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*J Clin Invest.* 1996;98(1):36-42. https://doi.org/10.1172/JCI118774.

**Research Article**

One of the earliest events in atherosclerosis is interaction of circulating mononuclear leukocytes and the endothelium. Endothelial cell (EC) activation by cytokines results in expression of adhesion molecules and production of chemotactic factors, augmenting leukocyte adhesion and recruitment, respectively. The incidence of atherosclerosis in premenopausal women is significantly less than that observed in age-matched males with similar risk profiles. Because estrogen has gene regulatory effects, we investigated whether 17beta-estradiol (E2) can inhibit cytokine-mediated EC adhesion molecule transcriptional activation. Cultured human umbilical vein EC (estrogen receptor-positive) were propagated in gonadal hormone-free medium and were E2-pretreated for 48 h before IL-1 activation. Detected by FACS analysis, E2 strongly (60-80%) inhibited IL-1-mediated membrane E-selectin and vascular cell adhesion molecule-1 induction, and intercellular adhesion molecule-1 hyperinduction. 17alpha-estradiol (an inactive E2 stereoisomer) had no effect. This inhibition correlated with similar reductions in steady state-induced E-selectin mRNA levels, and was abrogated by the E2 antagonist ICI 164,384, demonstrating a specific, estrogen receptor-mediated effect. Nuclear run-offs confirmed suppression at the transcriptional level. The implications of these results for the cardiovascular protective role of estrogen are discussed.



# Effects of 17β-Estradiol on Cytokine-induced Endothelial Cell Adhesion Molecule Expression

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## Abstract

**One of the earliest events in atherosclerosis is interaction of circulating mononuclear leukocytes and the endothelium. Endothelial cell (EC) activation by cytokines results in expression of adhesion molecules and production of chemotactic factors, augmenting leukocyte adhesion and recruitment, respectively. The incidence of atherosclerosis in premenopausal women is significantly less than that observed in age-matched males with similar risk profiles. Because estrogen has gene regulatory effects, we investigated whether 17β-estradiol ($E_2$) can inhibit cytokine-mediated EC adhesion molecule transcriptional activation. Cultured human umbilical vein EC (estrogen receptor–positive) were propagated in gonadal hormone–free medium and were $E_2$-pretreated for 48 h before IL-1 activation. Detected by FACS® analysis, $E_2$ strongly (60–80%) inhibited IL-1–mediated membrane E-selectin and vascular cell adhesion molecule-1 induction, and intercellular adhesion molecule-1 hyperinduction. 17α-estradiol (an inactive $E_2$ stereoisomer) had no effect. This inhibition correlated with similar reductions in steady state–induced E-selectin mRNA levels, and was abrogated by the $E_2$ antagonist ICI 164,384, demonstrating a specific, estrogen receptor–mediated effect. Nuclear runoffs confirmed suppression at the transcriptional level. The implications of these results for the cardiovascular protective role of estrogen are discussed. (*J. Clin. Invest.* 1996. 98: 36–42.) Key words: atherosclerosis • estrogen receptor • vascular endothelium • leukocyte • gene regulation**

## Introduction

Atherosclerosis bears several histopathologic similarities to chronic inflammation. In animal models and humans, a recognizable early atherosclerotic lesion involves an inflammatory process consisting of intimal T lymphocytes and lipid-laden macrophages, also known as the fatty streak. Beyond barrier function, the endothelium is a dynamically regulated tissue which actively contributes to the development of local vascular immune and inflammatory responses. Intercellular adhesion between circulatory leukocytes and vascular endothelial cells (EC)[1] constitutes the initial trigger for many of these responses (1). These interactions are mediated by an array of leukocyte adhesion receptors and their respective EC counterreceptors, many of which are cytokine inducible (2). In the cholesterol-fed, Watanabe-heritable hyperlipidemic rabbit model of atherosclerosis, it has been reported that an induced EC adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), is expressed in a localized fashion by aortic EC overlying very early atherosclerotic lesions (3). These areas of EC activation are associated with the presence of subendothelial macrophages that presumably adhere to and transmigrate the endothelium (4).

The incidence of coronary atherosclerosis in premenopausal women is half that observed in age-matched males (5). In contrast, postmenopausal women are not "protected," although estrogen replacement can reproduce those differences in cardiovascular disease seen in the premenopausal age group (6). This apparent protective effect of estrogen on the vascular wall is poorly understood. Estrogen affects cholesterol metabolism and deposition, generally inhibiting atherosclerotic plaque formation. However, the magnitude of the lipid changes cannot fully account for the observed cardiovascular protective effects (7). Estrogen studies in animal models have demonstrated inhibition of platelet aggregation (8), diminished lipoprotein-induced arterial smooth muscle cell proliferation (9), inhibition of myointimal proliferation associated with mechanical injury or stress (10), decreased foam cell formation (11), and increased arterial smooth muscle prostacyclin production (12). The significance of these effects in human atherosclerosis is not known.

We have been interested in cytokine-mediated endothelial activation, as it relates to leukocyte adhesion. Because estrogen has important gene regulatory effects (13–15) and because cytokine-induced expression of endothelial cell adhesion molecules (CAMs) requires transcriptional activation (16–18), we used a gonadal hormone–free EC culture medium to address whether 17β-estradiol ($E_2$) has detectable effects on IL-1–mediated induction of human EC E-selectin, intercellular adhesion molecule-1 (ICAM-1), and VCAM-1. We demonstrate that in

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*Received for publication 25 July 1995 and accepted in revised form 10 April 1996.*

J. Clin. Invest.

0021-9738/96/07/36/07 $2.00
Volume 98, Number 1, July 1996, 36–42

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; $E_2$, 17β-estradiol; EC, endothelial cells; ER, estrogen receptor; ERE, estrogen response element; GHS, gelding horse serum; HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

estrogen receptor (ER)-positive EC, pretreatment with $E_2$ dramatically reduces surface expression and mRNA levels of endothelial CAMs. Nuclear run-offs confirm that this estrogen effect is mediated at the transcriptional level. We discuss the possible molecular mechanisms and implications in atherogenesis.

