17β-Estradiol inhibits cyclic strain-induced endothelin-1 gene expression within vascular endothelial cells

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CARDIOVASCULAR DISEASES are the leading cause of mortality for postmenopausal women in industrialized countries, accounting for >30% of deaths (30). Conflicting results existed in the literature regarding actions of estrogen on the cardiovascular system (3, 19, 32, 35). A better understanding of the effects of estrogen on vascular cells might contribute to optimization of the role of estrogen in cardiovascular system. Literature provides the evidence that estrogen inhibits endothelin (ET)-1 production (17, 45). However, a shortcoming of previous work is that investigations were carried out in static cultures, which is not typical for blood vessels (47). Therefore, this study examines the mechanism by which estrogen modulates ET-1 in the conditions of cyclic strain, simulating the environment in the vasculature.

Endothelial cells are constantly under the influence of mechanical forces, including cyclic strain, as a consequence of blood vessel contraction and relaxation. The activation of several signal transduction systems has previously been demonstrated to arise as a consequence of hemodynamic forces within endothelial cells (18, 38, 39). Recently, it has been proposed that a function of reactive oxygen species (ROS) is as second messengers within cells exposed to various stimuli (13, 18, 26, 37). Studies have further demonstrated that intracellular ROS levels are elevated within endothelial cells after cyclic strain treatment (5, 48). We have also found that ROS mediate cyclic strain-induced ET-1 expression via a Ras/Raf/extracellular signal-regulated kinase (ERK) signaling pathway within endothelial cells (6), although it is not yet clear as to whether estrogen inhibits strain-induced ET-1 expression within vascular endothelial cells.

17β-Estradiol (E₂) is the terminal, biologically active, natural estrogen with the highest affinity for the estrogen receptor (9). Our study was conducted to examine whether E₂ inhibits strain-induced ET-1 gene expression and also to identify signaling protein kinase cascades that may be responsible for the putative effect of estrogen in cardiovascular system. In the present study, we clearly demonstrate that E₂ inhibits strain-induced ET-1 gene expression, activator protein-1 (AP-1) binding activity, and ERK phosphorylation in part via the attenuation of strain-induced ROS generation within endothelial cells. Thus this study delivers important new insight into the molecular pathways that may contribute to the proposed beneficial effects of estrogen in regard to cardiovascular disease.
MATERIALS AND METHODS

Materials. Imubind ET-1 ELISA kits were purchased from Biochemica (ENDOTHELIN, Biomedica). ET-1 cDNA was obtained from a human endothelial cell cDNA library as previously described (41). A full length of the ET-1 promoter region (4.4 kb) was fused to the chloramphenicol acetyltransferase (CAT) reporter gene (6). PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing the CAT gene only) were constructed as previously described (7). 2',7'-Dichlorofluorescin diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR). H2O2 was purchased from Acros Organics (Pittsburgh, PA). E2, N-acetylcysteine (NAC), and all other reagent-grade chemicals, were purchased from Sigma Chemical (St. Louis, MO). ICI-182,780 was purchased from Tocris Cookson (Ballwin, MO).

Endothelial cell culture. To avoid gender-related differences in the response to estrogen, human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords of newborn females as described previously (6). After 3 days of culture in medium 199 (GIBCO-BRL) containing 20% fetal calf serum, endothelial cells (2.0×10³ cells/well) were seeded on the flexible membrane base. A cell culture well (Flex 1, Flexcell; Mckeesport, PA). They were cultured for a further 3 days until the monolayer became confluent. The medium for the cultured endothelial cells was then changed to the same medium containing 2% fetal calf serum, and the cells were incubated overnight before the conduct of the experiment. The transformed human endothelial cell line ECV304 (ATCC CRL-1998) was purchased from the American Type Culture Collection (Bethesda, MD) and maintained in culture in DMEM supplemented in 2% serum DMEM, ECV304 cells were cultured under various conditions as indicated for a period of 48 h. ECV304 cells were assayed for luciferase activity using a luciferase reporter assay kit (Strategene). The specific firefly luciferase activity, which was the case for the specific AP-1 transcriptional activity, was normalized for transfection efficiency to its respective β-galactosidase activity and expressed as activity relative to the control.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed using one-way ANOVA or the unpaired Student’s t-test. P values < 0.05 at difference were considered to represent statistical significance.

