Abstract—Venous complications have been implicated in the adverse effects of hormone replacement therapy. This study investigated acute effects of the natural estrogen, 17β-estradiol, on function, estrogen receptors/GPR30 expression, and kinase activation in vascular rings and cultured smooth muscle cells from arteries and veins of patients with coronary artery disease. Changes in vascular tone of internal mammary arteries and saphenous veins exposed to the steroid were recorded. 17β-Estradiol caused concentration-dependent, endothelium-independent relaxation in arteries (*P*<0.05 versus solvent control) but not in veins (*P* not significant). 17β-Estradiol enhanced contractions to endothelin-1 in veins but not in arteries. The novel membrane estrogen receptor GPR30 was detected in both vessels. Moreover, gene expression of estrogen receptor β was 10-fold higher than that of estrogen receptor α or GPR30 (*P*<0.05). Expression of all 3 of the receptors was reduced after exposure to 17β-estradiol in arteries but not in veins (*P*<0.05). Basal phosphorylation levels of extracellular signal-regulated kinase were higher in venous than in arterial smooth muscle cells and were increased by 17β-estradiol in arterial cells only. In summary, this is the first study to report that, in human arteries but not in veins, 17β-estradiol acutely affects vascular tone, estrogen receptor expression, including GPR30, and extracellular signal-regulated kinase phosphorylation. These data indicate that effects of natural estrogens in humans differ between arterial and venous vascular beds, which may contribute to the vascular risks associated with menopause or hormone therapy. (Hypertension. 2007;49:1358-1363.)

Key Words: aromatase ■ bypass graft ■ clinical study ■ gender ■ hormone replacement therapy ■ human ■ 5α-reductase

Endogenous estrogens have been implicated in protection from cardiovascular disease in premenopausal women, and accordingly lack of estrogens is thought to be in part responsible for accelerated development of atherosclerosis in men and postmenopausal women. 1 Although epidemiological studies have suggested a protective effect of postmenopausal hormone therapy on the arterial vasculature, 2 this concept has been challenged recently based on the results of randomized clinical trials using conjugated equine estrogens that were shown recently that ERα protein also localizes to the cell membrane. 16–18 In addition, a G protein–coupled, 7-transmembrane receptor termed “GPR30” was identified recently as a protein structurally unrelated to ERα or ERβ binding 17β-estradiol with high affinity. 19,20 Whether and at what level GPR30 is expressed in human blood vessels is not known. Also, there is no information about whether 17β-estradiol similarly affects ER expression, vasoreactivity, or intracellular signaling pathways in arteries and veins in blood vessels.

Vanhoutte and coworkers 10,11 reported that vascular reactivity of veins showing a high release of vasoconstrictor prostanoids differs from that of arteries. The same group also showed that chronic administration of sex steroids differently affects vasoreactivity in arterial and venous vascular beds of rabbits and pigs. 12,13 Acute effects of estrogens involve membrane-associated estrogen binding sites independent of nuclear activation of ERα and ERβ, 14,15 and it has been shown recently that ERα protein also localizes to the cell membrane. In addition, a G protein–coupled, 7-transmembrane receptor termed “GPR30” was identified recently as a protein structurally unrelated to ERα or ERβ binding 17β-estradiol with high affinity. Whether and at what level GPR30 is expressed in human blood vessels is not known. Also, there is no information about whether 17β-estradiol similarly affects ER expression, vasoreactivity, or intracellular signaling pathways in arteries and veins in blood vessels.

In nonatherosclerotic human coronary arteries, 17β-estradiol induces rapid, endothelium-independent vasodilation 7 and enhances endothelium-dependent relaxation to bradykinin. 9 Vanhoutte and coworkers 10,11 reported that vascular reactivity of veins showing a high release of vasoconstrictor prostanoids differs from that of arteries. The same group also showed that chronic administration of sex steroids differently affects vasoreactivity in arterial and venous vascular beds of rabbits and pigs. 12,13 Acute effects of estrogens involve membrane-associated estrogen binding sites independent of nuclear activation of ERα and ERβ, 14,15 and it has been shown recently that ERα protein also localizes to the cell membrane. 16–18 In addition, a G protein–coupled, 7-transmembrane receptor termed “GPR30” was identified recently as a protein structurally unrelated to ERα or ERβ binding 17β-estradiol with high affinity. Whether and at what level GPR30 is expressed in human blood vessels is not known. Also, there is no information about whether 17β-estradiol similarly affects ER expression, vasoreactivity, or intracellular signaling pathways in arteries and veins in blood vessels.
humans, which would be important for the understanding of effects and adverse effects of estrogen therapy.

