Effect of estrogen replacement therapy on distribution of myocardial blood flow in female anesthetized rabbits


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Received 2 April 2001; accepted in final form 27 May 2001

The incidence of adverse cardiovascular events in women increases substantially after menopause (31, 42). Estrogen replacement therapy (ERT) in postmenopausal women has been shown to reduce the incidence of adverse coronary events and associated mortality (7) but not the risk of stroke (38). In women with confirmed coronary artery disease, treatment with 17β-estradiol (E2β) results in a significant improvement of exercise duration (34). Other benefits that could contribute to vascular protection in women include improvement in serum lipid profiles (2). However, many studies support the hypothesis that estrogen has a direct influence on regulation of vascular function (11, 18) and intracellular signaling pathways in vascular smooth muscle (16, 27). Chronic (26, 28) and acute (8, 40) administration of E2β improves vasorelaxation via either endothelium-dependent (19, 44) or -independent mechanisms (6, 17). Furthermore, estrogen therapy has been reported to significantly reduce myocyte injury (10) and the incidence of arrhythmias during ischemia-reperfusion injury (29).

Other steroids such as dehydroepiandrosterone (DHEA) and its sulfate may influence vasoregulation but they have not been well studied. DHEA therapy may attenuate progression or development of vascular disease (13) because it may be a precursor of estrogen and androgen in tissues. Greater serum DHEA levels have been associated with several major cardiovascular risk factors; however, they appear to be unrelated to the risk of fatal cardiovascular events in women (3).

There is accumulating evidence that ERT modulates multiple mechanisms within the vessel wall that contribute to vasoregulation. Accordingly, we studied distribution of myocardial blood flow in cycling/ovariectomized (Ovx) rabbits treated with either E2β or DHEA and during vascular challenge with either endothelium-dependent or -independent agonists.

Materials and Methods

All procedures used in the present study are in accordance with the “Guide to the Care and Use of Experimental Animals” of the Canadian Council on Animal Care. The Laval University Animal Ethics Committee also approved these studies. Female New Zealand White rabbits (2.2–2.7 kg body wt) obtained from Charles River Laboratories were used in these studies. The rabbits were acclimatized for several days in our animal housing facilities with free access to food and water and were kept on a strict 12:12-h dark-light cycle.

Drugs. Acetylcholine (ACH), isoproterenol (Iso), E2β, and DHEA were purchased from Sigma (St. Louis, MO). Chromonar (Chr), a selective coronary vasodilator, was a generous gift from Aventis. All drugs were prepared fresh on the day of the study and were dissolved in saline.

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Surgical preparation. Rabbits were premedicated with acepromazine maleate (5 mg/kg im) and anesthetized with intravenous pentobarbitone sodium (25 mg/kg). Butorphanol (0.22 mg/kg im) was administered for analgesia. The ovariotomy was performed via a bilateral ventral incision as previously described (24); incisions were closed in layers with sutures. Sham animals were handled in the same way as Ovx animals, with the exception that ovariectomy were not removed. E$_2$DHEA was administered with the use of subcutaneous implants (2.0 cm ¥ 1.4 mm and 5.0 cm ¥ 1.65 mm, respectively) positioned in the ventralateral region. Implants were made from Silastic tubing and packed with crystalline E$_2$DHEA; tubing was plugged with silicone adhesive. These Silastic implants have previously been used to constant release and maintenance of uniform circulating levels of estrogens (22); empty implants were used as placebo.

After 2 wk, rabbits were anesthetized and blood samples were collected for determination of plasma levels of E$_2$ and DHEA. Rabbits were intubated and mechanically ventilated with 12 ml/kg 75% oxygen-25% room air by using a positive-pressure small animal ventilator (MDI; Mobile, AL); respiratory rate and tidal volume were adjusted to maintain arterial blood gases within physiological values. The chest was opened via a left thoracotomy; Silastic catheters were positioned in the ventralateral region. Implants were made from Silastic tubing and packed with crystalline E$_2$DHEA; tubing was plugged with silicone adhesive. These Silastic implants have previously been used for constant release and maintenance of uniform circulating levels of estrogens (22); empty implants were used as placebo.

