Progesterone Antagonizes the Vasoprotective Effect of Estrogen on Antioxidant Enzyme Expression and Function

Kerstin Wassmann, Sven Wassmann, Georg Nickenig

Abstract—Oxidative stress plays an important role in the pathogenesis of atherosclerosis and can be effectively influenced by radical scavenging enzymes. Estrogens exert antioxidative effects in the vasculature; however, cotreatment with progesterone may abrogate the vasoprotective effects of estrogen. Therefore, the effects of progesterone on the production of reactive oxygen species (ROS) and expression and function of antioxidant and oxidant enzymes were investigated in cultured vascular smooth muscle cells (VSMCs) and vascular tissue of mice. Progesterone time- and concentration-dependently downregulated extracellular superoxide dismutase (ecSOD) and manganese superoxide dismutase (MnSOD) expression and enzyme activity and reversed 17β-estradiol–induced overexpression of ecSOD and MnSOD in VSMCs. Nuclear run-on assays revealed that progesterone decreases MnSOD and ecSOD transcription rates. Consequently, progesterone increased ROS release in VSMCs that was prevented by concomitant treatment with 17β-estradiol. Estrogen deficiency in ovariectomized mice was associated with an increase in vascular superoxide release and NADPH oxidase activity. Estrogen replacement prevented this increase, whereas progesterone substitution enhanced ROS production and NADPH oxidase activity. The modulation of superoxide release coincided with decreased expression of ecSOD and MnSOD and upregulation of the p22phox and p67phox subunits of the NADPH oxidase complex in progesterone-treated animals. Furthermore, administration of progesterone to ovariectomized mice treated with 17β-estradiol abrogated the antioxidative effects of estrogen. Progesterone antagonizes the vasoprotective effects of estrogen on ecSOD and MnSOD expression and increases NADPH oxidase activity. These findings may in part explain why hormone replacement therapy with estrogen plus progesterone displayed no beneficial effect on cardiovascular event rates in the prospective clinical trials. (Circ Res. 2005;97:1046-1054.)

Key Words: progesterone ■ estrogen ■ superoxide dismutase ■ oxidative stress ■ atherosclerosis

A large body of evidence has been accumulated suggesting that increased oxidative stress is an initial step in the development of endothelial dysfunction and atherosclerosis.1–3 Classical coronary risk factors such as smoking and hypercholesterolemia lead to increased oxidative stress.2,4 Reactive oxygen species (ROS) mediate endothelial cell apoptosis, inflammatory processes, proliferation of vascular smooth muscle cells (VSMCs), and destabilization of atherosclerotic plaques.4–6 These harmful effects of ROS are counterbalanced by nitric oxide (NO).2,6,7 NO is currently believed to be the main component responsible for endothelial function and integrity.1–4 In addition to the bioavailability of NO, the redox state is determined by the balance between ROS-generating and ROS-eliminating systems. As a defense against enhanced ROS production, mammalian cells have a complex network of antioxidant enzymes, such as superoxide dismutases (SODs), glutathione peroxidase (GPX), and catalase. Enhanced expression and function of these ROS-eliminating enzymes leads to diminished oxidative stress and increased bioavailability of NO.

Sex hormones are known to influence numerous molecular processes occurring in the vascular wall that are involved in the initiation and progression of atherosclerosis. In particular, the antioxidative properties of estrogen are well established. Estrogen enhances the bioavailability of NO by modulation of the function and expression of endothelial NO synthase (eNOS) and inhibits the ROS-generating NADPH oxidase by downregulating the expression of several functional important subunits of the enzyme complex.8–11 In addition, we have recently demonstrated that estrogen leads to an overexpression of extracellular (ecSOD) and manganese SOD (MnSOD).12

Menopause is the natural state of estrogen deficiency and is associated with a steep increase in cardiovascular diseases in females.13–15 Estrogen treatment was shown to improve endothelial function in postmenopausal women.16–19 In contrast to many retrospective analyses, however, recent randomized prospective clinical trials revealed that hormone replacement therapy was not associated with a decrease in cardiovascular complications in postmenopausal women.16–22 In this context, addition of progestins to estrogen replacement therapy, which is necessary to prevent endometrium cancer in women with an intact uterus, is speculated to deteriorate at least partly...
Cell Culture

VSMCs were isolated from rat thoracic aorta (female Sprague Dawley, 6 to 10 weeks old, Charles River, Sulzfeld, Germany) by enzymatic dispersion and cultured over several passages. Cells were grown in a 5% CO₂ atmosphere at 37°C in Dulbecco’s modified Eagles medium without phenol, supplemented with 100 μL of penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (free of steroid hormones, S-15-M, c.c. Pro). Experiments were performed with cells from passages 5 to 10.

