In Vivo Estrogen Manipulations on Coronary Capillary Network and Angiogenic Molecule Expression in Middle-Aged Female Rats

Subrina Jesmin, Ichiro Sakuma, Yuichi Hattori, Akira Kitabatake

Objective—Estrogen replacement therapy (ERT) ameliorates symptoms in postmenopausal women with syndrome X. We hypothesized that estrogen deprivation and replacement may modulate coronary expressions of angiogenic molecules, thereby modifying the coronary capillary network in perimenopausal women.

Methods and Results—Middle-aged (40-week-old) female rats were subjected to sham surgery, ovariectomy, or ovariectomy with ERT. Using immunohistochemical and in situ hybridization techniques, we showed that protein and gene expressions of estrogen receptor β, but not α, in coronary vessels were regulated by in vivo estrogen manipulations. Morphometric analysis showed a reduction in total coronary capillary density with decreased arteriolar capillaries after ovariectomy. ERT resulted in normalization of total capillary number with increased venular capillaries. Coronary expressions of vascular endothelial growth factor (VEGF) and its angiogenic receptor (fetal liver kinase-1) were diminished after ovariectomy, and ERT restored it to intact levels. Higher expressions of VEGF and fetal liver kinase-1 in middle-aged compared with young female rats were associated with an accumulation of hypoxia-inducible factor-1 protein, which was highly expressed in middle-aged female rats.

Conclusions—The coronary capillary network in middle-aged women may be regulated by physiological angiogenesis via VEGF, and reduction in coronary VEGF expression by estrogen deficiency could play a role as a molecular pathogenesis in the development of coronary heart disease in postmenopausal women. (Arterioscler Thromb Vasc Biol. 2002;22:)

Key Words: angiogenesis ■ coronary capillary network ■ estrogen ■ vascular endothelial growth factors ■ middle-aged female rats

It is widely held that the incidence of coronary heart disease in women dramatically increases after menopause. Many postmenopausal women with typical chest pain and ischemic changes on the exercise test are less likely than men to have atherosclerotic coronary lesions. Syndrome X is a term now frequently used to indicate a diagnostic level for patients with exertional angina, a positive response to exercise testing, and angiographically normal coronary arteries. Although syndrome X is heterogeneous with multiple pathogenic entities, an increased prevalence of the syndrome in postmenopausal women postulates a possible link between estrogen deficiency and this syndrome. This hypothesis could be supported by a significant reduction in the frequency of chest pain in estrogen-deficient women with syndrome X receiving estrogen replacement therapy (ERT).

The potential mechanism(s) for the symptomatic benefits of ERT in syndrome X remains poorly understood. Reduced coronary vasodilator reserve is proposed to explain syndrome X. Furthermore, some investigators have invoked a disturbance of coronary microvascular function as a central feature of this syndrome. Although the direct effects of estrogen on the vasculature are now well recognized, among the vascular effects of estrogen, one of the most important is its angiogenic property. Thus, it is possible that the presence of estrogen may play an important role in the development of the coronary capillary network in association with the regulation of physiological angiogenesis, which would be expected to bring about normalization of coronary blood flow reserve with ameliorated microvascular function.

In the present study, we used an experimental model of estrogen deficiency induced by ovariectomy (OVX) in middle-aged female rats, which can be expected to exhibit the same changes in coronary capillary network of the heart as observed in postmenopausal women. Our working design was to determine whether in vivo estrogen manipulations affect the cardiac expressions of several kinds of molecules that are pertinent as possible mediators of angiogenesis. In part, the hormonal influences were also investigated with the use of...
male and young female rats. The present experiments should contribute to an understanding of the molecular mechanisms that may involve cardiac alterations occurring in the setting of estrogen deficiency in elderly women.

Methods

See supplementary Methods section for details (which can be accessed online at http://atvb.ahajournals.org).