## Methods

*Materials.* 17β-estradiol, 17α-estradiol, and gelding horse serum (GHS) were purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant interferon-γ was obtained from Collaborative Biochemical Products (Bedford, MA). Human recombinant IL-1β was generously provided by S. Gillis (Immunex, Seattle, WA). The antiestrogen ICI 164,384 was a kind gift from A.E. Wakeling (ZENECA Pharmaceuticals, Macclesfield, United Kingdom). Monoclonal antibodies (mAbs) H4/18 (anti–E-selectin), E1/6 (anti–VCAM-1), RR1.1 (anti–ICAM-1), and CA141 (anti–HLA-DR) were generous gifts of J. Pober (Yale University), M. Bevilacqua (University of California, San Diego), T. Springer (Harvard University), and E. Engleman (Stanford University), respectively.

*Cell culture.* Human umbilical vein endothelial cells (HUVEC) were isolated from female donor umbilical veins as previously described (19). HUVEC were serially passaged on gelatin-coated flasks in phenol red–free DME (Gibco Laboratories, Grand Island, NY) containing 15% heat-inactivated GHS and supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), porcine heparin (100 μg/ml), and endothelial cell growth factor (ECGS 50 μg/ml). For some experiments, cells were propagated in 15% FBS rather than GHS. Cells were used within six passages and were identified as endothelial by their characteristic cobblestone morphology and presence of Factor VIII antigen. Complete medium was replaced with heparin- and ECGS-free medium 48 h before experiments. Unless otherwise specified, $E_2$ pretreatment was performed as a single dose 48 h before cell activation. Ishikawa human endometrial adenocarcinoma cells, known to contain a high level of ER, and HeLa cells derived from human cervical carcinoma that do not express the ER, were maintained in the DME/GHS media.

*Northern blot analysis.* Total RNA was prepared from cells by extraction with TRISOLV™ (Biotecx Laboratories, Houston, TX) according to the method of Chomczynski and Sacchi (20). RNA (20 μg/lane) was fractionated on an 0.8% agarose gel containing 0.4 M formaldehyde, transferred onto nylon membrane filters (Hybond-N; Amersham, Arlington Heights, IL), and hybridized overnight at 42°C with random primed [α-$^{32}$P]dCTP-labeled probes by standard methods (21). Membranes were washed twice in SSC, 0.1% SDS × 15 min at room temperature, once in 2× SSC, 0.1% SDS × 30 min at 50°C, and in 0.1× SSC, 0.1% SDS × 30 min at 65°C. Signals were visualized by autoradiography and densitometric analysis was performed on an Image Quant densitometric scanner (Molecular Dynamics, Sunnyvale, CA). The cDNA probes used were: ELAM/pi3HM (a gift of M. Bevilacqua, University of California, San Diego), human p35ER (gift from Dr. Geoffrey Green, University of Chicago, Chicago, IL), and a 1.0-kb fragment of human GAPDH (Clontech Laboratories, Inc., Palo Alto, CA). Probes were labeled with [α-$^{32}$P]dCTP (1–2 × $10^9$ cpm/mg) using a random-primed DNA labeling kit (United States Biochemicals Corp., Cleveland, OH). Steady state E-selectin mRNA levels were quantified after normalization to GAPDH (which was not significantly affected by cytokine or $E_2$ treatments). To generate means and standard errors between experiments results within each experiment were expressed as percentage of IL-1–induced E-selectin mRNA, as absolute cpm varied between experiments.

*Nuclear run-off.* HUVECs were incubated with or without $E_2$ for 48 h. Cells were then treated with IL-1 (20 U/ml × 1.5 h) or medium control, and nuclei isolated from 2 × $10^7$ cells per sample with lysis buffer containing 0.05% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ at 4°C. Nuclei were counted and an equivalent number was used for each experimental condition. The run-off was performed in buffer containing [α-$^{32}$P]UTP (3,000 Ci/mmol) and 4 mM rCTP, rATP, rGTP at 26°C for 30 min. The labeled nuclear RNA was isolated and hybridized overnight on nitrocellulose filters containing 2 μg of linearized, denatured ELAM/pi3HM, CD1.8 (ICAM-1/CDM8, a gift of T. Springer), VCAM-1E11/CDM8 (a gift of L. Osborn, Biogen, Cambridge, MA), α-actin/pBR322, or pBluescript DNA. The filters were washed and autoradiography was performed. Signals were quantified with an Image Quant densitometric scanner and normalized to γ-actin signals (not significantly affected by cytokine or $E_2$ treatments).

*Flow cytometry.* Immunofluorescent staining was performed using unconjugated murine mAbs followed by a secondary polyclonal goat anti–mouse IgG-FITC as previously described (22). Cytofluorographic analysis was performed on a FACSort® (Becton Dickinson, San Jose, CA), using Lysis II software. 5,000 events were analyzed per experimental sample. Fluorescence units (FU) = (mean channel fluorescence$[MCF]_{positive} - MCF_{background}$) × percentage positive. Percent inhibition was calculated as follows: 1 − [(FU $E_2$/IL-1–treated)/(FU $E_2$-treated)] × 100.

*Adhesion assays.* EC were grown to confluence in 96-well microtiter plates. PBMC were labeled with 5 μCi $Na_2CrO_4$ (New England Nuclear, Wilmington, DE)/$10^6$ cells; $10^5$ cells, approximately a five leukocyte to one EC ratio, were added per well and incubated for 90 min at 37°C. Each well was then washed four times, the remaining cells were lysed, and the lysate was counted in a gamma counter (5500B; Beckman Instruments, Inc., Fullerton, CA). The cpm were compared with maximum cpm from $10^5$ labeled cells, and percent adherence was calculated as previously described (23). Samples were performed in triplicate.

