Endogenous Estrogen Deficiency Reduces Proliferation and Enhances Apoptosis-Related Death in Vascular Smooth Muscle Cells

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Background—Altered proliferation and death of vascular smooth muscle cells (VSMCs) are important mechanisms in vascular growth and remodeling. This study examined the effect of endogenous estrogens on VSMC proliferation.

Methods and Results—An estrogen-deficient animal model, the aromatase-knockout (ArKO) mouse, was used. Primary cultures of VSMCs were established from aortas of 11-week-old male and female ArKO and wild-type (WT) mice. In ArKO cells, the absence of aromatase cytochrome P450 mRNA expression was demonstrated by reverse transcription–polymerase chain reaction; Western blotting showed normal expression of estrogen receptor protein. Proliferative responses to serum or platelet-derived growth factor-BB were lower in ArKO than WT cells; 17β-estradiol (E2, 10 nmol/L) enhanced the response to platelet-derived growth factor-BB in ArKO cells but inhibited the response in WT cells. E2 inhibited activity of mitogen-activated protein kinase ERK1/2 in WT but not ArKO cells. Apoptosis-related death caused by tumor necrosis factor-α was greater in ArKO than in WT cells; this effect in ArKO was attenuated by E2. Differences in VSMC proliferation between ArKO and WT occurred in both sexes. Morphological studies in aortas derived from male mice at 1 year of age demonstrated that medial smooth muscle area was ≈10% less in ArKO than WT mice at this age.

Conclusions—Deficiency of endogenous estrogens reduces proliferation and enhances apoptosis-related death in VSMCs; exogenous E2 corrects these abnormalities. (Circulation. 2004;109:537-543.)

Key Words: apoptosis ■ hormones ■ muscle, smooth ■ proliferation

Gender differences in cardiovascular disease have been attributed to hormonal differences between women and men. Cessation of ovarian hormone production in women is accompanied by an acceleration of atherosclerosis and an increased incidence of vascular dysfunction.1 Although recent data from clinical trials do not support the purported cardiovascular benefit of hormone replacement therapy in postmenopausal women,2,3 there is substantial evidence of direct vascular actions of sex steroids. Although genomic and nongenomic actions of estrogens have been described, the precise mechanisms of sex steroid actions on the cardiovascular system remain to be fully elucidated.

Abnormal proliferation and death of vascular smooth muscle cells (VSMCs) are important mechanisms in vascular growth and remodeling. Several lines of evidence suggest an effect of estrogens on these processes. Estrogens decrease VSMC proliferation induced by growth factors or mechanical stress via inhibitory effects on signaling pathways such as mitogen-activated protein (MAP) kinases or growth-response genes.4-9 Studies using estrogen receptor-knockout (ERKO) mice have also explored a role for estrogens in vascular function. Arteries from ERα-knockout mice show reduced basal nitric oxide release10 and increased VSMC proliferation after vascular injury11; antiproliferative effects of exogenous estrogen have been demonstrated in ERα-knockout,11 ERβ-knockout,12 and even ERα,β (double)-knockout mice.13 The vascular role of endogenous estrogen, by contrast, may best be understood in a model in which estrogens are not synthesized. We used an estrogen-deficient animal model, the aromatase-knockout (ArKO) mouse, to examine the effect of endogenous estrogens on proliferation and apoptosis of VSMCs. We hypothesized that VSMCs from ArKO mice would display abnormal patterns of proliferation and/or death resulting from their inability to synthesize estrogens. We found that VSMCs from ArKO mice grow more slowly in response to growth stimuli and are more susceptible to...
apoptosis and that these abnormalities are corrected by exogenous estrogen supplementation.

