Original Article

Estrogen Reduces Angiotensin II-Induced Acceleration of Senescence in Endothelial Progenitor Cells

Toshio IMANISHI, Takuzo HANO, and Ichiro NISHIO

The interaction among estrogen, angiotensin II (Ang II), and oxidative stress in endothelial progenitor cells (EPCs) remains unknown. We therefore investigated the potential effect of estrogen on Ang II-induced EPC oxidative stress and senescence in EPCs. EPCs were isolated from peripheral blood and characterized. Both reverse transcription (RT)–polymerase chain reaction (PCR) and Western blotting were used to assess gp91phox and angiotensin type 1 receptor (AT_1R) expression. Immunofluorescence of nitrotyrosine provided evidence of peroxynitrite formation. Our data indicate that Ang II increased the expression of gp91phox mRNA and protein, and these effects were attenuated by 17β-estradiol (E_2). The exposure of cultured EPCs to Ang II (100 nmol/l) significantly accelerated the rate of senescence compared to that in control cells during 14 days in culture as determined by acidic β-galactosidase staining, and this effect was significantly inhibited by E_2 ($p<0.01$). Because cellular senescence is critically influenced by telomerase, which elongates telomeres, we measured telomerase activity by using a PCR-ELISA-based assay. Ang II significantly diminished telomerase activity, although the effect was significantly reduced by pre-treatment with E_2 ($p<0.01$). Because we previously demonstrated that both the up-regulation of gp91phox and the acceleration of cellular senescence in Ang II-stimulated EPCs could be abolished by pre-treatment with the AT_1R-specific antagonist, valsartan, we also explored the effect of estrogen on AT_1R expression. Ang II increased AT_1R mRNA and protein expression, and these increases were prevented by E_2, suggesting that AT_1R may at least partially mediate the inhibitory effect of E_2 on Ang II-induced acceleration of senescence in EPCs. In conclusion, estrogen reduces Ang II-induced acceleration of senescence in EPCs partially through down-regulation of AT_1R expression. (*Hypertens Res* 2005; 28: 263–271)

***Key Words*: endothelial progenitor cell, estrogen, angiotensin II, senescence, oxidative stress**

Introduction

Recent studies have provided increasing evidence that the functional regeneration of ischemic tissue by improved neovascularization and possibly tissue repair is critically dependent on the mobilization and integration of endothelial progenitor cells (EPCs) into the ischemic tissue (*1*, *2*). However, ageing or senescence may limit the ability of progenitor cells to sustain ischemic tissue and repair. Indeed, Edelberg *et al*. (*3*) demonstrated that only transplantation of young bone marrow-derived cells restored age-associated impaired neovascularization, whereas bone marrow of aged mice was not effective. Moreover, the migratory capacity of peripheral blood-derived EPCs of elderly patients with coronary artery disease (CAD) is significantly reduced (*4*, *5*). Very recently,

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Scheubel *et al*. (*6*) demonstrated that the number of circulating CD34/CD133 cells, which are a subpopulation of immature hematopoietic precursor cells, and VEGF levels were reduced as a function of age in 50 patients (age 43 to 80 years) who were scheduled to undergo coronary artery bypass graft surgery (CABG). In this group of patients, the increase in VEGF and EPC levels after CABG was consistently depressed. Similarly, it has been shown that patients with CAD have reduced levels and functional impairment of EPCs, both of which correlate with atherosclerotic risk factors, including hypertension (*4*, *5*). These findings highlight a potentially relevant feature of the endogenous repair process and pose an interesting question as to the value of therapies based on expansion of endogenous EPCs in either elderly patients or patients with atherosclerotic risk factors.

An important question with respect to potential cell therapeutic approaches is whether the endogenous EPCs pool can be revitalized *in vivo* with pharmacological agents, cytokines, or even gene therapy. Previous studies have shown that VEGF gene transfer efficiently mobilizes EPCs in humans (*7*). Interestingly, Murasawa *et al*. (*8*) have demonstrated that the overexpression of human telomerase reverse transcriptase (hTERT) by adenovirus-mediated gene delivery could result in delay by senescence and a recovery/enhancement of the regenerative properties of EPCs. Moreover, Min *et al*. (*9*) have demonstrated that angiotensin-converting enzyme inhibitor therapy with ramipril augmented circulating EPCs with enhanced functional activity in patients with stable CAD.

