Reciprocal Antagonism Between Estrogen Receptor and NF-κB Activity In Vivo

Mark J. Evans, Amy Eckert, KehDih Lai, Steven J. Adelman, Douglas C. Harnish

Abstract—The functional interaction, or “cross-talk,” between estrogen receptor (ER) and the proinflammatory transcription factor nuclear factor (NF)-κB demonstrated in vitro has been suggested to play a role in estrogen prevention of cardiovascular disease. Here, we demonstrate that this reciprocal cross-talk occurs in vivo. Ovariectomized C57BL/6 mice fed an atherogenic diet had increased hepatic levels of active NF-κB and numerous inflammatory genes, including MHC invariant chain (Ii), vascular cell adhesion molecule-1, tumor necrosis factor-α, and RANTES. Treatment with 17α-ethinylestradiol (EE) strongly blocked induction of these genes but had no effect on their basal expression levels. ER was required for this activity, because the antagonist ICI 182,780 completely blocked the inhibitory activity of EE. Gene activation by EE was not required for inhibition of inflammatory gene expression, because both the phytoestrogen genistein and low doses of EE were effective in blocking inflammatory gene induction without inducing marker genes such as intestinal trefoil factor (ITF) or myo-inositol-1-phosphate synthase (IPS). The in vivo transcriptional interference was reciprocal, with EE induction of ITF and IPS greatly reduced in animals fed the atherogenic diet versus chow-fed controls. This interference was specific to the liver, because diet had no effect on uterine weight increases produced by EE. Transfection experiments confirmed that the extent of inhibition of ER-mediated transcription by inflammatory stimuli correlated with the extent of NF-κB activation. These results suggest that the cross-talk between ER and NF-κB does occur in vivo and may indeed contribute significantly to the cardioprotective effects of estrogen. (Circ Res. 2001; 89:●●●●●●.)

Key Words: estrogen replacement therapy ■ liver ■ transcription factors ■ atherosclerosis ■ nuclear factor-κB

Coronary artery disease is the leading cause of mortality for women and increases dramatically after menopause. Observational and epidemiological studies consistently indicate that estrogen replacement therapy (ERT) substantially lowers the risk for cardiovascular events. One clear benefit of ERT is favorable changes in the lipoprotein profile, including decreased LDL, increased HDL, and decreased lipoprotein (a) concentrations. The magnitude of these lipid changes, however, suggests that they are probably responsible for less than half of the reduction in coronary artery disease (reviewed by Mendelsohn and Karas). Estrogen treatment also inhibits lesion development in several animal models, even in the absence of plasma cholesterol lowering. Localization of estrogen receptors (ERs) in the endothelial cells and vascular smooth muscle cells suggests that the protective effects of estrogen may be due to direct effects on the vasculature.

Inflammation is now recognized to be a key component of atherosclerosis development, with the transcription factor nuclear factor (NF)-κB involved in both the early and late stages of the inflammatory-proliferative process. Both activated NF-κB and elevated expression of NF-κB–dependent proinflammatory gene products, including adhesion molecules, cytokines, and chemokines, are present in endothelial cells, macrophages, and smooth muscle cells within the human atheroma. Treatment of postmenopausal women with estrogens reduces plasma levels of adhesion molecules and other markers of endothelial activation. In hypercholesterolemic rabbits, estradiol both inhibits monocyte adhesion and decreases vascular cell adhesion molecule (VCAM)-1 expression. Estradiol also inhibits VCAM-1 expression in cultured endothelial cells through interference of NF-κB activity. These results suggest that part of the cardiovascular benefits of estrogen may be due to its ability to interfere with NF-κB–mediated inflammatory gene activation in the vasculature.