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Experimental protocol. The following four groups were studied: 1) sham (i.e., cycling females), 2) Ovx, 3) Ovx + E$_2$, and 4) Ovx + DHEA.

Cardiac hemodynamics and myocardial blood flow were assessed by using 15-µm spheres labeled with $^{113}$Ce, $^{114}$In, $^{115}$Sn, $^{86}$Sr, $^{90}$Nb, or $^{46}$Sc, under steady-state conditions, 3) ACh (2 mg bolus), and 4) Chb (8 mg/kg bolus). Drug dosages were determined from preliminary experiments in our laboratory (data not shown). ACh was used to assess endothelium-dependent vasodilatation. Isolated was used to assess metabolic-induced vasodilatation, and Chb was used to produce maximal pharmacological vasodilatation via endothelium-independent mechanisms (43). The same sequence of drug administration was replicated in each experiment; Chb was administered last because of its long-lasting and maximal pharmacological vasodilatory effects.

At the end of the experiment, a supplemental dose of pentobarbitone sodium was administered to ensure profound anesthesia; saturated KCl was then injected intra-aerially to arrest the heart during diastole. The heart was removed and fixed by immersion in 10% (vol/vol) neutral formalin. After the heart was fixed, fat and vascular tissues were trimmed away. The atria were removed and the ventricles and septum were divided into subendocardial (Endo) and subepicardial (Epi) tissue layers. Radioactivity was measured as previously described (20).

Measurements of steroids. Determination of steroids was performed as previously described (4). For blood samples, 5 ml of ethanol were added to 1 ml of plasma and centrifuged at 2,000 g for 15 min. To maximize steroid recovery, the resulting pellet was further washed with 2 ml of ethanol and then centrifuged; the two extracts were combined and evaporated with nitrogen. Unconjugated steroids were separated by C-18 columns (Bond Elut, Amersham; Bucks, UK) chromatography; columns were conditioned by successive passage of 10 ml methanol, 10 ml water, and 10 ml methanol-water (5:95; solution A). Extracts were solubilized in 2 ml of solution A and deposited on the column. After the C-18 column was washed with 2 ml of solution A, 5 ml of methanol-water (40:60) were added to elute the glucuronide and sulfate derivatives. The addition of 5 ml of methanol-water (85:15) enabled the elution of nonconjugated steroids. Both fractions were completely evaporated (Speed Vac Evaporator; Savant Instruments; Farmingdale, NY). Unconjugated steroids were then solubilized in 1 ml of isooctane-to-luene-methanol (90: 5:5) and deposited on Sephadex LH-20 columns (Pharmacia; Uppsala, Sweden). Steroids were measured by specific radioimmunoassay with rabbit antibodies; radioimmunoassay data were analyzed as described previously (33).

Calculations and data analysis. Transmural myocardial blood flow was expressed in ml/min $^1$100 g$^{-1}$. Maximal coronary conductance (ml-min$^{-1}$·mmHg$^{-1}$) was calculated as the quotient of blood flow (ml/min) and diastolic aortic pressure (mmHg) during pharmacologically induced vasodilatation. Cardiac output (CO; ml/min) was determined using the microsphere method (1) and calculated using the equation $Q_{uv} = I(Q_{uv}l_{uv})$, where $I$ is the amount of radioactivity initially injected, $Q_{uv}$ is the blood flow of the arterial reference sample, and $l_{uv}$ is the radioactivity in the arterial reference sample. A good correlation with other dye techniques and the microsphere method has previously been reported (12).

The heart rate-arterial blood pressure product (beats/min·mmHg $^1$10$^{-5}$), calculated as the product of systolic aortic pressure and heart rate, was used as an index of myocardial oxygen demand.

