Estrogen replacement restores flow-induced vasodilation in coronary arterioles of aged and ovariectomized rats

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Submitted 27 March 2009; accepted in final form 5 October 2009

The risk for cardiovascular disease (CVD) and heart failure increase with advancing age; however, sexual dimorphism exists in the chronological development of these risks (22, 47). Although the chronological rate of aging is independent of sex, mechanisms that regulate the cardiovascular system across the lifespan may differ dramatically between men and women. The risk for CVD in men begins to increase at approximately the same age that flow-mediated vasodilation begins to decline (5). Women also exhibit this age-related impairment of flow-mediated vasodilation; however, significant reduction of flow-mediated dilation becomes apparent at the age of menopause, more than a decade later than in men (5). The cardioprotection that women experience until menopause is presumably due to the presence of ovarian estrogen and results in a sex-related delay of the expression of CVD (49).

Chronic estrogen treatment has been shown to enhance endothelial function in a number of vascular beds (27, 31, 39), in part, through a pathway involving activation of Akt/PKB and subsequent phosphorylation of endothelial nitric oxide synthase (eNOS) (3, 10, 15, 43, 44). Endothelium-dependent vasodilation to acetylcholine in the peripheral vasculature is preserved or potentiated with chronic estrogen treatment in male, ovariectomized (OVX) and sham-operated rats (26, 27, 34, 52). A number of studies have shown that chronic estrogen treatment enhances endothelium-dependent vasodilation in large peripheral arteries of postmenopausal women (7, 24, 38).

In contrast to these reports of the cardioprotective effects of estrogen, the Women’s Health Initiative (37) and Heart and Estrogen/Progesterin Replacement Study (19) found an increased risk for coronary heart disease and stroke in postmenopausal women taking hormone-replacement therapy (HRT) compared with nonusers of HRT. This negative effect of HRT in postmenopausal women in the randomized clinical trials prompted investigations into the disparate results of animal studies, which have found a beneficial effect of chronic estrogen treatment (2, 26, 52). Even in humans, postmenopausal women have shown improved endothelium-dependent function in both small and large coronary arteries after short-term administration of estrogen (11, 13), raising questions as to why long-term HRT increases the risk for coronary heart disease in women after menopause. Discrepancies in the timing of hormone-replacement initiation, type and dose of estrogen/progesterone, and the age and health of women in clinical studies (32) warrants an optimized, pertinent animal model that more closely resembles reproductive aging in women. The present study utilizes a unique model, which allows identification of age-related changes in the coronary resistance vasculature while incorporating interventions such as the loss of ovarian hormones and/or estrogen replacement at appropriate time-points in the reproductive lifespan.

The purpose of this study was to determine how advancing age, ovariectomy, and estrogen-replacement affect endothelium-dependent, flow-induced vasodilation in the coronary resistance vasculature. We hypothesized that increasing age and lack of ovarian hormones would decrease flow-induced dilation in coronary arterioles. This study specifically investigated whether alterations in nitric oxide (NO) signaling underlie
age- and estrogen-mediated changes in endothelium-dependent vasodilation.

METHODS

Animals. Young (4 mo), middle-aged (12 mo), and old (22 mo) female Fischer-344 rats were obtained from Harlan (Indianapolis, IN). At the time of arrival, rats were maintained with ovariates intact (Intact), ovariectomized (OVX), or ovariectomized + estrogen-replaced (OVE) and housed for 6 to 8 wk postoperatively. Ages at the time of death were 6, 14, and 24 mo for young, middle-aged, and old rats, respectively. All procedures were approved by the Institutional Animal Care and Use Committee at West Virginia University and the University of Florida and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, Washington D.C., rev. 1996). Rats were housed individually at 23°C and were maintained on a 12:12-h light-dark cycle. All rats were fed a phytosterrogen-free rat chow and water ad libitum. Prior to death, at least two complete estrous cycles were monitored in all female rats by daily vaginal smears. At the time of death, 3 ml of blood were collected into chilled tubes containing dipotassium EDTA. Blood samples were centrifuged at 10,000 rpm for 4°C for 5 min, and the plasma was collected and stored at −80°C for future analysis. (in intact females), uterine, and cervix were dissected and trimmed of connective tissue and fat to obtain uterine weight (UW). Plasma estradiol was determined with ELISA immunonassay (Estradiol EIA kit, Oxford Biomedical; Oxford, MI).

