Modulation of Antioxidant Enzyme Expression and Function by Estrogen

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Abstract—Oxidative stress plays a pivotal role in the pathogenesis of atherosclerosis and can be effectively influenced by radical scavenging enzyme activity and expression. The vasoprotective effects of estrogens may be related to antioxidative properties. Therefore, effects of 17β-estradiol on production of reactive oxygen species and radical scavenging enzymes were investigated. 17β-estradiol diminished angiotensin II–induced free radical production in vascular smooth muscle cells (DCF fluorescence laser microscopy). 17β-estradiol time- and concentration-dependently upregulated manganese (MnSOD) and extracellular superoxide dismutase (ecSOD) expression (Northern and Western blotting) and enzyme activity (photometric assay). Nuclear run-on assays demonstrated that 17β-estradiol increases MnSOD and ecSOD transcription rate. Half-life of MnSOD mRNA was not influenced, whereas ecSOD mRNA was stabilized by estrogen. Copper-zinc SOD, glutathione-peroxidase, and catalase were not affected by estrogen. Estrogen deficiency in ovariectomized mice induced a downregulation of ecSOD and MnSod expression, which was associated with increased production of vascular free radicals and prevented by estrogen replacement or treatment with PEG-SOD. In humans, increased estrogen levels led to enhanced ecSOD and MnSOD expression in circulating monocytes. Estrogen acts antioxidative at least to some extent via stimulation of MnSOD and ecSOD expression and activity, which may contribute to its vasoprotective effects. (Circ Res. 2003;93:170-177.)

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

The natural state of estrogen deficiency, the menopause, is associated with a steep incline of cardiovascular risk in women.1,2 Besides lipid lowering, interactions with the renin-angiotensin system, and calcium-channel blockade, estrogens exert their potentially vasoprotective effects through decreased vascular oxidative stress.3,4

Increased oxidative stress is a key event in the pathogenesis of atherosclerosis.5-7 Hydrogen peroxide, superoxide and hydroxyl radicals, and peroxynitrite exert direct cellular toxicity, leading to endothelial dysfunction, proliferation, and apoptosis of vascular smooth muscle cells (VSMCs) and putatively to the destabilization of atherosclerotic plaques.5-8 These harmful effects of reactive oxygen species (ROS) are counterbalanced by nitric oxide (NO), which acts atheroprotective.5-7 Increased production of ROS causes a diminished bioavailability of NO, which is associated with vascular damage.9-13 Several authors have suggested that estrogens may act vasoprotective by enhancement of the bioavailability of NO.5,14-17 Classical coronary risk factors such as smoking and hypercholesterolemia alter this delicate balance in favor of ROS.5,7,18,19 Superoxide radicals are of special interest, because these molecules react rapidly with NO, leading to scavenging of NO and to formation of peroxynitrite.20 Therefore, elimination of superoxide within the vessel wall is imperative and is realized by SODs, which dismutase superoxide to hydrogen peroxide.20-22 In this context, catalase, glutathione peroxidase (GPX), and the superoxide dismutase (SOD) isoforms are of special interest for antioxidative actions within the vessel wall.20-22 Three isoforms of the SOD have been identified, the cytosolic copper/zinc SOD (Cu/ZnSOD), the mitochondrial manganese SOD (MnSOD), and the extracellular SOD (ecSOD).20-22 Expression and function of SODs is subject to regulation. The expression of the ecSOD isoform, for example, is increased by NO, angiotensin II, endothelin, and heparin and is reduced by growth factors.23-30 Vascular Cu/ZnSOD is downregulated by high-salt diet, whereas MnSOD is reduced in liver tissue by insulin-like growth factor and growth hormone and is induced by protein kinase C–dependent or rac1-dependent pathways.31-33 Atherosclerosis depresses ecSOD gene expression,25 whereas treatment with AT1 receptor antagonists as well as ACE inhibitors increases ecSOD expression levels.14 Of note, exercise training seems to enhance ecSOD expression by NO-dependent pathways.39 However, there is little known about regulation of radical scavenging enzyme activity and expression in the vascular
cells by sex steroids. Estrogens are reported to exert radical-scavenging effects through increased NO production, decreased AT1 receptor expression, and modulation of NADPH-oxidase enzyme activity. In human endometrium, progesterone increases Cu/ZnSOD as well as MnSOD, whereas estrogen has only weak effects. Arnal et al observed decreased superoxide release in endothelial cells under estrogen treatment. This effect was independent of endothelial NO synthase induction, suggesting a regulatory effect on SOD activity or expression in these cells. Therefore, we hypothesized that estrogens may directly influence radical scavenging enzymes residing in vascular smooth muscle cells, resulting in diminished oxidative stress.