Measurement of Reactive Oxygen Species

Intracellular ROS production in a culture of monolayer VSMCs was measured by 2',7'-dichlorofluorescein (DCF; 10 μmol/L) fluorescence using fluorescence microscopy techniques as described previously. Images were collected using single rapid scans and identical parameters, such as contrast and brightness, for all samples. Five groups of 25 cells for each sample were randomly selected from the image and fluorescent intensity was measured. The relative fluorescence intensity is an average value of all experiments.

Superoxide release in intact aortic segments was determined by L-012 chemiluminescence as described previously. L-012 is a luminol deriviate with high sensitivity for ROS that does not exert redox cycling itself. Superoxide release is expressed as relative chemiluminescence per mg aortic tissue.

Superoxide Dismutase Activity Assay

SOD activity was measured by the modified nitroblue tetrazolium (NBT) method of Spitz and Oberly as described previously. This is an indirect assay based on the competition reaction between SOD and the indicator molecule NBT. MnSOD activity was quantified in the presence of 5 mmol/L NaN₃, which only inhibits copper-zinc-SOD (czSOD). For ecSOD activity measurement, cell culture medium was replaced by HEPES buffer before stimulation, and enzyme activity was quantified in 1 mL of this buffer as described above. One unit of activity was defined as the concentration of SOD that reduces the NBT reaction to one-half of the maximum.

Measurement of NADPH Oxidase Activity

NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay in buffer B containing phosphate 50 mmol/L (pH 7.0), EGTA 1 mmol/L, protease inhibitors (Complete, Roche), sucrose 150 mmol/L, lucigenin 0.005 mmol/L, and NADPH 0.1 mmol/L. Tissue was mechanically lysed using a glass/tetlon potter in ice-cold buffer B lacking lucigenin and substrate. Total protein concentration was adjusted to 1 mg/mL. Aliquots of the protein sample (100 μL) were measured over 10 minutes in quadruplicates using NADPH as substrate in a scintillation counter (Berthold Lumat LB 9501) in 1-minute intervals.

mRNA Isolation, Northern Analysis, and Polymerase Chain Reactions

After the indicated treatments, culture medium was aspirated and VSMCs were lysed with 1 mL RNA-Clean, scraped, and processed according to the manufacturer’s protocol to obtain total cellular RNA. Ten-μg aliquots were electrophoresed and Northern analysis was performed with a [32P]-dCTP-labeled rat ecSOD, MnSOD, czSOD, catalase, and GPX cDNA probe, as described previously.

For assessment of vascular gene expression, aortas were isolated, quickly frozen in liquid nitrogen, and homogenized with a motorized homogenizer. RNA was isolated with RNA-Clean. Two μg of the isolated total RNA was reverse transcribed using random primers and Moloney murine leukemia virus reverse transcriptase for 60 minutes at 42°C and 10 minutes at 75°C. The single stranded cDNAs were amplified by polymerase chain reactions using Taq DNA polymerase. Real-time quantitative reverse transcription-polymerase chain reaction was performed with the TaqMan system (ABI Prism 7700 Sequence Detection System, PE Biosystems). For sequences of sense and antisense primers, polymerase chain reaction (PCR) conditions, and amplification fragment lengths see the online-only Data Supplement at http://circres.ahajournals.org.
Western Blotting
After treatment, VSMCs were homogenized in ice-cold lysis buffer containing additional leupeptin and aprotinin. Membrane proteins were isolated and 25-μg aliquots were separated on SDS/PAGE as described previously. Blot membranes were stained with Ponceau red to verify appropriate protein transfer and equal loading for each lane. Immunoblotting was performed with a MnSOD rabbit polyclonal immunoglobulin G antibody (1:1000 dilution overnight with agitation at 4°C, Upstate). Immunodetection was accomplished using a goat anti-rabbit secondary antibody (1:2000 dilution for 1 hour at room temperature, Sigma Chemical, Deisenhofen, Germany) and the enhanced chemiluminescence kit (Amersham).

