Differential Effects of Estrogen and Progesterone on AT1 Receptor Gene Expression in Vascular Smooth Muscle Cells

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Background—The beneficial vasoprotective effects of a postmenopausal estrogen replacement therapy may be prevented by a concomitant administration of progestins. To investigate the differential effects of estrogens and progesterone, we examined their influence on AT1 receptor gene expression in vascular smooth muscle cells (VSMCs).

Methods and Results—17β-Estradiol caused downregulation of AT1 receptor mRNA expression to 46±14%, whereas progesterone led to a significant upregulation to 201±29%, as assessed by Northern analysis. Western blots revealed that estrogen induced a downregulation and progesterone an upregulation of the AT1 receptor protein. Estrogen-induced decrease of AT1 receptor expression was mediated through activation of estrogen receptors. Nuclear run-on assays revealed that 17β-estradiol did not alter AT1 receptor mRNA transcription rate, whereas progesterone caused an enhanced AT1 receptor mRNA transcription rate. 17β-Estradiol decreased the AT1 receptor mRNA half-life from 5 to 2 hours, whereas progesterone induced a stabilization of AT1 receptor mRNA to a half-life of 10 hours. Preincubation of VSMCs with PD98059, SB203580, herbimycin, wortmannin, or Nω-nitro-L-arginine suggested that 17β-estradiol caused AT1 receptor downregulation through nitric oxide–dependent pathways. Progesterone caused AT1 receptor overexpression via P1,kinase activation. Angiotensin II–induced release of reactive oxygen species was inhibited by estrogens. Progesterone itself enhanced the production of reactive oxygen species.

Conclusions—Because AT1 receptor regulation plays a pivotal role in the pathogenesis of hypertension and atherosclerosis, the differential effects of estrogen and progesterone on the expression of this gene may in part explain the potentially counteracting effects of these reproductive hormones on the incidence of postmenopausal cardiovascular diseases. (Circulation. 2000;102:1828-1833.)

Key Words: receptors ■ angiotensin ■ muscle, smooth ■ hypertension ■ atherosclerosis ■ hormones

Several lines of epidemiological evidence indicate that estrogens play an important role in the pathogenesis of hypertension and atherosclerosis. First, at a young age, women suffer considerably less cardiovascular disease than men. Second, after menopause, the natural state of estrogen deficiency, the incidence of cardiovascular disease rises steeply in women.1,2 Finally, hormone replacement therapy potentially prevents the onset of cardiac events in postmenopausal women.3–5 The pathways by which estrogens interact with the cardiovascular physiology are not completely understood. Estrogens lower plasma lipoproteins, influence the renin-angiotensin system,6–7 exert antioxidative properties,8 and may act as calcium blocking agents.9 In addition, estrogens exert direct effects on the vessel wall, such as an increase of vascular NO production and modulation of expression of endothelial constitutive NO synthase (ecNOS).10–12

Numerous retrospective studies have shown that estrogen replacement therapy may be useful in the primary prevention of cardiovascular diseases in postmenopausal women.3–5 However, the first prospective and randomized study on that subject, the HERS trial,13 showed no advantage of hormone replacement therapy in terms of a lowered incidence of cardiac events in postmenopausal women who suffer from coronary heart disease. Among other things, this surprising outcome has been attributed to the addition of progesterone to the therapy regimen. Progesterone could display a number of potential adverse effects on the cardiovascular system that might overcome the beneficial influence of estrogens. In this context, reduction of HDL levels, downregulation of estrogen receptors, decreased carbohydrate tolerance, and reduced blood flow and vasodilatation have been reported.14–17 In any event, the molecular mechanisms by which a concomitant progesterone therapy could counteract the estrogen replacement treatment are not known in detail and are the subject of controversy, because some study results could not support the deleterious effects of progesterone.1,2

The AT1 receptor mediates many biological effects of the renin-angiotensin system (RAS), such as vasoconstriction, water and sodium retention, free radical release, and cell growth.18 Therefore, activation of the AT1 receptor has been implicated in the pathogenesis of cardiovascular disease. Recently, it was

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shown that estrogen deficiency causes AT₁ receptor overexpression in vivo, leading to enhanced biological effects of the RAS, which could in part serve as explanation for the increase in cardiac events after menopause in women.²⁹

We hypothesized that potentially counteracting effects of estrogens and progestins on the cardiovascular system could take place at the level of AT₁ receptor regulation. To further investigate the direct effects of these reproductive hormones on vascular cells, we examined their influence on AT₁ receptor gene expression in vascular smooth muscle cells (VSMCs) and sought to clarify the underlying molecular mechanisms.