Materials and Methods

Materials
Angiotensin II, lucigenin, taq DNA polymerase, nucleotides, salts, and other chemicals were purchased from Sigma Chemical. MMLV reverse transcriptase was obtained from Gibco BRL. RNA Clean was purchased from AGS.

Cell Culture
VSMCs were isolated from rat thoracic aorta (strain, female Sprague-Dawley, 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, supplemented with 100 U/mL of penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids (×100), and 10% FBS (free of steroid hormones, S-15-mol/L, c.c.Pro GmbH, Neu- stadt/W, Germany). Experiments were performed with cells from passage 5 to 10. Human endothelial cells and endothelial cell medium were obtained from PromoCells GmbH (Heidelberg, Germany).

Measurement of ROS
Intracellular ROS production in VSMCs was measured by 2′,7′-dichlorofluorescein (DCF; 10 μmol/L) fluorescence using confocal laser scanning microscopy techniques as described previously. Images were collected by 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 intensities were averaged for all experiments.

Superoxide release in intact aortic segments was determined by lucigenin chemiluminescence, as described previously. Aortic ring preparations were transferred into scintillation vials containing Krebs-HEPES buffer with 5 μmol/L lucigenin. Chemiluminescence was assessed over 10 minutes in a scintillation counter (Lumat LB 9501, Berthold) in 1-minute intervals. The vessel segments were then dried, and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue.

Superoxide Dismutase Activity Assay
SOD activity was measured by the modified NBT method described by Spitz and Obery. This is an indirect assay based on the competition reaction between SOD and the indicator molecule, NBT. The rate of increase at 560 nm over a 5-minute time period indicates the reduction of NBT by superoxide. Varying amounts of total protein were added to the reaction until maximal inhibition was obtained as determined by spectrophotometry. Total SOD activity was determined as the amount of protein necessary for half-maximal inhibition of the NBT reaction. VSMCs were treated for 24 hours with 1 μmol/L 17β-estradiol, and MnSOD activity was quantified in the presence of 5 mmol/L NaN₃, which only inhibits copper-zinc-SOD. For extracellular SOD activity measurement, cell culture medium was replaced by HEPES buffer before stimulation and the 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.

mRNA Isolation, Northern Analysis, and Polymerase Chain Reactions
After the indicated treatments, culture medium was aspirated and the cells were lysed with 1 mL RNA-Clean, scraped, and processed according to the manufacturer’s protocol to obtain total cellular RNA. Aliquots of 10 μg were electrophoresed, transferred on Hybond N membranes in 20× SSC (3 mol/L sodium chloride, 300 mmol/L sodium citrate), and cross-linked to the membranes using a Stratalinker 1800 (Stratagene). 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-primer, [32P]-dCTP-labeled cDNA probe in the same buffer but without Denhardt’s solution.

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. Isolated total RNA (2 μg) was reverse transcribed using random primers and MMLV reverse transcriptase for 60 minutes at 42°C and 10 minutes at 75°C. The single-stranded cDNAs for Northern blot hybridization were amplified by PCR using Taq DNA polymerase. Sequences for sense (S) and antisense (A) primers, PCR conditions, and amplification fragment lengths were as follows: MnSOD: S: 5′-TGG-GGACAATACACAAGG-3′; A: 5′-TAGCAGGAGCAGATGAG-3′, 60 seconds, 94°C; 60 seconds, 57°C; 90 seconds, 72°C; 649 bp; copper-zinc SOD: S: 5′-TGG-GGACAATACACAAGG-3′; A: 5′-TAGCAGGAGCAGATGAG-3′, 60 seconds, 94°C; 60 seconds, 53°C; 90 seconds, 72°C, 359 bp; eSOD: S: 5′-GGGAGAGCTTGTCAGGTG-3′; A: 5′-GGGAGAGCTTGTCAGGTG-3′, 60 seconds, 94°C; 60 seconds, 57°C; 90 seconds, 72°C; 736 bp; GPX: S: 5′-TACTACCCCAACAGCTT-3′; A: 5′-ATCGGGAAATGGCAGAAC-3′, 60 seconds, 94°C; 45 seconds, 57°C; 90 seconds, 72°C, 314 bp. The expression levels of the particular gene were normalized to the RNA loading.

