Gender-Specific Reduction in Contraction is Associated with Increased Estrogen Receptor Expression in Single Vascular Smooth Muscle Cells of Female Rat

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Key Words

Estrogen • Sex hormones • Blood vessels • Cardiovascular disease

Abstract

Gender differences in the incidence of cardiovascular disease have been related to plasma estrogen levels; however, the role of vascular estrogen receptor (ER) subtypes in these sex differences is less clear. We tested whether the gender differences in vascular smooth muscle (VSM) function reflect differential expression/activity of ER α , ER β and the newly-identified GPR30. Single aortic VSM cells (VSMCs) were freshly isolated from male and female Sprague-Dawley rats, and their contraction to phenylephrine (PHE, 10^{-5} M), AngII (10^{-7} M) and membrane-depolarization by KCl (51 mM) was measured in the absence or presence of 10^{-6} M 17 β -estradiol (E2, stimulant of most ERs), PPT (ER α agonist), DPN (ER β agonist), and ICI 182,780 (an ER α /ER β antagonist with GPR30 agonistic properties). The cells were fixed and fluorescently labeled with ER α , ER β or GPR30 antibody, and the subcellular distribution of ERs was examined using digital imaging microscopy. The mRNA expression and protein amount of aortic ER subtypes was examined using RT-PCR and Western

blots. PHE, AngII, and KCl caused less contraction in VSMCs of females than males. Pretreatment of VSMCs with E2 reduced PHE-, AngII- and KCl-induced contraction in both males and females. PPT caused similar inhibition of PHE-, AngII- and KCl-induced contraction as E2, suggesting a role of ER α . DPN mainly inhibited PHE and KCl contraction, suggesting an interaction between ER β and Ca $^{2+}$ channels. ICI 182,780 did not reduce aortic VSMC contraction, suggesting little role for GPR30. RT-PCR and Western blots revealed greater expression of ER α and ER β in VSMCs of females than males, but similar amounts of GPR30. The total immunofluorescence signal for ER α and ER β was greater in VSMCs of females than males, and was largely localized in the nucleus. GPR30 fluorescence was similar in VSMCs of males and females, and was mainly in the cytosol. In PPT treated cells, nuclear ER α signal was enhanced. DPN did not affect the distribution of ER β , and ICI 182,780 did not significantly increase GPR30 in the cell surface. Thus, ER subtypes demonstrate similar responsiveness to specific agonists in VSMCs of male and female rats. The reduced contraction in VSMCs of females could be due to gender-related increase in the expression of ER α and ER β .

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Introduction

Cardiovascular disease is more common in men and postmenopausal women than in premenopausal women, suggesting vascular protective effects of endogenous estrogen (E2) [1-6]. The beneficial vascular effects of E2 have been ascribed to modification of circulating lipoproteins [3, 7], changes in blood coagulation [8], inhibition of intravascular accumulation of collagen [9], as well as genomic and non-genomic effects on the blood vessels [6, 10]. Genomic effects of E2 include stimulation of endothelial cell growth and inhibition of vascular smooth muscle (VSM) proliferation [2, 6]. E2 causes rapid non-genomic vasodilation of blood vessels via activation of endothelium-dependent vascular relaxation pathways [11, 12]. E2 also causes relaxation of endothelium-denuded blood vessels, suggesting direct action on VSM cells (VSMCs) [13, 14].

Many of the effects of E2 are mediated via estrogen receptors (ERs) [15]. ERs have been characterized in the female reproductive tract, mammary glands and other tissues including blood vessels [15-18]. Two major ERs, ER α and ER β , have been suggested to mediate many of the genomic effects of E2 [15, 17, 18]. Surface membrane ERs have also been implicated in the rapid vasodilator effects of E2 [19]. Also, a novel trans-membrane G protein-coupled receptor GPR30 has been shown to bind E2 and to mediate some of the rapid effects of E2 [20-23].

Sex differences in vascular function have been demonstrated in the aorta, coronary, mesenteric and renal arteries of mice, rats and pigs [24-31]. We have previously shown that the aortic contraction is reduced in female compared with male rats [25, 28]. Also, studies from our laboratory and others have suggested that the sex differences in arterial contraction are likely due to the high plasma levels of E2 in females [25, 28, 32, 33]. However, the role of vascular ER subtypes in these sex differences is less clear.

The purpose of this study was to test the hypothesis that the gender differences in VSM contraction reflect differences in the expression, subcellular distribution, and/or activity of specific ER subtypes. Because E2 may affect various types of vascular cells, studying the role of gender and ER in a multicellular vascular preparation could be difficult. Therefore, this study was performed on single VSMCs freshly isolated from the aorta of male and female rats.