In the ageing population, particularly in postmenopausal women, the prevalence of hypertension is increased (*10*). Estrogen has been shown to down-regulate angiotensin II (Ang II) type 1 receptor (AT_1R) expression in mature endothelial cells (*11*), and it has been postulated that estrogen deficiency may be associated with hypertension, because the AT_1R receptor subtype plays a key role in the regulation of blood pressure (*12*). To our knowledge, the interaction among estrogen, Ang II, and oxidative stress in EPCs has not been investigated. In view of our previous findings that Ang II promotes EPCs senescence through enhanced oxidative stress, which in turn mediates up-regulation of gp91phox (*13*), we further investigated the potential effect of estrogen on Ang II-induced EPC senescence. Our data demonstrated that estrogen reduces Ang II-induced oxidative stress and senescence in EPCs partially through down-regulation of AT_1R expression.

Methods

Isolation of Mononuclear Cells and Cell Culture

EPCs were cultured according to a previously described technique (*14*, *15*). Briefly, peripheral blood mononuclear cells (PB-MNCs) were isolated from healthy volunteers by density gradient centrifugation with Histopaque 1077 (Sigma Chemical Co., St. Louis, USA). After purification with 3 washing steps, 10×10^6 PB-MNCs were plated on fibronectin-coated 6-well plates. Cells were cultured in endothelial basal medium-2 (EBM-2) (Clonetics; Walkersville, USA) with supplements (3 μg/ml bovine brain extract, 30 μg/ml gentamycin, 50 μg/ml amphotericin B, 10 μg/ml human epidermal growth factor, and 5% fetal bovine serum [FBS]). The 6-well plates were replated at day 4. At day 8 of the culture, the PB-MNCs were pre-treated for 24 h with one of several concentrations of 17β-estradiol (E_2). After an additional 24 h, and every 48 h thereafter, Ang II in one of several concentrations was added to the PB-MNCs.

RNA Isolation and Reverse Transcription (RT)–Polymerase Chain Reaction (PCR)

To investigate the effect of E_2 on Ang II-stimulated gp91phox and AT_1R expression, EPCs were pre-treated with one of several concentrations of E_2 for 24 h before Ang II-stimulation (100 nmol/l) for 1 h. Total RNA was extracted by using an RNeasy RNA extraction kit (Qiagen, Chatsworth, USA). Briefly, cells were lysed in guanidinium isothiocyanate buffer, and RNA was purified following the manufacturer's instructions. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)-treated H_2O. To generate cDNA, 1 μg of total RNA was treated with DNaseI (Ambion, Austin, USA) to remove any contaminating genomic cDNA. The DNase-treated RNA (100 ng) was then converted into cDNA by using murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Bethesda, USA). The transcribed cDNA was then used for PCR amplification to estimate the expression of gp91phox. Two specific primers matching the published sequences were used to identify and amplify gp91phox (sense primer, 5′-CAACAAGAGTTCGAAGA CAA-3′; antisense primer, 5′-CCCCTTCTTCTTCATCT GTA-3′). The PCR product was 689 bp in length. The amplification conditions were as follows: 44 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s. The effect of E_2 on Ang II-stimulated AT_1R mRNA expression was also evaluated. For this purpose, EPCs that had been pre-treated with E_2 at 100 nmol/l for 24 h, as well as EPCs that were not pre-treated, were incubated with Ang II at 100 nmol/l for 24 h, and then AT_1R mRNA expression was examined. For amplification, a primer pair specific for human AT_1R (sense, 5′-GTCATGAT TCCTACTTTATACAGTATC-3′; antisense pair, 5′-AGC CAGGTATCGATCAATGCTGAGACA-3′) was used. The PCR product was 304 bp in length. The amplification conditions were as follows: 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The PCR products were visualized on 1.5% ethidium bromide-stained agarose gels. β-Actin was amplified as a reference.

Western Blotting

The general protocol for Western blotting was the same as

that described previously (*16*). Briefly, EPC protein was size-separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro-transferred to a PVDF membrane (Millipore, Bedford, USA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline/Tween20 (TBST) and incubated with rabbit anti-human angiotensin type 1 (AT_1) receptor (N-10; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) anti-serum at a dilution of 1:300 for 12 h at 4°C. Goat polyclonal antibody against human gp91phox (Santa Cruz Biotechnology, Inc.) was also used as described previously (*13*). Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK). The profile of each band was plotted using NIH imaging software, and the densitometric intensity corresponding to each band was measured as an intensity value. Both AT_1R and gp91phox protein intensity was normalized using α-tubulin expression.

