Molecular Basis of Estrogen-Induced Cyclooxygenase Type 1 Upregulation in Endothelial Cells

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Abstract—Estrogen upregulates cyclooxygenase-1 (COX-1) expression in endothelial cells. To determine the basis of this process, studies were performed in ovine endothelial cells transfected with the human COX-1 promoter fused to luciferase. Estradiol (E_2) caused activation of the COX-1 promoter with maximal stimulation at 10^{-8} mol/L E_2, and the response was mediated by either ERα or ERβ. Mutagenesis revealed a primary role for a putative Sp1 binding motif at −89 (relative to the ATG codon) and lesser involvement of a consensus Sp1 site at −111. Electrophoretic mobility shift assays yielded a single complex with the site at −89, and supershift analyses implicated AP-2α and ERα, and not Sp1, in protein-DNA complex formation. In endothelial cells with minimal endogenous ER, the transfection of ERα mutants lacking the DNA binding domain or primary nuclear localization signals caused 4-fold greater stimulation of promoter activity with E_2 than wild-type ERα. In contrast, mutant ERα lacking the A-B domains was inactive. Thus, estrogen-mediated upregulation of COX-1 in endothelium is uniquely independent of direct ERα-DNA binding and instead entails protein-DNA interaction involving AP-2α and ERα at a proximal regulatory element. In addition, the process may be initiated by cytoplasmic ERα, and critical receptor elements reside within the amino terminus. **(*Circ Res*. 2005;96:518-526.)**

Key Words: cyclooxygenase ■ endothelium ■ estrogen ■ estrogen receptor

Evidence from multiple paradigms indicates that estrogen has potent impact on endothelial cell function. The hormone activates signaling by endothelium, and it modifies endothelial cell growth, migration, and apoptosis. These actions are primarily mediated by estrogen receptor (ER) subtypes α and β, which classically function as transcription factors.[1–3] We have demonstrated that ERα and ERβ are expressed in endothelium, and that subpopulations of ERα and ERβ reside in endothelial caveolae/lipid rafts.[4,5] One of the most thoroughly delineated targets of endothelial ER action is endothelial nitric oxide synthase (eNOS). eNOS transcription is upregulated by the activation of classical ER and estrogen-related receptor α_1 in processes that involve diverse regulatory elements within the 5′ flanking region of the eNOS gene.[6,7] In addition, eNOS activity is rapidly stimulated by ERα or ERβ ligand activation in endothelial caveolae.[4,5] Through these multiple mechanisms estrogen enhances the production of the important atheroprotective molecule NO.[8]

In contrast to the detailed understanding of the basis of estrogen action on endothelial NO production, considerably less is known about the equally potent ability of the hormone to stimulate endothelial prostacyclin synthesis. This process is critical to the overall impact of estrogen on vascular health.[9] We have previously demonstrated that estrogen acutely activates endothelial prostacyclin synthesis through an ERβ-dependent, calcium-dependent process.[10] We have also shown that estrogen upregulates the expression of the rate-limiting enzyme in prostacyclin synthesis, cyclooxygenase type 1 (COX-1) in endothelial cells, resulting in enhanced basal and agonist-stimulated prostacyclin synthesis. We have further demonstrated that this is mediated by increases in steady-state COX-1 mRNA levels, which are not related to changes in mRNA degradation, thus implicating transcriptional processes.[11] However, the molecular mechanisms by which COX-1 is upregulated by estrogen are unknown.

The purpose of the present study was to determine the processes by which COX-1 expression is upregulated in endothelium by estrogen. The 5′ flanking sequence of the COX-1 gene lacks estrogen response elements (EREs) or ERE half-palindrome motifs.[12] The hypothesis was therefore raised that estrogen activates COX-1 gene transcription via the involvement of putative Sp1 elements, which are prevalent in the COX-1 promoter and which mediate the estrogen responsiveness of certain genes.[13] In addition to testing this hypothesis, experiments were performed to determine the role

Original received October 17, 2004; revision received January 3, 2005; accepted January 27, 2005.

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Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000158967.96231.88**

of ERα and ERβ in this process. The specific *cis* DNA sequences involved in the estrogen response were also identified, along with nuclear proteins that are involved. Furthermore, studies were done to begin to determine the features of ERα that play a role in mediating COX-1 expression.

Materials and Methods

Cell Culture

Experiments were performed in primary ovine endothelial cells obtained as previously described and studied at passage 4 to 6.[14] These cells have been used previously in studies of COX-1 regulation.[11,15] In experiments evaluating the actions of estrogen receptor mutants, primary endothelial cell cultures were specifically selected that had negligible expression of endogenous ERα or ERβ by immunoblot analysis.

Construction of Reporter and Receptor Plasmids and Mutagenesis

The basis of estrogen-induced activation of COX-1 transcription was investigated using a 2075-base pair fragment (−2095 to −21 relative to the ATG codon, A as +1) of the human COX-1 5′ flanking region inserted into *Kpn*I/*Hin*dIII sites of the luciferase reporter gene plasmid, pGL2 (Promega Corp) to yield the full-length promoter-reporter plasmid denoted as −2095COX-1-Luc. Two 5′ deletion mutants were also studied including 1241 (−1261 to −21) or 117 (−137 to −21) base pairs of 5′ flanking sequence of COX-1, designated −1261COX-1-Luc and −137COX-1-Luc, respectively. The COX-1 genomic sequences for these plasmids were obtained from COX-1 promoter reporter constructs originally made using pXP1.[12] Site-directed mutants were also generated of the putative Sp1 element at −111, the putative Sp1 element at −89, or of both elements (see online data supplement available at http://circres.ahajournals.org).[12]

In studies of the role of estrogen receptors in COX-1 gene transcription, cells were cotransfected with cDNAs for wild-type human ERα inserted into pCDNA3.1[4] or deletion mutants of ERα. These included a mutant lacking the two primary NLS, NLS 2 and 3 (ERαΔ250–274), a mutant lacking the DNA binding domain (ERβΔ 185–251), and an N-terminal deletion mutant lacking residues 1 to 175 (ERβΔ1–175) (see online data supplement and online Figure I). In selected experiments, cells were transfected with mouse ERβ cDNA cloned into pCDNA3.1.[5]

