Estrogen Receptor-α Mediates the Protective Effects of Estrogen Against Vascular Injury

Gary Pare, Andrée Krust,* Richard H. Karas,* Sonia Dupont, Mark Aronovitz, Pierre Chambon, Michael E. Mendelsohn

Abstract—Blood vessel cells express the 2 known estrogen receptors, α and β (ERα, ERβ), which are thought to mediate estrogen inhibition of vascular injury and atherosclerosis, but the relative role of ERα and ERβ in these events is controversial. Estrogen inhibits the vascular injury response to the same extent in ovariectomized female wild-type mice and in the original single gene knockout mice for ERα (ERαKOChapel Hill [ERαKOCh]) and ERβ (ERβKOChapel Hill [ERβKOCh]). In double gene knockout mice generated by crossing these animals (ERαβKOCh), estrogen no longer inhibits medial thickening after vascular injury, but still inhibits vascular smooth muscle cell proliferation and increases uterine weight. The partial retention of estrogen responsiveness in ERαβKOCh mice could be due either to the presence of a novel, unidentified estrogen receptor or to functional expression of an estrogen receptor-α splice variant in the parental ERαKOCh mice. To distinguish between these possibilities, we studied recently generated mice fully null for estrogen receptor α (ERαKOStrasbourg [ERαKOSt]) and examined the effect of estrogen on the response to vascular injury. In the present study, we show that after vascular injury in ovariectomized ERαKOSt mice, estrogen has no detectable effect on any measure of vascular injury, including medial area, proteoglycan deposition, or smooth muscle cell proliferation. These data demonstrate that estrogen receptor-α mediates the protective effects of estrogen on the response to vascular injury. (Circ Res. 2002;90:1087-1092.)

Key Words: estrogen ■ hormones ■ vascular injury ■ receptors ■ animal models

The cardiovascular effects of steroid hormones are an area of intense interest at present. Estrogen has known systemic effects on circulating factors and has more recently been established to have direct effects on the blood vessel wall. Estrogen causes both rapid vascular dilatation and prolonged effects on gene expression in vascular cells (reviewed in Mendelsohn and Karas). At physiologically relevant concentrations of estrogen, studies support that estrogen receptors (ERs) α and β mediate both the rapid and the long-term cardiovascular effects of estrogen. ERα and ERβ are expressed in both vascular endothelial and smooth muscle cells, but their physiological roles in the vasculature are incompletely understood.

Using wild-type and estrogen receptor knockout mice, we have previously studied the role of ERα and ERβ in mediating the vascular protective effects of estrogen in a mouse carotid artery injury model. Studies of mice harboring single gene deletions of either ERα or ERβ showed that treatment of ovariectomized female mice with nanomolar concentrations of 17β-estradiol (E2) inhibits the response to vascular injury to equivalent levels in wild-type mice, ERαKOChapel Hill (ERαKOCh) and ERβKOCh. These findings suggested that ERα and ERβ are able to complement one another such that each receptor alone is sufficient to mediate the vascular protective effects of estrogen, or that the vascular protective effects of estrogen are mediated by an ERα/ERβ-independent pathway. To distinguish between these 2 hypotheses, studies of vascular injury in ERαβKOCh (double) estrogen receptor knockout mice were performed. However, the effect of estrogen on vascular injury in these mice was complex. Although E2 no longer inhibited the increases in medial carotid area after injury in the ERαβKOCh mice, E2 still significantly inhibited vascular smooth muscle cell (VSMC) proliferation after injury. In addition, E2 also caused a significant increase in uterine weight in the ERαβKOCh mice. These data showed that the role of estrogen receptors could diverge for specific components of the vascular injury response in the ERαβKOCh mice. However, the results left unresolved what is responsible for estrogen inhibition of VSMC proliferation and the increase in uterine weight in the ERαβKOCh mice. These could be due to an unidentified third estrogen receptor or to residual function of protein from an ERα splice variant known to be expressed in the parental ERαKOCh mice. To resolve the question as to how estrogen
inhibits vascular injury in the mouse carotid injury model, we have studied the effects of estrogen on the response to vascular injury in the newly created ERαKOStrasbourg (ERαKOSt) mice.