Several groups have identified the potential for a reciprocal transcription inhibition between agonist-bound ERα and activated NF-κB. The mechanisms of this cross-talk are cell type–dependent and involve direct protein-protein interactions, inhibition of DNA binding, induction of IkB expression, or coactivator sharing. Although the cross-talk between ER and NF-κB has been demonstrated in a number of cell types in vitro, its occurrence in vivo has not been observed. In C57BL/6 mice fed a high-fat diet, development of the fatty streak in the aorta is positively correlated with estrogens.
with induction of NF-κB binding activity and proinflammatory gene expression in the liver, suggesting that NF-κB activation in the liver may also contribute indirectly to atherosclerosis. Because liver expresses ERα, this model can provide a means to assess NF-κB and ER cross-talk in a physiologically relevant context. Using this model, we demonstrate here a reciprocal in vivo inhibition between NF-κB and ER-mediated gene expression in liver. Treatment of ovariectomized C57BL/6 mice with 17β-estradiol (EE) resulted in a dose-dependent, receptor-dependent inhibition of NF-κB-directed gene expression induced in the liver by an atherogenic diet. Reciprocally, ER-mediated gene activation was inhibited in the liver by the atherogenic diet. These results suggest an anti-inflammatory role for estrogen, which may be a fundamental component of its activity.

Materials and Methods

Mice
Ovariectomized C57BL/6 mice (16 to 20 g; Taconic) were separated into groups of 8. After 5 to 7 days of recovery, the mice were fed a chow diet, a western diet (20% fat, 0.15% cholesterol), or an atherogenic diet (15.75% fat, 1.25% cholesterol, 0.5% sodium cholate) (Purina dipts Nos. 5892 and 21539, respectively). EE, genistein, or ICI 182,780 (ICI) was administered once daily subcutaneously or by gavage in a 10% EtOH/corn oil vehicle (0.1 mL per mouse) for up to 5 weeks. At the end of the experimental period, the liver was collected and eterine wet weight was recorded.

RNA Analysis
Liver total RNA was prepared by use of Trizol reagent (BRL). For microarray studies, polyA RNA was purified with the polyATtract system (Promega). Double-stranded cDNA was synthesized from 1 μg polyA RNA by use of the SuperScript System (BRL). The cDNA was purified by phenol chloroform extraction, precipitated, and transcribed in vitro with T7 RNA polymerase. The cRNA was purified by RNeasy column (Qiagen) and fragmented by incubation in 40 mmol/L Tris (pH 8.1), 100 mmol/L potassium acetate, 30 mmol/L magnesium acetate buffer at 94°C for 35 minutes, after which 15 μg of fragmented RNA was hybridized sequentially to Mu11KsubA and Mu11KsubB GeneChips (Affymetrix) according to the manufacturer’s recommendations. The hybridized chips were washed as recommended and scanned and analyzed with GeneChip 3.1 (Affymetrix). Expression levels in animals receiving the high-fat diet plus vehicle or high-fat diet plus EE were determined by a paired Student’s t test.

To determine the ability of estrogens to regulate the development of diet-induced inflammation in the liver, gene expression changes resulting from the atherogenic diet were monitored by a global analysis of ~11,000 genes with silicon chip–based microarrays. As expected, feeding the atherogenic diet to vehicle-treated mice repressed expression of the LDL receptor and the cholesterol biosynthetic pathway genes present on the chip (Figure 1, top), with the exceptions of HMG-CoA reductase and mevalonate phosphate decarboxylase. HMG-CoA reductase is known to be subject to extensive posttranscriptional regulation, which may provide the basis for the lack of regulation of HMG-CoA reductase mRNA levels seen here. In addition, the atherogenic diet strongly induced the expression of numerous inflammatory genes (Figure 1, bottom), including members of the MHC family (MHC class II α- and β- and invariant [Ii] chain), monocyte/macrophage specific markers (CD14, CD18, CSF-1 receptor, lysozyme M), chemokines (RANTES), cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1β), and adhesion molecules (VCAM-1). Treatment of these mice with 10 μg · kg⁻¹ · d⁻¹ EE generally resulted in no regulation of cholesterol metabolism gene expression. In contrast, inflammatory gene induction by the atherogenic diet was consistently diminished by treatment with EE. The nuclear content of the NF-κB subunit p65 and the DNA binding activity of NF-κB were increased in mice fed the atherogenic diet (Figure 2) and were specific for NF-κB, because SP1 levels were unchanged (data not shown). EE treatment did not affect NF-κB nuclear translocation or DNA binding, suggest-
ing that ER regulation occurs through interference of NF-κB transcriptional activity.

