Age-Related Reduction in Estrogen Receptor–Mediated Mechanisms of Vascular Relaxation in Female Spontaneously Hypertensive Rats

Fanisha L. Wynne, Jason A. Payne, Ashley E. Cain, Jane F. Reckelhoff, Raouf A. Khalil

Abstract—Hypertension increases with aging, and changes in vascular estrogen receptors (ERs) may play a role in age-related hypertension in women. We tested whether age-related increases in blood pressure in female spontaneously hypertensive rats (SHRs) are associated with reduction in amount and/or vascular relaxation effects of estrogen and ER. Arterial pressure and plasma estradiol were measured in adult (12 weeks) and aging (16 months) female SHRs, and thoracic aorta was isolated for measurement of active stress, $^{45}$Ca$^{2+}$ influx, and ERs. Arterial pressure was greater and plasma estradiol was less in aging females than in adult females. In aorta of adult females, Western blots revealed $\alpha$- and $\beta$-ERs that were slightly reduced in aging rats. In endothelium-intact vascular strips, phenylephrine (Phe; $10^{-5}$ mol/L) caused greater active stress in aging rats (9.3±0.2) than in adult rats (6.2±0.3×10$^4$ N/m$^2$). 17$\beta$-estradiol (E2) caused relaxation of Phe contraction and stimulation of vascular nitrite/nitrate production, which was reduced in aging rats. In endothelium-denuded strips, E2 still caused relaxation of Phe contraction, which was smaller in aging rats than adult rats. KCl (51 mmol/L), which stimulates Ca$^{2+}$ influx, produced greater active stress in aging rats (9.1±0.3) than in adult rats (5.9±0.2×10$^4$ N/m$^2$). E2 caused relaxation of KCl contraction and inhibition of Phe- and KCl-induced $^{45}$Ca$^{2+}$ influx, which were reduced in aging rats. Thus, aging in female SHR is associated with reduction in ER-mediated NO production from endothelial cells and decrease in inhibitory effects of estrogen on Ca$^{2+}$ entry mechanisms of smooth muscle contraction. The age-related decrease in ER-mediated vascular relaxation may explain the increased vascular contraction and arterial pressure associated with aging in females. (Hypertension. 2004;43[part 2]:405-412.)

Key Words: hormones ■ endothelium ■ muscle, smooth, vascular ■ calcium ■ 

Hypertension is a major health problem in the industrialized world. The greater incidence of hypertension in men than in women of similar age has suggested gender-related, possibly estrogen-mediated, vascular protective effects.1-4 The incidence of hypertension is also greater in postmenopausal women compared with premenopausal women.5-7 Because the plasma estrogen levels are reduced during menopause, several studies have suggested that estrogen replacement therapy may have potential vascular benefits in aging postmenopausal women.8-9 However, this view has recently been challenged, and some studies suggest that estrogen replacement may not have beneficial vascular effects in elderly hypertensive women.10-13 The decreased vascular effects of estrogen in aging females may be because the vascular effects of estrogen are dependent not only on the plasma levels of estrogen but also on possible age-related changes in the amount of estrogen receptors (ERs) and/or the signaling mechanisms downstream from ER stimulation.

Estrogen is known to interact with specific ER. Both ER$\alpha$ and ER$\beta$ have been identified in several vascular beds in humans and in experimental animals such as rats.14-17 Estrogen has long been known to interact with cytosolic/nuclear receptors and stimulate gene transcription and thereby produce long-term genomic effects. Recent evidence suggests that estrogen may also have rapid nongenomic vascular effects.1,4 The nongenomic effects of estrogen have been ascribed to activation of both endothelium-dependent and endothelium-independent vascular relaxation.14,15,18-20

