Vascular Endothelial Estrogen Receptor α Is Modulated by Estrogen Status and Related to Endothelial Function and Endothelial Nitric Oxide Synthase in Healthy Women

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Context and Objective: Estrogen receptor α (ERα), a potent transcription factor expressed in vascular endothelial cells, plays a key role in regulating vascular function and health. We determined whether vascular endothelial cell expression of ERα is influenced by estrogen status and is related to vascular endothelial function in healthy women.

Methods: ERα protein expression was measured (quantitative immunofluorescence) in endothelial cells from peripheral veins of 16 healthy, premenopausal women during the early follicular (EF) and late follicular (LF) phases of the menstrual cycle and 17 estrogen-deficient postmenopausal women. Endothelial-dependent dilation (EDD; brachial artery flow-mediated dilation) and endothelial nitric oxide synthase (eNOS) expression and activation were also measured in a subgroup of women.

Results: In premenopausal women (n = 10), ERα expression was 30% lower ($P < 0.001$) during the EF (low estrogen) compared with the LF (high estrogen) phase of the menstrual cycle. In postmenopausal women, ERα expression was 33% lower ($P < 0.001$) compared with the LF phase of the menstrual cycle in premenopausal women. ERα expression was strongly related (r = 0.67; $P < 0.001$) to EDD, which was reduced in postmenopausal women. ERα abundance was positively related to expression of eNOS (r = 0.54; $P = 0.009$; n = 21) and ser1177 phosphorylated eNOS (r = 0.59; $P = 0.006$; n = 20).

Conclusions: These results provide the first evidence that expression of ERα in vascular endothelial cells is modulated by estrogen status and may be a key determinant of vascular endothelial function in healthy pre- and postmenopausal women. ERα expression may influence vascular endothelial function in women by affecting protein levels and activation of eNOS. **(*J Clin Endocrinol Metab* 94: 3513–3520, 2009)**

The prevalence of cardiovascular diseases (CVDs) is lower in premenopausal women than in men of similar age but increases markedly in women after menopause (1), presumably due in part to estrogen deficiency. The increased risk of CVD in estrogen-deficient postmenopausal women is associated with vascular endothelial dysfunction, as indicated by a reduction in endothelium-dependent dilation (2, 3). Thus, changes to the vascular endothelium as a result of reductions in circulating estrogen seem to contribute to increased CVD risk after menopause.

A key mechanism linking estrogen status to the function and health of the vascular endothelium may be estrogen receptors (ER) α and β. ERα is more highly ex-

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Abbreviations: BMI, Body mass index; CVD, cardiovascular disease; EDD, endothelial-dependent dilation; EF, early follicular; eNOS, endothelial NO synthase; ER, estrogen receptor; FMD, flow-mediated dilation; HUVEC, human umbilical vein endothelial cell; LF, late follicular; NO, nitric oxide.

pressed in endothelial cells than ERβ and is believed to play a more important role in mediating the effects of estrogen in the vascular endothelium (4–6). Findings from cell culture and ERα knockout mice indicate that estrogen may regulate vascular endothelial function via ERα modulation of endothelial nitric oxide synthase (eNOS) (7, 8), the enzyme responsible for nitric oxide (NO) synthesis in endothelial cells.

Vascular endothelial ER expression is plastic. In animals, endothelial ERα abundance decreases after ovariectomy (7, 9). In culture, endothelial cell ERα expression can increase within hours of incubation with physiological concentrations of estrogen (10). Taken together, these observations suggest that estrogen status may modulate endothelial ERα expression *in vivo* in women and influence vascular endothelial function, possibly via effects on eNOS. However, there is no direct evidence in humans supporting this possibility.

In the present study, we tested this hypothesis in healthy women. Using a novel translational approach, we obtained endothelial cells from peripheral veins of premenopausal women during low and high estrogen phases of their menstrual cycles and from postmenopausal women, and we measured ERα expression using quantitative immunofluorescence. We also explored the relations between endothelial cell ERα expression, endothelial-dependent dilation (EDD), and eNOS expression and activation in a subgroup of women.