Nuclear Run-On Assays
The nuclei of treated VSMCs were isolated and nuclear run-on assays were performed as described previously. Two μg of ecSOD, MnSOD, and GAPDH cDNA were applied to nylon membranes using a dot blot apparatus. The relative intensity of the ecSOD and MnSOD signal was determined as the ratio of ecSOD/MnSOD to GAPDH intensity.

Animals
Six-week-old female wild-type mice (C57BL6) were fed standard chow and were ovariectomized or sham operated. One group of ovariectomized mice received hormone replacement therapy with 17β-estradiol pellets, one group received therapy with 17β-estradiol plus progesterone, and one group received therapy with progesterone pellets (containing 0.18 mg 17β-estradiol and/or 10 mg progesterone each, 60 day release, Innovative Research). Pellets were administered subcutaneously with a 10-gauge trochar. The mice were euthanized by decapitation, and tissue and blood samples were collected immediately. Animal experiments were performed in accordance with the German animal protection law.

Progesterone and Estradiol Concentrations
Progesterone and estradiol concentrations were determined in plasma using commercial radioimmunoassay kits (progesterone: R&D Systems; estradiol: Cayman Chemical Company). Samples were analyzed in duplicates.

Statistical Analysis
Data are presented as mean±SE. Statistical analysis was performed using the ANOVA test. P<0.05 indicates statistical significance.

Results
Effects of Progesterone and 17β-Estradiol on Cellular ROS Production
VSMCs were incubated for 24 hours with 1 μmol/L progesterone and/or 1 μmol/L 17β-estradiol. Intracellular ROS production was assessed by DCF fluorescence microscopy. Quantitative analysis revealed that stimulation of VSMCs with either 1 μmol/L PMA or angiotensin II led to an increased ROS production compared with control cells (PMA: 185±27% of control; angiotensin II: 169±30% of control; data not shown). In addition, treatment with progesterone
enhanced (10 nmol/L: 135±7% of control; 1 μmol/L: 149±7% of control) whereas 17β-estradiol decreased (10 nmol/L: 83±7% of control; 1 μmol/L: 72±5% of control) intracellular ROS release after 24 hours of stimulation. Moreover, 17β-estradiol prevented progesterone-induced ROS production (82±3% of control). Figure 1A shows a representative scan and Figure 1B the quantitative analysis of 3 experiments.

Effect of Progesterone on ecSOD and MnSOD mRNA expression
VSMCs were incubated with 1 μmol/L progesterone for 0 to 24 hours before RNA was isolated and ecSOD and MnSOD mRNA expression were quantified by Northern analysis. Figure 2A and 2B shows representative autoradiograms and the densitometric analysis. Both ecSOD and MnSOD mRNA expression were downregulated to a minimum after 24 hours of 43±11% of control for ecSOD and 29±9% of control for MnSOD. Figure 2C and 2D shows the concentration dependency of progesterone-induced downregulation of ecSOD and MnSOD. VSMCs were incubated with 0.1 to 10 μmol/L progesterone for 24 hours. The maximal effect was reached at a concentration of 1 μmol/L progesterone for ecSOD (33±4% of control) and at a concentration of 10 μmol/L progesterone for MnSOD (21±2% of control). We found that 18S rRNA remained unaltered during the time course of the experiment. Progesterone had no effect on czSOD, GPX, or catalase mRNA levels (data not shown).

Effect of Progesterone on SOD Enzyme Activity
Incubation of VSMCs with 1 μmol/L progesterone for 24 hours decreased ecSOD activity from 2.8±0.5 to 1.1±0.2 U/mg protein and decreased MnSOD activity from 1.4±0.2 to 0.8±1 U/mg protein (Figure 3A and 3B).

Effect of Progesterone on Estrogen-Induced SOD Overexpression
Incubation of VSMCs with 1 μmol/L 17β-estradiol for 24 hours increased ecSOD (154±19% of control) and MnSOD (158±6% of control) mRNA expression. Coincubation with equimolar doses of progesterone antagonized estrogen-induced overexpression of SODs (Figure 4A and 4B).

MnSOD protein expression was quantified by Western blotting in VSMCs preincubated with 1 μmol/L progesterone and/or 1 μmol/L 17β-estradiol. Figure 4C displays that in response to estrogen, MnSOD expression was enhanced to 185±12% of control levels, whereas progesterone treatment decreased MnSOD protein levels to 51±4% of control. Progesterone abrogated estrogen-induced overexpression of MnSOD protein (105±7% of control).

