Estrogens and Glucocorticoids Inhibit Endothelial Vascular Cell Adhesion Molecule-1 Expression by Different Transcriptional Mechanisms

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Abstract—The antiatherogenic effect of estrogen is mediated, in part, by inhibitory effects on endothelial vascular cell adhesion molecule-1 (VCAM-1) expression. To determine the mechanism by which estrogen regulates VCAM-1 expression, we compared the effect of 17β-estradiol (E2) and of the glucocorticoid dexamethasone (Dex) on lipopolysaccharide (LPS)–induced VCAM-1 expression in human endothelial cells. E2 decreased LPS-induced VCAM-1 mRNA and protein expression to a greater extent than Dex. Dex, but not E2, stabilized VCAM-1 mRNA. This correlated with inhibition of monocytoid U937 cell adhesion to endothelial cells. Transfection of endothelial cells with a functional VCAM-1 promoter construct showed that E2 inhibited LPS-induced VCAM-1 gene transcription more potently than did Dex. However, using a truncated construct containing only the nuclear factor-κB (NF-κB)–responsive elements but lacking the consensus sequences for activator protein-1 (AP-1) and GATA, E2 and Dex had similar inhibitory effects. Consistently, gel-shift assays showed that E2 and Dex comparably inhibit LPS-induced activation of NF-κB, whereas E2 inhibited LPS-induced activation of AP-1 and GATA to a greater extent than Dex. E2 inhibition of NF-κB after LPS treatment was associated with decreased inhibitor κB (IκB) kinase activity and with a stabilization of the NF-κB inhibitor IκBa. These results indicate that E2 decreases VCAM-1 gene expression through the inhibition of NF-κB, AP-1, and GATA and suggest novel mechanisms for the antiatherogenic effect of estrogen on the vascular wall. (Circ Res. 2000;87:19-25.)

Key Words: estrogen ■ glucocorticoid ■ endothelial-leukocyte adhesion molecules ■ nuclear factor-κB ■ endothelial cells

The molecular pathways by which estrogen acts on the cardiovascular system remain largely unknown despite recent interest in using these agents in postmenopausal women for prevention of cardiovascular diseases.1 The cardioprotective effect of estrogen is only partially explained by favorable lipid profile modifications,2 and recent evidence suggests that the direct vascular effects of estrogen may actually account for most of their protective action.3

Atherosclerosis is considered a chronic inflammatory process,4 and the endothelium plays an important role in its initiation.5 Various atherogenic stimuli, such as modified LDLs, oxidative free radicals, homocysteine, and infectious agents, lead to the development of endothelial dysfunction.4 Many of these stimuli cause endothelial cell activation, which is defined as functional and antigenic changes in endothelial cells promoting monocyte adhesion.6 Endothelial activation involves the coordinated induction of genes encoding for leukocyte adhesion molecules (such as vascular cell adhesion molecule-1 [VCAM-1] and intercellular adhesion molecule-1), chemotactic factors (such as monocyte chemoattractant protein-1 [MCP-1]), and growth factors (such as macrophage colony-stimulating factor).6 Endothelial VCAM-1 is an important mediator of mononuclear cell adhesion, given that its cognate ligand, the integrin very late antigen-4 (VLA4), is selectively expressed on monocytes (and on some T lymphocytes) but not on neutrophils.7

In different animal models of atherosclerosis,8,9 17β-estradiol (E2) protects endothelial integrity and function and reduces leukocyte adhesion and intimal accumulation.10 In vitro, E211 and other estrogenic compounds12 decrease cytokine-induced expression of adhesion molecules and of monocyte chemoattractants.13 Glucocorticoids also inhibit the expression of endothelial cell adhesion molecules.14 However, the mechanism for these effects of estrogen, and whether estrogen and glucocorticoid have a common mechanism of action, is still not known. The purpose of this study, therefore,
is to determine and compare the mechanism by which estrogen and glucocorticoid regulate endothelial cell adhesion molecule expression.

Materials and Methods

Cell Cultures
Human saphenous vein endothelial cells (HSVECs) and bovine aortic endothelial cells (BAECs) were harvested and maintained as described. HSVECs were obtained by aortocoronary grafts from both male and female donors with an age range from 36 to 87 years. In preliminary experiments, no differences were noted regarding the investigated parameters. HSVECs were grown in the presence of 5% FBS, whereas for BAECs 10% FBS was used. All media were phenol red-free. Before treatments, cells were shifted to media containing FBS treated with charcoal stripping to remove steroid hormones.