Methods

Aromatase-Deficient (ArKO) Mice
Cytochrome P450 aromatase (P450arom), encoded by the Cyp19 gene, catalyzes the final step in the biosynthesis of estrogens. Mice lacking a functional aromatase (ArKO) have been generated by targeted disruption of exon IX of the Cyp19 gene. Young mature ArKO mice show an undetectable level of estradiol (±6 to 8 pg/mL), basal levels of progesterone, high levels of testosterone and luteinizing hormone in both females and males and increased follicle-stimulating hormone in females.

Cell Culture
VSMCs were prepared from aortas of 11-week-old mice, 6 males (3 ArKO and 3 wild-type [WT]) and 2 females (1 ArKO and 1 WT). Aortic segments were placed into ice-cold DMEM. All external fat and connective tissue was detached, the adventitia carefully removed, the vessel cut longitudinally, and the endothelial layer removed by scraping with forceps. Strips of media were transferred to 60-mm dishes anchored under 9% CO2 in air at 37°C. Culture media were changed every 3 days, and VSMCs were observed to be migrating and growing on the dishes. Cells were confirmed to be VSMCs with their typical characteristics under the phase-contrast microscope, and expression of the VSMC marker gene, smooth muscle (SM) actin, by reverse transcription–polymerase chain reaction (RT-PCR) and Western blot analyses. Cells at passages 2 to 5 were used in this study.

Determination of DNA Synthesis
Subconfluent cells in 24-well plates were subjected to growth stimulation by serum-rich medium or platelet-derived growth factor (PDGF)-BB for 24 hours. Cells were incubated with 0.2μCi/well of [3H]thymidine (ICN Biomedicals) during the last 5 hours, washed 3 times with 0.2N HClO4, incubated with 0.5 mL/well of 0.2N NaOH in 37°C for 1 hour, and neutralized with 0.2 mL/well of 6% acetic acid. Contents of the wells were transferred into scintillation vials with 3 mL Scintillation Liquid Instagel (Bio-Rad) and counted for 2 minutes per vial in a β-counter.

Cell Proliferation Assay
Subconfluent cells in 24-well plates were subjected to growth stimulation by serum-rich medium or PDGF-BB for 48 hours. Cells were harvested by 0.5% trypsin digestion and counted by an automatic cell counter (S.ST.II/ZM, Coulter Electronics).

RT-PCR Analysis of Cyp19 Gene Expression
Total RNA was isolated from cultured cells with the RNAagents Total RNA Isolation System (Promega). The RT-PCR was performed by use of a Qiagen OneStep RT-PCR kit. Oligonucleotide primers used for P450 aromatase exons IV–V1 were 5'-TGGAGAACACGCCCTTTCT-3' and 5'-TGGTTTGATGAGGAGTTG-3', which amplify a 273-bp fragment from the exons IV–V1 mRNA and for P450 aromatase exon IX were 5'-GTGACAGAGACATAAAGATCG-3' and 5'-TCACTGTGGAGGAGTACTGCTGATCTTCT-3' to amplify a 375-bp fragment from the exon IX mRNA. The PCR products along with the 100-bp molecular-weight markers were separated on a 1% agarose gel and visualized by ethidium bromide staining.

Analysis of Protein by Western Blotting
Total proteins (20 μg) isolated from the cells were electrophoresed on 10% SDS-polyacrylamide gels and transferred to Hybond ECL filters (Amersham Corp). After blocking with 10% nonfat dry milk in TBS (20 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, and 0.1% Tween-20) overnight, filters were incubated with the primary antibody for 1 hour and the horseradish peroxidase–conjugated secondary antibody for 1 hour. Filters were then incubated for 1 minute with enhanced chemiluminescence reagents (Amersham) and exposed to x-ray films for 1 to 10 minutes to obtain ideal exposure. Relative levels of protein signals were estimated by densitometry. Primary antibodies to ER-α, ER-β, ERK1/2, phosphorylated ERK1/2 (pERK), PKC-α, protein kinase C (PKC)-β1, Sp-1, and Egr-1 were purchased from Santa Cruz Biotechnology; those to bcl-2 and caspase-1α from ACN Biosciences; and those to SM actin from Dako Corp.