Immunofluorescence

Confirmation of endothelial-cell lineage was performed as previously described (*14*, *15*). Briefly, indirect immunostaining was performed with the use of endothelial-specific antibodies against vascular endothelial growth factor 2 and CD31 or the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acethylated low density lipoprotein (acLDL) or the binding of FITC-labeled Ulex europaeus agglutinin 1 (UEA-1) (data not shown). EPCs were immunostained using polyclonal antibody against nitrotyrosine (1:100; Upstate Biotechnology, Lake Placid, USA), a marker for peroxynitrite. To verify selectivity of nitrotyrosine staining for peroxynitrite, some cells were pre-treated with superoxide dismutase (SOD) conjugated to polyethylene glycol (Sigma Chemical Co.), which prevented Ang II-mediated increases in peroxynitrite formation (data not shown). Incubation with the secondary antibody dilution was performed at 4°C overnight. Incubation with FITC-conjugated goat anti-rabbit IgG (1:200) was performed for 90 min in the dark. Coverslips were placed over the cells and sealed with nail polish. A Vectorshield H-1200 Mounting Kit (Vector Laboratories, Burlingame, USA) was used for immunofluorescence. Cells were visualized by fluorescence microscopy with standard FITC excitation/emission filter combinations.

Measurements of Extracellular Signal-Related Kinase (ERK1/2) and p38 Mitogen-Activated Protein Kinases (MAPKs)

For analyzing the phosphorylation of ERK1/2, and p38 MAPK, a Cellular Activation of Signaling ELISA (CASE™) kit (SuperArray Bioscience Corp., Frederic, USA) was used according to the manufacturer's protocol. In the CASE™ assay, EPCs were seeded onto a 96-well plate, then pretreated with E_2 (100 nmol/l), or PD98059 (Calbiochem, La Jolla, USA), an ERK1/2 kinase inhibitor, or SB203580 (Calbiochem), an inhibitor of p38 MAPK, for 30 min before addition of Ang II (100 nmol/l). The cells were fixed with 4% formaldehyde to preserve any phosphorylation modification. Both anti-phospho-protein specific antibody (Ab) and anti-pan-protein specific Ab were used as primary Abs. Following incubation with primary and secondary Abs, the amount of bound protein in each well was determined using a developing solution and an ELISA Plate Reader. The absorbance readings were then normalized to the relative cell number as determined by cell staining and an ELISA Plate Reader. The amount of phosphorylated protein, after normalization to the amount of total protein, was then used directly as an index of the degree of activation of the downstream pathway.

Senescence-Associated β-Gal Activity Assay

Senescence-associated β-Gal (SA-β-Gal) activity was measured as previously described (*17*). Briefly, EPCs were washed in PBS, fixed for 3 min in 2% paraformaldehyde at room temperature, washed again, and incubated for 24 h at 37°C (no CO_2) with fresh SA-β-Gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indyl β-D-galactopylanoside (X-Gal), 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, 150 mmol/l NaCl, 2 mmol/l $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet-40). EPCs were counterstained with 4′,6-diamino-phenylindole (DAPI) (0.2 µg/ml in 10 mmol/l Tris-HCl, pH 7.0, 10 mmol/l EDTA, 100 mmol/l NaCl) for 10 min to count the total cell number.

Telomeric Repeat Amplification Protocol (TRAP) Assay

For quantitative analyses of telomerase activity, a TRAP assay, in which the telomerase reaction product is amplified by PCR, was performed using a *TeloTAGGG* PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol as previously described (*13*, *18*).

Cell Death ELISA

A quantitative ELISA that detects DNA fragmentation was used following the manufacturer's instructions (Cell Death Detection ELISA kit; Roche Molecular Biochemicals). This ELISA detects mono- and oligo-nucleosomal DNA using the cytoplasmic fractions of cell lysates. Briefly, anti-histone antibody was coated onto a microtitre plate. After a washing step, the cells were incubated with 200 µl incubation buffer for 30 min. The cells were then washed again and incubated with 100 µl sample for 90 min at room temperature. Following another washing step, the cells were incubated with 100 µl anti-DNA peroxidase for an additional 90 min. The addition of substrate solution produces a color change after 15 min. The plate was read at 405 nm on an automated plate reader. This assay can detect apoptotic DNA from as little as 40 cells/

