Estrogen therapy induces collateral and microvascular remodeling

Kathryn G. Lamping,1,3,4 Lance P. Christensen,2,3 and Robert J. Tomanek2,3

1Departments of Internal Medicine and Pharmacology, 2Department of Anatomy and Cell Biology, and 3The Cardiovascular Center, University of Iowa Roy J. and Lucille A. Carver College of Medicine, and 4Department of Veterans Affairs, Iowa City, Iowa 52246

Submitted 2 May 2003; accepted in final form 6 July 2003

ALTHOUGH RECENT CLINICAL TRIALS suggest that estrogen does not prevent the occurrence of secondary events if administered after the development of cardiovascular disease (12, 13), a large body of epidemiological studies suggests that the incidence of cardiovascular disease is reduced in premenopausal women compared with age-matched men (9). Reasons for the negative results of recent clinical trials may include the initiation of estrogen treatment long after the onset of menopause and the development of cardiovascular disease and the use of combination therapies, including progesterone, as opposed to estrogen alone. In contrast, experimental evidence suggests that estrogen and other gonadal hormones play important roles in physiological and pathophysiological angiogenesis (19).

In the human reproductive system, gonadal steroids tightly regulate cyclic uterine cell proliferation and vascular growth. The normal physiological cycling of the female reproductive tract involves the development and regression of a vascular network that is associated with fluctuations in estrogen levels. Several actions of estrogen may promote angiogenesis, including increased levels of vascular endothelial growth factor (VEGF) and its receptor Flk-1 (10, 14, 26), basic fibroblast growth factor (bFGF) (25), endothelial nitric oxide synthase and nitric oxide (28), adhesion molecules, and matrix metalloproteins (24). However, the relation between estrogen and vascular development in organs other than the uterus is not clearly established.

In vitro or in vivo administration of estrogen promotes the formation of new vessels by promoting endothelial cell proliferation and migration (20). This is in marked contrast to in vitro studies of vascular smooth muscle, where estrogen inhibits proliferation and migration (3, 7, 8). Estrogen enhances vessel growth in a bFGF-coated sponge implanted in ovariec
tomized mice (20). Indirect evidence suggests that estrogen promotes collateral vessel development in the ischemic hindlimb of rabbits (18). However, the effects of estrogen on coronary vascular growth in a model of coronary occlusion have not been studied. The objective of the present study was to determine the effects of estrogen on myocardial blood vessel growth in a model of gradual chronic coronary occlusion. We hypothesized that estrogen would improve the ability of the heart to increase vascular growth in response to a gradual decrease in coronary flow.

METHODS

Ovariectomy of rabbits. All surgical procedures and protocols used in this study conform to the “Guiding Principles for the Care and Use of Animals” approved by the Council of the American Physiological Society and were reviewed and ap-
proved by the Veterans Affairs Medical Center and the University of Iowa Animal Care and Use Committee. Adult female New Zealand White rabbits (2–3 kg) were treated with penicillin (300,000 units) before surgery. After induction of anesthesia with ketamine and xylazine (35 and 5 mg/kg im, respectively) and then with halothane (0.75–1.5%), the rabbits were mechanically ventilated, and their ovaries were removed using sterile surgical techniques after a midline incision in the abdomen (OVX, n = 11). 17β-Estradiol was administered once per week (1 mg/kg im) to another group (OVX-ES, n = 12) (1, 2, 11, 18).