Real-Time RT-PCR
Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed with the TaqMan system (Prism 7700 Strehlow et al Estrogen and SOD

Figure 1. Effect of 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 17β-estradiol or vehicle, followed by a 3-hour incubation with 1 μmol/L angiotensin II. Free radical production is visualized by DCF fluorescence laser scanning microscopy.
Sequence Detection System, PE Biosystems). For ecSOD the primers were 5'-GTGTGACCTAGCAGACCAGC and 3'-CAGGGCAAGTCGAGCTTAC. The MnSOD primers were 5'-GCAGATCTCGCGGTAA and 3'-GCCCTCCAGACCTGCTT. For GAPDH, the primers were 5'-CCTGGACCACCCAGCCAGCAA and 3'-TGTTATGGGGTCTGGGATA. For quantification, ecSOD/MnSOD mRNA expression was normalized to the expressed housekeeping gene GAPDH.

Western Blotting

Total proteins from VSMCs were isolated, and 25-μg aliquots were separated on SDS-PAGE, as described previously. Immunoblotting was performed using a MnSOD polyclonal antibody (MnSOD, rabbit polyclonal IgG, BIOMOL 1:500 dilution). Immunodetection was accomplished using a goat anti-rabbit secondary antibody (1:10 000 dilution) and the enhanced chemiluminescence kit (Amersham).

Nuclear Run-On Assays

VSMCs were collected and washed. After lysis for 10 minutes on ice, nuclei were isolated by centrifugation through 0.6 mol/L sucrose. The nuclei (3 to 5×10^6/20 μL reaction) were used to carry out the transcription in a reaction mixture containing 40% glycerol, 50 mmol/L Tris/HCl, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.5 mmol/L levels of CTP, GTP, and ATP, and 0.2 to 0.3 μmol/L [32P]-UTP (<3000 Ci/mmol) at 30°C for 30 minutes. Reactions were terminated by addition of RNA-clean, and the radioactive RNA was dissolved in hybridization solution (100 mmol/L TES, pH 7.4, 0.3 mol/L NaCl, and 100 μg/mL Escherichia coli tRNA). cDNA (2 μg) from RT-PCR for ecSOD, MnSOD, and GAPDH was applied to nylon membranes using a dot blot apparatus. These membranes were prehybridized for 2 hours at 42°C in 100 mmol/L TES, 0.3 mol/L NaCl, 100 μg/mL E coli tRNA, and 5× Denhard’s solution and were then hybridized at 42°C for 15 hours. Membranes were exposed to film, and autoradiographic signals were quantified by laser densitometry. 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 (C57-BL6) were given a standard chow diet and were ovariectomized or sham-operated. One group of ovariectomized mice received a hormone replacement therapy with 17β-estradiol pellets (containing 0.18 mg each, 60-day release, Innovative Research). The pellets were administered subcutaneously with a 10-gauge trochar. Four weeks after surgery, a group of ovariectomized mice were treated with 4000 U/kg PEG-SOD intravenously on 3 subsequent days. Control animals were treated with vehicle (NaCl 0.9%). The mice were euthanized by decapitation, and tissue samples were collected immediately. Animal experiments were performed in accordance with the German animal protection law.

Patients

Blood samples of five patients from the gynecology outpatient clinic subjected to planned in vitro fertilization, who had consented to the study, were investigated. Controlled ovarian hyperstimulation as described by long protocol was initiated in all patients with a GnRH analogue (Decapeptyl 0.3 SC daily) starting in the midluteal phase of the previous cycle until pituitary desensitization was achieved. Then
gonadotropin therapy to induce follicular growing was given using recombinant FSH 150 to 200 (Gonal-F; Serono). GnRH analogue injection was continued up to and including the day of ovulation induction (day 10 to 12). EDTA plasma 30 mL was taken before and after 6 to 10 days of FSH treatment. Estradiol levels were evaluated, and monocytes were isolated. Studies were approved by the local ethical committee of the University of the Saarland.

Isolation of Monocytes

Blood samples were drawn, and mononuclear cells were separated immediately by Ficoll gradient centrifugation. Monocytes were isolated with magnetic CD14 micro beads using the Monocyte Isolation Kit (Miltenyi Biotech Inc) according to the manufacturer’s protocol.

Statistical Analysis

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

Results

Antioxidative Effect of 17β-Estradiol

VSMCs were incubated for 12 hours with 1 μmol/L 17β-estradiol before 1 μmol/L angiotensin II was added for 3 hours. Intracellular ROS production was assessed and quantified by DCF-fluorescence laser scanning microscopy. Quantitative analyses of four experiments revealed that angiotensin II increased ROS release to 249±16% of control. Stimulation of VSMCs with estradiol had no effect (104±2.5% of control) but prevented angiotensin II–induced ROS production (112±8% of control). Figure 1 shows a representative scan.