The vascular endothelium is known to release endothelium-derived relaxing factors such as nitric oxide (NO),21-23 NO diffuses into the smooth muscle, where it stimulates the enzyme guanylate cyclase, leading to increased cGMP production and smooth muscle relaxation.21-25 Estrogen has been suggested to affect the NO–cGMP vascular relaxation pathway by changing the amount and activity of endothelial NO synthase (eNOS).26-31 Estrogen also causes rapid relaxation in de-endothelialized vascular strips, suggesting that it affects other mechanisms in addition to the classic genomic pathway of steroid action,
possibly involving effects on the cellular mechanisms of vascular smooth muscle contraction.\textsuperscript{4,18–20} It is widely accepted that vascular smooth muscle contraction is triggered by increases in intracellular Ca\textsuperscript{2+}.\textsuperscript{32,33} Also, previous studies have suggested that estrogen may cause long-term and short-term changes in intracellular Ca\textsuperscript{2+} of vascular smooth muscle.\textsuperscript{20,33,34}

Although the estrogen/ER-mediated effects on the endothelium-dependent and endothelium-independent mechanisms of vascular relaxation have been reported,\textsuperscript{1,4,15} little information is available on whether the vascular effects of estrogen and ER are modified with aging in females, especially in aging hypertensive females. The purpose of the present study was to test the hypothesis that age-related increases in blood pressure in female spontaneously hypertensive rats (SHRs) are associated with reduction in the amount and/or the vascular relaxation effects of estrogen and ERs. To test this hypothesis, we compared the vascular effects of estrogen in aging (16 months) and adult (12 weeks) female SHRs. Experiments were designed to investigate (1) whether estrogen-induced endothelium-dependent vascular relaxation is reduced in aging compared with adult female SHRs; (2) whether the changes in estrogen-induced endothelium-dependent vascular relaxation involve alterations in the effects of estrogen on the NO-cGMP pathway; (3) whether estrogen-induced endothelium-independent vascular relaxation is reduced in aging compared with adult female SHRs; and (4) whether the changes in estrogen-induced endothelium-independent vascular relaxation involve alterations in the effects of estrogen on the Ca\textsuperscript{2+} mobilization mechanisms of vascular smooth muscle contraction, ie, Ca\textsuperscript{2+} release from the intracellular stores and Ca\textsuperscript{2+} entry from the extracellular space.

Methods

Animals

Aging female SHRs (Taconic Farms, Germantown, NY) were obtained at 8 months of age. The baseline mean arterial pressure was measured in the rats at 8 months of age, and the average value was 172±3 mm Hg. The aging rats (n = 6) were maintained for 8 months on standard rat chow (1% salt) and tap water ad libitum in an environment with a 12-hour light/12-hour dark cycle. Data from the aging rats were compared with data obtained in parallel in adult (12 weeks) female SHR (n=12) and maintained in the animal facility for a 1-week acclimation period. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee at the University of Mississippi Medical Center.

Measurement of Mean Arterial Pressure

On the day of the experiment, each rat was anesthetized with the thiobarbiturate Inactin (110 mg/kg; Research Biomedical), placed on a temperature-regulated surgery table, and underwent a surgical procedure for catheter implantation. A PE-50 arterial catheter was placed in the femoral artery and connected to a pressure transducer (Cobe model CDX III, Sema), and mean arterial pressure was recorded on a Grass polygraph (model 7D, Astro-Med). Mean arterial pressure was measured acutely in anesthetized rats and was averaged over a 40-minute period to indicate mean arterial pressure value for each rat. Although effects of Inactin on blood pressure have been reported,\textsuperscript{35} blood pressure measurements were compared in Inactin-anesthetized adult and aging rats under the same conditions.

Measurement of Plasma Estradiol-17β Levels

On the day of the experiment, blood samples (0.5 mL) were collected for measurement of plasma estradiol-17β concentrations in adult and aging rats by using a radioimmunoassay kit according to the manufacturer’s instructions (ICN Biomedicals). The assay reactivity with estradiol-17β is 100%. Cross reactivity with estrone, estradiol, and other steroids is 6%, 1.45%, and <0.01%, respectively.

The plasma estradiol levels were not determined at specific stages of the ovarian cycle because synchronization of the adult rats at specific stages of the ovarian cycle would require administering exogenous estrogen and progesterone and abortifacient drugs such as prostaglandin F2α, which could change the vascular reactivity and thus affect the measurements of the contractile response in the vascular strips. Therefore, adult rats were studied by using random selection 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 rats should cancel out any possible fluctuations in estradiol levels at specific stages of the ovarian cycle and should, roughly, represent the average changes in plasma estradiol during all stages of the ovarian cycle.

