expression of vascular cell

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From the Departments of Obstetrics and Gynecology (L.N., S.P., R.S., G.C.) and Molecular and Medical Pharmacology (R.S., G.C.), UCLA School of Medicine, University of California, Los Angeles, Calif, and Department of Pathobiology and Program in Nutritional Sciences (M.R.), University of Washington, Seattle, Wash.

Correspondence to Gautam Chaudhuri, MD, PhD, Departments of Obstetrics and Gynecology, and Molecular and Medical Pharmacology, UCLA School of Medicine, 10833 LeConte Ave, 27-139 CHS, University of California, Los Angeles, CA 90095-1740. E-mail gchaudhu@obgyn.medsch.ucla.edu

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adhesion molecule-1 (VCAM-1). The rabbit was selected as the animal model, because the female of this species is in persistent estrus\textsuperscript{14}; therefore, the animals are under the constant influence of E\textsubscript{2}, with no compounding influence of progesterone. Furthermore, we took advantage of previous studies of the time course of lesion development in hypercholesterolemic rabbits\textsuperscript{15} to focus this investigation on those time points at which monocyte adhesion and subendothelial migration are known to first occur.

### Materials and Methods

#### Materials

Rabbit chow (modified laboratory rabbit diet 5321, with 0.5% cholesterol) was purchased from Purina Mills, Inc. E\textsubscript{2} pellets were purchased from Innovative Research of America. Hybond-polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence reaction detection reagent, and autoradiography film (HybontrxTM, Amersham Life Science, Inc.) were obtained from Hyclone Laboratories, Inc. The GeneAmp RNA PCR kit was obtained from Invitrogen. Goat F(ab\textsuperscript{9})\textsubscript{9} anti-mouse IgG FITC conjugate was obtained from Caltag Laboratories. Gelatin for coating the culture plates and all other reagents were obtained from Sigma Chemical Co. ICI-182780 was supplied by Dr. G. Rubanyi (Berlex Biosciences, Richmond, Calif). VCAM-1 antibody for cell culture studies was supplied by Dr M.I. Cybulsky (Brigham and Women’s Hospital, Boston, Mass) and for Western blot was obtained from Santa Cruz Biotechnology, Inc. GAPDH monoclonal antibodies were obtained from Chemicon.

#### Animals

Male and female New Zealand White (NZW) rabbits weighing 3 to 3.5 kg were used. All animals were initially fed regular chow for 2 weeks. Certain animals then underwent oophorectomy (OVX) with placement of either placebo or E\textsubscript{2} pellets (1.5 mg, 60-day release). Briefly, animals were placed under anesthesia with ketamine and xylazine (40 mg/kg, IM) and the placebo or E\textsubscript{2} pellets were placed subcutaneously. All animals received prophylactic antibiotics (ceflazolin, 500 mg IM). The animals were allowed to recover from surgery for the next 7 to 10 days, during which time they were fed normal chow (NC). They were then continued on NC or placed on a 0.5% cholesterol diet (HC) for 4 weeks. Animals developing evidence of postoperative infection were excluded from the study. An aliquot of blood was collected into tubes containing EDTA from each animal on a weekly basis from a peripheral ear vein to assess cholesterol, HDL, and LDL levels at weeks 0, 1, 2, 3, and 4, respectively. Only tissues from those animals with comparable AUCs were selected for assessment of monocyte adhesion and expression of VCAM-1 protein.

### Experimental Protocols

#### Protocol 1: Gender Differences in Monocyte Adhesion in Gonad-Intact Rabbits Fed Normal or Cholesterol-Enriched Chow

Animals were divided into the following 4 groups: (1) gonad-intact females fed NC for 4 weeks (n=5), (2) gonad-intact males fed NC for 4 weeks (n=8), (3) gonad-intact females fed HC for 4 weeks (n=8), and (4) gonad-intact males fed HC for 4 weeks (n=8).

#### Protocol 2: Effects of E\textsubscript{2} Supplementation on Monocyte Adhesion in Animals Fed a Cholesterol-Enriched Diet

Animals were divided into the following 2 groups: (1) OVX females supplemented with placebo and fed HC for 4 weeks (n=7) and (2) OVX females supplemented with E\textsubscript{2} and fed HC for 4 weeks (n=6).