Materials and Methods

Animals

Age-matched male and female Sprague-Dawley rats (12 weeks old, Charles River laboratory, Wilmington, MA) were housed in the animal facility and maintained on *ad libitum* standard rat chow and tap water in 12 hr/12 hr light/dark cycle. Adult female rats were randomly selected regardless of the stage of the ovarian cycle. Because the ovarian cycle in rats is frequent (every 4 to 5 days) and the estrous stage is short (12 hours), the average data from all adult female rats should cancel out any possible fluctuations in estradiol levels at the specific stages of the ovarian cycle and should roughly represent the average changes in VSMC contraction during all stages of the ovarian cycle. Rats were euthanized by inhalation of CO₂, and the thoracic aorta was rapidly excised. All procedures followed the guidelines of the Animal Care and Use Committee at Harvard Medical School and the American Physiological Society.

Tissue preparation

The thoracic aorta was placed in oxygenated Krebs solution, cleaned of connective and adipose tissue, opened by cutting along its longitudinal axis, then sectioned into 2 x 2 mm strips.

Single cell isolation

Single aortic VSMCs were freshly isolated as previously described with a few modifications, specifically avoiding aspiration through a pipette or centrifugation [28, 34]. Rat aortic strips (50 mg) were placed in a siliconized flask containing a tissue digestion mixture of collagenase type II (236 U/mg protein activity, Worthington, Freehold, NJ), elastase grade II (3.25 U/mg protein activity, Boehringer Mannheim, Indianapolis, IN), and trypsin inhibitor type II-soybean (10,000 U/ml, Sigma, St. Louis, MO) in 7.5 ml of Ca²⁺- and Mg²⁺-free Hanks solution supplemented with 30% bovine serum albumin (Sigma). The tissue was incubated 3 consecutive times in the tissue digestion mixture to yield 3 batches of cells. For the first batch, the tissue was incubated with 6 mg collagenase, 4 mg elastase and 147 μ l trypsin inhibitor for 90 min. For batch 2 and 3, the collagenase was reduced to 3 mg, the trypsin inhibitor was reduced to 122 μ l and the incubation period was reduced to 30 min. The tissue preparation was placed in a shaking water bath at 34°C in an atmosphere of 95% O₂ and 5% CO₂. At the end of each incubation period, the preparation was rinsed with 12.5

ml of Hanks with albumin and poured over ice-cooled wells with glass bottom (Biotechs, Butler, PA) (for cell contraction studies), or over glass cover slips placed in 6-well plates (for immunofluorescence studies). By using the gravitational force, single VSMCs were allowed to settle and adhere to the glass coverslips. Ca^{2+} was gradually added back to the preparation in order to avoid the “calcium paradox” [35]. The cell isolation procedure consistently yielded spindle-shaped and viable VSMCs that showed significant contraction in response to contractile stimuli. Although differences between female and male cell adhesion to the glass coverslips may affect contraction, such possibility was not tested in the present study.

Cell contraction

Freshly isolated aortic VSMCs in the glass-bottom wells were placed on a slide warmer at 37°C (Biotechs) and on the stage of an inverted Nikon Eclipse-300 microscope. The cells were viewed using a 20X or 40X objective, and cell images were acquired using a Nikon digital camera and image acquisition software. Three different VSM activators were used. The α -adrenergic agonist phenylephrine (PHE) was used to stimulate both Ca^{2+} release from the intracellular stores and Ca^{2+} entry from the extracellular space [36]. AngII was used as another activator of receptor-mediated mechanisms via the AT_1 receptor. Membrane depolarization by high KCl solution was used to activate Ca^{2+} entry from the extracellular space through voltage-gated Ca^{2+} channels [28, 36]. The changes in cell length in response to PHE (10^{-5} M), Ang II (10^{-7} M) and high KCl depolarizing solution (51 mM) were measured and the magnitude of cell contraction was expressed as $((L_i - L)/L_i) \times 100$, where L_i is the initial cell length and L is the final cell length. To test the role of ERs, the PHE, AngII and KCl contraction was measured in VSMCs pretreated with 10^{-6} M 17β -estradiol (E2, activator of most ERs). We have previously examined the dose-response curve for E2 in isolated vascular strips and VSMCs and demonstrated that E2 causes significant relaxation at 10^{-6} M concentration or higher [13, 28]. Based on these previous observations we used E2 (10^{-6} M) in the present study. We should note that these E2 concentrations are significantly higher than the nM plasma levels observed *in vivo*. However, E2 is a lipophilic compound, and the plasma levels of E2 may not reflect the concentration of E2 in the vascular tissue. To further examine the role of specific ER subtypes, the PHE, AngII and KCl contraction was measured in VSMCs pretreated with 10^{-6} M 4,4',4''-(4-propyl-[1H]-

pyrazole-1,3,5-triyl)-tris-phenol (PPT, selective ER α agonist) [37, 38], diarylpropionitrile (DPN, selective ER β agonist) [39], and ICI 182,780 (fulvestrant, an ER α /ER β antagonist that also possesses agonistic activity at GPR30) [40, 41].