Animal Models

Male and female Wistar rats, aged 12 and 44 weeks, were used. Female rats aged 8 or 40 weeks were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) under aseptic conditions. Some female rats were ovarioctomized by making a small incision in the lower abdomen and removing both ovaries, as previously described. Sham-operated (intact) female rats received only laparotomy. Some O VX rats were given 17β-estradiol (10 μg/d) subcutaneously through an osmotic pump that was implanted in the back. Female rats were euthanized 4 weeks after surgery. Blood samples were collected from the inferior vena, and the plasma 17β-estradiol level was determined by radioimmunoassay. Only young female rats that were in the proestrus to the estrus stage (based on vaginal smear findings) when they were killed were used as controls. Middle-aged female rats did not exhibit an obvious estrous cycle, as confirmed by vaginal smears. On the day of the experiments, rats were anesthetized with diethyl ether and killed by exsanguination (20 animals for each group). The heart was removed quickly after the opening of the chest.

Staining of Capillary Morphology

Serial sections (16 μm thick) were cut from the frozen left ventricle (LV). Double staining of sections was carried out to discriminate arteriolar and venular capillaries, as previously described. Arteriolar capillaries were stained blue because they contained alkaline phosphatase; venular capillaries were stained red because they contained dipeptidylpeptidase IV.

Capillary density was assessed light-microscopically on 6-μm-thick deparaffinized tissue sections that were immunostained with anti–von Willebrand factor (factor VIII) antibody (Dako). The antibody was made visible by a secondary exposure of the sections to Cy3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories).

Immunohistochemistry

Five- to 8-μm-thick frozen cryostat sections were fixed in acetone and air-dried. The sections were incubated with primary antibodies, followed by exposure to a suitable secondary antibody coupled to hors eradish peroxidase. Immunostains were visualized by light microscopy with diaminobenzine. The specificity of the immunoreaction was confirmed that the nuclei of cells stained positively for ERs. In coronary vessels of sham-operated intact female rats, ERα and ERβ were expressed almost equivalently, resulting in an ERα/ERβ ratio of 0.95 ± 0.06. When females were subjected to O VX, coronary ERβ expression was evidently diminished, and the ERα/ERβ ratio was changed to a significantly higher level (1.41 ± 0.03, P < 0.01). Treatment of O VX rats with 17β-estradiol enhanced coronary ERβ expression, as seen in intact female rats, and reversed the ERα/ERβ ratio (0.91 ± 0.03, P < 0.01). In situ hybridization studies showed that ERβ mRNA was decreased in middle-aged rats after O VX (Figure 1B). When the numbers of mean mRNA grains per section of coronary vessel were calculated (10 fields ×20 samples), O VX reduced the numbers of grains per section from 62 ± 3 to 31 ± 1 (P < 0.01). The OVX-induced reduction in the number of mRNA grains was significantly prevented by ERT (68 ± 3 grains per section, P < 0.001). There was no significant difference in the level of ERα gene expression among the 3 groups (57 ± 3 grains per section for intact rats, 55 ± 4 grains per section for O VX rats, and 58 ± 3 grains per section for O VX + ERT rats; Figure 1C).

Positive staining for ERβ in coronary vessels was observed more strongly in young female rats than in middle-aged female rats (Figure 1D). Because quantification of immunoreactivity was assessed by pixel intensity, the value for coronary ERβ in young female rats (3.67 ± 0.21) was significantly higher than that in middle-aged female rats (2.57 ± 0.10, P < 0.001), but the values for coronary ERα did not differ between young and middle-aged female rats.
**Figure 1.** A, Confocal images showing immunofluorescence double labeling for ERα (red) and ERβ (green) in LV sections from intact (sham-operated), OVX, and OVX+ERT middle-aged female rats. Positive staining is focused on coronary vessels (inner diameter <100 μm). B and C, In situ hybridization analysis showing gene expression for ERβ (B) and ERα (C) in LV sections from intact, OVX, and OVX+ERT middle-aged female rats. Nuclei in coronary vessels (inner diameter <100 μm) were stained with hematoxylin as bluish-violet. The presence of mRNA is shown by black grains in the field. Original magnification ×400.

(2.49±0.11 versus 2.45±0.11, respectively). Thus, young female rats exhibited a significantly lower ERα/ERβ ratio (0.68±0.05, P<0.05). OVX caused a marked reduction in coronary ERβ expression, resulting in a significant increase in the ERα/ERβ ratio (1.49±0.06, P<0.001).

Compared with middle-aged female rats, male rats showed essentially the same coronary expressions of ERα and ERβ (please refer to online Figure 1, which can be accessed at http://atvb.ahajournals.org). The ERα/ERβ ratio was 0.93±0.06. No age difference was found in coronary expressions of ERα and ERβ in male rats.