#### Protocol 3: Effects of E\textsubscript{2} Supplementation on VCAM-1 Protein Expression in Animals Fed a Cholesterol-Enriched Diet

Animals were divided into the following 3 groups: (1) OVX females supplemented with placebo and fed NC for 4 weeks (n=5), (2) OVX females supplemented with placebo and fed HC for 4 weeks (n=5), and (3) OVX females supplemented with E\textsubscript{2} and fed HC for 4 weeks (n=5).

#### Protocol 4: Effects of E\textsubscript{2} on VCAM-1 Protein Expression in Cultured Endothelial Cells Stimulated With Lysophosphatidylcholine (Lyso-PC)

The cell groups were as follows: (1) cells incubated with vehicle, (2) cells incubated with vehicle for 48 hours followed by stimulation with 100 \textmu M lyso-PC for 15 hours (10 \textmu M stock in ethanol), (3) cells incubated with 1000 \textmu g/mL E\textsubscript{2} for 48 hours followed by stimulation with 100 \textmu M lyso-PC for 15 hours, and (4) cells incubated with 3000 \textmu g/mL E\textsubscript{2} for 48 hours followed by stimulation with 100 \textmu M lyso-PC for 15 hours. Before treating the cells, the presence of estrogen receptors on the cells was confirmed with the use of reverse transcription–polymerase chain reaction (RT-PCR) as described below. In some experiments, ICI-182780 (9 ng/mL), an estrogen receptor antagonist,\textsuperscript{16} was added in the culture medium just before addition of 3000 \textmu g/mL E\textsubscript{2}. Cells were then prepared for fluorescence-activated cell sorting (FACS) as described below.

#### Perfusion Fixation

Perfusion fixation was carried out according to the method described by Rosenfeld et al.\textsuperscript{15} After establishment of deep anesthesia, one of the femoral veins was exposed and cannulated for perfusion runoff. A cannula connected to a perfusion apparatus was inserted into the right carotid artery. One of the femoral arteries was also cannulated for placement of a pressure manometer to measure mean arterial perfusion pressure. Immediately before exsanguination, the animals were given a bolus injection of sodium pentobarbital, and the blood was flushed out with lactated Ringer’s solution at a flow rate of \( \approx 100 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \) body weight to yield an average intra-arterial perfusion pressure of 100 mm Hg. When the runoff was clear of blood, 2 to 4 liters of 2.5% glutaraldehyde in 0.1N phosphate buffer was perfused through at the above flow rate. After perfusion, the femoral vein and carotid artery were clamped, and the arteries were fixed under pressure for an additional 30 minutes. After perfusion fixation, the entire aorta and common iliac arteries were dissected from each animal. The vessels were cleaned of adherent fat and fascia, and a 1-cm area of aorta surrounding the celiac bifurcation was isolated and prepared for scanning electron microscopy.

#### Calculation of Lipid Exposure

To assure that differences in cholesterol exposure during the feeding period could not account for differences in monocyte adhesion between the groups, cholesterol exposure during the 4-week feeding period was calculated for each animal and compared with that of the other animals in the respective groups. This was done by calculating the total cholesterol, HDL, and LDL areas under the curve (AUCs) for each animal, with the following formula: \( \text{AUC}=\int_{0}^{Y}(Y/Y0)(Y1+Y2+Y3+Y4)/2 \), where \( Y0, Y1, Y2, Y3, \) and \( Y4 \) were total cholesterol, HDL, and LDL levels at weeks 0, 1, 2, 3, and 4, respectively. Only tissues from those animals with comparable AUCs were selected for assessment of monocyte adhesion and expression of VCAM-1 protein.
The segments of aorta surrounding the celiac bifurcation were opened lengthwise and pinned out on polytetrafluoroethylene. The tissue was washed in buffer, postfixed in 1% OsO₄ in PBS for 3 hours, followed by 1 hour in 1% thiocarbohydrazide and finally again in 1% aqueous OsO₄ for 2 hours, all at room temperature. The tissue was next dehydrated in ethanol, critical point-dried with CO₂ and sputter-coated with a 300-Å layer of gold palladium. A standardized area of each specimen measuring 13.5 mm² (Figure 1A) was then examined in a blinded fashion with a JEOL 35C scanning electron microscope at 15 kV (JEOL, Inc). The numbers of both adherent monocytes (Figure 1B, 1D, and 1F) and monocytes covered by endothelium (Figure 1C, 1E, and 1G) were counted.