Materials and Methods

Generation of ERαKOStrasbourg Mice

Full details have been described recently.8 In brief, to create mice fully null for ERα, a targeting vector containing exon 3 of ERα, a TKneo cassette, and a loxp site was generated using PCR-based site-directed mutagenesis, as described.8 The targeting vector was electroporated into 129/SvPas H1 embryonic stem (ES) cells, and after expansion of G418 neomycin-resistant clones, ES cells containing a targeted ERα allele were identified and injected into C57BL/66 blastocysts. These were introduced into pseudopregnant hosts to obtain chimeric males that transmitted the mutation through crosses with C57BL/66 females, yielding heterozygous mice, which were next bred with homozygous CMV-Cre transgenic mice to generate heterozygous mice in which both ERα exon 3 and the selectable marker were deleted. Inbreeding of heterozygous ERα mice yielded mice homozygous for the deletion of ERα exon 3 (ERαKOSt mice),8 in numbers consistent with Mendelian expectations. Institutional guidelines of the IACUC and DLAM were followed in the care and use of animals in this study.

Mouse Model of Carotid Arterial Injury

The mouse carotid injury model used in this study has been described and validated in detail.2,3,5,6 The general study design, as previously, begins with ovariectomies of 10- to 12-week-old female mice 7 to 10 days before the start of the experiment. At day 0, vehicle- or E2-releasing pellets (0.1 mg, 21-day release pellets, Innovative Research of America) were implanted in each animal. At Day 0, carotid injuries were performed, followed by implantation of osmotic minipumps calibrated to release BrdU over the course of the experiment (25 mg BrdU/kg per day), to allow measurement of the extent of vascular cell proliferation. Mice were killed at day 14. Bloods were harvested and blood vessels were fixed at physiological pressures (100 to 120 mm Hg), followed by analyses for each of the endpoints described, as previously.2,3,5,6

Morphometry and Immunohistochemistry

Parallel sections from all 92 carotids (46 injured, 46 contralateral unjured) were stained as described previously with hematoxylin-eosin and elastin, and area measurements were made using a computerized morphometric analysis system on the elastin-stained sections.3 BrdU-labeled cells were identified by immunostaining and were counted in all sections. Two independent observers fully blinded to treatment made all measurements. To distinguish between vascular cell types, immunostaining also was performed on parallel carotid artery sections using endothelial cell–specific (factor VIII–related antigen) and VSMC-specific (α-actin) antibodies.3 BrdU-positive cells were categorized as endothelial cells if they were positive for factor VIII–related antigen and negative for α-actin, and as VSMCs if the converse pattern was seen. Proliferation Index is calculated as the ratio of BrdU-positive VSMCs to unstained medial nuclei in each section.6 Proteoglycan deposition was quantified using Movat’s pentachrome staining8 and computerized quantification of positive (blue) regions (Figure 2). For all statistical analyses, genotype and treatment groups were independent variables and thus analyzed using a 2-factor ANOVA, with post hoc pairwise comparisons made with the Student-Newman-Keuls test.

Results and Discussion

ERαKOSt mice and their wild-type (WT) littermates were used to study the effect of estrogen on the vascular response to injury.2,3,5,6 Complete ablation of ERα in ERαKOSt mice was confirmed by the absence in their uteri of any ERα polypeptide immunoreactive with antibody directed against ERα C-terminus, as well as by the absence of mRNA for ERα-containing transcripts of any exons located downstream of exon 2.8 The mouse carotid injury model involves the uniform passage of a fine wire into the common carotid artery, causing endothelial denudation and an injury response characterized by increases in medial area and VSMC proliferation.3,10

The response to vascular injury was studied in 46 normocholesterolemic, ovariectomized adult female mice (21 ERαKOSt and 25 WT littermates) randomized to receive vehicle alone or 17β-estradiol (E2), as described.2,6 No differences in the total or HDL cholesterol levels or triglycerides were noted between the experimental groups (Table). Estrogen levels were undetectable in vehicle-treated mice. Estrogen-treated animals attained mean circulating estrogen levels of 90 to 117 pg/mL (0.33 to 0.43 nmol/L). Uterine weights were low in both vehicle-treated WT and ERαKOSt mice (Table and Figure 1). In WT mice, E2 caused an increase in uterine weight from 9.4±0.5×10^-5 to 110.5±7.2×10^-5 g (P<0.001). In contrast, E2 had no effect on ERαKOSt uterine weights (Figure 1).