RESULTS

Effects of E2 on strain-induced ET-1 expression. HUVECs experiencing cyclic strain for a period of 48 h were observed to

![Figure 1](http://ajpheart.physiology.org/)
proportional (percent) change determined for experimental groups compared with untreated controls.

Effects of E2 on strain-increased NADPH oxidase and intracellular ROS levels. Previous studies have shown that cyclic strain increases NADPH oxidase activity (4, 24, 25) and ROS formation in endothelial cells (4, 5, 24, 48). As a consequence, we examined whether E2 prevents the observed strain-increased NADPH oxidase activity and intracellular generation of ROS. HUVECs were treated with E2 (1–100 nM) in the absence or presence of strain treatment. The pretreatment of E2 (1–100 nM) with cultured endothelial cells significantly inhibited strain-induced NADPH oxidase activity and ROS levels as measured after strain treatment for a period of 1 h (Fig. 3, A and B). The pretreatment of cultured endothelial cells

demonstrate an increase in their ET-1 secretion into the culture medium to a level of about double that of the unstrained control analog (Fig. 1A). The preincubation of HUVECs with E2 at physiological concentrations (1–100 nM) for a period of 12 h before strain treatment for 48 h resulted in a concentration-dependent decrease in strain-induced ET-1 protein secretion (Fig. 1A). Unlike E2, 17α-estradiol (100 nM) appeared to elicit no effect on strain-induced ET-1 secretion, whereas the preincubation of HUVECs with the estrogen receptor antagonist ICI-182,780 (1 μM) prevented the E2-mediated downregulation of strain-induced ET-1 secretion (Fig. 1B).

To determine whether E2 inhibits strain-induced ET-1 gene expression, HUVECs were pretreated with E2 (1–100 nM) for a period of 12 h followed by a 6-h period of cyclic strain. HUVECs experiencing cyclic strain for 6 h demonstrated a significant increase in the ET-1 mRNA level compared with unstrained controls (Fig. 2A). Pretreatment of HUVECs with E2 (1–100 nM) significantly reduced the strain-induced ET-1 mRNA levels. Unlike E2, pretreatment of HUVECs with 17α-estradiol (100 nM) resulted in no effect on strain-induced ET-1 mRNA levels, whereas preincubation of HUVECs with the estrogen receptor antagonist ICI-182,780 (1 μM) prevented the E2-mediated downregulation of strain-induced ET-1 mRNA levels (Fig. 2B). To further investigate whether the effect of E2 on strain-induced ET-1 gene expression is a transcriptional event, a plasmid containing an ET-1 upstream sequence in a CAT reporter construct (~4.4 k CAT) was cotransfected with pSV-β′-galactosidase into ECV304 endothelial cells. As shown in Fig. 2C, increased ET-1 promoter activity was observed in endothelial cells experiencing cyclic strain for 24 h. For endothelial cells pretreated with E2 (1–100 nM), the strain-induced ET-1 promoter activity appeared to have been significantly reduced (P < 0.05; Fig. 2C). Consistently, 17α-estradiol (100 nM) exerted no effect on strain-induced ET-1 promoter activity, whereas preincubation with the estrogen receptor antagonist ICI-182,780 (1 μM) prevented the E2-mediated downregulation of strain-induced ET-1 promoter activity (Fig. 2D).

