Estrogen Reduces Angiotensin II–Induced Nitric Oxide Synthase and NAD(P)H Oxidase Expression in Endothelial Cells

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Objective—Angiotensin II (AII) has been shown to increase endothelial NAD(P)H oxidase activity, which is a source of superoxide anion that in turn may induce the formation of peroxynitrite. Estrogen (E₂) has been reported to have vascular protective effects. In this study, we hypothesized that E₂ reduces the AII-induced expression of NAD(P)H oxidase and peroxynitrite in endothelial cells.

Methods and Results—Endothelial cells were cultured and stimulated with AII in the absence or presence of E₂. Western blots were used to assess nitric oxide synthase (NOS) and NAD(P)H oxidase expression. Immunofluorescence of nitrotyrosine provided evidence of peroxynitrite formation. Our data indicate that AII increased the expression of endothelial NOS, inducible NOS, and NAD(P)H oxidase in a dose-dependent manner, which was attenuated by incubation with either E₂, superoxide dismutase, or the AII type 1 receptor (AT₁R) inhibitor candesartan. Estrogen as well as superoxide dismutase also inhibited AII-induced AT₁R expression and nitrotyrosine staining. The effects of E₂ on the AII responses were not inhibited by the E₂ receptor antagonist ICI-182,780.

Conclusions—AII stimulation of endothelial cells increases expression of NAD(P)H oxidase and NOS, which may contribute to oxidative stress, as evidenced by peroxynitrite formation. E₂ inhibits these AII effects, possibly through reduced AT₁R expression. (Arterioscler Thromb Vasc Biol. 2003;23:38-44.)

Key Words: estrogen ■ angiotensin ■ oxidative stress ■ endothelium ■ peroxynitrite

Cardiovascular disease is the leading cause of death worldwide to date, and hypertension and atherosclerosis are significant risk factors. An important regulator of these risk factors is the vasoactive peptide angiotensin II (AII). Although AII is a potent vasoconstrictor by itself, it has other important functions in the vasculature. For instance, AII is known to enhance free radical production, such as superoxide anion, through activation of NAD(P)H oxidase.¹ ² This action of AII to promote oxidative stress may be contributing to vascular endothelial dysfunction. Indeed, oxidative stress along with endothelial cell dysfunction is associated with hypertension³ and atherosclerosis,⁴ ⁵ which are precipitating factors for ischemic heart disease.

Nitric oxide (NO) is a free radical, and it is a potent vasodilator produced from NO synthase (NOS). AII stimulates release of NO to modulate the vasoconstrictor actions of AII in the short term.¹⁰ However, AII infusion for 7 days in rats caused increased endothelial NOS (eNOS) expression and enhanced NAD(P)H oxidase expression in aortic tissue that resulted in impaired endothelial-dependent function.¹¹ AII has also been shown to increase peroxynitrite formation,¹² which is produced by the combination of NO and superoxide anion.¹³ In addition to reducing NO bioavailability for vascular relaxation, the decrease in NO bioavailability and increase in peroxynitrite production may have other implications. NO inhibits NOS activity, and it has been suggested that NO serves as a negative feedback regulator of eNOS expression.¹⁴ ¹⁵ Therefore, if there is less NO available, the expression of eNOS may be upregulated because of the attenuation of the negative feedback effect.

In the aging population, particularly in postmenopausal women (a state of natural estrogen [E₂] deficiency), the prevalence of hypertension is increased.¹⁶ E₂ has been shown to downregulate AII type 1 receptor (AT₁R) expression in vascular smooth muscle cells, and it has been postulated that E₂ deficiency may be associated with hypertension, because this receptor subtype plays a key role in the regulation of
blood pressure.\textsuperscript{17} Also, it has been suggested that E\textsubscript{2} stimulates the increase in bioavailability of NO, perhaps through a decrease in endothelium-derived production of superoxide anion.\textsuperscript{18–20} However, the effects of E\textsubscript{2} on AT\textsubscript{1}R and AII-induced NOS and NAD(P)H oxidase expression in endothelial cells have not been studied extensively. E\textsubscript{2} alone may increase the expression of eNOS\textsuperscript{21,22} and decrease NAD(P)H oxidase expression in endothelial cells,\textsuperscript{23} but the interactions between E\textsubscript{2}, AII, and oxidative stress have not, to our knowledge, been studied previously.

