Antioxidant Effect of Estrogen on Cytomegalovirus-Induced Gene Expression in Coronary Artery Smooth Muscle Cells

Edith Speir, MS; Zu-Xi Yu, MD, PhD; Kazuyo Takeda, MD, PhD; Victor J. Ferrans, MD, PhD; Richard O. Cannon III, MD

Background—Pathogens infecting the arterial wall with resultant inflammation may contribute to atherogenesis. Human coronary artery smooth muscle cells (SMCs) infected with human cytomegalovirus (CMV) demonstrate a rapid increase in reactive oxygen species (ROSs), with activation of genes involved in viral replication and inflammation. Because estrogen appears to have antioxidant properties, we wished to determine whether this hormone attenuates SMC responses to CMV infection.

Methods and Results—Using confocal microscopy and an intracellular fluorescent dye activated by ROSs, we found that 17\(\beta\)-estradiol (0.1 to 10 nmol/L) and its stereoisomer 17\(\alpha\)-estradiol (which has low affinity for the estrogen receptor) dose-dependently inhibited ROS generation in CMV-infected SMCs. These effects were not blocked by the estrogen receptor inhibitor ICI 182,780. 3-Methoxyestrone, which lacks the phenolic hydroxyl group, did not interfere with ROS generation. We found that 17\(\beta\)-estradiol and 17\(\alpha\)-estradiol, but not 3-methoxyestrone, prevented binding of nuclear factor (NF)-k\(\beta\) to DNA. Furthermore, in SMCs transfected with the reporter constructs 3Xk\(\beta\)-CAT, MIEP-CAT, or ICAM-CAT, cotransfection with a CMV-IE72 expression plasmid caused promoter and CAT activation. Treatment with 17\(\beta\)-estradiol and 17\(\alpha\)-estradiol, but not 3-methoxyestrone, inhibited CAT activity and, in CMV-infected SMCs, prevented IE72 and ICAM-1 protein expression and cytopathic effects.

Conclusions—These findings indicate that estrogen molecules with an A-ring hydroxyl group have estrogen receptor–independent anti-CMV effects at physiological concentrations by inhibiting ROS generation, NF-k\(\beta\) activation, NF-k\(\beta\)–dependent transcription, and viral replication. To the extent that chronic infection of the vascular wall with CMV contributes to atherogenesis, these antioxidant actions of estrogen may be of therapeutic importance. (Circulation. 2000;102:2990-2996.)

Key Words: atherosclerosis \| viruses \| hormones \| antioxidants \| adhesion molecules

An atheroprotective effect of estrogen therapy in healthy postmenopausal women has been suggested by several studies.\(^1,2\) Although most actions of estrogen are receptor-dependent, nongenomic effects may also be important. Thus, 17\(\beta\)-estradiol (E\(_2\)) has antioxidant effects in isolated microsomes and protects neurons from oxidative stress, and E\(_2\) or 17\(\alpha\)-estradiol (17\(\alpha\)-E\(_2\)) can be effective in physiological doses.\(^3,5\) Furthermore, estradiol inhibits LDL peroxidation in postmenopausal women.\(^6\) The antioxidant effect of estradiol in neurons and lipid membranes appears to be dependent on the presence of the hydroxyl group in the C3 position of the A-ring of the steroid molecule.\(^5\)

Human cytomegalovirus (CMV) may contribute to the development and progression of atherosclerosis.\(^7\) We previously detected accumulated amounts of the tumor suppressor protein p53 in human coronary artery smooth muscle cells (SMCs) of restenosis atherectomies, which correlated with the presence of intracellular CMV.\(^8\) We further demonstrated that CMV infects SMCs and initiates viral replication, with generation of reactive oxygen species (ROSs),\(^9\) ROSs are important for activation of NF-k\(\beta\),\(^10\) a transcription factor that initiates expression of CMV proteins such as IE72 and IE84, and of mediators of inflammation such as intercellular adhesion molecule-1 (ICAM-1).\(^11\) CMV infection causes release of arachidonic acid, stimulation of xanthine/xanthine oxidase,\(^9\) and calcium influx, which contribute to ROS generation.