**Tissue Preparation for Scanning Electron Microscopy**

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Western Blot
After exsanguination, the entire aorta and iliac arteries were dissected out from the animal and cleared of adherent fat and fascia. The abdominal aorta, including the renal artery branch points, was then isolated, and VCAM-1 and, in some experiments, GAPDH were then analyzed by Western blot with a polyclonal goat, anti-human VCAM-1 mouse monoclonal anti-rabbit GAPDH antibody. The tissues were homogenized in 50 mmol/L tetraethylammonium-HCl (pH 7.4) containing (in mmol/L) EGTA 0.1, EDTA 0.1, and DTT 0.5, as well as 1 mmol/L pepstatin A and 2 mmol/L leupeptin, at 4°C with the aid of a tissue grinder fitted with a polytetrafluoroethylene pestle. Protein concentration was measured by the Bradford Coomassie brilliant blue method as described by Bio-Rad Laboratories. The samples were then diluted in loading buffer (10% glycerol, 2% SDS, and 62.5 mmol/L Tris-HCl [pH 6.8], 5% β-mercaptoethanol, and 0.1% bromphenol blue). Fifty micrograms of protein in tissue homogenates was loaded in each lane, separated on 7.5% SDS-polyacrylamide gels, and transferred to Hybond PVDF membrane electrophoretically using a semidry transfer system (Pharmacia Biotech). Equal loading in each lane was confirmed by staining the PVDF membrane with the male (18–9) and female (18–6) rabbits fed NC. The entire descending thoracic aorta was excised, cleaned, and incubated with 0.08% collagenase at 37°C for 15 minutes. The membranes were then exposed to autoradiography film (Hyperfilm), and the relative intensities of the bands were quantified by densitometric analysis (Personal Densitometer SI, Molecular Dynamics). For reprobing for GAPDH, the membranes were stripped at 50°C for 30 minutes in buffer containing 2% SDS, 62.5 mmol/L Tris-HCl (pH 6.8), and 100 mmol/L β-mercaptoethanol. The membranes were immunoblotted with GAPDH antibody at 1:5000 dilution.

Cell Culture
Rabbit aortic endothelial cells (RAECs) were isolated from NZW rabbits fed NC. The entire descending thoracic aorta was excised, cleaned, and incubated with 0.08% collagenase at 37°C for 15 minutes. The aorta was rinsed with PBS, and the endothelial cells were flushed out and seeded in plastic plates precoated with 0.1% gelatin and cultured in DMEM-low glucose supplemented with 10% FBS, 12.5 μg/mL endothelial cell growth supplement, 25 μg/mL heparin, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Culture plates with 90% confluent endothelial cells were subsequently trypsinized with 0.25% trypsin-EDTA for 5 minutes and reseeded in gelatin-coated (0.1%) plates, and cells from the third passage were used for experiments. Cells were identified as endothelial by their characteristic cobblestone morphology. Before the cells were used, they were incubated with E2 for 48 hours and tested for the presence of estrogen receptors with RT-PCR as described below. Cells were placed in medium containing phenol red–free DMEM and charcoal-extracted FBS. Cells were then treated as described in protocol 4.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)
RAECs (10⁶ cells) were harvested, and total RNA was extracted using Tri-Reagent as described by the Molecular Research Center. Reverse transcription was performed with 3 μg of total RNA, 50 units of Moloney murine leukemia reverse transcriptase, and 100 pmol of oligo-dT and running the reaction at 42°C for 20 minutes. The resulting cDNA samples were PCR-amplified using the GeneAmp RNA PCR kit and 100 ng each of estrogen receptor sense and antisense primers in a 100-μL reaction mixture. The amplification procedure consisted of initial heat denaturation at 94°C for 5 minutes followed by PCR amplification of estrogen receptor cDNA for 40 cycles each of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The following set of primers was used for estrogen receptor: sense, 5′-AGGAAGAGCTTCCAGGCTT-3′, and antisense, 5′-CCAGTTGATCATGTAACCATAGC-3′. The primers were obtained from Custom Primers (Gibco-BRL). The amplified DNA fragment obtained was 381 bp, as expected. The PCR product was cloned into TA vector pCR 2.1, and its identity as estrogen receptor was confirmed by DNA sequencing (data not shown).