## Results

The detection of ERs on blood vessels has been variable, dependent upon the vascular bed and technique used. To ascertain that HUVEC could be utilized for estrogen studies, the presence of ERs was evaluated at the mRNA and protein level. By Northern analysis, HeLa cells were ER negative (Fig. 1, lane *1*) as previously described (24). Surprisingly, mRNA harvested from HUVEC propagated in gonadal hormone–free medium also failed to hybridize with the ER probe. HUVEC pretreated with $E_2$ for 3 and 24 h were also apparently ER-negative (Fig. 1, lanes *2* and *3*, respectively). After 48 h of $E_2$ pretreatment (Fig. 1, lane *4*), an mRNA signal comparable with that detected in the ER-positive Ishikawa cell line (Fig. 1, lane *5*) was observed. It is likely that HUVEC grown in hormone-free conditions do have a level of functional ER but the mRNA is undetectable by Northern analysis because we have demonstrated acute $E_2$ responses in those conditions (Caulin-

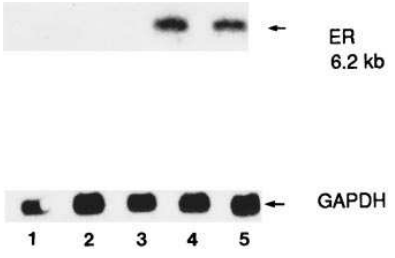


*Figure 1.* Northern blot analysis of steady state ER mRNA levels in HUVEC. HUVEC were propagated in $E_2$-free medium, followed by treatment with a single dose of $E_2$ 1,000 ng/ml × 3 h (lane *2*), 24 h (lane *3*), or 48 h (lane *4*). ER-negative HeLa (lane *1*) and ER-positive Ishikawa (lane *5*) cells were used as controls. 20 μg of total RNA was hybridized per sample. GAPDH mRNA levels were detected as internal controls. Vehicle treatment (no $E_2$) × 48 h appeared identical to lanes *2* and *3* (not shown).

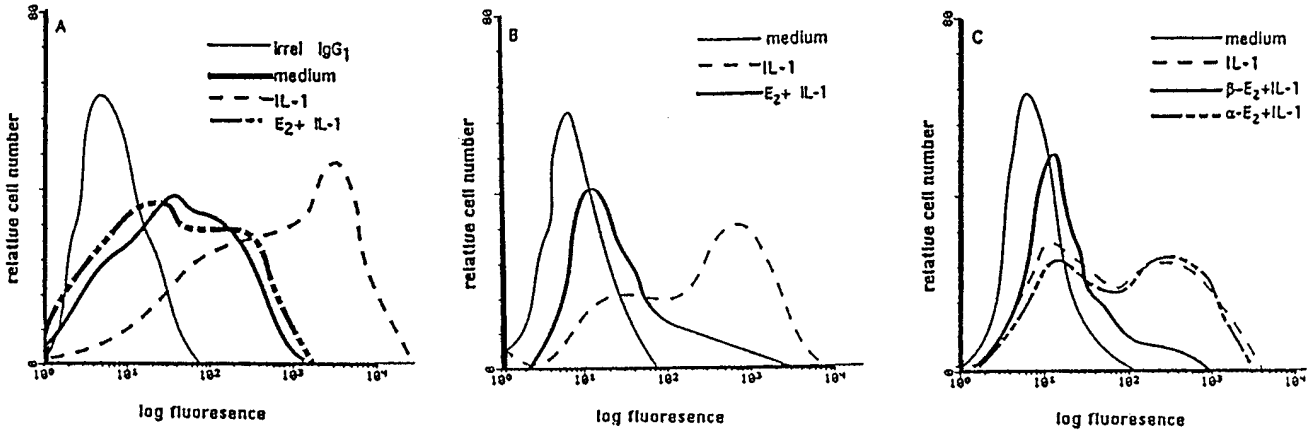


*Figure 2.* Flow cytometric analysis of $E_2$ effects on IL-1–mediated endothelial CAM induction. HUVEC were propagated in $E_2$-free medium and treated for 48 h with medium (vehicle), single dose $E_2$ 1,000 ng/ml (β-$E_2$), or α-estradiol 1,000 ng/ml (α-$E_2$), followed by a 4-h activation with IL-1 20 U/ml. Curves represent conditions as noted in each panel. *A* represents ICAM-1 membrane expression. *B* and *C* represent E-selectin expression. 5,000 events were analyzed per sample.

Glaser, T., W. Sessa, and J.R. Bender, unpublished observation), and the induction of ER message by $E_2$ may proceed via functional receptor. Nevertheless, the 48 h required for induction of significant levels may partially explain the dosing requirements in the following experiments (see below). Immunofluorescent analysis of 48-h $E_2$-pretreated HUVEC using an anti-ER mAb demonstrated intense nuclear and variable cytoplasmic staining consistent with the described predominant location of the receptor (not shown).

To evaluate the effect of $E_2$ on cytokine-mediated endothelial CAM induction, EC were grown in phenol red– and gonadal hormone–free medium, and induced with IL-1 20 U/ml after a 48-h pretreatment with $E_2$. Fig. 2 delineates the membrane expression of ICAM-1(*A*) and E-selectin (*B* and *C*), demonstrated by FACS® analysis. $E_2$ completely abrogates the IL-1–mediated EC ICAM-1 hyperinduction (Fig. 2 *A*), and greatly inhibits E-selectin induction (Fig. 2 *B*). To address whether the observed inhibition was due to a nonspecific steroid effect, ECs were pretreated with 17α-estradiol, which neither binds to nor activates ERs, instead of 17β-estradiol. Fig. 2 *C* demonstrates that, at an identical dose, 17α-estradiol had no effect on E-selectin induction, supporting a level of specificity for 17β-estradiol, presumably mediated via ERs. Furthermore, 17β-estradiol had no effect on IFN-γ–mediated EC class II HLA (HLA-DR) induction (not shown). Thus, $E_2$ does not inhibit all cytokine-mediated EC activation (transcriptional) events.

Results displayed in Fig. 2 were obtained with "high dose" $E_2$, i.e., 1,000 ng/ml. Using this culture system of EC propagation in $E_2$-free medium and one time $E_2$ dosing × 48 h, an inhibitory dose response trend was observed, down to 50 ng/ml (Fig. 3 *A*). As demonstrated in Fig. 1, significant levels of ER mRNA were only detected after 48 h of $E_2$ treatment. Furthermore, it is possible that $E_2$ is unstable in our experimental system and/or only fractionally available for cellular uptake, requiring high concentrations for maximal inhibitory effect at 48 h. Instead of a single $E_2$ dose for 48 h in $E_2$-depleted cells, parallel experiments were performed in which cells were propagated in