Cell Transfection and Reporter Activity

Cell transfection was performed using methods that have been previously reported.[16] Twenty four hours later, the cells were placed in media containing charcoal-stripped serum and were treated with either vehicle, 17β-estradiol (E_2, 10^{-12} to 10^{-6} mol/L), or E_2 plus ICI 182,780 (10^{-5} mol/L) for 48 hour. The cells were lysed, extracts were centrifuged at 10 000*g*, and luciferase and β-galactosidase activity were measured.[17,18] The results are normalized as relative luciferase light units/β-galactosidase activity. In selected wells, the cells were transfected with pGL2-Control Vector (Promega Corp) to serve as a positive control for luciferase expression. In preliminary studies, 17α-estradiol had no effect on promoter activity. In experiments focused on the role of ER, sham plasmid or cDNAs for wild-type or mutant ER were cotransfected with the promoter-reporter constructs and pSV-β-Gal, and equal expression of ER forms was confirmed by immunoblot analysis.[4]

Electrophoretic Mobility Shift Assays

After 0 to 48 hours treatment of endothelial cells with vehicle or 10^{-8} mol/L E_2, nuclear extracts were prepared.[16] Oligonucleotide probes were generated and added to incubations of nuclear extracts using methods that have been previously reported.[16] In competition studies, excess wild-type, mutant, or unrelated oligonucleotides were added in 2- to 200-fold molar excess before the addition of the ^{32}P-labeled probe. The wild-type −111 Sp1 probe was 5′-GAGGGAGGAGCGGGGGTGGAGCCGGGGGAA-3′ (upper strand) and the mutant probe was 5′-GAGGGAGGAGCGGTTTTAGAGCCGGGGGAA-3′. The wild-type −89 Sp1 probe was 5′-GGGGGAAGGGTGGGGAGGGGATGGGCTGGA-3′ (upper strand), and the mutant probe was 5′-GGGGGAAGGGTGTTTAAGGGATGGGCTGGA-3′.

To identify the nuclear proteins that bound to the putative Sp1 domains, supershifting of the DNA-protein complex was performed. For ERα, 2 μL of MAB461 (Chemicon International Inc) or unrelated IgG was added. For Sp1, Sp3, AP-2α, and AP-2γ, 2 μL of antiserum to the respective transcription factors was added. The antibodies were 2 μg/μL from Santa Cruz Biotechnology, Inc, and with the exception of Sp3, all were monoclonal. To confirm effective supershifting with antibody to Sp1, additional experiments were performed with an Sp1 probe from the eNOS promoter (5′-GGATAGGGGCGGGGCGAGG-3′, upper strand).[16] All nuclear protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels containing 1× Tris borate/EDTA buffer. Dried gels were exposed to Kodak XAR film for autoradiograpy.

Statistical Analysis

Data for promoter activity were analyzed by ANOVA and Neuman-Keuls post hoc testing.[19] Results are expressed as mean±SEM. All stated differences achieved statistical significance at the 0.05 level of probability or less.

Results

COX-1 Promoter Activation by Estrogen

To determine whether estrogen activates COX-1 transcription, the effects of the hormone on the activation of the full-length promoter (−2095COX-1-Luc) were determined. The levels of basal promoter activity obtained with −2095COX-1-Luc was at least 3-fold greater than with vector alone, and activity with vector alone was not altered by hormone treatment (data not shown). In cells transfected with −2095COX-1-Luc, treatment with 10^{-8} mol/L E_2 for 48 hours caused a 2.6-fold increase in promoter activity that was fully reversed by concomitant treatment with ICI 182,780 (Figure 1A). In additional studies, the dose-response to E_2 was evaluated (Figure 1B). Activation was observed at 10^{-10} mol/L E_2, and maximal response occurred with 10^{-8} mol/L E_2. Minimal stimulation of promoter activity was seen with 10^{-6} mol/L E_2, indicating a biphasic dose-response.

To further determine the role of ER in COX-1 gene modulation, experiments were done in endothelial cells overexpressing either ERα or ERβ (Figure 1C). Relative to the responses seen with endogenous ER action in sham-transfected cells, there was a 2.3-fold greater E_2-mediated promoter activity after ERα overexpression. Similarly, there was a 2.2-fold enhancement of the E_2 response with overexpression of ERβ. As such, both ER subtypes are capable of activating COX-1 transcriptional transactivation.

Promoter Elements Required for Regulation by Estrogen

To determine the elements in the COX-1 promoter required for estrogen responsiveness, the activities of 5′ deletion mutants were evaluated in endothelial cells. Basal promoter activity fell by 63% with deletion from −2095 to −1261, and it fell a comparable 70% with deletion from −2095 to −137 (Figure 2A). However, the relative capacity for E_2-stimulated promoter activity was conserved with deletion from −2095 to −1261, with the hormone causing similar 2.4-fold and 2.6-fold increases in activity over basal, respectively. In addition,