Stimulation of collateral growth. Two weeks after ovariotomy, rabbits were anesthetized first with ketamine and xylazine (35 and 5 mg/kg im, respectively) and then with halothane (0.75–1.0%), intubated, and mechanically ventilated, their hearts were exposed through a left thoracotomy, and an incision was made in the pericardial sac. A segment of the proximal left circumflex (LCx) or left anterior descending (LAD) coronary artery was isolated, and an ameroid constrictor (1.0 mm ID) was placed around the vessel. The pericardium and incision were closed, the chest was evacuated, and the animals were allowed to recover a week. General preparation. Four weeks after implantation of the ameroid occluder, the rabbits were anesthetized with ketamine and xylazine (35 and 5 mg/kg im, respectively) and then with pentobarbital sodium (10 mg/kg iv). In a subset of rabbits, systemic hemodynamics and myocardial blood flow were measured. Rabbits were intubated and ventilated. Both femoral arteries and a vein were cannulated for measurement of aortic pressure, withdrawal of reference blood samples for measurement of myocardial blood flow, and administration of drugs or fluids. The heart was exposed by an incision in the fifth intercostal space and suspended in a pericardial cradle. The left atrium was cannulated for injection of microspheres for determination of myocardial perfusion.

Measurement of myocardial perfusion. Myocardial perfusion was measured using neutron-activated microspheres (23). Microspheres (1–2 × 10^6, 15 μm diameter) labeled with samarium, gold, rhenum, or lutetium were vortexed, injected into the left atrium, and then flushed with saline. Before injection and for 90 s after injection, two reference flow samples were withdrawn at a constant rate (1.23 ml/min) with a pump from both femoral arteries (−4 ml each). Myocardial perfusion was measured before (resting) and during maximal vasodilation with adenosine (1 mg·kg⁻¹·min⁻¹). A snare on the aorta was used to maintain mean arterial pressure at resting levels (±10 mmHg) during infusion of adenosine. At the completion of the study, the heart was arrested with lidocaine and removed, the aorta was cannulated, and the heart was flushed with Locke’s solution containing lidocaine. The heart was perfusion fixed with paraformaldehyde-glutaraldehyde (1.5 and 0.2%, respectively) in phosphate buffer (0.2 M) at 100 mmHg pressure. Two to three samples were excised from the LAD and LCx regions (0.5–1.0-g samples). Closure of the ameroid constrictor was verified by visual inspection and after injection of a gelatin-dye mixture (see below). Tissue and reference flow samples were dried overnight, and microsphere radioactivity was measured by a Germanium detection system after neutron activation (BioPal) (23).

Analysis of capillaries, collateral arteries, and noncollateral arteries. After perfusion fixation, a gelatin-dye mixture was injected into the left main coronary artery (0.2 ml India ink and 0.36 g procine skin gelatin in 10 ml warmed physiological salt solution) (17). This procedure allowed visualization of collateral arteries between the LAD and the LCx, i.e., arterial-arterial connections. Collateral vessels were selected as the segments between the LAD and the LCx with the smallest diameter. These collateral vessels, as well as arteries from collateral-independent regions of similar size, were excised and embedded in Spurr’s plastic, and 1.0-μm-thick sections were stained with Richardson’s solution (27). Images were digitized, and lumen diameter, wall thickness, and wall-to-lumen ratio were determined using the Image Pro image analysis program. Diameter and wall thickness of collateral and noncollateral vessels were determined at magnifications of ×68 and ×1,380, respectively. Lumen diameter was considered the shortest luminal axis of the collateral or noncollateral vessel; wall thickness was measured at two points where the line defining the lumen diameter contacted the wall. The mean of the two measurements of wall thickness values was recorded. We also prepared ultrathin sections that were stained with uranyl acetate and lead citrate and viewed with an electron microscope (model 7000, Hitachi) (4).

Tissue samples for capillary analyses were dissected from midmyocardium in 1) the region distal to the ameroid occluder (collateral-dependent region) and 2) the normal region. Semithin (1 μm thick) sections of specimens embedded in Spurr’s plastic were cut perpendicular to the long axis of the muscle fibers. Sections were placed on glass slides and stained with Richardson’s solution (azure II and methylene blue). Capillary images were projected onto the image analysis monitor, and several fields (including a total of 300–400 capillaries) were selected for measurements. Our program (Image Pro) is designed to determine a variety of capillary parameters, as previously described (27). Length density (L_v), i.e., the total capillary length in 1 mm³ of myocardium, was calculated from the long (a) and short (b) axes and capillary numerical density (N_v) as follows: L_v (mm/mm³) = (a/b)N_v. Length density provides an indicator of growth of the vascular tree. This parameter represents the total vessel length in a unit volume of tissue and, unlike numerical density, is not affected by plane of sectioning. Volume density (V_v) was derived from the sum of lumen cross-sectional areas times 1 (tissue section thickness).