To compare the concentrations of EE required for suppression of inflammatory gene expression to known EE inductions, ovariectomized C57BL/6 mice were fed the atherogenic diet and treated with increasing concentrations of EE (Figure 3). Real-time RT-PCR analysis confirmed the atherogenic diet induction of mRNA for Ii, VCAM-1, RANTES, and TNF-α. As before, treatment with 10 μg·kg⁻¹·d⁻¹ EE strongly suppressed the induction of these genes. Furthermore, doses as low as 0.1 μg·kg⁻¹·d⁻¹ EE also significantly attenuated atherogenic diet induction of all these genes, although not to the same magnitude as higher doses of EE. As markers for EE induction of gene expression in the liver, we quantified expression of intestinal trefoil factor (ITF) and myo-inositol-1-phosphate synthase (IPS). These 2 genes are induced by estrogen in other systems, and our GeneChip analysis confirmed that these genes were also strongly induced in the mouse liver by EE treatment (data not shown). In contrast to the effects on inflammatory gene induction, ITF and IPS were induced in the liver only with the 10-μg·kg⁻¹·d⁻¹ EE dose. Uterine size is another highly sensitive marker for estrogenic effects. Significant increases in uterine weight were observed with the 1- and 10-μg·kg⁻¹·d⁻¹ EE treatments. Thus, inhibition of inflammatory gene expression could occur at EE doses lower than those necessary to stimulate gene expression in the liver or to induce uterine proliferation.

Estrogens have multiple mechanisms of action, including not only ER-mediated genomic and nongenomic actions but also receptor-independent activities due to the physicochemical antioxidant properties of estrogens. The antioxidant pyrrolidine dithiocarbamate inhibits NF-κB activation of gene expression in the rat liver, suggesting that the antioxidant properties of EE could be important for activity in this system. To verify that ER was necessary for EE inhibition of inflammatory gene induction, mice on the atherogenic diet were treated with EE, the ER antagonist ICI, or EE plus a 100-fold excess of ICI (Figure 4). ICI alone did not reduce atherogenic diet induction of Ii, VCAM-1, RANTES, or TNF-α. The EE-mediated downmodulation of these genes was completely blocked by ICI coadministration, however, demonstrating that ER mediates the inhibitory activity of EE.

Inhibition of ER Activity by Inflammatory Signals

Inhibition of diet-induced gene expression by EE raised the possibility that the converse might also occur: that EE induction of gene expression could be inhibited by inflam-

---

**Figure 1.** Atherogenic diet regulation of gene expression in the liver. Ovariectomized C57BL/6 mice were fed a chow or atherogenic diet with subcutaneous treatment with vehicle (gray bars) or 10 μg·kg⁻¹·d⁻¹ EE (solid bars). After 5 weeks, liver total RNA was prepared and subjected to microarray analysis. The bars denote expression levels of each gene, with the expression level of each gene in mice fed the chow diet plus vehicle treatment defined as 1. Top, Expression of several genes involved in cholesterol metabolism; bottom, expression of several genes involved in inflammatory processes.

**Figure 2.** Atherogenic (Athero) diet induction and EE regulation of NF-κB in the mouse liver. Ovariectomized female C57BL/6 mice were fed either chow or atherogenic diet and treated with 10 μg·kg⁻¹·d⁻¹ EE. After 5 weeks, nuclear extracts were prepared and analyzed for p65 protein levels by Western blot (top) or for NF-κB binding activity by gel mobility shift analysis with NF-κB oligonucleotide probe (bottom). Results shown are from 3 representative animals. The indicated composition of the retarded complexes was determined by antibody supershift experiments.
matory transcription factors, such as NF-κB, induced by the diet. To address this possibility, the induction of ITF and IPS mRNA levels in the liver by EE was monitored in ovariectomized C57BL/6 mice fed the chow, western, or atherogenic diet (Figure 5). The induction of both ITF and IPS was strongly reduced in animals fed the atherogenic diet, and the western diet significantly reduced induction of ITF and partially inhibited induction of IPS. In contrast, the increase in uterine wet weights produced by EE was not altered by diet, suggesting that the inflammatory response in the liver mediated the diminishment of EE activity. The induction of ITF and IPS is completely dependent on the presence of ERα and is completely eliminated in ERα-knockout mice (data not shown). Because the acute-phase inflammatory response decreases expression of several hepatic transcription factors, including RXRa, PPARα, and LXRa, it was possible that reduced induction of ITF and IPS was due to diminished levels of ERα. Equivalent ERα protein levels, however, were detected by Western blot analysis of nuclear extracts prepared from mice fed the different diets. Together, this indicates that reduction of ITF and IPS gene induction by EE in mice fed the atherogenic diet probably also occurs through interference with ERα transcriptional activity.