Tissue Preparation

After measuring the arterial pressure, the rats were euthanized by overdose of Inactin. The thoracic aorta was rapidly excised, placed in oxygenated Krebs solution, and cleaned of connective tissue. The aorta was cut into 3-mm-wide rings. Aortic rings were cut open into strips. For endothelium-intact aortic strips, extreme care was taken to avoid injury of the endothelium. For endothelium-denuded aortic strips, the endothelium was removed by gently rubbing the vessel interior with wet filter paper. Removal of the endothelium was verified by the absence of acetylcholine relaxation in tissues precontracted by submaximal concentrations of phenylephrine (Phe).

Isometric Contraction

One end of the aortic strip was attached to a glass hook by using a thread loop, and the other end was connected to a Grass force transducer (FT03). Aortic strips were stretched to Lmax (1.5 the unloaded initial length, L). The strips were allowed to equilibrate for 1 hour in a water-jacketed, temperature-controlled tissue bath filled with 50 mL Krebs solution continuously bubbled with 95% O2/5% CO2 at 37°C. The changes in isometric contraction were recorded on a Grass polygraph (model 7D).

A control contraction was elicited by applying Phe (10\textsuperscript{-3} mol/L) to the tissue bath solution. The tissue was rinsed with Krebs 3 times for 10 minutes. The whole procedure of contraction and washing was repeated 2 times. The tissue was then stimulated with Phe (10\textsuperscript{-4} mol/L) or 51 mmol/L KC1 to elicit a contraction. Once contraction reached a plateau, the tissue was treated with increasing concentrations of 17β-estradiol (E2) in the absence or presence of the ER antagonist ICI-182780, or with 17α-estradiol, and the extent of vascular relaxation at steady state (15 minutes) was measured. Control experiments in the presence of equal concentrations of the vehicle ethanol showed no significant effect on Phe or KC1 contraction. In other experiments, the tissues were pretreated for 30 minutes with N\textsuperscript{3}-nitro-l-arginine methyl ester (l-NNAME, 10\textsuperscript{-5} mol/L), to inhibit NO synthase, or with 1H-[1,2,4]oxadiazolo[4,3-quinolin-1-one (ODQ; 10\textsuperscript{-5} mol/L), to inhibit cGMP production in smooth muscle,\textsuperscript{36–38} and the effects on E2-induced relaxation of Phe contraction were observed.

Nitrile/Nitrate Production

Endothelium-intact aortic strips were placed in test tubes containing 2 mL Krebs solution aerated with 95% O2/5% CO2 at 37°C, and the solution was changed every 30 minutes for 1 hour. Samples for basal accumulation of nitrite (NO\textsubscript{2}) formed from released NO were first inhibited in synthesis, or with 1H-[1,2,4]oxadiazolo[4,3-quinolin-1-one (ODQ; 10\textsuperscript{-5} mol/L), to inhibit cGMP production in smooth muscle,\textsuperscript{36–38} and the effects on E2-induced relaxation of Phe contraction were observed.
triplicate) were mixed in a 96-well microtiter plate with 100 μL of the Griess reagent. The chromophore generated by the reaction with nitrite was detected spectrophotometrically (553 nm) by using a microtiter plate reader (THERMOMax, Molecular Devices). The concentration of nitrite was calculated by using a calibration curve with known concentrations of NaNO$_2$.