Subjects and Methods

Subjects

Thirty-three healthy women were studied: 16 eumenorrheic premenopausal women (18–33 yr of age), and 17 estrogen-deficient postmenopausal women (48–63 yr of age). All subjects were normotensive, nonsmokers, not taking any medications, sedentary or recreationally active, and free of overt chronic diseases as assessed by medical history and physical examination. Postmenopausal women were further evaluated by standard blood chemistries and hematological evaluation and by electrocardiogram and blood pressure responses during incremental treadmill exercise to exhaustion. All postmenopausal women were at least 1 yr without menses and had not taken hormone therapy for at least 6 months. All subjects gave their written informed consent to participate. All procedures were reviewed and approved by the University of Colorado at Boulder Human Research Committee and the Colorado Multiple Institutional Review Board and were performed in the University of Colorado at Boulder and the University of Colorado Denver General Clinical Research Centers.

Study design

To determine the effects of estrogen-deficient states *vs.* estrogen-replete states on ERα expression, peripheral venous endothelial cells and blood were obtained in 10 premenopausal women across one menstrual cycle: during the early follicular (EF; 2–6 d after onset of menses), and late follicular [LF; 24–48 h after positive ovulation test measured using urine ovulation prediction kits (ClearPlan Easy; Unipath Diagnostics, Waltham, MA)] phases and in the postmenopausal women. To explore the relations among menopause-associated reductions in EDD and ERα expression and eNOS expression and activation, EDD was measured, and vascular endothelial cells were obtained in the postmenopausal women and in six additional premenopausal women who were studied during the early to midfollicular phase (2–8 d after onset of menses).

Study procedures

All women were studied after a 10–12 h overnight fast (water only) and with no vigorous physical activity over the prior 20+ hours.

Subject characteristics

Body mass index (BMI) was measured from height and body mass. Arterial blood pressure and heart rate were measured over the brachial artery during seated rest using a semiautomated device (Dynamap XL, Johnson & Johnson, Langhorne, PA).

Vascular endothelial cell protein expression

The procedures used for the collection and measurements of venous vascular endothelial cells have been described in detail previously (11–16). Briefly, endothelial cells were collected from an antecubital vein using two sterile J-wires advanced (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter. The wires were transferred to a dissociation buffer solution, and cells were recovered by washing and centrifugation. Cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine coated slides (Sigma Chemical, St. Louis, MO).

For immunofluorescence staining, cells were rehydrated with PBS and rendered permeable using 0.1% Triton X-100 (Alfa Aesar, Ward Hill, MA). After blocking nonspecific binding sites with 5% donkey serum (Jackson Immunoresearch, West Grove, PA), cells were incubated with monoclonal antibodies for one of the following: ERα (Santa Cruz Biotechnology Inc., Santa Cruz, CA), eNOS (Transduction Laboratories, Lexington, KY), and phosphorylated-eNOS-Ser1177 (Calbiochem, San Diego, CA). Cells were next incubated with a CY3-conjugated secondary antibody (Jackson Immunoresearch).

Slides were systematically scanned using a fluorescence microscope (Eclipse 600, Nikon, Melville, NY) to identify endothelial cells (positive staining of von Willebrand factor), and nuclear integrity was confirmed using DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) staining. Endothelial cell images (Figs. 1 and 2) were digitally captured by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Inc., Tucson AZ) and analyzed using Metamorph Software (Universal Imaging Corp., Downingtown, PA) to quantify the intensity of CY3 staining (*i.e.* average pixel intensity). The software program allowed for systematic quantification of staining intensity and eliminated human subjectivity in analysis. Values are reported as ratios of endothelial cell protein expression/human umbilical vein endothelial cell (HUVEC; control cells) protein expression. Reporting ratios minimizes the potential confound of differences in intensity of staining among different staining sessions. Technicians were blinded to subject identity during the staining and analysis procedures.

