Role of Aging Versus the Loss of Estrogens in the Reduction in Vascular Function in Female Rats

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Although aging is known to lead to increased vascular stiffness, the role of estrogens in the prevention of age-related changes in the vasculature remains to be elucidated. To address this, we measured vascular function in the thoracic aorta in adult and old ovariectomized (ovx) rats with and without immediate 17β-estradiol (E2) replacement. In addition, aortic mRNA and protein were analyzed for proteins known to be involved in vasorelaxation. Aging in combination with the loss of estrogens led to decreased vasorelaxation in response to acetylcholine and sodium nitroprusside, indicating either smooth muscle dysfunction and/or increased fibrosis. Loss of estrogens led to increased vascular tension in response to phenylephrine, which could be partially restored by E2 replacement. Levels of endothelial nitric oxide synthase and inducible nitric oxide synthase did not differ among the groups, nor did total nitrite plus nitrate levels. Old ovx exhibited decreased expression of both the α and β-subunits of soluble guanylyl cyclase (sGC) and had impaired nitric oxide signaling in the vascular smooth muscle. Immediate E2 replacement in the aged ovx prevented both the impairment in vasorelaxation, and the decreased sGC receptor expression and abnormal sGC signaling within the vascular smooth muscle. (Endocrinology 150: 212–219, 2009)

Aging is a progressive process characterized by a reduction in protective responses, an increase in inflammatory mediators, and impairment of organ function. In females the loss of estrogens may further compound these changes (1). Results from the World Health Initiative study cast doubts on the protective effects of estrogens in postmenopausal women. Estrogen replacement led not only to an increased risk of breast cancer but also to increased cardiovascular disease (2). However, many of the patients enrolled in these studies had been without estrogens for 10 yr or more, and this late addition of estrogens may have had unintended or unexpected results (3). The initiation of hormone replacement therapy (HRT) closer to menopause tended to reduce coronary heart disease risk among postmenopausal women compared with women who begin HRT later in menopause (4).

The fundamental age-related change in arterial function is an impairment of relaxation, which is associated with an increase in pulse wave velocity (5). In males, this age-related decrease in vascular relaxation is characterized by reduction in the nitric oxide (NO)-dependent vasodilator response to acetylcholine (ACh) (6). This impaired response to ACh is thought to be primarily due to down-regulation in the activation and expression of endothelial nitric oxide synthase (eNOS) (7); however, downstream changes within the vascular smooth muscle (VSM) can similarly impair vasodilation. Aging has also down-regulated the NO receptor, soluble guanylyl cyclase (sGC), as well as protein kinase G (PKG)-I activation in adult male rat aortas (8–10). The underlying mechanisms mediating abnormal vascular function with aging remain controversial.

Estrogens, in contrast to aging, are known to be vasoprotective. In the endothelium, 17β-estradiol (E2) activates eNOS nongenomically through the phosphatidylinositol-3-kinase/AKT pathway (11, 12). This leads to NO production within the endothelium, which then diffuses to the VSM and causes vasorelaxation. Studies in female estrogen receptor-α knockout mice have demonstrated reduced NO release and decreased relaxation in response to E2 treatment compared with wild type (13). In addition, estrogens can modulate the release of other vasoactive substances, such as prostacyclin and endothelin (14). In the

Abbreviations: ACh, Acetylcholine; CCAAT, cytidine-cytidine-adenosine-adenosine-thymidine; eNOS, endothelial nitric oxide synthase; E2, 17β-estradiol; HRT, hormone replacement therapy; iNOS, inducible nitric oxide synthase; NO3, nitrate; NO, nitric oxide; NO2, nitrite; ovx, ovariectomized; ns, not significant; PE, phenylephrine; PKG, protein kinase G; sGC, soluble guanylyl cyclase; SMHC, smooth muscle myosin heavy chain; SNP, sodium nitroprusside; VASP, vasodilator-stimulated phosphoprotein; VSM, vascular smooth muscle.
VSM, estrogens reduce intracellular Ca\(^{2+}\) concentrations and hyperpolarize the cell by opening potassium channels (15).