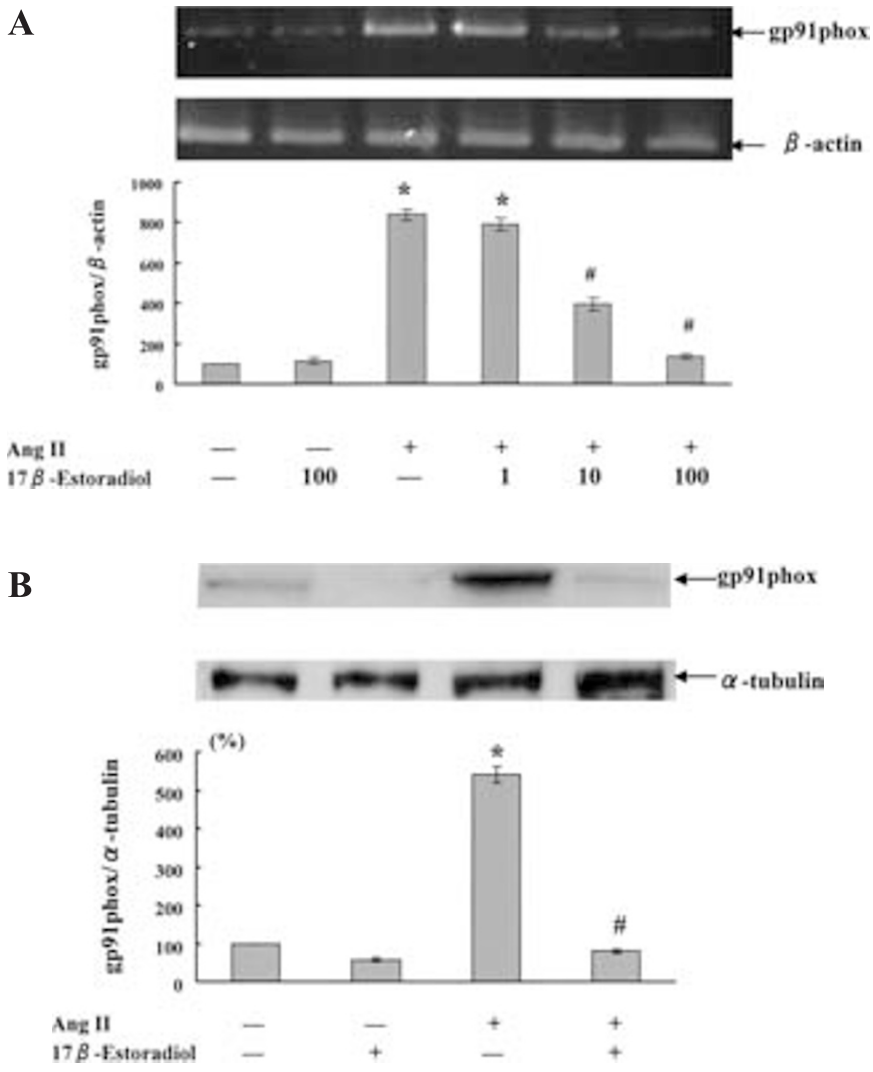

Fig. 1. *Effect of 17β-estradiol on Ang II-stimulated gp91phox mRNA and protein of NAD(P)H oxidase in EPCs. EPCs were pre-treated with the indicated doses of 17β-estradiol (E_2) for 24 h before Ang II stimulation (100 nmol/l). E_2 significantly diminished the Ang II-induced increase in gp91phox mRNA (A) and protein (B) expression. Representative images from five independent experiments are shown (top). Bar graphs show gp91phox mRNA and protein normalized by β-actin and α-tubulin, respectively (bottom). Data are expressed as a ratio of the test value to the value for unstimulated cells (set at 100%). Bars represent the mean±SEM;* n = *5 separate experiments.* *$p < 0.05$ vs. *the control cells.* #$p < 0.05$ vs. *EPCs treated with Ang II alone.*

well.

Statistical Analysis

Data were expressed as the mean±SEM based on at least five independent experiments. Statistical analysis was performed by 1-way ANOVA (LSD test) for multiple testing. Probability values were considered statistically significant at $p < 0.05$.

Results

We previously demonstrated that Ang II promotes human EPC senescence through enhanced oxidative stress, which in turn mediates up-regulation of gp91phox NAD(P)H oxidase (*13*). Therefore, in the present study, we further investigated the potential effect of estrogen on these Ang II-induced effects on human EPCs.

Estrogen Reduces Ang II-Induced gp91phox Expression in Human EPCs

We previously demonstrated that Ang II-induced gp91phox mRNA expression occurred dose-dependently with a maximal effect achieved at 100 nmol/l (*13*). Therefore, in this study, we examined whether estrogen modulates Ang II-induced gp91phox mRNA expression in human EPCs. Our