Surgical procedures. OVX surgeries were performed as described previously (35). Briefly, rats were anesthetized with isoflurane (3%/O2 balance). Bilateral dorsolateral incisions were made through the top layer of skin and the underlying muscles were bluntly dissected to locate the ovary and fallopian tube. The fallopian tube was ligated with absorbable suture, and the ovary was removed. The surrounding fat pad was returned to the abdominal cavity, and the musculature and outer layer of skin were sutured. Nonabsorbable suture used to suture the skin was removed 7–10 days following surgery. The ovaries were weighed while the rats were anesthetized (isoflurane 5%/O2 balance) and euthanized by an overdose of pentobarbital sodium (Abbott Laboratories, Chicago, IL). All rats were fed a phytoestrogen-free rat chow and water ad libitum. Prior to death, at least two complete estrous cycles were monitored in all female rats by daily vaginal smears.

Microvessel preparation. Six to eight weeks after surgery, rats were anesthetized with isoflurane (3%/O2 balance) and euthanized by an overdose of pentobarbital sodium. All procedures were approved by the Institutional Animal Care and Use Committee at West Virginia University and the University of Florida and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, Washington D.C., rev. 1996). Animals were anesthetized with isoflurane (3%/O2 balance). Bilateral dorsolateral incisions were made through the top layer of skin and the underlying muscles were bluntly dissected to locate the ovary and fallopian tube. The fallopian tube was ligated with absorbable suture, and the ovary was removed. The surrounding fat pad was returned to the abdominal cavity, and the musculature and outer layer of skin were sutured. Nonabsorbable suture used to suture the skin was removed 7–10 days following surgery. OVE was performed simultaneously with the OVX procedure. OVE was performed with TaqMan probes (Applied Biosystems, Foster City, CA) specific for rat Akt-1 (Applied Biosystems). Custom TaqMan probes were designed from the published sequences for rat eNOS (eNOS primers at exon 8–9 junction: forward, GTG ACC CTC ACC GAT ACA ACA TAC; reverse, TGT CCG GTG GTC TAG ATC CAT). The fluorescent signal from the probe (FAM-labeled reporter dye; NFQ labeled-quencher dye) was measured by the ABI Prism 7900HT Fast Real-Time PCR system, as described previously (45).

Levels of the target sequence and levels of coamplified 18S ribosomal RNA were quantified relative to the cycle number (cycle threshold, CT), at which the target and 18S reach a fixed threshold as described previously (45).

Protein expression. Segments were dissected off of the LAD (±150 µm Dm; ~1,000 µm in length) in cold PSS solution (Albumin) (4°C), snap frozen (four per tube), and stored at −80°C. After addition of 20 µl Price-Laemmli lysis buffer, arterioles were solubilized and sonicated. Protein determination was assessed using NanoOrange (Molecular Probes, Carlsbad, CA). Equal volumes of whole vessel homogenates were electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Following blocking, membranes were incubated with primary antibodies for eNOS (1:1,000) (BD Transduction Laboratories, Lexington, KY), or p-Akt serine 473 (1:500), p-eNOS serine 1177 (1:500), Akt (1:1,000), or β-actin (1:5,000) (Cell Signaling Technology, Beverly, MA) overnight (4°C). Antibody binding was assessed by enhanced chemiluminescence (Super Signal; Pierce, Rockford, IL) following the manufacturer’s instructions. p-Akt (Serine 473) and p-eNOS (Serine 1177) were detected by using secondary anti-rabbit or anti-mouse antibodies as appropriate (1 h). Exposure was the same for each specific protein across all blots. Densitometric analysis of immunoblot films was performed using National Institutes of Health ImageJ 1.38 X Analysis Software (National Institutes of Health, Bethesda, MD). Data were normalized by expressing p-eNOS, p-Akt, eNOS, and Akt values relative to the β-actin loading control. p-eNOS and p-Akt were expressed relative to the β-actin loading control to distinguish between absolute differences in protein levels in the absence of possible age-related changes to either total eNOS or Akt.

Solutions and chemicals. Albumin was purchased from USB Chemicals (Cleveland, OH, USA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Data analysis. Analysis of variance was performed using the PROC MIXED procedure of the SAS system (version 9.1; SAS Institute, Cary, NC). Data were analyzed using analysis of variance (ANOVA) with repeated measures on one factor to detect statistically significant flow rates from 5 to 50 nl/s (28).

Responses to Dea-NONOate. Concentration-response relations to cumulative addition of the nitric oxide donor Dea-NONOate [2-(N,N-diethyl-aminono)-diazenolate-2-oxide, sodium salt] (3 × 10−4 M–1 × 10−8 M) were determined to determine responsiveness of the vascular smooth muscle to NO.