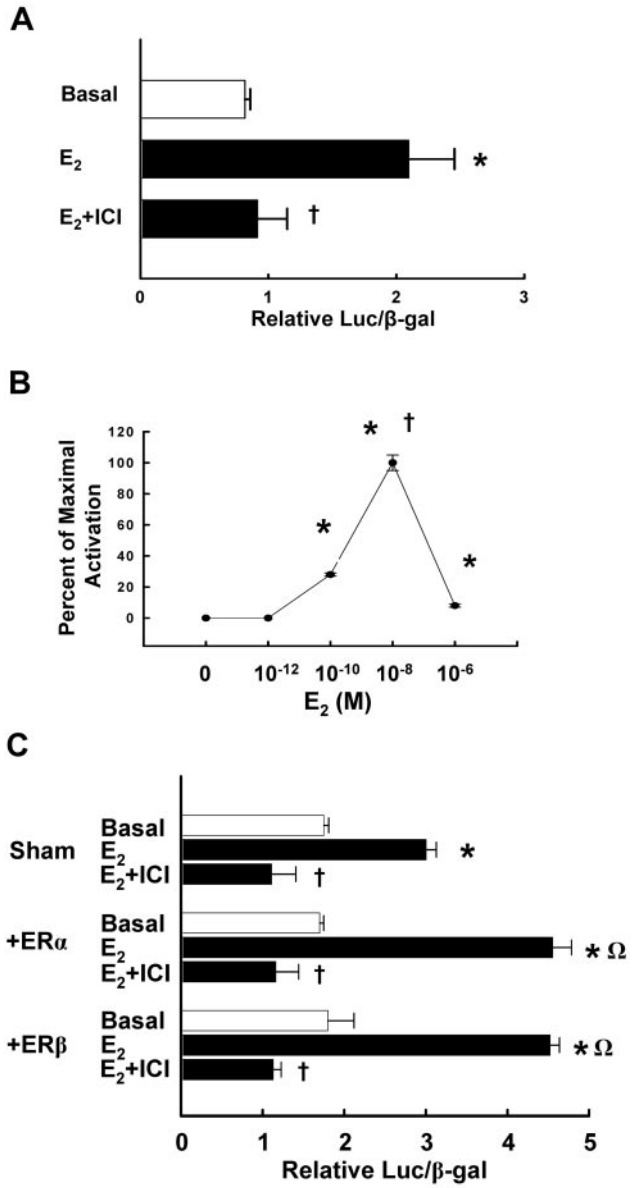

Figure 1. E_2 activation of COX-1 gene promoter activity in endothelial cells. A, COX-1 promoter-reporter gene constuct −2095COX-1-Luc was cotransfected with SV40-driven β-galactosidase plasmid (β-gal), cells were treated with control media or media containing 10^{-8} mol/L E_2 with or without 10^{-5} mol/L ICI 182,780 added, and relative activities (Luc/β-gal) were determined in cell lysates 48 hours later. *$P<0.05$ vs basal, †$P<0.05$ vs E_2 alone. B, Using the same approach, the dose-response to E_2 (10^{-12} to 10^{-6} mol/L) was evaluated over 48 hours. *$P<0.05$ vs no E_2, †$P<0.05$ vs 10^{-10} and 10^{-6} mol/L E_2. C, Cells were also cotransfected with either sham plasmid, ERα cDNA, or ERβ cDNA to determine the effect of ER overexpression. Responses to 10^{-8} mol/L E_2 with or without 10^{-5} mol/L ICI 182,780 present were determined over 48 hours. *$P<0.05$ vs basal, †$P<0.05$ vs E_2 alone, Ω$P<0.05$ vs sham transfection for receptor cDNA. For A, B, and C, values are mean±SEM, n=4 to 6.

there was continued conservation of E_2 activation of the promoter with deletion to −137, with −137COX-1-Luc displaying a 2.8-fold response to hormone. Importantly, ER antagonism with ICI 182,780 reversed estrogen responses in all cases. To further implicate the proximal promoter in hormone action, additional experiments were done using −2095COX-1-Luc and −137COX-1-Luc in cells cotransfected with ERα cDNA (Figure 2B). With ERα overexpression, E_2-stimulated activity of −2095COX-1-Luc was raised by 2.4-fold. Similarly, with overexpression of the receptor the E_2-induced activity of −137COX-1-Luc increased by 2.2-fold.

Inspection of the proximal COX-1 promoter reveals a lack of estrogen response elements (ERE) or ERE half-palindrome motifs known to mediate the estrogen responsiveness of certain genes.[20] However, there is a potential Sp1 binding site at −111 and another at −89,[12] and Sp1 is known to mediate estrogen responsiveness in the absence of ERE-like motifs in certain paradigms.[13] Because E_2-induced activation of the promoter in endothelial cells was conserved with 5′ deletions, which retained these domains, site-directed mutants of either or both of these putative Sp1 elements within −2095COX-1-Luc were tested. First, the basal activity of mutated promoter sequences was evaluated (Figure 2C). Compared with full-length wild-type promoter (−2095COX-1-Luc), mutation of the −111 Sp1 caused a 58% decline in activity, mutation of the −89 Sp1 yielded a 72% fall, and mutation of both elements caused a 53% decrease. Estrogen-stimulated promoter activity expressed relative to the level of basal activity for the same construct is shown in Figure 2D. Mutation of the Sp1 element at −111 caused a decline in E_2-induced activity of 43%, and mutation of −89 Sp1 resulted in a 73% decline in the E_2 response. Mutation of both domains yielded an 88% fall in E_2-induced promoter activity.

Evaluation of Relevant Nuclear Proteins

To evaluate the nuclear proteins in endothelial cells involved in estrogen actions mediated by the consensus Sp1 elements of the COX-1 promoter at −111 and at −89, electrophoretic mobility shift assays were performed. Incubation of nuclear extracts from E_2-treated endothelial cells (48 hours) with a double-stranded oligonucleotide probe encompassing the putative Sp1 site at −111 resulted in the appearance of one major DNA-protein complex that was diminished by 200-fold molar excess of unlabeled probe (Figure 3A, left). In contrast, three minor complexes were formed with mutated −111 Sp1 site probe. The use of mutated oligonucleotide as a competitor did not prevent the formation of the major complex by the wild-type probe (Figure 3A, right). Incubation of nuclear extracts with a probe containing the putative Sp1 site at −89 resulted in the appearance of one major DNA-protein complex that were prevented by 200-fold molar excess of unlabeled probe (Figure 3B, left). The complex was minimally formed with mutated −89 Sp1 DNA probe, and the mutated oligonucleotide only partially prevented the formation of the complex by the wild-type probe (Figure 3B, right).