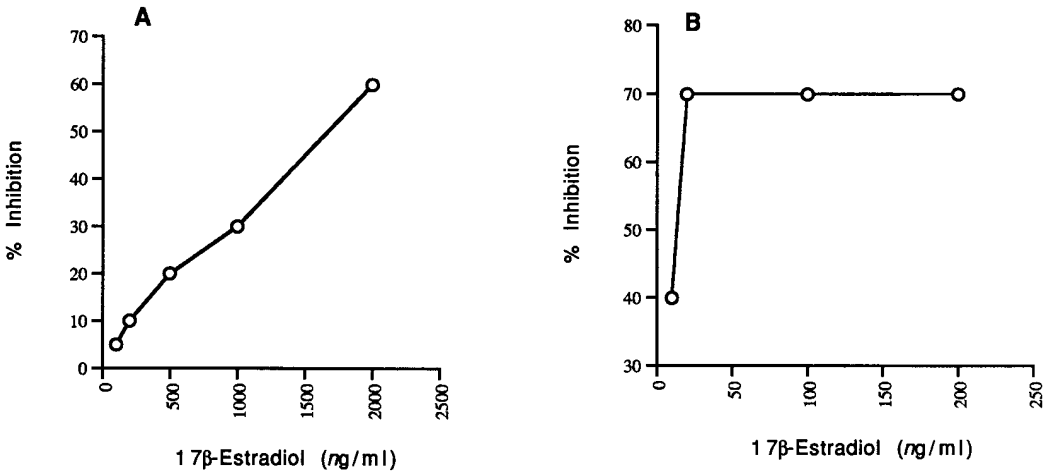


*Figure 3.* Dose response of $E_2$ inhibitory effects on IL-1–mediated endothelial E-selectin induction. HUVEC were propagated in $E_2$-free medium and pretreated with a single dose of the indicated concentrations of $E_2$ for 48 h before activation (*A*), or propagated in 15% FBS-containing medium (final [$E_2$] 5 ρg/ml), switched to $E_2$-free medium for 24 h, followed by treatments with the indicated concentrations of $E_2$ every 4 h × 48 h (medium plus $E_2$ was replaced every 4 h) before activation (*B*). During the last 4 h of $E_2$ treatment, cells were treated with IL-1 20 U/ml and subsequently analyzed for membrane E-selectin expression by flow cytometry, with 5,000 events analyzed per sample. Data are displayed as percent inhibition of IL-1–induced E-selectin membrane expression at the various $E_2$ concentrations, calculated as described in Methods.

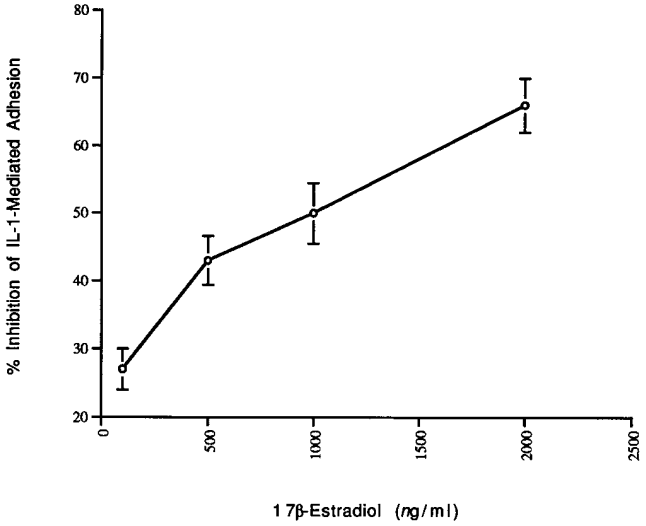


*Figure 4.* $E_2$ effect on IL-1–mediated augmentation of PBMC-EC adhesion. HUVEC propagated in gonadal hormone–free medium were pretreated (one dose) × 48 h with the indicated concentrations of $E_2$, followed by a 16-h IL-1 (20 U/ml) activation. After washing out the $E_2$ and IL-1, $10^5$ $^{51}$Cr-labeled PBMCs (preactivated with PMA 50 nM × 30 min at 37°C) were added per well, and adhesion assays were performed as described in Methods. IL-1 treatment (no $E_2$) resulted in a 150% increase in binding. Data are represented at each $E_2$ concentration as percent inhibition of IL-1–mediated adhesion, i.e., the 150% augmentation noted above. Samples were performed in quadruplicate. This experiment represents means±SE for three separate experiments.

phenol red–free DME/15% FBS (final $E_2$ concentration 5 ρg/ml), switched to $E_2$-free medium for 24 h, followed by repeated treatments with medium(vehicle) or $E_2$ every 4 h × 48 h. Fig. 3 *B* displays a representative $E_2$ dose response curve with such a protocol and demonstrates a 70% reduction in IL-1–induced E-selectin membrane expression by $E_2$ 20 ng/ml. Simultaneous treatment with $E_2$ and the $E_2$ antagonist ICI 168,384 resulted in the expected abrogation (up to 90%) of $E_2$'s inhibitory effects (not shown). Thus, cells grown in the presence of some $E_2$ and repeatedly dosed over 48 h are equally sensitive to the inhibitory effects of 100-fold less $E_2$ than cells grown as in Fig. 3 *A*. These lower concentrations are similar to those used ($\sim 10^{-8}$ M) in many estradiol studies (25, 26).

These CAM induction inhibitory effects were confirmed at the functional level in phorbol ester–activated PBMC-EC adhesion assays. HUVEC pretreatment with $E_2$ 2 μg/ml resulted in a 60% reduction of the augmented adhesion induced by 16 h of IL-1 exposure (Fig. 4). 16-h IL-1 activation results in hyperinduction of ICAM-1 and induction of VCAM-1 membrane expression. Although these functional inhibitory effects of $E_2$ could result from alternative, unexplored mechanisms, the dose response demonstrated in Fig. 4 parallels the inhibitory dose response for membrane CAM induction (Fig. 3 *A*).