45Ca$^{2+}$ Influx

Endothelium-denuded vascular strips were incubated in Krebs solution and then stimulated with Phe (10$^{-5}$ mol/L) or 51 mmol/L KCl for 10 minutes in the absence or presence of E2 (10$^{-5}$ mol/L). The tissues were transferred to the respective radioactive 45Ca$^{2+}$-labeled solution (specific activity, 5 μCi/mL, ICN) for 90 seconds. Preliminary experiments have shown that the relationship between 45Ca$^{2+}$ uptake versus time is linear during 15-, 30-, 60-, and 90-second exposures to the 45Ca$^{2+}$ label. The tissues were transferred to ice-cold Ca$^{2+}$-free Krebs for 45 minutes to quench extracellular 45Ca$^{2+}$ label. The vascular strips were weighed and placed in 2 mL hypotonic (5 mmol/L) EDTA for 24 hours at 4°C to disrupt the cell membranes and release the intracellular content of 45Ca$^{2+}$ label. The vascular strips were then incubated in PBS-Tween and then incubated in horseradish peroxidase for 15 minutes in PBS-Tween and then incubated in horseradish peroxidase for 1 hour and then incubated in the antibody solution at 4°C overnight. PBS-Tween contained the following (in mmol/L): 80 Na$_2$HPO$_4$, 20 NaH$_2$PO$_4$, 100 NaCl, and 0.05% Tween. To quantify ERs, monoclonal anti-ER and anti-estrogen (Sigma) were prepared in distilled water. ODQ (Calbiochem) was dissolved in DMSO. The final concentration of the vehicle ethanol in solution was 0.01%. All other chemicals were of reagent grade or better.

Western Blots

Aortic strips were transferred to a homogenization buffer containing 20 mmol/L 3-(N-morpholino)propanesulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol, 1.2 mmol/L EDTA, 0.02% BSA, 5.5 mmol/L leupeptin, 5.5 mmol/L pepstatin, 2.15 μmol/L aprotinin, and 20 μmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride. The tissue was homogenized by using a 2-mL tight-fitting homogenizer for 15 minutes in PBS-Tween and then incubated in horseradish peroxidase for 1 hour and then incubated in the antibody solution at 4°C overnight. PBS-Tween contained the following (in mmol/L): 80 Na$_2$HPO$_4$, 20 NaH$_2$PO$_4$, 100 NaCl, and 0.05% Tween. To quantify ERs, monoclonal anti-ERs (1:100, Santa Cruz Biotech) and polyclonal anti-ERβ (1:1000, Affinity Bioreagents) were used. To quantify NOS III, monoclonal anti-noE1 antibody (1:1000, Transduction Laboratory) was used. To maintain the labeling conditions constant, we used the same antibody titer and protein concentration (10 μg) in all tissue samples. These antibody titers and protein concentration produced significant immunoreactive bands while remaining on the linear portion of the titration curve. Control experiments were performed in the absence of primary antibody. Nitrocellulose membranes were washed 5 times for 15 minutes in PBS-Tween and then incubated in horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (1:3000) for 1.5 hours. The blots were visualized with enhanced chemiluminescence detection system (Amersham). To verify equal loading of sample protein, the immunoblots were stripped in stripping solution (100 mmol/L β-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris HCl at pH 6.8) at 60°C for 60 minutes and reprobed with monoclonal anti-β-actin antibody (Sigma, 1:5000). The reactive bands were analyzed quantitatively by optical densitometry by using a GS-700 imaging densitometer (Bio-Rad), and the amount of ER and eNOS was normalized to the β-actin signal.

Solutions, Drugs, and Chemicals

Normal Krebs contained the following (in mmol/L): 120 NaCl, 5.9 KCl, 5 NaHCO$_3$, 1.2 Na$_2$HPO$_4$, 11.5 dextrose, 1.2 MgCl$_2$, 2.5 CaCl$_2$, at pH 7.4. For Ca$^{2+}$-free Krebs, CaCl$_2$ was omitted and replaced with 2 mmol/L EGTA. High-KCl depolarization solution was prepared as Krebs but with equimolar substitution of NaCl with KCl. Stock solutions of L-Phe HCl, acetylcholine, sodium nitroprusside, and L-NAME (Sigma) were prepared in distilled water. ODQ (Calbiochem) was dissolved in DMSO. The final concentration of DMSO in solution was <0.1%. Stock solution of 17β-estradiol (Sigma) was prepared as 5×10$^{-2}$ mol/L in 100% ethanol. 17α-Estradiol (Sigma) and ICI-182780 (Tocris) were prepared as 10$^{-2}$ mol/L stock solutions in 100% ethanol. The final concentration of the vehicle ethanol in solution was <0.01%. Caffeine (Sigma, 25 mmol/L) was prepared in Ca$^{2+}$-free Krebs. All other chemicals were of reagent grade or better.