To identify the endothelial nuclear proteins involved in complex formation with the presumptive −111 Sp1 and the −89 Sp1COX-1 promoter elements, supershift analyses were done. Two different antisera to Sp1 did not cause supershifting of either the −111 Sp1 protein-DNA complex or the −89 Sp1 protein-DNA complex (Figure 4A and 4B, left). To provide a control for supershifting of Sp1, a study was performed using the same endothelial nuclear proteins and the Sp1 element from the proximal eNOS promoter. With the Sp1 domain from eNOS, supershifting was observed as

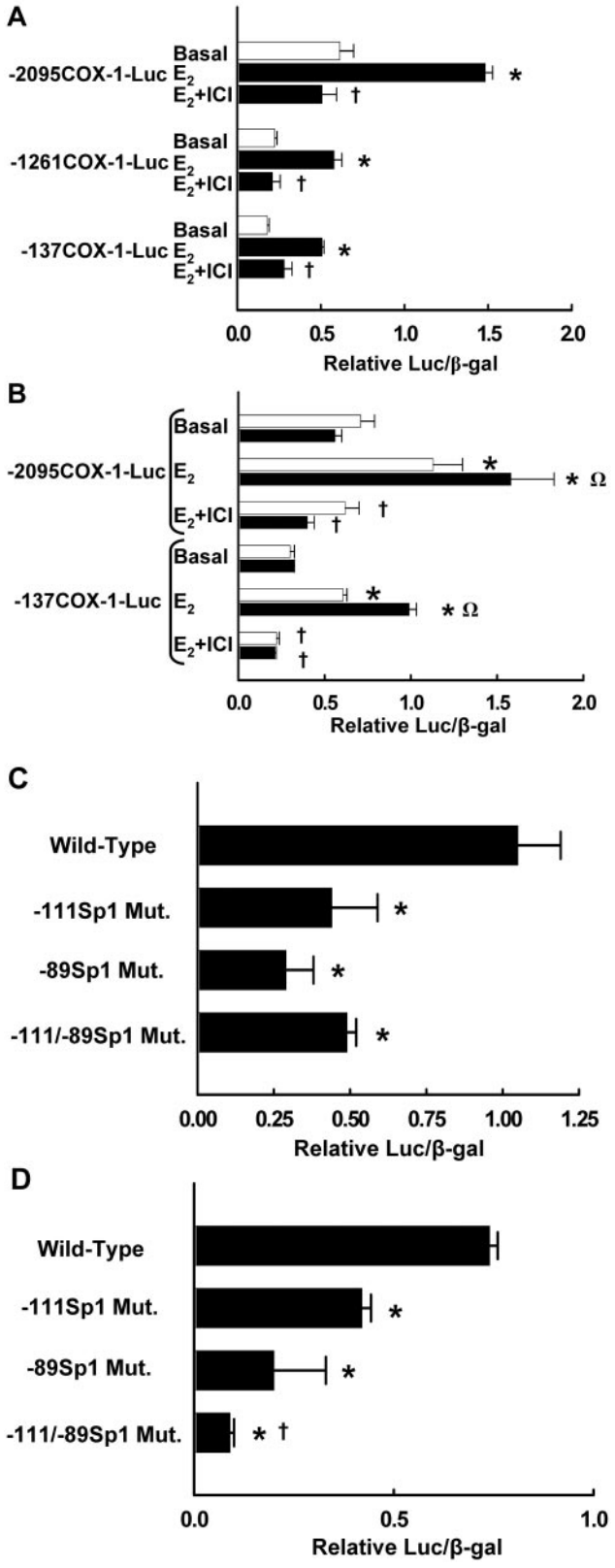

Figure 2. E_2-stimulated activity of 5′ deletion and consensus Sp1 site mutants of the COX-1 gene promoter. A, COX-1 promoter-reporter gene constructs −2095COX-1-Luc, −1261COX-1-Luc, or −137COX-1-Luc were cotransfected with SV40-driven β-galactosidase plasmid (β-gal), cells were treated with control media or media containing 10^{-8} mol/L E_2 with or without 10^{-5}M ICI 182,780 added, and relative activities (Luc/β-gal) were determined in cell lysates 48 hours later. *$P<0.05$ vs basal, †$P<0.05$ vs E_2 alone. Under all conditions tested, results for −1261COX-1-Luc and −137COX-1-Luc differed from those for −2095COX-1-Luc. B, In studies of −2095COX-1-Luc and −137COX-1-Luc, cells were also cotransfected with either sham plasmid (open bars) or ERα cDNA (closed bars) to determine the effect of ER overexpression. Responses to 10^{-8} mol/L E_2 with or without 10^{-5} mol/L ICI 182,780 present were determined over 48 hours. *$P<0.05$ vs basal, †$P<0.05$ vs E_2 alone, Ω$P<0.05$ vs sham transfection for receptor cDNA. C, Basal activity of the −2095COX-1-Luc wild-type construct and site-directed mutants (Mut.) of either the putative Sp1 binding motif at −111, the consensus Sp1 binding motif at −89, or both were compared. *$P<0.05$ vs wild-type. D, E_2-stimulated promoter activity (10^{-8} mol/L for 48 hours) expressed relative to basal activity for the same four promoter-reporter constructs. *$P<0.05$ vs wild-type. †$P<0.05$ vs −111Sp1 Mut. For A, B, C, and D, values are mean±SEM, n=4 to 6.

previously described (data not shown).[16] In an effort to identify other potential nuclear proteins relevant to gene regulation by Sp1 elements, the effects of antisera to Sp3 were also assessed, and supershifting was not evident for either the −111 Sp1 protein-DNA complex or the −89 Sp1 protein-DNA complex (Figure 4A and 4B, middle). In studies of potential involvement of AP-2α or AP-2γ, which have consensus binding sequences similar to Sp1,[21–24] antisera to either protein did not cause supershifting of the −111 Sp1 protein-DNA complex (Figure 4A, right). However, antisera to AP-2α caused supershifting of the −89 Sp1 protein-DNA complex (Figure 4B, right). In experiments comparing nuclear proteins from endothelial cells treated with vehicle vs 10^{-8} mol/L E_2 for 48 hours, there was no difference in the overall formation of the −111 Sp1 protein-DNA complex or the −89 Sp1-protein DNA complex, and the supershifting of AP-2α in the −89 Sp1 protein-DNA complex was also similar (data not shown).

The contribution of ERα to complex formation was also assessed by supershift analyses. Using nuclear proteins from cells treated with E_2 for 48 hours, antiserum to ERα shifted both the −111 Sp1 protein-DNA complex and the −89 Sp1 protein-DNA complex, whereas an unrelated IgG did not (Figure 5A and 5B, left). In evaluations of the temporal features of ERα involvement in complex formation, studies of nuclear proteins from cells treated with E_2 for 0 versus 6 hours yielded comparable supershifting of ERα from the −111 Sp1 complex (Figure 5A, right). In contrast, the supershifted bands for ERα in the −89 Sp1 complex differed at 0 and 6 hours of E_2 exposure; there was less prevalence of a rapidly migrating supershifted complex and greater abundance of a slowly-migrating supershifted complex after 6 hours of E_2 treatment versus control (Figure 5B, right).