Monocytoid Cell Adhesion Assays
Endothelial cells were pretreated with steroids for 48 hours, after which lipopolysaccharide (LPS; 100 ng/mL) was added for 18 hours. In some experiments, endothelial cells were treated with a VCAM-1 blocking antibody (E1/6). The adhesion assay was performed by adding U937 cells (10^6/mL) to each monolayer under rotating conditions (63 rpm) at 21°C, as described. At least 8 wells were used for each cell antigen, was used in control studies. Enzyme immunoassays E1/1, recognizing a constitutive, non–cytokine-inducible endothelial cell antigen, was used in control studies. Enzyme immunoassays were carried out as described. At least 8 wells were used for each condition.

Northern Analysis
Endothelial cells were pretreated for 48 hours with E2 or dexamethasone (Dex) followed by a 4-hour stimulation with LPS (100 ng/mL). Northern analysis was performed as described.

Western Blotting
Proteins (40 μg/lane) were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp). Membranes were hybridized with standard technique. Immunodetection was accomplished with enhanced chemiluminescence (Amersham).

Transfection Assays
Human VCAM-1 promoter constructs have been described. BAECs were used because of their higher transfection efficiency (15% versus 1% for human cells) with the calcium phosphate precipitation method. Transfection, chloramphenicol acetyltransferase (CAT), and β-galactosidase assays were performed as described.

Electrophoretic Mobility Shift Assays
Nuclear and cytosolic extracts were prepared as described. Oligonucleotide probes corresponding to the nuclear factor-κB (NF-κB) (5'-TGGGAGTTCCCTCCCTGAGGATTTTCTCC-3'), activator protein-1 (AP-1) (5'-CGCTGTGAGCTCAGCGGAA-3'), and GATA (5'-CAGTGTACCAGGGATCTCC-3') binding sites on VCAM-1 promoter were labeled, and DNA binding reactions were performed as described.

Immunofluorescence Analysis
Endothelial cells were grown on glass slides precoated with 2% gelatin. Cells were fixed with acetone and blocked with 3% normal goat serum. Cells were incubated with an antibody directed against the NF-κB subunit Rel A (Santa Cruz Biotechnology). A biotinylated antibody was used as secondary antibody. Streptavidin-FITC was added, and immunofluorescence was analyzed and photographed by an investigator blinded as to the experimental conditions.

Inhibitor αB (IκB) Kinase Assay
After preincubation, endothelial cells were treated with LPS, and cell lysates were prepared as described. After immunoprecipitation with an antibody versus IκB kinase α (Santa Cruz Biotechnology), 1 μg of glutathione S-transferase (GST)–fusion IκBα was added, and [γ^32P]dATP was used as phosphate donor. The reaction products were run on SDS-PAGE, which was dried and exposed for autoradiography.

Statistical Analysis
Multiple comparisons were performed by 1-way ANOVA, and individual differences were tested by the Fisher protected least significance difference test after the demonstration of significant intergroup differences by ANOVA. Two-group comparisons were performed by the unpaired Student t test.

Results

Effect of E2 and Dex on Monocyte Adhesion
To determine the effect of estrogen on endothelial cell–monocyte interactions, we performed adhesion assays. LPS treatment increased U937 cell adherence to endothelial surface (Figure 1). Pretreatment with E2 (10 μmol/L, 48 hours) decreased U937 cell adhesion by >50%, whereas Dex (10 μmol/L, 48 hours) reduced adhesion to a lesser degree. A blocking anti–VCAM-1 monoclonal antibody produced a 37% decrease in cell adhesion (not shown), suggesting that some of the E2 effect may be mediated by factors other than VCAM-1.

Effect of E2 and Dex on LPS-Induced VCAM-1 Expression
In a concentration-dependent manner, treatment with E2 and Dex inhibited LPS-induced VCAM-1 expression (Figure 2A).
The steroids also inhibited VCAM-1 expression induced by interleukin-1α or TNF-α (not shown). Consistent with monocyte adhesion findings, E2 was more potent than Dex. The 2 compounds did not produce cellular toxicity, as assessed by cell number and viability (ie, morphology and trypan blue exclusion) and were specific, because they did not affect the expression of the constitutive surface antigen E1/116 (not shown). VCAM-1 protein reduction corresponded to a comparable decrease in mRNA levels (Figure 2B).

E2 inhibition of VCAM-1 expression was competitively blocked by the pure estrogen receptor antagonist Imperial Chemical Industries (ICI) 182,780, suggesting that the effect is mediated through 1 or both estrogen receptors (Figure 2A), which are both expressed in our culture conditions (Figure 2C). In addition, the inactive E2 stereoisomer, αE2, had no effect on VCAM-1 expression (data not shown), indicating that the estrogen effect is not mediated by intrinsic chemical properties (eg, antioxidant effects).