Figure 1. Expression of aromatase and ER in ArKO VSMCs. A, Expression of exon IX of Cyp19 gene on agarose gels analyzed by RT-PCR. M indicates DNA marker; C1, positive control; C2, negative control; 1, 2, 3, WT cells; 4, 5, 6, ArKO cells. B, Expression of estrogen receptor proteins ER-α and -β on Western blots. MCF7, human breast cancer cells; IMA, VSMCs from human internal mammary artery; 1, 2, 3, WT cells; 4, 5, 6, ArKO cells.

Figure 2. Proliferation of ArKO VSMCs in serum-enriched media. Cell proliferation was assessed by [3H]thymidine incorporation after 24 hours and cell numbers after 48 hours. WT cell is indicated by circles and ArKO by squares.
Annexin-V–FITC/Propidium Iodide Stain
Cells were stained by incubation (37°C, 10 to 15 minutes) with annexin-V–Fluos label solution, prepared shortly before use by prediluting 20 μL annexin-V–FITC (Roche Molecular Biochemicals) and 20 μL propidium iodide (PI) (50 μg/mL, Sigma) in 1 mL HEPES buffer (10 mmol/L HEPES/NaOH, pH 7.4; 140 mmol/L NaCl; 5 mmol/L CaCl₂). The numbers of total cells, annexin-V positive but PI negative (FITC+/PI−), and both annexin-V and PI positive (FITC+/PI+) cells were analyzed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson).

Morphological Analysis of Whole Aortas
Thoracic aortas from 1-year-old male mice (6 ArKO and 6 WT) were embedded in wax for section preparation. Four cross sections (5 μm thick) from each aorta were stained with hematoxylin and eosin, picrosirius red, or smooth muscle α-actin (immunohistochemical staining) as described previously. The arterial lumen, medial smooth muscle layer, and adventitia were measured under the light microscope with Optimas image analysis software (Media Cybernetics).

Statistical Analysis
Data are presented as mean±SEM. All experiments were conducted using at least 1 pair of cell-culture plates (ArKO and WT). Most results were from 4 pairs of mice (ArKO and WT: 3 male, 1 female each), except for the results of experiments examining testosterone supplementation and steroid-free serum culture, in which 1 pair of cell culture plates (male ArKO and WT) was used, and the morphological analysis, in which aortas from 6 ArKO and 6 WT mice were compared. All comparisons were made by ANOVA, with post hoc testing by the Student-Newman-Keuls test where appropriate. Differences at a value of $P<0.05$ were considered significant.

Figure 3. Proliferative response to PDGF-BB in ArKO VSMCs. Cell proliferation in response to PDGF-BB assessed by [3H]thymidine incorporation after 24 hours and cell numbers assessed after 48 hours. WT data are indicated by circles and ArKO by squares.

Figure 4. Proliferation of ArKO VSMCs in steroid-free, serum-enriched media. VSMCs were cultured in DMEM containing steroid-free FBS at various concentrations, with and without 17β-estradiol (E₂, 10 nmol/L) for 3 days. Cell proliferation was assessed by [3H]thymidine incorporation after 24 hours of culture and cell numbers after 48 hours. WT data are indicated by circles and ArKO by squares.

Figure 5. Proliferative response to PDGF-BB in ArKO VSMCs with exogenous E₂ or testosterone supplementation. VSMC proliferative responses to PDGF-BB in absence or presence of 17β-estradiol (E₂+/−/+) or testosterone (T+/−, 10 nmol/L) for 3 days. Cell proliferation was assessed by counting cell numbers after 48 hours. WT data are indicated by circles and ArKO by squares.
Results

Expression of P450arom and ER in ArKO VSMCs
RT-PCR showed that ArKO VSMCs expressed transcripts containing exons IV–VI of the Cyp19 gene, as WT cells did (data not shown), but did not express exon IX, a key exon for production of functional P450arom (Figure 1A).