Maximal diameter. At the conclusion of the experiment, the vessels were washed with Ca2+-free PSS every 15 min for 1 h to obtain maximal passive diameter at 60 cm H2O.

Blockade of nitric oxide synthase and cyclooxygenase. In a second set of experiments, the contribution of nitric oxide to flow-induced vasodilation was reevaluated in the presence of N(G)-nitro-l-arginine methyl ester (t-NAME; 1 × 10−5 M), a nonspecific blocker of nitric oxide synthase (NOS). To determine the role of cyclooxygenase (COX) signaling, indomethacin (INDO; 1 × 10−5 M) or a combination of both inhibitors (t-NAME+INDO) was applied to vessels during exposure to flow. Inhibitor incubation was performed for a minimum of 30 min prior to beginning experiments.

mRNA levels. Arterioles were snap frozen and stored at −80°C. Arterioles were later pulverized in lysate buffer and total RNA was extracted using an aqueous and ethanol filter isolation method (RNAqueous Isolation Kit, Ambion, Austin, TX). Real-time PCR was performed with TaqMan probes (Applied Biosystems, Foster City, CA) specific for rat Akt-1 (Applied Biosystems). Custom TaqMan probes were designed from the published sequences for rat eNOS (eNOS primers at exon 8–9 junction: forward, GTG ACC CTC ACC GAT ACA ACA TAC; reverse, TGT CCG GTG GTC TAG ATC CAT). The fluorescent signal from the probe (FAM-labeled reporter dye; NFQ labeled-quencher dye) was measured by the ABI Prism 7900HT Fast Real-Time PCR system, as described previously (45).

Levels of the target sequence and levels of coamplified 18S ribosomal RNA were quantified relative to the cycle number (cycle threshold, CT), at which the target and 18S reach a fixed threshold as described previously (45).
differences within (flow rate or concentration) and between (experimental groups) factors. Pairwise comparisons were made by post hoc analysis (Bonferroni’s) when a significant main effect was found. Two-way ANOVA was used to determine group differences in body weight, heart weight, heart weight/body weight ratio, uterine weight, uterine weight/body weight ratio, spontaneous tone, and maximal diameter. One-way ANOVA was used to compare numeric plasma estradiol concentrations between treatments (Intact, OVX, OVE) within each age group. In some samples, estradiol concentrations were low enough to fall below the limit of detection of the assay (<10 pg/ml); therefore, uterine weight/body weight ratios and estradiol concentrations were transformed to rank order, and a nonparametric correlation coefficient using Spearman’s rho test was calculated to determine whether there was a significant predictive relationship between uterine weight/body weight ratio and plasma estradiol concentration. In all statistical analyses, n indicates the number of animals in each group. Significance was defined as P ≤ 0.05.

Values are expressed as means ± SE; n indicates number of rats. BW, body weight; HW, heart weight. *Indicates significant age-related difference vs. young intact. †Indicates significant OVX effect vs. age-matched intact; ‡Indicates significant OVE effect vs. age-matched intact. §Indicates significant OVE effect vs. age-matched OVX (P ≤ 0.05).

Table 1. Animal characteristics of young, middle-aged, and old intact, OVX, and OVE rats

<table>
<thead>
<tr>
<th></th>
<th>Young Intact</th>
<th>Middle-Age Intact</th>
<th>Old Intact</th>
<th>Young OVX</th>
<th>Middle-Age OVX</th>
<th>Old OVX</th>
<th>Young OVE</th>
<th>Middle-Age OVE</th>
<th>Old OVE</th>
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<tbody>
<tr>
<td>BW, g</td>
<td>211±3</td>
<td>266±4*</td>
<td>303±4*</td>
<td>242±5†</td>
<td>278±6</td>
<td>307±6</td>
<td>219±6§</td>
<td>249±43§</td>
<td>272±8§</td>
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<tr>
<td>n</td>
<td>(30)</td>
<td>(20)</td>
<td>(22)</td>
<td>(19)</td>
<td>(21)</td>
<td>(16)</td>
<td>(15)</td>
<td>(14)</td>
<td>(12)</td>
</tr>
<tr>
<td>HW, mg</td>
<td>587±10</td>
<td>707±13*</td>
<td>745±15*</td>
<td>617±11</td>
<td>700±16</td>
<td>767±18</td>
<td>617±17</td>
<td>744±15</td>
<td>824±362</td>
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<tr>
<td>HW/BW, mg/g</td>
<td>2.78±0.04</td>
<td>2.65±0.03*</td>
<td>2.47±0.05†</td>
<td>2.56±0.05†</td>
<td>2.52±0.04†</td>
<td>2.50±0.06</td>
<td>2.87±0.07§</td>
<td>2.99±0.04§</td>
<td>3.04±0.11‡</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of age, ovariectomy, and estrogen supplementation on estradiol, uterine weight/body weight, and estrous cycle. A: plasma estradiol measurements are positively correlated with uterine weight/body weight ratios (slope = 0.449; P = 0.001). B: relative frequencies of estrous cycle status at time of death in young, middle age, and old intact rats. C: estrous cycle status at time of death does not affect flow vasodilation measurements in young or old intact rats. D: plasma estradiol, uterine weights, and uterine weight/body weight [uterine wt/body weight (BW)] for intact, OVX, and OVE groups. OVX decreased uterine weight in all groups compared with age-matched intact females, whereas uterine weight was increased with estrogen replacement after OVX in all groups. Plasma estradiol was greater in young intact compared with middle-aged and old intact females. OVX decreased estradiol in young rats only. Estrogen replacement after OVX increased estradiol in old females compared with old intact, whereas OVE in young rats brought estradiol levels back to those seen in young intact. Values are expressed as means ± SE. *Significant compared with estrogen status-matched young group, †Significant compared with age-matched OVE group (P ≤ 0.05).
RESULTS