Uninjured (Uninj) carotid arteries from WT and ERαKOSt mice were indistinguishable by all morphometric criteria measured, whether treated with vehicle or E2. As expected for this vascular injury model, which is characterized by medial thickening and VSMC proliferation,2,5 only a negligible

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>E2 Level, nmol/L</th>
<th>Total Cholesterol, mg/dL</th>
<th>Triglyceride, mg/dL</th>
<th>HDL, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type, vehicle</td>
<td>ND</td>
<td>81</td>
<td>251</td>
<td>75</td>
</tr>
<tr>
<td>Wild type, +E2</td>
<td>0.43±0.02</td>
<td>70</td>
<td>267</td>
<td>66</td>
</tr>
<tr>
<td>ERαKOSt, vehicle</td>
<td>ND</td>
<td>71</td>
<td>214</td>
<td>68</td>
</tr>
<tr>
<td>ERαKOSt, +E2</td>
<td>0.33±0.01</td>
<td>65</td>
<td>205</td>
<td>63</td>
</tr>
</tbody>
</table>

ND indicates not detectable.
vascular wall cells. We quantified the extent of deposition and deposition of matrix proteins elaborated by vascular injury is due to an increase in both cellular proliferation (Figure 3A).

In the WT mice, the medial area per section increased from 18.5 ± 0.8 × 10^{-3} to 22.0 ± 0.8 × 10^{-3} mm² after injury (P < 0.001). As in previous studies, estrogen replacement significantly inhibited the injury-induced increase in medial area in the WT mice to levels comparable to uninjured animals (to 20.8 ± 1.3 × 10^{-3} mm², P < 0.001 versus Inj, -E₂; P = NS versus Uninj) (Figure 3A). In ERαKO₆ mice, significant injury was also noted, with the medial area per section increasing from 18.5 ± 0.6 × 10^{-3} to 22.0 ± 0.8 × 10^{-3} mm² after injury (P < 0.05). However, estrogen replacement failed to inhibit this response in ERαKO₆ mice (Inj, +E₂ = 22.6 ± 1.2 × 10^{-3} mm², P = NS versus Inj, -E₂) (Figure 3A). The medial area thickening after injury in the vehicle-treated ERαKO₆ mice was significantly less than that of the vehicle-treated, WT mice (P = 0.007) (Figure 3A).

The increase in medial area that occurs in response to vascular injury is due to an increase in both cellular proliferation and deposition of matrix proteins elaborated by vascular wall cells.\(^{11}\) We quantified the extent of deposition of proteoglycan, one of the principal matrix substances elaborated, in carotid sections from all mice in the study (Figures 2d through 2f and 3B). In WT mice, the proteoglycan deposition per section rose from undetectable levels in uninjured vessels to 24.2 ± 3.8% of total medial area after injury. Estrogen replacement significantly inhibited proteoglycan deposition in WT mice (to 7.6 ± 1.9% of total area, P < 0.001) (Figure 3B). In ERαKO₆ mice, proteoglycan deposition per section rose from undetectable levels in uninjured vessels to 16.3 ± 2.8% of total medial area. Estrogen replacement failed to significantly inhibit this response in ERαKO₆ mice (Inj, -E₂ = 16.3 ± 2.8% versus Inj, +E₂ = 13.5 ± 2.1% of total medial area, P = NS; Figure 3B). The proteoglycan deposition per section in vehicle-treated ERαKO₆ mice was less than that in WT, vehicle-treated mice (P = 0.02).

The extent of endothelial cell and VSMC proliferation over the 14 days after injury was quantified by immunostaining for 5-Bromo-2'-deoxyuridine (BrdU) in sections from both injured arteries and the contralateral uninjured vessels (Figures 2g through 2i and 4). Injury induced significant increases in endothelial cell labeling in both WT and ERαKO₆ mice, but the extent of endothelial cell labeling was not different for these groups, nor was it affected by estrogen treatment (cf. references 2, 3, 5, and 6). Medial VSMC cell proliferation was also quantified (Figure 4). In uninjured vessels from WT mice, very few (mean, <1 cell/section) cells were labeled.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Representative sections stained for measurement of medial area (elastin stain, a through c), detection of proteoglycan deposition (Movat’s pentachrome stain, d through f), or VSMC proliferation (BrdU immunostain, g through i). In a, arrows depict the internal (open arrow) and external (black arrow) elastic membranes used to demarcate the media for computerized morphometric analysis. In f, yellow arrow and dotted lines indicate a region of blue staining for proteoglycan deposition, which is also quantified by computerized morphometric analysis. VSMC proliferation is quantified by counting BrdU-labeled cells and identifying VSMCs and endothelial cells by cell-specific immunostaining in parallel sections. In i, yellow arrow indicates a BrdU-labeled VSMC. For further details, see text and references 2, 3, 5, and 6.