Features of ERα Required for Modulation of COX-1 Transcription

There are multiple mechanisms by which ERα regulates gene transcription.[13,20] To begin to elucidate the features of the receptor necessary for modulation of COX-1, experiments were performed in a primary endothelial cell culture selected to have negligible endogenous ERα or ERβ into which ERα mutant constructs were cotransfected along with −2095COX-1-Luc. In cells transfected with sham plasmid for wild-type

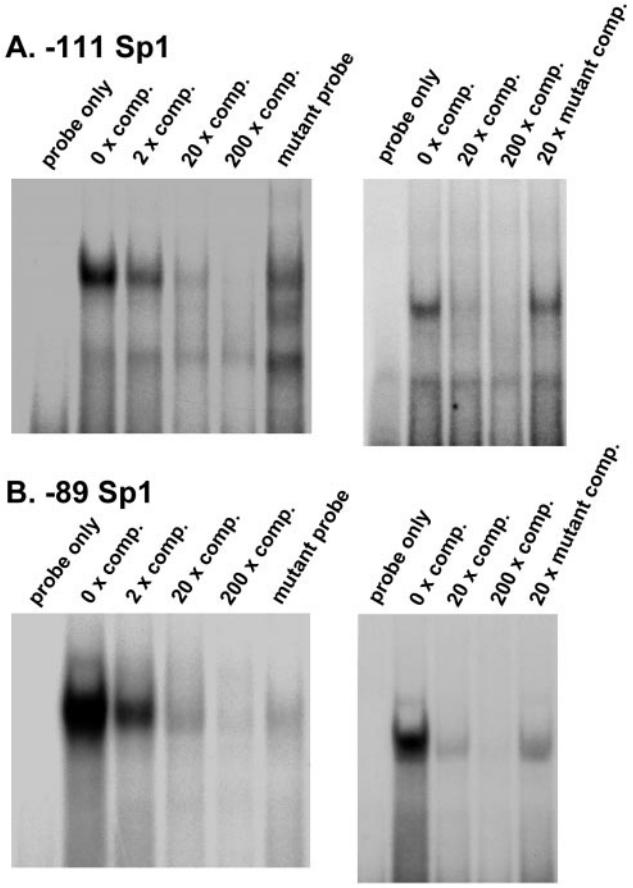

Figure 3. Electrophoretic mobility shift assays with nuclear extracts from E_2-treated (10^{-8} mol/L for 48 hours) endothelial cells. Extracts were incubated with labeled double-stranded oligonucleotide probes containing either the COX-1 gene consensus Sp1 binding motif at −111 (A) or the putative Sp1 binding motif at −89 (B) or their corresponding mutant probes. Competition reactions were performed with either unlabeled, wild-type competitor (comp.) at a 2, 20, or 200-fold molar excess or with unlabeled mutant competitor at a 20-fold molar excess. Observations were confirmed in three independent experiments.

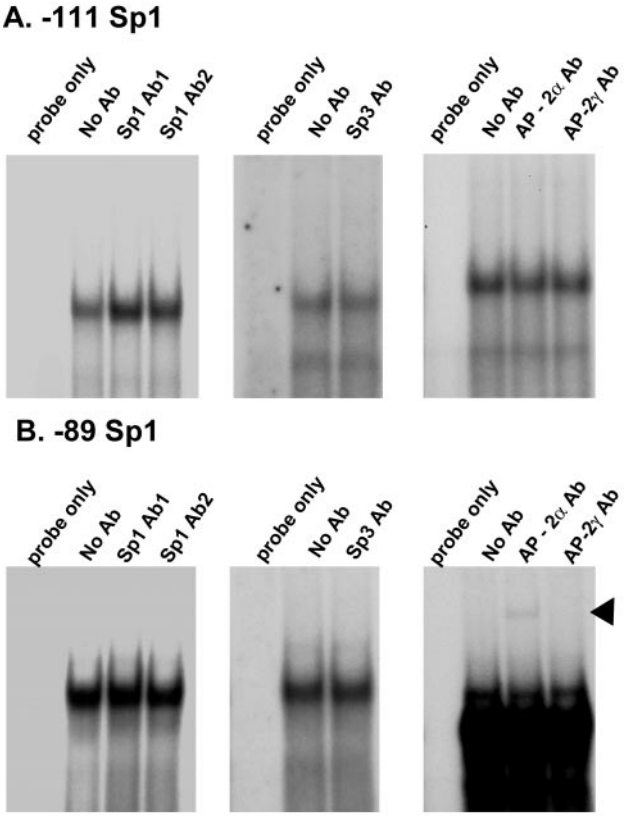

Figure 4. Supershift analyses of nuclear protein-COX-1 promoter DNA complexes in E_2-treated (10^{-8} mol/L for 48 hours) endothelial cells. Two different antisera to Sp1, an antiserum to Sp3, an antiserum to AP-2α, or an antiserum to AP-2γ was added to the DNA-nuclear protein complexes formed using oligonucleotide probes containing the consensus Sp1 binding motif at −111 (A) or the putative Sp1 binding motif at −89 (B) before electrophoresis. Supershifted complex for AP-2α is identified with an arrowhead. Observations were confirmed in three independent experiments.

ERα, E_2 caused no change in the activity of −2095COX-1-Luc (Figure 6A). In contrast, cells transfected with wild-type ERα displayed a 2.5-fold increase in promoter activity with E_2. In simultaneous comparisons of wild-type ERα and ER$\alpha\Delta$185–251, E_2 caused a 1.8-fold increase in promoter activity in cells expressing wild-type receptor and there was a 7-fold increase in activity with E_2 in cells expressing ER$\alpha\Delta$185–251 (Figure 6B). Additional independent studies revealed a 2.3-fold stimulation of promoter activity with E_2 via wild-type ERα and a markedly greater 9.2-fold stimulation in cells expressing ER$\alpha\Delta$250–274 (Figure 6C). In contrast, the N-terminal truncation mutant of ERα, ER$\alpha\Delta$1–175, was incapable of E_2-induced activation of the COX-1 promoter (Figure 6D).