Effect of 17β-Estradiol on ecSOD and MnSOD mRNA Expression

Cells were incubated with 1 μmol/L 17β-estradiol for 0 to 24 hours before RNA was isolated, and ecSOD and MnSOD mRNA were quantified by Northern analysis. Figures 2A and 2B show representative autoradiograms and the densitometric analyses. Both ecSOD and MnSOD mRNA were upregulated with a maximum for ecSOD of 219±15% after 24 hours and a maximum for MnSOD of 255±52% of control after 12 hours. Concentration dependency was tested after a 24-hour incubation with 0.1 to 10 μmol/L 17β-estradiol. Figures 2C...
and 2D reveal that the maximal effect was reached for ecSOD and for MnSOD at 1 μmol/L 17β-estradiol. 18S RNA remained unaltered during the time course of the experiment. Estrogen had no effect on copper-zinc SOD, GPX, or catalase mRNA levels as well as on SOD expression in endothelial cells (data not shown).

Effect of 17β-Estradiol on SOD Enzyme Activity and Protein Expression
A 24-hour incubation with 1 μmol/L 17β-estradiol enhanced ecSOD activity from 2.5±0.5 to 7.6±0.6 U/mg protein of control and increased MnSOD activity from 1.4±0.2 to 2.9±0.1 U/mg protein of control level (Figures 3A and 3B). In addition, MnSOD protein expression was quantified by Western blotting in VSMCs preincubated with 1 μmol/L 17β-estradiol. Figure 3C displays that MnSOD expression was enhanced in response to estrogen to 185±12% of control levels.

Receptor Dependency of Estrogen Effects
To gain insight into involved signaling cascades, VSMCs were incubated with 1 μmol/L 17β-estradiol in the presence of 1 μmol/L of the estrogen receptor antagonist ICI 182,780 followed by RNA isolation and ecSOD and MnSOD mRNA quantification by Northern blotting. Figure 4 shows that upregulation of ecSOD as well as MnSOD expression is inhibited by ICI 182,780.

Transcription Rate and mRNA Half-Life
VSMCs were incubated with 1 μmol/L 17β-estradiol for 12 hours before RNA polymerase II was inhibited by 5,6-dichlorobenzimidazole (DRB). RNA decay was assessed by Northern blotting 0 to 18 hours after addition of DRB. Figure 5A shows that ecSOD mRNA was stabilized by estrogen treatment, whereas MnSOD mRNA half-life was not altered (Figure 5B). In addition, cells were incubated for 24 hours with 1 μmol/L 17β-estradiol before nuclei were isolated and nuclear run-on assays were performed. The densitometric analysis of 3 experiments is demonstrated. Signal intensities of ecSOD, MnSOD, and GAPDH mRNA were calculated. ecSOD/GAPDH (C) and MnSOD/GAPDH (D) ratio is shown (mean±SE, n=3, *P<0.05).

Regulation of ROS Production and ecSOD and MnSOD Expression in C57-BL6 Mice
Four weeks after ovariectomy, estrogen levels of C57-BL6 mice dropped to less than 5 pg/mL (sham-operated mice 121±20 pg/mL). Estrogen supplementation increased estrogen levels to 125±18 pg/mL (n=10 per group). Vascular superoxide release measured by lucigenin chemiluminescence in intact aortic rings was increased in ovariectomized mice to 191±28% compared with sham-operated mice (Figure 6A). Hormone replacement therapy normalized vascular ROS production (113±30%). In addition, experiments in ovariectomized mice treated with PEG-SOD also led to a significant reduction of vascular superoxide release (70±4%).

Moreover, aortic SOD mRNA expression was assessed by real-time PCR. Figures 6B and 6C show that ecSOD and MnSOD mRNA expression was reduced to 63±3% and 43±6% of control levels, respectively. Estrogen replacement therapy prevented this downregulation (ecSOD, 106±23%; MnSOD, 84±11%).

SOD Expression in Human Monocytes
In agreement with these data, ecSOD and MnSOD expression in human monocytes was estrogen-dependently regulated,
measured by real-time PCR. An increase in estrogen levels from 29±21 to 284±46 pg/mL (Figure 7A) led to an upregulation of ecSOD mRNA expression to 272±38% of control (Figure 7B). In addition, MnSOD mRNA expression was increased to 202±38% of control (Figure 7C).