Animal characteristics. Middle-age and old intact rats exhibited a greater body weight and heart weight than young intact rats (Table 1). The ratio of heart weight (HW) to body weight (BW) was lower in middle-aged and old intact rats compared with young intact rats (Table 1). OVX induced a significant increase in BW in young females and decreased HW/BW ratio in young and middle-aged females (Table 1). Estrogen replacement caused a decrease in BW and an increase in HW/BW ratio compared with OVX in all age groups. In middle-aged and old females, BW decreased and HW/BW increased in OVE compared with age-matched intact (Table 1). Serum estradiol measurements were positively correlated with UW/BW ratios (slope = 0.450, P = 0.001, Fig. 1A). Vaginal smears performed for eight consecutive days prior to death revealed that the majority of old intact females are in constant diestrous at the time of death compared with regularly cycling young intact females (Fig. 1B). Subsequent analysis of the estrous cycle at time of death revealed no significant effect of cycle on vascular reactivity (Fig. 1C), which is consistent with findings reported in rat aorta (46). Uterine weight decreased and HW/BW increased in OVE compared with age-matched intact females (Table 1). OVX did not alter plasma estradiol in middle-age OVX females was slightly, but not significantly, different compared with intact females of the same age (Fig. 1D). Estrogen replacement increased estradiol in old females compared with old intact rats (Fig. 1D).

Vessel characteristics. Maximal diameter was similar among arterioles from all female groups except young OVE rats, in which maximal diameter was reduced (Table 2). Spontaneous tone was not altered by age or changes in estrogen status. Treatment with L-NAME increased tone to a similar degree in coronary arterioles from all groups compared with OVX rats of all age groups (Fig. 1D). Young intact females had greater plasma estradiol compared with middle-aged and old intact females (Fig. 1D). OVX decreased plasma estradiol in young intact, and estrogen-replacement restored estradiol to levels to those of young intact females (Fig. 1D). OVX did not alter plasma estradiol in middle-aged and old females compared with the age-matched intact rats (Fig. 1D). Estrogen replacement increased estradiol in old females compared with old intact rats (Fig. 1D).

Coronary arterioles from old females dilated less to flow than arterioles from young females. Values are expressed as means ± SE. *Significant age-related difference vs. young intact (P ≤ 0.05).

Fig. 2. Flow-induced dilation in young, middle-aged, and old intact females. Coronary arterioles from old females diluted less to flow than arterioles from young females.

Table 2. Vessel characteristics of isolated coronary arterioles in young, middle-aged, and old intact, OVX, and OVE rats