The purpose of this study is to determine changes in protein expression of NOS and NAD(P)H oxidase in endothelial cells over long-term stimulation of AII. We hypothesized that AII increases eNOS, inducible NOS (iNOS), and/or NAD(P)H oxidase expression in endothelial cells and these changes result in increased production of peroxynitrite. Moreover, the changes in protein expression will be attenuated using an AT\textsubscript{1}R inhibitor as well as E\textsubscript{2} pretreatment, demonstrating the important interactions between these two hormones.

## Methods

### Reagents

L-glutamine, trypsin, α-minimum essential media (α-MEM) with and without phenol red, and horse serum were obtained from GIBCO. 17-estradiol, gentamycin, kanamycin, AII, and superoxide dismutase (SOD) conjugated to polyethylene glycol were purchased from Sigma Chemical Co. Candesartan was obtained from Astra Pharma Inc. ICI-182,780 was obtained from Tocris Cookson Inc.

### Endothelial Cell Culture

A bovine coronary microvascular endothelial cell line, obtained from Gensia Inc, was selected on the basis of the significance of the coronary microvasculature in cardiovascular disease. Moreover, these cells express eNOS and iNOS, NAD(P)H oxidase, AT\textsubscript{1}R, and E\textsubscript{2}α- and β-receptors. Other cellular characteristics include growth as a monolayer, a cobblestone morphology at confluence, positive immunostaining for von Willebrand factor–related antigen, and the presence of receptors for acetyl low-density lipoproteins.

Cells were grown at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/95% air with α-MEM containing 0.6 mmol/L L-arginine, 10% horse serum, 2 mmol/L L-glutamine, gentamycin (5 μg/mL), and kanamycin (20 μg/mL). Cells were plated at confluence in 6-well tissue culture plates at 5×10\textsuperscript{4} cells per mL (1 mL per well). After attaching to plates, cells were quiesced in phenol-red free media with 0.1% BSA for 24 hours before experimental stimulation. Immediately before stimulation with AII (log doses ranging from 0.01 to 10 μmol/L for 24 hours), this phenol-red free media was replaced with fresh media. In a separate set of experiments, the cells were pretreated with a physiological dose of 17-estradiol (E\textsubscript{2}; 1 nmol/L) for 24 hours in the absence or presence of the E\textsubscript{2} receptor (ER) antagonist ICI-182,780 (10 μmol/L) before AII stimulation (0.1 μmol/L). This dose of E\textsubscript{2} was chosen based on our previous work.\textsuperscript{24} In a third set of experiments, candesartan, an AT\textsubscript{1}R-selective antagonist (10 μmol/L), was added to the cells 3 hours before AII stimulation (0.1 μmol/L). In a fourth set of experiments, cells were pretreated with SOD (50 U/mL) 3 hours before AII stimulation.

At the end of the stimulation period, the cells were rinsed with PBS, scraped from the 6-well plates, and collected in a total volume of 200 μL of homogenizing buffer. Immediately after collection, the cells were sonicated for ~5 seconds. The samples were then stored at ~80°C until further analysis. Total protein for cells was measured by the Bradford method, with BSA used as a standard.

### Western Immunoblot

The general protocol for Western immunoblotting was performed as described earlier.\textsuperscript{24} Primary rabbit polyclonal antibodies were used for eNOS (1:1000), iNOS (1:1000), and AT\textsubscript{1}R (1:1000). For AT\textsubscript{1}R, there was a band at ~41 kDa, which agrees with previous reports in the literature for AT\textsubscript{1}R in bovine cells as well as rat heart and vascular smooth muscle cells.\textsuperscript{25–27} In addition, peptide competition experiments for the AT\textsubscript{1}R confirm that the antibody was specific for the receptor subtype. Polyclonal antibodies (1:100) were also used to assess the subunits of NAD(P)H oxidase. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Imaging was conducted using a Fluor-S MultiImager (BioRad).

### Immunofluorescence

The cells were plated in accordance with the cell culture protocol on 8-chamber slides. Six triplicate groups of cells were quiesced for 24 hours. Three of the triplicate sets were stimulated with either AII at 0.1 μmol/L alone or pretreated with E\textsubscript{2} (1 nmol/L) in the absence or presence of ICI-182,780 (10 μmol/L). The other 3 sets consisted of a control group (no treatment), E\textsubscript{2} (1 nmol/L) alone, and ICI-182,780 (10 μmol/L) alone. Cells were immunostained using polyclonal antibodies against nitrotyrosine (1:100; Upstate Biotechnology), a marker for peroxynitrite. To verify selectivity of nitrotyrosine staining for peroxynitrite, some cells were pretreated with SOD, which prevented AII-mediated increases in peroxynitrite formation. Incubation with the nitrotyrosine antibody dilution occurred at 4°C