Statistical analysis. Values are means ± SE. Hemodynamics and myocardial conductance data were compared using a repeated-measures analysis of variance. Collateral, noncollateral, and capillary data were compared using a two-way analysis of variance. Post hoc comparisons were performed using Student-Newman-Keuls test with P < 0.05 for statistical significance.

RESULTS

Systemic hemodynamics. There were no differences in the body weights of OVX and OVX-ES rabbits: 3.17 ± 0.10 kg (n = 11) and 3.29 ± 0.11 kg (n = 12), respectively. Hemodynamics and myocardial blood flow were measured in a subset of rabbits in each group (Table 1). Although, under resting conditions, systemic blood pressure tended to be less in OVX-ES (n = 5) than in OVX (n = 7) rabbits, there were no significant differences. During infusion of adenosine, systolic and mean pressure decreased more in OVX-ES than in OVX rabbits. Heart rates were not different between the two groups.

Estrogen increases coronary conductance in collateral-dependent myocardium. Coronary blood flow was measured in OVX (n = 5) and OVX-ES (n = 7) rabbits at rest and during maximal vasodilation with adenosine. Coronary conductance was calculated by dividing
transmural myocardial perfusion (ml·min⁻¹·100 g⁻¹) by mean arterial pressure (mmHg). At rest, conductance was similar in normal and collateral-dependent myocardium in OVX rabbits (Fig. 1). During maximal vasodilation with adenosine, conductance was increased approximately fivefold in normal and collateral-dependent myocardium in OVX rabbits. There was no difference in conductance between normal and collateral-dependent myocardium of OVX rabbits during maximal vasodilation with adenosine. Conductance in collateral-dependent myocardium was twofold greater in OVX-ES than in OVX rabbits during maximal vasodilation with adenosine (Fig. 1). Although conductance was twofold higher in normal myocardium in OVX-ES than in OVX rabbits, the difference was not significant. These data suggest that estrogen enhanced flow in collateral-dependent myocardium.

**Estrogen stimulates collateral vessel remodeling.** Collateral vessels between the LAD and the LCx, identified after injection of a gelatin-dye mixture into the left main coronary artery, were located at the border between the two perfusion territories. Collateral and noncollateral vessels of similar size (60–470 μm diameter) were selected from OVX [noncollateral (n = 11) and collateral (n = 13)] and OVX-ES [noncollateral (n = 8) and collateral (n = 12)] rabbits. Mean diameter of collateral and noncollateral vessels from OVX and OVX-ES rabbits was similar (Fig. 2A).

In OVX rabbits, the wall-to-lumen ratios of collateral and noncollateral vessels were similar in vessels of similar size (Fig. 2B). Wall-to-lumen ratios of collateral vessels from OVX-ES rabbits were greater than those from OVX rabbits (Fig. 2B). As seen in the electron micrographs in Fig. 3, the thicker media in collaterals from OVX-ES rabbits was due to more muscle layers. In addition, the wall-to-lumen ratio was greater for collateral vessels than for noncollateral vessels in OVX-ES rabbits. These data suggest that estrogen affected remodeling of the collateral vessels, which resulted in increased thickness of the tunica media of collateral vessels without an increase in vessel diameter. The effect of estrogen was specific for collateral vessels, because the wall-to-lumen ratio of noncollateral vessels was not increased.