<table>
<thead>
<tr>
<th></th>
<th>Young Intact</th>
<th>Middle-Age Intact</th>
<th>Old Intact</th>
<th>Young OVX</th>
<th>Middle-Age OVX</th>
<th>Old OVX</th>
<th>Young OVE</th>
<th>Middle-Age OVE</th>
<th>Old OVE</th>
</tr>
</thead>
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<tr>
<td>Maximum diameter, μm</td>
<td>136±3</td>
<td>138±5</td>
<td>143±5</td>
<td>131±5</td>
<td>142±5</td>
<td>144±5</td>
<td>123±5</td>
<td>141±4</td>
<td>136±5</td>
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<td>n</td>
<td>(31)</td>
<td>(23)</td>
<td>(33)</td>
<td>(28)</td>
<td>(31)</td>
<td>(31)</td>
<td>(23)</td>
<td>(29)</td>
<td>(29)</td>
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<td>Spontaneous tone, %</td>
<td>43±3</td>
<td>49±3</td>
<td>46±3</td>
<td>49±4</td>
<td>51±3</td>
<td>52±3</td>
<td>45±5</td>
<td>52±3</td>
<td>45±3</td>
</tr>
<tr>
<td>Pre-L-NAME</td>
<td>(14)</td>
<td>(14)</td>
<td>(14)</td>
<td>(16)</td>
<td>(15)</td>
<td>(13)</td>
<td>(14)</td>
<td>(16)</td>
<td>(15)</td>
</tr>
<tr>
<td>Post-L-NAME</td>
<td>54±5†</td>
<td>59±3†</td>
<td>67±5†</td>
<td>62±7†</td>
<td>70±4†</td>
<td>68±3†</td>
<td>60±8†</td>
<td>63±5†</td>
<td>63±6†</td>
</tr>
<tr>
<td>Pre-INDO</td>
<td>(14)</td>
<td>(14)</td>
<td>(14)</td>
<td>(16)</td>
<td>(15)</td>
<td>(13)</td>
<td>(14)</td>
<td>(16)</td>
<td>(15)</td>
</tr>
<tr>
<td>Post-INDO</td>
<td>39±5</td>
<td>37±3</td>
<td>34±6</td>
<td>30±5</td>
<td>42±4</td>
<td>34±7</td>
<td>30±3</td>
<td>46±5</td>
<td>37±4</td>
</tr>
<tr>
<td>Pre-L-NAME + INDO</td>
<td>(5)</td>
<td>(5)</td>
<td>(8)</td>
<td>(8)</td>
<td>(8)</td>
<td>(5)</td>
<td>(6)</td>
<td>(7)</td>
<td>(8)</td>
</tr>
<tr>
<td>Post-L-NAME + INDO</td>
<td>39±5</td>
<td>39±3</td>
<td>34±7</td>
<td>30±5</td>
<td>41±5</td>
<td>35±4</td>
<td>31±3</td>
<td>46±6</td>
<td>36±4</td>
</tr>
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</table>

Values are expressed as means ± SE; n indicates number of vessels. *Indicates significant OVE effect vs. age-matched intact. †Indicates significant inhibitor effect vs. preinhibitor (control) (P ≤ 0.05).
replacement augmented flow-induced dilation to a level significantly greater than that of arterioles from either intact or OVX rats (Old OVE: 54.5% ± 6.5, Old OVX: 23.2% ± 8.2, Old Intact: 30.2% ± 5.8) (Fig. 3C).

**NOS inhibition.** To determine whether NO contributed to flow-induced dilation in coronary arterioles, dilation to flow was assessed in the presence of a nonspecific inhibitor of NOS (l-NAME). In intact females, only arterioles from young rats exhibited a decrease in flow-induced dilation after l-NAME treatment (Fig. 4A), indicating a loss of NO contribution to

Fig. 3. Effects of ovariectomy (OVX) and ovariectomy with estrogen replacement (OVE) on flow-induced vasodilation of coronary arterioles. A: flow-induced dilation is decreased after OVX in young females but restored to intact levels after OVE. B: OVX does not decrease flow-induced dilation in middle-age females, but OVE increases flow-induced dilation compared with OVX. C: OVE increases flow-induced dilation in old females. Values are expressed as means ± SE. †Significant OVX effect vs. intact. ‡Significant OVE effect vs. age-matched intact. §Significant OVE effect vs. OVX (P < 0.05).

Fig. 4. Flow-induced dilation in the presence and absence of l-NAME (1×10⁻³ M), a nitric oxide synthase (NOS) inhibitor, in young, middle-age, and old intact females. Flow-induced dilation of arterioles from young females was reduced by pretreatment with l-NAME (A). l-NAME did not alter flow-induced dilation of arterioles from middle-age (B) or old (C) females. Values are expressed as means ± SE. *Significant effect of l-NAME vs. control (P < 0.05).
flow with advancing age. Flow-induced dilation in OVX females of all ages was impervious to prior incubation with L-NAME (Fig. 5A). Conversely, blockade with L-NAME abolished dilation to flow in all OVE females, indicating a reliance on NO-dependent vasodilation after estrogen replacement, regardless of age (Fig. 5B).