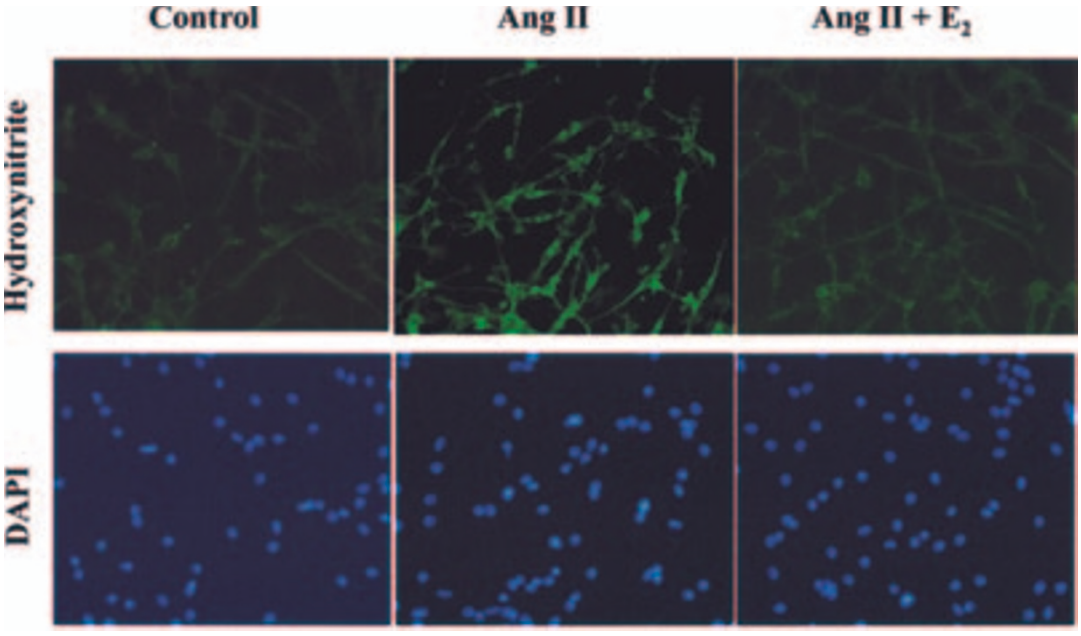

Fig. 2. *Representative immunofluorescence of nitrotyrosine staining (a marker of peroxynitrite) of EPCs exposed to either buffer (control), Ang II (100 nmol/l) alone or Ang II+E_2 (100 nmol/l). E_2 pre-treatment attenuated Ang II-induced nitrotyrosine formation.*

results showed that estrogen alone had no effect on the expression of gp91phox mRNA (Fig. 1A). On the other hand, estrogen markedly diminished the Ang II-induced increase in gp91phox mRNA expression (Fig. 1A). Similarly, E_2 markedly diminished the Ang II-induced increase in gp91phox protein expression (Fig. 1B).

Estrogen Inhibits Ang II-Induced Peroxynitrite Formation in Human EPCs

Ang II markedly increased nitrotyrosine staining, suggesting an increase in peroxynitrite formation (Fig. 2). The Ang II-induced peroxynitrite production was prevented by estrogen pre-treatment (Fig. 2).

Estrogen Reduces Ang II-Induced EPC Senescence

To assess the onset of senescence, acidic β-galactosidase was detected as a biochemical marker of the acidification that occurs at the onset of cellular senescence. Prolonged cultivation of EPCs resulted in an increase in SA-β-Gal-positive cells (Fig. 3). Coincubation with Ang II accelerated the increase in SA-β-Gal-positive cells. By day 14, the proportion of SA-β-Gal-positive cells in 100 nmol/l Ang II-treated EPCs was markedly increased compared to that in the control cells (33±1% *vs.* 13±1%, respectively; $p<0.01$) (Fig. 3). As shown in Fig. 3, the Ang II-induced increase in SA-β-Gal-positive cells was significantly attenuated by the pre-treatment with estrogen (Fig. 3). Under certain conditions, Ang II can induce apoptosis in human endothelial cells (*19*). Therefore, the apoptotic rate of EPCs at day 14 was considered to rule out the possibility that Ang II-induced acceleration of senescence in EPCs was secondary to the induction of apoptosis. However, in this setting, Ang II at concentrations ≤100 nmol/l did not increase the rate of EPC apoptosis, as measured by a cell death ELISA (data not shown). These data suggest that Ang II had a specific, non-toxic effect on the acceleration of senescence in EPCs.

Effects of Ang II on Telomerase Activity in EPCs

Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the telomere length reduction induced by each cell division. Therefore, we measured telomerase activity by a *TeloTAGGG* Telomerase PCR ELISA. As demonstrated in Fig. 4, 100 nmol/l Ang II significantly diminished telomerase activity by about 50%, and this effect was significantly abolished by pre-treatment with estrogen.

Estrogen Inhibits Ang II-Induced AT_1R Expression

As demonstrated in Fig. 5, Ang II stimulated a significant increase in AT_1R mRNA and protein expression, and pre-treatment with estrogen prevented these Ang II-induced effects.