IL-1 promotes E-selectin gene activation and hyperinduces endothelial ICAM-1 transcription. To address whether $E_2$ affects induced mRNA levels, Northern blot analyses were performed on mRNA harvested from EC with (Fig. 5 *A*, lanes *2* and *4*) or without (Fig. 5 *A*, lanes *1* and *3*) 48-h $E_2$ pretreatment, hybridized with E-selectin and GAPDH probes. As expected, there is no basal E-selectin mRNA (Fig. 5 *A*, lane *1*). E-selectin transcript levels induced by IL-1 (20 U/ml × 2 h, Fig. 5 *A*, lane *3*) were significantly reduced by $E_2$ pretreatment (Fig. 5 *A*, lane *4*). When densitometrically normalized for GAPDH levels, this represents a 64% decrease. $E_2$ treatment had no effect on GAPDH transcript levels, demonstrating that its effect is not the result of general transcriptional inhibition. This is consistent with the absence of $E_2$ effect on basal EC ICAM-1 (Fig. 2 *A*) or class I HLA (not shown) levels, or the induction of HLA-DR, as noted above. The histogram displayed in Fig. 5 *B* represents the densitometric analysis of a similar (separate) Northern analysis, in which $E_2$ treatment resulted in a 65% reduction of IL-1–induced E-selectin mRNA levels. Simultaneous use of the $E_2$ antagonist ICI 164,384, which alters $E_2$ interaction with the ER (27), abrogates $E_2$'s in-

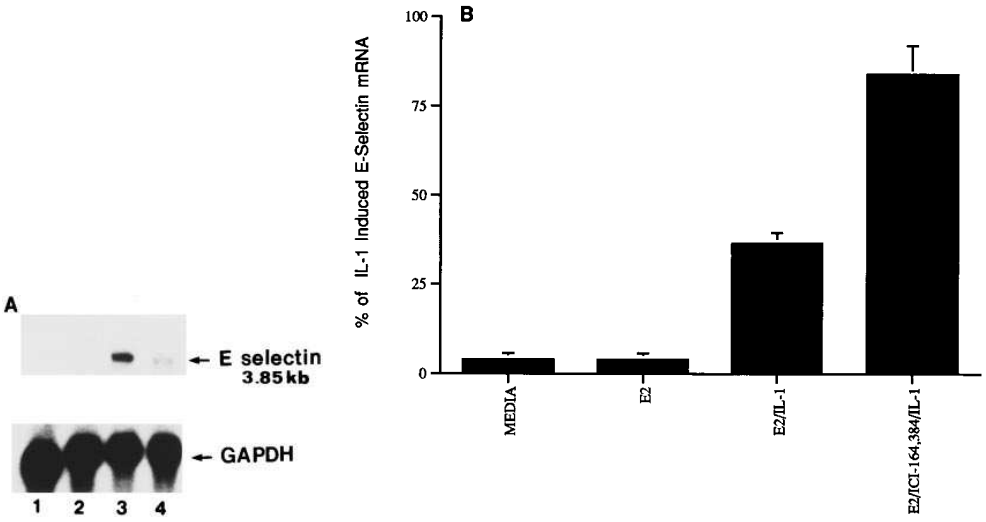


*Figure 5.* Northern blot analysis of $E_2$ effects on EC E-selectin induction. (*A*) HUVEC were propagated in $E_2$-free medium and treated for 48 h with fresh medium (containing vehicle) (lanes *1* and *3*) or $E_2$ 1 μg/ml (lanes *2* and *4*), before a 2-h activation with IL-1 20 U/ml (lanes *3* and *4*) or no cytokine (lanes *1* and *2*). 20 μg total RNA was hybridized per sample. GAPDH mRNA levels were used as internal controls. When densitometrically analyzed, lane *4* signal ($E_2$ + IL-1) represents a 64% decrease from lane *3* signal (IL-1). (*B*) Separate experiment with identical conditions as in *A*, with the addition of ICI 164,384 ($E_2$ antagonist) in one of the experimental samples, as noted. Histograms represent phosphorimage-analyzed, GAPDH-normalized, percent of IL-1–induced E-selectin mRNA levels by Northern blot. $E_2$ treatment results in a 65% decrease in IL-1–induced steady state E-selectin mRNA levels. The inhibition is largely abrogated by ICI 164,384. This experiment represents means±SE for three separate experiments.

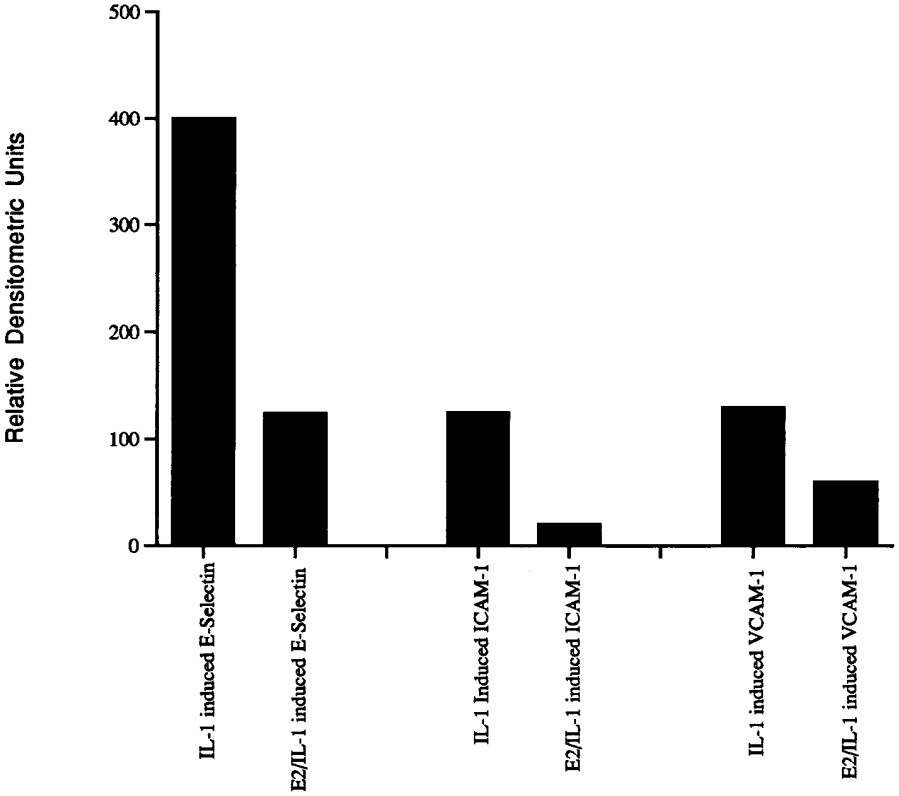


*Figure 6.* $E_2$ effects on endothelial CAM gene expression by nuclear run-off transcription. HUVEC were propagated in $E_2$-free medium and treated for 48 h with fresh medium (vehicle only) or $E_2$ 1 μg/ml before a 1.5-h activation with IL-1 20 U/ml or no cytokine, after which $2 \times 10^7$ nuclei per sample were harvested and radiolabeled RNA was used to probe slot blots containing E-selectin, ICAM-1, VCAM-1, γ-actin, or Bluescript DNA. Histograms represent background (e.g., basal ICAM-1 signal)-subtracted, actin transcript–normalized densitometric analysis, comparing IL-1–induced nuclear transcripts in the absence or presence of $E_2$.

hibitory effect, suggesting that its specific receptor is involved. $E_2$ pretreatment also partially abrogated the IL-1–mediated increase in monocyte chemoattractant protein-1 mRNA levels (data not shown), suggesting that estrogen affects a spectrum of inflammatory responses in EC, not limited to CAM expression, and also potentially antiatherogenic (28, 29).