### Effect of E2 and Dex on VCAM-1 mRNA Stability

To determine whether E2 or Dex may regulate VCAM-1 through posttranscriptional effects, we pretreated endothelial cells for 48 hours with E2, Dex, or vehicle, and then we stimulated them with LPS for 4 hours to induce a maximal synthesis of VCAM-1 mRNA. Thereafter, we added the RNA polymerase inhibitor actinomycin-D. With Northern analysis, we studied VCAM-1 mRNA stability, examining samples harvested at 1-hour intervals. E2 did not affect VCAM-1 mRNA half-life ($t_{1/2}=164±21$ minutes versus $183±26$ minutes). Instead, Dex prolonged VCAM-1 mRNA half-life ($t_{1/2}=248±37$ minutes) (Figures 3A and 3B), thus potentially posttranscriptionally increasing VCAM-1 production.

### Effect of E2 and Dex on VCAM-1 Gene Promoter Region

Steroid hormones usually act as transcription factors, but VCAM-1 promoter contains no estrogen or glucocorticoid response elements.17 To determine whether E2 and Dex regulate VCAM-1 promoter and to identify which regions are involved, we transfected deletional VCAM-1 promoter constructs linked to the chloramphenicol acetyl transferase reporter gene17 into BAECs. F0.CAT is the VCAM-1 promoter construct containing the AP-1, GATA, and tandem kB sites (Figure 4A). F3.CAT contains the kB sites, and F4.CAT contains only a TATA box (Figure 4A). LPS increased F0.CAT activity by 5-fold compared with unstimulated cells (Figure 4B). Pretreatment with E2 or Dex (10 μmol/L, 48 hours) inhibited LPS induction by 60% and 30%, respectively (Figure 4B). In cells transfected with F3.CAT, however, E2 and Dex produced similar decreases in promoter activity (Figure 4B). F4.CAT was only minimally induced by LPS (Figure 4B). These findings suggest that E2 and Dex inhibition of VCAM-1 expression is transcriptional and potentially due to regulation of different transcription factors and that the different steroid potencies may be the consequence of distinct modulations of AP-1 and/or GATA.

### Effect of E2 and Dex on NF-κB Activation

To determine whether E2 and Dex regulate VCAM-1 expression by inhibiting NF-κB, we performed gel-shift assays using an oligonucleotide corresponding to the tandem kB
sites on VCAM-1 promoter. Both steroids (10 μmol/L, 48 hours) markedly decreased the intensity of the shifted band produced by nuclear extracts treated with LPS (Figure 5).

Specificity of the complexes was determined with antibodies versus NF-κB subunits Rel A (p65), p50, and c-Rel, which supershifted the complexes, and by competition with an excess of unlabeled oligonucleotide. Because cytoplasmic extracts from the same cells showed no differences, the changes in nuclear NF-κB binding are probably due to inhibition of NF-κB nuclear translocation. In agreement with transfection studies, the 2 steroids had similar potency with respect to NF-κB inhibition, furthermore suggesting that other cis-acting elements within the VCAM-1 promoter mediate the differential inhibitory effect of E2 versus Dex.

Immunoblot of nuclear and cytoplasmic extracts confirmed that E2 and Dex decrease nuclear localization of both p50 and Rel A (p65) (Figure 6A). Immunofluorescent staining for p65 NF-κB subunit. Pictures are representative microscopic fields at different magnifications. E2 and Dex concentration was 10 μmol/L. The experiment was repeated 2 times with equal results.

Effect of E2 on IκB Degradation and IκB Kinase Activity

To clarify whether the E2 effect on NF-κB nuclear translocation is due to regulation of the NF-κB inhibitor IκBα, we pretreated endothelial cells with E2 and then added LPS for different times to follow LPS-induced IκBα degradation. E2 slightly increased basal IκBα levels and markedly reduced IκBα degradation after LPS treatment, whereas αE2 had no effect (Figure 7A).

Because IκB degradation is triggered by phosphorylation by IκB kinase (IKK), we studied whether E2 treatment was
associated with inhibition of IKKα. LPS markedly increased IKKα activity (Figure 7B). Pretreatment with E2, but not with αE2, decreased LPS-induced IKKα kinase activity nearly to baseline level. As controls, samples were immunoprecipitated with an irrelevant antibody (versus endothelial NO synthase), and others received a mutated IκBα with differential inhibition of NF-κB activity, mRNA, and protein synthesis. This leads to reduced endothelial adhesiveness toward monocytoid cells and may be the mechanism for some of the antiatherogenic effects of estrogen.

VCAM-1 regulation by E2 has been described with contrasting results, and none of the previous reports clarified the underlying molecular mechanisms. Our findings confirm that E2 decreases VCAM-1 expression in human endothelial cells. E2 and Dex decreased LPS-induced endothelial VCAM-1 protein and mRNA levels, as well as monocytoid cell adhesion. E2, however, was consistently more potent than Dex, suggesting distinct mechanisms. Indeed, the 2 steroids differentially modulate NF-κB, AP-1, and GATA, reducing their ability to activate VCAM-1 promoter.