**Morphometric Changes**

Micrographs of coronary capillaries in LV sections by the double-staining method showed that the arteriolar capillary portion, which was stained blue, was evidently abundant in intact middle-aged female rats (Figure 2A). There was a marked reduction in arteriolar capillaries after OVX. Thus, OVX significantly diminished the proportion of arteriolar capillaries without changing the venular capillary proportion (Figure 2B). This resulted in a remarkable decrease in the density of labeled capillaries (Figure 2C). After OVX, the total capillary density was reduced to 76% of that in intact middle-aged female rats (Figure 2D). ERT in OVX rats significantly improved the total capillary density to the intact level (Figure 2C and 2D). However, the venular capillary portion, which was stained red, became evident in OVX+ERT rats (Figure 2A). Thus, ERT caused a 2-fold increase in the proportion of venular capillaries, and the arteriolar capillary proportion remained at the reduced level (Figure 2B). As a result of the capillary morphometric changes, the capillary domain area (an area where 1 capillary provides oxygen) was significantly (P<0.01) increased (from 468±8 to 595±6 μm²) after OVX, and this increase was reversed by ERT (458±7 μm²).

In young female rats, the total capillary density (1765±4/mm²) was significantly lower than that in middle-aged female rats (75%, P<0.001). This was associated with a lower proportion of arteriolar capillaries, but a higher venular proportion was evident (see online Figure 1B). In young female rats, OVX reduced the total coronary capillary density, with decreases in arteriolar and venular capillaries, and ERT resulted in restoration of the total number of capillaries with an increased venular proportion. Young and middle-aged male rats also exhibited a low value of the total capillary density (1920±6/mm², 87% that of in middle-aged female rats), and the venular capillary portion was found to be much pronounced (see online Figure 1B).

**Expression of VEGF and Its Receptors**

Immunofluorescent staining for vascular endothelial growth factor (VEGF) showed that its expression was evident in coronary vessels of LV sections from middle-aged female rats (Figure 3A). VEGF was weakly stained after OVX, and its expression was reduced to the same level found in male rats (Figure 3B). The reduced VEGF expression level seen in OVX female rats was completely reversed by ERT (Figure 3A and 3B). VEGF expression was less pronounced in young female rats compared with middle-aged female rats, but OVX caused a further reduction in its expression (Figure 3B). VEGF mRNA was evidently decreased in middle-aged female rats after OVX (Figure 3C). When the numbers of mean mRNA grains per section of coronary vessel were calculated (10 fields×20 samples), VEGF mRNA was decreased to 40% of that in intact female rats by OVX (P<0.001). The OVX-induced decrease in VEGF mRNA was significantly prevented by ERT (P<0.001).

Reduced immunofluorescent staining for fetal liver kinase-1 (Flik-1) was detected in LV sections from OVX compared with intact middle-aged female rats (Figure 4A). Positive staining for Flik-1 was primarily in coronary vessels.
ERT increased the expression of Flk-1 protein in coronary vessels to the level obtained in intact female rats (Figure 4A and 4B). Male rats exhibited a level of Flk-1 expression similar to that in OVX females (Figure 4B). Flk-1 was less abundantly expressed in young female rats than in middle-aged female rats, although its expression was significantly reduced after OVX (Figure 4B). The decrease in positive staining for Flk-1 protein in coronary vessels of OVX middle-aged female rats was correlated with a decrease in Flk-1 mRNA, which was obtained from in situ hybridization experiments (Figure 4C). The results of quantitative analysis showed a 53% decrease in Flk-1 mRNA expression after OVX \((P<0.001)\). ERT significantly increased its gene expression nearly to the level obtained in intact middle-aged female rats \((P<0.001)\). The expression of another VEGF receptor, \(fms\)-like tyrosine kinase-1 (Flt-1), was moderately detected in coronary vessels of rat LV sections. In contrast to the Flk-1 level, the expression level of Flt-1 was not significantly affected by OVX, sex, and age.

