Estrogen receptor-mediated enhancement of venous relaxation in female rat: Implications in sex-related differences in varicose veins

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Background: **A greater incidence of varicose veins has been reported in premenopausal women than in men. We hypothesized that the sex differences in venous function reflect reduced constriction and enhanced venous dilation in women due to direct venous relaxation effects of estrogen on specific estrogen receptors (ER).**

Methods: **Circular segments of inferior vena cava (IVC) from male and female Sprague-Dawley rats were suspended between two wires, and isometric contraction (in mg/mg tissue) to phenylephrine, angiotensin II (AngII), and 96 mM KCl was measured. To investigate sex differences in venous smooth muscle, Ca^{2+} release from the intracellular stores, and Ca^{2+} entry from the extracellular space, the transient phenylephrine contraction in 0 Ca^{2+} Krebs was measured. Extracellular $CaCl_2$ (0.1, 0.3, 0.6, 1, 2.5 mM) was added, and the $[Ca^{2+}]_e$-dependent contraction was measured. To investigate sex differences in venous endothelial function, acetylcholine-induced relaxation was measured. To test the role of specific ERs, the amount of venous tissue ERs was measured using Western blots, and the venous relaxation in response to 17β-estradiol (E2, activator of most ERs), 4,4,′4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-tris-phenol (PPT; ERα agonist), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; ERβ agonist), and ICI 182,780 (ERα/ERβ antagonist, and G protein-coupled receptor 30 [GPR30] agonist) was measured in IVC segments nontreated or treated with the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME).**

Results: **Phenylephrine caused concentration-dependent contraction that was less in female (max 104.2 ± 16.2) than male IVC (172.4 ± 20.4). AngII (10^{-6})-induced contraction was also less in female (81.0 ± 11.1) than male IVC (122.5 ± 15.0). Phenylephrine contraction in 0 Ca^{2+} Krebs was insignificantly less in female (4.8 ± 1.8) than male IVC (7.2 ± 1.7), suggesting little difference in the intracellular Ca^{2+} release mechanism. In contrast, the $[Ca^{2+}]_e$-dependent contraction was significantly reduced in female than male IVC. Also, contraction to membrane depolarization by 96 mM KCl, which stimulates Ca^{2+} influx, was less in female (129.7 ± 16.7) than male IVC (319.7 ± 30.4), supporting sex differences in Ca^{2+} entry. Acetylcholine relaxation was greater in female (max 80.6% ± 4.1%) than male IVC (max 48.0% ± 6.1%), suggesting sex differences in the endothelium-dependent relaxation pathway. Western blots revealed greater amounts of ERα, ERβ, and GPR30 in female than male IVC. ER agonists caused concentration-dependent relaxation of phenylephrine contraction in female IVC. E2-induced relaxation (max 76.5% ± 3.4%) was more than DPN (74.8% ± 9.1%), PPT (71.4% ± 12.5%), and ICI 182,780 (67.4% ± 7.8%), and was similar in L-NAME-treated and nontreated IVC.**

Conclusion: **The reduced α-adrenergic, AngII, depolarization-induced, and $[Ca^{2+}]_e$-dependent venous contraction in female rats is consistent with sex differences in the Ca^{2+} entry mechanisms, possibly due to enhanced endothelium-dependent vasodilation and increased ER expression/activity in female rats. E2/ER-mediated venous relaxation in female rats is not prevented by NOS blockade, suggesting activation of an NO-independent relaxation pathway. The decreased venous contraction and enhanced E2/ER-mediated venous relaxation would lead to more distensible veins in female rats. (J Vasc Surg 2010;51:972-81.)**

Clinical Relevance: **Studies have demonstrated an increase in the incidence of varicose veins in women compared with men, suggesting potential role of endogenous sex hormones. However, little is known regarding the difference in function of venous tissues of women and men and their vasoreactive responses to various agonists and to specific estrogenic compounds. The present data in rat veins demonstrate a gender-related decrease in venous tissue contraction and an enhanced endothelium-dependent venous relaxation pathway, possibly due to increased expression of venous tissue estrogen receptor-α, estrogen receptor-β, and G protein-coupled receptor 30, and downstream activation of the nitric oxide-independent pathway. The enhanced estrogen-mediated venous relaxation pathways may promote venous dilation and lead to more distensible veins in female rats. Future studies in human veins should investigate the possibility of gender differences in estrogen receptor-mediated venous relaxation pathways and thereby explain the higher prevalence of varicose veins in women.**

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Varicose veins is a common disease of the lower extremity characterized by valve degeneration and vein wall dilation and tortuousity.[1] Several predisposing factors have been implicated in varicose veins, including diabetes, obesity, smoking, and age.[2,3] Gender could also influence the incidence of varicose veins. Sex differences in the prevalence of varicose veins have been reported, with a greater incidence in women compared with men.[4,5]

Prevalence estimates of varicose veins could be as low as 1% to 2% in men and women, but could reach 56% in men and as high as 73% in women.[2,3,6] Specifically, the incidence rate of varicose veins in the Framingham Heart Study was greater in women (2.6%) than in men (1.9%).[7] The San Diego Population Study, a cross-sectional study of a multiethnic sample of 2211 men and women, demonstrated varicose veins in 28% of adult women compared with 15% of adult men.[8]

Gender differences could be related to hormonal influence as evidenced by the increased incidence of varicose veins in women during pregnancy and with the use of oral contraceptives containing estrogen (E2).[2,9-12] However, the venous mechanisms underlying the gender differences and the role of sex hormones in the incidence of varicose veins are unclear.