This study provides a comparison of the effects of pharmacological concentrations of estrogen and Dex. The effects of estrogen on VCAM-1 surface expression were apparent already at physiological (nanomolar range) concentrations. Most of our other studies, however, were performed using higher concentrations (10 µmol/L), at which inhibitory effects were more evident. This discrepancy between in vitro and in vivo concentrations of estrogen has been noted previously. This suggests the relevance of an intact vascular microenvironment, or of the prolonged endothelial exposure to estrogens, as occurring in chronic replacement studies, in decreasing the concentration requirement for the inhibitory effect on adhesion molecule expression. Concentrations of Dex during anti-inflammatory dosing are in this concentration range, for which inhibitory effects on inflammatory transcription factors have been reported.

The inhibition of nuclear translocation and DNA binding of NF-κB by E2 represents a potentially important mechanism for the vascular protective effects of estrogens. NF-κB is a proinflammatory transcription factor upregulating several
genes involved in endothelial activation. The action of E2 and DEX on NF-κB may thus explain their effects on adhesion molecule expression and on other mediators implicated in monocyte adhesion, such as MCP-1. Furthermore, because NF-κB activation is a common pathway for many atherogenic stimuli (such as oxidized LDL, reactive oxygen species, and cytokines), NF-κB inhibition by E2 may further explain its antiatherogenic properties. Indeed, a recent in vivo study confirms that estrogens reverse aortic VCAM-1 upregulation and leucocyte adhesion and intimal accumulation in hypercholesterolemic rabbits.

Glucocorticoids inhibit NF-κB, inducing the synthesis of NF-κB inhibitor IκB, as well as through protein-protein interactions between the ligand-bound glucocorticoid receptor and NF-κB subunit Rel A. E2 blocks NF-κB activation in an osteoblast cell line through binding of the estrogen receptor α with Rel A and p50 subunits. Our results indicate that in human endothelial cells, NF-κB inhibition by E2 is associated with decreased IκBα degradation, possibly as a result of inhibition of LPS-induced IκKe, proposing a novel mechanism for regulation of the -κB transcription factor family by estrogens.

Our results demonstrate that inhibition of VCAM-1 by E2 and DEX is associated with a reduction of AP-1 and GATA binding to VCAM-1 promoter and that differences in the potency of these steroids correlate with differences in the inhibition of these transcription factors.

Glucocorticoid receptor interacts with the c-Jun/c-Fos heterodimer, resulting in a mutual inhibition of DNA binding activity. Less is known about estrogen, but, consistent with our findings, E2 regulates AP-1 nuclear binding in a tissue-selective way. Moreover, E2 downregulates cytokine-induced TNF expression by inhibiting Jun NH2-terminal kinase (JNK), resulting in reduced c-Jun and JunD expression and binding on TNF gene promoter. In endothelial cells, AP-1 is activated by various proinflammatory stimuli, such as LDL and cytokines, and induces several genes involved in monocyte adhesion, such as MCP-1. The simultaneous inhibition of AP-1 and NF-κB by estrogens may thus result in a synergic endothelial atheroprotective effect, further enhanced by likely decreased interaction of AP-1 with NF-κB, which increases NF-κB activation of VCAM-1 promoter in endothelial cells.

A new and intriguing finding of our study is E2 inhibition of GATA binding to its consensus motif. Recent evidence suggests that some of the GATA transcription factors (GATA-4 and GATA-6) may be under hormonal control in reproductive tissues. Moreover, in erythroid precursor cells, estrogen receptor inhibits GATA-1 by ligand-dependent protein-protein interaction, downregulating several erythroid-specific genes. GATA-2 may represent a potential target for E2 in endothelial cells. In fact, endothelin-1 gene promoter is driven by GATA-2 binding on 2 GATA sites, and estrogen has been shown to repress endothelin-1 production from endothelial cells. Glucocorticoid interactions with GATA transcription factors have been reported in different systems, in which interactions of the glucocorticoid receptor with GATA-1 have been documented.

Interestingly, DEX, but not estrogen, prolongs VCAM-1 mRNA half-life. DEX would therefore exert 2 opposing actions on VCAM-1 expression, reducing transcription through inhibition of NF-κB and GATA-dependent promoter activation on the one hand and acting at the posttranscriptional level increasing VCAM-1 mRNA stability on the other. These data agree with other reports describing posttranscriptional regulations of the expression of various genes by glucocorticoids in mammalian cells, although no studies are available on the molecular mechanisms of these actions. The lack of such an effect for E2 may contribute, in part, to its greater inhibitory effect on VCAM-1 expression compared with DEX.

In conclusion, our findings characterize the mechanisms through which estrogen and glucocorticoid decrease cytokine-induced endothelial activation, demonstrating differential inhibitions of NF-κB, AP-1, and GATA transcription factors, and help explain the molecular basis of some of the antiatherogenic effects of estrogen on the vascular wall.

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