Expression of HIF Family

Immunofluorescent staining for 2 hypoxia-inducible factor-1 (HIF-1) subunits, HIF-1\(\alpha\) and HIF-1\(\beta\), showed that their protein expressions were markedly enhanced in coronary

![Figure 2](image-url)
vessels of LV sections from middle-aged female rats compared with young female rats (Figure 5A and 5B). Although HIF-1α and HIF-1β proteins were present in the nucleus in young female rats, these proteins were increased at nuclear and cytoplasmic levels in middle-aged female rats, as analyzed by immunohistochemistry. As assessed by the quantification of immunoreactivity with the use of pixel intensity, HIF-1α and HIF-1β proteins were both increased 2-fold in middle-aged female rats compared with young female rats (P < 0.001). The increased expression levels of HIF-1α and HIF-1β in middle-aged female rats were unmodified by OVX (see online Figure IIIA, which can be accessed at http://atvb.ahajournals.org). In middle-aged male rats, HIF-1α and HIF-1β protein expressions were maintained at the same levels as found in young male and female rats (see online Figure IIIA). HIF-2α protein expression levels did not significantly differ among middle-aged female rats (intact and OVX), middle-aged male rats, and young female rats (Figure 5C; see online Figure IIIA).

In situ hybridization studies showed no difference in HIF-1α and HIF-1β mRNAs between middle-aged and young female rats (see online Figure IIIB). Also, the gene expression of HIF-2α remained unchanged with age.

**Discussion**

Estrogen has been shown to regulate ER expression in tissues relevant to gonadal function, such as breast and uterus. Radioligand binding studies indicate that ERs are also expressed in tissues not classically defined as estrogen targets, including the vasculature. To date, 2 ERs have been described, ERα and ERβ, but their physiological relevance in vasculature is incompletely understood. We found the presence of ERα and ERβ in the coronary vessels of rat hearts at mRNA and protein levels by using immunofluorescence and in situ hybridization. OVX resulted in a marked decrease in coronary ERβ in middle-aged female rats. ERT restored coronary ERβ in OVX rats to levels similar to those in sham-operated rats. A notable reduction in coronary ERβ was
also obtained when young female rats were subjected to OVX. These results suggest that gene and protein expression of ERβ in coronary vessels can be strongly regulated by circulating estrogen. This idea is consistent with the finding that young female rats, compared with middle-aged female rats, exhibit more abundant expression of coronary ERβ, with a much higher level of circulating estrogen. No significant changes were seen in gene and protein expression levels of ERα in coronary vessels after estrogen deprivation or treatment compared with intact levels regardless of whether the animals were young or middle-aged. This result agrees with the reported findings that reverse transcription–PCR has shown no significant differences in the expression levels of ERα mRNA in the hearts of sham-operated, OVX, and OVX+ERT middle-aged female rats.15

Figure 4. A, Confocal images showing immunofluorescence labeling for Flk-1 in LV sections from intact, OVX, and OVX+ERT middle-aged female rats. Immunostaining was mainly focused on coronary vessels (inner diameter <100 μm). B, Quantification of immunoreactivity shown by pixel intensity. The averaged pixel intensity was calculated in 20 randomly selected coronary vessels per sample (20 samples). Data are mean ± SEM. *P<0.001 vs intact middle-aged female rats; †P<0.001 vs OVX middle-aged female rats; and ‡P<0.01 vs intact young female rats. C, In situ hybridization analysis showing gene expression for Flk-1 in LV sections from intact, OVX, and OVX+ERT middle-aged female rats. Original magnification ×400.

Figure 5. Confocal images showing immunofluorescence labeling for HIF-1α (A), HIF-1β (B), and HIF-2α (C) in LV sections from intact middle-aged (top) and young (bottom) female rats. Immunostaining was mainly focused on coronary vessels (inner diameter <100 μm). Original magnification ×400.
Among the many roles of estrogen, one of the most important is its angiogenic property. Our findings that the total density of coronary capillaries in middle-aged female rats significantly declined after OVX and returned to intact levels with ERT are interpreted to indicate that estrogen participates in promoting the formation of new capillaries from preexisting coronary vessels in middle-aged female rat hearts. The role of estrogen in uterine angiogenesis, mediated by ERα, has been suggested by the demonstration that angiogenesis is impaired in ERα knockout mice. However, the striking changes in ERβ expression in parallel with the total capillary density after in vivo estrogen manipulations raise the possibility that ERβ may mediate coronary angiogenesis in response to estrogen. Recent research using endometrial adenocarcinoma cells transfected with VEGF luciferase vectors and expression vectors encoding either ERα or ERβ has demonstrated that estrogen-regulated VEGF gene transcription is dependent on ERα and ERβ. The contribution of each ER subtype to the angiogenic effect of estrogen may be tissue specific. The recent development of ERβ-specific antagonists and the ERβ knockout mouse would allow the crucial role of ERβ in mediating coronary angiogenesis by estrogen to be studied directly.