Because of the known effects of lipid and neuronal antioxidant properties and of reported interaction with calcium and cAMP, we asked whether estradiol might have effects with regard to ROS generation and cAMP activation and thereby inhibit viral and cellular gene expression. The partial estrogen antagonist tamoxifen has been shown to have lipid membrane antioxidant effects by decreasing membrane fluidity\(^4\) and by inhibiting protein kinase (PK) Ca\(\alpha\)\(^12\) an activator of NF-k\(\beta\). Because interaction of CMV particles...
with the cell membrane leads to activation of PKC.\(^1\) tamoxifen may interfere with CMV-dependent activation of the host cell by several mechanisms. Accordingly, we included tamoxifen, previously associated with reduced cardiovascular risk, in our experiments.\(^14\)

**Methods**

**Cells, Viruses, Plasmids, Antibodies, and Drugs**

Human coronary SMCs (38-year-old female donor) and optimal medium (SmGM including 5% FBS) were purchased from Clonetics. Cells and virus (CMV, Towne strain) were grown as described elsewhere.\(^9\) Virus and the plasmids pHDI101CAT3 (MIEP-CAT), pRC/RSVIE72, and pRSVIE72 were gifts from E.S. Huang, University of North Carolina at Chapel Hill; 3X-kB-CAT and 3X-mutxB-CAT were gifts from A.S. Baldwin Jr, University of North Carolina at Chapel Hill; the ICAM-1 constructs (\(-277/+1\) and \(-182/+1\); the former contains an essential NF-κB site, which is deleted in the latter) were gifts from S.W. Caughman, Emory University, Atlanta, Ga.\(^1\) The following supplies were purchased: E2, 17α-E2, 3-methoxyestrone (3-ME), and tamoxifen from Sigma; forskolin, (R)-p-adenosine-3',5'- cyclic phosphorothioate (Rpispo), and the calcium ionophore A23187 from Calbiochem. The estrogen receptor (ER) inhibitor IC1 182,780 was kindly supplied by Zeneica Pharmaceuticals. Biotinylated ICAM-1 antibody was obtained from R&D Systems, and fluorescein-streptavidin and anti-streptavidin reagents were obtained from Vector Laboratories. Fluo 3-AM was obtained from Molecular Probes.

**Assessment of H2O2-, CMV-, or cAMP-Dependent Intracellular Redox State**

Generation of ROSs after administration of H2O2 (200 nmol/L) to SMCs or during the first stages of CMV infection in SMCs was detectable after incubation with DCFH-DA for 5 minutes. One slide for ICAM-1 and 3-ME, and tamoxifen from Sigma; forskolin, (R)-p-adenosine-3',5'-cyclic phosphorothioate (Rpispo), and the calcium ionophore A23187 from Calbiochem. The estrogen receptor (ER) inhibitor IC1 182,780 was kindly supplied by Zeneica Pharmaceuticals. Biotinylated ICAM-1 antibody was obtained from R&D Systems, and fluorescein-streptavidin and anti-streptavidin reagents were obtained from Vector Laboratories. Fluo 3-AM was obtained from Molecular Probes.

**Immunocytochemistry and Northern Blot Analysis for ICAM-1**

**Transfections and CAT Assays**

SMCs were grown in optimal medium (5% FBS). For CAT assays, cells were seeded in 100-mm plates (1 x 10^6) and transfected 20 hours later in serum-free SmGM with 0.1 µg (per 3 mL total volume per dish) of MIEP-CAT. For cotransfections, we used 0.1 µg MIEP-CAT plus 1 µg pRC IE72; 1 µg of 3X-kB-CAT or 3X-mutxB-CAT plus 0.5 µg of the IE72; and 2 µg ICAM-1-CAT or kB-deleted ICAM-1-CAT plus increasing amounts of IE72. DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylene sulfate; Boehringer Mannheim) lipofection agent was used as described above.\(^9\) The assay was repeated twice.

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each was pretreated with E2 or 3-ME (1 nmol/L), with N-acetylcysteine (NAC, 10 mmol/L, Sigma), or with vehicle for 1 hour in serum-free medium. CMV infection was performed as described above. SMCs were incubated with growth medium until harvested at 3 hours after infection by scraping into 15 mL Trizol (phenol and guanidine isothiocyanate, Gibco/BRL). The suspension was extracted with chloroform according to directions supplied with the reagent. Total RNA (15 to 20 µg/lane) was resolved on a 1% agarose/formaldehyde gel and transferred overnight to nylon membranes. With ICAM-1 oligo-cocktail (R&D Systems) end-labeled with $^{32}$P, $1 \times 10^7$ cpm of probe was used for overnight (12 to 16 hours) hybridization at 42°C. Three experiments were performed, with similar results.