Differences in cardiac hemodynamic and myocardial blood flow between groups and interventions were assessed by analysis of variance with an interaction effect. The Student-Newman-Keuls multiple-range test, with $\alpha = 0.05$, was performed on all main-effect means to locate significant differences within groups and interventions. Normality and variance assumptions were fulfilled. $P < 0.05$ was used to confer statistical significance. Analyses were performed using the SAS statistical package (SAS Institute; Cary, NC). Data are presented as means ± SE.

RESULTS

Animal population. Thirty-nine rabbits were entered into the study. Five rabbits died during surgical preparation and were not included in the data analysis. Data are presented for nine rabbits in the Ovx + E$_2$ and Ovx + DHEA treatment groups, respectively; eight rabbits are included in the sham and Ovx groups, respectively.

Biochemical determinations. Plasma estradiol levels were measured in an additional series of rabbits; in Ovx rabbits, plasma estradiol levels were lower than in the sham group (95 ± 5 vs. 144 ± 31 pmol/l). Plasma estradiol levels were higher in Ovx + E$_2$ (579 ± 40 vs. 144 ± 31 pmol/l in sham; $P = 0.001$) and Ovx + DHEA (184 ± 34 vs. 144 ± 31 pmol/l in sham; $P = 0.001$) rabbits; in fact, supraphysiological plasma estradiol levels were achieved with E$_2$ treatment. Plasma DHEA levels were also significantly greater in Ovx + DHEA rabbits (23 ± 1 nmol/l) compared with the Ovx + E$_2$ group (0.6 ± 0.1 nmol/l; $P = 0.001$); these data indicate that treatment with DHEA restores...
plasma estradiol levels to near-normal physiological values for the rabbit. Androgen (i.e., testosterone) levels were not determined in these studies.

Cardiac hemodynamics. Cardiac hemodynamic data for each experimental group and intervention are summarized in Table 1. Heart rate was slower (P = 0.001) in Ovx compared with sham; it was similar in Ovx + E\(_{2}\)\(_{\beta}\) and Ovx + DHEA groups but faster than in both sham and Ovx groups. During Iso, heart rate increased significantly (compared with baseline values with Veh); this parameter decreased to near baseline values after administration of Chr. Aortic blood pressure during systole was lower (P = 0.001) in Ovx compared with sham; it was similar in Ovx + E\(_{2}\)\(_{\beta}\) and Ovx + DHEA groups but significantly different from both sham and Ovx groups. Aortic blood pressure during systolic blood pressure was lower than Veh (P = 0.001) during ACh, Iso, and Chr interventions. CO was less in Ovx (P = 0.003) compared with all other groups; no significant differences in CO were observed during each pharmacological intervention. Aortic blood pressure during diastole (i.e., coronary perfusion pressure) was lower (P = 0.001) in Ovx compared with the other groups; coronary perfusion pressure was significantly reduced (compared to Veh) during ACh and Iso and was further reduced during Chr (P = 0.001 compared with Veh, ACh, and Iso). Heart rate-arterial blood pressure product, an index of myocardial oxygen demand, is shown in Fig. 1 and was significantly lower in Ovx compared with the other groups; treatment with either E\(_{2}\)\(_{\beta}\) or DHEA restored heart rate-arterial blood pressure product to levels observed in the sham group.

Distribution of myocardial blood flow. During autoregulation (i.e., administration of Veh), myocardial blood flow was not different between groups, as shown in Fig. 2, top; diastolic perfusion pressure in Ovx rabbits was significantly lower than sham but restored to baseline values after either E\(_{2}\)\(_{\beta}\) or DHEA treatments. The leftward shift to a lower coronary perfusion pressure on the pressure-flow curve in Ovx rabbits would suggest reduced vascular reserve in this group (35). Pharmacological intervention with ACh and Iso shifted the coronary perfusion pressure values towards the

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**Table 1. Hemodynamic measurements and myocardial blood flow**

