Increased Expression of Estrogen Receptor-β mRNA in Male Blood Vessels After Vascular Injury

Volkhard Lindner, Sung K. Kim, Richard H. Karas, George G.J.M. Kuiper, Jan-Åke Gustafsson, Michael E. Mendelsohn

Abstract—Estrogen exerts direct effects on vascular endothelial and smooth muscle cells that are important for vascular protection. Estrogen receptor-α (ERα) is expressed in vascular cells from males and females and may mediate some of the effects of estrogen on vascular tissue. However, we recently found that estrogen is able to protect against vascular injury in ovariectomized female ERα knockout mice. These mice express the newly described estrogen receptor-β (ERβ) in their aortas, suggesting that ERβ may also mediate some of the direct effects of estrogen on the vasculature. In this study, the level of expression of ERα and ERβ mRNA in male rat aortas was examined before and after vascular injury using en face (Häutchen) preparations and in situ hybridization. Little or no change in ERα expression was observed after vascular injury in either vascular endothelial or smooth muscle cells at any time point. In contrast, ERβ mRNA was found to be expressed markedly after balloon injury. In endothelial cells, ERβ was increased by 2 days after injury, and high levels of expression were maintained at 8 and 14 days. Furthermore, ERβ expression was high in luminal smooth muscle cells at 8 and 14 days after injury and had decreased to low levels by 28 days after injury. These data demonstrate the presence of ERβ in male vascular tissues and the induction of ERβ mRNA expression after vascular injury, supporting a role for ERβ in the direct vascular effects of estrogen. (Circ Res. 1998;83:224-229.)

Key Words: estrogen receptor ■ vasculature ■ knockout mouse ■ vascular injury ■ endothelium

Ischemic cardiovascular events are the leading cause of morbidity and mortality in Western society. These diseases are uncommon in women before menopause, and postmenopausal estrogen replacement therapy decreases their incidence markedly, suggesting that estrogen protects against vascular disease.1-3 Although the beneficial effects of estrogen have been attributed previously to indirect effects of estrogen on classic risk factors, recent data suggest that these do not account for the majority of the protective cardiovascular effects of estrogen.4,5 Indeed, estrogen is now recognized to have direct effects on the blood vessel wall that are central to the beneficial effects of estrogen on vascular physiology and disease (reviewed in References 9 and 10).

Many of the effects of estrogen on its nonvascular target cells are mediated through the first ER identified, a ligand-activated transcription factor now called ERα (reviewed in References 11 to 13). Very recently, a second ER capable of activated transcription factor now called ERα (reviewed in References 11 to 13). Very recently, a second ER capable of

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mg/kg, AnaSed, Lloyd Laboratories) and ketamine (50 mg/kg body wt, Ketaset, Avecoo Co, Inc). Early time points of wounded endothelium (10 hours and 2 days) were studied in the partially denuded rat aorta by passing an uninflated catheter along the vessel. Later time points (8 days, 2 weeks, and 4 weeks) were examined in vessels from which the endothelium was removed by passing an inflated embolectomy catheter along the vessel as described by Clowes et al.\textsuperscript{22} We have previously published illustrations of this procedure,\textsuperscript{29,30} which in the aorta leads to outgrowth of endothelium from the intercostal arteries and the appearance of smooth muscle cells in the intima within 7 days after denudation. Since the normal rat aorta and carotid artery have no intimal smooth muscle cells, endothelial cells (and a rare inflammatory cell) are the only cell types present in the intima at time points earlier than 4 days. All rats were injected with Evans blue before they were killed to identify the areas of endothelial outgrowth. On en face preparations, endothelial cells were readily distinguished from smooth muscle cells by use of the following criteria: Endothelial cells are identifiable as a single coherent layer of cells originating from the intercostal arteries with oblong nuclei equidistant from each other and oriented with blood flow.\textsuperscript{29,30} Smooth muscle cells, on the other hand, appear anywhere within the denuded area, have randomly oriented nuclei that frequently overlap, and appear in multiple layers. The Häutchen technique reliably removes the entire luminal cell layer for examination; smooth muscle cells appear on the en face preparations as they migrate into the region of the lumen, which occurs only after day 4 in this model. The vasculature of all animals was perfusion-fixed with phosphate (0.1 mol/L, pH 7.4) buffered with 4% paraformaldehyde. For in situ hybridization and immunostaining, rats were killed at the indicated times after injury (between 4 hours and 4 weeks).

**In Situ Hybridization**

For en face preparations, vessel segments were cut open longitudinally, and the tissue was pinned out flat on polytetrafluoroethylene (Teflon) cards (luminal side facing up). Incubation with protease K (1 \(\mu\)g/mL, 37°C, Boehringer Mannheim Corp) took place for 15 minutes, followed by prehybridization for 2 hours with 0.3 mol/L NaCl/20 mol/L Tris (pH 7.5)/200 U/mL RNase A (20 \(\mu\)g/mL, 37°C, Boehringer Mannheim Corp) took place for 15 minutes at 37°C, Sigma Chemical Co), and washed in 2× SSC (as above), followed by a high-stringency wash at 55°C for 2 hours (0.1× SSC/10 mmol/L \(\beta\)-mercaptoethanol/1 mmol/L EDTA). Subsequent steps followed the protocol as described.\textsuperscript{29} The Häutchen procedure for en face preparations was carried out after the probe hybridization. Slides were coated with autoradiographic emulsion (Kodak, NTB2), exposed for 3 weeks, and then developed (Kodak, D-19). Vessel preparations were observed under the light microscope using dark-field, bright-field, and a combination of epituliminescence and bright-field illumination (refractive light). Estimates of ER\(\alpha\) and ER\(\beta\) mRNA were made by comparison of sense or background signal with those at each of the various time points and quantified according to an arbitrary scale (see Table legend) by an experienced observer (V.L.) blinded to probe identity and animal treatment.