Receptor Dependency of Progesterone Effects
To test whether the effects of progesterone were mediated via stimulation of its receptor, VSMCs were incubated with 1 μmol/L progesterone in the presence of 1 μmol/L of the progesterone receptor antagonist RU486, followed by ecSOD and MnSOD mRNA quantification by Northern blotting. Figure 5 shows that downregulation of ecSOD as well as MnSOD expression was inhibited by RU486.

Figure 3. Effect of progesterone on ecSOD and MnSOD enzyme activity. A and B, Incubation of VSMCs with 1 μmol/L progesterone for 24 hours enhanced ecSOD (A) and MnSOD (B) activity measured in a photometric assay. Values are mean±SE, n=4. *P<0.05.

Effect of Progesterone on SOD Transcription Rates
VSMCs were incubated with 1 μmol/L progesterone for 24 hours before nuclei were isolated and de novo mRNA synthesis was assessed. Figure 6A and 6B demonstrates that progesterone significantly decreased the gene transcription rate of ecSOD to 68±12% of control and of MnSOD to 46±16% of control.

Intracellular Signaling Pathways of Steroid Hormone-Induced Regulation of ecSOD and MnSOD mRNA Expression
VSMCs were incubated with 1 μmol/L progesterone or 1 μmol/L 17β-estradiol for 24 hours in the absence or presence of pharmacological inhibitors of p42/44 MAP kinase (PD98059, 20μmol/L), p38 mitogen-activated protein (MAP) kinase (SB203580, 10μmol/L), phosphoinositide-3 (PI3) kinase (Wortmannin; 0.1μmol/L), tyrosine kinase (Herbimycin; 1μmol/L), and NO synthase (LNN; 1μmol/L). Inhibition of p42/44 MAP kinase and PI3 kinase prevented the progesterone effect on MnSOD expression (progesterone: 52±1% of control; PD98059: 94±2%; PD98059 plus progesterone: 88±3%; Wortmannin: 88±4%; Wortmannin plus progesterone: 77±11%), whereas none of the investigated signaling pathways was involved in the regulation of ecSOD expression by progesterone (data not shown). In the case of estrogen, activation of PI3 kinase and NO synthase were essential for the regulation of ecSOD.
Vascular ROS Production and NADPH Oxidase Activity and Expression of NADPH Oxidase Subunits, ecSOD, and MnSOD in C57BL6 Mice

Six-week-old C57BL6 mice were ovariectomized or sham operated. One group of ovariectomized mice received hormone replacement therapy with 17β-estradiol, one group received therapy with 17β-estradiol plus progesterone, and one group received therapy with progesterone. Four weeks after ovariectomy, estrogen levels of C57BL6 mice dropped to 12.7 ± 2.9 pg/mL (sham-operated mice 88.0 ± 10.4 pg/mL). Estrogen and estrogen plus progesterone supplementation increased estrogen levels (estrogen-treated mice: 106.7 ± 10.9 pg/mL; estrogen plus progesterone-treated mice: 113.6 ± 13.1 pg/mL). Progesterone treatment slightly enhanced estrogen plasma levels compared with ovariectomized mice (48.9 ± 5.1 pg/mL; n = 6 per group).

No significant differences in progesterone levels were found between sham-operated (18.8 ± 1.0 ng/mL), ovariectomized (19.0 ± 1.2 ng/mL), and estrogen-treated mice (19.5 ± 1.4 ng/mL). In contrast, progesterone levels were significantly higher in plasma collected from progesterone-treated mice (24.8 ± 1.0 ng/mL).

Vascular superoxide release measured by L-012 chemiluminescence in intact aortic rings was increased in ovariectomized mice to 229 ± 81% of control compared with sham-operated mice (control group; Figure 7A). Hormone replacement with 17β-estradiol decreased ROS production (54 ± 12% of control), whereas progesterone supplementation increased superoxide release (334 ± 10% of control). Treatment of ovariectomized mice with 17β-estradiol plus progesterone reduced vascular superoxide production but prevented the profound reduction of ROS release by estrogen alone (122 ± 24% of control).