<table>
<thead>
<tr>
<th>Groups and Interventions</th>
<th>n</th>
<th>HR, beats/min</th>
<th>PAoS, mmHg</th>
<th>CO, ml/min</th>
<th>PAOD, mmHg</th>
<th>Endo, ml·min(^{-1})·100 g(^{-1})</th>
<th>Epi, ml·min(^{-1})·100 g(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>8</td>
<td>237 ± 9(^a)</td>
<td>81 ± 2(^a)</td>
<td>440 ± 38</td>
<td>64 ± 3(^a)</td>
<td>3.1 ± 0.2</td>
<td>2.9 ± 0.2(^c)</td>
</tr>
<tr>
<td>ACh</td>
<td>8</td>
<td>229 ± 9(^b)</td>
<td>64 ± 3(^b)</td>
<td>406 ± 21</td>
<td>37 ± 3(^b)</td>
<td>2.8 ± 0.2</td>
<td>3.1 ± 0.2(^c)</td>
</tr>
<tr>
<td>Iso</td>
<td>8</td>
<td>263 ± 17(^A)</td>
<td>69 ± 3(^A)</td>
<td>455 ± 52</td>
<td>39 ± 3(^A)</td>
<td>3.4 ± 0.4</td>
<td>4.7 ± 0.5(^A)</td>
</tr>
<tr>
<td>Chr</td>
<td>8</td>
<td>222 ± 13(^B)</td>
<td>62 ± 2(^B)</td>
<td>385 ± 36</td>
<td>33 ± 3(^C)</td>
<td>4.1 ± 0.7</td>
<td>6.1 ± 0.6(^A)</td>
</tr>
<tr>
<td>Ovx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>8</td>
<td>204 ± 6(^B)</td>
<td>71 ± 4(^a)</td>
<td>324 ± 30</td>
<td>56 ± 3(^a)</td>
<td>3.7 ± 0.5</td>
<td>3.4 ± 0.6(^c)</td>
</tr>
<tr>
<td>ACh</td>
<td>8</td>
<td>203 ± 5(^B)</td>
<td>54 ± 3(^B)</td>
<td>357 ± 33</td>
<td>33 ± 1.1(^B)</td>
<td>3.3 ± 0.6</td>
<td>3.6 ± 0.7(^c)</td>
</tr>
<tr>
<td>Iso</td>
<td>8</td>
<td>218 ± 15(^A)</td>
<td>56 ± 2(^B)</td>
<td>351 ± 26</td>
<td>28 ± 4(^B)</td>
<td>2.8 ± 0.3</td>
<td>3.9 ± 0.4(^B)</td>
</tr>
<tr>
<td>Chr</td>
<td>8</td>
<td>192 ± 10(^B)</td>
<td>52 ± 3(^B)</td>
<td>442 ± 42</td>
<td>25 ± 3(^B)</td>
<td>3.1 ± 0.5</td>
<td>5.6 ± 0.8(^B)</td>
</tr>
<tr>
<td>Ovx + E(<em>{2})(</em>{\beta})</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>9</td>
<td>254 ± 8(^B)</td>
<td>75 ± 3(^B)</td>
<td>375 ± 39</td>
<td>62 ± 3(^a)</td>
<td>3.4 ± 0.4</td>
<td>3.0 ± 0.4(^C)</td>
</tr>
<tr>
<td>ACh</td>
<td>9</td>
<td>254 ± 7(^B)</td>
<td>57 ± 3(^B)</td>
<td>458 ± 29</td>
<td>36 ± 1(^B)</td>
<td>3.8 ± 0.3</td>
<td>3.0 ± 0.2(^C)</td>
</tr>
<tr>
<td>Iso</td>
<td>9</td>
<td>283 ± 2(^A)</td>
<td>63 ± 2(^B)</td>
<td>510 ± 50</td>
<td>34 ± 3(^B)</td>
<td>3.2 ± 0.2</td>
<td>4.3 ± 0.2(^B)</td>
</tr>
<tr>
<td>Chr</td>
<td>9</td>
<td>257 ± 6(^B)</td>
<td>59 ± 3(^B)</td>
<td>500 ± 30</td>
<td>28 ± 1(^C)</td>
<td>3.1 ± 0.2</td>
<td>5.6 ± 0.3(^B)</td>
</tr>
<tr>
<td>Ovx + DHEA</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>9</td>
<td>263 ± 14(^B)</td>
<td>82 ± 5(^A)</td>
<td>419 ± 47</td>
<td>67 ± 5(^A)</td>
<td>3.6 ± 0.5</td>
<td>3.3 ± 0.4(^C)</td>
</tr>
<tr>
<td>ACh</td>
<td>9</td>
<td>268 ± 12(^B)</td>
<td>56 ± 3(^B)</td>
<td>454 ± 40</td>
<td>32 ± 2(^B)</td>
<td>3.0 ± 0.3</td>
<td>3.2 ± 0.3(^C)</td>
</tr>
<tr>
<td>Iso</td>
<td>9</td>
<td>286 ± 12(^A)</td>
<td>61 ± 2(^B)</td>
<td>442 ± 27</td>
<td>32 ± 2(^B)</td>
<td>3.4 ± 0.4</td>
<td>4.6 ± 0.5(^B)</td>
</tr>
<tr>
<td>Chr</td>
<td>9</td>
<td>277 ± 7(^B)</td>
<td>58 ± 2(^B)</td>
<td>502 ± 46</td>
<td>28 ± 1(^C)</td>
<td>3.5 ± 0.3</td>
<td>6.2 ± 0.4(^A)</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of rabbits. HR, heart rate; PAoS, systolic aortic pressure; CO, cardiac output; PAOD, diastolic aortic pressure; Endo and Epi, subendocardial and subepicardial blood flow, respectively; Ovx, ovarioctomized; Veh, vehicle; ACh, acetylcholine; Iso, isoproterenol; Chr, chromonar; E\(_{2}\)\(_{\beta}\), 17β-estradiol; DHEA, dehydroepiandrosterone; NS, not significant. Statistical analysis: 4 × 4 ANOVA with replications between groups and interventions (Inter). When statistical significance was achieved, the Student-Newman-Keuls multiple-range test, with a ≤ 0.05, was performed on all main-effect means to determine significant differences within groups (A–C, horizontally) and interventions (A–C, vertically); means with similar letters are not different. P < 0.05.
line of maximal pharmacological vasodilatation obtained with Chr (line shown in Fig. 2, top).

