Estrogen inhibits the vascular injury response in estrogen receptor β-deficient female mice

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The protective effects of estrogen in the cardiovascular system result from both systemic effects and direct actions of the hormone on the vasculature. Two estrogen receptors have been identified, ERα and ERβ. We demonstrated previously that estrogen inhibits the response to vascular injury in both wild-type and ERα-deficient mice, and that ERβ is expressed in the blood vessels of each, suggesting a role for ERβ in the vascular protective effects of estrogen. In the present study, we examined the effect of estrogen administration on mouse carotid arterial injury in ERβ-deficient mice. Surprisingly, in ovariectomized female wild-type and ERβ knockout mice, 17β-estradiol markedly and equally inhibited the increase in vascular medial area and the proliferation of vascular smooth muscle cells after vascular injury. These data demonstrate that ERβ is not required for estrogen-mediated inhibition of the response to vascular injury, and suggest that either of the two known estrogen receptors is sufficient to protect against vascular injury, or that another unidentified estrogen receptor mediates the vascular protective effects of estrogen.

17β-estradiol | vascular smooth muscle

The cardiovascular protective effects of estrogen are well established, and the direct effects of the hormone on vascular tissues are now well recognized (1, 2). Using a model of carotid arterial injury in a mouse, we have shown previously that administration of physiologic levels of 17β-estradiol (E2) completely inhibits the vascular injury response in ovariectomized female wild-type (w.t.) mice (3, 4). Subsequent studies demonstrated that E2 continues to protect against vascular injury in ERα knockout mice (αERKO) (4), indicating that estrogen can inhibit the vascular injury response by an ERα-independent pathway. Another estrogen receptor, termed ERβ, was identified recently (5), suggesting the possibility that ERβ mediates the protective effects of E2 on the vasculature. Studies demonstrating that ERβ is expressed in vascular cells and tissues (4, 6–11), and, in contrast to ERα, that ERβ mRNA expression increases in vascular endothelial and smooth muscle cells after vascular injury (11, 12), prompted widespread speculation that ERβ may place a central role in mediating the cardiovascular effects of E2 (10, 13, 14). To test directly the hypothesis that ERβ mediates the vasoprotective effects of estrogen, we have now developed knockout mice in which the ERβ gene is disrupted by gene targeting (βERKO) (15). In the present study, we examine the effect of estrogen on the response to vascular injury in these βERKO mice.

Methods

Animals. The βERKO mice were generated by targeted disruption of exon three of the ERβ gene as described (15). The βERKO mice are normal in appearance, but the females are subfertile because of impaired ovarian function (15). For vascular injury studies, 3- to 4-month-old F_2 littermates bred from heterozygote matings of F_1 animals (129 × C57BL/6J) were used. The mice were fed a normal diet *ad libitum,* as previously described (3, 4).

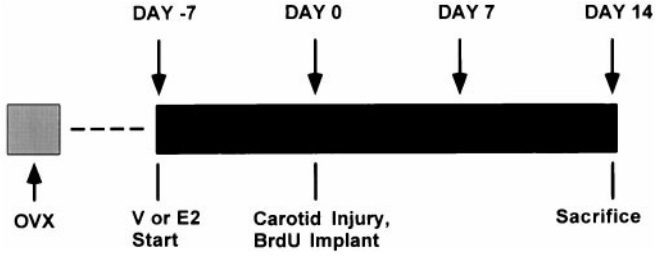

Fig. 1. Design of the carotid artery injury study. All animals were ovariectomized, and then allowed to recover for 7–10 days. Estrogen-containing (E2) or vehicle-containing (V) pellets were implanted s.c. one week before the animals underwent unilateral carotid injury and implantation of an osmotic minipump containing BrdU. On day 14 after injury, the animals were anesthetized and killed; both carotid arteries were perfusion-fixed, harvested, and embedded in paraffin. Vessels were then sectioned transversely and stained to allow morphometric and immunohistochemical analysis of the response to injury. Further details of the procedure have been published previously (3, 4).

Carotid Artery Injury. Ovariectomized F_2 female mice (30 w.t. and 27 βERKO) underwent unilateral carotid artery injury under the protocol described in detail previously (3, 4) (Fig. 1). Briefly, w.t. and βERKO mice were ovariectomized, allowed to recover, and then randomized to receive either placebo pellets (−E2) or E2-containing pellets (+E2) (Innovation Research, Sarasota, FL) to reestablish circulating physiological levels of E2 (3, 4). One week after pellet implantation, endothelial denudation vascular injury was produced by intraluminal introduction of a thin wire into the left common carotid artery. Two weeks after injury, the animals were anesthetized and killed and the uninjured and injured vessels were harvested.