Estrogen deficiency increased NADPH oxidase enzyme activity in homogenates of aortic tissue of ovariectomized mice (146 ± 18% of control), which was prevented by supplementation of estrogen (112 ± 17% of control). In contrast, progesterone treatment led to a profound increase in NADPH oxidase activity (182 ± 15% of control), which was not

Figure 4. Effect of progesterone on estrogen-induced overexpression of ecSOD and MnSOD. A and B, VSMCs were incubated for 24 hours with 1 μmol/L progesterone and/or 1 μmol/L 17β-estradiol. ecSOD (A) and MnSOD (B) mRNA expression were quantified by Northern blotting (mean±SE, n=5, *P<0.05 vs control; **P<0.05 vs estrogen). C, Western blot of proteins isolated from VSMCs stimulated for 24 hours with 1 μmol/L progesterone and/or 1 μmol/L 17β-estradiol. Representative autoradiogram and densitometric analysis of MnSOD protein expression (mean±SE, n=3). Prog indicates progesterone. *P<0.05 vs control; **P<0.05 vs Prog.
affected by estrogen cotreatment (179±12% of control; Figure 7B).

Aortic SOD mRNA expression was assessed by real-time PCR. Figure 8A and 8B shows that ecSOD and MnSOD mRNA expression was reduced in ovariectomized mice to 63±5% and 46±6% of control levels, respectively. Estrogen replacement therapy prevented this downregulation (ecSOD: 111±24%; MnSOD: 103±24% of control), whereas progesterone treatment led to a further downregulation of both enzymes (ecSOD: 32±7%; MnSOD: 29±4% of control). In ovariectomized mice treated with estrogen plus progesterone, ecSOD mRNA expression was slightly decreased beyond control level (85±14%) and MnSOD mRNA expression was reduced to 53±4% of control.

In addition, estrogen deficiency by ovariectomy led to an increase of aortic p22phox (141±15% of control) and p67phox (189±32% of control) mRNA expression (Figure 8C and 8D). Replacement of estrogen prevented this effect (p22phox: 117±8%; p67phox: 122±19% of control), whereas progesterone treatment induced a significant overexpression of these NADPH oxidase subunits (p22phox: 261±23%; p67phox: 306±75% of control). Cotreatment with estrogen and progesterone decreased mRNA expression of p67phox to control level (141±32% of control), whereas estrogen only partially prevented progesterone-induced mRNA overexpression of p22phox (167±29% of control). Neither estrogen nor progesterone treatment had any significant effect on Nox2 and Nox4 expression (data not shown).

Discussion

Steroid hormones influence numerous molecular processes in the arterial wall that are involved in the pathogenesis of atherosclerosis. Because of the increased incidence of cardiovascular diseases in women after menopause, the effect of estrogen on the vasculature has been of special interest. There is plentiful evidence that estrogens are modulators of proliferation and migration of vascular cells, as well as of immune and inflammatory processes, all of which have been impli-
ROS-eliminating enzymes such as the SODs. In expression and function of NADPH oxidase, eNOS, and Estrogen exerts antioxidative properties by modulating the cated in the initiation and progression of atherosclerosis. Estrogen exerts antioxidative properties by modulating the expression and function of NADPH oxidase, eNOS, and ROS-eliminating enzymes such as the SODs. Increased oxidative stress is believed to be an initial and important step in the development of endothelial dysfunction and atherosclerosis. Although experimental evidence indicates that estrogen exerts multiple potentially vasoprotective effects and thereby retards atherosclerosis progression and reduces the risk of clinical ischemic events, all recently published prospective clinical trials, in particular the Women’s Health Initiative trial and the Heart and Estrogen/Progestin Replacement Study (HERS), demonstrated that hormone replacement with combined estrogen plus progestin treatment increased the incidence of myocardial infarction, stroke, and thromboembolism in women older than 65 years. In contrast to the atheroprotective effects of estrogens, which are well established in experimental settings, the influence of the coadmin-istration of progestins, which is necessary for the prevention of endometrial cancer, remains unclear.

Multiple molecular findings indicate that progesterone, like estrogen, may have a direct impact on several processes involved in atherogenesis. Moreover, some findings suggest that progesterone may at least in part abrogate the potentially beneficial effects of estrogen. In animal studies, progesterone antagonized estrogen-induced increases in coronary blood flow and vasodilation. In addition, it was demonstrated that concomitant administration of medroxyprogesterone reversed estrogen-induced endothelium-dependent vasorelaxation in postmenopausal women.