**Estrogen increases capillary diameter and volume density.** Mean lumen diameter of capillaries and capillary volume density in OVX rabbits (n = 8) in normal myocardium was not different from that in collateral-dependent myocardium (Fig. 4A). Thus gradual occlusion alone had no effect on capillary size. In OVX-ES rabbits (n = 8), mean capillary diameter in normal and collateral-dependent myocardium was significantly increased. Estrogen treatment increased capillary diameter in normal myocardium by ~15% and in collateral-dependent myocardium by ~18%. Associated with the increase in capillary diameter was an increase in capillary volume density in normal and collateral-dependent myocardium of OVX-ES rabbits (Fig. 4B). These data suggest that estrogen increased mean capillary diameter in all regions of the heart and did not specifically target ischemic tissue.

**Capillary length density in normal myocardium was not different from that in collateral-dependent myocardium of OVX rabbits (Fig. 4C).** Estrogen treatment had no significant effect on capillary length density in normal or collateral-dependent myocardium (Fig. 4C). These data indicate that, in a model of flow reduction, estrogen stimulates vascular remodeling, resulting in an overall increase in capillary diameter and volume, but does not facilitate angiogenesis, as indicated by capillary length density.

**DISCUSSION**

The goal of these studies was to determine the effects of estrogen on growth of the collateral circulation and

<table>
<thead>
<tr>
<th></th>
<th>Resting</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OVX</td>
<td>OVX-ES</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>OVX-ES</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>94 ± 11</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>62 ± 6</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>76 ± 9</td>
<td>69 ± 5</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>160 ± 20</td>
<td>196 ± 22</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals. OVX, ovariectomized; OVX-ES, ovariectomized and treated with 17β-estradiol (1 mg·kg⁻¹·wk⁻¹); BP, blood pressure; SBP, systolic BP; DBP, diastolic BP. *P < 0.05 vs. OVX.
on the capillary bed in a model of gradual coronary artery occlusion. Three major findings are revealed from the data of this study. 1) Estrogen treatment increased capillary diameter and capillary volume density in normal and collateral-dependent myocardium. Because capillary length density was not altered in normal or collateral-dependent myocardium, angiogenesis was not documented. 2) Myocardial vascular conductance was increased in collateral-dependent myocardium of OVX-ES rabbits, with maximal vasodilation with adenosine. The fact that conductance during maximal vasodilation was greater in estrogen therapy indicates that maximal perfusion was elevated, thus providing for a greater perfusion during high O\textsubscript{2} demand. 3) In collateral vessels of similar size, the wall-to-lumen ratio was increased in OVX-ES compared with OVX rabbits. This finding indicates that estrogen stimulated remodeling of collateral channels. Most importantly, our data show that estrogen treatment improves perfusion to collateral-dependent myocardium.

Rabbit model of coronary occlusion. Several models have been utilized to study development of the coronary collateral circulation. Although far more studies on development of the collateral circulation have been performed in dogs or pigs with slow, progressive occlusion or repetitive occlusions of a proximal coronary artery, the rabbit model of collateral vessel development is attractive, because age-matched animals of a known strain, weight, and gender are easily obtained. Previous studies in rabbits have used both approaches with conflicting results. Although Cohen and co-workers (6) failed to demonstrate evidence of collateral vessel growth in the heart after only 2 wk of repetitive occlusions, Operschall et al. (21) used a model similar to our model with progressive occlusion of the proximal coronary artery for a longer duration and demonstrated development of an extensive collateral circulation. Approximately 3 wk after implantation of an ameroid-type occluder, complete occlusion of the coronary artery was confirmed. Resting flow in epicardium and endomyocardium in the collateral-dependent region was similar to flow before occlusion. During maximal vasodilation with adenosine, increases in epicardial flow to normal and collateral-dependent myocardium were similar to preocclusion levels, whereas flow to endocardium in the collateral-dependent region was diminished. Thus, during progressive occlusion of the proximal artery, collateral vessels in the rabbit heart can develop to maintain normal flow under baseline conditions.