The Endo-to-Epi blood flow ratio (Endo/Epi) for the experimental groups during each pharmacological intervention is shown in Fig. 2, bottom. In Ovx rabbits, the Endo/Epi was maintained at a lower coronary perfusion pressure; treatment with E₂ or DHEA induced a rightward shift on the Endo/Epi diastolic aortic pressure curve. Vasodilatation induced by ACh shifted the Endo/Epi leftward towards the autoregulatory break point. With the use of Iso, Endo/Epi and diastolic aortic pressure were reduced; these data points fall directly on the line of maximal vasodilatation obtained with Chr.

Recruitable coronary vascular reserve was available to each group because coronary vascular conductance increased significantly during ACh, Iso, and Chr (ACh < Iso < Chr), as shown in Fig. 3. Coronary vascular conductance was higher (P = 0.03) in Ovx
than in sham animals and was restored to near-baseline values with either E2β or DHEA treatment.

**DISCUSSION**

The incidence of cardiovascular disease in women increases significantly after menopause; ERT is widely considered to be cardioprotective (30, 40). DHEA has been reported (21, 41) as being catabolized to either androgen or estrogen by various enzymes (i.e., 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase, 17β-hydroxysteroid dehydrogenase, 5α-reductase, and aromatase) in animals and humans. The cardiac effects of DHEA are not well described.