The present study investigated whether progesterone has an influence on vascular ROS production and antioxidant and oxidant enzyme expression. The data derived from cultured VSMCs reveal that progesterone leads to increased oxidative stress by decreasing the expression and activity of MnSOD and ecSOD by transcriptional mechanisms. Moreover, progesterone antagonizes the estrogen-induced upregulation of ecSOD and MnSOD. Progesterone mediates this effect on SOD expression by stimulation of the progesterone receptor, because this action is blocked by coincubation with the steroid hormone receptor antagonist RU486. The expression of other antioxidative enzymes such as ecSOD, catalase, or GPX is not altered by progesterone. Regarding the intracellular signaling pathways of steroid hormone-dependent regulation of ecSOD and MnSOD expression, activation of PI3 kinase and p42/44 MAP kinase seem to play an important role. Pharmacological inhibition of PI3 kinase and p42/44 MAP kinase prevented progesterone-induced downregulation of MnSOD expression. In the case of estrogen, activation of PI3 kinase and NO synthase seem to be essential for the regulation of ecSOD and MnSOD expression, because treatment with Wortmannin and LNNA blocked the effect of estrogen-induced overexpression of both SOD isoforms. Additionally, the effect of estrogen on ecSOD expression was abrogated by inhibition of p42/44 MAP kinase. These data suggest that PI3 kinase and p42/44 MAP kinase are essential for steroid hormone-induced regulation of SOD isoforms. It may be speculated that the differential effect of estrogen and progesterone on the expression of these SODs is mediated by an estrogen-induced increase of NOS activity.

The in vitro effects of progesterone on SOD expression and activity were evoked with concentrations comparable to the physiological setting. The physiological plasma levels of progesterone in mice are 10 to 15 ng/mL, which equals a concentration of approximately 50 nmol/L. The physiological plasma levels of progesterone in women reach from 0.15 ng/mL to 25 ng/mL (0.5 to 80 nmol/L), depending on the day of menstrual cycle. The average plasma levels in men are 0.3 to 2 ng/mL (0.9 to 6 nmol/L). Progesterone affected SOD expression in a dose-dependent manner, with a maximum at 1 μmol/L, but significantly decreased the expression of both SOD enzymes already at a concentration of 10 nmol/L. Thus, the demonstrated effects of progesterone may be of physiological relevance.
teract the favorable effects of estrogen.\textsuperscript{12,15,23,26,30} To confirm that physiological concentrations of progesterone may abrogate the potentially vasoprotective effect of estrogen on ecSOD and MnSOD, the expression of these enzymes, as well as the expression and function of the NADPH oxidase complex, were investigated in ovariectomized mice. During estrogen deficiency, vascular ecSOD and MnSOD expression is decreased. Hormone replacement with 17$\beta$-estradiol prevents these potentially harmful effects, whereas progesterone administration leads to a further decline of SOD expression. This downregulation of antioxidant enzyme expression is associated with an increased vascular superoxide release in progesterone-treated animals. Importantly, progesterone suppresses the estrogen-induced overexpression of ecSOD and MnSOD and inhibits the antioxidative properties of estrogen.

Moreover, estrogen deficiency led to an increased vascular expression of the p22phox and p67phox subunits of the NADPH oxidase enzyme complex. Replacement of estrogen prevented this effect, whereas progesterone treatment induced a significant overexpression of these subunits. Cotreatment with estrogen and progesterone decreased mRNA expression of p67phox to control levels, whereas estrogen only partially prevented progesterone-induced mRNA overexpression of p22phox. Neither estrogen nor progesterone had any significant effect on Nox2 and Nox4 subunit expression. The regulation of p22phox and p67phox subunit expression was associated with an altered NADPH oxidase enzyme activity.

Estrogen deficiency increased NADPH oxidase activity in the aorta of ovariectomized mice, and supplementation of estrogen prevented this effect. In contrast, progesterone treatment led to an increased enzyme activity.

The in vitro and in vivo data of the present study support the hypothesis that progesterone may exert potentially harmful effects on the vasculature and counteracts the beneficial antioxidative properties of estrogen.