In vivo, transcription factors other than NF-κB could be induced by the atherogenic diet. To verify the in vivo results and confirm that NF-κB itself could inhibit ERα stimulation of gene expression, HepG2 cells were cotransfected with an ERα expression vector and a luciferase reporter driven by either an estrogen response element (ERE.LUC; Figure 6A) or an NF-κB response element (NF-κB.LUC; Figure 6B). Treatment with the cytokines IL-1β and TNF-α repressed 17β-estradiol (E2)-induced transactivation of the ERE reporter, whereas treatment with IL-6 had only a modest effect. The relative ability of these cytokines to interfere with ERα transactivation correlated with their ability to stimulate expression from the NF-κB reporter. Finally, overexpression of the p65 subunit of NF-κB both strongly repressed ERα transactivation and strongly induced expression of the NF-κB reporter plasmid. Thus, NF-κB (p65) itself was able to inhibit ERα activity.

Genistein Can Inhibit the Diet-Induced Elevation of Inflammatory Gene Expression

The reciprocal antagonism of ER- and NF-κB-mediated gene expression implies that in vivo, ER can antagonize inflammatory gene expression without the requirement for ER-dependent gene activation, because the transcriptional activity of ERα itself is diminished by NF-κB, and inhibition of atherogenic diet–induced gene expression occurred in vivo at EE doses that failed to activate gene expression (Figure 3).
This possibility was addressed by treating mice with the weaker ER agonist genistein, a phytoestrogen with favorable effects on lipid profiles and with inhibitory activity for adhesion molecule expression (Takahashi et al.; M.J.E., data not shown). Genistein treatment of ovariectomized C57BL/6 mice fed the atherogenic diet inhibited induction of i, VCAM-1, RANTES, and TNF-α to the same extent as EE treatment (Figure 7). Unlike EE, however, genistein did not stimulate ITF (Figure 7) or IPS (data not shown). Although we cannot exclude the possibility that genistein induced some genes, these results suggest that ER activation of gene expression in general is not required for inhibition of diet gene inductions.

**EE Prevents but Does Not Reverse High-Fat Diet–Induced Inflammation**

Emerging clinical data suggest that ERT is most effective at preventing cardiovascular disease when used early in the atherosclerotic process (reviewed by Genazzani and Gambacciani). To determine whether the animal model established here might share this feature, we investigated whether treatment with EE after inflammatory gene expression was already induced would be as effective as treatment with EE from the initiation of the experiment. Induction of VCAM-1 (Figure 8) and other genes (not shown) was shown to occur as early as 2 weeks after the mice were switched to an atherogenic diet. Ovariectomized C57BL/6 mice were fed an atherogenic diet and treated with either vehicle throughout the 5 weeks; orally administered 10 mg · kg⁻¹ · d⁻¹ EE throughout the 5 weeks (prevention mode); or orally administered 10 μg · kg⁻¹ · d⁻¹ EE administered from week 3 to week 5 (treatment mode, Figure 8). Oral administration of EE in the prevention mode inhibited induction of i, VCAM-1, and TNF-α as completely as seen previously with subcutaneous administration of EE. In contrast, mRNA levels for these genes were not decreased when EE was administered in the treatment mode. This was not due to a general loss of ER activity, because ITF induction still occurred in the treatment mode. Thus, EE treatment dramatically blocked the development of inflammation induced by the atherogenic diet but was not effective at reducing inflammatory gene expression (at least in a 3-week period) once the inflammatory process was already initiated.