Western blotting (Figure 1B) showed that ArKO VSMCs expressed primarily ER-β protein, with ER-α protein at relatively low levels compared with human breast cancer cells (MCF7). There was no difference in ER expression in cells from ArKO and WT or male and female mice.

Proliferative Characteristics of ArKO VSMCs
In normal growth medium (DMEM with 10% serum), ArKO VSMCs grew more slowly than WT cells, as shown by DNA synthesis and cell numbers (Figure 2). This slower proliferation rate occurred in cells from both male and female ArKO mice, although cells from male ArKO and WT mice appeared generally to grow faster than those from females. Like the findings in serum-rich cultures, ArKO VSMCs showed less increase in DNA synthesis and cell numbers with PDGF-BB treatment than WT cells (Figure 3). In cells cultured in media with steroid-free serum, results were the same as in steroid-supplemented medium (Figure 4).

To further test the effects of exogenous steroids on cell proliferation, 17β-estradiol (E₂, 10 nmol/L) or testosterone (10 nmol/L) was added to the culture medium for 3 days before growth stimulation. In ArKO cells, E₂ supplementation (Figures 4 and 5A) significantly increased ArKO but decreased WT cell proliferation; the difference in proliferation between the ArKO and WT cells (from both males and females) was abolished by E₂. Testosterone did not significantly influence these responses (Figure 5B).

Studies of Proliferation-Related Signaling Molecules and Genes
Western blotting analysis showed that MAP kinase ERK1/2 activity, assessed by pERK protein, was induced by PDGF-BB treatment for 15 minutes in all cells; this activation was reduced in ArKO (1.46-fold) compared with WT (1.93-fold) VSMCs; E₂ supplementation attenuated the activation of ERK1/2 in WT cells but increased it slightly in ArKO cells (Figure 6). Proteins of the transcription gene Sp-1, early growth response gene (Egr-1), and PKC-α and -β were not significantly different in cells from ArKO and WT, with or without PDGF-BB or E₂ supplementation (data not shown).

Studies of Cell Death
Annexin-V–FITC/PI staining (Figure 7) in 10% FBS-DMEM showed that positively stained (damaged) cells were infrequently seen (<2%) in both ArKO and WT VSMCs. With tumor necrosis factor (TNF)-α (20 ng/mL) treatment (24 hours), the number of stained cells did not change in WT cells but increased ~2-fold in ArKO cells (3.75±0.98% of FITC+/PI− and 0.22±0.02% of FITC+/PI+, stained cells, P<0.05). E₂ supplementation protected ArKO cells from death, reducing FITC+/PI− cells to 1.81±0.04% (P<0.05) and FITC+/PI+ cells to 0.14±0.03% (P<0.05). There was no difference in protein levels of bcl-2 or caspase-1 between WT and ArKO VSMCs; neither bcl-2 nor caspase-1 protein was changed by PDGF or E₂ in these VSMCs (data not shown).

Morphological Study of Aortas
The arterial intimal, medial, and adventitial layers were clearly identifiable in picrosirius red–stained sections (Figure 8A). There was no difference in the luminal or adventitial areas between ArKO and WT aortas, but the medial smooth
The recent development of animal models of estrogen deficiency and estrogen resistance has permitted research not only into the reproductive effects of sex hormones but also into their cardiovascular actions. Estrogens exert their biological effects primarily through ER; ERKO mice exhibit some features of lack of estrogenic actions, despite normal levels of circulating estrogens. Although no data using cultured ERKO vascular cells have been reported to date, vascular protection by exogenous estrogens has also been reported in ERKO model studies, presumably via unidentified estrogen receptors or other unidentified pathways. As an estrogen-deficient model, the ArKO mouse has some specific advantages. ArKO mice have no detectable estradiol and an increase in testosterone levels in their circulation; they do not synthesize their own estrogens, yet are responsive to the actions of exogenously administered estrogens. These properties differentiate this model from those in which responsiveness to estrogen action is blocked. Changes in the reproductive and skeletal systems have been investigated in humans with natural mutations of the aromatase gene and in ArKO mice. In previous studies of ArKO mice, cardiovascular structures like the large arteries and the heart were subjected to naked-eye examination and light microscopy, and no significant changes were reported. To date, few data are available on cardiovascular function in the ArKO model.