**Results**

**ER\(\alpha\) and ER\(\beta\) mRNA Expression in Male Animals After Vascular Injury**

We have shown previously using RT-PCR methods that aortic vessels from female wild-type and ER\(\alpha\)-disrupted (ER\(\alpha\)KO) mice express mRNA for ER\(\beta\) at qualitatively similar levels.\textsuperscript{25} To examine the expression of mRNA for ER\(\alpha\) and ER\(\beta\) after vascular injury, the surface cells of uninjured or balloon-injured rat aortas were studied using the en face (Häutchen) technique.\textsuperscript{29} This procedure allows study of the surface endothelial and intimal smooth muscle cells migrating into the region as a single monolayer and provides a sensitive method for studying gene expression in these 2 cell populations. The en face technique was used to study expression of both ER\(\alpha\) and ER\(\beta\) in uninjured endothelial cells (the intact and normal vascular lining) as well as in endothelial cells localized to the advancing endothelial cell edge at various time points after injury. Since smooth muscle cells are observed in such preparations only at 4 to 8 days after injury,\textsuperscript{29} ER\(\alpha\) and ER\(\beta\) expressions in smooth muscle cells in the en face section were examined and compared at 8, 14, and 28 days after balloon injury. The Table summarizes the level of expression of ER\(\alpha\) and ER\(\beta\) in endothelial cells and smooth muscle cells in these experiments. ER\(\alpha\) expres-
sion by vascular endothelial cells was very modest in all cases and was barely above background at all time points examined (Table and Figure 1). Similarly, smooth muscle cells also expressed very little ERα mRNA after injury (Table and Figure 1).

In contrast, ERβ was clearly expressed in both vascular endothelial cells and smooth muscle cells in the aortas of male rats after injury. For endothelial cells, ERβ mRNA was moderately increased at the leading edge of endothelial cells by 2 days after balloon injury, and by 8 days leading edge endothelial cells showed high levels of expression of ERβ mRNA (Table and Figure 2). This high level of expression of ERβ in endothelial cells at the leading edge persisted at day 14 after injury. Expression levels of ERβ behind the leading edge were similar to those seen in normal (uninjured) endothelium in sections examined (data not shown).

Expression of ERα and ERβ in luminal smooth muscle cells was also examined. The en face technique does not allow determination of baseline gene expression in smooth muscle cells of normal vessels and allows study only of smooth muscle cells that migrate into the region of the luminal surface ≥4 days after vascular injury. This population of smooth muscle cells thus differs from other intimal or medial smooth muscle cell populations in the vessel wall. However, once smooth muscle cells have appeared on the luminal surface (ie, 8 days and 2 and 4 weeks), comparison of ERα and ERβ mRNA expression between time points is possible. After injury, ERβ mRNA was moderately abundant in luminal smooth muscle cells at both 8 and 14 days, and in all cases, ERβ message was significantly greater than that for ERα in these cells (Table and Figure 3). By 28 days, the level of expression of ERβ mRNA in the smooth muscle cell population had decreased to very low levels compared with those seen at the 8-day and 2-week time points.

**Discussion**

The present study demonstrates the expression of ERβ mRNA in male rat aortas before and after balloon denudation injury. The level of expression of mRNA for the ERα and ERβ after balloon injury of male rat aortas was strikingly different in these studies. ERα mRNA was expressed only at very low levels in vascular endothelial and smooth muscle cells after injury. ERβ mRNA expression, however, increased to high or very high levels in vascular endothelial cells after injury. In the smooth muscle cells that appeared at the luminal surface after injury, ERβ mRNA was abundant at both 8 days and 2 weeks but declined to near baseline levels by 28 days after injury.
Estrogen inhibits the response to vascular injury in a variety of animal models. In particular, rat vascular tissues express functional estrogen, and estradiol inhibits myointimal proliferation after carotid balloon injury in gonadectomized male rats, the animal studied in these experiments. The high levels of ERβ expression in vascular cells of injured male vessels in these studies, without significant changes in ERα expression, are striking and raise the possibility that ERβ may mediate some of the protective effects of estrogen in the setting of vascular injury. This hypothesis is consistent with the presence of ERβ in the aortas of ERαKO mice and the ability of estradiol to inhibit the vascular response to injury in these animals to the same degree as in their wild-type littermates.

Upstream regulatory regions of the ERβ gene important to the observed induction of ERβ message after vascular injury remain to be defined, and the potential growth factors or other stimuli induced by balloon injury that lead to increased ERβ expression also are presently unclear. However, the observed increase in mRNA for ERβ after balloon injury suggests that study of ERβ may be important in the understanding of the direct vascular protective effects of estrogen. It will be important in subsequent studies to define carefully the relative abundance of ERβ.
mRNA and protein in vascular cells and tissues from intact and ovariectomized female animals after injury. Furthermore, careful analysis of ERα and ERβ mRNA protein in vascular cells and tissues under various conditions will be important, including human cells and tissues from men, premenopausal women, and postmenopausal women, both untreated and on hormone replacement therapy. It will be especially important to examine cell culture expression of the ERs and to correlate in vitro with in vivo expression. Preliminary RT-PCR studies indicate that ERβ is expressed in male and female vascular cells (data not shown), but it is already apparent that expression levels of ERβ mRNA, like those of ERα, vary with culture conditions, passage, and vascular bed of origin (see Reference 23). It will also be important to understand regional differences in the vascular expression of ERβ in vivo and to examine specifically the role of ERβ in mediating the vascular protective effects of estrogen. The recent development of ERβ-specific antagonists and the ERβ knockout mouse will allow this latter topic to be studied directly.

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