There is a lack of literature addressing changes in arterial relaxation in aged females with the loss of estrogens. These two factors, aging and estrogen loss, are critical to our understanding of how menopause leads to an increased risk of hypertension, atherosclerosis, and cardiovascular disease. Despite the body of literature addressing changes in aging and/or estrogen supplementation, much remains to be elucidated. The purpose of this study was to investigate how estrogen loss vs. aging affected vascular function, and whether any observed changes would be attenuated by E2 replacement. To address these questions, we used Norwegian brown rats, as an aging model, and compared changes in the vasculature after ovariectomized (ovx) with and without E2 replacement in young and old rats.

Materials and Methods
The animal protocol was approved by the University of California, Davis, Animal Research Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Norwegian brown rats were obtained from the National Institute of Aging and housed under standard conditions in female rat only housing. Aged (19–20 months) and adult (3–4 months) female rats were ovx under sterile conditions using standard methods. In one half of the ovx rats, hormone replacement was done with 0.36 mg E2 90 d slow-release pellets (Innovative Research, Sarasota, FL) implanted sc as previously described (16). Nine weeks after ovariectomy (i.e. at 5–6 and 21–22 months), body weight was measured, and the rats were euthanized by exsanguination. Before exsanguination, plasma samples were collected by cardiac puncture for measurement of NO metabolites, cytokines, and E2. The descending thoracic aorta was excised, cleansed of adhering tissue, and cut into 3- to 4-mm long aortic rings. For each rat a segment of the aorta was harvested for vascular contraction and relaxation studies, and the remaining segment was stored at −80°C for subsequent RNA and protein studies.

Isometric tension studies
Isometric tension was measured in 3- to 4-mm long descending thoracic aortic rings from rats as previously described (17). Individual aortic rings were suspended from an isometric transducer (Radnoti Glass Technology, Inc., Monrovia, CA) in oxygenated tissue baths containing bicarbonate-buffered Krebs-Henseleit solution [118 mM NaCl, 4.6 mM KCl, 27.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.75 mM CaCl₂, 0.03 mM Na₂EDTA, and 11.1 mM glucose (pH 7.4)]. According to length-tension curves previously established in our laboratory, a passive load of 2.0 g was applied, and the aortic segments were allowed to equilibrate for approximately 1 h with frequent readjustment of tension until reaching a stable baseline. Two KCl (70 mM)–induced contractions were performed to train the vessels to constrict. Rings were then washed and allowed to equilibrate for 40 min. To determine the maximum contractile response, a concentration-response curve to l-phenylephrine (PE) (1 nM to 3 μM) was obtained. To evaluate the vasodilatory response, a single concentration of PE (100 μM) was used to develop similar tension values in all groups, and, subsequently, cumulative concentrations of either Ach (1 nM to 3 μM) or sodium nitroprusside (SNP) (1 nM to 3 μM) were added to the tissue bath to induce endothelial cell-dependent or endothelial cell-independent relaxation, respectively. At least two aortic rings were assayed from each animal for multiple compounds. Drugs were added with an interval of approximately 1 min between them. All aortas were studied with intact endothelium. Data were collected and analyzed using PowerLab software (ADInstruments, Colorado Springs, CO).

Quantification of NO metabolites in plasma
Quantitative measurements of plasma nitrite (NO₂⁻) and nitrate (NO₃⁻) were performed as an index of local NO production according to the procedure outlined by Van Der Vliet et al. (18). Briefly, this procedure is based on acidic reduction of NO₂ and NO₃ to NO by vanadium (III) and purging of NO with helium into an Antek 7020 NO detector (Antek Instruments, Houston, TX). At room temperature, vanadium (III) only reduces NO₂, whereas NO₃ and other redox forms of NO such as S-nitrosothiols are also reduced after the solution is heated to 80–90°C, so that both NO₃ and total nitrogen oxides (primarily NO₂) can be measured. Data were quantified by comparison with standard solutions of NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃. Detection limits for NO₂ and NO₃.