Morphometry and Immunohistochemistry. The response to vascular injury was evaluated by measuring the medial area and by immunohistologic assessment of vascular smooth muscle cell (VSMC) proliferation (BrdU incorporation). Parallel sections from all 114 carotid arteries were stained for hematoxylin/eosin and for elastin, and area measurements were made by using a computerized morphometric analysis system on the elastin-stained sections (3, 4). For each animal, two sections were analyzed, and the medial area measurements were averaged. BrdU-labeled cells were identified by immunostaining, and counted, as described (3, 4). All measure-

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Abbreviations: E2, 17β-estradiol; ER, estrogen receptor; βERKO, mice with the ERβ gene disrupted by gene targeting; RT, reverse transcription; VSMC, vascular smooth muscle cell; w.t., wild type.

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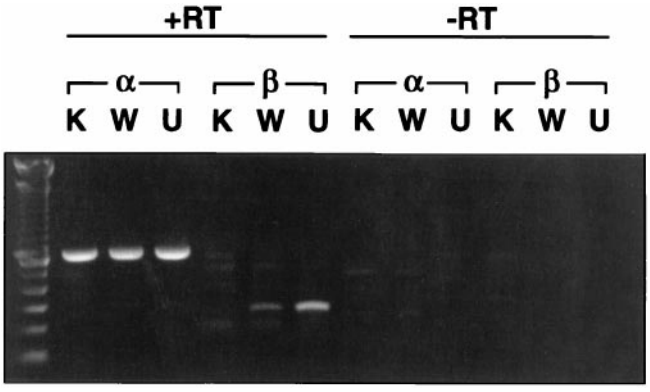

Fig. 2. Reverse Transcriptase-PCR analysis of ERα and ERβ expression in w.t. and βERKO aortae. PCR was performed by using either ERα- or ERβ-specific primers on the product of an RT reaction with RNA derived from w.t. (W) or βERKO (K) aortae, or w.t. mouse uterus (U). The RT reaction was carried out in the absence (−) or presence (+) of reverse transcriptase. Primer pairs were selected to produce PCR products of 240 bp (ERβ) or 611 bp (ERα). The PCR products were then separated by agarose gel electrophoresis. A 100-bp DNA ladder (leftmost lane) is provided as a comparison for evaluating the size of the PCR products.

ments were made by two independent observers fully blinded to treatment. To distinguish between different vascular cell types, immunostaining was also performed on parallel carotid artery sections with endothelial cell-specific factor VIII-related antigen (Sigma) and VSMC-specific α-actin antibodies (Sigma) (3, 4).

Reverse Transcriptase-PCR. Random hexamer-primed reverse transcription (RT) reactions were carried out with 1.25 μg of total RNA (TriReagent, Sigma), derived from the aortae of w.t. or βERKO mice, or from w.t. uterus. The primers for PCR included: (*i*) ERβ-specific forward primers, 5′-25 (base pairs 25–46) and 5′-38 (base pairs 38–59); (*ii*) ERβ-specific reverse primers, 3′-299 (base pairs 299–278) and 3′-798 (base pairs 798–787); (*iii*) ERα-specific forward primers, 5′-506 (base pairs 506–526) and 5′-536 (base pairs 536–556); and (*iv*) ERα-specific reverse primers, 3′-1185 (base pairs 1185–1155) and 3′-1147 (base pairs 1147–1127). These primers are based on the published coding sequences for murine ERα (16) and ERβ (5). The specificity of the primers was confirmed by PCR with plasmids containing ERα and ERβ cDNAs. Negative controls, in which the RT enzyme was omitted, were included in all experiments. The following PCR protocol was employed: 94°C for 5 min, followed by 32 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final 5-min extension at 72°C. In all cases, nested PCR was then carried out on the initial PCR product by using receptor-specific internal primers.

Statistical Analyses. For all analyses, within-group differences were assessed with one-factor ANOVA; post hoc comparisons were tested with the Student–Newman–Keuls test. $P < 0.05$ was considered significant.