Immunofluorescence

VSMCs nontreated or treated with PPT, DPN, or ICI 182,780 were fixed for 10 min in 2% paraformaldehyde in PBS (pH 7.4). The excess fixative was quenched two times 5 min each with 0.1 mM glycine in 1% bovine serum albumin (BSA) Hanks. The cells were permeabilized with 0.1% Triton X-100 in 1% BSA Hanks for 10 min and then washed 3 times 5 min each with 0.05% Triton X-100 in 1% BSA Hanks. Cells were blocked in 1% BSA Hanks supplemented with 2% goat serum for 45 min and then reacted with polyclonal specific antibody to ER α (1:200), ER β (1:500) or GPR30 (1:200) (Affinity Bioreagents, Golden, CO). Cells were washed three times 10 min each in 1% BSA Hanks, then incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (Sigma) for 30 min. For double staining of the nucleus the DNA marker ethidium dimer (1:1000, Invitrogen, Carlsbad, CA) was used. Excess label was removed by washing in 1% BSA Hanks. All procedures were performed at 22°C.

Fluorescently-labeled cells were viewed on a Nikon microscope using a 40X objective. For FITC, an excitation filter at 485 nm, a dichroic filter at 500 nm, and a long-pass filter at 530 nm were used. For ethidium dimer, a Texas Red excitation filter at 488 nm, a dichroic filter at 500 nm, and a long-pass filter at 615 were used. An excitation light shutter was opened only when necessary for taking measurements. Fluorescent images were acquired using a Quantix cooled intensified charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) and analyzed using Metamorph Imaging System software (Universal Imaging Corporation, West Chester, PA). Images were background subtracted. Average cell fluorescence intensity was measured by tracing the area along the borders of the cell image, integrating the total cell fluorescence intensity in the cell image, and dividing by the number of pixels in the cell image. To test for subcellular localization of ERs at the cell surface, a line was traced along the cell border and the average pixel intensity was determined. Nuclear staining was determined by tracing the area of ethidium dimer fluorescence. We have used a similar fluorescence microscopy approach to determine the subcellular distribution of protein kinase C (PKC) in VSMCs and

demonstrated that PKC activation with PHE causes translocation of PKC from the cytosol to the vicinity of the surface membrane [42, 43]. In these VSMCs, the surface membrane area was identified using the cell membrane marker 7-decylBODIPY-1-propionic acid, and the nuclear area was identified using ethidium dimmer. The difference between the total fluorescence and the combined fluorescence in the surface membrane and nucleus represented the cytosolic fluorescence. To test for localization at the cell surface, the peripheral cell surface/cytosolic fluorescence ratio was measured. To test for localization in the nucleus, the nuclear/cytosolic fluorescence ratio was measured.

Real-Time RT-PCR analysis

RNA was isolated from endothelium-denuded aortic tissue samples using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA). 1 µg of total RNA was used for reverse transcription to synthesize single-strand cDNA in a 20 µl-reaction mixture according to the protocol of First-Strand cDNA Synthesis Kit (Amersham Biosciences, Pittsburgh, PA). 2 µl of cDNA dilution (1:5 for ERα and ERβ, and 1:25 for α-actin) of reverse transcription (RT) product was applied to 20 µl RT-PCR reaction. Quantification of gene expression was performed using real-time quantitative RT-PCR machine (Mx4000 Multiplex Quantitative PCR System, Stratagene, La Jolla, CA) and employing published oligonucleotide primers specific for ERα and ERβ (Integrated DNA Technologies (IDT), Coralville, IA), and the Bio-Rad iQ SYBR Green Supermix, which employs the fluorescein compound *SYBR-Green* for amplicon detection (Bio-Rad, Hercules, CA). α-Actin primer with expected product length of 285 base pairs was included in the RT-PCR reaction as internal standard to normalize the results.

Primers Sequences

ERα	Forward	5'- AATTCTGACAATCGACGCCAG -3'
	Reverse	5'- GTGCTTCAACATTCTCCCTCCTC -3'
ERβ	Forward	5'- AAAGCCAAGAGAAACGGTGGGCAT -3'
	Reverse	5'- GCCAATCATGTGCACCAAGTTCCTT -3'
α-actin	Forward	5'- GACACCAGGGAGTGATGGTT -3'
	Reverse	5'- GTTAGCAAGGTCGGATGCTC -3'

PCR was carried out with 1 cycle for 10 min at 95°C then 40-45 cycles of 30 sec denaturation at 95°C, 45 sec of annealing at 56°C, and 30 sec of extension at 72°C, followed by 1 min of final extension step at 95°C. The number of PCR cycles varies according to the expression level of the target gene. An appropriate primer concentration and number of cycles was determined to ensure that the PCR is taking place in the linear range

and thereby guarantees a proportional relationship between input RNA and the cycles readout. The relative amount of gene expression was calculated by comparison of cycle thresholds with the housekeeping gene α-actin.