**Methods**

**Cell Culture**

VSMCs were isolated from rat thoracic aorta (female Wistar-Kyoto, 6 to 10 weeks old, Charles River GmbH, Sulzfeld, Germany) by enzymatic dispersion and cultured over several passages. Cells were grown in a 5% CO₂ atmosphere at 37°C in DMEM without phenol and 10% FBS (free of steroid hormones, S-15-M, c.c.pro GmbH).

**mRNA Isolation and Northern Analysis**

Culture medium was aspirated, and RNA was isolated with 1 mL RNA-Clean. Ten-microgram aliquots were electrophoresed, transferred onto Hybond N membranes, and UV–cross-linked. Northern blots were prehybridized for 2 hours at 42°C in a buffer containing 50% deionized formamide, 0.5% SDS, 6× SSC, 10 μg/mL denatured salmon sperm DNA (Sigma Aldrich Chemicals), and 5× Denhardt’s solution and were then hybridized for 15 hours at 42°C with a random-primed, [³²P]dCTP-labeled rat AT₁ receptor cDNA probe in the same buffer but without Denhardt’s solution.

**Nuclear Run-On Assay**

After treatment, VSMCs were dispersed with trypsin and washed. The cell pellet was lysed for 10 minutes on ice, and the nuclei were isolated by centrifugation. The nuclear pellet was resuspended in a buffer containing 40% glycerol, 50 mmol/L Tris, 0.5 mmol/L MgCl₂, and 0.1 mmol/L EDTA. Nuclei (~5×10⁶ to 20×10⁶ nuclei per reaction) were used to carry out the transcription in a reaction mixture containing 40% glycerol, 50 mmol/L Tris, 5 mmol/L MgCl₂, 0.1 mmol/L/EDTA, 0.5 mmol/L of CTP, GTP, and ATP, and 0.2 to 0.3 μmol/L [³²P]UTP (≥3000 μCi/mmol) at 30°C for 30 minutes. Reactions were terminated, and radioactive RNA was isolated and purified. [³²P]UTP-labeled RNA (5×10⁶ to 1×10⁷ cpm) was dissolved in hybridization solution. Membranes were prehybridized for 2 hours at 42°C and hybridized at 42°C for 16 hours.

**Western Blotting**

VSMC samples were homogenized. Equal volumes of 2× SDS gel loading buffer (100 mmol/L Tris-HCl [pH 6.8], 200 mmol/L dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) were added, and samples were heated to 95°C for 10 minutes. The samples were then sonicated and spun at 10,000 g for 10 minutes at room temperature. Twenty-five micrograms of protein of the supernatant was run through a 10% polyacrylamide gel. Western blotting of proteins was performed in a semidry blotting chamber (Pharmacia). Culture dishes were transferred to a Zeiss Axiovert 135 inverted microscope (Carl Zeiss), equipped with a ×25, numerical aperture 0.8, oil-immersion objective (Plan-Neofluar, Carl Zeiss) and Zeiss LSM 410 confocal attachment, and ROS generation was detected as a result of the oxidation of H₂DCF (excitation, 488 nm; emission long-pass LP515-nm filter set). Pixel images (512×512) were collected by single rapid scans. In 4 separate experiments, 5 groups of 25 cells each were randomly selected from the image, and fluorescence intensity was taken. The relative fluorescence intensities are average values of all experiments, and each value reflects measurements performed on a minimum of 100 cells for each sample.

**Statistical Analysis**

Data are presented as mean±SEM. Statistical analysis was performed with the ANOVA test.