Fluorescence-Activated Cell Sorting (FACS)
After various incubations as described in protocol 4, the cells were trypsinized, collected in tubes, and washed 3 times with PBS. Cells (5×10⁶) were resuspended in 50 μL PBS. Rabbit VCAM-1 mouse monoclonal antibody (Rb 1/9; 2 μg) was added to each tube, which was kept shaking for 2 hours on ice. The cells were washed with cold PBS 4 times and incubated with FITC-conjugated goat F(ab′)₂ anti-mouse IgG at a dilution of 1:25 in PBS for 1 hour in ice and covered from light. After washing 4 times with PBS, the cells were fixed with 1% paraformaldehyde and analyzed by FACScan (Becton Dickinson) analysis. Results were plotted as intensity of fluorescence versus cell numbers.

Lipid Assays
Plasma total cholesterol, HDL, and LDL were measured in each animal. All samples were analyzed in triplicate using enzymatic colorimetric assays as described previously. The assays were performed in the Molecular Biology Institute Lipid and Lipoprotein Laboratory (University of California, Los Angeles, CA). This laboratory is certified by the Centers for Disease Control and Prevention–National Heart, Lung and Blood Institute Lipid Standardization Program and meets their criteria for precision and accuracy.

Radioimmunoassay for E₂
The plasma concentrations of E₂ were measured by radioimmunoassay as previously described. Briefly, 0.6 to 0.8 mL plasma containing known amounts (∼400 cpm) of [³H]E₂ were extracted with 7 mL fresh dimethyl ether, and the extract was evaporated to dryness. The dried plasma extract was dissolved in isoctane and then applied to a column of insuforial earth (Celite) for chromatoGraphic separation of E₂, as described by Brenner et al. Steroid fractions collected from the column were dried and reconstituted with assay buffer for radioimmunoassay. The results showed that peaks of radioactive and immunoreactive estrogens coincided in Celite column fractions.

Statistics
Values are expressed as mean±SEM. Comparisons of means were made by using the Student t test for unpaired values; when more than 2 means were compared, an ANOVA with repeated measurements was used. If a significant F value was found, the Scheffé test for multiple comparisons was used to identify differences among groups. Values were considered significant when P<0.05.

Results
Protocol 1: Gender Differences in Monocyte Adhesion in Gonad-Intact Rabbits Fed Normal or Cholesterol-Enriched Chow
The mean cholesterol AUCs for gonad-intact female and male rabbits fed NC for 4 weeks were 482±131 and 277±94 mg/dL, respectively, and were not significantly different from one another (P>0.5). Mean HDL and LDL AUCs were not different between groups (data not shown). The mean E₂ levels were higher in the female (25±2 pg/mL) as compared with the male (18±1 pg/mL) rabbits (P<0.05). The mean numbers of adherent monocytes per 13.5 mm² in females and
males were 0 and 1.4±1.2 monocytes per 13.5 mm², respectively, and were not significantly different from one another.

The mean cholesterol AUCs for gonad-intact female and male rabbits after 4 weeks of HC were not significantly different from one another throughout the feeding period (P>0.05) (Figure 2). Mean HDL and LDL AUCs were not different between groups (data not shown). The mean number of monocytes adherent to the endothelial surface and the number that migrated subendothelially were significantly lower in the female as compared with the male animals (Figure 3), and the mean E2 levels were higher in the female as compared with the male animals (Figure 3), indicating an inverse relationship between E2 levels and monocyte adhesion and subendothelial migration in hypercholesterolemic animals.

Protocol 2: Effects of E2 Supplementation on Monocyte Adhesion in Ovariectomized Animals Fed a Cholesterol-Enriched Diet

The mean cholesterol AUCs throughout the feeding period for animals supplemented with OVX/placebo and OVX/E2 were not significantly different from one another (P>0.05) (Figure 2). Mean HDL and LDL AUCs were also not different between groups (data not shown). The mean number of monocytes adherent to the endothelial surface and the number that migrated subendothelially were significantly greater in the placebo-supplemented than in the E2-supplemented animals (Figure 4), and the mean E2 levels were significantly lower in the placebo-supplemented than in the E2-supplemented animals (Figure 4), again demonstrating an inverse relationship between E2 levels and monocyte adhesion and subendothelial migration in animals fed a cholesterol-enriched diet. Representative photomicrographs depicting this relationship are shown in Figure 1.