Sex differences in arterial function have been demonstrated in the aorta, coronary, mesenteric, and renal arteries.[13-20] We have previously shown that aortic contraction is reduced in female compared with male rats.[16,17] Studies from our laboratory and others have also suggested that the sex differences in arterial vasoconstriction are likely due to the high levels of E2 in females.[16,17,21-23] E2 induces long-term genomic effects involving up-regulation of endothelium-dependent vascular relaxation pathways such as nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF).[24,25] E2 also induces down-regulation of the mechanisms of vascular smooth muscle contraction, including Ca^{2+} channels and protein kinase C.[17,26] In addition to its long-term effects, E2 promotes rapid nongenomic effects involving activation of endothelium-derived vasodilators and inhibition of the Ca^{2+}-dependent mechanisms of arterial contraction.[23-25,27,28]

Estrogen receptors (ERs) have been characterized in the female reproductive tract, mammary glands, and other tissues.[29] ERs have also been identified in blood vessels.[30-32] Two major ERs, ERα and ERβ, have been suggested to mediate many of the genomic effects of E2.[29,31,32] Surface membrane ERs have also been implicated in the rapid vasodilator effects of E2.[27] A new transmembrane G protein-coupled receptor, GPR30, has also been shown to bind E2 and to mediate some of its rapid effects.[33-36]

Although numerous studies have examined the sex difference and the effects of E2 on arterial function, little is known regarding the sex differences and the effects of E2 on venous function. Also, although ERα, ER_β, and GPR30 have been characterized in numerous arterial preparations from human and experimental animals,[15,30-32,35] the amount and functional significance of ERs in venous tissues are less clear.

Recent experiments from our laboratory have demonstrated a reduction in $[Ca^{2+}]_i$ signaling and Ca^{2+}-dependent contraction in female rat inferior vena cava (IVC) segments loaded with the Ca^{2+} indicator fura-2.[37] The present study was designed to test the hypothesis that the sex differences in venous function reflect reduced venous constriction as well as enhanced venous dilation in female rats compared with male rats, partly due to direct effects of E2 on specific ERs in venous tissues and downstream activation of ER-mediated venous relaxation pathways.

METHODS

All procedures in this study followed the National Institutes of Health guide for the Care of Laboratory Animal Welfare Act and the guidelines of the Animal Care and Use Committee at Harvard Medical School.

Animals and tissues. Male (n = 6) and female (n = 6) Sprague-Dawley rats (age, 12 weeks old; weight, 250-300 g; Charles River Lab, Wilmington, Mass) were maintained on ad libitum standard rat chow and tap water in a 12 h light/12 h dark cycle.

Rats were euthanized by inhalation of CO_2. The IVC was rapidly excised, placed in oxygenated Krebs solution, and carefully dissected and cleaned of connective tissue under microscopic visualization. The IVC was portioned into 3-mm rings in preparation for isometric contraction experiments. Four 3-mm-long segments were obtained from each rat IVC. All vein segments were obtained from the IVC below the renal veins. Extreme care was taken throughout the tissue isolation and dissection procedure to minimize injury to the endothelium and the vein wall.

In all experiments, the IVC was harvested freshly and dissected immediately in normal Krebs solution. The time between harvesting the IVC, removing the fat and connective tissue, and sectioning it into 3-mm rings was approximately 45 minutes. One vein segment from each rat was used to perform one experiment and obtain one data point. Data from one to four vein segments from different rats were used to calculate the average data for each arm of the study.

Isometric contraction. Circular segments of IVC were suspended between two stainless-steel hooks, one hook was fixed at the bottom of a tissue bath, and the other hook was connected to a Grass force displacement transducer (FT03, Astro-Med Inc, West Warwick, RI). Vein segments were stretched under 0.5 g of resting tension and allowed to equilibrate for 45 minutes in a tissue bath filled with 50 mL Krebs solution continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. We have previously constructed the relationship between basal tension and the contraction to 96 mM KCl in rat IVC, and demonstrated that 0.5 g basal tension produced maximal KCl contraction. Further increases in basal tension did not cause any significant increases in IVC contraction in response to KCl.[38] The changes in isometric contraction/relaxation were recorded on a Grass polygraph (Model 7D, Astro-Med Inc).[38,39]

Control IVC contraction in response to 96 mM KCl was first elicited. Once the KCl maximum contraction was reached and a plateau achieved (within 10 to 15 minutes) the tissue was washed three times in Krebs, 10 minutes each. The control contraction to 96 mM KCl was repeated twice before further experimentation.

IVC segments were then stimulated with increasing concentrations (10^{-9} to 10^{-5} M) of phenylephrine (PHE), and the contractile response was measured. To correct for any undetectable differences in the vein segment size, all contraction measurements were normalized for the weight of the vein segments and presented as mg/mg tissue weight. The contractile response at each PHE concentration was also presented as the percentage of the maximum PHE contraction. The individual PHE concentration-response curves were analyzed using a nonlinear regression curve (best-fit sigmoidal dose-response curve) using SigmaPlot software (Systat Software, Inc, San Jose, CA). The effective concentration that produced half the maximal contraction (ED50) was presented as pED50 (–log M). In other experiments, the tissues were treated with angiotensin II (AngII, 10^{-6} M), and the maximal peak contraction was measured.