**Results**

The influence of estrogen and progestin on AT₁ receptor mRNA in VSMCs was measured. Cells were incubated for 0 to 24 hours with 1 μmol/L of either 17β-estradiol or progesterone, and AT₁ receptor mRNA was measured by Northern blotting. Figure 1, A and B, shows that 17β-estradiol caused a significant decrease of AT₁ receptor mRNA after 2 hours, reaching the maximum after 4 hours of 46±14% of control levels (P<0.05). In contrast, progesterone led to an upregulation of AT₁ receptor mRNA, with a maximal effect of 201±29% reached after 12 hours (Figure 1, A and B). Control experiments in which VSMCs were incubated with vehicle showed that AT₁ receptor and GAPDH mRNA levels remained stable over the experimental period of 24 hours (data not shown). Both 17β-estradiol and progesterone led to concentration-dependent modulation of AT₁ receptor mRNA in VSMCs (Figure 1C). After a 4-hour treatment, the maximal effect was measured at 1 μmol/L 17β-estradiol, which led to a reduction of AT₁ receptor mRNA to 41±6% of control cells (P<0.05). The maximal progesterone effect was reached at a concentration of 1 μmol/L after a 12-hour incubation (215±7%). In all experimental setups, GAPDH mRNA and 18S mRNA levels were not altered (data not shown).

To assess whether this modulation of AT₁ receptor mRNA expression translates into comparable changes in AT₁ receptor mRNA protein levels, VSMCs were incubated for 4 and 12 hours with 1 μmol/L 17β-estradiol and for 12 and 24 hours with 1 μmol/L progesterone. Total protein was isolated, and Western analysis was used to quantify AT₁ receptor protein. Figure 2 illustrates that 17β-estradiol caused a significant downregulation (48±7% and 32±5% of control levels), whereas progesterone induced an upregulation (173±4% and 226±32% of control levels), of AT₁ receptor protein expression. Control experiments had shown that a 0- to 24-hour incubation with vehicle did not modulate AT₁ receptor expression (data not shown).

The specificity of the 17β-estradiol effect on the expression of the AT₁ receptor mRNA was tested by use of the
biologically inactive stereoisomer 17α-estradiol and the estrogen receptor antagonists ICI 182,780 and tamoxifen. VSMCs were incubated for 4 hours with 1 μmol/L 17α-estradiol, 1 μmol/L tamoxifen, 1 μmol/L ICI 182,780, and either ICI 182,780 or tamoxifen before AT1 receptor mRNA expression was measured by Northern analysis. Whereas 17α-estradiol, ICI 182,780, and tamoxifen exerted no effect on AT1 receptor mRNA expression, 17β-estradiol caused a significant downregulation of AT1 receptor mRNA. Tamoxifen as well as ICI 182,780 abolished the 17β-estradiol-induced effects on AT1 receptor mRNA (Figure 3A). In addition, the involvement of progesterone receptor activation was tested by coincubation of 1 μmol/L progesterone and 1 μmol/L RU486. Figure 3B shows that the progesterone receptor antagonist completely abolished the effect of progesterone.

To investigate the potential mechanism mediating regulation of AT1 receptor mRNA expression, we evaluated the effect of either 17β-estradiol or progesterone on the AT1 receptor gene transcription rate. VSMCs were treated for 4 hours with 1 μmol/L 17β-estradiol or vehicle for 12 hours with 1 μmol/L progesterone or vehicle. Nuclei were then isolated, and nuclear run-on assays were performed. Figure 4 shows a representative autoradiogram of radiolabeled, de novo synthesized mRNA to the AT1 receptor, GAPDH, and plasmid DNA, and the densitometric analysis of 3 separate experiments. The relative intensity of the AT1
indicated time points, and AT1 receptor mRNA was quantified by Northern analysis. Mean±SEM, n=3.

Figure 4. Effect of 17β-estradiol and progesterone on AT1 receptor mRNA transcription rate. VSMCs were incubated with vehicle (4 and 12 hours), 1 μmol/L 17β-estradiol (4 hours), or 1 μmol/L progesterone (12 hours). Nuclear were isolated and nuclear run-on assays performed. A representative autoradiogram and densitometric analysis are demonstrated. Signal intensities of AT1 receptor and GAPDH mRNA were calculated. AT1 receptor/GAPDH ratio is shown. Mean±SEM, n=3.