Discussion

Estrogen causes upregulation of COX-1 expression in endothelial cells that is instrumental to the impact of the hormone on vascular function.[9] We have previously demonstrated that this is mediated by an increase in steady-state levels of COX-1 mRNA, and that the degradation of the mRNA is unaltered.[11] In the present study, we show that E_2 activates COX-1 gene transcription in an ER-dependent manner. We further demonstrate that the process entails unique ERE-independent mechanisms, providing new insights into the complex nature of E_2 actions in endothelium.

Role of ERα and Regulatory Elements on COX-1 Gene

In transient transfection experiments in endothelial cells with a construct containing the human COX-1 promoter fused to luciferase, we have observed E_2-induced promoter activation of sequence residing between −2095 and −21 of the 5′ flanking sequence relative to the ATG codon. Experiments with ICI 182,780 indicated that the process is ER-dependent, and the dose-response was biphasic with a threshold concentration of 10^{-10} mol/L and maximal activation at 10^{-8} mol/L E_2. The biphasic dose-response mimics that found previously for changes in COX-1 mRNA abundance with E_2.[11] Interestingly, the nongenomic activation of prostacyclin synthesis by E_2 does not display a biphasic dose response,[10] suggesting quite different kinetics for genomic versus nongenomic E_2 modulation of prostacyclin synthesis in endothelial cells. Cotransfection studies further indicated that the COX-1 promoter response can be

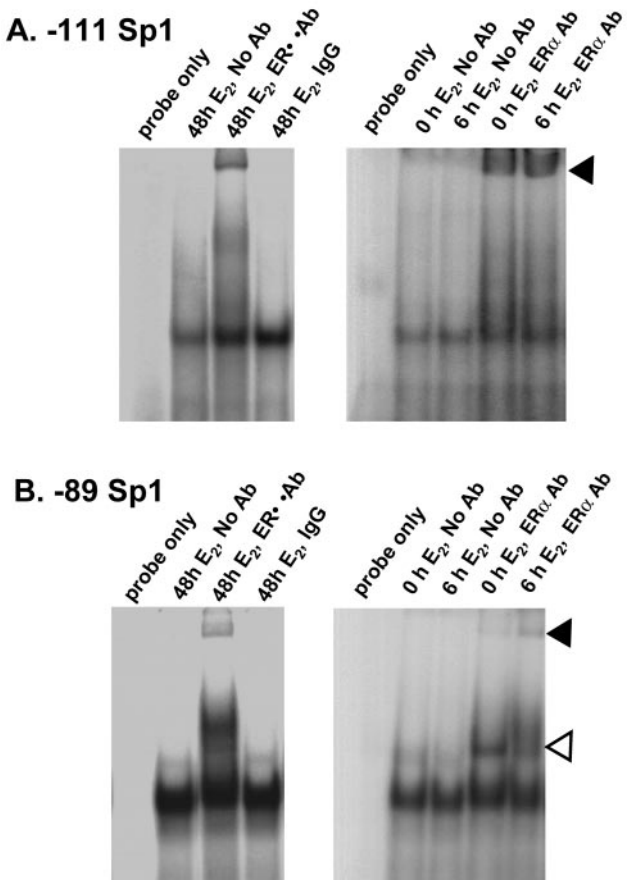

Figure 5. Supershift analyses for ERα in nuclear protein-COX-1 promoter DNA complexes in endothelial cells treated with control media or media containing 10^{-8} mol/L E_2 for 0, 6, or 48 hours. Antiserum to ERα, or unrelated IgG, was added to the DNA-nuclear protein complexes formed using oligonucleotide probes containing the consensus Sp1 binding motif at −111 (A) or the putative Sp1 binding motif at −89 (B) before electrophoresis. Supershifted complexes for ERα are identified with an open or closed arrowhead. Observations were confirmed in three independent experiments.

mediated by either ERα or ERβ. Thus, COX-1 regulation by E_2 occurs at a physiological concentration of the hormone[8] and via either of the ER subtypes.

To determine the regulatory elements within the COX-1 promoter required for estrogen responsiveness, the activities of progressive 5′ deletion mutants were evaluated. The capacity for E_2-stimulated promoter activity was fully conserved with 5′ deletion from −2095 to −1261 and also to −137. In addition, the enhancement in E_2 response with ERα overexpression was similar for −2095COX-1-Luc and −137COX-1-Luc. These findings indicate that the estrogen responsiveness of the COX-1 gene resides within the proximal promoter.

Because the 5′ flanking sequence of the COX-1 gene lacks EREs or ERE half-palindrome motifs, alternative modes of estrogen regulation were contemplated. Within the proximal COX-1 promoter, there are potential Sp1 binding sites at −111 and at −89,[12] and Sp1 mediates estrogen responsiveness in the absence of ERE-like motifs in certain paradigms. Sp1 physically associates with ERα, resulting in increased binding of Sp1 to its DNA site.[13] With mutation of −111Sp1, E_2-induced promoter activity was attenuated by 43%, and it was decreased by over 70% with mutation of −89Sp1 alone and by almost 90% with mutation of both −111Sp1 and −89Sp1. These findings indicate that putative Sp elements within the core COX-1 promoter are critical to E_2-induced upregulation.

Identification of Nuclear Proteins Mediating COX-1 Response

To evaluate the nuclear proteins involved in binding to the proximal consensus Sp1 domains of the COX-1 promoter, electrophoretic mobility shift assays were performed with nuclei from E_2-treated endothelial cells and oligonucleotide probes encompassing either −111Sp1 or −89Sp1. With −111Sp1, one major DNA-protein complex was formed with wild-type probe that was less apparent with mutant probe. Because the identical mutation of −2095COX-Luc yielded a modest decrease in E_2 responsiveness, it suggests that the complex with −111Sp1 plays a minor role in hormonal regulation of COX-1 expression. With −89Sp1, a single major DNA-protein complex was formed with wild-type but not mutant probe, and the same mutation in −2095COX-Luc prevented the majority of E_2 responsiveness either alone or in combination with mutation of −111Sp1. As such, the complex with −89Sp1 most likely depicts the principal protein-DNA interaction mediating E_2 induction of the COX-1 gene in endothelium.