Results

The expression of ERα and ERβ mRNA in the vasculature of the w.t. and βERKO mice was investigated by using reverse transcriptase-PCR. ERβ mRNA was expressed in low abundance in

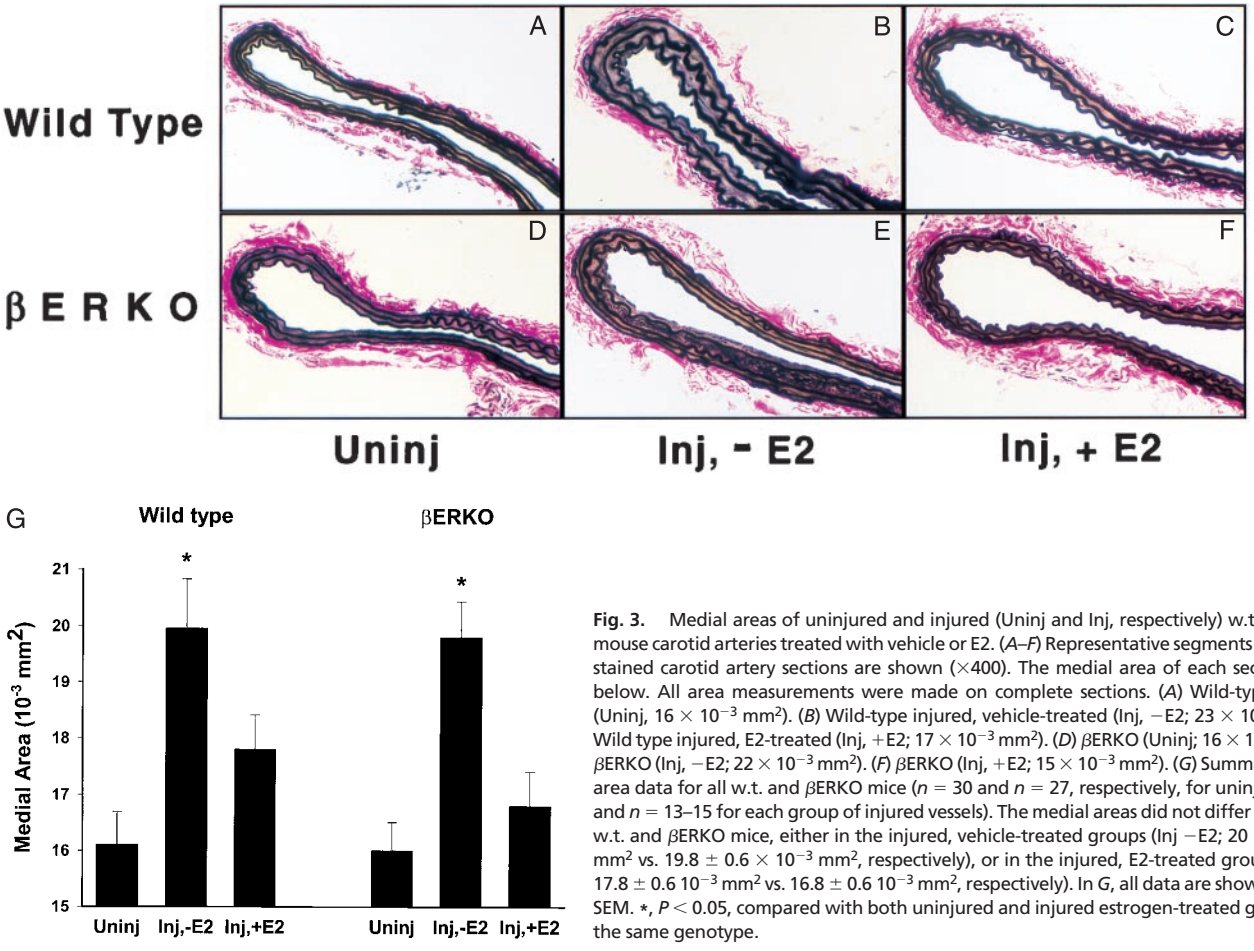

Fig. 3. Medial areas of uninjured and injured (Uninj and Inj, respectively) w.t. and βERKO mouse carotid arteries treated with vehicle or E2. (*A–F*) Representative segments from elastin-stained carotid artery sections are shown (×400). The medial area of each section is listed below. All area measurements were made on complete sections. (*A*) Wild-type uninjured (Uninj, 16×10^{-3} mm^2). (*B*) Wild-type injured, vehicle-treated (Inj, −E2; 23×10^{-3} mm^2). (*C*) Wild type injured, E2-treated (Inj, +E2; 17×10^{-3} mm^2). (*D*) βERKO (Uninj; 16×10^{-3} mm^2). (*E*) βERKO (Inj, −E2; 22×10^{-3} mm^2). (*F*) βERKO (Inj, +E2; 15×10^{-3} mm^2). (*G*) Summary of medial area data for all w.t. and βERKO mice ($n = 30$ and $n = 27$, respectively, for uninjured vessels, and $n = 13$–15 for each group of injured vessels). The medial areas did not differ between the w.t. and βERKO mice, either in the injured, vehicle-treated groups (Inj −E2; $20 \pm 0.9 \times 10^{-3}$ mm^2 vs. $19.8 \pm 0.6 \times 10^{-3}$ mm^2, respectively), or in the injured, E2-treated groups (Inj, +E2; $17.8 \pm 0.6\ 10^{-3}$ mm^2 vs. $16.8 \pm 0.6\ 10^{-3}$ mm^2, respectively). In *G*, all data are shown as mean ± SEM. *, $P < 0.05$, compared with both uninjured and injured estrogen-treated groups within the same genotype.