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Medial areas (A) and matrix deposition (B) in uninjured and injured carotid arteries from wild-type and ERαKO₆ mice treated with vehicle or 17β-estradiol. Estrogen replacement significantly inhibited the injury-induced increase in medial area (A) and proteoglycan deposition (B) to levels comparable to the uninjured animals in wild-type mice (P < 0.001), but failed to inhibit these responses in ERαKO₆ mice (P = NS). The medial area and proteoglycan deposition were determined by computerized morphometric analysis of full circumference sections from each animal. Bars represent the mean ± SEM for each group. *P < 0.05 versus the uninjured (Uninj) group within the same genotype.

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Medial areas (A) and matrix deposition (B) in uninjured and injured carotid arteries from wild-type and ERαKO₆ mice treated with vehicle or 17β-estradiol. Estrogen replacement significantly inhibited the injury-induced increase in medial area (A) and proteoglycan deposition (B) to levels comparable to the uninjured animals in wild-type mice (P < 0.001), but failed to inhibit these responses in ERαKO₆ mice (P = NS). The medial area and proteoglycan deposition were determined by computerized morphometric analysis of full circumference sections from each animal. Bars represent the mean ± SEM for each group. *P < 0.05 versus the uninjured (Uninj) group within the same genotype.
Injury resulted in a marked increase in the proliferation of medial VSMCs in WT animals (mean Proliferation Index* (PI) = 2.8 ± 0.8, *P < 0.01; Figure 4). E2 inhibited this increase significantly in the WT mice (to PI = 0.9 ± 0.3, *P < 0.05; Figure 4), although not completely to levels of uninjured vessels. Medial VSMC labeling also was negligible in the uninjured vessels from ERαKOSt mice (<1 cell/section) and increased significantly after carotid artery injury (to 1.1 ± 0.3, *P < 0.01; Figure 4). In contrast to their WT littermates, VSMC proliferation was not inhibited significantly by estrogen treatment in the ERαKOSt mice (P = NS; Figure 4). The extent of VSMC proliferation was less in injured blood vessels from vehicle-treated ERαKOSt mice than in those from WT, vehicle-treated mice (PI = 1.1 ± 0.3 versus 2.8 ± 0.8, respectively, *P < 0.05; Figure 4). Thus, for all 3 measures of vascular injury response, the ovariectomized, vehicle-treated WT mice demonstrated greater injury responses than ERαKOSt mice, and the protective effects of estrogen observed in the WT mice were absent in ERαKOSt mice.

Numerous studies to date implicate estrogen receptors in the regulation of the vascular protective effects of estrogen,1,3–10 but remain inconclusive as to the relative roles of ERα, ERβ, and/or a potential third estrogen-responsive receptor in mediating this protection. ERαKOCh, the first ERα knockout mouse to be generated, were created by insertion of a neo gene into exon 2 of ERα.4 ERαKOCh mice have marked reproductive phenotypes,4,12 but also display a low level of residual, high-affinity estradiol binding, as well as expression of two ERα-derived transcripts, one of which encodes a truncated ERα with intact DNA- and hormone-binding domains. In contrast, ERαKOSt mice are fully null.7,8 ERαKOSt mice are protected against vascular injury equally well by physiological estrogen replacement as their WT littermates.5 Finally, ERαβKOCh mice,5 generated by crossing ERαKOCh and ERβKOCh mice, retain some estrogen responses in both uterine and vascular tissues.6

The present data show that ERα mediates the inhibitory effects of E2 on all injury responses measured in the mouse carotid vascular injury model. This resolves a longstanding debate in the literature regarding whether ERs mediate the vascular protective effects of estrogen at all, and addresses the relative importance of ERα and ERβ in these effects. ERα and ERβ are transcription factors that alter gene expression in both reproductive and nonreproductive target tissues, including liver, bone, brain, and the cardiovascular system.1,14–16 The present findings highlight the potential importance of ERα and ERα-regulated genes in the protective effects of estrogen against vascular injury. Some likely ERα-regulated gene candidates include estrogen-induced genes, such as endothelial NOS,17 cyclooxygenase-1,18 and matrix metalloproteinase-2,19 estrogen-inhibited genes such as endothelin-1,20,21 vascular adhesion molecules,22 and the angiotensin II receptor AT1,23 (see also summary Tables in references1,24,25). The loss of estrogen protection in the ERαKOCh mice thus may be due in part to the altered expression of ERα-regulated genes responsible for estrogen-mediated enhancement of endothelial cell proliferation.