Therefore, in the present study, we investigated effects of 17β-estradiol, a nonselective agonist of ERα, ERβ, and GPR30,21 in human mammary arteries and saphenous veins. We also investigated acute effects on gene expression of ERα, ERβ, GPR30, and enzymes involved in estrogen synthesis. Finally, basal phosphorylation levels and effects of 17β-estradiol on phosphorylation of the kinases extracellular signal-regulated kinase (ERK)1/2 and Akt were determined. The results indicate differences in ER expression and pronounced heterogeneity in the responsiveness to 17β-estradiol between arteries and veins. Unlike arteries, human veins display higher levels of basal ERK1/2 phosphorylation and were devoid of any changes in vascular tone, gene expression, or ERK1/2 phosphorylation on exposure to 17β-estradiol.

Methods
An Expanded Methods section is available in a data supplement available online at http://hyper.ahajournals.org.

Patients and Vascular Function Studies
Human internal mammary arteries (IMAs) and saphenous veins (SVs) were obtained from patients undergoing coronary artery bypass graft surgery. The study and the experiments were reviewed and approved by the institutional ethics committee, and informed consent was obtained from patients before surgery. The study conformed with the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. Clinical and laboratory data were collected from patient records, and low-density lipoprotein concentrations were calculated using the Friedewald formula.22 Patient demographics, clinical parameters, and laboratory data values are shown in Table S1. Vascular function experiments were performed as described.7 For experimental details see the data supplement.

Quantitative Real-Time PCR Gene Expression Studies
Selected rings were snap-frozen in liquid nitrogen after incubation with either 17β-estradiol or solvent control for 3 hours at 37°C and kept at −80°C until further analysis. For experimental details of RNA isolation, reverse transcription and real-time PCR, and primer sequences, see the data supplement.

Effects of 17β-Estradiol on Phosphorylation of ERK1/2 and Akt
Internal mammary artery and SV smooth muscle cells were explanted using the explant technique as described23 and cultured in petri dishes using phenol-red free DMEM and Ham’s F-12 medium (1:1, vol/vol; Bioconcept) supplemented with 10% FCS (Sigma Aldrich). Cells were identified by their bill and valley morphology using phase-contrast microscopy and immunofluorescence staining for α-actin.23 Cells were passaged after treatment with 0.05% trypsin (weight/vol)/0.02% EDTA (weight/vol) in PBS. Subconfluent cells of passages 2 to 4 were used for experiments. Cells were serum-starved for 24 hours and then exposed to 17β-estradiol for 10 minutes. Western blot analysis experiments are described in the data supplement.

Calculations and Statistical Analyses
Data are expressed as means ± SEM. Vasoconstrictor responses are given as percentage of contraction to KCl, and vasodilator responses were calculated as percentage of relaxation of precontraction as described.7 For time-course experiments, curves are shown, but presented values (group means ± SEM) were obtained at indicated time points after 17β-estradiol administration and were analyzed with unpaired Student’s t test or, if data were not normally distributed, the Mann–Whitney U test was used. Concentration–response curves were analyzed by a 2-way ANOVA followed by posthoc unpaired multiple comparison test (Bonferroni test). Gene expression is expressed as arbitrary units (ΔΔCt method).24 Comparisons of group means were performed using the unpaired Student’s t test or the Mann–Whitney U test if data were not normally distributed. Statistical significance was accepted at P < 0.05.

Results
Direct Effects of 17β-Estradiol on Vascular Tone
In precontracted IMA rings, 17β-estradiol evoked a concentration-dependent relaxation starting at 30 nmol/L (−31 ± 4%; P < 0.05 versus solvent control; Figure 1A) reaching a maximal response of −70 ± 3% at 3 μmol/L after 40 minutes (P < 0.05 versus solvent control; Figure 1A). Similar dilating effects of 17β-estradiol were observed in endothelium-denuded IMA rings after 40 minutes (−74 ± 3% at 3 μmol/L; P < 0.05 versus solvent control; Figure 1B, “−E”). Onset of the relaxation was within 5 minutes after application of the hormone (P < 0.05 versus solvent control; Figure 1A). In contrast, 17β-estradiol showed no relaxant effect in SV rings even after 40 minutes of exposure (Figure 2B).

Figure 1. A, Relaxant effects to different concentrations of 17β-estradiol (0.3, 30, and 3000 nmol/L) compared with solvent control (CTL) after 40-minute exposure in human IMA. B, Denudation of arteries (−E) had no effect on maximal relaxant effect of 17β-estradiol after 40-minute exposure (−74 ± 3% vs −70 ± 3%; +E). Data are mean ± SEM; *P < 0.05 vs solvent control (CTL).