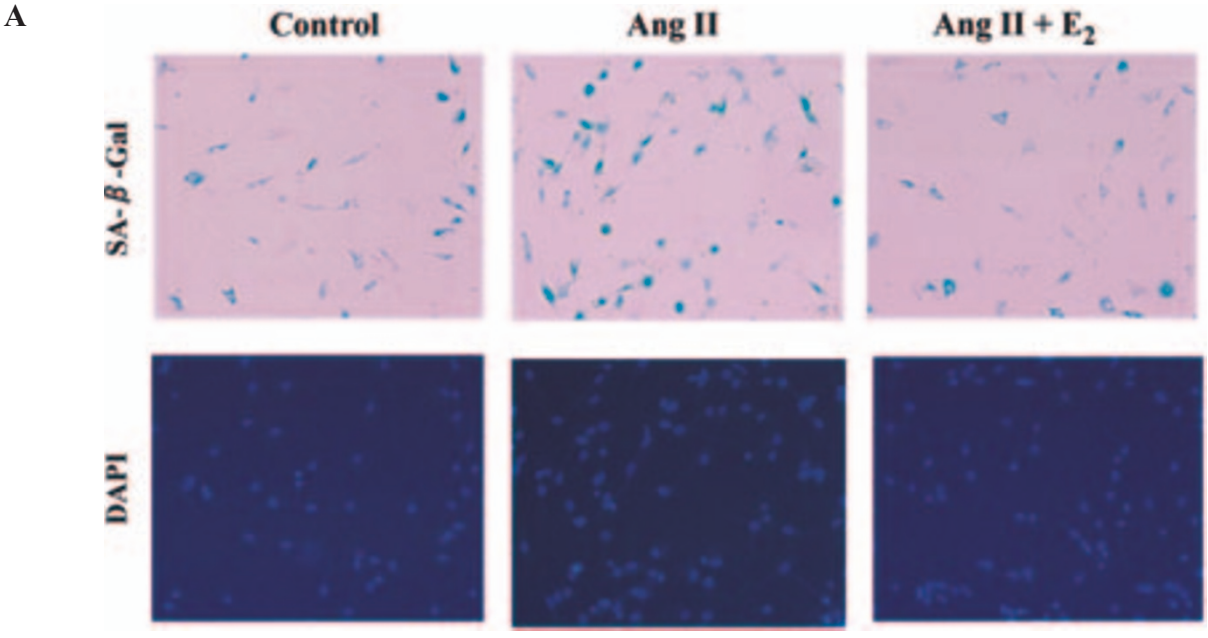

Fig. 3. *Effect of E_2 on Ang II-induced EPC senescence. (A) EPC senescence was evaluated by senescence-associated β-galactosidase (SA-β-Gal) staining. Representative photomicrographs of EPCs exposed to either buffer (control group), Ang II (100 nmol/l) alone, or Ang II+E_2 (100 nmol/l) at day 14. (B) At day 14 after culture, the number of SA-β-Gal-positive cells (blue cells) was counted manually from a total of 200 cells. Data are expressed as the mean±SEM (*n=5). *$p<0.01$ compared with the control (untreated) cells; #$p<0.01$ compared with treatment by Ang II (100 nmol/l) alone.*

Estrogen Inhibits Ang II-Induced Phosphorylation of ERK1/2 and p38 MAPK

Because it is well known that numerous signaling pathways are influenced by Ang II, we examined the effect of E_2 on the intercellular pathways involving Ang II, particularly those for the phosphorylation of MAPKs. The phosphorylated levels of MAPKs in EPCs were evaluated by CASE™ assay. Ang II increased the phosphorylation of ERK1/2 and p38 MAPK in EPCs compared with the levels in the untreated control EPCs (3.2- and 2.4-fold increase, respectively) (Fig. 6). Pre-treatment with E_2 significantly blunted the increase in phosphorylated MAPKs induced by Ang II, although E_2 itself had no significant effect on the phosphorylation of EPC1/2 and p38 MAPK (Fig. 6).

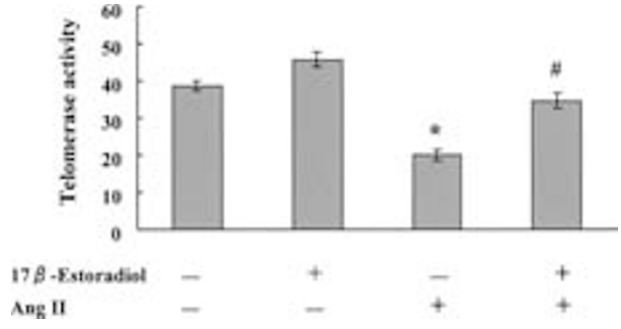

Fig. 4. *Effect of E_2 on Ang II-induced telomerase activity. Freshly isolated mononuclear cells were cultivated in endothelial basal medium-2 (EBM-2) supplemented with EGM-2MV single aliquots. The six-well plates were replated at day 4. After an additional 3 days of cultivation, Ang II with or without pre-treatment with E_2 was then added for 24 h and telomerase activity was measured. Data are expressed as the mean±SEM (n=5). *$p<0.01$ compared with the control (untreated) cells; #$p<0.01$ compared with Ang II-treated cells (100 nmol/l).*