Protocol 3: Effects of E2 Supplementation on VCAM-1 Protein Expression in Ovariectomized Animals Fed a Cholesterol-Enriched Diet

OVX/placebo-supplemented animals placed on HC demonstrated an increase in VCAM-1 protein expression as compared with that seen in OVX/placebo-supplemented animals placed on NC. In all animals, this increase was attenuated by supplementation with E2. In Figure 5 is shown a Western blot from representative animals in each group, and in Figure 6 is a scatterplot of the arbitrary densitometric units of data from 5 individual animals in the cholesterol-fed groups that received either placebo or E2 when the Western blot was run on the same gel. In some experiments, we compared the expression of VCAM-1 with GAPDH. We consistently observed a decrease in VCAM-1 expression in E2-supplemented animals with no change in GAPDH expression (data not shown).

Protocol 4: Effects of E2 Supplementation on VCAM-1 Protein Expression in Cultured Endothelial Cells Stimulated With Lyso-PC

Lyso-PC at 100 μmol/L increased the cellular content of VCAM-1 protein by 248% as compared with vehicle (data not shown). E2 inhibited this increase in a concentration-dependent fashion (Figure 7). Figure 8 shows the representative traces of FACS analysis from 2 separate experiments. ICI-182780 abolished the effects of E2.

Discussion

The mechanisms by which females are protected from the development of atherosclerosis compared with males and the role that estrogen plays in this process are not known. The primary objective of this study was to assess whether there was a gender difference in the adhesion of monocytes to endothelial cells in vivo in animals fed a cholesterol-enriched diet. The mean cholesterol AUCs for gonad-intact female and male rabbits after 4 weeks of HC were not significantly different from one another (P>0.05) (Figure 2). Mean HDL and LDL AUCs were also not different between groups (data not shown). The mean number of monocytes adherent to the endothelial surface and the number that migrated subendothelially were significantly lower in the female as compared with the male animals (Figure 3), and the mean E2 levels were higher in the female as compared with the male animals (Figure 3), indicating an inverse relationship between E2 levels and monocyte adhesion and subendothelial migration in hypercholesterolemic animals.

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diet and whether this can be explained on the basis of circulating E₂.

Our studies demonstrate in vivo that adhesion of monocytes to endothelial cells is retarded in females as compared with males. This difference was not mediated by any changes in circulating lipids, as the male and female animals used for the study had comparable lipid levels. Thus, reduced monocyte adhesion in females may be a partial explanation for the gender differences in the extent of atherosclerosis previously reported in this species.¹¹,¹²

To elucidate whether the higher E₂ levels seen in the ovary-intact females accounted for the gender difference in monocyte adhesion in vivo, we studied this phenomenon in ovariectomized rabbits either supplemented with placebo or supplemented with 1.5-mg, 60-day release E₂ pellets. These pellets provide near-physiological concentrations of E₂, which are concentrations comparable with those seen in ovary-intact rabbits. In ovariectomized rabbits supplemented with E₂, there were both significantly fewer monocytes adherent to endothelial cells and fewer subendothelial monocytes when compared with ovariectomized animals supplemented with placebo at 4 weeks of hypercholesterolemia. Again, this action of E₂ was not due to any E₂-induced changes in circulating lipids. It is interesting to note that the number of monocytes adherent to the endothelium in ovariectomized animals supplemented with placebo was significantly higher as compared with gonad-intact males. The role of circulating androgens on monocyte endothelial interactions was not addressed in this study. However, it is possible that androgens such as testosterone are converted into E₂ by the aromatase activity present in endothelial cells.²³