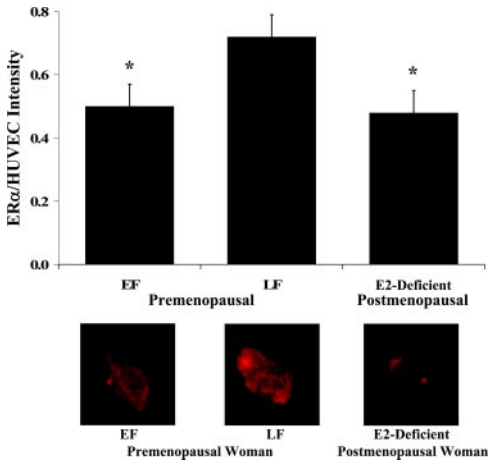

FIG. 1. ERα protein expression in endothelial cells collected during the EF and LF phases of the menstrual cycle in premenopausal women and in estrogen-deficient postmenopausal women. Values are mean ± SE. Representative immunofluorescent images from a premenopausal woman measured during the EF and LF phases of the menstrual cycle and a postmenopausal woman are shown. *, $P < 0.001$ *vs.* LF.

The present technique of quantitative immunofluorescence of human endothelial cells was originally validated against immunoblotting previously by Colombo *et al.* (11) with an excellent correlation coefficient (r = 0.99), and more recently by our laboratory (correlation coefficient r = 0.97; $P = 0.007$) (17).

Vascular endothelium-dependent dilation

Ultrasound measurements of brachial artery flow-mediated dilation (FMD) were performed according to the method originally described by Celermajer *et al.* (18) and as previously described by our laboratory (15, 19, 20). The dilation of the brachial artery in response to the stimulus of forearm ischemia has been shown to be dependent on the release of vasodilators, predominantly NO, from the vascular endothelium (21).

Sex hormone concentrations

Serum concentrations of estradiol and progesterone were measured via RIA by the University of Colorado General Clinical Research Center Core Laboratory.

Statistical analysis

To determine the effect of menstrual cycle phase (EF *vs.* LF) on the primary outcome, endothelial ERα expression, and sex hormone levels, a paired *t* test was used. An unpaired *t* test was used to determine the effect of age/menopausal status (postmenopausal *vs.* premenopausal measured at the EF and LF menstrual cycle phases) on endothelial ERα expression. Univariate correlation analyses were used to determine the relation between endothelial ERα expression and EDD, eNOS expression, phosphorylated-eNOS expression, and other variables of interest (*i.e.* estrogen concentrations). Statistical significance was defined as $P \leq 0.05$. All data are reported as mean ± SE. Data analysis was performed with SPSS software, version 16.0 (SPSS Inc., Chicago, IL).

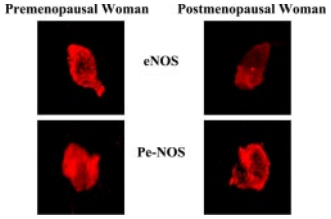

FIG. 2. Representative immunofluorescent images of eNOS and phosphorylated-eNOS-Ser1177 (Pe-NOS) proteins from a premenopausal woman and a postmenopausal woman.

Results

The characteristics of the subject groups are presented in Table 1. Postmenopausal women were older and had a higher body mass, BMI, and systolic blood pressure compared with the premenopausal women (all $P < 0.05$). Postmenopausal women were on average 7 ± 1 yr postmenopause (range, 1–20 yr). Thirteen of the women were prior hormone therapy users (mean, 3.2 ± 1.0 yr; range, 0.5–10 yr) but were estrogen-deficient for 3.5 ± 1.0 yr (range, 0.6–10 yr).