Western blot analysis

Endothelium-denuded aortic strips from the same rats used for the cell isolation studies were homogenized in a buffer containing 20 mM 3-[N-morpholino]propane sulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol, 1.2 mM EDTA, 0.02% BSA, 5.5 µM leupeptin, 5.5 µM pepstatin, 2.15 µM aprotinin and 20 µM 4-(2-aminoethyl)-benzenesulfonyl fluoride, using a 2 ml tight-fitting homogenizer (Kontes Glass). The homogenate was centrifuged at 10,000g for 2 min and the supernatant was used as whole tissue fraction. Protein concentration was determined using a protein assay kit (Bio-Rad).

Aortic tissue homogenate was subjected to electrophoresis on 8% SDS polyacrylamide gel then transferred electrophoretically to nitrocellulose membrane. The membrane was incubated in 5% dried non-fat milk for 1 hr, then treated with polyclonal antibody to ERα, (1:100), ERβ (1:1000) and GPR30 (1:200) (Affinity bioreagents) for 24 hr. α-Actin was used as an internal control and was detected using a very specific monoclonal anti-actin antibody at an extremely low titer (1:500000, Sigma). The nitrocellulose membrane was washed 5 times 15 min each in TBS-Tween then incubated in horseradish peroxidase-conjugated secondary antibody (1:1000) for 1.5 hr. The blots were visualized with Enhanced Chemi Luminescence (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.

Solutions

Normal Krebs solution was used for dissecting the tissue and contained (in mM): 120 NaCl, 5.9 KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 11.5 dextrose, 2.5 CaCl₂, and 1.2 MgCl₂ at pH 7.4. Hank's solution was used for cell isolation and for performing the experiments and contained (in mM): 137 NaCl, 5.4 KCl, 0.44 KH₂PO₄, 0.42 Na₂HPO₄, 4.17 NaHCO₃, 5.55 dextrose and 10 HEPES. Hank's solution was bubbled for 30 min with a 95% O₂ 5% CO₂ mixture and the pH was adjusted to 7.4. The high KCl (51 mM) depolarizing solution had the same composition as normal Hank's solution with equimolar substitution of NaCl with KCl.

Fig. 1. Effect of PHE, AngII and membrane depolarization by high KCl on contraction of VSMCs of male and female rats. Freshly isolated aortic VSMCs from male and female rats were viewed on the stage of a Nikon microscope and images of the single cells at rest were acquired. The cells were stimulated with PHE (10^{-5} M) (A), AngII (10^{-7} M) (B) or KCl (51 mM) (C) for 10 min and the cell contraction was measured as described in the Methods. Total magnification = 400. Calibration bar in pictures = $10\mu\text{m}$. Cumulative data ($n= 11$ to 28 cells from 5 to 7 different rats) were presented as the means $\pm$ SEM in bar graphs. * Measurement in females are significantly different ($p<0.05$) from corresponding measurements in males.

Drugs and chemicals

Stock solutions of PHE (10^{-1} M, Sigma) and AngII (10^{-2} M, Sigma) were prepared in distilled water. Stock solution of 17β -estradiol (2,3,5[10]-Estratriene-3,17 β -diol, Sigma) was prepared as 5×10^{-2} M in 100% ethanol. Stock solutions of PPT, DPN, and ICI 182,780 (10^{-1} M, Tocris) were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in the experimental solution was $<0.1\%$. All other chemicals were of reagent grade or better.

Statistical analysis

Data were collected from 2 to 7 cells from each single rat for each single experiment, and the cumulative data from the total number of cells “n” from 5 to 7 different rats for each experimental protocol were used for analysis. The data were first analyzed using ANOVA. When a statistical difference was observed, the data were further analyzed using Student’s *t*-test for unpaired data for comparison of two means. Data were presented as means $\pm$ S.E.M, and differences were considered statistically significant if $p<0.05$.

Results

The cell isolation procedure produced cells of variable lengths. Only spindle-shaped cells $\geq 50\mu\text{m}$ in length were selected for this study. In resting cells of male rats, the average cell length was $61.86 \pm 0.86\mu\text{m}$, and was not significantly different from the resting cell length in female

Fig. 2. Representative images demonstrating the effect of ER modulators on cell contraction. VSMCs from male (A,B,C) or female rats (D,E,F) were either at rest (A,D) or pretreated with (10^{-6} M) of 17β -estradiol (E2, activator of most ERs) (E), PPT (ER α agonist) (B), DPN (ER β agonist) (F), or ICI 182,780 (ER α /ER β antagonist, and GPR30 stimulant) (C), for 10 min then stimulated with PHE (10^{-5} M) and the effects on cell contraction were observed. Total magnification = 400. Calibration bar in pictures = $10\mu\text{m}$.

rats $60.17 \pm 0.91\mu\text{m}$. Aortic VSMCs of male rats were responsive to contractile stimuli. PHE (10^{-5} M) and

Fig. 3. Effect of ER modulators on PHE, AngII and KCl-induced contraction in VSMCs of male and female rats. VSMCs of male (left panels) or female rats (right panels) were stimulated with PHE (10^{-5} M) (A), AngII (10^{-7} M) (B) or KCl (51 mM) (C) in the absence or presence of (10^{-6} M) of 17 β -estradiol (E2), activator of most ERs), PPT (ER α agonist) (C), DPN (ER β agonist) (D), or ICI 182,780 (ER α /ER β antagonist, and GPR30 stimulant), and the cell contraction was measured. Data represent the means $\pm$ SEM (n= 10 to 32 cells from 5 to 7 different rats). * Measurements in the presence of E2, PPT, DPN, or ICI 182,780 are significantly different ($p<0.05$) from corresponding measurements in the absence of the ER modulator.