Effects of E2 on strain-induction of ET-1 promoter activity. The results shown are means ± SE; n = 3; *P < 0.05 vs. control; †P < 0.05 vs. strain-treated cells (by ANOVA). A: inhibition of strain-induced ET-1 mRNA by E2. HUVECs were either untreated controls (lane 1) or treated with various different concentrations of E2 (1–100 nM) in the absence (lanes 2–4) or presence of strain treatment (lanes 6–8). 18S RNA was used to normalize the RNA applied to each lane. Data are presented as the proportional (percent) change determined for experimental groups compared with untreated controls. B: effects of the ER antagonist ICI on the E2-mediated downregulation of strain-induced ET-1 mRNA and the corresponding absence of any effect from α-E administration to endothelial cells before strain-induced ET-1 mRNA. Cells were preincubated with α-E (100 nM), E2 (100 nM), or the combination of E2 plus the ER antagonist ICI (1 μM) and then stimulated with cyclic strain for a period of 6 h vs. no strain application. C: E2 (1–100 nM) inhibits strain-induced ET-1 promoter activity. ECV304 cells were pretreated with E2 (1–100 nM) for 12 h and then treated with cyclic strain for 24 h. C, no drugs; chloramphenicol acetyltransferase (CAT)2 and CAT3 are shown as positive and negative controls. CAT activity subsequent to normalization relative to that of β-galactosidase activity is shown as relative activity compared with corresponding values for control groups. D: effects of the ER antagonist ICI on the E2-mediated downregulation of strain-induced ET-1 promoter activity and the relative absence of any effect of α-E on strain-induced ET-1 promoter activity. Cells were pretreated with α-E (100 nM), E2 (100 nM), or the combination of E2 plus the ER antagonist ICI (1 μM) and then stimulated with cyclic strain for a period of 24 h vs. no cyclic strain.

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Effects of E2 on strain-increased AP-1 transcriptional activity within endothelial cells. To evaluate the effect of E2 on strain-increased AP-1 activation, which is involved in ET-1 gene induction (7, 15, 24), we used an electromobility shift assay. The extent of AP-1 binding to the consensus AP-1 binding sequence was assayed for HUVECs that had undergone strain treatment for a period of 6 h (Fig. 5A). Pretreating these cells with E2, NAC, or DPI attenuated the strain-stimulated AP-1 binding activity. The effects of E2 on strain-induced AP-1 functional activity were also assessed in a reporter gene assay. Cyclic strain appeared to exert no effect on the luciferase activity of the background vector containing no AP-1 binding site (data not shown). Contrasting this, however, cyclic strain significantly increased AP-1-luciferase activity in a time-dependent manner (data not shown). Furthermore, we examined the effects of E2, NAC, and DPI on AP-1 activity and noted that E2 (100 nM), NAC (10 mM), or DPI (10 μM) significantly attenuated the level of strain-induced AP-1 reporter activation (Fig. 5B; such results suggest that E2 inhibits strain-increased AP-1 activation.

Effects of E2 on strain-activated ERK phosphorylation within endothelial cells. To gain insight into the mechanism of action of E2, we thus examined whether E2 affects intracellular protein kinase signaling pathways. Given that the ERK signaling pathway is involved in strain-induced ET-1 expression (6), we further investigated whether E2 inhibits the ERK pathway for strain-treated HUVECs. We examined the phosphorylation of ERK in endothelial cells exposed to E2 (1–100 nM) in either the absence or the presence of strain treatment. As shown in Fig. 4A, endothelial cells that were exposed to strain treatment for 30 min rapidly activated phosphorylation of ERK, although endothelial cells pretreated with E2 (1–100 nM) exhibited a significant reduction in strain-induced ERK phosphorylation compared with their non-E2-pretreated analogs. Endothelial cells pretreated with E2 (100 nM), NAC (10 mM), or DPI (10 μM) all revealed a significant decrease in strain-induced ERK phosphorylation.