The authors of the Women’s Health Initiative trial have recently shown that treatment with conjugated equine estrogens alone could not reduce the risk of cardiovascular events compared with placebo treatment.\textsuperscript{20} In contrast to the elevated risk after treatment with conjugated equine estrogens plus medroxyprogesterone, however, the risk was not increased in this study group. Moreover, subgroup analyses showed that patients who received estrogens at young age, between 50 and 59 years, benefit from hormone replacement with estrogens alone, indicating that hormone therapy may only be effective in women who have not already accumulated high cardiovascular risk or manifested atherosclerosis because of advanced age and prolonged risk factor disposition.

Taken together, the demonstrated effects of progesterone on vascular ecSOD, MnSOD, and NADPH oxidase expression and function do not only support the hypothesis that concomitant administration of progestins may antagonize potentially beneficial effects of estrogens, but also give a molecular understanding of possible underlying mechanisms of this phenomenon.
Acknowledgments

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References

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Kerstin Wassmann, MD; Sven Wassmann, MD; Georg Nickenig, MD

**Online Supplement - Methods**

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<tr>
<th>Enzyme Type</th>
<th>Primer Sequences</th>
<th>Temperature Parameters</th>
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| manganese SOD (MnSOD) (Northern blot) | S: 5´-TGA-CCT-GCC-TTA-CGA-CTA-TG-3´  
A: 5´-GCT-GCA-ATG-CTC-TAC-AC-3´ | 60 s, 94°C  
60 s, 57°C  
90 s, 72°C  
649 bp |
| copper-zinc SOD (czSOD) (Northern blot) | S: 5´-TGG-GGA-CAA-TAC-ACA-AGG-3´  
A: 5´-TAG-CAG-GAC-AGC-AGA-TGA-G-3´ | 60 s, 94°C  
60 s, 53°C  
90 s, 72°C  
359 bp |
| extracellular SOD (ecSOD) (Northern blot) | S: 5´-GGG-AGA-GCT-TGT-CAG-GTG-TGG-3´  
A: 5´-GCC-GCT-TCT-TGC-GCT-CCT-TTG-3´ | 60 s, 94°C  
60 s, 60°C  
90 s, 72°C  
736 bp |
| glutathione peroxidase (GPX) (Northern blot) | S: 5´-AGG-AGA-ATG-GCA-AGA-ATG-AAG-3´  
A: 5´-ATC-GGG-AAT-GGA-CGA-GAA-C-3´ | 45 s, 94°C  
45 s, 57°C  
90 s, 72°C  
505 bp |
| catalase (Northern blot) | S: 5´-TAC-TAC-CCC-AAC-AGC-TT-3´  
A: 5´-GCT-AAG-CCC-TAA-TCT-TTA-A-3´ | 45 s, 94°C  
45 s, 57°C  
90 s, 72°C  
589 bp |
| p22phox (PCR) | S: 5´-GAC-GCT-TCA-CGC-AGT-GGT-ACT-3´  
A: 5´-CAC-GAC-CTC-ATC-TGT-CAC-TGG-3´ | 60 s, 94°C  
60 s, 65°C  
90 s, 72°C  
40 cycles |
| p67phox (PCR) | S: 5´-AGA-CAC-CTT-GAA-CTA-GCA-TCC-3´  
A: 5´-CTG-CTC-TTC-TGC-TTCT-CC-3´ | 60 s, 94°C  
60 s, 60°C  
90 s, 72°C  
30 cycles |
| Nox4 (PCR) | S: 5´-CAT-TTG-GCT-GCT-CCT-AAA-CG-3´  
A: 5´-AAC-AAA-CCA-CCT-GAA-AAC-TGC-3´ | 60 s, 94°C  
60 s, 56°C  
90 s, 72°C  
40 cycles |
| Nox2 (PCR) | S: 5´-CCT-ATG-ACT-TGG-AAA-TGG-AT-3´  
A: 5´-CAG-AGC-CAG-TAG-AAG-TAG-AT-3´ | 30 s, 94°C  
30 s, 58°C  
45 s, 72°C  
40 cycles |
| GAPDH (PCR) | S: 5´-ACC-ACA-GTC-CAT-GCC-ATC-AC-3´  
A: 5´-TCC-ACC-ACC-CTG-TTG-CTG-TA-3´ | 30 s, 94°C  
45 s, 55°C  
45 s, 72°C  
23 cycles |
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**Table 1.** Sequences for sense (S) and antisense (A) primers, PCR conditions, cycle counts, and amplification fragment lengths.