Further experiments measured the effect of either 17β-estradiol or progesterone on AT1 receptor mRNA stability. VSMCs were preincubated with either vehicle, 1 μmol/L 17β-estradiol (4 hours), or 1 μmol/L progesterone (12 hours); transcription was blocked with 50 μg/mL 5,6-dichlorobenzimidazole (DRB), and total RNA was isolated 0 to 8 hours after the addition of DRB. Figure 5 shows the quantification of AT1 receptor mRNA. Under control conditions, the AT1 receptor mRNA half-life was measured at 5 hours. 17β-Estradiol destabilized AT1 receptor mRNA, resulting in an AT1 receptor mRNA half-life of 2 hours; progesterone stabilized AT1 receptor mRNA to a half-life of ≈10 hours. GAPDH mRNA levels remained stable over the experimental period (data not shown).

By means of various pharmacological inhibitors, the involved signal transduction pathways were characterized. VSMCs were preincubated with 1 μmol/L PD98059 (p42/44 MAP kinase inhibitor), 1 μmol/L SB203580 (p38 MAP kinase inhibitor), 1 μmol/L of the tyrosine kinase inhibitor herbimycin, 1 μmol/L of the phosphatidylinositol (PI3)- kinase inhibitor wortmannin, or the NO inhibitor Nω-nitro- L- arginine (L-NNA, 10 μmol/L) followed by a coincubation with 1 μmol/L 17β-estradiol (4 hours) or 1 μmol/L progesterone (12 hours). The densitometric analysis of the Northern blots quantifying AT1 receptor mRNA is depicted in Figure 6. The estrogen-mediated downregulation of AT1 receptor mRNA was inhibited only by L-NNA, suggesting an NO-dependent downregulation of the AT1 receptor. In contrast, the progesterone-caused AT1 receptor upregulation was abolished by coinubation with wortmannin, which supports the concept that PI3-kinase is involved in this induced modulation of AT1 receptor gene expression. All inhibitors were also used in concentrations from 10 nmol/L to 10 μmol/L. PD98059, SB203580, and genistein did not blunt the effects of 17β-estradiol or progesterone on AT1 receptor expression, even if applied in high concentrations (data not shown).

AT1 receptor activation causes free radical release in VSMCs. To evaluate the functional relevance of the AT1 receptor regulation, cells were preincubated for various time points with either 1 μmol/L 17β-estradiol or 1 μmol/L.
The incidence of cardiovascular disease is low in premenopausal women, but it increases steadily in postmenopausal women. In addition, postmenopausal hormone replacement therapy may reduce this rise of cardiovascular events, as suggested by retrospective studies. The vasoprotective potential of estrogens has been attributed primarily to its effects on serum lipid concentrations. However, an increasing body of evidence indicates that direct effects of estrogens on blood vessels contribute significantly to their cardio-protective effects. Activation of estrogen α- and β-receptors causes long-term effects on cellular gene expression programs, which are thought to be mediated by genomic effects of the activated steroid receptors. Intriguingly, estrogens also induce nongenomic, rapid effects in vascular cells that obviously occur independently of modulation of gene expression. One of the most prominent features seems to be a rapid vasodilatory effect of estrogen, which is elicited through endothelium-dependent and endothelium-independent mechanisms. Current data suggest that estrogens enhance the bioavailability of NO through stimulation of NOS and NO release and potentially also through the antioxidant properties of estrogens. Such as stimulation of NO release, may also cause longer-lasting effects on vascular cells that ultimately contribute to the atheroprotective effects of estrogens.

Figure 7. Effect of progesterone and 17β-estradiol on angiotensin II–induced intracellular production of ROS in VSMCs. Representative microscopic scan. VSMCs were preincubated for 12 hours with 1 μmol/L progesterone or 17β-estradiol or vehicle, followed by a 4-hour incubation with 1 μmol/L angiotensin II. Free radical production is visualized through DCF fluorescence.

Progesterone, followed by a 4-hour incubation with 1 μmol/L angiotensin II. Production of free radicals was assessed via DCF fluorescence. Figure 7 shows that angiotensin II led to a profound increase of ROS, which was inhibited by a 12-hour preincubation with 17β-estradiol. 17β-Estradiol itself had no effect on the basal release of free radicals. In addition, only a long-term preincubation with estrogens (12 to 24 hours) lowered angiotensin II–caused production of ROS (data not shown). Progesterone itself significantly enhanced the release of free radicals. No further increase of angiotensin II–induced production of ROS was detected.