To evaluate whether the $E_2$ effect on cytokine-induced mRNA levels is transcriptional or posttranscriptional, nuclear run-offs were performed with nuclei harvested from medium or $E_2$-treated IL-1–induced EC. Fig. 6 demonstrates the background-subtracted, actin transcript–normalized densitometric data for E-selectin, ICAM-1, and VCAM-1 nuclear transcripts which closely parallel the findings at the membrane protein (Fig. 2) and mRNA (Fig. 5) levels. That is, $E_2$ pretreatment promotes 74, 83, and 60% reductions in E-selectin, ICAM-1, and VCAM-1 induced nuclear transcription, respectively.

## Discussion

The assessment of hormone responses in vitro is complicated by incomplete definition of critical culture conditions. In this study, gonadal hormone and phenol red–free medium was used to exclude any basal estrogen effect and artifactual ER binding, respectively. In fact, absolute estrogen "deprivation" led to ER mRNA levels undetectable by Northern analysis. ER message is apparently $E_2$-inducible, although the kinetics are relatively slow (48 h). This is consistent with prior reports of murine uterine ER mRNA induction coincident with proestrus, when $E_2$ levels are highest (30). Despite the variable difficulty in vascular ER demonstration in vivo, these human EC have ER mRNA (and protein) levels equal to a highly $E_2$-responsive cell line at the time all assays were performed in this study.

We demonstrate that $E_2$ markedly inhibits IL-1–mediated endothelial CAM transcription, also reflected in reduced membrane protein expression and IL-1–induced leukocyte adhesion. The specificity of $E_2$'s effect is evident in that transcription is not generally inhibited, 17α-estradiol at equimolar concentrations was ineffective, and the estrogen antagonist ICI 164,384 efficiently abrogates the inhibition. The antagonist efficacy indicates that $E_2$'s inhibitory effect(s) is mediated, at least in part, via the endothelial ER.

Interactions between ligand-activated steroid hormone receptors and specific genomic sequences are well described (13) and are believed to be the dominant mechanism whereby this class of hormones exerts its molecular effects. ERs preferentially bind to a 13-bp palindromic DNA sequence, the estrogen response element (ERE). This element can be sufficient to enhance transcription, as can "imperfect" or half-EREs, which can be located a great distance from the transcription start site (31). Although traditionally thought to be enhancing, there are accumulating reports of estradiol negatively affecting transcription (32). As does ours, these reports speculate about ERE interactions. The negative effect of $E_2$ on IL-1–mediated IL-6 gene activation has recently been studied (32). Although inhibition involved the ER, high-affinity ER binding to the IL-6 promoter could not be demonstrated (32), nor does recombinant ER bind to the critically involved 225-bp promoter fragment in gel mobility shift assays (32). Overexpression of the NF-κB subunit p65 or, to a lesser extent, NF-IL-6, overcomes $E_2$'s inhibitory effect (32). These results suggest that $E_2$-activated ER interacts with critical transacting factors, perhaps to form an inactive intermolecular complex. Several

other examples of ER interactions with ubiquitous transcription factors exist, including AP-1 (33) and SP1 (34). κB-like proteins induced by $E_2$ in rat uterus have been reported (14). Although a single trend has not been demonstrated, it is likely that tissue, cell-type, and gene specificities exist which determine whether such induced interactions exert positive or negative regulatory effects.

There is minimal information regarding hormonal regulation of CAM expression. Via the glucocorticoid receptor, dexamethasone has been shown to reduce IL-1–induced E-selectin mRNA levels (35). Similarly, a recent report demonstrated reduced VCAM-1 mRNA and membrane protein levels in $E_2$-pretreated, IL-1–activated cells (36). These studies are consistent with our current findings. Another report described an enhancement in TNF-mediated endothelial CAM induction by estradiol (37). Although apparently opposite to our results, there were several important differences in that study. The EC culture conditions were different, potentially critical in these hormone studies. This includes the use of charcoal-stripped calf serum, rather than GHS used in our study. Steroid hormone removal by stripping can be incomplete and/or can remove other serum components which may be important for EC metabolic processes. Also, in the prior study, $E_2$ pretreatment was not done. In the $E_2$-treated experimental samples, $E_2$ and cytokine were added simultaneously at time 0. TNF was used and the observed enhancement was specifically dependent upon EC plating on laminan, whereas IL-1 and collagen, respectively, were used in our study. Although other steroid hormones were used as specificity controls in the prior study, ER specificity was not addressed, as in the current study. Finally, the observed estradiol enhancement was marginal by FACS® and Northern analyses at 4 h, and $E_2$ actually appeared inhibitory at 6 h.

E-selectin, ICAM-1, and VCAM-1 5'-regulatory regions contain both distinct and common elements (38–42). All three promoters contain at least one κB site, which is required for cytokine-mediated gene activation. The aforementioned studies involving the IL-6 promoter are consistent with the notion that ER complexes interact with components of the NF-κB signaling pathway, negatively affecting transcription (32). $E_2$ could modulate human endothelial NF-κB nuclear translocation, DNA binding, or transactivation, thereby affecting transcription of all three aforementioned genes.

$E_2$ has antioxidant activity and has been shown to inhibit low-density lipoprotein oxidation in vivo (43) and in vitro (44). This is postulated to be one of estrogen's important cardioprotective effects. Furthermore, antioxidants partially inhibit cytokine-mediated E-selectin and VCAM-1 gene activation, and diminish induced NF-κB binding activity on gel mobility shift assays (45). Although we cannot exclude a component of antioxidation, prior in vitro studies have demonstrated that 17α-estradiol is an effective antioxidant (46). Furthermore, this $E_2$ effect is thought to be ER independent. As demonstrated here, 17α-estradiol does not inhibit adhesion molecule expression. The profound abrogation of $E_2$'s inhibitory effect by the receptor antagonist ICI 164,384 indicates that the ER is involved, and that this inhibition is not mediated predominantly via an antioxidant pathway.