AngII (10^{-7} M) caused cell contraction that reached steady-state in ~ 5 min (Fig. 1). Also, membrane depolarization by 51 mM KCl caused significant contraction in VSMCs of male rats (Fig. 1). The PHE, AngII and KCl-induced contraction was significantly reduced in VSMCs of female compared with male rats (Fig. 1).

Pretreatment of VSMCs with E2 (10^{-6} M) or the ER α agonist PPT (10^{-6} M) for 10 min did not cause significant change in resting cell length, but significantly

Fig. 4. Expression and protein amount of ER α , ER β and GPR30 in VSM of male and female rats. Real Time RT-PCR were used to measure the mRNA expression (A) and Western blot analysis was used to measure protein amount (B) of ER α , ER β , and GPR30 in aortic tissue homogenate from male and female rats. Measurements were normalized to the house keeping gene or protein actin. Data represent the means $\pm$ SEM (n= 3 to 4). * $p<0.05$.

reduced PHE, AngII and KCl-induced contraction in cells of male and female rats (Fig. 2 and 3). Pretreatment of VSMCs with the ER β agonist DPN (10^{-6} M) significantly reduced PHE and KCl contraction, but insignificantly reduced AngII-induced contraction in cells of male and female rats (Fig. 2 and 3). In contrast, pretreatment of the cells with ICI 182,780 did not significantly affect PHE, AngII or KCl-induced contraction in VSMCs of male or female rats (Fig. 2 and 3).

Fig. 5. Immunofluorescence of ER α , ER β and GPR30 in VSMCs of male and female rats. Resting aortic VSMCs from male and female rats were fixed, permeabilized and labeled with antibodies to ER α (1:200) (A), ER β (1:500) (B) and GPR30 (1:200) (C). Images of fluorescently-labeled cells were acquired, the total cell fluorescence was divided by the number of pixels in the cell image, and the average cell fluorescence was measured. Total magnification = 400. Calibration bar in pictures = 10 μ m. Data represent the means $\pm$ SEM (n= 15 to 41 cells from 5 to 7 different rats). * p<0.05.

Fig. 6. Subcellular distribution of ER α , ER β and GPR30 in VSMCs of male and female rats. Resting aortic VSMCs from male and female rats were fixed, permeabilized and labeled with primary antibodies to ER α (1:200) (A), ER β (1:500) (B) and GPR30 (1:200) (C), and FITC-labelled IgG (Green). The center of the cell was determined by co-staining with the DNA marker ethidium dimer (Red). Co-localization of the specific ER with the DNA marker ethidium dimer in the nucleus is presented in yellow. The cell image was dissected into 3 components: the cell surface, the center (corresponding to the nucleus), and the cytosol (total fluorescence - combined peripheral and central fluorescence) and the average pixel intensity in each segment was measured. Total magnification = 400. Calibration bar in pictures = 10 μ m. Data represent the means $\pm$ SEM (n= 15 to 41 cells from 5 to 7 different rats).

RT-PCR analysis revealed greater expression of ER α and ER β in the aorta of female compared with male rats (Fig. 4A). Western blot analysis of aortic tissue homogenate from male and female rats revealed

immunoreactive bands corresponding to ER α at 64 kDa, ER β at 55 kDa, and GPR30 at 50 kDa. Optical density analysis revealed greater protein amount of ER α and ER β , but similar amount of GPR30 in aortic

Fig. 7. Effect of specific ER modulators on the subcellular distribution of ER α , ER β and GPR30 in VSMCs of male and female rats. VSMCs from male and female rats were nontreated or treated for 10 min with PPT (ER α agonist), DPN (ER β agonist) or ICI 182,780 (ER α /ER β antagonist, and GPR30 stimulant). The cells were fixed, permeabilized and labeled with antibody to either ER α (1:200) (A and D), ER β (1:500) (B and E) or GPR30 (1:200) (C and F). The average fluorescence in the cell surface and cytosol was used to measure the periphery/cytosol ratio (Top panels). The average fluorescence in the nuclear area (as determined by ethidium dimer) and the cytosol was used to measure the nuclear/cytosol ratio (Bottom panels). Data represent the means $\pm$ SEM (n= 14 to 43 cells from 5 to 7 different rats). * $p < 0.05$.

tissue homogenate of female compared with male rats (Fig. 4B).

Immunofluorescence experiments indicated that in resting VSMCs the average fluorescence signal for ER α or ER β was greater in cells of female compared with male rats. In contrast, the GPR30 signal was not significantly different in VSMCs of female and male rats (Fig. 5).