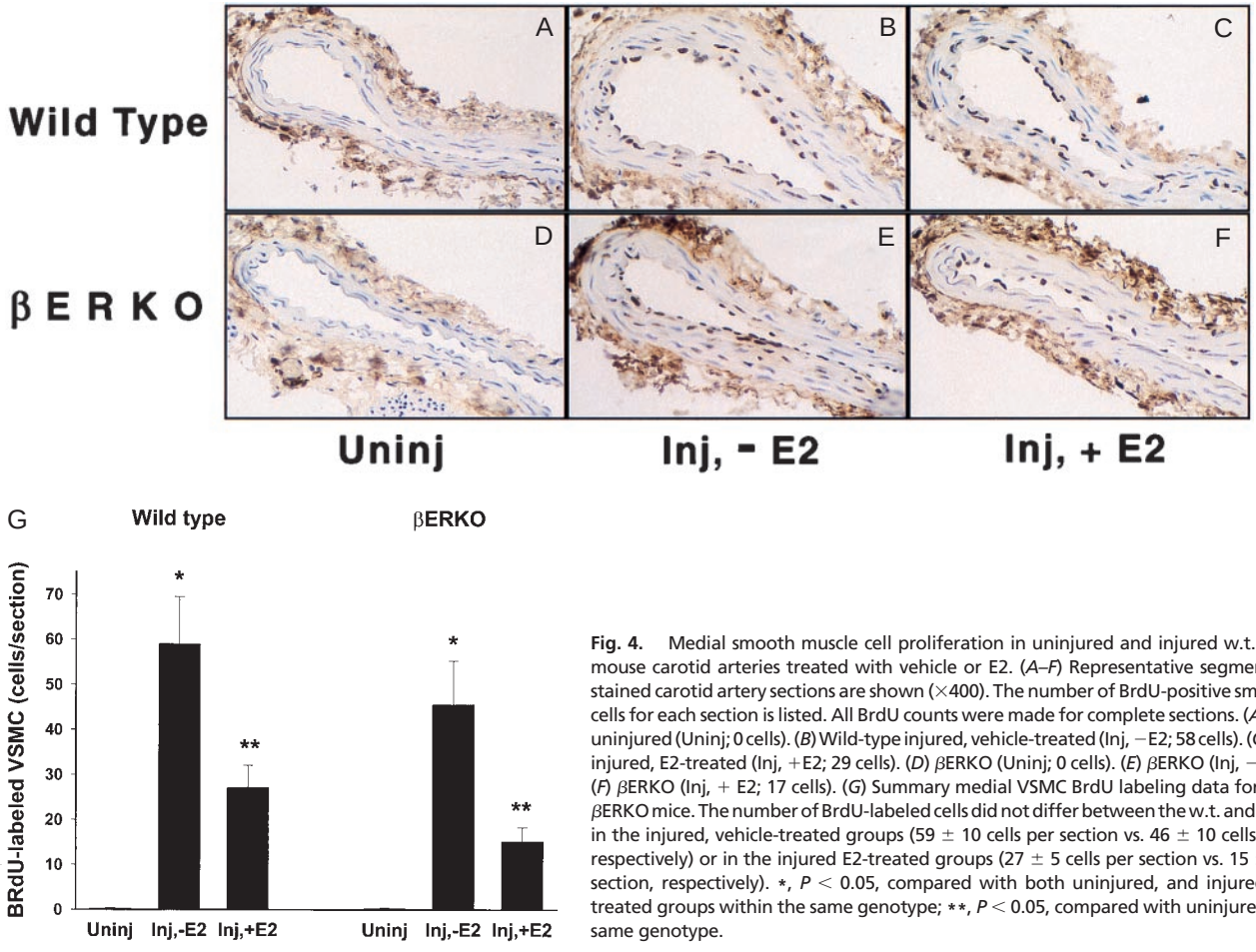

Fig. 4. Medial smooth muscle cell proliferation in uninjured and injured w.t. and βERKO mouse carotid arteries treated with vehicle or E2. (*A–F*) Representative segments of BrdU-stained carotid artery sections are shown (×400). The number of BrdU-positive smooth muscle cells for each section is listed. All BrdU counts were made for complete sections. (*A*) Wild-type, uninjured (Uninj; 0 cells). (*B*) Wild-type injured, vehicle-treated (Inj, −E2; 58 cells). (*C*) Wild-type, injured, E2-treated (Inj, +E2; 29 cells). (*D*) βERKO (Uninj; 0 cells). (*E*) βERKO (Inj, −E2; 45 cells). (*F*) βERKO (Inj, + E2; 17 cells). (*G*) Summary medial VSMC BrdU labeling data for all w.t. and βERKO mice. The number of BrdU-labeled cells did not differ between the w.t. and βERKO mice in the injured, vehicle-treated groups (59 ± 10 cells per section vs. 46 ± 10 cells per section, respectively) or in the injured E2-treated groups (27 ± 5 cells per section vs. 15 ± 3 cells per section, respectively). *, $P < 0.05$, compared with both uninjured, and injured, estrogen-treated groups within the same genotype; **, $P < 0.05$, compared with uninjured within the same genotype.

the vasculature of the w.t. mice (Fig. 2), confirming our previous findings (4, 11, 12). As expected, ERβ mRNA was not detectable in the βERKO vessels. Furthermore, disruption of the ERβ gene in the βERKO mice had no effect on the level of vascular expression of ERα mRNA (Fig. 2).