Statistical Analysis

The developed force was corrected for the cross-sectional area of each individual strip and expressed as active stress (N/m$^2$) by using the following equation: stress=force/cross-sectional area, where cross-sectional area= wet weight/(tissue/×length of the strip), and tissue density=1.055 g/cm$^3$. Data were analyzed and expressed as mean±SEM. Data were compared by using ANOVA with multiple classification criteria (rat type [aging versus adult], condition [endothelial [intact versus denuded]], and vascular treatment [nontreated versus treated with estrogen, and nontreated versus pretreated with l-NAME or ODQ]) followed by Bonferroni post test to compare selected groups. Differences were considered statistically significant if P<0.05.

Results

Mean arterial pressure was 156±3 mm Hg in adult rats and was significantly greater in aging rats (192±3 mm Hg, P<0.001). The plasma estradiol in adult females (averaged for estrus phases) was 118.8±17.7 pg/mL and was significantly reduced in aging females (53.6±7.2 pg/mL, P=0.023).

In endothelium-intact vascular strips, Phe (10$^{-5}$ mol/L) caused greater increases in active stress in aging rats (9.3±0.2) than in adult rats (6.2±0.3×10$^3$N/m$^2$, P<0.001). E2 caused concentration-dependent relaxation of Phe contraction. The E2-induced inhibition of Phe contraction was significantly reduced in aging rats compared with adult rats (Figure 1). The inhibitory effects of E2 on Phe contraction were abolished in tissues pretreated with the ER antagonist ICI-182780 (10$^{-6}$ mol/L) (Figure 1). 17α-Estradiol did not cause any significant inhibition of Phe contraction (Figure 1).

Western blot analysis on whole-tissue homogenate of vascular strips of adult females, and using anti-ERs and -ERβ antibodies, revealed prominent bands at 66 and 54 kDa, corresponding to ERα and ERβ, respectively. Measurement of the optical density of the immunoreactive bands suggested...
that the amount of ERα and ERβ was not significantly different between aging and adult rats (P = 0.695 and P = 0.747, respectively) (Figure 2).

The E2-induced relaxation of Phe contraction was significantly reduced, but not abolished, in endothelium-denuded compared with endothelium-intact vascular strips of adult females (Figure 3A). In vascular strips of aging rats, a slight reduction in E2-induced relaxation of Phe contraction was observed in endothelium-denuded compared with endothelium-intact vascular strips; however, the difference did not reach significant level (Figure 3A). Pretreatment of endothelium-intact strips with L-NAME (10⁻⁴ mol/L), to inhibit NO synthase (Figure 3B), or with ODQ (10⁻⁵ mol/L), to inhibit cGMP production in smooth muscle (Figure 3C), inhibited E2-induced relaxation significantly in adult rats but only slightly and not significantly in aging rats.

Western blot analysis by using homogenates of endothelium-intact vascular strips and anti-eNOS antibody showed a prominent band at ~140 kDa. Measurement of the optical density of the immunoreactive bands was determined and presented as the mean±SEM of measurements in 6 tissue samples from 6 rats of each group. *Measurements in control Endo strips, or +Endo strips in the presence of L-NAME or ODQ are significantly different (P<0.05) from corresponding measurements in control +Endo vascular strips.

To test whether the vascular smooth muscle of adult and aging rats is sensitive to relaxation by NO, the effects of sodium nitroprusside (SNP), an exogenous NO donor and a standard guanylate cyclase activator, were investigated. In endothelium-denuded vascular strips of adult rats, SNP caused concentration-dependent relaxation of Phe contraction. The SNP-induced relaxation of Phe contraction was slightly reduced, but not significantly different in vascular strips from aging compared with adult rats (Figure 5).