Figure 2. Time-dependent relaxant effects of 17β-estradiol in human IMA and SV at individual time points. Incubation with 17β-estradiol (E2; 3 μmol/L) evoked time-dependent relaxations in precontracted IMA rings (A). In contrast, 17β-estradiol had no relaxant effect in SVs (B). Data are mean ± SEM; n = 18 to 22 per group for IMA; n = 7 to 11 for group in SV; *P < 0.05 vs solvent control (CTL); †P < 0.05 vs IMA.
Effects of 17β-Estradiol on Contractile Responses

Contractions to endothelin-1 (0.01 nmol/L to 0.1 μmol/L) were stronger in IMA than SV rings (maximal response: 102±6% versus 71±4%; P<0.05). In SV rings, 17β-estradiol potentiated contractions to endothelin-1 (P<0.05 versus solvent control) but had no effect on contractions in IMA rings (Figure 3). Contractions to norepinephrine (0.1 to 10 μmol/L) were unaffected by 17β-estradiol in IMA and SV rings (data not shown).

ER Gene Expression: Effects of 17β-Estradiol

In both IMA and SV, mRNA transcripts of ERα, ERβ, and GPR30 genes were detected. In IMA and SV, gene expression levels of ERβ were >10-fold higher than mRNA levels of ERα or GPR30 (Table). Moreover, expression levels of ERα and ERβ in IMA were 2.1-fold and 1.8-fold higher than in SV (P<0.05), whereas GPR30 was expressed at similar levels in both vessels. These differences in expression levels were also evident in rings not exposed to the solvent control (data not shown). Further, ERα, ERβ, and GPR30 genes were expressed in cultured smooth muscle cells derived from IMA or SV (data not shown). Exposure to 17β-estradiol reduced ERα, ERβ, and GPR30 gene expression in IMA (P<0.05 versus solvent control; Table), whereas 17β-estradiol had no effect in SV (P not significant; Table). In both IMA and SV, transcripts of aromatase and 5α-reductase type 1 were detected at comparable expression levels and unaffected by 17β-estradiol (P not significant; Table). 5α-Reductase type 2 mRNA was not detected in any of the samples investigated.

Effects of 17β-Estradiol on Phosphorylation of ERK1/2 and Akt

Phosphorylation of the kinases Akt (protein kinase B) and ERK1/2 was analyzed in IMA and SV smooth muscle cells after exposure to 17β-estradiol (10 to 1000 nmol/L) for 10 minutes by immunoblotting with phosphospecific antibodies. Basal phosphorylation of ERK1/2 was higher in unstimulated SVs than in IMA smooth muscle cells despite a similar level of total ERK1/2 (Figure 4). 17β-Estradiol enhanced ERK1/2 phosphorylation in IMA at low concentrations (10 nmol/L; Figure 5, left) but had no effect in SV smooth muscle cells (Figure 5, right). Akt phosphorylation was unaffected by 17β-estradiol in IMA and SV smooth muscle cells (Figure 5). In contrast, insulin caused strong phosphorylation of Akt (data not shown).

Discussion

This study presents several new findings contributing to the understanding of vascular action of estrogens in the human vasculature. The results demonstrate that SVs completely lack vasodilator effects, changes in ER gene expression, or kinase phosphorylation in response to 17β-estradiol; vasoconstriction to endothelin-1 was increased. In contrast, in mammary arteries, short-term exposure to 17β-estradiol results in endothelium-independent relaxation, ERK1/2 phosphorylation, and downregulation of ERα, ERβ, and GPR30 gene expression. To the best of our knowledge, this study is also the first demonstrating that human blood vessels express the novel ER GPR30 and that arteries and veins differently

<table>
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<tr>
<th>Gene Expression of ERs, GPR30, Aromatase, and 5α-Reductase Type 1</th>
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5αRed1 indicates 5α-reductase type 1. Data are mean±SEM and are expressed as arbitrary units=ΔΔCt, of gene of interest and housekeeping gene GAPDH; n=7 to 12 per group.

*P<0.05 vs ERα; †P<0.05 vs ERβ; ‡P<0.05 vs control; and §P<0.05 vs IMA.
respond to the natural estrogen 17β-estradiol at both the functional and molecular level.