The salient finding of the present in vivo study is that coronary vascular reserve is present but significantly reduced in untreated Ovx rabbits. Coronary pressure-flow data from this group are shifted towards a lower coronary perfusion pressure limit during autoregulation. This is probably because blood flow remains unchanged despite the reduced myocardial demand indices in this group. We also observed a higher level of coronary vascular conductance in Ovx rabbits; possible explanations for these results include reduced extravascular compressive forces in relation to the lower heart rate, arterial pressure, and CO (Table 1) and altered vascular receptor sensitivity to autacoids (25). Chronic administration of either E2β or DHEA after oophorectomy were equally efficient in restoring the oxygen supply-demand equilibrium and shifted coronary pressure-flow data to levels observed in animals with intact ovaries. Alterations in the autoregulatory plateau are known to occur in relation to adjustments in left ventricular pressure and volume (36).

The physiological actions of E2β are probably produced by receptor-mediated effects because functional estrogen receptors are present in both endothelial and vascular smooth muscle (23, 32). Estrogen also affects gene expression of cardiac growth factors and cytokines after myocardial infarction possibly via an endothelin-mediated mechanism; upregulation of myocardial endothelin β-type receptors with estrogen treatment has been shown (37) after ischemia-reperfusion injury.

Cardioprotective effects of E2β appear to involve multiple mechanisms, including enhanced synthesis of nitric oxide via nitric oxide synthase (15), a calcium channel blocker effect (14), inhibition of vascular smooth muscle α2-adrenergic responses (9), increased synthesis of prostaglandins (5), and activation of intracellular signaling mechanisms (i.e., cGMP-dependent phosphorylation) (45). In the present study, circulating estradiol levels after DHEA remained within the range of control animals (~100 pmol/l), whereas in E2β-treated animals, serum estradiol levels reached supra-physiological levels (~600 pmol/l). The positive cardioprotective effect of DHEA may be due to direct conversion to estradiol within tissues.

In vivo studies in dogs have shown that acute intracoronary administration of supraphysiological doses of estrogen induces dilation of conductance and resistance vessels. This vasodilatory effect is likely to be endothelium independent, mediated by a direct nongenomic effect (i.e., ATP-sensitive K+ and/or Ca2+ channels) different from classic intracellular estrogen receptors (39). Immediate vasodilator effects of acute estrogen are observed when plasma levels are >0.1 μM/L; physiological levels of estrogen in the plasma are between 100 and 500 pmol/l (plasma E2β levels were >500 pmol/l in the present study).

The long-term effects of estrogen therapy on myocardial blood flow distribution in normal coronary vessels in vivo remain poorly defined. In the present study, the maximal pharmacological vasodilatation observed during Chr occurred within the Epi tissue layer. These results suggest that the Endo tissue layer was already maximally vasodilated; this phenomenon may be inherent in this animal species. Although blood flow in the endocardium was preserved, higher blood flow levels in this tissue layer may not have been achievable due to the substantial reduction in perfusion pressure.

In conclusion, hearts from Ovx rabbits function at lower levels on the myocardial oxygen supply-demand relation; coronary vascular reserve is present but significantly reduced because coronary pressure-flow data are shifted toward the lower pressure limit of autoregulation. This may be due to preserved myocardial blood flow, although myocardial oxygen demand indices are lower. ERT with either E2β or DHEA restores the myocardial oxygen supply-demand relation to normal levels and shifts coronary pressure-flow data to levels observed in animals with intact ovaries. Whether these beneficial effects are mediated by specific vascular estrogen receptor subtypes should be examined in future studies.

We thank Serge Simard for statistical analyses and Nathalie Rodrigue and Lynn Atton for technical assistance. This study was supported by a grant from the Heart and Stroke Foundation of Canada and by Medical Research Council of Canada Grant MT-13664. A. Dagnault was supported by a Medical Scientist fellowship from the Heart and Stroke Foundation of Canada.

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AJP-Heart Circ Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpheart.org