The effects of E2 on the endothelium-independent mechanisms of vascular smooth muscle contraction were also investigated in endothelium-denuded vascular strips. To investigate whether the differences in E2-induced vascular relaxation between aging and adult rats reflect changes in ⁴⁵Ca²⁺ release from the intracellular stores, caffeine-induced contraction in ⁴⁵Ca²⁺-free (2 mmol/L EGTA) Krebs was measured. In ⁴⁵Ca²⁺-free Krebs, caffeine (25 mmol/L) caused a transient increase in contraction in vascular strips of adult rats, which was not significantly different from that in aging rats (Figure 6A). Pretreatment of the vascular strips with E2 (10⁻⁵ mol/L) did not cause any significant change in caffeine-induced contraction in vascular strips of aging or adult rats (Figure 6A).

Membrane depolarization by 51 mmol/L KCl, which stimulates ⁴⁵Ca²⁺ entry from the extracellular space, produced an increase in active stress that was significantly greater in aging rats (9.1±0.3) than in adult rats (5.9±0.2×10⁴ N/m², P<0.001). E2 caused concentration-dependent relaxation of KCl contraction that was significantly reduced in aging rats compared with adult rats (Figure 6B). The inhibitory effects of E2 on KCl contraction were abolished in tissues pretreated with the ER antagonist ICI-182780 (10⁻⁶ mol/L) (Figure 6B).

To further investigate the role of ⁴⁵Ca²⁺ entry in mediating the vascular effects of E2, ⁴⁵Ca²⁺ influx was measured. In vascular strips of adult rats, the basal ⁴⁵Ca²⁺ was 13.8±1.6 μmol/kg per minute, and both Phe (10⁻⁵ mol/L) and KCl (51 mmol/L) caused significant increase in ⁴⁵Ca²⁺.
The basal and Phe- and KCl-induced $^{45}$Ca$^{2+}$ influx were greater in aging rats than in adult rats (Figure 7). E2 did not significantly affect the basal $^{45}$Ca$^{2+}$ influx but caused significant inhibition of Phe- and KCl-induced $^{45}$Ca$^{2+}$ influx. In vascular strips of adult rats, E2 ($10^{-5}$ mol/L) caused significant inhibition of Phe- (44%) and KCl-induced $^{45}$Ca$^{2+}$ influx (60%) to levels not significantly different from the basal levels (Figure 7). In vascular strips of aging rats, E2 caused smaller, yet significant, inhibition of Phe- (22%) and KCl-induced $^{45}$Ca$^{2+}$ influx (23%), but to levels still significantly greater than the basal levels (Figure 7). The inhibitory effects of E2 on Phe and KCl-induced $^{45}$Ca$^{2+}$ influx were abolished in vascular strips treated with the ER antagonist ICI-182780 ($10^{-6}$ mol/L) (Figure 7).

Discussion

The main findings of the present study are as follows: (1) E2-induced endothelium-dependent vascular relaxation is reduced in aging rats compared with adult female SHRs, (2) the reduced E2-induced endothelium-dependent vascular relaxation in aging female SHRs is associated with a decrease in the E2-mediated activation of endothelial NO-cGMP pathway, (3) E2-induced endothelium-independent vascular relaxation is reduced in aging rats compared with adult female SHRs, and (4) the reduction in E2-induced endothelium-independent vascular relaxation is associated with a decrease in the inhibitory effects of estrogen on the Ca$^{2+}$ entry mechanism of vascular smooth muscle contraction.

The present study showed that the arterial pressure was greater in aging rats than in adult female SHRs. These observations are consistent with previous reports that the arterial pressure is greater in aging rats than in adult male and female SHRs. We have previously shown that the increased arterial pressure in aging male SHR is associated with age-related enhancement of vasoconstriction. In the present study, we tested whether the vascular control mechanisms could play a role in the observed increase in arterial pressure in aging female SHRs. We found that the vascular contraction to Phe was enhanced in aging compared with adult female SHRs. Because estrogen has been shown to promote vascular relaxation via ERs, the enhanced vascular contraction in aging compared with adult female rats could be owing to changes in the plasma levels of E2, the amount of vascular ERs, or the E2/ER-mediated mechanisms of vascular relaxation.