Natural and synthetic estrogens reduce the development of atherosclerosis in animal models (47–50), and play a cardiovascular protective role in women. Our studies demonstrate that estradiol inhibits cytokine-mediated endothelial activation at the transcriptional level in vitro. Future work will address whether this negative effect of $E_2$ is direct, via ER-CAM gene promoter interactions, or indirect, via "adsorption" and/or transcriptional modulation of critically involved transacting factors.

## Acknowledgments

We express gratitude to Lynn O'Donnell, Louise Benson, and Gwen Davis for assistance with cell culture, and Julie Sevanick for help with manuscript preparation. We thank all those who provided generous gifts of valuable reagents. We are grateful to the Milford General Hospital Delivery Room nursing staff for obtaining umbilical cords.

This work was supported by National Institutes of Health grant HL-43331 (J.R. Bender), the Raymond and Beverly Sackler Foundation Inc., and the European Community grant CT950875 (R. Pardi). T. Caulin-Glaser was a Fellow of the Patrick and Catherine Weldon Donaghue Medical Research Foundation at the time this study was performed. C.A. Watson is an Associate Research Scientist of the Raymond and Beverly Sackler Foundation Inc. J.R. Bender is a Raymond and Beverly Sackler Foundation Scholar.

## References

1. Ross, R. 1986. Medical progress: the pathogenesis of atherosclerosis – an update. *N. Engl. J. Med.* 314:488–500.

2. Cotran, R., and J. Pober. 1990. Cytokine-endothelial interactions in inflammation, immunity and vascular injury. *J. Am. Soc. Nephrol.* 1:225–235.

3. Cybulsky, M.I., and M.A. Gimbrone. 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science (Wash. DC).* 251:788–791.

4. van der Wal, A., P. Das, A. Tigges, and B. Ae. 1992. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am. J. Pathol.* 141:1427–1433.

5. Gossland, I.F., V. Wynn, D. Crook, and N.E. Miller. 1987. Sex, plasma lipoproteins and atherosclerosis: prevailing assumptions and outstanding questions. *Am. Heart J.* 114:1467–1503.

6. Stampfer, M.J., G.A. Colditz, W.C. Willett, J.E. Manson, B. Rosner, F.E. Speizer, and C.H. Hennekens. 1991. Postmenopausal estrogen therapy and cardiovascular disease. *N. Engl. J. Med.* 325:756–762.

7. Adams, M.R., T.B. Clarkson, J.R. Kaplan, and D.R. Kortnik. 1990. Ovarian secretions and atherosclerosis. *In* Ovarian Secretions and Cardiovascular and Neurological Function. F. Naftolin, J.N. Gutmann, A.H. DeCherney, and P.M. Sarrel, editors. Raven Press, New York. 151–159.

8. Sarrel, P.M. 1988. Effects of ovarian steroids on the cardiovascular system. *In* Circulation in the Female. J. Ginsburg, editor. Parthenon Press, Carsforth, United Kingdom. 117–141.

9. Wolinsky, H. 1992. Effects of estrogen and progestogen treatment on the response of the aorta of male rats to hypertension: morphological and chemical studies. *Circ. Res.* 30:341–349.

10. Williams, J.K., M.R. Adams, and H.S. Klopfenstein. 1990. Estrogen modulates responses of atherosclerotic coronary arteries. *Circulation.* 81:1680–1687.

11. Wagner, J.D., T.B. Clarkson, R.W. St. Clair, D.C. Schwenke, C.A. Shively, and M.R. Adams. 1991. Estrogen and progesterone replacement therapy reduces low density lipoprotein accumulation in the coronary arteries of surgically postmenopausal cynomolgus monkeys. *J. Clin. Invest.* 88:1995–2002.

12. Wakasugi, M., T. Noguchi, Y.-I. Kazama, Y. Kanemaru, and T. Onaya. 1989. The effects of sex hormones on the synthesis of prostacyclin (PGI 2) by vascular tissue. *Prostaglandins.* 37:401–410.

13. Beato, M. 1989. Gene regulation by steroid hormones. *Cell.* 56:335–344.

14. Shyamala, G., and M.C. Guiot. 1992. Activation of kB-specific proteins by estradiol. *Proc. Natl. Acad. Sci. USA.* 89:10628–10632.

15. Gaub, M.P., M. Bellard, I. Scheuer, P. Chambon, and P. Sassone-Corsi. 1990. Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex. *Cell.* 63:1267–1276.

16. Montgomery, K.F., L. Osborn, C. Hession, R. Tizard, D. Goff, C. Vassallo, P.I. Tarr, K. Bomsztyk, R. Lobb, J.M. Harlan, and T.H. Pohlman. 1991. Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. *Proc. Natl. Acad. Sci. USA.* 88:6523–6527.

17. Iadermarco, M., J. McQuillan, G. Rosen, and D. Dean. 1992. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). *J. Biol. Chem.* 267:16323–16329.

18. Degitz, K., L. Lian-Jie, and S.W. Caughman. 1991. Cloning and characterization of the 5′ transcriptional regulatory region of the human intercellular adhesion molecule 1 gene. *J. Biol. Chem.* 266:14024–14030.

19. Gimbrone, M.A. 1976. Culture of vascular endothelium. *In* Progress in Hemostasis and Thrombosis. T.H. Spaet, editor. Grune and Stratton Inc., New York. 1–28.

20. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

21. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 7.38–7.52.

22. Lainier, L.L., E.G. Engleman, P. Gatenenby, G.F. Babcock, N.L. Warner, and L.A. Herzenberg. 1983. Correlation of functional properties of human lymphocyte subsets and surface marker phenotype using multiparameter analysis and flow cytometry. *Immunol. Rev.* 74:143–148.

23. Bender, J.R., R. Pardi, M.A. Karasek, and E.G. Engleman. 1987. Phenotypic and functional characterization of lymphocytes that bind in vitro. *J. Clin. Invest.* 79:1679–1688.