**Discussion**

Atherosclerosis is now recognized as an inflammatory process dependent primarily on monocyte infiltration into the vascular wall. Feeding C57BL/6 mice a high-fat diet has been...
shown previously to induce inflammation in the mouse liver, which correlates to fatty-streak formation in the aorta. Here, we have used microarray analysis to characterize this inflammatory process. Numerous inflammation-associated genes were induced by the diet, including antigen presentation proteins, chemokines, cytokines, and adhesion molecules. These markers were all indicative of a primarily monocytic activation. No markers of T-cell infiltration or activation, such as CD3 or IL-2 receptor, or markers of neutrophils, such as elastase, were upregulated by the diet. In addition, diet induction of genes such as RANTES, VCAM-1, and TNF-α occurred to a similar extent in C57BL/6 scid mice lacking T and B cells (data not shown). Thus, the diet-induced inflammation in the liver, like the atherosclerotic lesion, appears to be a predominantly monocyte-driven process.

Although the in vitro cross-talk between ER and NF-κB has been demonstrated in a number of cell types, the in vivo physiological existence of this effect has not been clearly established. NF-κB DNA binding activity was greatly increased in animals consuming the atherogenic diet, and NF-κB is known to directly activate many of the genes stimulated by the diet, including Il, RANTES, VCAM-1, and TNF-α. Here, we show that treatment of ovariectomized mice with EE blocked diet induction of all of these genes and was dependent on the ER. No loss of NF-κB nuclear localization or DNA binding was observed, however, suggesting that ER is targeting at the transcriptional level, consistent with previous observations in cultured hepatocytes, in which competition for limiting amounts of the coactivator CREB-binding protein (CBP) was implicated. No regulation of CBP was observed in the mice fed the atherogenic diet with or without EE treatment (data not shown), consistent with the possibility that CBP may become limiting in hepatocytes when both ER and NF-κB are activated.

The inhibition of NF-κB and ER-mediated gene expression was reciprocal. Microarray analysis identified ITF and IPS as being significantly induced in the liver by EE treatment. ITF expression is also strongly stimulated by estrogen treatment of human breast cancer cells, whereas IPS stimulation has been demonstrated in the rat uterus. EE induction of both ITF and IPS was greatly reduced in mice fed the atherogenic diet. The Western diet did partially reduce EE induction of ITF, but this may have been due to the ability of short-chain fatty acids to inhibit ITF gene expression. No loss of EE action was observed in the uterus of the mice fed the atherogenic diet, suggesting that only those tissues undergoing an inflammatory response were affected. Direct evidence for the interference of ERα activity by NF-κB was confirmed by transfection experiments demonstrating cytokine interference of E2-mediated reporter activity in HepG2 cells. The extent of ERα inhibition by the various cytokines correlated with their ability to activate an NF-κB reporter, implying a direct involvement of NF-κB. In fact, overexpression of the NF-κB subunit p65 also inhibited ERα-mediated reporter activity. Thus, in vitro data concur with the in vivo results,
suggesting an in vivo transcriptional interference between NF-κB and ER.

The loss of ER-mediated gene activation during an inflammatory response while maintaining the ability to interfere with NF-κB-directed gene expression implies that the predominant mechanism of action of estrogen during inflammation is gene repression. Two lines of evidence argue that ER stimulation of gene expression is not required for inhibition of inflammatory gene induction. First, repression of i, RANTES, and TNF-α occurred at EE doses that did not stimulate IPS or ITF gene activation. Second, treatment with the weak ER agonist genistein repressed inflammatory gene expression to the same extent as EE, yet did not stimulate IPS or ITP expression in the liver. Genistein is also known to inhibit tyrosine kinases, but it requires concentrations >10 μmol/L,41 which were not achieved here. Genistein interaction with ERα has been shown to inhibit NF-κB in vitro17 and is mechanistically consistent with reports demonstrating reduced intimal lesion area in C57BL/6 mice fed a high-cholesterol, isoflavone-rich diet42 and the ability of genistein to inhibit neointima proliferation in a rat carotid injury model.43