Western blot analysis
Western blotting was performed and analyzed as previously described (19). Briefly, aortic tissue was homogenized in radioimmuno-precipitation assay buffer [50 mM Tris (pH 7.4), 0.5% Nonidet P-40, 0.2% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM NaF]. Aortic homogenates were then centrifuged at 2300 × g for 5 min to remove insoluble material. Primary antibodies were all rabbit polyclonal antibodies, and dilutions were as follows: 1:300 anti-eNOS, 1:500 and 1:250 inducible nitric oxide synthase (iNOS), 1:250 anti-p50, 1:500 phospho (p)-vasodilator-stimulated phosphoprotein (VASP) (Ser239), and 1:1,000 total VASP (Cell Signaling Technologies, Beverly, MA); 1:100 anti-α and 1:500 anti-sGC β1 (Cayman Chemical, Ann Arbor, MI); 1:500 anti-cytidine-cytidine-adenosine-adenosine-thymidine (CCAT) enhancer-binding protein (BioLegend, San Diego, CA); 1:10,000 β-actin (Novus Biologicals, Littleton, CO); 1:1,000 α-actin (Sigma-Aldrich Corp., St. Louis, MO); and 1:200 anti-smooth Muscle Myosin (Biomedical Technologies, Inc., Stoughton, MA). Membranes were washed, incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:10,000) in 2% milk-Tris-buffered saline with Tween 20 (Amersham Biosciences Inc., Piscataway, NJ). Membranes were washed, incubated with a chemiluminescent substrate (West Pico; Pierce, Rockford, IL), and visualized using CL-Xposure Film (Fuji Photo Film, Düsseldorf, Germany). Denitrosylation was performed using a Hewlett-Packard Scanjet model G3010 (Hewlett-Packard Co., Palo Alto, CA) and Un-Scan-It software (Silk Scientific, Inc., Orem, UT). Relative intensities for eNOS, α- and sGC β1 were normalized to β-actin, as a loading control. P-VASP (Ser 239) was normalized to total VASP.

Real-time PCR
Frozen tissue was ground in liquid nitrogen with a porcelain mortar and pestle. Total RNA was extracted by the modified guanidine isothiocyanate method (20). After isolation, RNA was processed with the RNeasy mini kit, according to the manufacturer’s protocol (QIAGEN, Inc., Valencia, CA). First-strand cDNA was generated using 2 μg total RNA and the high-capacity cDNA reverse transcription system (Applied Biosystems, Foster City, CA). Primers were purchased from SuperArray Biosystems (Frederick, MD).

PCRs were set up in 25-μl vol, consisting of 2.5 μl cDNA, 0.5 μl forward and 0.5 μl reverse 10 μM primers, 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), and 9 μl ribonuclease-deoxyribonuclease free water. For all primer sets, a denaturing step at 94°C for 10 min was followed by 40 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 45 sec, and 72°C extension for 30 sec. Real-time PCR was performed using an ABI Prism 7900HT Sequence Detector, and data were analyzed using SDS 2.1 software (Applied Biosystems). The relative concentration of the corresponding mRNA was measured as the number of cycles of PCR required to reach threshold fluorescence and normalized against that of an internal standard gene (β-actin).
Drugs and chemicals

All chemicals were purchased from Sigma-Aldrich except for Bay 41-2272 (Calbiochem, San Diego, CA).

Statistical analysis

Results from vascular contraction-relaxation studies were analyzed by both a two-way ANOVA followed by Bonferroni’s post hoc test to compare individual means and a sum of squares F test for whole curve comparison (GraphPad Prism, version 4.0b; GraphPad Software Inc., San Diego, CA). IC₅₀ was calculated from individual concentration-response curves after fitting the data to sigmoidal dose response curves (OriginLab, Northampton, MA). Western blot and NO metabolite data were analyzed by a one-way ANOVA or ANOVA on Ranks, where appropriate, followed by a Student-Newman-Keuls test. Data are presented as mean ± SEM. P < 0.05 was considered significant.