In the present study, we focused on the effects of 4 wk of estrogen therapy on collateral vessel development and capillary growth and remodeling after implantation of the ameroid occluder to ensure complete occlusion of the proximal artery. The dose of estradiol

![Fig. 2. Average diameter (A) and wall-to-lumen ratio (B) of noncollateral and collateral vessels in OVX (open bars) and OVX-ES (solid bars) rabbits. Collateral [OVX (n = 13) and OVX-ES (n = 12)] and noncollateral [OVX (n = 11) and OVX-ES (n = 8)] vessels of similar size (100–675 μm diameter) were selected for comparison. Wall-to-lumen ratio was much greater for collateral vessels from OVX-ES than OVX rabbits. Wall-to-lumen ratio was greater for collateral vessels in OVX-ES rabbits than for noncollateral vessels. Values are means ± SE. *P < 0.05, OVX-ES vs. OVX; †P < 0.05, OVX-ES collateral vs. noncollateral vessels.](AJP-Heart Circ Physiol • VOL 285 • NOVEMBER 2003 • www.ajpheart.org)
The ability of estrogen to promote cardiac perfusion.

Utilizing physiological levels of estradiol for rabbits (1, 2, 11, 18), the present study was sufficient to obtain physiological levels of estradiol for rabbits (1, 2, 11, 18).

Estrogen stimulates capillary remodeling and myocardial perfusion. The ability of estrogen to promote growth of new vessels or development of preexisting vessels may involve a variety of angiogenic factors. Estrogen upregulates expression of VEGF and its receptors (14, 26), bFGF (25), and nitric oxide synthase (28). Each of these factors may regulate vascular growth of new vessels or development of preexisting vessels. In the nonischemic rabbit heart, ovariectomy resulted in a decrease in total capillary density in peripheral tissue. In the nonischemic rabbit heart, ovariectomy resulted in a decrease in total capillary density in peripheral tissue. In the ischemic rabbit hindlimb, estrogen facilitated an increase in capillary density in collateral-dependent myocardium compared with OVX rabbits. Estrogen had no effect on length density. Values are means ± SE. *P < 0.05, OVX-ES vs. OVX.

This study has documented a favorable response of high O2 demand. This is the first study to examine the effect of estrogen on development of collateral vessels in an in vivo model. Four weeks after implantation of the ameroid occluder, growth of the collateral vessels in ovariec-
tomized rabbits normalized the wall-to-lumen ratio. Wall-to-lumen ratio was greater in collateral vessels from OVX-ES rabbits than in collateral vessels from OVX rabbits. This would suggest that, in vessels undergoing remodeling, estrogen enhances proliferation of vascular smooth muscle, resulting in a thicker vessel. The finding that the wall-to-lumen ratio of collateral vessels was greater in the estrogen-treated group indicates a remodeling process. This morphogenic adaptation is consistent with an increase in flow and pressure in these vessels coupled with the effects of estrogen. The effect of estrogen appeared to be specific for collateral vessels, because it did not increase the wall-to-lumen ratio of noncollateral vessels. Thus estrogen facilitates remodeling in collateral vessels that experience increased flow. Enhancement of the wall-to-lumen ratio would serve to normalize wall stress. This is consistent with previous work that documented outward hypertrophic remodeling in response to hyperperfusion in small mesenteric arteries (22).

Summary. This study has documented a favorable role of estrogen during a progressive coronary occlu-
sion. Our data are the first to show that estrogen treatment increases conductance to the collateral-dependent myocardium. The decreased resistance to flow may be related to an increased capillary diameter and, most importantly, volume density and expansion of the cross-sectional area of resistance vessels. In the rabbit model of gradual coronary occlusion, estrogen did not cause capillary proliferation, because capillary length density was not increased. However, the increased myocardial perfusion attributed to estrogen suggests growth in large vessels, as well as remodeling of collateral vessels, because wall thickness was augmented in the latter. Although in the short term (4 wk) estrogen appears to be beneficial, the long-term effects and mechanisms responsible for the dramatic reduction in vascular resistance need further investigation.

DISCLOSURES

These studies were supported by National Heart, Lung, and Blood Institute Grants HL-39050 and HL-62587 and the American Heart Association.

REFERENCES