**Discussion**

In the present study, we tested whether estrogens could act antioxidative by direct impact on antioxidative defense mechanisms. Our data in cultured VSMCs reveal that expression as well as activity of MnSOD and ecSOD are enhanced by estrogens by transcriptional pathways. The impact of estrogens on these SODs is mediated by estrogen receptor activation, because it is blocked by costimulation with the estrogen receptor antagonist ICI 182,780. The effects of estrogens on SOD expression seem to be selective, because other antioxidative enzymes such as catalase or GPX are not altered by 17β-estradiol. Interestingly, Cu/ZnSOD is not affected by estrogen treatment. Several publications describe Cu/ZnSOD as a NO-regulated gene.41,42 On the other hand, it is known that SOD isoforms can be differentially regulated.43,44 With regard to the findings of estrogen on transcription rate of ecSOD and MnSOD, it may be speculated that estrogen enhances promoter activity of ecSOD and MnSOD but not CuZnSOD. However, additional studies are needed regarding...
the mechanisms and role of the differential regulation of SOD isoforms.

Nuclear run-on experiments and experiments with the RNA polymerase II inhibitor DRB indicate that estrogen-induced ecSOD overexpression is transcriptionally as well as posttranscriptionally regulated in VSMCs, whereas MnSOD expression was mediated by an increased transcription rate. However, the detailed intracellular transduction pathways remain to be clarified. Nitric oxide, phosphatidylinositol-3 (PI-3) kinase, and mitogen-activated protein kinase may be promising candidates in this cascade. Estrogens are known to stimulate NO release as well as PI-3 kinase activation.\textsuperscript{4,15–17,45} PI-3 kinase stimulation leads to activation of the inducible as well as endothelial NO synthase.\textsuperscript{26} Therefore, estrogens could possibly enhance PI-3 kinase in VSMCs, leading to NO release. In addition, Dos Santos et al\textsuperscript{37} found that estrogen rapidly activates p42/44 mitogen-activated protein kinase in rat adipocytes in a nongenomic manner. At least for the ecSOD isoform, it is known that nitric oxide plays a fundamental role in the regulation of its gene expression.\textsuperscript{26}

Estrogen deficiency counts as an independent coronary risk factor. Our results of estrogen-deficient animals confirm a potentially important interaction of sex steroids with ecSOD and MnSOD expression in the in vivo situation. In ovariectomized mice, ecSOD and MnSOD expression was decreased and superoxide release was increased. Hormone replacement as well as SOD substitution prevented these potentially harmful effects. To investigate whether SOD regulation in the endothelium is involved in increased superoxide release in estrogen-deficient mice, we performed cell culture experiments with human endothelial cells. In agreement with Wagner et al,\textsuperscript{37} we found no regulatory effect of estrogen on SOD expression in endothelial cells. Moreover, to extend our observation to humans, we investigated ecSOD and MnSOD expression in monocytes of young women recruited for in vitro fertilization. With increasing estrogen levels, ecSOD and MnSOD expression was upregulated in isolated monocytes. The women were treated with a GnRH analogue until pituitary desensitization was achieved, which led to a suppression of estrogen release. Subsequent to GnRH treatment, women received recombinant FSH. Under this treatment, estrogen levels increased substantially. The increasing estrogen levels correlated with ecSOD and MnSOD expression on circulating monocytes. Little is known about the effects of GnRH or FSH on the vasculature or oxidative status. Behl and Pandey\textsuperscript{48} described an activation of catalase by FSH in granulose cells. Although we cannot exclude that GnRH or FSH treatment affected SOD expression in circulating monocytes of these women, based on our findings in vitro and in vivo, the observed upregulation of ecSOD and MnSOD may at least partially be explained with increasing estrogen levels.

The data are in agreement with several authors who have suggested that estrogens may act vasoprotective by antioxidative effects.\textsuperscript{4,14–17,37} Nevertheless, the prospective clinical trials published so far could not show any beneficial effect of a hormone replacement therapy with conjugated estrogens and medroxyprogesterone. Besides the advanced age of the women included in these trials, the findings of Arnal et al\textsuperscript{39} that 17β-estradiol but not ethinylestradiol decreases superoxide release in endothelial cells may give an explanation for the disappointing results.\textsuperscript{49–51} It may be speculated that antioxidative properties of estrogens and beneficial vascular effects depend on the type of substituted steroid hormone.

EcSOD and MnSOD induction by estrogens in vitro and in vivo is a novel mechanism by which these reproductive hormones may act atheroprotective. These findings reveal not only an intriguing additional cellular action of estrogens on vascular cells but may also initiate the development of innovative treatment strategies on the level of antioxidative defense mechanisms.

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References


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