Optical dissection of the subcellular distribution of ER α and ER β revealed a low fluorescence signal in the cell periphery, a greater fluorescence in the cytosol, and a prominent fluorescence signal in the center of the cell coinciding with the nucleus (Fig. 6A and 6B). In contrast, the subcellular distribution of GPR30 was mainly in the

cytosol, with less fluorescence signal in the periphery and the center of the cells (Fig. 6C). The distribution pattern of ER α , ER β and GPR30 in the periphery, cytosol, and nuclear segments was not significantly different in VSMCs of female and male rats.

To test whether ER activation is associated with its translocation between the cell surface and the cytosol, the periphery/cytosol fluorescence ratio was measured at rest and during stimulation by a specific ER agonist. An increase in periphery/cytosol ratio would indicate increased localization at the cell surface. Also, to test whether ER activation is associated with its translocation from the cytosol to the nucleus, the nuclear/cytosol fluorescence ratio was measured at rest and during

activation by a specific ER stimulant. An increase in the nuclear/cytosol ratio would indicate increased localization in the nucleus. The ER α agonist PPT did not cause significant change in the distribution of ER α at the cell surface of VSMCs of male or female rats (Fig. 7A). In contrast, PPT significantly increased the nuclear distribution of ER α in VSMCs of female rats (Fig. 7D). The ER β agonist DPN did not significantly increase the distribution of ER β at the cell surface (Fig. 7B) or the nucleus (Fig. 7E) of VSMCs of male or female rats. Also, no significant increase in the distribution of GPR30 at the cell surface (Fig. 7C) or the nucleus (Fig. 7F) was observed in VSMCs of male or female rats when treated with the GPR30 stimulant ICI 182,780.

Discussion

The main findings of the present study are: 1) VSMC contraction to three different modes of stimulation is reduced in cells of female compared with male rats, 2) ER α and ER β agonists cause similar inhibition of VSMC contraction in males and females, 3) The expression of ER α and ER β is greater in VSMCs of females compared with males, and is localized mainly in the nucleus, 4) GPR30 demonstrates similar expression in males and females, is mainly in the cytosol, and its activation does not appear to affect VSM contraction or its subcellular location.

Gender differences in vascular function have been demonstrated in numerous blood vessels from various species [24-31]. We have previously demonstrated gender-related reduction in contraction of aortic strips isolated from female compared with male rats [25, 44]. The aortic VSM contraction was enhanced in ovariectomized compared with intact females, but not different in castrated compared with intact male rats. These studies suggested that the sex differences are more likely due to differences in the amount/activity of estrogen and ER, rather than testosterone and the androgen receptor. Also, several studies have examined the gender differences and the effects of E2 in arterial rings [13, 24, 27]. However, the multicellular nature of blood vessels has made it difficult to discriminate between the specific roles of the endothelium and VSM in the gender differences in vascular function. The present observations in freshly isolated single aortic VSMCs of Sprague-Dawley rats are consistent with our previous report in Wistar-Kyoto and spontaneously hypertensive rats [28] and demonstrate gender-specific reduction in contraction

in VSMCs of female compared with male rats. The reduction in both PHE and AngII-induced contraction in female VSMCs suggests a reduction in a common-receptor mediated mechanism of VSM contraction. We have previously demonstrated that the transient cell contraction and $[Ca^{2+}]_i$ in response to caffeine and PHE in Ca^{2+} -free medium were not different in male and female WKY rat aortic VSMCs, suggesting that the gender differences in cell contraction are not related to differences in the intracellular Ca^{2+} stores. Membrane depolarization by high KCl is known to stimulate mainly Ca^{2+} entry from the extracellular space through voltage-gated Ca^{2+} channels [28, 36]. We have previously demonstrated that the maintained PHE and KCl-induced contraction and $[Ca^{2+}]_i$ were reduced in VSMCs of female compared with male WKY rats, suggesting differences in the Ca^{2+} -entry mechanisms from the extracellular space [28]. The observed reduction in KCl contraction in female Sprague-Dawley VSMCs is consistent with the possibility that the gender-specific reduction in VSMC contraction is related to differences in the Ca^{2+} entry mechanism of VSM contraction.

Gender differences in vascular contraction have been partly related to differences in the plasma levels of E2, being higher in females compared with males [25, 28, 32, 33]. Therefore, the results from the VSMC contraction experiments should be interpreted with caution as the adult female rats were randomly selected regardless of the stage of the estrous cycle. Because the plasma levels of E2 are known to change during various stages of the ovarian cycle and E2 could affect VSMC reactivity, studies on VSMCs isolated from female rats at the specific stage of the estrous cycle as monitored by vaginal cytology should be examined in future studies.

