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The Activation Function-1 of Estrogen Receptor Alpha Prevents Arterial Neointima Development Through a Direct Effect on Smooth Muscle Cells

Natalia F. Smirnova, Coralie Fontaine, Mélissa Buscato, Adrien Lupieri, Alexia Vinel, Marie-Cécile Valera, Maeva Guillaume, Nicole Malet, Jean-Michel Foidart, Isabelle Raymond-Letron, Françoise Lenfant, Pierre Gourdy, Benita S. Katzenellenbogen, John A. Katzenellenbogen, Muriel Laffargue, Jean-François Arnal

Rationale: 17 β -Estradiol (E2) exerts numerous beneficial effects in vascular disease. It regulates gene transcription through nuclear estrogen receptor α (ER α) via 2 activation functions, AF1 and AF2, and can also activate membrane ER α . The role of E2 on the endothelium relies on membrane ER α activation, but the molecular mechanisms of its action on vascular smooth muscle cells (VSMCs) are not fully understood.

Objective: The aim of this study was to determine which cellular target and which ER α subfunction are involved in the preventive action of E2 on neointimal hyperplasia.

Methods and Results: To trigger neointimal hyperplasia of VSMC, we used a mouse model of femoral arterial injury. Cre-Lox models were used to distinguish between the endothelial- and the VSMC-specific actions of E2. The molecular mechanisms underlying the role of E2 were further characterized using both selective ER α agonists and transgenic mice in which the ER α AF1 function had been specifically invalidated. We found that (1) the selective inactivation of ER α in VSMC abrogates the neointimal hyperplasia protection induced by E2, whereas inactivation of endothelial and hematopoietic ER α has no effect; (2) the selective activation of membrane ER α does not prevent neointimal hyperplasia; and (3) ER α AF1 is necessary and sufficient to inhibit postinjury VSMC proliferation.

Conclusions: Altogether, ER α AF1-mediated nuclear action is both necessary and sufficient to inhibit postinjury arterial VSMC proliferation, whereas membrane ER α largely regulates the endothelial functions of E2. This highlights the exquisite cell/tissue-specific actions of the ER α subfunctions and helps to delineate the spectrum of action of selective ER modulators. (*Circ Res.* 2015;117:770-778. DOI: 10.1161/CIRCRESAHA.115.306416.)

Key Words: estradiol ■ estrogen receptor alpha ■ mice ■ muscle, smooth, vascular ■ myocytes, smooth muscle

Neointimal hyperplasia essentially arises when cells positive for smooth muscle markers cross the internal elastic lamina then migrate and proliferate.^{1,2} In human pathology, this process frequently occurs after the treatment of symptomatic atherosclerosis, which involves mechanical endovascular ballooning (angioplasty) followed by stenting. Neointimal hyperplasia leads to a narrowing of the arterial lumen and is thus termed restenosis.³

On the basis of both experimental and clinical data, we found that estrogens have been proposed to exert several

protective arterial effects. In particular, 17 β -estradiol (E2), the main endogenous estrogen, has a dual beneficial effect on the 2 facets of vascular healing after angioplasty because it both accelerates endothelial regrowth and inhibits the proliferation of vascular smooth muscle cells (VSMC), which otherwise leads to the narrowing of the arterial lumen (restenosis).⁴ Consistent with these functions, E2 has been shown to prevent neointimal hyperplasia in response to endovascular injury in various animal models and species, including rats, pigs, and sheep,⁵⁻⁷ but this action has not been reported to

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Nonstandard Abbreviations and Acronyms

AF	activation function
E2	17 β -Estradiol
EDC	estrogen dendrimer conjugate
ER	estrogen receptor
MISS	membrane-initiated steroid signaling
SMA	smooth muscle actin
SMC	smooth muscle cell
VSMC	vascular smooth muscle cell

date in a mouse model and our understanding of the underlying mechanisms are limited.