Effects of E2 on strain-increased NADPH oxidase activity and ROS generation within HUVECs. The results shown are means ± SE; n = 6. *P < 0.05 vs. control; #P < 0.05 vs. strain-treated cells (by ANOVA). A: effect of E2 (1–100 nM) on strain-increased NADPH oxidase activity. Cells were preincubated with E2 (1–100 nM) for 12 h and then stimulated with cyclic strain for a period of 1 h vs. no strain application. B: effect of E2 (1–100 nM) on strain-induced ROS generation. Cells were preincubated with E2 (1–100 nM) for 12 h and then stimulated with cyclic strain for a period of 1 h vs. no strain application. C: effects of the ER antagonist ICI or antioxidants on E2-mediated downregulation of strain-induced ROS generation. Cells were preincubated with E2 (100 nM) or the combination of E2 plus the ER antagonist ICI (1 μM) and the antioxidants N-acetylcysteine (NAC; 10 mM) or diphenylene iodonium (DPI; 10 μM) and then stimulated with cyclic strain for a period of 1 h or not at all. H2O2 (25 μM) was used as a positive control.

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Fig. 3. Effect of E2 on strain-increased NADPH oxidase activity and ROS generation within HUVECs. The results shown are means ± SE; n = 6. *P < 0.05 vs. control; #P < 0.05 vs. strain-treated cells (by ANOVA). A: effect of E2 (1–100 nM) on strain-increased NADPH oxidase activity. Cells were preincubated with E2 (1–100 nM) for 12 h and then stimulated with cyclic strain for a period of 1 h vs. no strain application. B: effect of E2 (1–100 nM) on strain-induced ROS generation. Cells were preincubated with E2 (1–100 nM) for 12 h and then stimulated with cyclic strain for a period of 1 h vs. no strain application. C: effects of the ER antagonist ICI or antioxidants on E2-mediated downregulation of strain-induced ROS generation. Cells were preincubated with E2 (100 nM) or the combination of E2 plus the ER antagonist ICI (1 μM) and the antioxidants N-acetylcysteine (NAC; 10 mM) or diphenylene iodonium (DPI; 10 μM) and then stimulated with cyclic strain for a period of 1 h or not at all. H2O2 (25 μM) was used as a positive control.