Results

ovx rats had plasma E₂ levels of 27.3 ± 4.8 pg/ml, whereas E₂ replacement resulted in levels of 59.48 ± 9.6 pg/ml. Old shams had E₂ levels of 58.0 ± 8.1 pg/ml. Body weight among groups did not differ significantly (adult ovx 225.6 ± 15.6, adult ovx plus E₂ 196.2 ± 12.6, old ovx 269.2 ± 6.9, old ovx plus E₂ 246.6 ± 13.5 g), as would be expected with Norway Brown rats.

Aging and the loss of estrogens lead to enhanced vasoconstriction

To determine the effects of aging and withdrawal of estrogens on both the endothelium and the surrounding smooth muscle, we measured the contractile-relaxant responses of isolated thoracic aortic segments. Data are summarized in Fig. 1A (adult ovx n = 5, adult ovx plus E₂ n = 5, old ovx n = 4, and old ovx plus E₂ n = 4). When normalized to maximal constriction, aged animals had increased vasomotor responses to L-PE (P < 0.05), specifically at 30, 100, and 300 nM. E₂ replacement did not affect the normalized vasoconstrictor response in either the adult or old aortas (Fig. 1A). Although vasomotor responses are usually presented as normalized values, we also analyzed changes in absolute grams of tension developed in each group. Both adult and old ovx animals had greater tension development, specifically at 1 and 3 μM compared with similarly aged ovx rats with E₂ replacement, indicating increased vessel tension after ovariectomy (Fig. 1B). Maximal tension generated per aortic ring was measured in response to 3 μM PE. Although there was a trend for adult and old ovx animals to have slightly higher maximal tension with PE administration as seen in Fig. 1C, this was not statistically significant.

Aging plus estrogen deficiency impairs ACh-induced relaxation

To investigate the effect of aging vs. estrogen loss on vasorelaxation, we tested vasomotor responses to ACh (1 × 10⁻⁹ M to 3 × 10⁻⁶ M). We observed no differences between adult ovx and adult ovx plus E₂ in response to ACh, as seen in Fig. 2A (adult
ovx n = 7, adult ovx plus E2 n = 5, old ovx n = 8, and old ovx plus E2 n = 7). However, old ovx demonstrated impaired relaxation in response to ACh in comparison to old ovx plus E2 at 100 and 300 nM (P < 0.05; Fig. 2B). Absolute grams of tension were also analyzed in all groups (data not shown) and had the same result, that there was no difference between adult ovx and adult ovx plus E2, whereas old ovx had greater tension vs. old ovx plus E2 by whole curve comparison (P < 0.05). The IC$_{50}$ (Fig. 2C) was higher in the old ovx vs. all others (adult ovx 1.3e$^{-7}$ ± 2.6e$^{-8}$ M, adult ovx plus E2 2.9e$^{-8}$ ± 3.5e$^{-8}$ M, old ovx vs. old ovx plus E2 1.0e$^{-8}$ ± 2.9e$^{-8}$; P < 0.05).

### Aging plus estrogen deficiency necessary for endothelial-independent impaired relaxation

To identify whether the dysfunction was occurring in the endothelium or smooth muscle, we repeated the vasorelaxation experiments using SNP (1e$^{-9}$ to 3e$^{-6}$ M) as an NO donor. Adult ovx and adult ovx plus E2 showed no differences in relaxation (Fig. 3A). As seen in Fig. 3B, old ovx exhibited impaired relaxation in response to SNP at 1 and 3 nM (P < 0.05), indicating that the vascular dysfunction was endothelium independent and was occurring within the smooth muscle (adult ovx n = 5, adult ovx plus E2 n = 4, old ovx n = 6, and old ovx plus E2 n = 6). Normalized and absolute tension curves (data not shown) demonstrated impaired relaxation for old ovx in comparison to old ovx plus E2 by whole curve comparison (P < 0.05), whereas adult and adult ovx plus E2 did not differ. The SNP IC$_{50}$ (Fig. 3C) was higher in the old ovx vs. all others (adult ovx 1.5e$^{-7}$ ± 4.3e$^{-10}$ M, adult ovx plus E2 1.2e$^{-9}$ ± 3.6e$^{-11}$ M, old ovx 2.9e$^{-9}$ ± 5.2e$^{-10}$ M, old ovx plus E2 1.8e$^{-9}$ ± 2.3e$^{-10}$; P < 0.05).