![Figure 1](image-url)

Figure 1. A, B, and C, Increased eNOS, iNOS, and gp91\textsuperscript{phox} subunit of NAD(P)H oxidase expression, respectively, in endothelial cells after stimulation with AII (0.01 to 10 μmol/L) for 24 hours. Results are normalized to control (no stimulation). Bars represent mean ± SE; n = 3 to 6 separate experiments. *P < 0.05 vs control.
overnight. Incubation with the secondary antibody took place for 30 minutes in the dark. Coverslips were placed and sealed with nail polish over the cells. The Vectashield H-1200 Mounting Kit (Vector Laboratories) was used for immunofluorescence.

Data Analysis

Data are summarized as mean±SEM of 6 individual experiments containing 2 replicates per condition. All data are expressed as a percentage of the control condition. One-way ANOVA with Tukey's test was used to determine statistical differences among 3 or more groups. Differences were considered significant at $P<0.05$.

Results

As shown in Figure 1, endothelial protein expression of eNOS (A), iNOS (B), and the gp91$^\text{phox}$ subunit of NAD(P)H oxidase (C) was increased after a 24-hour stimulation with AII. Other subunits of NAD(P)H oxidase (p22$^\text{phox}$, p47$^\text{phox}$, and p67$^\text{phox}$) were also significantly increased in a trend similar to that of gp91$^\text{phox}$ (see Figure I online at http://atvb.ahajournals.org). Pretreatment of these cells with 10 $\mu$mol/L candesartan, an AT$_1$R antagonist, abolished the AII-mediated increase in protein expression of eNOS, iNOS, and gp91$^\text{phox}$ subunit of NAD(P)H oxidase (Figures 2A, 2B, and 2C). The AII-induced expression of the other NAD(P)H oxidase subunits (p22$^\text{phox}$, p47$^\text{phox}$, and p67$^\text{phox}$) was also prevented by pretreatment with candesartan (see Figure II online at http://atvb.ahajournals.org). These results demonstrate that AT$_1$Rs mediated AII-induced expression of eNOS, iNOS, and components of NAD(P)H oxidase.

Next, we determined whether E$_2$ modulates AII effects on endothelial cells. E$_2$ treatment alone slightly increased the expression of eNOS, iNOS, and NAD(P)H oxidase. Inhibition of ERs with either tamoxifen (data not shown) or ICI-182,780 prevented E$_2$-induced increase in eNOS without AII but did not change iNOS expression (Figures 3A and 3B). Estrogen markedly diminished the AII-induced increases in eNOS, iNOS, and gp91$^\text{phox}$ subunit of NAD(P)H oxidase expression (Figures 3A, 3B, and 3C, respectively). The

![Figure 2](image-url) Effect of AT$_1$R inhibition on protein expression of eNOS (A), iNOS (B), and gp91$^\text{phox}$ subunit of NAD(P)H oxidase (C) in endothelial cells. Endothelial cells were treated for 3 hours with 10 $\mu$mol/L of the AT$_1$R-selective antagonist candesartan (CN), followed by stimulation with 0.1 $\mu$mol/L AII for 24 hours. Bars represent mean±SE; n=3 to 6 separate experiments. *$P<0.05$ vs control; †$P<0.05$ vs All.

![Figure 3](image-url) The effect of E$_2$ alone or with AII stimulation of eNOS (A), iNOS (B), and gp91$^\text{phox}$ subunit of NAD(P)H oxidase (C) protein expression in endothelial cells. Cells were pretreated with a physiological dose of 17-$\beta$-estradiol (E$_2$; 1 nmol/L) for 24 hours in the absence or presence of the ER antagonist ICI-182,780 (10 $\mu$mol/L) before AII stimulation (0.1 $\mu$mol/L). Bars represent mean±SE; n=3 to 6 separate experiments. *$P<0.05$ vs control; †$P<0.05$ vs All.
AII-induced expression of the p22phox and p67phox NAD(P)H oxidase subunits was also prevented by pretreatment with estrogen (see Figure III online at http://atvb.ahajournals.org). Interestingly, AII-induced expression for eNOS, iNOS, or NAD(P)H oxidase was not altered by the ER inhibition with ICI-182,780 (Figure 3 and Figure III online).