Discussion

Recent studies have demonstrated that atherosclerotic risk factors inversely correlate with the number of EPCs (*4*, *5*). Moreover, angiotensin-converting enzyme inhibitor therapy

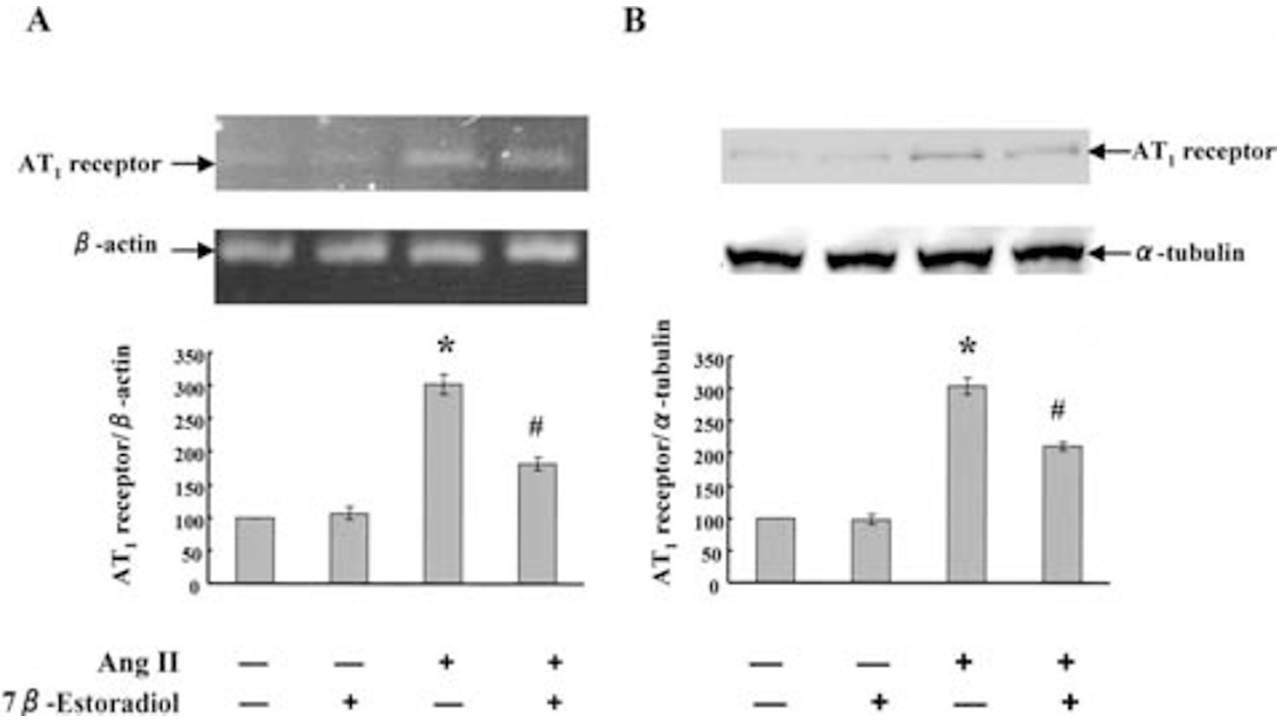

Fig. 5. *Effect of E_2 on AT_1 receptor expression. Ang II (100 nmol/l) stimulated a significant increase in AT_1R mRNA (A) and protein (B) expression, whereas pre-treatment with E_2 (100 nmol/l) reduced these increases. Data are expressed as the mean±SEM (*n = 5). *$p < 0.01$ compared with the control (untreated) cells; #$p < 0.01$ compared with Ang II-treated cells (100 nmol/l).*

with ramipril has been shown to augment circulating EPCs with enhanced functional activity in patients with stable coronary artery disease (*9*). Given that EPCs play a role in postnatal neovascularization, an improved understanding of the mechanisms that regulate EPC biological activity may provide new insights into the pathogenesis of vasculogenesis. We previously demonstrated that Ang II promotes EPC senescence through enhanced oxidative stress, which in turn mediates up-regulation of gp91phox (*13*). Here we have shown that estrogen inhibits Ang II-stimulated gp91phox expression in EPCs, which may contribute to the inhibitory effect of estrogen on Ang II-induced oxidative stress, as evidenced by peroxynitrite formation. Secondarily, estrogen reduces Ang II-induced acceleration of senescence in EPCs, at least in part through the down-regulation of AT_1R.