Consistent with previous reports in animal models of surgical menopause, we found that the plasma E2 levels were reduced in aging compared with adult female SHRs. Because E2 causes vascular relaxation, the decreased plasma E2 with aging may well explain, at least in part, the enhanced vascular contraction in aging rats. However, when isolated vessels from aging and adult rats were treated with the same concentrations of E2, a smaller inhibition of vascular contraction was observed in aging compared with adult rats. The vascular effects of E2 appear to be specific because 17α-estradiol did not cause significant inhibition of vascular contraction. Also, ICI-182780 is a selective ER antagonist. The observation that the vascular effects of E2 were abolished in tissues treated with the ER antagonist ICI-182780 lends support to the contention that they are ER mediated. The decreased E2-induced inhibition of vascular contraction in aging rats could then be owing to reduction in the amount of ER or in the E2/ER-mediated mechanisms of vascular relaxation. Measurement of the total amount of vascular ERα and ERβ have shown no significant difference between aging and adult rats, suggesting that the decreased E2-induced inhibition of vascular contraction in aging rats is more likely due to reduction in E2/ER-mediated mechanisms of vascular relaxation.
E2 has been shown to promote vascular relaxation via both endothelium-dependent and endothelium-independent mechanisms. In search for possible changes in endothelium-dependent E2-mediated mechanisms of vascular relaxation in aging rats, we found that removal of the endothelium significantly reduced E2-induced relaxation in adult rats but had minimal effects in aging rats. These results provide evidence that an endothelium-dependent E2-mediated relaxation pathway is active in adult rats but inhibited in aging rats.

E2 has been shown to stimulate the release of relaxing factors such as NO from the vascular endothelium. The reduced E2-induced relaxation in aging rats could be owing to a decrease in the synthesis/release of NO from endothelial cells or may reflect age-related change in the sensitivity of vascular smooth muscle to relaxation by NO. The sensitivity of vascular smooth muscle to relaxation by NO could be evaluated by its sensitivity to relaxation by exogenous NO donors such as SNP. The observation that the relaxation of endothelium-denuded vascular strips by SNP was slightly but not significantly different between aging and adult rats suggests that the decreased E2-induced relaxation in aging rats is not owing to decreased vascular smooth muscle sensitivity to NO but more likely is owing to changes in the synthesis/release of NO.

Pretreatment of the vascular strips with l-NAME, which blocks NO synthesis, inhibited E2-induced vascular relaxation in adult but not aging rats, suggesting that E2-induced NO synthesis/release by endothelial cells is impaired in aging rats. This is supported by the observation that both the basal and E2-induced NOx production were reduced in vascular strips from aging rats compared with adult rats. The observed decrease in the amount of eNOS in vascular strips of aging rats could explain, at least in part, the decreased E2-induced vascular NOx production. Interestingly, the amount of eNOS in aging rats appeared to be reduced to only 72% of that in adult rats. On the other hand, the E2-induced NOx production in aging rats was reduced to 53% of that in adult rats. Even though the protein levels of eNOS were only reduced by ~30% in aging rats, the inhibition of eNOS activity could be even greater. Other factors in addition to the amount of eNOS could play a role because changes in protein levels detected by Western analysis are dependent on the quality of the antibody and the conditions of the assay. Also, protein levels may not reflect the eNOS enzyme activity on a 1:1 basis owing to many factors, including posttranslational regulation. E2 has been shown to stimulate translocation, palmitoylation as well as mitogen-activated protein kinase and protein kinase B/Akt-induced phosphorylation, and full activation of eNOS. Whether these E2-mediated mechanisms of eNOS activation are reduced with aging are unclear and should be the subject of future investigations.

In agreement with previous studies on porcine coronary artery, E2 still caused relaxation of endothelium-denuded rat aortic strips stimulated with Phe or KCl. The E2-induced relaxation in endothelium-denuded vascular strips was reduced in aging rats compared with adult rats. These data suggest that the reduction in E2-induced vascular relaxation in aging rats may involve changes in the effects of E2 on...
endothelium-independent mechanisms of vascular smooth muscle contraction.