The ratio of venular to total coronary capillaries was much lower in middle-aged female rats than in young female rats, and arteriolar capillary portions were markedly increased in middle-aged female rats. As a result of the decreased total capillary density after OVX, the capillary domain area was increased, indicating low LV perfusion. Although ERT significantly improved the decreased total capillary density in middle-aged female rats after OVX, this resulted from an increase in venular capillaries. Thus, the ratio of arteriolar to total capillaries remained low even after ERT in OVX middle-aged female rats. However, such a change in the capillary proportion was essentially similar to that seen in intact young female rats. It may be stated that ERT could result in a trend of rejuvenation rather than normalization of the capillary network. Because capillary angiogenesis is usually initiated from the venular site, the increased venular capillary density would reduce the intercapillary distance, leading to facilitation of the oxygen supply to the surrounding tissues.

The possibility that the angiogenic action of estrogen is mediated indirectly, via the production of VEGF, has been suggested by the finding that VEGF expression in the endometrium is increased by estrogen. The mRNA and protein expression levels of VEGF in coronary vessels were significantly decreased with estrogen deprivation, but with estrogen replacement, these levels increased to levels similar to those seen in the control group, supporting the suggestion that estrogen directly regulates VEGF gene transcription. Expression of Flk-1, a receptor that mediates the angiogenic effect of VEGF, was significantly downregulated at mRNA and protein levels after OVX in coronary vessels, and ERT prevented the OVX-induced change in Flk-1 expression. On the other hand, no significant changes were seen in the expression of another VEGF receptor, Flt-1, which is devoid of angiogenic activities, in coronary vessels after estrogen deprivation or treatment. This specific regulation of Flk-1 expression by estrogen deprivation or treatment suggests that expression of the VEGF angiogenic receptor is a contributory factor to estrogen-regulated VEGF-dependent angiogenesis. Interestingly, despite the fact that the circulating estrogen level in middle-aged female rats was much lower than that in young female rats, expressions of VEGF and Flk-1 were significantly higher in middle-aged female rats, implying that alterations in circulating estrogen cannot totally account for the mechanisms responsible for VEGF and Flk-1 expressions in middle-aged female rats.

HIF-1α and HIF-1β proteins were highly expressed in the coronary vessels of middle-aged compared with young female rat hearts regardless of whether the animals were subjected to OVX. HIF-1α and HIF-1β are the subunits of HIF-1, which is a transcriptional factor under hypoxic conditions. Whereas HIF-1β is constitutively expressed, HIF-1α is a specific factor responsible for hypoxic responses. Under hypoxic conditions, HIF-1α protein is stabilized without being degraded through oxygen-dependent proteolysis and initiates a multistep pathway of activation, including dimerization with its partner, HIF-1β. Thus, HIF-1 activities are not regulated at the mRNA level but at the level of protein stability. This could explain the lack of HIF-1α- and HIF-1β mRNA induction in middle-aged female rats. Because coronary expressions of HIF-1α and HIF-1β proteins remained unchanged in middle-aged male rats, the female heart might be rendered ischemic from the perimenopausal stage, leading to increased HIF-1 expression. However, there could be factors other than hypoxia involved that are able to induce HIF-1α protein expression. Recent work has demonstrated that HIF-1α protein is present in the nuclei of different tissues, including the heart, under normoxic conditions. Furthermore, we did not detect any increase in coronary expression of HIF-2α protein, which is also subject to oxygen-dependent proteosomal destruction, in middle-aged female rats. The activation of HIF-1 regulates the VEGF gene by its binding to a hypoxia-responsive element in the 5′-flanking region of the VEGF gene. Thus, HIF-1 is a strong inducer of VEGF mRNA expression. Therefore, it seems likely that the increased expression levels of HIF-1α and HIF-1β proteins in middle-aged female rats may have contributed to increased VEGF expression.