AII increases nitrotyrosine staining, suggesting an increase in peroxynitrite formation (Figure 4). The AII-induced peroxynitrite production is prevented with E2 pretreatment. Similar to the above findings, ER antagonism did not prevent this effect (Figure 4). AII stimulated a significant increase in AT1R expression, whereas pretreatment with E2 prevented this AII-induced expression of AT1R (Figure 5). Similarly, SOD also prevented AII-induced expression of AT1R (Figure 6).

**Discussion**

There is an increase in endothelial expression of eNOS, iNOS, and NAD(P)H oxidase expression with increasing concentrations of AII. In the presence of the AT1R antagonist candesartan, AII-induced increases in eNOS, iNOS, and NAD(P)H oxidase protein expression are significantly reduced. In addition, peroxynitrite formation was enhanced with AII exposure. Pretreatment with E2 attenuated the AII-mediated responses. However, inhibition of ERs did not alter the effects of E2 on AII-dependent responses. AII also increased AT1-R expression, which was prevented by E2, suggesting that AT1-R may mediate the inhibitory effect of E2 on AII stimulation of eNOS, iNOS, and NAD(P)H oxidase expression.

It is intriguing that our data in endothelial cells demonstrated AII-induced increase in AT1-R. Data from other cell...
types suggest that there is a downregulation after prolonged AII stimulation. Additionally, reactive oxygen species, such as superoxide anion, have been suggested to play a role in this downregulation.\textsuperscript{28,29} However, many of these experiments were done in cultured vascular smooth muscle cells, whereas our study focused on endothelial cells. A possible explanation for our study may be that increased concentration of AII increases the enzymes in the endothelium that are responsible for the formation of NO and superoxide anion leading to peroxynitrite formation. The production of superoxide anion [possibly by NAD(P)H oxidase] would scavenge NO, and it has been suggested that NO directly decreases transcription of AT,Rs by binding to DNA.\textsuperscript{30} Therefore, a decrease in NO levels may allow for increased AT,R. It has also been suggested that activation of nuclear factor \(kB\) (NFkB) is important in the upregulation of AT,R expression.\textsuperscript{31} Because NFkB is redox-sensitive, an increase in oxidative stress would activate this transcription factor and increase expression of AT,Rs. Indeed, a major signaling pathway of AII is through activation of NFkB.\textsuperscript{32} We have previously reported that peroxynitrite activates NFkB, leading to induction of iNOS in endothelial cells.\textsuperscript{33} Thus, a positive feedback may ensue, where greater production of peroxynitrite attributable to AII leads to activation of NF-kB to upregulate AT,Rs and iNOS and additionally heightens the production of peroxynitrite. In our study, we demonstrated that both peroxynitrite and AT,R upregulation by AII could be reduced by SOD, a superoxide anion scavenger. These data additionally support our speculation that AII upregulation of its own receptor is attributable, in part, to oxygen free radicals that additionally exacerbate vascular function. Indeed, AII stimulation on endothelial cells that results in peroxynitrite production could enhance vasoconstriction by isoprostane formation\textsuperscript{34,35} in addition to the loss of the vasodilating capacity of NO (attributable to scavenging effects of superoxide anion). Thus, these pathways may be mechanisms for modulating AII-mediated vasoconstriction.

AII may also increase oxidative stress in the short term by immediate activation of NAD(P)H oxidase to form superoxide anion.\textsuperscript{36–38} Indeed, Zhang et al\textsuperscript{39} demonstrated that AII induces immediate release of superoxide anion in endothelial cells via activation of NAD(P)H oxidase. A recent study suggested that AII increases NOS activity, resulting in increased NO and peroxynitrite production in endothelial cells,\textsuperscript{12} and the increase in peroxynitrite is associated with endothelial dysfunction associated with cardiovascular disease.\textsuperscript{40} Heitsch et al\textsuperscript{41} have also suggested that increased NO and peroxynitrite production may be attributable Angiotensin-(1-7), a product of angiotensin II/II enzymatic cleavage, which can be produced by endothelial cells.\textsuperscript{42} However, these studies did not evaluate AII-induced effects on expression of enzymes that are major contributors to oxidative stress, which was one of the aims of the present study. Indeed, our data indicate that AII induces both eNOS and iNOS as well as NAD(P)H oxidase expression, which likely contributes to peroxynitrite formation.