Vascular endothelial cell ERα protein expression is related to circulating estradiol in premenopausal women

In the 10 premenopausal women studied serially during their menstrual cycles, serum estradiol and progesterone concentrations were 36 ± 7 and 83 ± 17 pg/ml (130 ± 24 and 301 ± 62 pmol/liter, respectively; $P = 0.005$) and 0.4 ± 0.1 and 4.0 ± 1.7 ng/ml (2.2 ± 0.2 and 12.8 ± 5.4 nmol/liter, respectively) during the EF and LF phases, respectively ($P = 0.10$). Vascular endothelial cell ERα expression was 30% lower (0.51 ± 0.07 *vs.* 0.72 ± 0.07 ERα intensity/HUVEC intensity; $P < 0.001$) during the EF phase compared with the LF phase (Fig. 1). ERα expression was positively related to serum estradiol concentrations during the EF phase (r = 0.62; $P < 0.05$) and when the phases were pooled (r = 0.43; $P < 0.05$). These results indicate that endothelial cell ERα protein expression is modulated by circulating estrogen during the menstrual cycle in healthy premenopausal women.

TABLE 1. Subject characteristics

Variable	Postmenopausal	Premenopausal
n	17	16
Age (yr)	57 ± 1	24 ± 1
Height (cm)	164 ± 1	166 ± 2
Body mass (kg)	71.6 ± 3.7	59.4 ± 4[a]
BMI (kg/m^2)	26.6 ± 1.2	21.6 ± 1.4[a]
Systolic BP (mm Hg)	116 ± 3	107 ± 3[a]
Diastolic BP (mm Hg)	68 ± 3	64 ± 3

Data are expressed as mean ± SE. BP, Blood pressure.

[a] $P < 0.05$ *vs.* postmenopausal.

Endothelial ERα expression is reduced in estrogen-deficient postmenopausal women

In the postmenopausal women, serum estradiol was 30 ± 6 pg/ml (110 ± 13 pmol/liter), which was not different from concentrations in the premenopausal women during the EF phase but was lower than concentrations during the LF phase. Serum progesterone concentration was 0.3 ± 0.8 ng/ml (0.9 ± 0.1 nmol/liter) in the postmenopausal women, which was lower ($P < 0.001$) than the concentrations measured during the EF and LF phases of the premenopausal women [0.7 ± 0.1 (2.2 ± 0.20) and 4 ± 1.7 ng/ml (12.8 ± 5.4 nmol/liter), respectively]. Consistent with their lower circulating estradiol concentrations, endothelial cell ERα expression was 33% lower (0.48 ± 0.07 ERα intensity/HUVEC intensity; $P < 0.001$) in the postmenopausal women compared with expression in the LF phase of the menstrual cycle in premenopausal women (Fig. 1). Similarly, ERα expression in the postmenopausal women was equivalent to expression measured in the EF phase of the premenopausal women (Fig. 1). Among postmenopausal and premenopausal (LF phase) women, ERα protein expression was positively related to circulating estrogen (r = 0.42; $P < 0.05$) but not progesterone (r = 0.08; r = 0.71) concentrations. These observations suggest that ERα expression is reduced in estrogen-deficient postmenopausal women and is related to their lower circulating concentrations of estrogen.

Endothelial cell ERα expression is related to EDD and endothelial cell eNOS expression

Brachial artery FMD was 30% lower in the postmenopausal compared with premenopausal vascular function controls (n = 6) (5.0 ± 0.6% *vs.* 7.2 ± 0.7%; $P < 0.05$). Brachial artery FMD was positively related to endothelial ERα expression both in the overall group (r = 0.67; $P < 0.001$; Fig. 3) and among the postmenopausal women (r = 0.70; $P = 0.002$). In the overall group, ERα expression also was positively related to endothelial cell expression of eNOS protein (r = 0.56; $P = 0.009$; n = 21) and phosphorylated-eNOS-Ser1177 (r = 0.59; $P = 0.006$; n = 20) (Fig. 4). Among the postmenopausal women, brachial artery FMD, ERα expression, and endothelial cell expression of eNOS and phosphorylated-eNOS-Ser1177 were not related to plasma total cholesterol, low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, or any other subject characteristic or risk factor. These results indicate that the reduced endothelial cell ERα expression in estrogen-deficient postmenopausal women is related to their impaired EDD, reduced expression of eNOS protein, and decreased eNOS activation.