To investigate sex differences in Ca^{2+} release from the intracellular stores, IVC segments were incubated in Ca^{2+}-free (2 mM ethyleneglycotetraacetic acid [EGTA]) Krebs for 5 minutes and then in nominally 0 Ca^{2+} Krebs for 5 minutes. The IVC segments were then stimulated with PHE (10^{-5} M), and the peak transient contraction was measured. To investigate sex differences in Ca^{2+} influx, increasing extracellular $CaCl_2$ concentrations (0.1, 0.3, 0.6, 1, 2.5 mM) were added and the $[Ca^{2+}]_e$-contraction relationship was constructed.

To investigate sex differences in venous endothelial function, IVC segments precontracted with PHE (10^{-5} M) were treated with increasing concentrations (10^{-9} to 10^{-5} M) of acetylcholine (Ach), and the percentage of venous relaxation was measured. To test the role of ERs, female IVC segments precontracted with PHE (10^{-5} M) were treated with increasing concentrations (10^{-12} to 10^{-5} M) of 17β-estradiol (E2, activator of most ERs), 4,4,′4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-tris-phenol (PPT, selective ERα agonist),[40,41] 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, selective ERβ agonist),[42] and ICI 182,780 (fulvestrant, a known ER antagonist that also possesses agonistic activity at GPR30),[43,44] and the percentage of venous relaxation was measured. To test the role of ER-mediated endothelium-dependent NO, experiments were repeated in IVC segments treated with the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4}M).

Western blot analysis. The venous tissue homogenate was subjected to electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gel and then transferred electrophoretically to nitrocellulose membrane. The membrane was incubated in 5% dried nonfat milk for 1 hour and then treated with polyclonal antibody to ERα, (1:100), ERβ (1:1000), and GPR30 (1:100; Affinity Bioreagents, Golden, Colo) for 24 hours. α-Actin was used as an internal control and detected by a monoclonal antiactin antibody (1:500000, Sigma, St. Louis, Mo).

The nitrocellulose membranes were washed five times for 15 minutes each in Tris-buffered saline–Tween then incubated in horseradish peroxidase-conjugated secondary antibody (1:1000) for 1.5 hours. The blots were visualized with enhanced chemiluminescence (ECL) Western Blotting Detection Reagent (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and the reactive bands were analyzed quantitatively by optical densitometry. The densitometry values represented the pixel intensity and were normalized to α-actin to correct for loading.[38]

Solutions, drugs, and chemicals. Normal Krebs solution contained (in mM) NaCl, 120; KCl, 5.9; $NaHCO_3$, 25; NaH_2PO4, 1.2; dextrose, 11.5 (Fisher Scientific, Fair Lawn, NJ), $CaCl_2$, 2.5 (BDH Laboratory Supplies Poole, England), and $MgCl_2$, 1.2 (Sigma). Krebs solution was bubbled with 95% O_2 and 5% CO_2 for 30 minutes at an adjusted pH 7.4. For nominally 0 Ca^{2+} Krebs, $CaCl_2$ was omitted. For Ca^{2+}-free Krebs, $CaCl_2$ was omitted and 2 mM EGTA (Sigma) was added. KCl (96 mM) was prepared as normal Krebs but with equimolar substitution of NaCl with KCl. Stock solutions of PHE (10^{-1} M), AngII, (10^{-2} M), Ach (10^{-1} M), and L-NAME (10^{-1}M, Sigma) were prepared in deinoized water, 17β-estradiol (E2, 10^{-2}M, Sigma) in ethanol, and PPT, DPN, and ICI 182,780 (10^{-1}M, Tocris, Ellisville, Mo) in dimethylsulfoxide. The final concentration of ethanol or dimethylsulfoxide was <0.1%. All other chemicals were of reagent grade or better.

Statistical analysis. Data were analyzed and presented as means ± standard error of the mean and compared using the *t* test for unpaired data. Differences were considered statistically significant if $P < .05$.

RESULTS

PHE caused a concentration-dependent contraction in rat IVC that reached a maximum at 10^{-5}M. The maximum PHE contraction (mg/mg tissue) was significantly reduced ($P = .012$) in females (104.2 ± 16.2) compared with males (172.4 ± 20.4; Fig 1). When the PHE response was measured as percentage of maximum contraction and the EC50 calculated, PHE was equally potent in female (pEC50 5.35% ± 0.37%) compared with male IVC segments (pEC50 5.22% ± 0.37%; Fig 1).

AngII (10^{-6} M) caused a transient contraction in rat IVC that returned to baseline in 5 to 10 minutes. The peak AngII contraction was significantly less in female compared with male IVC (Fig 2, *A*). Membrane depolarization by 96 mM KCl, which stimulates Ca^{2+} influx, caused a significant and rapid initial IVC contraction, followed by a maintained steady-state contraction. Both the initial and steady-state KCl contraction were reduced in female compared with male IVC (Fig 2, *B*). In nominally 0 Ca^{2+} Krebs, PHE (10^{-5} M) caused a small transient contraction representing the response to Ca^{2+} release from the intracellular stores. The PHE contraction in 0 Ca^{2+} Krebs was insignificantly less in female (4.8 ± 1.8) than male IVC (7.2 ± 1.7 mg/mg tissue; Fig 2, *C*). The gradual addition of extracel-