### NOS expression and systemic NO production

To determine whether changes in the signaling cascade proteins were the reason for impaired relaxation, we measured expression of the NOS isoforms, eNOS and iNOS, and circulating NO levels. Aging and/or E2 replacement did not affect basal protein expression of eNOS or iNOS among the four groups (P not significant [ns]), as shown in Fig. 4, A and B. Expression of the NOS isoform, neuronal NOS, could not be detected, although our internal control was detected. To corroborate these findings, we quantified levels of circulating NO metabolites in the plasma of the animals. As shown in Fig. 4C, circulating levels of NO did not differ among groups (adult ovx 22.4 ± 2.0, adult ovx plus E2 22.5 ± 2.6, old ovx 21.6 ± 1.7, and old ovx plus E2 23.5 ± 3.5 MUM; P = ns), which is consistent with the protein results.

### Aging and withdrawal of estrogens impair response of VSM to NO

Because old ovx had impaired relaxation in response to SNP, we hypothesized that old ovx had abnormalities in the signaling cascade downstream of eNOS. We examined expression of the NO receptor sGC. As shown in Fig. 5, A and B, mRNA and protein levels of both the sGC a1 (P < 0.05) and sGC B1-isoforms (P < 0.05) were decreased in old ovx compared with all other groups. In addition, we investigated whether VASP, a downstream target of sGC and a biochemical endpoint in the NO signaling pathway, was activated in the aortas of the aforementioned groups. Expression of VASP in the aortas of the respective groups. Expression was normalized to -actin on the same blot. Lanes for both blots: 1, adult ovx; 2, adult ovx plus E2; 3, old ovx; 4, old ovx plus E2 (n = 4–5 per group) (P = ns). Representative Westerns in lower panel showing eNOS and -actin on the same blot. B, Graph summarizes iNOS expression in aortic tissue from respective groups. Expression was normalized to -actin. Representative Westerns in lower panel showing iNOS and -actin on the same blot. Lanes for both blots: 1, adult ovx; 2, adult ovx plus E2; 3, old ovx; and 4, old ovx plus E2 (n = 4–5 per group) (P = ns). C, Plasma concentration of the circulating NO metabolites, NO$_2$ and NO$_3$ (n = 8–12 per group) (P = ns).
tioned groups. Phospho-serine 239, the major phosphorylation site activated in response to PKG activation, was examined. In support of the sGC data, we found decreased p-VASP in old ovx compared with all other groups (\(P < 0.05\)), whereas no differences were observed in the total levels of VASP (\(P/n=ns;\) Fig 6, A and B). To assess whether changes in VSM cell phenotype could be a cause of the impaired sGC function, we measured expression of \(\alpha\)-actin and smooth muscle myosin heavy chain (SMHC). No changes were detected in either \(\alpha\)-actin or SMHC expression among the groups (Fig. 6C).

**BAY 41-2278 rescues smooth muscle function in old ovx rats**

To assess whether the smooth muscle abnormalities can be corrected with an sGC agonist, we compared vasorelaxation in the old ovx in the presence of BAY 41-2272 (an sGC \(\alpha\)-agonist; Calbiochem) from \(1 \times 10^{-9} M\) to \(3 \times 10^{-6} M\). As shown in Fig. 7, stimulation of the sGC receptor restores the vasorelaxation response in old ovx plus ACh to that seen in old ovx plus E2 plus ACh (\(P < 0.05;\) old ovx n = 8, old ovx plus Bay-41 n = 4, and old ovx plus E2 n = 7).