Discussion

The results of the present study provide novel insight into the role of estrogen status on *in vivo* ERα protein expression in vascular endothelial cells and its functional relation to EDD in healthy women. First, we found that states of short-term and chronic estrogen deficiency, *i.e.* a low-estrogen phase of the menstrual cycle and postmenopausal status, are associated with reduced endothelial cell ERα expression. Second, the abundance of endothelial ERα is positively related to EDD. Third, endothelial ERα expression also is positively related to both eNOS protein and an activated state of eNOS. Taken together, these findings support the idea that in healthy women endothelial ERα expression is regulated by physiological concentrations of

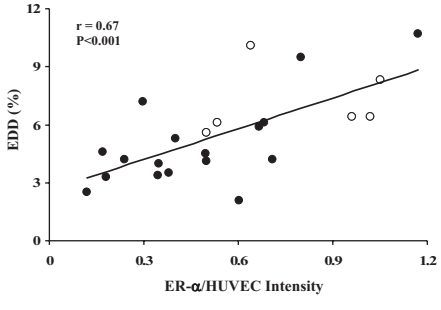

FIG. 3. Relation between EDD and ERα expression in vascular endothelial cells obtained from premenopausal (*open circles*) and postmenopausal women (*solid circles*). Each *symbol* represents the response of an individual woman.

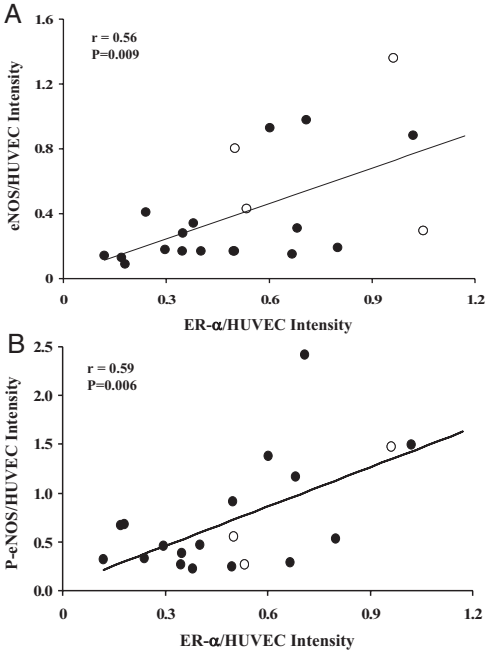

FIG. 4. Relation between eNOS (A) and phosphorylated-eNOS-Ser1177 (Pe-NOS) (B) and ERα expression in vascular endothelial cells obtained from premenopausal (*open circles*) and postmenopausal women (*solid circles*). Each *symbol* represents the response of an individual woman.

estrogen and that ERα influences vascular endothelial function, in part by modulating eNOS and its state of activation.

Menstrual cycle phase and endothelial cell ERα protein expression

Because of its varying sex hormone state, the menstrual cycle provides a natural physiological setting in which to isolate the independent effect of estrogen on vascular function without the confounding effects of changes with menopause, aging, or other pathophysiological influences. EDD varies during the menstrual cycle, with lower EDD observed in the EF compared with the LF phase suggesting a modulatory effect of estrogen on vascular endothelial function (22, 23). Consistent with this, we found lower ERα expression in endothelial cells collected during the low estrogen (EF) phase compared with the high estrogen (LF) phase of the menstrual cycle in healthy premenopausal women. ERα was positively related to circulating estrogen concentrations, providing further evidence for a direct influence of circulating estrogen on endothelial cell ERα expression. Our data are in agreement with previous studies demonstrating cyclic changes in ERα within the reproductive tract during the menstrual cycle in women (24, 25). In these studies, ERα was higher in the endometrial vasculature and myometrium during the late proliferative and early secretory phases of the menstrual cycle and was related to the preovulatory estrogen surge.