In our study, E\(_2\) prevented AII-induced activation of endothelial cells. In particular, we observed that E\(_2\) reduced AII-induced expression of NAD(P)H oxidase; however, E\(_2\) alone had little effect on this enzyme. Another recent study investigated the effects of E\(_2\) on NAD(P)H oxidase expression in endothelial cells,\textsuperscript{23} which resulted in similarities as well as differences with our findings. For instance, in both the present study and the study by Wagner et al.,\textsuperscript{23} eNOS expression increased with treatment of E\(_2\) alone, but gp91\textsuperscript{phox} expression decreased with E\(_2\) treatment in their study, which is in contrast with our findings. A possible difference could be that we used endothelial cells from the coronary microcirculation of the heart, whereas they used umbilical vein endothelial cells. The primary finding in our study is that E\(_2\) prevents AII-induced activation of endothelial cells, which may represent a protective role of E\(_2\) in the presence of AII. This is the first study to our knowledge that looks at the interaction between E\(_2\) and AII with regards to oxidative stress, particularly in endothelial cells specifically, although it has been suggested that use of E\(_2\) and an AT,R blocker has additive antioxidant effects on low-density lipoproteins.\textsuperscript{43}

In our study, E\(_2\) alone (without AII) increased eNOS, which was prevented by an ER antagonist, ICI-182,780. In contrast, E\(_2\) inhibited AII-mediated increases in eNOS, iNOS, NAD(P)H oxidase, and nitrotyrosine staining. These data are intriguing and suggest that E\(_2\) may have effects through reduction of the AT,R as we demonstrated. Interestingly, these effects of E\(_2\) on endothelial cells were independent of an estrogen receptor. Previous studies using cultured vascular smooth muscle cells demonstrated that E\(_2\) as well as the selective estrogen receptor modulator idefoxifene reduced AT,R.\textsuperscript{44} Although ultimately E\(_2\) reduces AT,R in both endothelial and vascular smooth cells, the role of estrogen receptors mediating these effects are dissimilar, depending on the vascular cell type.

The effect of estrogen to prevent AII-mediated responses in endothelial cells may involve nonspecific effects, such as altering the physicochemical membrane properties. However, previous reports of nonspecific (receptor-independent) actions of estrogen have used supraphysiological concentrations of steroids (\(>10\ \mu\text{mol/L}\)),\textsuperscript{45} whereas in our study we used a physiological dose (1 nmol/L) of estrogen. Nonetheless, it is possible that estrogen may have altered membrane properties that altered the binding capacity of AT,R receptor, thereby preventing AII regulation of its own receptor. Another possibility is that estrogen acts as an antioxidant independent of its receptor.\textsuperscript{46,47} Indeed, we showed that SOD also prevents AII-mediated increases in AT,R, suggesting that free radicals have a role in upregulation of AT,R in endothelial cells. Surprisingly, there is paucity of data regarding the mechanisms of AII regulation of its own receptor in endothelial cells. Our study provides the foundation for future studies to address these specific cellular mechanisms.

The interesting finding that E\(_2\) reduces AII-induced NOS expression may seem to contradict the evidence of enhanced NO production by E\(_2\). A possible interpretation for our study may be that although E\(_2\) inhibits the AII-induced increase in NOS expression by downregulating the AT,Rs, it also increases the bioavailability of NO\textsuperscript{18,19} by limiting the formation of superoxide anion by mechanisms either dependent on NAD(P)H oxidase expression or through direct antioxidant effects.\textsuperscript{46,47} Indeed, in our study, E\(_2\) prevented AII-induced
expression of NAD(P)H oxidase as well as reduced peroxynitrite formation (as evidenced by reduced nitrotyrosine staining).

In postmenopausal women, there may be an exaggerated response to E2 attributable to an increase in AT1R expression due to age and lack of E2, because it has been suggested that this receptor subtype is upregulated with age.48 Therefore, another cardioprotective role of E2 may be its interaction with the AT1Rs. Indeed, a decrease in AII response by decreasing another cardioprotective role of E2 may be its interaction with endothelial cells, especially through regulating the proteins involved with formation of peroxynitrite. The effects of E2 are comparable with the effects of the AT1R blocker candesartan on eNOS, iNOS, and NAD(P)H oxidase expression in the endothelial cells, suggesting that the ability to inhibit long-term effects induced by AII is similar.

In summary, our data demonstrate the important interactions between E2 and AII and their role in oxidative stress on endothelial cells, especially through regulating the proteins involved with formation of peroxynitrite. One effect of E2 may be manifested through the downregulation of AT1Rs. Although most actions of AII on vascular smooth muscle, the effects on endothelial cells cannot go unrecognized. AII can potentially damaging effects through increasing oxidative stress in the endothelium, but these effects may be alleviated by E2 treatment.

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