In conclusion, to the best of our knowledge, this is the first report demonstrating that the effects of in vivo estrogen manipulations on the coronary capillary network in middle-aged female rats are strictly associated with VEGF expression in coronary vessels. We propose that VEGF may be a critical regulatory molecule for physiological coronary angiogenesis that constitutes a naturally occurring, compensatory change under the hypoestrogenic condition with aging. The dramatic reduction in VEGF expression in middle-aged female rats after OVX suggests that subtle changes from critical concentrations of estrogen at this age could affect the expression level of VEGF. Moreover, the transcription factor HIF-1, whose expression level greatly increases in middle-aged female rats, appears to lead to an additive action on VEGF expression. The findings observed in our animal models of hypoestrogenic female rats may have clinical implications for postmenopausal women with syndrome X. The understanding
of the regulation by estrogen deprivation or replacement of the coronary VEGF expression mechanisms in middle-aged women deserves consideration and can lead to a potential therapeutic strategy for this clinical setting.

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References
9. Liu M-Y, Hattori Y, Fukao M, Sato A, Sakuma I, Kanno M. Alterations of the regulation by estrogen deprivation or replacement of VEGF expression mechanisms in middle-aged women deserves consideration and can lead to a potential therapeutic strategy for this clinical setting.
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Methods

Animal Models

The experimental design was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Male and female Wistar rats, 12 and 44 weeks old, were used. Female rats at 8 or 40 weeks of age were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) under aseptic condition. Some female rats were ovariectomized by making a small incision in the lower abdomen and removing both ovaries as previously described. Sham-operated (intact) female rats received only laparotomy. Some OVX rats were given 17β-estradiol (10 µg/day) subcutaneously through an osmotic pump which was implanted in the back. Female rats were euthanized 4 weeks after surgery. Blood samples were collected from the inferior cava and the plasma 17β-estradiol level was determined by radioimmunoassay. Only young females staying in the proestrus to estrus stage, based on the vaginal smear findings, at the day when they were killed, were used as control. Middle-aged females did not exhibit an obvious estrous cycle, as confirmed by vaginal smears. On the day of the experiments, rats were anesthetized with diethyl ether and killed by exsanguinations (20 animals for each group). The heart was removed quickly following opening of the chest. Some portion of left ventricle (LV) was dipped into optimum cutting temperature (OCT) compound and immediately frozen in liquid nitrogen. The
remaining portion was preserved at –80°C without OCT compound or was fixed in 4% paraformaldehyde and then embedded in paraffin.

**Staining of Capillary Morphology**

Sixteen-µm-thick serial sections were cut from the frozen LV, and the capillary morphology was analyzed in the subendocardial region. Double staining of sections was carried out to discriminate arteriolar and venular capillaries as previously described. Arteriolar capillaries were stained blue because they contained alkaline phosphatase, while venular capillaries were stained red because they contained dipeptidylpeptidase IV.

Capillary density was assessed light-microscopically on 6-µm-thick deparaffinized tissue sections that were immunostained by anti-von Willebrand factor (FVIII) antibody (Dako). The antibody was made visible by a secondary exposure of the sections to CyTM3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories).

**Immunohistochemistry**

Immunohistochemical studies were performed with the following commercially available antibodies: anti-human ERα polyclonal antibody (Santa Cruz Biotechnology), ERβ monoclonal antibody (Chemicon International), vascular endothelial growth factor (VEGF) polyclonal antibody
(Immunobiological Laboratories), fms-like tyrosine kinase (Flt-1) polyclonal antibody (Santa Cruz Biotechnology), fetal liver kinase (Flk-1) polyclonal antibody (Santa Cruz Biotechnology), hypoxia-inducible factor-1α (HIF-1α) monoclonal antibody (Novus Biologicals), HIF-1β monoclonal antibody (Novus Biologicals), and HIF-2α polyclonal antibody (Santa Cruz Biotechnology).