**Immunoblotting**

SMCs were pretreated for 1 hour with the estrogen compounds or tamoxifen and then infected with 2 MOI of CMV for 16 hours. Cells were then lysed, and equal aliquots of protein were subjected to SDS-PAGE as described in detail elsewhere. Whole-cell extracts were prepared for blotting with IE72 at 16 hours after CMV infection.

![Figure 1](image1.png)

**Figure 1.** SMCs were pretreated for 1 hour with indicated doses of E2, $17\alpha$-E2 (17a), 3-ME, or tamoxifen (TAM). Cells were then infected for 1 hour with CMV at 5 MOI, followed by addition of DCFH-DA fluorescent dye. Both stereoisomers of estradiol and tamoxifen, but not 3-ME, dose-dependently inhibited fluorescence, as determined by confocal laser microscopy.

![Figure 2](image2.png)

**Figure 2.** Intracellular cAMP levels at 0.5 hour (lanes 1 to 4) and 1 hour (lanes 5 to 8) after treatment of CMV-infected cells with vehicle, forskolin (FSK; 5 µmol/L), E2, and $17\alpha$-E2 (both 10 nmol/L).

![Figure 3](image3.png)

**Figure 3.** Electrophoretic mobility shift assay was performed as described in Methods after SMCs were pretreated for 1 hour with hormones and then infected for 1 hour with CMV, $17\alpha$-E2 and E2 attenuated NF-κB/DNA complex formation, whereas 3-ME had little effect compared with vehicle-treated infected SMCs (lane 2). Lane 1, Labeled DNA probe; lane 2, CMV+vehicle (0.1% ethanol); lane 3, CMV+$17\alpha$-E2 (10 nmol/L); lane 4, CMV+3-ME; lanes 5 and 6, CMV+E2 (1 nmol/L and 10 nmol/L, respectively). Top band is nonspecific (NS). Migration of 2 bands is characteristic for p65/p50 heterodimers (solid arrow) and p50/50 homodimers (open arrow).
Viral Infectivity Assay and Cytopathic Effects

SMCs were infected with 5 MOI of CMV, as described before. Estrogen compounds, tamoxifen, ethanol vehicle, or medium was added to SMCs at 1 hour before infection. Free virus was then removed, cells were washed, and growth medium was added. The medium was renewed the next day and every 48 hours thereafter. Cytopathic effects and plaque formation were assessed at 3 to 5 days after infection by counting the number of foci (plaques) of infected cells exhibiting cytomegalic changes.

Cell Viability

After each experiment described above, SMCs were returned to the incubator for 3 to 7 days and monitored for cytopathic effects. Extra wells and dishes were prepared for cell counts and trypan blue exclusion. None of the treatments caused cell death.

Statistics

All experiments were performed in duplicate or triplicate with 3 to 6 separate SMC cultures, except for the immunoblots and Northern blots, which were performed twice from 2 separate cultures. Data are presented as mean±SD. InStat3 software was used for calculation of probability values by 1-way ANOVA and for Tukey-Kramer multiple comparison test.

Results

**Effects of Estrogen on ROS-Dependent Fluorescence**

We examined the effect of estradiol on ROS generation by SMCs in response to exogenous H2O2 (substrate for DCFH-DA) or to CMV infection. E2 and its stereoisomer 17α-E2 dose-dependently inhibited ROS-dependent fluorescence in SMCs (0.1, 0.5, and 1.0 nmol/L), whether induced by exogenous H2O2 (Table) or by CMV infection (Figure 1, Table). Similar inhibitory effects on ROS generation were seen with the partial ER antagonist tamoxifen (50 to 500 nmol/L), but not with 3-ME. Treatment with the ER inhibitor ICI 182,780 (1 μmol/L) had little effect on reduction of fluorescence by E2 (Table). These data indicate that in SMCs, estradiol has dose-dependent and ER–independent inhibitory effects on ROSs derived from H2O2, the substrate for DCFH-DA. CMV infection causes cellular release of ROSs and arachidonic acid and stimulates PKC.13 We also assessed the effects of E2 on calcium by loading SMCs with the calcium-specific fluorescence probe fluo 3-AM. Intracellular calcium, depicted as fluo 3-AM fluorescence, was increased at 1 to 60 minutes after SMCs had been exposed to (1) 50 nmol/L calcium chloride in the presence of the ionophore A23187 and (2) CMV. E2 inhibited calcium-dependent fluorescence in each case. ROSs can induce calcium overload, loss of cAMP, and smooth muscle contraction, and providing cAMP or antioxidants can restore cAMP-dependent PKA, which can counteract ROS-induced effects.16,17 E2 has been shown to...
increase intracellular cAMP levels, and we found that treatment of CMV-infected cells with E2 or 17α-E2 increased intracellular cAMP levels at 0.5 to 1 hour after infection. We used forskolin (5 μmol/L), an adenylyl cyclase activator that increases PKA, as a positive control (Figure 2). Increased cAMP has beneficial effects on calcium homeostasis and cellular redox state, and this could be one of the inhibitory mechanisms of E2 on SMCs activated by CMV.