It is intriguing that our data in EPCs demonstrated an Ang II-induced increase in AT_1R. Data from other cell types suggest that there is a down-regulation after prolonged Ang II stimulation. Additionally, reactive oxygen species, such as superoxide anion, have been suggested to play a role in this down-regulation (*20*, *21*). However, many of these experiments were done in cultured vascular smooth muscle cells, whereas our study focused on EPCs. Indeed, Gragasin *et al*. (*11*) have shown that Ang II stimulates a significant increase in AT_1R expression in bovine coronary microvascular endothelial cells. It has been suggested that activation of nuclear factor κB (NFκB) is important in the up-regulation of AT_1R expression (*21*). Because NFκB is redox-sensitive, an increase in oxidative stress would activate this transcription factor and increase the expression of AT_1R (*22*). Furthermore, Cooke *et al*. (*23*) have demonstrated that peroxynitrite activates NFκB. Taking these results together, one possible explanation for the present findings may be that greater production of peroxynitrite attributable to Ang II leads to activation of NFκB to up-regulate AT_1R. However, our experiments did not clarify the mechanisms by which Ang II up-regulates AT_1R expression, and further studies will be needed on this subject.

The mechanism by which estrogen reduces Ang II-induced acceleration of senescence in EPCs also remains to be determined. We previously demonstrated that both the up-regulation of gp91phox and the acceleration of cellular senescence in Ang II-stimulated EPCs could be abolished by pre-treatment with an AT_1-specific receptor antagonist, valsartan (*13*). In the present study, estrogen partially prevented the Ang II-induced AT_1R mRNA and protein expression. Furthermore, we have also shown that E_2 significantly inhibited Ang II-induced activation of MAPKs, although E_2 itself had no significant effect on the phosphorylation of ERK1/2 and p38 MAPK. Taking these findings together, it appears that the

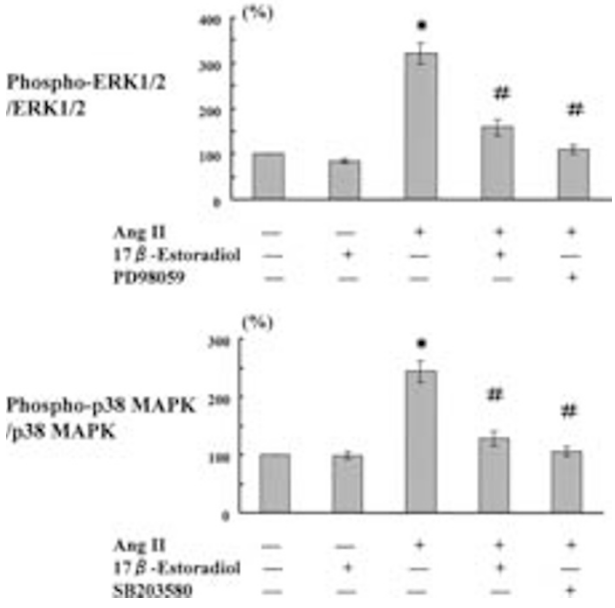

Fig. 6. *Effect of E_2 on Ang II-induced phosphorylation of ERK1/2 and p38 MAPK in EPCs. EPCs were pre-treated with E_2 (100 nmol/l for 30 min), or PD98059 (30 μmol/l for 30 min), or SB203580 (10 μmol/l for 30 min) and then stimulated with Ang II (100 nmol/l) for 30 min. Phosphorylated MAPKs were measured 30 min after treatment. Statistical results of levels of phosphorylated ERK1/2 (A) and p38 MAPK (B) were obtained from 5 independent experiments. The levels of phosphorylated ERK1/2 (A) and p38 MAPK (B) in the control cells were defined as 100%. Data are expressed as the mean±SEM (*n = 5). *$p < 0.01$ compared with the control (untreated) cells; #$p < 0.01$ compared with the Ang II-treated cells (100 nmol/l).*

inhibitory effect of estrogen on Ang II-induced senescence may be partially due to the AT_1R down-regulation induced by estrogen. However, the prevention of Ang-II-mediated responses by estrogen may involve other effects, such as direct effects on the senescence process. Our study provides the foundation for future studies to address the mechanisms of Ang II-induced senescence in EPCs.

In postmenopausal women, there may be an exaggerated response to Ang II attributable to an increase in AT_1R expression due to age and lack of E_2, because it has been suggested that this receptor subtype is up-regulated with age (*24*). Therefore, another cardioprotective role of E_2 may be its interaction with AT_1R. Indeed, a decrease in Ang II response induced by a decrease in the expression of the AT_1R would attenuate the stimulation of superoxide anion production and peroxynitrite formation. To our knowledge, this is the first study to examine the interaction between E_2 and Ang II with regard to gp91phox expression and peroxynitrite formation in EPCs.

In conclusion, given that EPCs may contribute to postnatal neovascularization, the protective effect of E_2 on Ang II-induced cellular senescence in EPCs may affect the process of vascular remodeling.

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