The response to vascular injury in the w.t. and βERKO mice was quantified by measuring both the carotid medial area and the VSMC proliferation 2 weeks after endothelial denudation (3, 4). There were no differences observed in the medial areas of the uninjured vessels of the w.t. and βERKO mice. In the w.t. mice, injury induced an increase in medial area from $16.1 \pm 0.6 \times 10^{-3}$ mm^2 in the w.t. uninjured to $20.0 \pm 0.9 \times 10^{-3}$ mm^2 in the w.t. injured −E2; $P < 0.05$ (Fig. 3). E2 replacement inhibited the increase in the medial area after injury in the w.t. mice to a value indistinguishable from that of the uninjured vessels ($17.8 \pm 0.6 \times 10^{-3}$ mm^2; $P < 0.05$ vs. w.t. injured −E2). In the βERKO mice, injury also caused a significant increase in carotid medial area (from $16.8 \pm 0.6 \times 10^{-3}$ mm^2 in the βERKO uninjured to $19.8 \pm 0.6 \times 10^{-3}$ mm^2 in βERKO injured −E2; $P < 0.05$ for injured vs. uninjured; see Fig. 3). Just as in the w.t. mice, E2 treatment prevented the increase in medial area after injury in the βERKO animals ($16.8 \pm 0.6 \times 10^{-3}$ mm^2 βERKO injured +E2; $P < 0.05$ for injured +E2 vs. injured −E2; P is not significant for injured +E2 vs. uninjured). There were no significant differences between the w.t. and βERKO mice for either the increase in carotid medial area after injury or for suppression of this response by E2 treatment.