COX inhibition. In arterioles from young and middle-aged rats, indomethacin treatment had no effect on flow-induced dilation, regardless of estrogen status (data not shown). In arterioles from old intact females, indomethacin treatment increased maximal flow-induced vasodilation (Fig. 6), suggesting that a COX-dependent constrictor pathway limits flow-induced dilation at an advanced age (Fig. 6). In contrast, indomethacin reduced flow-induced dilation in arterioles from old OVE females (Fig. 6), suggesting that estrogen replacement increases the contribution of COX-mediated signaling to flow-induced dilation in arterioles from old rats.

Combined NOS and COX inhibition. In all groups, combined NOS and COX inhibition on flow-induced dilation in coronary arterioles was similar to NOS inhibition alone (data not shown).

Vasodilator responses to DEA-NONOate. To determine whether the age-related impairment of vasodilation in coronary arterioles was due to a decrease in smooth muscle responsiveness to NO, vasodilation to DEA-NONOate was measured. DEA-NONOate elicited similar dilation in coronary arterioles from young, middle-aged, and old intact females (Fig. 7A). In young and old rats, neither OVX or OVE altered dilation of coronary arterioles to DEA-NONOate (Fig. 7, B and D). Coronary arterioles from middle-aged OVE rats exhibited increased sensitivity to DEA-NONOate compared with those from middle-aged OVX rats (Fig. 7C; IC$_{50}$: Middle-age OVX = 2.31 × 10$^{-6}$ M, Middle-age OVE = 7.01 × 10$^{-7}$ M).

**AKT and eNOS mRNA levels.** In coronary arterioles from intact females, Akt mRNA expression declined with age (Fig. 8A). OVX decreased (vs. intact), whereas OVE increased (vs. OVX) AKT mRNA in coronary arterioles from both young and middle-aged females (Fig. 8A). In coronary arterioles from middle-aged and old OVX rats, estrogen-replacement increased Akt mRNA expression to levels greater than those of arterioles from age-matched intact rats (Fig. 8A).

Similar to Akt mRNA, advancing age also caused a decrease in eNOS mRNA in coronary arterioles from middle-aged and old intact females compared with arterioles from young females; however, OVX did not alter eNOS mRNA in any age group (Fig. 8B). OVE upregulated eNOS mRNA in both young and middle-aged females compared with intact and OVX (Fig. 8B). Surprisingly, OVE did not alter eNOS mRNA in coronary arterioles from old rats (Fig. 8B).

**p-eNOS, p-Akt, eNOS, and Akt protein levels.** Basal p-Akt was undetectable in coronary arterioles from all groups. Total Akt and eNOS protein levels of coronary arterioles were not altered by age or changes in ovarian hormones (Fig. 9). Coronary arterioles from young females exhibited no changes in total eNOS protein or p-eNOS after OVX or OVE (Fig. 9B). In middle-aged females, neither OVX nor OVE altered total
eNOS protein levels (Fig. 9C); however, there was a 93% increase in p-eNOS after OVE treatment in middle-aged females (Fig. 9C). Similarly, total eNOS did not change with OVX or OVE in coronary arterioles from old females (Fig. 9D), but estrogen replacement increased p-eNOS levels by 45% in arterioles from old OVE females compared with arterioles from old OVX rats.

DISCUSSION

Although there are numerous reports in the literature detailing the beneficial influence of estrogen replacement on the vasculature in young animals (4, 10, 14, 41), to our knowledge this is the first study to examine the effects of estrogen supplementation on the coronary vasculature in female rats of an advanced age. The foremost finding of this study is that age reduces flow-induced, NO-mediated vasodilation of coronary arterioles of female rats, and estrogen replacement improves flow-induced dilation in coronary arterioles of old female rats, indicating a favorable coronary endothelial response to estrogen replacement in this aged population. This improvement in endothelium-dependent, NO-mediated dilation is accompanied by an increase in phosphorylation of eNOS protein after estrogen replacement. Congruent with the finding that estrogen replacement improved flow-induced dilation in rats with low levels of circulating estradiol, ovariectomy reduced flow-induced, NO-mediated vasodilation in coronary arterioles from young females, and estrogen replacement restored NO-mediated dilation to flow in coronary arterioles from young and middle-age ovariectomized rats. Together, these findings indicate that the age-related loss of endothelium-dependent dilation to flow in coronary arterioles occurs, in part, as a result of declining levels of ovarian estrogen, and that estrogen supplementation can improve flow-induced dilation, even at an advanced age.