Based on the present study and very recent work from several laboratories, a molecular explanation is now possible for the results reported here and previously.2,6 The Korach laboratory7 originally reported that their ERαKOCh mice still encodes a variant ERα transcript, corresponding to a partial deletion in the A/B domain of 64 amino acids and an insertion of 7 amino acids encoded by the neomycin insert. In heterologous expression studies, they showed that this transcript, which would encode a truncated, 55-kDa ER (ERα55), retained estrogen-dependent transcriptional activity, although protein expression was not evaluated in the murine tissues. Recently, Flouriot and colleagues26 characterized a new 46-kDa endogenous isoform of ERα in nonvascular cells. This isoform, encoded by an ERα transcript lacking the first coding exon, is missing the N-terminal 173 amino acids of full-length ERα, but retains transcriptional activity in the appropriate cell type. A separate study published while this article was under review and including those authors of this work responsible for construction of the ERαKOSt mice (A.K., P.C.),27 confirmed the presence of ERα mRNA variants in ERαKOCh mice. In the aorta of ovariectomized wild-type mice, full-length ERα transcript and another 540-bp PCR product were detected, the latter resulting from splicing of exon 1 such that it would encode an ERα46 isoform deleted for the A/B domain, as reported previously by the Gannon laboratory.26 No RT-PCR products were generated from mRNA obtained in the aorta of the ERαKOSt mutant mice in this study, but 3 unique RT-PCR products were detected in the aorta of ERαKOCh mice.27 Sequencing revealed that 1 was identical to the 540-bp splice variant found in wild-type mice and 2 were those reported previously by Couse et al.,27 including 1 predicted to encode chimeric ERα55. These investigators go on to show expression of a
55-kDa protein in both uterus and aorta of ERαKOCH mice using a C-terminal ERα antibody.27

Taken together, the available data now support that the ERαKOCH mice express a truncated isoform of ERα in the vasculature that can mediate several physiological functions. The isoform expressed in ERαKOCH mice, which lacks the AF-1 of ERα, is sufficient to mediate the effect of E2 on the uterine response (present study and Pendaries et al27), the endothelial production of NO,27 and the inhibition of smooth muscle proliferation, but not the carotid medial thickening in response to injury.2,6 It may therefore be useful to reexamine muscle proliferation, but not the carotid medial thickening in unliganded ERα vasculature.

The isoform expressed in ERαKOCH mice demonstrates widespread vascular abnormalities and develop hypertension as they age.30 Thus, both ERα and ERβ mediate physiologically important effects in the vasculature. Wild-type mice uniformly displayed the largest vascular injury response for all parameters measured in the studies reported here. These animals, unlike the other groups, have an unliganded ERα, which is known to be activated by estrogen-independent pathways that can lead to changes in the expression of a different set of genes than those recruited by estrogen-bound ERα.31–33 This raises the possibility that ligand-independent activation of ERα contributes to the pronounced vascular injury response observed in vehicle-treated WT mice. This might be relevant to the recognized increase in ischemic cardiovascular diseases that occurs in postmenopausal women,34–36 in whom there is an analogous unliganded estrogen receptor. In summary, the present study shows that ERα mediates the inhibition of the vascular injury response by estrogen. These data help resolve the longstanding debate regarding the role of estrogen receptors, and the relative roles of ERα and ERβ, in the protective effects of estrogen against the vascular injury response. They also suggest that identification of novel vascular cell-specific ERα agonists may prove to be useful therapeutic agents in cardiovascular diseases.

Acknowledgments
This work was supported in part by NIH SCOR in Ischemic Heart Disease P50 HL63494; NIH R01 HL55309 and NIH R01 HL56069 (M.E.M.); NIH R01 HL61928 (R.H.K.); and by funds from the Center National de la Recherche Scientifique, The Institut National de la Santé et de la Recherche Médicale, the Collège de France, The Hôpital Universitaire de Strasbourg, The Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Human Frontiers Science Program, the Ministère de la Recherche, and the European Economic Community. We thank Sharon M. Lynch for expert technical assistance. Requests for animals should be addressed to P. Chambon (E-mail chambon@igbmc.u-strasbg.fr).

References
Estrogen Receptor-α Mediates the Protective Effects of Estrogen Against Vascular Injury
Gary Pare, Andrée Krust, Richard H. Karas, Sonia Dupont, Mark Aronovitz, Pierre Chambon and Michael E. Mendelsohn

Circ Res. 2002;90:1087-1092; originally published online May 9, 2002;
doi: 10.1161/01.RES.0000021114.92282.FA
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/90/10/1087

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/