In the present study, VSMC cultures were derived from young mature (11-week-old) ArKO mice. We chose animals at this age because hormonal changes are well established but animals are still young, so that cell cultures can be more easily obtained than in older animals. ArKO VSMCs at low passages are normal in their morphology and expression of SM α-actin and ER. The cells express normal transcripts containing levels of exons IV–VI of the Cyp19 gene but not exon IX, the key part of the gene to produce functional P450arom; therefore, VSMCs derived from ArKO mice cannot produce estrogens.

Exogenous estrogen does not always inhibit VSMC proliferation, although in most studies it does. Ovarian ablation promotes aortic intimal hyperplasia in sheep. However, the effects of estrogens can be modulated by hormonal status and cellular phenotypes; in the present study in ArKO VSMCs and in some other previous studies, estrogens were also...
shown to enhance VSMC DNA synthesis and cell proliferation. The variable effects of estrogens on VSMCs are probably mediated by different signaling pathways. MAP kinase ERK is important to VSMC proliferation, and inhibition of this enzyme is a key mechanism underlying the effects of estrogen on VSMC proliferation. In the present study, upregulation of ERK activity, assessed by its phosphorylated protein (Figure 6), was involved in the proliferation of cells exposed to PDGF, and estrogen-induced growth inhibition in WT VSMCs seems to be mediated via this pathway. However, estrogen-induced enhancement of proliferation in ArKO VSMCs may be effected via an ERK-independent pathway, as shown by the lack of change in ERK1/2 activity (Figure 6); other reports indicate that PKC is possibly involved. It is also possible that the proliferative effects of estradiol in ArKO VSMCs may be related to more generalized abnormalities of vascular development related to the lack of estradiol.

TNF-α, an inflammatory cytokine, plays an important functional role in the arterial response to injury. TNF-α has been shown to induce apoptosis in VSMCs, together with other cytokines, such as interleukin-1β and interferon-γ. In the present study, TNF-α caused apoptotic damage in VSMCs, and ArKO cells were more sensitive to this damage than normal (WT) cells; furthermore, ArKO cells seemed to be relatively resistant to this damage after estrogen supplementation. Our findings indicate that endogenous estrogens protect VSMCs from cytokine-induced apoptosis and most likely play a protective role against vascular injury and senescence. Two other recent studies from our laboratory have shown that endogenous estrogens contribute to normal endothelial function and vascular tone in males. Taken together, our studies provide evidence of a role for aromatase-derived estrogens in vascular health.

In morphological studies of aortas from male ArKO mice 1 year of age, the arterial lumen and wall seemed largely normal, but a decreased medial smooth muscle area was observed compared with WT mice. These data are consistent with a slower growth pattern in smooth muscle cells derived from ArKO mice at younger ages. Additional in vivo studies are needed to assess arterial function in ArKO mice and to determine the consequences of impaired smooth muscle proliferation in this model.

In summary, VSMCs derived from animals with a genetic deficiency of endogenous estrogens (ArKO mice) show altered cell proliferation and death patterns compared with WT mice, with delayed proliferation in serum- or growth factor–enriched cultures and increased apoptotic death induced by TNF-α stimulation. These differences from WT mice exist in the ArKO artery from both males and females and can be corrected by supplementation of exogenous E₂. The results suggest a role for endogenous estrogens in cell proliferation and apoptosis in the vasculature of males and females.

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