Five to eight-µm-thick frozen cryostat sections were fixed in acetone and air-dried. The sections were incubated with primary antibodies, followed by exposure to a suitable secondary antibody coupled to horseradish peroxidase. Immunostains were visualized by a light microscopy with diaminobenzine. The specificity of the immunoreaction was evaluated in comparison with the negative control specimen in which non-immune IgG was used instead of the primary antibodies. Quantification of immunoreactivity by pixel intensity was analyzed using an image-analyzing software, as previously described.¹⁰

For immunohistochemical determination of ERs, tissue specimens were fixed in 4% buffered formalin solution, dehydrated and then embedded in paraffin. The preparations were cut in 5 µm sections transversely, deparaffinized and treated with citrate buffer (pH 6.0) in a microwave oven before the immunostaining. In preliminary experiments, the dilutions of the primary antibodies were titrated in each preparation to achieve optimal nuclear staining with minimal unspecific binding. In our hands, the ERα and ERβ
antibodies used for these studies produced the most prominent robust signal with immunofluorescence. Controls included omission of the primary antibodies, omission of the secondary antibody, absorption of the primary antibodies with their respective antigen, and cross-absorption controls. The ratio of ERα/ERβ was calculated by dividing the mean level of ERα by that of ERβ in the same coronary vessel, as determined by immunohistochemistry. The background was adjusted by obtaining the optimum background color during the diaminobenzine reaction and the serial sections showing the same pixel intensity value for the background color were used for calculation of the ERα/ERβ ratio.

We confirmed that protein expressions of the molecules studied herein were expressed more abundantly in coronary vessels compared with cardiomyocytes, and we did not find a clear variation in positive staining between arteriolar and venular microvessels.

Immunofluorescence and Confocal Analysis

Immunodetection of target proteins was also performed using fluorescence secondary antibodies, as previously described.10 For double-label immunofluorescent staining of the two ER subtypes, ERα and ERβ, the sections were incubated with ERα rabbit polyclonal antibody, followed by Cy™3-conjugated anti-rabbit IgG, and then incubated with ERβ
mouse monoclonal antibody, followed by fluorescein-conjugated anti-mouse IgG. Immunofluorescent images were observed under Laser Scanning Confocal Imaging System.10

In Situ Hybridization

In situ hybridization was performed on 10- to 15-µm-thick tissue sections using 35S-labeled synthetic oligonucleotides, as previously described.10 The probes were complementary to nucleotide residues 301 to 346 of ERα cDNA (accession number Y 00102), 45 to 90 of ERβ cDNA (accession number U 57439), 61 to 106 of VEGF cDNA (accession number AF 239170), 541 to 586 of Flk-1 cDNA (accession number U 93306), 961 to 1016 of Flt-1 cDNA (accession number D 28498), 1681 to 1726 of HIF-1α cDNA (accession number U 22431), 541 to 586 of HIF-1β cDNA (accession number AF 348088), and 301 to 346 of HIF-2α cDNA (accession number AX 323419). The specificity of in situ hybridization was confirmed by the disappearance of signals when excessive doses of the corresponding cold oligonucleotides were added to the hybridization fluid. The mRNA grains per blood vessel were quantified using an image-analyzing software.10

Statistical Analysis

Data are shown as means ± SEM. Means were compared by ANOVA,
followed by Fisher’s protected least significance $t$ test for multiple comparisons.

Differences were considered significant at $P<0.05$. 

Online Figure I
A  Young Female

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Online Figure II
**Figure I-online.** Confocal images showing immunofluorescence double labeling for ERα (red) and ERβ (green) in LV sections from intact and OVX young females as well as from young male. Positive staining is focused on coronary vessels (inner diameter: <100 µm). Magnification ×400.

**Figure II-online.** Photomicrographs of LV sections from intact, OVX, and OVX+ERT young females (A) as well as young males (B) by the double staining method (upper panels in A and left panel in B) and by the FVIII staining method (lower panels in A and right panel in B). Magnification ×400.

**Figure III-online.** A, Confocal images showing immunofluorescence labeling for HIF-1α, HIF-1β, and HIF-2α in LV sections from OVX middle-aged females (upper panels) and middle-aged males (lower panels). Immunostaining was mainly focused on coronary vessels (inner diameter: <100 µm). B, In situ hybridization analysis showing gene expression for HIF-1α, -1β, and –2α in LV sections from intact middle-aged (upper panels) and young (lower panels) females. Magnification ×400.