Vascular smooth muscle contraction is triggered by increases in intracellular Ca\(^{2+}\) owing to Ca\(^{2+}\) release from the intracellular stores and Ca\(^{2+}\) entry from the extracellular space.\(^{32-34}\) The vascular contraction in response to caffeine, which stimulates Ca\(^{2+}\) release from the intracellular stores, was not different between aging and adult rats. Also, E2 did not significantly affect caffeine-induced contraction, suggesting that changes in the caffeine-sensitive Ca\(^{2+}\) release mechanism from the intracellular stores may not be involved in the observed decrease in E2-induced vascular relaxation in aging rats. However, subtle effects of E2 on other Ca\(^{2+}\) release mechanisms cannot be ruled out.

Membrane depolarization by high KCl is known to stimulate Ca\(^{2+}\) entry from the extracellular space.\(^{32}\) The KCl-induced contraction and the Phe- and KCl-induced Ca\(^{2+}\) influx were enhanced in aging rats compared with adult rats, suggesting that the Ca\(^{2+}\) entry mechanisms of vascular contraction are enhanced in aging rats. E2 reduced KCl-induced contraction and the Phe- and KCl-induced Ca\(^{2+}\) influx. These data are consistent with previous reports that E2 inhibits the Ca\(^{2+}\) entry mechanisms in porcine coronary artery.\(^{20,33}\) The reduced E2-induced inhibition of Ca\(^{2+}\) influx in vascular strips of aging rats suggests reduction in the effects of E2 on the Ca\(^{2+}\) entry mechanisms of vascular contraction.

**Perspectives**

The present results suggest that aging in female SHRs is associated with not only a decrease in the plasma levels of E2 but also a decrease in E2/ER-mediated mechanisms of vascular relaxation. Previous studies have shown that aging (16-month-old) postmenopausal SHRs could be a suitable model for the study of postmenopausal hypertension observed in elderly female individuals.\(^7\) Thus, the present results in aging female SHRs may explain, in part, the increased vascular contraction and arterial pressure associated with aging in elderly females, as well as the refractoriness of the age-related vasoconstriction and hypertension to E2 replacement therapy.

The present results suggest that the total amount of aortic ER may not be different between aging and adult females. However, we should caution that the presence of several ER isoforms and variants may cause false-negative results in our tests of ER expression. Also, the amount and subcellular distribution of ER may vary in different vascular beds. In addition, the acute effects of E2 in ex vivo experiments may be different from the in vivo conditions in which E2 may promote additional genomic effects on the signaling pathways of vascular contraction/relaxation. This is particularly important because E2 concentrations higher than those predicted in vivo were necessary to elicit the acute effects of E2. In relation to this point, although the inhibitory effects of E2 on contraction of vascular strips of aging rats appeared to be minimal or not detectable at maximum Phe concentrations, the vascular effects of physiological concentrations of E2 could be greater at submaximal agonist concentrations encountered in vivo.

A reduction in E2/ER-mediated NO production from endothelial cells may explain the reduced E2-induced endothelium-dependent vascular relaxation in aging rats. However, it remains to be clarified whether this is only owing to reduction in eNOS expression with age or may involve posttranscriptional or posttranslational changes that affect eNOS activity, such as its binding to plasma membrane caveolin, or its palmitoylation and phosphorylation, which are required for its full activation. An additional reduction in the inhibitory effects of E2/ER on the Ca\(^{2+}\) entry mechanisms of vascular smooth muscle contraction may explain the reduced E2-induced endothelium-independent vascular relaxation in aging rats. E2 has been suggested to inhibit Ca\(^{2+}\) influx through Ca\(^{2+}\) channels by direct interaction with the Ca\(^{2+}\) channel\(^{46,47}\) or via activation of K\(^{+}\) channels, membrane hyperpolarization, and inhibition of Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels.\(^{48}\) The reduced E2-induced inhibition of Ca\(^{2+}\) influx with aging may then reflect age-related changes in the sensitivity of plasma membrane channels to the effects of E2 and may represent important areas for future investigation.

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