The temporal pattern of EE administration was critical to its ability to inhibit inflammatory gene expression. When EE was administered starting the same day the mice began to receive the atherogenic diet, inflammatory gene induction was suppressed. In contrast, if EE treatment did not begin until 2 weeks after the mice began eating the atherogenic diet, no repression of inflammatory gene expression could be detected. Mechanistically, the ability of EE to prevent but not reverse induction of i, VCAM-1, and TNF-α expression suggests either that regulation of other earlier genes in the inflammatory cascade may be critical or that once the inflammatory process is well established, liganded ER is no longer an effective inhibitor. This pattern is consistent with emerging clinical data suggesting that ERT may be most effective at the prevention of development of cardiovascular disease rather than the regression of established disease.44

Inhibition of NF-κB activity is a promising avenue of research for treatment of inflammatory diseases. Estrogen has negative effects for women, however, such as uterine hypertrophy, and is not currently considered for use in men. The demonstration of apparent ER inhibition of NF-κB activity in vivo by a mechanism that does not require gene induction suggests an alternative approach. Development of ligands that bind to the ER and induce a conformation able to mediate the negative cross-talk with the NF-κB pathway without inducing

Figure 6. In vitro competition between NF-κB and ER. A, HepG2 cells were cotransfected with 0.5 μg ERE.LUC reporter, 0.5 μg pRSVβ-galactosidase, and 0.2 μg pcDNA3-ERα expression vector. A pcDNA3.1-p65 expression vector (0.5 μg) was included in the transfection as indicated. After transfection, the cells were treated for 6 hours with 100 nmol/L E2 (+) or vehicle (−), along with IL-1β (100 U/mL), TNF-α (5 ng/mL), or IL-6 (200 U/mL) as indicated. Cell extracts were prepared and analyzed for luciferase expression. Values are mean±SEM after normalization for β-galactosidase activity. The activity of the ERE.LUC plasmid in the absence of E2 is defined as 1. *P<0.1 for difference from the unstimulated, E2-treated group. B, Transfections were conducted as above except with an NF-κB.LUC reporter in the presence of the various agents as indicated. The activity of the NF-κB.LUC reporter alone is defined as 1.0. *P<0.1 for difference from the untreated group.
ER-mediated gene expression may represent a new preventative for inflammatory diseases.

**Acknowledgment**
This work was supported by Wyeth-Ayerst Pharmaceuticals.

**References**

Figure 7. Genistein inhibits inflammatory gene expression. Ovariectomized C57BL/6 mice were fed either a chow or atherogenic diet and treated subcutaneously with vehicle (solid bars), 10 μg · kg⁻¹ · d⁻¹ EE (open bars), or 10 mg · kg⁻¹ · d⁻¹ genistein (gray bars). After 5 weeks, mRNA expression was determined by quantitative real-time PCR. All mRNA levels were normalized for GAPDH expression and are reported as mean ± SEM for each group, with the mean expression level in animals fed the chow diet defined as 1.* P < 0.1 vs atherogenic diet with no treatment.


**Figure 8.** EE does not reverse atherogenic diet–induced inflammation. A, Ovariectomized C57BL/6 mice were fed either a chow or atherogenic diet for the indicated period of time. The relative fold induction of VCAM-1 mRNA in animals receiving the atherogenic diet vs chow diet is indicated for each time point. B, Ovariectomized C57BL/6 mice were fed a chow (gray bars) or atherogenic diet, and control mice (solid bars) received vehicle by gavage for the entire 5 weeks. The prevention group (open bars) received 10 μg·kg−1·d−1 EE by gavage for the entire 5 weeks, whereas the treatment group (hatched bars) received vehicle for the first 2 weeks followed by 10 μg·kg−1·d−1 EE treatment for weeks 3 through 5. At the end of 5 weeks, liver gene expression was monitored by real-time PCR as above. *P<0.1 vs atherogenic diet with no treatment.


Reciprocal Antagonism Between Estrogen Receptor and NF-κB Activity In Vivo
Mark J. Evans, Amy Eckert, KehDih Lai, Steven J. Adelman and Douglas C. Harnish

Circ Res. published online September 13, 2001;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/early/2001/09/13/hh2101.098543.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/