24. Green, S., P. Walter, V. Kumar, A. Krust, J.-M. Bornert, P. Argos, and P. Chambon. 1986. Human estrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature (Lond.).* 320:134–139.

25. Keri, R.A., B. Andersen, G.C. Kennedy, D.L. Hamerik, C.M. Clay, A.D. Brace, T.M. Nett, A.C. Notides, and J.H. Nilson. 1991. Estradiol inhibits transcription of the human glycoprotein hormone α-subunit gene despite the absence of a high affinity binding site for estrogen receptor. *Mol. Endocrinol.* 5: 725–733.

26. Yuan, H., D.A. Bowlby, T.J. Brown, R.B. Hochberg, and N.J. Maclusky. 1995. Distribution of occupied and unoccupied estrogen receptors in the rat brain: effects of physiological gonadal steroid exposure. *Endocrinology.* 136:96–105.

27. Wakeling, A.E. 1990. Novel pure antiestrogens: mode of action and therapeutic prospects. *Ann. NY Acad. Sci.* 595:348–356.

28. Nelken, N.A., S.R. Coughlin, D. Gordon, and J.N. Wilcox. 1991. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* 88:1121–1130.

29. Sica, A., J.M. Wang, F. Colotta, E. Dejana, A. Mantovani, J.T. Oppenheim, C.G. Larsen, C.O.C. Zachariae, and K. Matsushima. 1990. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J. Immunol.* 144:3034–3038.

30. Bergman, M.D., B.S. Schachter, K. Karelus, E.P. Combatsiaris, T. Garcia, and J.F. Nelson. 1992. Up-regulation of the uterine estrogen-receptor and its messenger ribonucleic acid during the mouse estrous cycle: the role of estradiol. *Endocrinology.* 130:1923–1930.

31. Kumar, V., and P. Chambon. 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell.* 55:145–156.

32. Ray, A., K.E. Prefontaine, and P. Ray. 1994. Down-modulation of interleukin-6 gene expression by 17β-estradiol by the estrogen receptor. *J. Biol. Chem.* 269:12940–12946.

33. Weisz, A., and R. Rosales. 1990. Identification of an estrogen response element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucl. Acids Res.* 18:5097–5106.

34. Krishnan, V., X. Wang, and S. Safe. 1994. Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast tissue. *J. Biol. Chem.* 269:15912–15917.

35. Cronstein, B., S. Kimmel, R. Levin, F. Martiniuk, and G. Weissmann. 1992. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* 89:9991–9995.

36. Nakai, K., C. Itoh, K. Hotta, T. Itoh, M. Yoshizumi, and K. Hiramori. 1994. Estradiol-17β regulates the induction of VCAM-1 mRNA expression by interleukin-1β in human umbilical vein endothelial cells. *Life Sci.* 54:221–227.

37. Cid, M.C., H.K. Kleinman, D.S. Grant, H.W. Schnaper, A.S. Fauci, and G.S. Hoffman. 1994. Estradiol enhances leukocyte binding to tumor necrosis factor (TNF) stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1. *J. Clin. Invest.* 93:17–25.

38. Kaszubska, W., R.H. van Huijsduijnen, P. Ghersa, A.M. DeRaemy-Schenk, B.P. Chen, T. Hai, J.F. DeLamarter, and J. Whelan. 1993. Cyclic AMP-independent ATF family members interact with NF-κB and function in the activation of the E-selectin promoter in response to cytokines. *Mol. Cell Biol.* 13: 7180–7190.

39. DeLuca, L., D. Johnson, M. Whitley, T. Collins, and J. Pober. 1994. cAMP and tumor necrosis factor competitively regulate transcriptional activation through and nuclear factor binding to the cAMP-responsive element/activating transcription factor element of the endothelial leukocyte adhesion molecule-1 (E-selectin) promoter. *J. Biol. Chem.* 269:19193–19196.

40. Neish, A.S., A.J. Williams, H.J. Palmer, M.Z. Whitley, and T. Collins. 1992. Functional analysis of the human vascular cell adhesion molecule 1 promoter. *J. Exp. Med.* 176:1583–1593.

41. Hou, J., V. Baichwal, and Z. Cao. 1994. Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* 91: 11641–11645.

42. Read, M.A., M.Z. Whitley, A.J. Williams, and T. Collins. 1994. NF-κB and IκBα: an inducible regulatory system in endothelial activation. *J. Exp. Med.* 179:503–512.

43. Keaney, J.F., G.T. Shwaery, A. Xu, R.J. Nicolosi, J. Loscalzo, T. Foxall, and J. Vita. 1994. 17β-estradiol preserves endothelial vasodilator function and limits low density lipoprotein oxidation in hypercholesterolemic swine. *Circulation.* 89:2251–2259.

44. Negre-Salvayre, A., M.T. Pierassi, L. Mobile, and R. Salvayre. 1993. Protective effects of 17 β-estradiol against the cytotoxicity of minimally oxidized LDL to cultured bovine aortic endothelial cells. *Atherosclerosis.* 99:207–217.

45. Marui, N., M.K. Offermann, R. Swerlick, C. Kunsch, C.A. Rosen, M. Ahmad, R.W. Alexander, and R.M. Medford. 1993. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* 92:1866–1874.

46. Mooradian, A.D. 1993. Antioxidant properties of steroids. *J. Steroid Biochem. Mol. Biol.* 45:509–511.

47. Rhee, C.Y., T.H. Spaet, M.B. Stemerman, F. Lajam, and H.H. Shiang. 1977. Estrogen suppression of surgically induced vascular intimal hyperplasia in rabbits. *J. Lab. Clin. Med.* 90:77–84.

48. Foegh, M.L., B.S. Khirabadi, T. Nakanishi, R. Vargas, and P.W. Ramwell. 1987. Estradiol protects against experimental cardiac transplant atherosclerosis. *Transplant. Proc.* 19:90–95.

49. Williams, J.K., M.R. Adam, and H.S. Klopfenstein. 1990. Estrogen modulates responses of atherosclerotic coronary arteries. *Circulation.* 81:1680–1687.

50. Weigensberg, B.I., J. Lough, R.H. More, E. Katz, E. Pugash, and C. Peniston. 1984. Effects of estradiol on myointimal thickening from catheter injury and on organizing white mural non-occlusive thrombi. *Atherosclerosis.* 52: 253–265.