Products of lipid oxidation can activate the endothelium to support a specific increase in the adhesion of monocytes and T lymphocytes, but not polymorphonuclear leukocytes.¹³,²⁴,²⁵ VCAM-1 is likely to be the adhesion molecule responsible for providing this specificity.²⁶–²⁸ VCAM-1 is a member of the IgG superfamily²⁹ and interacts with its counterreceptor, the very late appearing antigen 4 (VLA-4), a β₁-integrin expressed on the surface of monocytes and lymphocytes but not on polymorphonuclear leukocytes.³⁰,³¹ In fatty streaks of rabbits with diet-induced or genetically determined hypercholesterolemia, the endothelial cells express VCAM-1 focally.³² Furthermore, the expression of VCAM-1 occurs in lesion-prone areas of the rabbit aorta as early as 1 week after initiation of a hypercholesterolemic diet before intimal macrophages accumulate.³² Our results demonstrated that E₂ administration at physiological concentrations to ovariectomized rabbits fed a cholesterol-enriched diet markedly decreased VCAM-1 expression when compared with ovariectomized rabbits implanted with a placebo pellet. We did not specifically evaluate whether the VCAM-1 was expressed predominantly in the endothelial cells or vascular smooth muscles. However, other investigators⁵³ have reported that in

**Figure 4.** Left, Number of adherent and subendothelial monocytes per standardized field (13.5 mm² at the celiac bifurcation in OVX/placebo (P)- and OVX/E₂-supplemented rabbits after 4 weeks of HC. Right, E₂ levels in the same rabbits depicted in the left panel at the end of the 4-week feeding period. Data are expressed as mean±SEM of 6 or 7 animals in each group. *P<0.05 denotes significant difference in number of adherent monocytes between OVX/placebo and OVX/E₂ animals (left) and in E₂ levels between OVX/placebo and OVX/E₂ animals (right).

**Figure 5.** Western blot analysis of VCAM-1 protein in aorta of female rabbits that were ovary-intact and fed NC (lane 1); ovariectomized, supplemented with placebo, and fed high-cholesterol diet (lanes 2 and 3); or ovariectomized, supplemented with E₂ and fed high-cholesterol diet (lanes 4 and 5). Each lane represents 1 animal, which was representative of its respective group (n=5).

**Figure 6.** Arbitrary densitometric units of Western blot analysis of the effects of E₂ on VCAM-1 protein expression in aorta of female rabbits. Animals were either OVX and supplemented with placebo (P) and fed HC (n=5) or OVX and supplemented with E₂ and fed HC (n=5). Samples from individual animals were run separately, all on the same gel.
rabbits fed a cholesterol-enriched diet for 6 weeks or less, VCAM-1 was expressed predominantly in endothelial cells. It is therefore likely that the differences in VCAM-1 expression observed in our studies were due to differences in endothelial cell VCAM-1 expression. Results from our studies do not indicate whether the effects of E2 in decreasing VCAM-1 expression in vivo are a result of its antioxidant properties or whether this action is mediated by the binding of E2 to the estrogen receptor.

To corroborate our in vivo data and to try and determine the mechanism by which E2 regulates VCAM-1 expression, we also investigated whether there was a corresponding effect of E2 in the presence of an estrogen receptor antagonist on VCAM-1 protein expression by RAECs in vitro. We used lyso-PC to activate our endothelial cells, as lyso-PC is a major chemotactic lipid component of both oxidized LDL and β-VLDL. In addition, the concentration of lyso-PC is increased in atherosclerotic arterial lesions in animals fed an atherogenic diet and has been demonstrated to be a selective chemoattractant for mononuclear leukocytes. Lyso-PC has also been shown to selectively induce both VCAM-1 and intracellular adhesion molecule-1 in RAECs. In keeping with previous studies, we also observed that lyso-PC (100 μmol/L) induced the expression of VCAM-1 on endothelial cells. Our results further indicated that E2 at concentrations of 1000 and 3000 pg/mL reduced the surface expression of VCAM-1 on endothelial cells in culture in the presence of lyso-PC. This action of E2 in attenuating VCAM-1 expression in vitro was abolished in the presence of the E2 antagonist, ICI-182780, which blocks the interaction of E2 with the estrogen receptor. This suggests that the action of E2 in regulating VCAM-1 expression was at least in part due to the interaction of E2 with its receptor and not via an indirect antioxidant effect of E2.