Endothelial cell ERα expression is reduced in estrogen-deficient postmenopausal women

The present findings demonstrate for the first time that *in vivo* endothelial cell ERα is reduced in healthy estrogen-deficient postmenopausal women compared with premenopausal women. The lower endothelial cell ERα appears to be related, in part, to lower circulating estrogen levels in that: 1) ERα expression was positively related to serum estrogen concentrations; and 2) differences in ERα expression between the postmenopausal and premenopausal women only are observed during the LF phase when serum estrogen concentrations differ between the groups.

These observations are consistent with ERα expression in reproductive tissue from premenopausal and postmenopausal women (25). Specifically, estrogen-deficient postmenopausal had lower expression of ERα compared with premenopausal women studied during periovulatory and secretory phases, but not the midproliferative phase of the menstrual cycle. Estrogen administration increased ERα expression in myometrium from postmenopausal women to levels observed in the premenopausal women. In turn, reductions in estrogen using a gonadotropin-releasing hormone agonist decreased ERα expression in myometrium from premenopausal women to levels observed in postmenopausal women (25).

Our findings also confirm in healthy women observations in animal and *in vitro* cell culture studies showing that ERα expression in vascular cells is regulated by endocrine status (7, 9, 10). For example, ERα expression is decreased with ovariectomy and increased with chronic estrogen treatment in female rat cerebral blood vessels and the aortic endothelium (7, 9). Moreover, long-term exposure to physiological concentrations of estrogen causes an increase in ERα protein in human umbilical vein endothelial cells (10). Taken together, the lower ERα protein in postmenopausal women appears to be mediated at least partly by estrogen deprivation.

We recognize that other mechanisms likely modulate ERα protein expression in postmenopausal women. Multiple cellular systems have been implicated in the regulation of ERα besides endocrine status, including changes in chromatin structure and pathological conditions including inflammation and diabetes (26, 27). For example, aging is associated with epigenetic changes including methylation of a cytosine- and guanine-rich area in promoter regions (CPG islands). DNA methylation is associated with permanent inactivation of gene transcription (28). In this regard, an age-related increase in ERα gene methylation has been reported in atrial tissues obtained from men and women undergoing coronary artery bypass surgery and in normal human colon (29, 30). Thus, it is possible that DNA methylation also contributed to the reduced endothelial ERα protein expression in our postmenopausal women. It also is plausible that vascular inflammation or related processes may have contributed to the reduced ERα protein expression (25). However, it is unlikely that pathological conditions such as diabetes, hypertension, or CVD were involved because we studied only healthy, normotensive postmenopausal women without major risk factors for CVD or evidence of overt clinical diseases.

Endothelial cell ERα expression, EDD, and eNOS

Consistent with previous studies (2, 3), we found that EDD is reduced in healthy estrogen-deficient postmenopausal women. Impaired EDD is characterized by a shift in the balance of endothelium-derived vasodilators/constrictors featuring reduced NO bioavailability (32). In women, the age-related decline in EDD only is evident after the menopause transition (2). Such observations suggest that the loss of circulating estrogen with menopause contributes to the impairment of vascular endothelial function in postmenopausal women.