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**DISCUSSION**

Important vascular proteins such as ET-1 have been previously suggested to promote the development of cardiovascular diseases (31). Estrogen regulates the production of ET-1 as a potential novel mechanism in the prevention of the development of vascular disease among women (29). Estrogen may regulate prepro-ET-1 at the transcriptional level within cultured porcine endothelial cells (43). Estrogen is able of inhibiting ET-1 production via negative transcriptional regulation.
Dynamic forces including shear effects of the estrogen are indeed estrogen receptor mediated. It has recently been shown that estrogen exposure could greatly impact cellular oxidative stress by regulating phase II enzyme activity through an antioxidant/electrophile response element-mediated pathway, thereby helping to explain why estrogen exposure is a risk factor for the induction of certain types of cancer. However, the data presented here and by others (40) have shown that E2 can exert antioxidative effects by inhibiting expression of NADPH oxidase in human endothelial cells, thereby inhibiting NADPH oxidase activity and ROS formation. Furthermore, recently, several lines of evidence suggest that hormone replacement therapy might be more effective in statin users (8, 21). Lipid profiles are more favorable in women on both statins and hormone replacement therapy than in women on either treatment alone (14). Estrogen and statins, although structurally unrelated to each other, both seem to possess similar action(s) on the inhibition of intracellular ROS generation at the molecular level (40, 44), which NADPH oxidase (40). As a result of the decrease in NADPH oxidase gene expression, phorbol ester-stimulated (i.e., NADPH oxidase mediated) superoxide anion formation appeared to be significantly attenuated for estrogen-treated endothelial cells (40). By way of peroxynitrite formation, NO and superoxide anions readily neutralize each other, thereby reducing the bioavailability of NO (23). Indeed, the results of our present study have demonstrated that E2 reduces the level of strain-induced ROS generation within endothelial cells, suggesting a reduction of intracellular ROS generation arises due to strain treatment. In particular, it has been demonstrated that the activation of ERK is redox sensitive (33, 34, 36) and that the suppression of ROS inhibits strain-induced ET-1 gene expression (6). In a study focusing on cultured bovine aortic endothelial cells, treatment of such cells with estrogen substantially inhibited angiotensin II stimulation of ERK activity (29). One possible explanation for the inhibitory effect of E2 on strain-induced ET-1 gene expression may thus be its ability to attenuate ROS formation within endothelial cells. Alternatively, E2 may inhibit strain-induced ET-1 gene expression by virtue of its ability to induce an increase in NO production (11, 16, 46). Transcriptional regulation is important for molecular signaling in cellular response, involving interactions between the proteins of the general transcriptional apparatus and proteins that bind gene-specific enhancer elements. The mechanisms regulating the transcription of the ET-1 gene have been previously studied within endothelial cells (20, 22). Two regions were found to be important for constitutive expression of this gene in endothelial cells in culture. A GATA motif is located at bp −148 to −117, and an AP-1-like sequence (GTGAC-TAA) is located at bp −129 to −98 of the ET-1 gene (20, 22). In our previous study, deletion mapping revealed constructs containing 143 bp of the ET-1 5′-flanking sequence allowing strain-induced transcription and the presence of responsiveness elements for ROS- or strain-induced ET-1 expression located within the first 143 bp upstream of the transcription initiation site (6). Recently, we have also determined that the activation of AP-1 is redox sensitive and might play a key role in ET-1 gene induction (24). Our present results indicate that E2 inhibits strain-induced AP-1 transcriptional activity. The inhibitory effect of estrogen on strain-induced AP-1 transcriptional activation suggests that the attenuation of strain-induced ROS by estrogens leads to the inhibition of AP-1.

Previous studies, including ours, have indicated that hemodynamic forces including shear flow (49) and pressure-induced strain (6, 42) can stimulate the level of ET-1 gene expression. Our collaborative research and that from the work of others has demonstrated that cyclic strain treatment of endothelial cells can induce intracellular ROS generation (5, 25, 48). There appears to be mounting evidence that NADPH oxidase, a major source of ROS, within endothelial cells (10, 27), plays a critical role in the development of atherosclerosis (28). In 2001, Matsushita et al. (25) further demonstrated a pivotal role for NADPH oxidase in cyclic strain-induced endothelial cell ROS production. Elevated ROS levels are involved in the release of ET-1, and it would appear that the induction of the ET-1 gene can be attenuated by antioxidant pretreatment of endothelial cells (6). Although several reports have suggested that the vascular protective effect of estrogen is mediated by an increase in the endothelial nitric oxide (NO) synthase gene expression and the production of NO (11, 16, 46), for human cultured endothelial cells, E2 also inhibits the expression of
may contribute to their vasoprotective effects. However, additional studies are needed regarding the mechanisms of hormone replacement therapy might be more effective in statin users.

Within the past decade, it has been repeatedly suggested that oxidative stress is related to, and may be a cause of, endothelial dysfunction in cases of atherosclerosis (12). The present study delivers important new insights into the molecular mechanisms of action of E2 for endothelial cells. Moreover, we show that E2 acts in part via the suppression of the ERK pathway to reduce strain-induced ET-1 gene expression. It is plausible that the strain-activated signaling pathway investigated herein consists of a number of redox-sensitive steps and that E2 treatment of endothelial cells could be involved in the modulation of the redox state of the cell. In summary, our data show that E2 inhibits strain-induced ET-1 gene expression, in part, via the attenuation of ROS formation and thereafter the suppression of the ERK pathway within endothelial cells. These findings support the suggested beneficial effects of estrogen on the cardiovascular system.

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