Endothelium-dependent vasodilation has been shown to correlate inversely with age in large epicardial coronary arteries (8). Vasodilation of coronary arteries in response to noradrenaline (29), adenosine (16), and testosterone (9) declines with age. Additionally, impaired endothelium-dependent vasodila-

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**Fig. 7.** Concentration-response curves to Dea-NONOate, an exogenous NO donor. Vasodilation to Dea-NONOate did not change with advancing age (A). Ovariectomy and ovariectomy with estrogen replacement did not alter vasodilator responses to Dea-NONOate in arterioles from young (B) or old (D) rats. Sensitivity (IC50) in coronary arterioles from middle-aged OVE was greater than those from middle-aged OVX (middle-age OVX: 2.31×10^{-6} M, Middle-age OVE 7.01×10^{-7} M) (C). Values are expressed as means ± SE. $Significant OVE effect vs. age-matched OVX ($P < 0.05$).

**Fig. 8.** mRNA RQ values for Akt (A) and endothelial NOS (eNOS) (B) for all female groups (n = 8 per group). Young (Y), Middle-age (M), or Old (O), and either Intact (I), OVX (O), or OVE (E). Values are expressed as means ± SE. *Significant age-related difference vs. young intact. †Significant OVX effect vs. age-matched intact. ‡Significant OVE effect vs. age-matched intact. §Significant OVE effect vs. age-matched OVX ($P < 0.05$).
tion of septal arteries (~200 μm) in middle-aged male rats has been reported (6); however, much of the current literature regarding aging effects in the coronary vasculature is confined to studies of larger resistance arteries from males. The novelty of this study is the examination of the coronary resistance vasculature at three critical stages in the reproductive lifespan of female rats: young adulthood, reproductive senescence, and senescence. The present study illustrates an age-related impairment in endothelium-dependent dilation in coronary arterioles from female rats, which is associated with a loss of NO-dependent dilation (Fig. 4) and a decrease in phosphorylation of eNOS protein (Fig. 9). These findings extend previous studies showing that flow-induced dilation in females decreases with advancing age in brachial arteries (5, 33) and mesenteric arteries (1) by demonstrating that age-related endothelial impairment also occurs in resistance arterioles where endothelial responsiveness is critical to flow distribution.

In the present study, young females were the only age group in which OVX decreased flow-induced dilation of coronary arterioles. L-NAME treatment reduced flow-induced dilation of arterioles from young females before OVX, but L-NAME had no effect on the response to flow after OVX. In contrast, L-NAME did not alter flow-induced dilation of arterioles from either intact or OVX middle-age and old females, suggesting that a loss of ovarian hormones, whether induced by ovariectomy or aging, leads to a decline in flow-induced NOS signaling. Estrogen replacement after OVX enhanced flow-induced, NO-mediated dilation of coronary arterioles from all age groups (Fig. 3). Restoration of NO-dependent dilation by estrogen replacement has been observed previously in cerebral arteries (34) and in the coronary microcirculation of guinea pigs (50) at a young age. Additionally, several human studies have shown that HRT in postmenopausal women improves flow-induced dilation in peripheral conduit arteries (7, 24, 38). The current data indicate that estrogen supplementation also improves flow-induced vasodilation in the coronary resistance vasculature at all ages, primarily through enhancement of NOS-mediated signaling.

Vasodilation to flow was unaltered by L-NAME treatment in coronary arterioles from OVX rats. In addition, substantial dilation to flow remained in arterioles from all groups of rats, even during simultaneous inhibition of NOS and COX signaling (data not shown). In the cerebral microcirculation, OVX abolished endothelium-dependent dilation through up-regulation of caveolin-1, a negative regulator of eNOS (34). Xu et al. (51) found that NO-mediated signaling decreased in cerebral arterioles from ovariectomized rats, whereas signaling through KCa2 channels increased following ovariectomy, suggesting a conversion to dependency on hyperpolarizing factor in the absence of estrogen. Golding and Kepler have shown that in cerebral arteries of control female rats, endothelium-derived
hyperpolarizing factor (EDHF)-mediated dilations are negligible but can be enhanced after OVX (12). Additionally, Huang et al. (17) showed that when eNOS was inhibited in murine gracilis arterioles of WT mice, EDHF substitutes for NO-dependent dilation in response to flow (17). We did not directly assess the EDHF contribution to flow-induced dilation of coronary arterioles; however, it seems plausible that a conversion from NO dependency to EDHF dependency of flow-induced dilation may occur when ovarian hormones decline. Future studies are necessary to assess the effects of age and ovarian hormones on the contribution of EDHF to flow-induced vasodilation in coronary arterioles.