**Discussion**

Our results show that aging in combination with the withdrawal of estrogens (menopause) leads to decreased vasorelaxation in response to ACh and SNP. The effected cascades are summarized in Fig. 8. Vasoconstriction is increased in both adult and old ovx animals in response to PE, and is decreased by E2 replacement. Levels of eNOS and iNOS did not differ among the groups, nor did total NO\(_2\) plus NO\(_3\) level, indicating that the abnormal relaxation was secondary to VSM dysfunction. Old ovx exhibited decreased expression of both the \(\alpha_1\) and \(\beta_1\) subunits of sGC. Immediate E2 replacement in the aged ovx prevented both the depressed vasorelaxation and the loss of the sGC expression.

In the current study, we examined changes in vascular function in adult and old ovx female Norway Brown rats with and without E2 replacement. The Norway Brown rat is a standard model of aging, allowing the selective study of aging independent of obesity. The purpose of our study was to investigate the effects of E2 replacement vs. aging in a menopausal model. Female rats do not undergo menopause but, instead, enter a stage of slowly declining ovarian function as they age. Intact, cycling animals display wide variations in estrogen levels during their cycles and as they age. We carefully considered the use of an intact control but thought there was no optimal point in the 4-d rat cycle that would be a good control in the adult rats, and in the aging rats,
the sustained estrus with slowly declining levels of estrogens over time did not represent a useful control. The addition of these multiple groups would complicate analysis and make it more difficult to discern the effect of E2 replacement after ovx, which was the principle question of interest. Therefore, we focused on adult and old rats with and without E2 replacement after ovariectomy.

Previously, we have observed in the hearts of female Sprague Dawley rats that a period of 9 wk was necessary for levels of the cardio-protective heat shock protein 72 to decrease to levels seen in age-matched males (16). Although clinical studies have demonstrated that the withdrawal of estrogens after 3 wk leads to increased blood pressure and decreases in forearm blood flow (21, 22), we believe that the acute loss of estrogens is not sufficient to induce the full cascade of changes that occur with loss of estrogen. An additional strength of our study design is the use of multiple groups would complicate analysis and make it more difficult to discern the effect of E2 replacement after ovx, which was the principle question of interest. Therefore, we focused on adult and old rats with and without E2 replacement after ovariectomy.

Increased vascular stiffness during aging can partly be attributed to enhanced sensitivity to vasoconstrictive agents. In mesenteric arteries of female Sprague Dawley rats, loss of estrogens has led to increased PE induced contractions through the up-regulation of the angiotensin II type I receptor (AT1R), which can be reduced by E2 supplementation (23). In contrast, others have found that E2 status did not affect constriction to PE but did enhance sensitivity to angiotensin II in thoracic aortic segments (24). We found in the descending thoracic aorta of Norway Brown rats that ovariectomy led to increased response to ACh in adult female rats with and without E2 supplementation, indicating no change in NO production by the endothelium. However, in aged female rats, ovariectomy impaired relaxation in response to ACh, which was prevented by immediate E2 replacement. As discussed previously, E2 replacement blocked these changes.

We found no difference in vascular relaxation in response to ACh in adult female rats with and without E2 supplementation, indicating no change in NO generation, such as AKT (8). Recent work suggests that along with decreased eNOS expression, in males aging leads to increased superoxide production, arginase I activity, and endothelial susceptibility to apoptotic stimuli, all which may lead to decreased bioavailability of NO (27–29). However, it is not known whether these same changes apply to females or the role of estrogens. In the current study in female rats, neither aging nor ovariectomy affected eNOS expression.