**Estrogen and CMV-Induced Binding of NF-κB**

CMV has been shown to cause ROS generation, with subsequent activation and nuclear translocation of NF-κB in a variety of cells. Pretreatment of SMCs for 1 hour with 17α-E2 or E2, but not with 3-ME, caused a marked decrease in CMV-induced NF-κB binding, as shown by electrophoretic mobility shift assay (Figure 3).

**Estrogen and NF-κB–Dependent Transcription**

Because transcriptional mechanisms play an important role in ROS/NF-κB stimulation of viral and inflammatory gene expression, we used transient transfections to examine E2 effects on transactivation by IE72 protein (Figure 4A through 4C) versus (1) reporters containing 3 wild-type or 3 mutated NF-κB–binding elements (3XκB-CAT, 3Xm-κB-CAT); (2) the CMV promoter MIEP, which contains 4 NF-κB binding sites and multiple other cellular transcription factor binding sites; and (3) the effect of E2-induced cAMP on NF-κB–dependent transcription. In SMCs transfected with 3XκB-CAT, cotransfection with IE72 caused a 5-fold increase in CAT activity, and this activity was inhibited by forskolin (20 μmol/L) or E2 (10 nmol/L; bars 5 and 7, respectively). Treatment of transfected cells with forskolin plus Rpiso (10 μmol/L, PKA inhibitor) or E2 plus Rpiso partially relieved the inhibition. These findings indicate signaling between E2 and cAMP that leads to anti-inflammatory effects in coronary SMCs. cAMP has been shown to interfere with NF-κB–dependent transcription in human endothelial cells. We now report that E2-induced cAMP reduces NF-κB–dependent transcription in SMCs.

**Estradiol Inhibits IE72 Protein Expression**

Immunoblotting of lysates of CMV-infected cells with an IE72-specific antibody demonstrated that treatment with E2 and 17α-E2 but not 3-ME reduced steady-state levels of IE72 protein at 16 hours after infection (Figure 5). These experiments show that estradiol attenuates the expression of IE72. This blocking effect is due, at least in part, to the antioxidant and NFκB-inhibitory effects of estradiol. Because IE72 is essential for the expression of the early and late genes of CMV, inhibition of IE72 effectively decreases viral productivity.

**Estradiol Inhibits ICAM-1 Transcription and Protein Expression**

CMV-IE72 transactivates the ICAM-1 promoter via the intact NF-κB binding site, because deletion of this site results in lack of response (Figure 6A). As shown by Northern blotting, ICAM message was detectable at 3 hours after CMV infection of SMCs, whereas baseline (0 hours) mRNA was undetectable (Figure 6B). Pretreatment of SMCs with NAC (10 mmol/L) or with E2 (10 nmol/L) reduced ICAM-1 mRNA, whereas 3-ME had little effect. Hybridization with the housekeeping gene glucose-3-phosphate-dehydrogenase
confirmed both viability of the cells and equal sample loading. As seen by immunofluorescence, pretreatment with E$_2$ but not with 3-ME, inhibits ICAM-1 protein expression at 24 hours after CMV infection (Figure 6C). These data indicate that E$_2$ attenuates CMV-induced ICAM-1 message by an antioxidant mechanism. This effect appears to be independent of ERs, because similar effects were obtained with 17α-E$_2$.

**Viral Replication and Cytopathology**

We next examined whether the inhibitory effect of estradiol on viral transcription and on expression of IE72 would affect viral replication. We infected SMCs with viral stock that had been treated with ethanol vehicle or with increasing concentrations of E$_2$, 17α-E$_2$, 3-ME, or tamoxifen. Infection in untreated and vehicle-treated wells was similar. In contrast, E$_2$ and 17α-E$_2$, but not 3-ME, each inhibited viral infectivity in concentration-related fashion, and maximally (10 nmol/L) by 65%, and 5 µmol/L tamoxifen reduced viral titer by 80% (Figure 7). These data suggest that estradiol and tamoxifen attenuate CMV infectivity and cytopathic effects in human coronary SMCs.