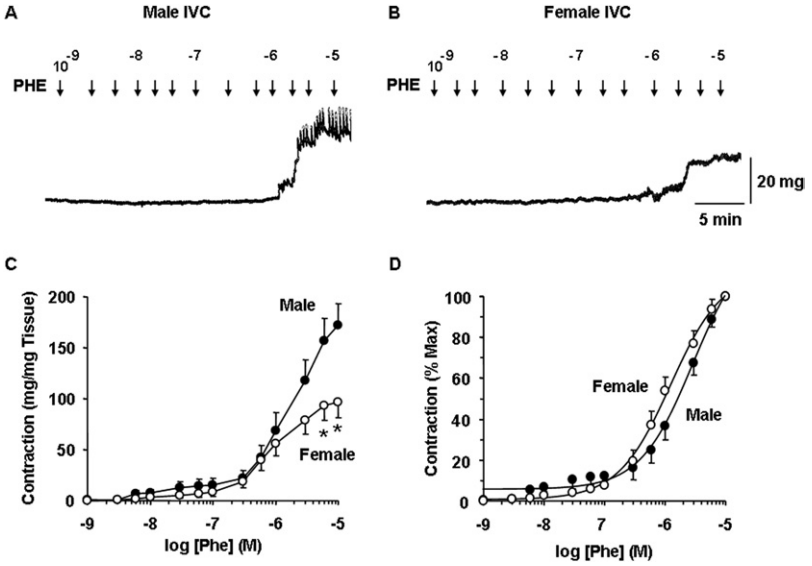

Fig 1. Effect of phenylephrine *(PHE)* on male and female rat inferior vena cava (*IVC)* incubated in normal Krebs (2.5 mM Ca^{2+}). After two control 96 mM KCl contractions, followed by washing in Krebs, IVC segments of **(A)** male and **(B)** female rats were stimulated with the α-adrenergic agonist PHE (10^{-9} to 10^{-5} M). The contractile response was recorded and presented as **(C)** mg/mg tissue or as **(D)** percentage of maximal contraction. The *unlabeled arrow* represents a threefold greater PHE concentration than the *preceding arrow*. Data represent the means ± standard error of the mean for 16 to 20 vein segments. *Significantly different ($P < .05$).

lular $CaCl_2$ caused corresponding increases in PHE contraction that reached a maximum at 2.5 mM $[Ca^{2+}]_e$ (Fig 2, *C*). The $[Ca^{2+}]_e$-dependent contraction was significantly reduced in female compared with male IVC.

To test for sex differences in endothelium-dependent relaxation, the response to Ach was compared in female and male IVC. In IVC segments precontracted with PHE (10^{-5} M), increasing concentrations of Ach (10^{-9} to 10^{-5} M) caused concentration-dependent relaxation that reached a maximum at 10^{-5}M. Ach-induced relaxation was significantly greater in female compared with male IVC (Fig 3).

Western blot analysis of IVC tissue homogenate revealed immunoreactive bands corresponding to ERα at 64 kDa, ERβ at 55 kDa, and GPR30 at 50 kDa. Optical density analysis revealed that the amount of ERα, ERβ, and GPR30 was significantly greater in female compared with male IVC (Fig 4).

To test the functionality of ERs in rat IVC, the effects of ER agonists on PHE contraction was measured (Fig 5). E2 (10^{-12} to 10^{-5} M) caused concentration-dependent relaxation of female IVC that reached a maximum at 10^{-5} M E2. PPT (ERα agonist), DPN (ERβ agonist), and ICI 182,780 (ERα/ERβ antagonist and GPR30 stimulant) also caused relaxation of female IVC that reached a maximum at 10^{-5} M (Fig 6). The maximal IVC relaxation was 76.5 ± 3.4 for E2, 74.8 ± 9.1 for DPN, 71.4 ± 12.5 for PPT, and 67.4 ± 7.8 for ICI 182,780. The venorelaxant effects of E2, DPN, and ICI 182,780 were reversible, and after washing the IVC segments three times with Krebs, a significant PHE contraction could still be elicited. In contrast, the venorelaxant effect of PPT was irreversible, and no PHE contraction could be measured after repeated washing with Krebs.

To determine the potential role of NO as a post-ER downstream venous relaxation mechanism, we examined the effect of treatment of female rat IVC with the NOS inhibitor L-NAME (3×10^{-4} M) for 15 minutes. E2, PPT, DPN, and ICI 182,780 caused similar relaxation of PHE contraction in L-NAME treated and nontreated IVC (Fig 7).

DISCUSSION

The main findings of the present study are (1) α-adrenergic-, AngII-, depolarization-induced, and $[Ca^{2+}]_e$-dependent contraction are reduced in female compared with male IVC, (2) Ach-induced relaxation is greater in female compared with male IVC, (3) female IVC has more ERs than male IVC, and (4) selective ER agonists cause relaxation of female IVC that is not blocked by NOS inhibitor.