Discussion

The AT₁ receptor, which induces vasoconstriction, cellular growth, and free radical release in the vessel, is regulated by angiotensin II, lipoproteins, growth factors, and insulin, among other things. It has recently been reported that estrogen deficiency leads to overexpression of the vascular AT₁ receptor, which is preventable by estrogen replacement therapy. Most likely, the underlying mechanism for these changes is the modulation of AT₁ receptor gene expression in VSMCs by estrogens, as shown in the present study. 17β-Estradiol leads to a dose- and time-dependent downregulation of AT₁ receptors in VSMCs. This effect is mediated by estrogen receptors and involves the destabilization of the AT₁ receptor mRNA. The latter seems to be the principal cellular mechanism by which AT₁ receptor expression is modulated. Although MAP kinase activation has been described on stimulation with estrogens, this signal transduction pathway is not participating in the estrogen-induced AT₁ receptor regulation. Estrogen-caused release of NO seems to be a prerequisite of the described regulative pathways, as shown by the inhibition of estrogen-induced AT₁ receptor downregulation by an NO inhibitor. This is in agreement with previous findings that NO is released on estrogen stimulation. Moreover, it has been shown that NO is involved in AT₁ receptor downregulation by, for example, cytokines in VSMCs. The effect of estrogen seems to be mediated by estrogen receptors, because the estrogen receptor antagonists ICI 182,780 and tamoxifen abolished the AT₁ receptor–downregulating effect of 17β-estradiol. 17α-Estradiol, which does not activate estrogen α- or β-receptors, showed no effect on AT₁ receptor expression.

Surprisingly, progesterone causes a profound upregulation of AT₁ receptor gene expression mediated through stabilization of the AT₁ receptor mRNA. In striking contrast to the mode of action of estrogen, this effect on AT₁ receptor gene expression is mediated through activation of PI₃-kinase. The current concept of heterologous AT₁ receptor regulation ascribes a pivotal role to posttranscriptional mechanisms. Stabilization or destabilization of AT₁ receptor mRNA seems to participate decisively in the modulation of AT₁ receptor expression. The signal transduction pathways
involved in this phenomenon are less clear. MAP kinase activation, cytolsic calcium release, cAMP accumulation, and NO have been implicated in transcriptional as well as posttranscriptional regulation of AT₁ receptor gene expression.21–24 Nevertheless, the factors downstream of this signaling event are unknown. There is evidence that mRNA binding proteins induce (de)stabilization of the AT₁ receptor mRNA, but the detailed mechanisms have not yet been elucidated.21 It may be speculated that PI3-kinase activation as well as NO release interact directly or indirectly with these binding proteins, leading to changes in AT₁ receptor mRNA degradation. Interestingly, NO obviously plays a role in the modulation of ecNOS stability.28 According to recent findings, ecNOS stability is dependent on distinct mRNA binding proteins. Therefore, NO could potentially play a role in the regulation of events involved in the AT₁ receptor mRNA processing, as suggested by the presented data.

The AT₁ receptor regulation by estrogens and progesterone is accompanied by functional alterations in VSMCs, as shown by the altered angiotensin II–induced release of ROS. In general, activation of the AT₁ receptor is a major source for ROS in the vessel wall, and these ROS are closely involved in the pathogenesis of atherosclerosis and hypertension. Our data indicate that the antioxidant properties of estrogens could be mediated at least in part through the downregulation of the AT₁ receptor. Furthermore, estrogen-induced AT₁ receptor downregulation could be related to a decreased vasoconstriction and cell growth. In contrast, progesterone-induced AT₁ receptor upregulation would lead to the opposite effect. Progesterone itself enhances the release of free radicals, suggesting a direct interaction of progesterone with enzymatic systems such as NAD(P)H oxidase. The latter, and the upregulation of the AT₁ receptor, are possible mechanisms by which administration of progesterone causes a decreased blood flow and an increased vascular resistance in postmenopausal women.29 The failure to show a reduction of endothelium-dependent vasorelaxation by estrogen in depolarized rat and mouse aorta: role of nuclear estrogen receptor and Ca²⁺ uptake. Circ Res. 1997;81:242–248.


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