Although NO levels did not differ among the groups, both sGC α1 and β1 were decreased in old ovx compared with old ovx plus E2, indicating that the reduced response to NO in old ovx was due to decreased receptor expression, not to deficits in NO generation. Similarly, we have found, as part of a microarray study of gene changes in the heart of aging female Norway Brown rats, that sGC α and β are decreased with ovariectomy (unpublished observations), whereas adult female Sprague Dawley rats showed no variation with ovariectomy (30).
Aging with concomitant hypertension has led to decreased expression of the sGC α and/or β isoforms in the aortas of male spontaneously hypertensive rats and Wistar-Kyoto rats (9, 31). To the best of our knowledge, this is the first study that demonstrates that chronic E2 replacement can preserve sGC expression and prevent impaired relaxation in aged ovx female rats. Previous studies have focused on the acute effects of E2 treatment on sGC function in female rat uterine and pituitary tissues, not on vascular tissue (32, 33). The reduction in sGC α1 and β1 in old ovx was accompanied by decreased downstream activation of VASP by phosphorylation at serine 239, verifying reduced activation of the NO signaling cascade in old ovx without E2 replacement. A limited number of old sham aortas were examined, and found to have no change in total eNOS, and similar sGC α1 and β1 expression to that seen in old ovx plus E2 (data not shown). This supports our findings that the combination of aging and E2 withdrawal lead to the aforementioned changes.

The exact cause of the down-regulation of sGC in our model remains unknown, but in other studies decreased expression has been linked to increased endotoxin and cytokine levels (34). Although we found no difference in circulating levels of IL-1 β, IL-6, or TNF-α in plasma of young and old with and without E2 supplementation (data not shown), localized inflammation could be one mechanism for the down-regulation of the sGC isoforms. Previous studies suggest that E2 replacement can reduce proinflammatory cytokine levels induced by ovariectomy; however, some controversy exists as to whether these changes are seen across all menopausal models with HRT (35, 36). The transcription factors nuclear factor κB and CCAAT-binding factor have also negatively regulated sGC α1 and β1 expression by binding to the promoter site (37, 38). In our model we did could not detect expression of the either p50 or CCAAT-binding factor in any of the groups tested. Further work needs to be done to determine how sGC α1 and β1 expression is being down-regulated. To exclude phenotypical changes impairing sGC expression, we measured smooth muscle heavy chain-2 and smooth muscle α-actin expression. No changes in expression were detected for either protein in the four groups.

Changes in the extracellular matrix can also affect vessel stiffness. Studies in aging male rats have shown increased collagen content and cross-linking, along with increases in fibronectin, and decreases in elastin, due to calcification and fragmentation (39). Recently, Qui et al. (40) found that aortic stiffness increased with age, but to a greater degree in aged male than aged female premenopausal and perimenopausal monkeys. Decreases in collagen type 8 and elastin were observed only in aged males and likely accounted for the greater increase in aortic stiffness compared with aged females (40). Further work needs to be done to determine whether aging and chronic loss of estrogens in females lead to changes in extracellular matrix proteins, such as collagen, fibronectin, and elastin.

Treatment of hypertension in postmenopausal women who has focused on vasodilators. In the current study, the sGC receptor was reduced by the combination of ovariectomy and aging, and this was accompanied by reduction in vascular relaxation in response to ACh and SNP. The impairment of vasorelaxation due to decreased expression of sGC is a potential target in the treat-ment of hypertension in postmenopausal women (41). Bay 41-2272, an agonist of sGC, which binds to the regulatory site of sGC α and stimulates the enzyme synergistically with NO, restored normal relaxation. sGC activators, such as Bay 41-2272, are a possible new therapeutic approach for elderly female patients. Recent work has shown that Bay 41-2272 can reduce systemic arterial and pulmonary hypertension in rat models, and also can inhibit leukocyte adhesion in vivo (42). Thus, this compound shows promise as a new therapy to treat hypertension in postmenopausal women.

In conclusion, estrogens are a powerful compound with a plethora of effects. Further work is needed to understand the effects of aging vs. the withdrawal of estrogens in the aging female. The availability of an increasing number of synthetic estrogen receptor modulators raises the possibility of potentially being able to exploit the positive properties of estrogen without the deleterious effects.

Acknowledgments

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This work was supported by National Institutes of Health Grant AG19327 (to A.A.K.), the Department of Veterans Affairs (to A.A.K.), and the American Heart Association Western States Affiliate (to J.P.S.). Disclosure Summary: The authors have nothing to disclose.

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