Although the male rat IVC is a very thin and delicate preparation, we have previously shown that it produces significant and consistent contraction in response to PHE and membrane depolarization by high KCl.[38,39] Therefore, the rat IVC was an appropriate preparation to study the sex differences in venous tissue function between males and females. The α-adrenergic agonist PHE produced concentration-dependent contraction that was significantly reduced in female compared with male IVC, sug-

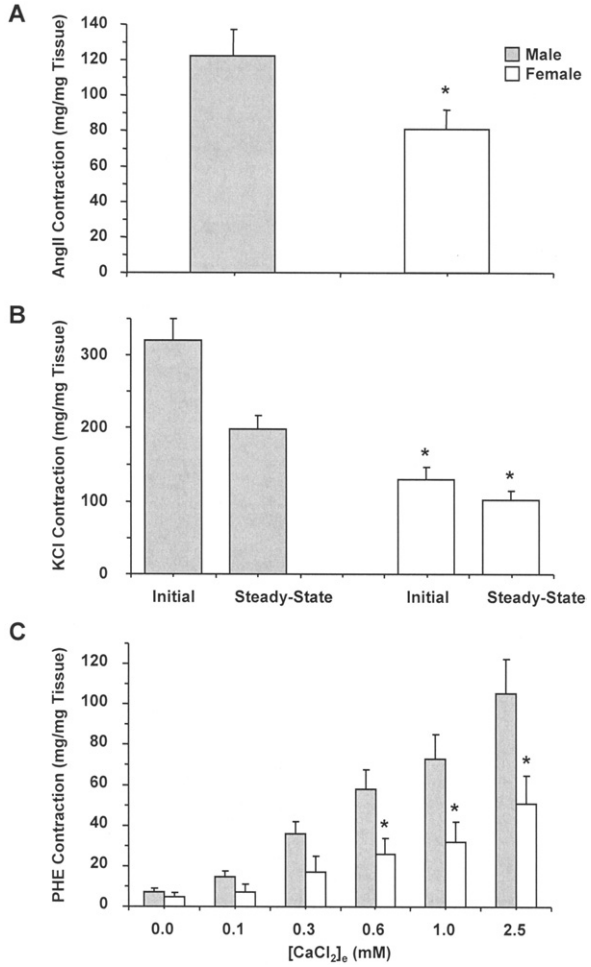

Fig 2. Angiotensin II *(AngII)*, potassium chloride *(KCl)* and calcium (Ca^{2+})-dependent contraction in male and female rat inferior vena cava (IVC) segments. IVC segments from male *(shaded bars)* and female rats *(white bars)* were incubated in normal Krebs (2.5 mM Ca^{2+}). The tissues were stimulated with the angiotensin type 1 receptor agonist AngII (10^{-6} M) and the **(A)** peak contractile response was measured or **(B)** with 96 mM KCl depolarizing solution and the initial maximal contractile response and steady-state contraction were recorded. **C,** Other IVC segments were incubated in Ca^{2+}-free (2 mM ethyleneglycotetraacetic acid) Krebs for 5 minutes, then nominally 0 Ca^{2+} Krebs for 5 minutes, then stimulated with phenylephrine *(PHE;* 10^{-5} M) and the transient contraction was measured. Increasing concentrations of extracellular $CaCl_2$ (0.1, 0.3, 0.6, 1, 2.5 mM) were added, and the contractile response at different $[Ca^{2+}]_e$ was recorded. Data represent the means ± standard error of the mean for 8 to 20 vein segments. *Significantly different ($P < .05$).

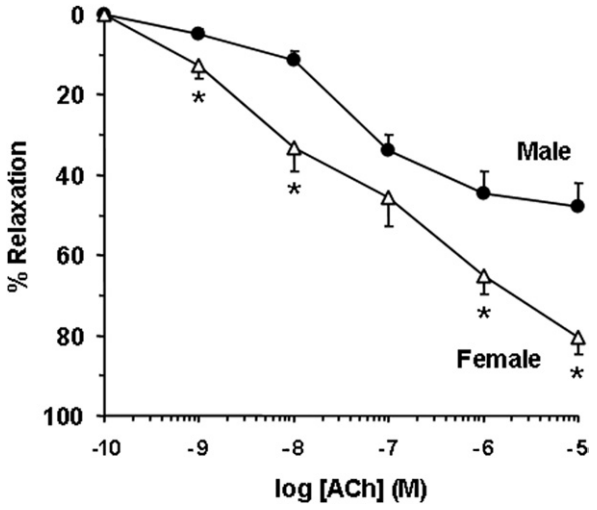

Fig 3. Acetylcholine (*Ach*)-induced relaxation is shown in male and female rat inferior vena cava (IVC) segments. IVC segments were stimulated with phenylephrine PHE (10^{-5} M), and the steady-state contraction was recorded. Increasing concentrations of Ach (10^{-9} to 10^{-5} M) were added, and the percentage relaxation of PHE contraction was compared in male and female IVC. Data represent the means ± standard error of the mean for 20 to 23 vein segments. *Significantly different ($P < .05$).

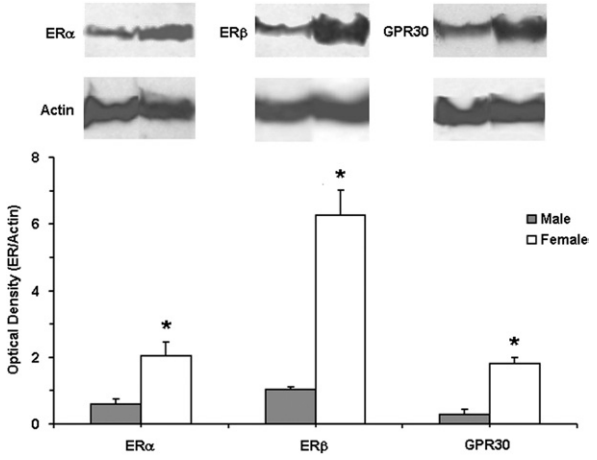

Fig 4. Expression of estrogen receptor *(ER)*α, ERβ, and G protein-coupled receptor 30 *(GPR30)*is shown in male and female rat inferior vena cava (IVC). Tissue homogenates of male and female IVC were prepared for Western blot analysis and the amount of ERs were measured using antibodies to ERα (1:100), ERβ (1:1000), and GPR30 (1:100). The intensity of the immunoreactive bands was analyzed using optical densitometry and normalized to the housekeeping protein actin. Data represent the means ± standard error of the mean for three experiments.*Significantly different ($P < .05$).