Flow-induced dilation was augmented in coronary arterioles from old intact females following pretreatment with indomethacin (Fig. 6), suggesting that COX-dependent vasoconstriction increases with age. Stewart and colleagues have established that a decline in circulating estrogen (2) and advancing age (48) enhances prostaglandin H synthase (PGHS)-2-dependent vasoconstriction in mesenteric arteries while simultaneously decreasing NO-dependent vasodilation. In mesenteric arteries from aged rats, an increase in circulating estrogen improved vasodilation by decreasing PGHS-dependent constriction in mesenteric arteries (2). Similarly, our results indicate that a decrease in COX-dependent vasoconstriction and an increase in COX-mediated vasodilation contributed to the enhancement of flow-induced vasodilation in coronary arterioles that occurred with estrogen supplementation in old rats.

Improvement of NO-mediated vasodilation by estrogen treatment may involve changes in expression and/or regulation of eNOS (4, 10, 15). Numerous reports have documented both the presence (25, 34, 36, 40) and absence (3, 20, 30) of estrogenic modulation of eNOS expression in various vascular beds. The present data show that the removal of ovarian estrogen does not alter eNOS mRNA expression in coronary arterioles at any age; however, eNOS mRNA is upregulated by estrogen replacement in coronary arterioles from young and middle-aged females (Fig. 8B). Surprisingly, but in keeping with findings reported in cerebral microvessels (20), mesenteric arteries (30), and left ventricular tissue (3), neither age nor estrogen status altered total eNOS protein in coronary arterioles. Although total eNOS was not affected by estrogen status, phosphorylation of eNOS protein (serine 1177) was increased in arterioles from middle-aged and old females after estrogen replacement. In coronary arterioles from male rats, we have previously reported that advancing age impairs flow-induced vasodilation through reductions in Flk-1 activation and PI3-kinase/Akt signaling (23). Similar to our findings in coronary arterioles from old male rats, our current results indicate that basal NO remained constant (Table 2) despite changes in NO-mediated dilation that occurred with advancing age and manipulation of circulating estrogen. Thus, our data suggest that changes in NO-mediated signaling that occur with age and alterations of estrogen status in female rats are more likely related to modifications in regulation of eNOS activity rather than adaptations in eNOS expression. Future studies in which phosphorylation of eNOS at other regulatory sites and feedback of NO and reactive oxygen species on eNOS transcription and translation will be needed to provide a more comprehensive understanding of the effects of age and estrogen status on regulation of eNOS expression and function in the coronary endothelium of female rats.

Although molecular evidence shows that Akt is responsive to the presence of estrogen (10, 18, 44), previous studies focused on the acute effects of estrogen exposure rather than the chronic estrogen supplementation. In endothelial cells, acute administration of estrogen causes phosphorylation of Akt within 1 min (10); however, we found that phosphorylated AKT was undetectable in coronary arteriolar samples under basal conditions. Bhuiyan et al. (3) found no changes in phosphorylated Akt or total Akt protein after OVX in left ventricular tissue from female Wistar rats. Although it would seem reasonable that circulating estrogen affects both Akt mRNA and Akt protein similarly, our data indicate that chronic changes in estrogen altered Akt mRNA expression, but not Akt protein levels. The results suggest that estrogen exerts differential effects on transcription, translation, and activation of Akt. Our results also suggest that modifications of Akt mRNA expression induced by alterations of estrogen status are unlikely to be a critical factor in the changes in NO signaling that occur with advancing age or estrogen supplementation.

**Perspectives and Significance**

The major finding from this study is that estrogen replacement following ovariectomy restores or enhances flow-induced dilation in coronary arterioles from all ages of female Fischer-344 rats. Estrogen replacement reversed the age-related impairment of flow-induced dilation in coronary arterioles from aged females by increasing the contribution of NO to flow-induced dilation. This estrogen-induced increase in NO signaling in arterioles from aged female rats was paralleled by an increase in phosphorylation of eNOS protein. These observations indicate the endothelium of coronary resistance vasculature retains its ability to increase NO signaling in response to elevation of circulating estrogen, even at an advanced age. Thus, estrogen supplementation could remain beneficial to the coronary resistance vasculature of postmenopausal women, depending on the timing of administration and the overall health of the endothelium upon initiation of replacement.

**ACKNOWLEDGMENTS**

The authors would like to thank Dr. Charles Wood for performance of plasma estradiol assays. The authors gratefully acknowledge the technical contributions of Yanduan Hu.

**GRANTS**

This work was supported by National Institutes of Health Grant R01HL077224.

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