To identify the nuclear proteins involved, supershift analyses were done. Antiserum to Sp1did not shift either the −111Sp1 or the −89Sp1 probe-nuclear protein complexes. Because Sp3 can interact with ERα to mediate Sp1 responses,[13] supershifts for Sp3 were also done that were negative. AP-2 was then considered because Sp1 and AP-2 have similar consensus binding sequences consisting of short GC-rich elements, and Sp1 and AP-2 bind to the same or overlapping regions within a variety of promoters.[21–24] Furthermore, AP-2α and AP-2γ are both known to be involved in E_2 modulation of gene expression.[25] Antisera to AP-2α or AP-2γ did not shift the −111Sp1 probe-nuclear protein complex. However, antiserum to AP-2α uniquely caused supershifting of the −89Sp1 probe-nuclear protein complex. Thus, AP-2α is likely to play an important role in the modulation of COX-1 gene expression by E_2.

The participation of ERα was also investigated. Antibody to ERα supershifted the −111Sp1 and the −89Sp1 probe-nuclear protein complexes, indicating that ERα is a component of both complexes. However, differences in overall complex formation were not observed in the absence versus presence of E_2 treatment for 6 or 48 hours, there were only modest changes in the relative quantities of supershifted bands for the −89Sp1 complex with ERα antibody at 0 versus 6 hours of E_2, and no changes in AP-2α recruitment were apparent. Thus, although the current approaches revealed important roles for the proximal Sp1-like elements and for ERα and AP-2α, the exact events activated by ER ligand binding are yet to be elucidated. Recent work using chromatin immunoprecipitation (ChIP) suggests that ER and coregulator association in gene promoters is temporally regulated. ChIP and cell imaging studies further show that members of the steroid receptor coactivator (SRC)-1 family and other coac-

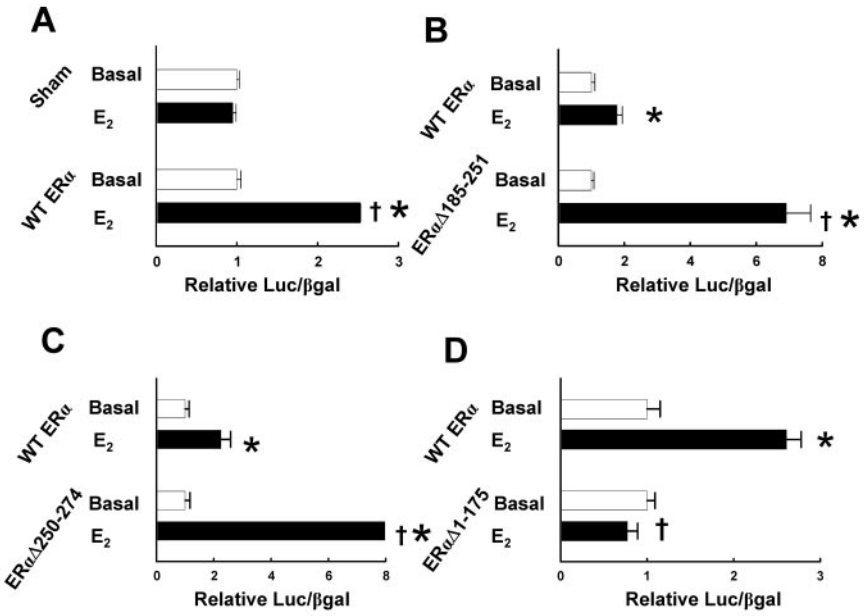

Figure 6. Comparison of COX-1 gene promoter activation by wild-type and mutant forms of ERα. Studies were performed in primary endothelial cells with minimal endogenous ER. A, −2095COX-1-Luc was cotransfected with SV40-driven β-galactosidase plasmid (β-gal) and sham plasmid or cDNA for wild-type ERα, cells were treated with control media or media containing 10^{-8} mol/L E_2, and relative activities (Luc/β-gal) were determined in cell lysates 48 hours later. *$P<0.05$ vs basal, †$P<0.05$ vs sham. In a similar manner, −2095COX-1-Luc activation by wild-type ERα vs ER$\alpha\Delta$185–251 (B), ER$\alpha\Delta$250–274 (C), or ER$\alpha\Delta$1–175 (D) were compared. For B, C, and D, *$P<0.05$ vs basal, †$P<0.05$ vs wild-type ERα. For A, B, C, and D, values are mean±SEM, n=4 to 6.

tivators may cycle on and off promoters with rapid kinetics.[20,26] Further experiments are now indicated using strategies including ChIP to interrogate this unique promodulatory role of AP-2α, which is collaborating with ER and other yet-to-be identified coactivators to target proximal regulatory elements on an important endothelial cell gene.

Features of ERα-Mediating COX-1 Response

The features of ERα required for COX-1 upregulation were also investigated. In studies of primary endothelial cells specifically selected to have minimal endogenous ER, the transient transfection of wild-type receptor caused COX-1 promoter responsiveness to E_2 comparable to that observed at endogenous levels of ER expression. In contrast, a mutant form of ERα lacking the DNA binding domain (ER$\alpha\Delta$185–251) yielded a 4-fold greater E_2 response than wild-type receptor. This finding indicates that E_2 upregulation of COX-1 is independent of direct ERα-DNA interaction, which is consistent with the involvement of Sp1-like elements and AP-2α. In addition, a mutant form of ERα lacking the two primary nuclear localization signals (ER$\alpha\Delta$250–274) displayed 5-fold greater E_2 responsiveness than wild-type receptor. Because ER$\alpha\Delta$250–274 (HE257G) has attenuated nuclear localization in the absence or presence of E_2,[27] this finding suggests that the process may be initiated primarily by cytoplasmic ERα, and that other mechanisms besides NLS must be responsible for nuclear import of the ERα-related transcriptional machinery in this paradigm. Furthermore, we found that mutant ERα lacking the A-B domains (ERα_{1-175}) was incapable of inducing COX-1 promoter activity. This observation parallels the findings in prior work indicating that the AF-1 region within the A-B domains of ERα is important for Sp1 activation.[13,28] Similar domains within AF-1 may bind Sp1 and AP-2α, and further detailed mutagenesis will be required to delineate these domains.