gesting sex differences in receptor-mediated mechanisms of venous constriction. To test whether the sex differences in PHE contraction represent differences in the α-adrenergic receptor affinity to PHE, we compared the PHE EC50 in female and male IVC. The lack of difference in PHE EC50 suggests that the reduced PHE contraction in female IVC is not due to a decrease in the α-adrenergic receptor binding or sensitivity to PHE.

To test whether the reduced venous contraction in female IVC is specific to α-adrenergic receptor-mediated

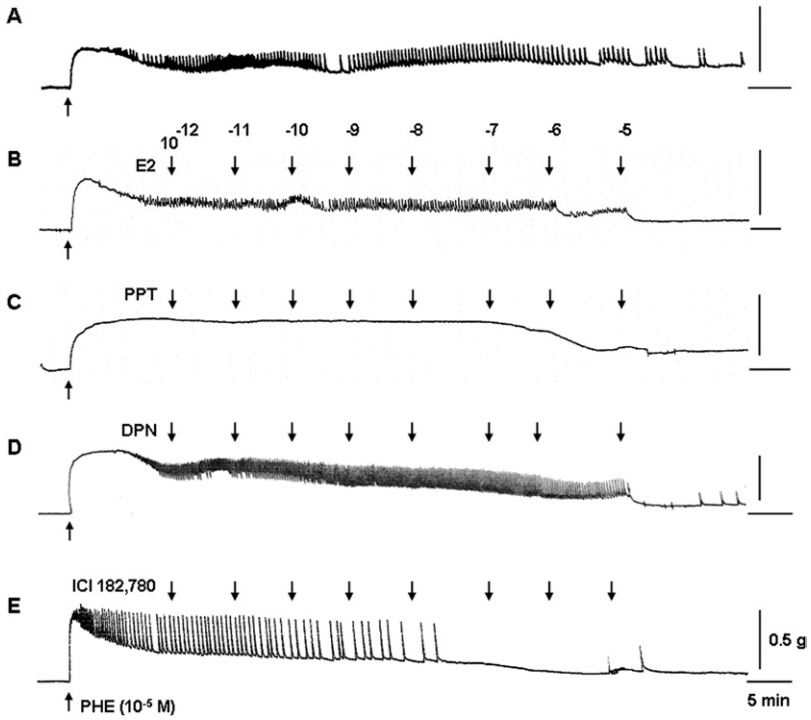

Fig 5. Representative traces of estrogen receptor (*ER*)-mediated relaxation in female rat inferior vena cava (IVC) are shown. Segments of female rat IVC were stimulated with phenylephrine (*PHE*; 10^{-5} M), and the steady-state contraction was recorded. IVC segments were **(A)** nontreated or were treated with increasing concentrations (10^{-12} to 10^{-5} M) of **(B)** 17β-estradiol (E2, activator of most ERs), **(C)** the ERα agonist 4,4,′4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-tris-phenol (*PPT*), **(D)** the ER_{β} agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (*DPN*), or **(E)** ICI 182,780, a ERα/ERβ antagonist and G protein-coupled receptor 30 stimulant. The effect on PHE contraction was observed. The *vertical bars* show 0.5 g, and the *horizontal bars* show 5 minutes.

effects, we investigated sex differences in another receptor-mediated vasoconstrictive response. AngII is known to cause vasoconstriction by stimulating angiotensin type 1 receptor.[45] The observation that AngII-induced venous constriction was also reduced in female compared with male IVC suggests that the sex differences in venous contraction are not receptor-specific and may involve reduction in postreceptor signaling mechanism.

The reduced contractile response in female compared with male veins is less likely due to difference in the vein thickness because the contractile response was normalized to the vein size. Future histologic analysis and morphometry should further measure the tunica media thickness and smooth muscle density in female compared with male veins.

Vascular smooth muscle (VSM) contraction is triggered by increases in $[Ca^{2+}]_i$ due to initial Ca^{2+} release from the intracellular stores and maintained Ca^{2+} entry from the extracellular space.[17,46] Agonist-induced VSM contraction in Ca^{2+}-free Krebs has been used as a measure of the intracellular Ca^{2+} release mechanism.[17] The relatively small PHE contraction in 0 Ca^{2+} Krebs suggests a small role of the Ca^{2+} release mechanism in IVC contraction. Although the PHE contraction in 0 Ca^{2+} Krebs was less in female compared with male IVC, the difference was not statistically significant, suggesting little gender differences in the intracellular Ca^{2+} release mechanism.