**Discussion**

We have previously reported that infection of coronary artery SMCs with human CMV results in a rapid increase in ROSs. Here we show that E$_2$, at physiological concentrations, inhibits ROSs induced by H$_2$O$_2$ or by CMV infection, E$_2$ also inhibits intracellular calcium after (1) uptake of exogenous calcium chloride in the presence of ionophore A23187 and (2) accumulation during CMV infection (Speir et al, unpublished observations). Interestingly, in a recent report, sex-specific inhibition of calcium entry during vasoconstriction was E$_2$-dependent. In a reperfusion model with nanomolar E$_2$ plasma concentrations, E$_2$ diminished hydroxyl radical production. In the present study, inhibition of ROSs by E$_2$ and 17α-E$_2$ is probably due to antioxidant properties, because 3-ME, a related compound that lacks the phenolic group, does not reduce ROSs. These properties of E$_2$ and 17α-E$_2$ are independent of ligand/receptor binding because (1) ERs in SMCs are not demonstrable by immunocytochemistry or immunoblotting (data not shown); (2) inhibition of H$_2$O$_2$- and CMV-induced ROSs are similar for E$_2$ and 17α-E$_2$, but the latter has weak ER affinity; and (3) coincubation with the ER blocker ICI 182,780 does not diminish the inhibitory actions of E$_2$ on ROS fluorescence. Estrogen also prevents SMC membrane peroxidation, but micromolar E$_2$ concentrations were required. Although high concentrations of E$_2$ are generally required for ROS scavenging, physiological doses of E$_2$ can be effective. This could depend on cell type and experimental conditions. For example, in our experience, human coronary SMCs do not tolerate H$_2$O$_2$ concentrations >1 µmol/L, whereas human aortic and rat SMCs tolerate 100 to 200 µmol/L H$_2$O$_2$. Importantly, CMV-infected SMCs are in a highly activated state: within minutes, CMV induces NF-κB, phospholipases, calcium influx, and release of arachidonic acid. Bioavailability of E$_2$ is another variable. Thus, physiological concentrations of estradiol increased nitric oxide bioavailability by inhibiting superoxide anion production; however, an ER-dependent mechanism was implied.

Another finding is linked to E$_2$ antioxidant actions: E$_2$ and 17α-E$_2$ stimulate intracellular cAMP (Figure 2), which inhibits NF-κB–induced transcription (Figure 4C). The effects of E$_2$ on cAMP could account for important ER-independent mechanisms of gene activation. Effects via hypothetical membrane receptors have been reported, and our findings suggest that redox effects, perhaps relieving oxidative modification of cAMP-dependent PKA may play a role. In neurons, protective effects by E$_2$ have been linked to increases in cAMP levels, and this was inhibited by antagonists of cAMP/PKA, but not by the ER antagonist ICI 182,780, involving E$_2$-binding sites that are distinct from both ERα and ERβ. It is possible that SMCs express binding sites similar to those in neurons. However, our data suggest that E$_2$ increased cAMP levels by scavenging ROSs and subsequent protection of PKA from oxidation. Further experiments are needed to support this hypothesis.

We demonstrate that estradiol inhibits important steps of viral gene expression. Activation of the MIEP by mitogenic stimuli leads to expression within 3 to 4 hours of IE72, which transactivates the MIEP via NF-κB, phospholipases, calcium influx, and release of arachidonic acid. Bioavailability of E$_2$ is another variable. Thus, physiological concentrations of estradiol increased nitric oxide bioavailability by inhibiting superoxide anion production; howev

**Figure 7.** Inhibition of IE72 by pretreatment with E$_2$, 17α-E$_2$, or tamoxifen (TAM) had further functional consequences by decreasing cytopathic effects at 96 hours after CMV infection of SMCs. Treatment with vehicle (0.1% ethanol) or with 3-ME had little effect. Shown are means of 3 separate experiments. P < 0.05.

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In latently infected vascular cells, frequent abortive reactivation occurs, characterized by activation of the MIEP and expression of IE72, which activates the MIEP and ICAM. Thus, suppression of NF-κB and IE72 prevents cell activation and progression of infection. As evidence for this, both stereoisomers of estradiol and tamoxifen, but not 3-methoxyestrone, reduced IE72 protein expression, viral titers, and cytopathic effects in SMCs. Our results suggest that estrogen treatment may inhibit viral and cellular inflammatory genes, which may contribute to atherogenesis in susceptible women.

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