Estrogen modulates vascular endothelial function in part by enhancing NO release and promoting vasodilation (5). Estrogen triggers the release of NO through ERα-

mediated genomic and nongenomic mechanisms. This occurs via activation of eNOS as well as by increasing eNOS protein via transcriptional regulation of the eNOS gene (33, 34). Functional ERα appears to be required for normal vascular function and influences the bioavailability of NO independent of the concentration of circulating estrogen (8, 35). Indeed, basal NO production is related to the number of estrogen binding sites in male mouse aorta regardless of circulating concentrations of estrogen (8). Additionally, reduced NO production and impaired EDD are observed in ERα knockout mice and a male patient with an ERα mutation, respectively (8, 36). Prolonged estrogen deprivation results in a marked reduction in ERα expression, resulting in a functional impairment of the ERα /eNOS signaling network (7). These data raise the possibility that eNOS and NO bioavailability could be influenced by ERα expression rather than the concentration of circulating estrogen. Our results show for the first time that endothelial cell ERα is positively related to EDD, eNOS protein, and a common phosphorylated state associated with eNOS activation (phosphorylated-eNOS-Ser1177) in healthy women.

Limitations

There are several limitations associated with the present study. Our measurements of protein expression were performed in endothelial cells collected from veins rather than arteries because of the difficulty of invasively obtaining endothelial cells from arteries on repeated measures across the menstrual cycle. However, we (15, 37) and others (11) have demonstrated that age- and CVD-related group differences in protein expression in arterial endothelial cells are consistently reflected in endothelial cells obtained from veins and that, among individual subjects, significant positive correlations exist in protein expression measured in endothelial cells obtained from peripheral veins and arteries. We recognize that only ERα was assessed and not ERβ, consistent with our study aim, and that quantitative immunofluorescence has limitations. However, quantitative immunofluorescence has been validated against immunoblotting by our laboratory and others (11, 17), and the number of endothelial cells that can be obtained from our human subjects does not yield enough total protein or mRNA to use alternative techniques such as Western blotting or real-time RT-PCR. Additionally, we are unable to quantify the intensity of the CY3 stain by endothelial cell location (*i.e.* membrane, cytosol, or nuclear). We wish to emphasize that despite these limitations, we were able to demonstrate differences in endothelial ERα protein expression between estrogen-deficient and estrogen-replete states, and that our experimental approach allowed us to characterize the influence of circulating estrogen status on ERα expression and other characteristics of the vascular endothelium in women in the absence of confounding clinical disease.

Clinical significance

Our findings have important clinical implications. CVD is now considered a major public health concern for women (38), and vascular aging featuring an impairment in endothelial function is recognized as a key factor in the etiology of CVD (39). Indeed, women endure a "double whammy" in that arteries are exposed to adverse changes in other risk factors during a time (menopause) of vulnerability to damage mediated by changes in the hormonal environment. As such, a better understanding of the cellular and molecular mechanisms regulating vascular health in women with aging clearly is needed. In this regard, the present findings provide the first evidence that *in vivo* endothelial ERα protein expression is influenced by circulating estrogen status, and that reduced ERα expression is related to impaired EDD in postmenopausal women. Our findings also provide insight into molecular mechanisms that may be involved, namely that endothelial cell ERα expression is positively related to eNOS protein and its state of activation in healthy women.

These observations are important in that a reduction/absence of ERα is found in atherosclerotic compared with normal coronary arteries of premenopausal and postmenopausal women (40), and premature coronary artery disease has been observed in a male patient with an ERα mutation, suggesting that ERα may play a role in protection against CVD (31, 36). Thus, a decrease or lack of functional ERα may be considered a novel risk factor for the development of CVD (8). Future studies need to determine whether chronic estrogen administration can preserve vascular ERα expression during menopause and postmenopause, allowing target tissues to remain responsive to estradiol (26).

Conclusions

In conclusion, our results provide experimental support to the idea that endothelial ERα expression is regulated by circulating estrogen concentrations, is reduced in states of estrogen deficiency, and is related to the impairment in NO bioavailability and EDD observed in estrogen-deficient postmenopausal women. Our findings have important implications for the development of CVD in postmenopausal women and for interpreting the cardiovascular outcomes of hormone replacement trials.

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