Rapid dilator effects to 17β-estradiol, which is a nonselective agonist of ERα, ERβ, and GPR30, involve nongenomic signaling and are thought to be mediated via membrane-bound estrogen binding sites. Confirming previous studies in human coronary arteries, the present study shows that 17β-estradiol causes endothelium-independent relaxation in human mammary arteries. The exact contributions of ERα and ERβ to the acute dilator response to 17β-estradiol in IMAs are currently unknown; however, recent work from our laboratory using epicardial coronary arteries suggests that the dilator response caused by selective activation of ERα is markedly different from nonselective ER activation. In contrast to mammary arteries, we observed no dilation in response to 17β-estradiol in human SVs. This has potentially important implications, because hormone therapy has been associated with venous complications. The mechanisms underlying this lack of responsiveness may be severalfold. Seminal work by Vanhoutte and coworkers has shown that veins display different response patterns to various vasoactive substances compared with arteries and that veins show a higher release of vasoconstrictor prostanoids. Recently, Eriksson et al reported that proinflammatory activity is greater in veins than in arteries. Similar to our previous findings in humans, we now compared the effects of 17β-estradiol and 17β-estradiol at both the functional and molecular level.

Based on our previous observation showing that 17β-estradiol acutely modulates the vascular activity of vasoconstrictors such as angiotensin or serotonin in human arteries, we now compared the effects of 17β-estradiol on endothelin-mediated contractility between human arteries and veins. Endothelin is regarded as one of the most potent and long-lasting vasoconstrictors. Although 17β-estradiol had no effect on contractions in internal mammary arteries, responses were enhanced in SVs, compatible with an indirect estrogen-mediated vasoconstrictor effect. Endogenous sex hormones not only regulate endothelin expression but also regulate venous endothelin receptor expression. It is also of interest to note that venoconstriction in response to endothelin-1 involves the release of vasoconstrictor prostanoids, and 17β-estradiol may even stimulate the formation of cyclooxygenase-derived vasoconstrictor prostanoids.

An important finding and to our knowledge the first demonstration that, in addition to the “classical” ERs, ERα and ERβ, was the observation that the novel membrane ER GPR30 is expressed in smooth muscle cells of human arteries and veins. Expression of ERβ was higher than that of ERα or GPR30; this is likely to be of relevance for vascular effects of estrogens and/or susceptibility to disease. It was shown that expression of ERβ, but not of ERα, correlates with coronary artery calcification in women. Surprisingly, in mammary arteries but not in veins, GPR30 mRNA, like ERα and ERβ mRNA, was downregulated after short-term exposure to 17β-estradiol. The mechanisms underlying this regulation are currently unclear. Interestingly, inactivation of transcription by methylation of ER genes differs between arteries and veins. Also possibly relevant for adverse effects of estrogen is the observation that high ERβ expression in veins is associated with growth of vascular smooth muscle.

In the present study, we demonstrate that human arteries and veins express aromatase and 5α-reductase type 1 but not 5α-reductase type 2. Given that testosterone is locally converted to 17β-estradiol by aromatase and that aromatase deficiency accelerates atherogenesis in males, it is possible that protective vascular effects of 17β-estradiol are not restricted to females. Indeed, androgens are converted to estrogens in males, and estrogen activity is important for the atheroprotective effects of androgens in males. This is further supported by the observation that the nonselective ER agonist 17β-estradiol inhibits experimental atherosclerosis in male mice. Together with our previous findings in humans and atherosclerotic mice, the present investigation indicates that arteries from female and from male patients respond to 17β-estradiol via rapid changes in vascular tone, ERK1/2 phosphorylation, and ER expression.

Basal levels of ERK1/2 phosphorylation may vary between smooth muscle cells from different arterial vascular beds, whereas no data on veins are available. We found that basal activation of ERK1/2, as measured by the phosphorylated protein, was higher in venous compared with arterial smooth muscle cells. We also found that 17β-estradiol induces ERK1/2 phosphorylation only in arterial but not in venous smooth muscle cells. Higher basal levels of phosphorylated ERK1/2 in veins may possibly explain the inability to further increase ERK1/2 phosphorylation in this vessel upon exposure to 17β-estradiol.
Perspectives
We have demonstrated marked differences in functional and molecular responsiveness between human veins and arteries in response to the nonselective ER agonist 17β-estradiol. The results reported herein might add to the understanding of how natural estrogens or conjugated equine estrogens (which, among other substances, contain 17β-estradiol) contribute to vascular protection and to vascular risk in humans.46,47

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Disclosures
None.

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Differential Effects of 17β-Estradiol on Function and Expression of Estrogen Receptor α, Estrogen Receptor β, and GPR30 in Arteries and Veins of Patients With Atherosclerosis

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