The extent of vascular cell proliferation was assessed 14 days after vascular injury. BrdU-labeled cells were rarely detected in the uninjured arteries of the w.t. and the βERKO mice (average of <1 labeled cell per vessel for both w.t. and βERKO). As shown in Fig. 4, carotid injury induced a marked increase in BrdU-labeled VSMCs in w.t. animals (to 59 ± 10 cells per section; $P < 0.05$ for injured, −E2 vs. uninjured). This increase in VSMC proliferation was attenuated by E2 treatment (to 27 ± 5 cells per section; $P < 0.05$ for injured +E2 vs. injured −E2). In the βERKO mice, the same pattern was seen; injury caused substantial VSMC proliferation in placebo-treated mice (to 46 ± 10 cells per section; $P < 0.05$ for βERKO injured −E2 vs. βERKO uninjured), and E2 again inhibited the injury-induced proliferation of VSMC in the βERKO mice (to 15 ± 3 cells per section; $P < 0.05$ for βERKO injured, +E2 vs. βERKO injured −E2; see Fig. 4). There were no significant differences in the amount of VSMC proliferation between the w.t. and βERKO animals within each treatment group. Thus, for this second independent measure of vascular injury response, E2 proved equally protective in the w.t. and βERKO mice. A similar increase in the extent of endothelial cell proliferation 14 days after injury was seen in both the placebo-treated w.t. group (38 ± 3 cells per section; $P < 0.05$ for injured vs. uninjured) and βERKO (34 ± 3 cells per section; $P < 0.05$ for injured vs. uninjured) carotid arteries. The extent of endothelial cell labeling was also similar for the w.t. and βERKO mice treated with E2 (39 ± 2 and 37 ± 2 cells per section, respectively). Thus, there were no differences in the extent of endothelial cell proliferation at 14 days after injury between any of the groups (P is not significant for all comparisons). As shown previously (3, 4), only a minority of vessels developed a detectable neointimal response after injury (9 of 57 animals or 16%). Thrombosis was observed in only 1 of the 57 injured vessels.

Discussion

The effects of estrogen on the cardiovascular system are diverse; they include indirect effects on systemic factors as well as direct effects on the vasculature (for review, see refs. 2, 17, and 18). The direct effects of E2 on the vasculature include rapid effects on vasomotor tone (19–22), and longer-term effects on atherosclerosis (3, 4, 17, 18, 23–26) and vascular cell proliferation and migration (17, 18, 27–32). The longer-term effects of estrogens result from alterations in gene expression, and are believed to be mediated by estrogen receptors, which are ligand-activated transcription factors (33, 34). The data presented here show that E2 inhibits equally well the response to vascular injury in ERβ-deficient mice and their littermate w.t. controls. We have previously shown that estrogen inhibits the response to carotid artery injury in w.t. mice (3, 4) and in mice devoid of ERα (4), demonstrating that this vasoprotective effect of estrogen also does not require ERα. After the identification of ERβ, studies demonstrated that ERβ is expressed in the heart (refs. 35, 36, and unpublished observations) as well as in the vasculature (4, 11, 36), and that ERβ, but not ERα, mRNA levels are markedly increased in vascular endothelial cells and in VSMCs after vascular injury in male rats (11, 12). These data suggested to many researchers that ERβ may mediate the vasoprotective effects of E2 (10, 13, 14). Several other observations also support different functional roles for ERβ and ERα. Although ERβ and ERα are highly homologous in some domains (e.g., the DNA-binding domains), other regions of the proteins (e.g., the N terminus) share little or no homology (5). In addition, the two receptors differ with respect to their patterns of cell-specific tissue expression (35–38), ligand-binding affinities (35, 39), and gene targets (refs. 40, 41, and unpublished observations), further supporting a potential role in the cardiovascular system for ERβ that is distinct from that for ERα.

The data presented here demonstrate equivalent inhibition by estrogen of the response to vascular injury in βERKO and w.t. mice, and show that ERβ is not required for estrogen-mediated vasoprotection in this model. One explanation for these findings is that ERα and ERβ play redundant roles in mediating vascular protection by estrogen, such that the presence of either ERα or ERβ is sufficient to achieve vascular protection. Such functional redundancy between ERα and ERβ might reflect the fundamental biological importance of the vasoprotective effect of estrogen. An alternative to this hypothesis is that another, still unidentified estrogen receptor (i.e., "ERγ") mediates the vasoprotective effects of estrogen. Differentiation of these two hypotheses will be addressed directly by studying the effects of estrogen on the response to injury in mice lacking both known estrogen receptors, when they become available. In summary, the present study shows that arterial injury leads to similar increases in carotid medial area and smooth muscle cell proliferation in w.t. and βERKO mice, and that these measures of vascular injury are inhibited significantly and to the same degree by E2 in both ERβ-deficient animals and their w.t. littermates. Our results suggest that either of the two known estrogen receptors, ERα or ERβ, is sufficient to protect against vascular injury, or that an unidentified receptor for estrogen exists that mediates the vasoprotective effects of estrogen.

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