To test for sex differences in the venous Ca^{2+} entry mechanisms, we measured the PHE-induced IVC contraction at increasing $[Ca^{2+}]_e$. The observation that the PHE-induced $[Ca^{2+}]_e$-dependent contraction was reduced in female compared with male IVC suggests reduction in the Ca^{2+} entry mechanisms of venous contraction and reduced VSM responsiveness to Ca^{2+} entry in female veins. Extracellular Ca^{2+} enters VSM cells through store-operated, ligand-gated, and voltage-gated Ca^{2+} channels.[17,47] Although agonists such as PHE and AngII may activate various types of Ca^{2+} channels, membrane depolarization by high KCl mainly activates voltage-gated Ca^{2+} channels. The observation that the KCl contraction was reduced in female compared with male IVC, suggests reduced amount or response to Ca^{2+} entry through voltage-gated Ca^{2+} channels. However, gender-related reduction in Ca^{2+} entry through store-operated or ligand-gated Ca^{2+} channels in female veins cannot be completely ruled out and should be further examined using electrophysiologic and patch clamp techniques.

Our results in rat IVC are consistent with reports that the Ca^{2+} entry mechanisms are reduced in female com-

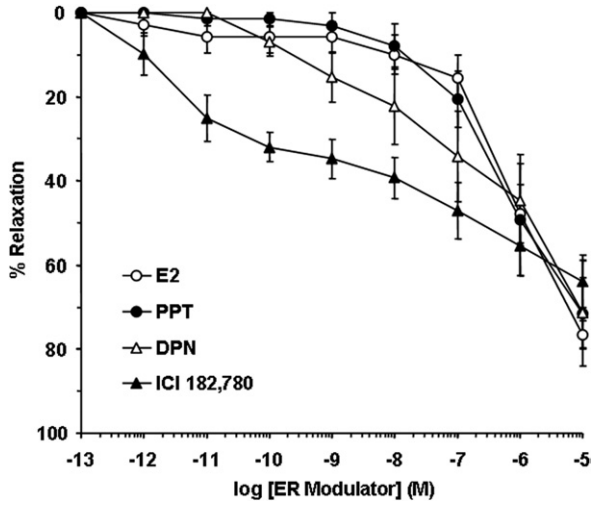

Fig 6. Estrogen receptor (ER)-mediated venous relaxation was tested in female rat inferior vena cava (IVC) segments. Segments of female rat IVC were stimulated with phenylephrine (*PHE*; 10^{-5} M), and the steady-state contraction was recorded. Increasing concentrations (10^{-12} to 10^{-5} M) of 17β-estradiol (E2, activator of most ERs), the ERα agonist 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-tris-phenol *(PPT)*, the ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile *(DPN)* or ICI 182,780, the ERα/ERβ antagonist and G protein-coupled receptor 30 stimulant, were added and the percentage relaxation of PHE contraction was measured. Data represent the means ± standard error of the mean for six vein segments. *Significantly different ($P < .05$).

pared with male rat aorta.[16,17] The present results are also consistent with our recent finding of reduced $[Ca^{2+}]_i$ and Ca^{2+}-dependent constriction in female rat IVC segments loaded with the Ca^{2+} indicator fura-2.[37] The reduced Ca^{2+} entry could be due to decreased expression/activity of Ca^{2+} channels in female IVC.

We asked whether the decreased Ca^{2+}-dependent mechanisms of venous contraction in females occur as a result of increased endothelium-dependent venous relaxation pathways. Ach-induced vascular relaxation is often used to test the endothelium-dependent mechanisms such as the NO-cyclic guanosine monophosphate (cGMP) vascular relaxation pathway.[48,49] The observation that Ach-induced relaxation was greater in female than male IVC suggests an enhanced endothelium-dependent venous relaxation mechanism in female IVC. The enhanced Ach-induced relaxation in female IVC could be due to enhanced release of endothelium-derived factors such as NO. However, an increased responsiveness of VSM of female rats to vasodilators cannot be excluded under these conditions.

We tested whether the sex differences in venous contraction and endothelium-dependent relaxation are related to the expression/activity of venous ERs. Most of our knowledge regarding the amount and function of the vascular ERs are derived from studies on arteries.[15,29-32] The present study demonstrates that ERs are expressed in rat IVC and that the amounts of ERα, ER_B, and GPR30 are greater in females than males.

These Western blot data in rat veins should be interpreted with extreme caution because they are difficult to extrapolate to human veins. Interestingly, previous immunohistochemical studies in human veins have demonstrated that ERs were more abundant in tissue sections of varicose segments than in the nonvaricose parts of the same vein, especially in women.[50] It has also been shown that smooth muscle cell hypertrophy in varicose veins is associated with increased expression of ERβ.[51] E2 binds ERs and induces both genomic and nongenomic effects that promote arterial vasodilation.[23-28] The observation that E2 caused significant relaxation of female rat IVC suggests that the venous ER are functional and are involved in venous relaxation. E2 binds to all ERs and the venorelxant effects of E2 likely involve activation of most ER subtypes.

The advent of specific agonists to ER subtypes allowed the examination of the contribution of specific ERs to the E2 effects. The observation that selective ERα and ERβ agonists produced significant relaxation in female rat IVC supports the contention that these ER subtypes contribute significantly to venous relaxation in the female rat. The venorelaxant effects of the ERβ agonist DPN were reversible, supporting specificity of the ERβ-mediated effects. In contrast, the vasorelaxant effects of the ERα agonist PPT were not reversible, suggesting that ERα activation causes persistent venous relaxation. However, a potential PPT-mediated nonspecific blockade of α-adrenergic receptors or direct inhibitory effects on the contractile myofilaments can not be excluded and should be further examined in future studies.