ER subtypes have also been identified in numerous vascular preparations [16-18, 21, 29, 45]. Previous imaging studies were able to localize ER α , but not ER β , in *en face* arterial endothelium of female rat vessels [46]. Also, studies from our laboratory and others have shown that the interaction of E2 with ERs causes direct vasodilation of blood vessels via endothelium-dependent and endothelium-independent mechanisms [5, 6, 10, 19]. The present study provides evidence for gender-related difference in the amount of ER α and ER β , being greater in VSMCs of females compared with males because: 1) RT-PCR analysis showed greater mRNA expression of ER α and ER β in female aorta, 2) Western blot analysis revealed increased protein amount of ER α and ER β in aortic tissue homogenate from female rats, and 3) Immunofluorescence experiments on single cells

demonstrated greater total fluorescence signal for ER α and ER β in female than male VSMCs. Although it is difficult to reach a definitive conclusion regarding the amount of ER based on any one of these techniques alone, the consistent findings using three different techniques raise the likelihood of meaningful data and support the conclusions regarding the amount of ER subtypes in VSM.

The present RT-PCR and Western blot data in the rat aortic VSMCs are consistent with our previous report that the expression of ER α and ER β is greater in venous tissues from female compared with male Sprague-Dawley rats [47]. We should note that some studies have reported that only the ER β isoform level showed a clear gender difference, being lower in VSMCs from female than in cells derived from male rats [48]. Other studies have shown that VSMCs from female rat express less ER α and ER β than VSMCs from male rats [49]. The causes of the differences in the results between those studies and the present study are unclear. However, in those reports the rat strain studied could not be verified. Also, those studies were performed on cultured aortic VSMCs, which are phenotypically different from the freshly isolated contractile VSMCs used in the present study.

Although the biochemical and immunofluorescence studies provide important information regarding the relative abundance of ERs in aortic VSM of female rats, they do not necessarily indicate whether these ERs are functional. ER α and ER β in VSM could mediate multiple functions including: 1) genomic effects on the expression of membrane channel or contractile protein that eventually affect VSM contraction, 2) rapid non-genomic effects that directly affect the mechanisms of VSM contraction, and 3) genomic nuclear events that are unrelated to VSM contraction and likely involved in modulating cell growth and proliferation. The reduced VSMC contraction to PHE, AngII and KCl, and the increased expression of ER α and ER β in female aorta are consistent with a possible genomic role of these ER subtypes on the Ca²⁺ entry mechanism of VSM contraction. To determine whether ER α and ER β also mediate rapid non-genomic effects in VSM we sought to test the effects of modulators of specific ER subtypes on VSMC contraction. Previous studies to evaluate the effects of ER subtypes on arterial tone have shown variable results. It has been shown that activation of either ER α or ER β decreases rat pulmonary arterial tone; however, the contribution of a specific ER subtype appears to be stimulus specific, with ER α primarily modulating PHE-induced vasoconstriction, while ER β inhibiting hypoxia-induced pulmonary vasoconstriction [50]. It has

also been shown that both PPT, activator of ER α [37, 38], and DPN, selective ER β agonist [39], cause acute relaxation in isolated rat mesenteric arteries, but PPT has a significantly greater vasodilatory effect [51]. Other studies demonstrated that E2 causes acute relaxation of rat aorta solely through ER α [52]. Also, studies on subcutaneous arteries from postmenopausal women have shown that ER β expression was enhanced in the vascular wall from women with coronary heart disease, while ER α was predominant in control women [53]. In the present study, both E2, an activator of most ERs, and PPT, activator of ER α [37, 38], caused significant reduction of PHE, AngII and KCl contraction, suggesting a role for ER α in mediating the inhibition of a common VSM signaling pathway. Because KCl contraction is mainly due to Ca²⁺ entry from the extracellular space [36], the ER α -mediated inhibition of VSM contraction is likely due to inhibition of Ca²⁺ entry mechanisms. This is supported by our previous report that E2 decreases KCl-induced Ca²⁺ influx in endothelium-denuded coronary artery strips and in isolated rat aortic VSMCs [13, 28]. We do not wish to draw conclusions on whether the E2- or PPT-ER α interaction inhibits Ca²⁺ entry by direct or indirect action on plasmalemmal Ca²⁺ channels. Some studies have shown that E2 activates Ca²⁺-activated K⁺ channels, leading to membrane hyperpolarization, and inhibition of Ca²⁺ entry through voltage-gated channels [54, 55]. Other studies have shown that E2 blocks Ca²⁺ channels in cultured A7r5 and aortic VSMCs [56, 57]. The similarity in the inhibitory effects of E2 and the ER α agonist PPT on VSMC contraction suggests that ER α is a major mediator of the effects of E2 in VSM. Also, the prominent inhibitory effects of E2 and PPT on contraction of VSMCs of both males and females suggest that ER α is functional and mediates rapid non-genomic effects in VSM of both genders.

Another approach to test for functionality of the VSM ERs is to track the effects of modulators of specific ER subtypes on the distribution of ERs in VSMCs. The effects of E2 on target tissues have classically been thought to involve genomic actions mediated through interaction with cytoplasmic ERs and translocation of the E2-ER complex to the nucleus [58]. Although a genomic role of ER α on the expression of the Ca²⁺ channels might underlie the observed reduced contractility of female aortic VSMCs, it is less likely to account for the acute inhibitory effects of E2 or PPT on cell contraction. The acute nature of the vasorelaxant effects of E2 and PPT may represent additional non-genomic effects on the mechanisms of Ca²⁺ entry into VSM.