The action of E2 in modulating VCAM-1 expression in endothelial cells has also been studied by other investigators, but only in vitro, and the results are controversial. In studies of human umbilical vein endothelial cells (HUVECs), E2 suppressed the induction of VCAM-1, E-selectin, and intracellular adhesion molecule-1 mRNA expression by interleukin-1β. In contrast, Cid et al observed that E2 did not affect basal VCAM-1 expression but caused a 30% to 50% increase in the presence of tumor necrosis factor (TNF) and had no effect when the HUVECs were stimulated with IL-1. It has also been reported that E2 increased the TNF-α–induced expression of E-selectin in endothelial cells in culture but had no effect on the expression of VCAM-1. These contrasting observations may in part be due to differences in the cell types used (HUVECs versus RAECs) and the degree to which they express estrogen receptors, differences in the concentrations of E2 administered (ranging from 50 to 5000 pg/mL), and differences in the agents used to induce VCAM-1 expression (lyso-PC, IL-1β, and TNF-α).

In this regard, in both the present study and in those previously reported, the concentrations of E2 required for inhibition of VCAM-1 expression in vitro were in the supraphysiological range. This is in sharp contrast to the E2-mediated inhibition of VCAM-1 expression in vivo.
occurred at physiological E2 concentrations. At present, it is unclear why there is this discrepancy between the concentration of E2 required for the suppression of VCAM-1 expression in vivo versus in vitro. It is possible that in vivo, E2 acts by the following 2 mechanisms: inhibition of LDL oxidation with a concomitant decrease in the amount of lyso-PC formed, and a direct estrogen receptor-mediated action on the endothelial cells. Estrogen receptors are present in both endothelial cells and vascular smooth muscles in vivo.44–47 However, the relative degree to which estrogen receptors are expressed by these cells under in vitro conditions has not yet been reported. A higher expression of estrogen receptors in vivo could partly explain why lower concentrations of circulating E2 inhibit VCAM-1 expression in vivo relative to the much higher concentrations of E2 required in vitro. It is also possible that E2 may be converted into a more active metabolite in vivo, which may also partially account for the higher concentrations of E2 required to suppress VCAM-1 expression in vitro. Further work is in progress to assess this aspect.

Finally, our data also demonstrated that E2 administration reduced the number of monocytes that migrated into the subendothelial space. This could simply be a result of the reduction in the number of adhesion sites. However, this may also be due to an E2-mediated inhibition of expression of monocyte-specific chemokines. For example, in preliminary studies of a similar group of hypercholesterolemic rabbits, we observed that E2 inhibits expression of the monocyte chemotactic protein-1 (MCP-1).48 Thus, estrogen appears to have the combined property of inhibiting both monocyte adhesion and the subsequent recruitment of monocytes into the arterial intima.

Monocyte adhesion and subendothelial migration are key cellular events generally associated with the initiation of the atherogenic process. In this regard, an estrogen-mediated inhibition of these processes could not account for the fact that the protection against CVD seen in women does not manifest itself until middle age, when inflammatory cell–rich fatty streaks have generally progressed to more advanced atherosclerotic lesions. However, monocyte recruitment is part of a fibroproliferative inflammatory response that is continuous throughout the entire atherogenic process. For example, VCAM-1 expression with an associated leukocyte infiltration has been demonstrated in microvessels within advanced human plaques.49 Furthermore, there is now ample evidence that advanced plaques most frequently rupture at sites enriched with inflammatory cells and lipid.50,51 Plaque rupture in turn leads to the formation of occlusive thrombosis and the clinical sequelae of CVD. Thus, the effect of estrogen in inhibiting VCAM-1 and MCP-1 expression and monocyte adhesion and subendothelial migration is likely to have a profound impact on all stages in the atherogenic process and may also account for the protective effects in women before menopause.

In conclusion, our results suggest that reduced inflammatory cell infiltration into the artery wall due to an estrogen-mediated inhibition of the expression of VCAM-1 and/or MCP-148 is in part responsible for the protection from atherosclerosis that is seen in women.

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Estradiol Inhibits Leukocyte Adhesion and Transendothelial Migration in Rabbits In Vivo: Possible Mechanisms for Gender Differences in Atherosclerosis

Lauren Nathan, Shehla Pervin, Rajan Singh, Michael Rosenfeld and Gautam Chaudhuri

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