The ability of estrogen to upregulate COX-1 expression in endothelium through transcriptional transactivation represents a novel mode of regulation of a gene for which there are few means of dynamic regulation.[29,30] Our collective observations indicate that this process is independent of direct ERα-DNA binding, and that it instead entails protein-DNA interaction involving AP-2α and ERα at proximal regulatory elements within the COX-1 promoter. In addition, cytoplasmic ERα may play a key role in this process, and critical receptor elements reside within the amino terminus. As such, ERα modulation of COX-1 entails mechanisms that are both shared and unique to the known processes whereby estrogen regulates a variety of gene targets.[20,26] Further studies in this realm will increase our understanding of both COX-1 regulation and the potent, diverse capacities of estrogen to alter endothelial cell function.

Acknowledgments

This work was supported by National Institutes of Health Grants HL53546 (to P.W.S.), and HL50675 and NS23327 (to K.K.W.). Partial support was also provided by the Crystal Charity Ball Center for Pediatric Critical Care Research and the Lowe Foundation. We are indebted to Marilyn Dixon for preparing the manuscript.

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Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Molecular Basis of Estrogen-Induced Cyclooxygenase Type 1 Upregulation in Endothelial Cells

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Circ Res. 2005;96:518-525; originally published online February 10, 2005;
doi: 10.1161/01.RES.0000158967.96231.88
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Online Data Supplement

Expanded Materials and Methods

To generate site-directed mutants of putative Sp1 elements at -111 or -89 of the COX-1 5' flanking sequence[1], recombinant PCR was performed with two rounds of amplification. The primers to mutate both presumptive Sp1 domains were 5'-GCGG**TTT**T**A**GAGCCGGGGGAAGGGTG**TTT**A**A**GGGA-3' (sense) and its reverse complement. The primers for mutation of the consensus Sp1 domain at –111 alone contained the first mutation but not the second. The primers for mutation of the consensus Sp1 domain at –89 alone contained the second mutation but not the first. The wild type sequence for the underlined bases is GGG T/A G and the mutations are shown in bold. The two PCR reactions for each construct contained one of the mutation primers and its flanking primer, with the latter primers containing either an internal DraIII site at –1177 or a HindIII site at 70bp in the pXp1 vector, and the –2095COX-1-Luc plasmid was used as template. The resulting fragments were gel purified, sequenced, and pooled in another PCR reaction using only the two outside primers. The PCR product generated containing the desired mutation was digested with DraIII and HindIII followed by gel purification. The –2095COX-1-Luc plasmid was also digested with DraIII and HindIII and the fragment containing the vector and promoter region from –2095 and –1177 was isolated, gel purified and ligated to one of the 3 mutated PCR products generated above. All three mutant promoter constructs were sequenced to verify that only the mutated bases differed from –2095COX-1-Luc.

For studies of ER∀ function, ER∀ mutants lacking the two primary NLS, NLS 2 and 3 (ER∀)250-274), the DNA binding domain (ER∀)185-251), or an N-terminal deletion mutant lacking residues 1-175 (ER∀)1-175) were generated as follows. The ER∀)250-274 mutant was

constructed by ligating two PCR products generated from wild-type human ER∀ cDNA template. The upstream PCR product was amplified using primers both directed to ER∀ (sense primer 5'-GCCGCCTACGAGTTCAACGC-3' and antisense primer 5'-CCATCGATTCCCACTTCGTAGCATTTGC-3'), whereas the downstream PCR product was made using sense primer 5'-GGATCGATGAGGGCAGGGGTGAAGTGGG-3' directed to the ER∀ D domain and antisense primer 5-TAGAAGGCACAGTCGAGGCTG-3' anchored in the pCDNA3.1 vector. The PCR products were ligated to each other using the Cla I site which was engineered into the primers, and into the ER∀ wild-type cDNA construct in pCDNA3.1 at the Sac II restriction site upstream and the Eco RV site located downstream in the vector. The resulting construct is comparable to HE257G [2]. ER∀)185-251, which is equivalent to HE11G [2], and ER∀)1-175 were kindly provided by Dr. Michael Mendelsohn of the Molecular Cardiology Research Institute, New England Medical Center, Tufts University School of Medicine. The nucleotide sequences of all ER constructs were determined to confirm that no errors had been introduced by cloning or mutagenesis.

Additional Figures and Supporting Information

Before testing the ability of ER∀ mutants to modulate COX-1 promoter activity, the classical actions of the mutants were tested in endothelial cells using the estrogen-responsive reporter plasmid ERE-Luc[3]. Whereas reporter activity was increased by 9-fold with E_2 in cells transfected with wild-type ER∀ and the response was prevented by ER antagonism, reporter activity was not stimulated by the hormone in cells expressing the DNA binding domain-deficient mutant, ER∀)185-251 (Supplemental Fig. 1A). In addition, reporter activity was not stimulated by E_2 in cells expressing ER∀)250-274, which lacks the two primary NLS, NLS2 and

NLS3 (Supplemental Fig. 1A). In separate experiments, ERE-related reporter activity was increased by 7-fold with E_2 in cells transfected with the N-terminal truncation mutant, ER∀)1-175, and the response was fully prevented by ICI 182,780, closely mimicking findings for wild-type receptor (Supplemental Fig. 1B).

Legend for Supplemental Fig. 1: Assessment of nuclear function of wild-type and mutant forms of ER∀ transfected into primary endothelial cells with minimal endogenous ER. A. The estrogen responsive reporter gene constuct ERE-Luc was cotransfected with SV40-driven ∃-galactosidase plasmid (∃-gal) and cDNA for either wild-type ER∀, ER∀)185-251 or ER∀)250-274, cells were treated with control media or media containing 10^{-8}M E_2 with or without 10^{-5}M ICI 182,780 added, and relative activities (Luc/∃-gal) were determined in cell lysates 48h later. B. In a similar manner, ERE-Luc activation by wild type ER∀ and ER∀)1-175 were compared. For A and B, values are mean ∀SEM, n=4-6, *$p<0.05$ vs. basal, ⊥$p<0.05$ vs. E_2 alone.

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Supplemental Fig. 1