GPR30 is a new transmembrane protein that binds E2 and mediates some of its rapid non-genomic effects.[33-36] Although ICI 182,780 is largely known as an ERα and ERβ antagonist, recent studies suggest that it may also activate GPR30.[43,44] The observation that ICI 182,780 caused significant relaxation of female IVC suggests an important role of GPR30 in E2-mediated venous relaxation in females.

E2 has been suggested to promote vasodilation by activating endothelium-dependent NO-cGMP, PGI_2-cyclic adenosine monophosphate (cAMP), and the EDHF relaxation pathway.[24,25] The observation that the NOS inhibitor L-NAME did not inhibit the E2-, PPT-, DPN-, or ICI 182,780-induced relaxation of rat IVC, suggests that the venous ER may not be coupled to activation of the NO-cGMP relaxation pathway. The present results can be explained by the possibility that venous ERs may be coupled to enhanced PGI_2-cAMP or EDHF-mediated hyperpolarization, or both, which in turn cause a reduction in VSM $[Ca^{2+}]_i$ and lead to venous relaxation.[52]

The present study focused on the effects of E2 and ERs on venous function. Progesterone is another female sex hormone that may affect venous function. Previous studies have demonstrated the expression of progesterone receptors in human saphenous vein, especially in women.[50,53] We have also demonstrated rapid inhibitory effects of progesterone on arterial smooth muscle function.[16,46] Studying the effects of progesterone on female veins and its

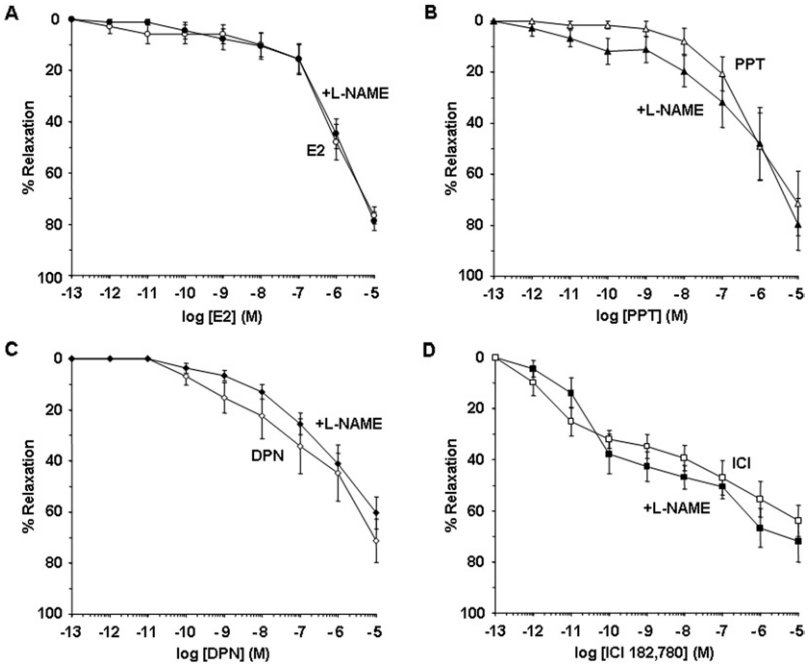

Fig 7. Effect of nitric oxide synthase (NOS) blockade on estrogen receptor (ER)-mediated venous relaxation was tested in female rat inferior vena cava (IVC). Segments of female rat IVC were nontreated or pretreated with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (*L-NAME*; 3×10^{-4} M) for 15 minutes and then stimulated with phenylephrine (PHE; 10^{-5} M). The steady-state contraction was recorded. Increasing concentrations (10^{-12} to 10^{-5} M) of **(A)** 17β-estradiol (E2), **(B)** 4,4,′4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-tris-phenol *(PPT)*, **(C)** 2,3-bis(4-hydroxyphenyl)-propionitrile *(DPN)*, or **(D)** ICI 182,780 were added and the percentage relaxation of PHE contraction was compared in L-NAME-treated and nontreated IVC segments. Data represent the means ± standard error of the mean for five to six vein segments.

potential role in the gender differences in venous function is an important area for future investigation.

CONCLUSIONS

α-Adrenergic, AngII, depolarization-induced, and $[Ca^{2+}]_e$-dependent venous contraction are reduced in female rat IVC possibly due to increased ER expression/activity and the enhanced endothelium-dependent but NO-independent relaxation pathway. The results suggest inherent sex differences in venous tissues, where the ER-mediated enhanced venous relaxation and decreased venous contraction would lead to more distensible veins in females. One limitation of the present study is that the experiments were conducted on rat IVC. Future experiments should examine whether the sex differences in venous function are also present in other rat veins, particularly those with more relevance to the lower extremity such as the iliac and femoral veins. We should also note that the rat is a consistent species, and studies on rat veins avoid the differences related to age, body weight, ethnic background, comorbidities, and other confounding factors that are often encountered in human studies.

The present study on rat veins provided critical mechanistic information and suggested sex-related differences in the mechanisms of venous contraction and relaxation. Performing similar venous function experiments to study the sex differences in human great saphenous vein and in varicose vs nonvaricose veins would add to our understanding of the mechanisms of venous disease.

AUTHOR CONTRIBUTIONS

Conception and design: JR, RK
Analysis and interpretation: JR, RK
Data collection: JR, XQ, KB, RK
Writing the article: JR, RK
Critical revision of the article: JR, RK
Final approval of the article: JR, XQ, KB, RK
Statistical analysis: JR, RK
Obtained funding: RK
Overall responsibility: RK

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