It is important to note that the distribution of ER α in resting VSMCs was mainly in the nucleus. Activation of ER α by PPT did not cause significant change in the distribution of ER α at the cell surface, suggesting that ER α -mediated inhibition of VSM contraction does not involve redistribution of ER α between the cytosol and the surface membrane. In contrast, treatment of VSMCs with PPT for 10 min caused significant increase in the nuclear localization of ER α . The rapid localization of ER α to the nucleus may not be related to its rapid inhibition of VSM contraction, but suggests potential ER α -mediated initiation of rapid nuclear events and genomic effects. This is supported by the recent report that estrogen directly and rapidly recruits specific transcriptional factors that then propagate distinct cascades of gene expression in blood vessels [59].

The ER β agonist DPN inhibited PHE and KCl-induced contraction, suggesting rapid non-genomic effect of ER β on Ca²⁺ entry mechanisms or downstream contraction pathways such as myosin light chain kinase. However, DPN did not inhibit AngII-induced contraction, suggesting that AngII could be activating other mechanisms that are not inhibited by ER β . AngII is known to stimulate AT1 receptors and to activate tyrosine kinase-dependent pathways in VSM [60-66], and the effects of ER β on these pathways need to be further examined. It is important to note that ER β was mainly localized in the nucleus, and its activation by DPN was not associated with increased localization at the cell surface, suggesting that the inhibitory effects of DPN on VSM contraction do not involve redistribution of ER β between the cytosol and the surface membrane. Also, DPN did not cause significant increase in the nuclear localization of ER β within the 10 min exposure time, suggesting that any potential ER β -mediated activation of nuclear events may be delayed beyond the 10 min of stimulation used in the present study.

GPR30 is a novel transmembrane G protein-coupled receptor that has been shown to bind E2 and to mediate some of its rapid non-genomic effects [20-23]. ICI 182,780 is a known ER α and ER β antagonist, that also possesses GPR30 agonistic properties [40, 41]. These unique properties make ICI 182,780 an interesting compound to test the effects of GPR30 while simultaneously blocking unwanted effects on ER α or ER β . The present experiments demonstrated that GPR30 is expressed at similar levels in male and female rats, is mainly distributed in the cytosol of VSMCs at rest,

and its activation by ICI 182,780 did not affect its subcellular location or VSMC contraction. We have performed preliminary experiments to test the effects of the GPR30 agonist G1 on female rat aortic strips. Our preliminary findings suggest that PHE (3×10^{-7} M) caused significant contraction in female rat thoracic aorta (0.64 ± 0.08 g/mg tissue weight), and that treatment with G1 (10^{-6} M) for 15 min did not cause any significant decrease in PHE contraction (0.61 ± 0.09 g/mg tissue weight, $94.18 \pm 3.38\%$) (Reslan & Khalil, unpublished observations). While these data suggest a little role of GPR30 in the gender differences and the effects of E2 on rat aortic VSM function, the results do not rule out potential role of GPR30 on other vascular cell types or other vascular beds. Future studies with G1 and other GPR30 agonists such as STX are needed to further clarify the role of GPR30 in the regulation of VSM contraction in various vascular beds. We should also note that the present subcellular distribution of ERs was determined using fluorescence microscopy. Given the limited resolution of this technique, future experiments should further verify the subcellular distribution of ERs in VSMCs using confocal microscopy.

In conclusion, aortic VSMCs of female rats exhibit gender-related reduction in contraction to three different modes of stimulation. The reduced VSMC contraction in females is likely related to increased expression of ER α and ER β . The localization of ER α and ER β mainly in the nucleus suggests genomic effects on the mechanisms of VSM contraction, and perhaps other long-term nuclear events. ER α and ER β demonstrate similar rapid responsiveness in male and female VSMCs, and specific ER α and ER β agonists may be useful for rapid non-genomic reduction of VSM contraction in vascular diseases associated with severe vasoconstriction. It is important to note that while there was an increase in ER expression in aorta from female compared to male rats, the inhibition of the contractile response induced by ER activation was similar in male and female VSMCs. This is likely related to differences in the sensitivity of the ER to activation possibly due to the differences in exposure to endogenous estrogen in females compared with males, and should be further examined in future experiments. GPR30 demonstrates similar expression in males and females, is mainly in the cytosol, and its activation does not affect its subcellular location or VSM contraction. More studies in other vascular beds are needed to further evaluate the role of GPR30 in VSM function.

Abbreviations

AngII (angiotensin II); DPN (diarylpropionitrile); E2 (17 β -estradiol); ER (estrogen receptor); PHE (phenylephrine); PPT (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)-tris-phenol); VSM (vascular smooth muscle); VSMC (vascular smooth muscle cell).

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