Estrogens mediate most of their actions through the binding and activation of the intracellular estrogen receptors (ER) α and β . Their roles have been explored in vivo using transgenic mouse models. We^{8,9} and others¹⁰ have demonstrated that ER α , but not ER β , is required for estrogen-dependent endothelial protection from vascular injury. Indeed, using a model of carotid artery electric injury, we demonstrated that ER α , and not ER β , mediates the stimulatory effect of E2 on re-endothelialization through the action of both endothelial and hematopoietic ER α .^{8,11} Both ER α and ER β act as transcription factors in the nucleus, where they modulate transcription by directly binding to estrogen response element sequences in the DNA. They can also modulate the activity of heterologous transcription factors through protein–protein interactions.¹² Two activation functions, ER α AF1 and ER α AF2, have been shown to play crucial roles in the transcriptional effects of ER α ⁹ through the recruitment of coactivators.^{13,14} Using mice selectively deficient for ER α AF1 or ER α AF2, we previously demonstrated that both of these functions are necessary for E2-mediated endometrial proliferation¹⁵ but are dispensable for the acceleration of reendothelialization by E2.^{16,17} In addition to the well-established role of the nuclear pool of ER α in its transcriptional (also named genomic) actions, a fraction of ER α is also present at or near the plasma membrane, where it has been found to elicit rapid nongenomic membrane-initiated steroid signaling (MISS) effects.^{18,19} Using a unique mouse model containing a disabled palmitoylation site within ER α that is essential for MISS, we recently demonstrated that MISS is essential for the endothelial effects of E2, including its acceleration of endothelial healing.^{15,20} In striking contrast, the responses of the uterus to E2, in particular epithelial proliferation and gene expression changes, depend on the action of nuclear ER α , whereas membrane ER α seems to play little if any role.^{15,20}

The molecular and cellular mechanisms of the action of E2 on endothelial healing have been extensively described,^{8,11,20,21} in particular through the use of the electric injury of the carotid artery model. In contrast, much less information is available on the effects of E2 on VSMC. The effect of E2 on neointimal hyperplasia has been reported mostly in large- or medium-sized animal models. We recently developed a model of endovascular mechanical injury of the femoral artery which, in contrast to the carotid artery site model, induces strong neointimal hyperplasia in mice.² The aim of this study was to

evaluate (1) the effect of E2 in a mouse model of neointimal thickening, (2) the role of ER α and its subfunctions (nuclear versus membrane), and the cellular targets involved in the action of E2 on neointimal hyperplasia.

Methods

An expanded Methods section is available in the Online Data Supplement.

Mice

Wild-type female mice with a C57Bl/6J background were purchased from Charles River Laboratories (France). *Tie2Cre*^{-/-} ER α ^{lox/lox} and *Tie2Cre*^{+/-} ER α ^{lox/lox} mice were generated as described previously,²² and are further referred to as *Tie2Cre*⁻ ER α ^{lox/lox} and *Tie2Cre*⁺ ER α ^{lox/lox}. ER α AF1^{+/+} and ER α AF1^{0/0} mice were generated as described previously.¹⁶ To generate mice carrying a specific deletion of the ER α -encoding gene expressed under the control of the smooth muscle actin (SMA) promoter, ER α ^{lox/lox} mice were crossed with SMACreER^{T2} transgenic mice,²³ further referred to as *SMACreER*^{T2+} ER α ^{lox/lox} (Figure 1B). *SMACreER*^{T2+} ER α ^{lox/lox} (control mice) and *SMACreER*^{T2+} ER α ^{lox/lox} mice were injected daily with tamoxifen (1 mg/mouse per day; Sigma, France) during 5 days from 3 weeks of age to induce activation of the Cre recombinase (Figure 1A). Throughout all protocols, mice were housed at the animal facility of Rangueil (US06, Toulouse, France) and kept under SPF conditions. Mice were housed in a temperature-controlled room with a 12:12-hour light-dark cycle and maintained with access to food and water ad libitum. All animal procedures were conducted in accordance with institutional guidelines on animal experimentation and were under a French Ministry of Agriculture license.

Ovariectomy and Treatments

Bilateral ovariectomy was performed at 4 weeks of age after anesthesia with a mixture of xylazine and ketamine, and mice concomitantly received estrogens or selective ER modulator treatments (Figure 1B and 1C; Online Figure 1). Mice were submitted to a femoral artery wire injury 2 weeks after the start of the treatment (Figure 1C; Online Figure 1).

Femoral Artery Wire Injury in Mice

The femoral artery wire injury was performed as previously described.² For subsequent neointimal hyperplasia analysis, mice were euthanized 28 days later.

Femoral Artery Processing and Morphometry

To assess neointimal hyperplasia in the injured arteries, morphometry was performed on sections from paraffin-embedded arteries. Intimal hyperplasia was expressed as a ratio of A_{NI}/A_{med} ; A_{NI} :neointimal area; A_{med} :medial area).

Immunohistochemistry

SMC and T cells were, respectively, immunostained with anti- α SMA and anti-CD3 antibodies, followed by a standard ABC-peroxidase/DAB protocol. Endothelial cells were immunostained with an anti-CD31 antibody, followed by a standard immunofluorescence protocol.

Analysis of mRNA Levels by Real-Time Quantitative Polymerase Chain Reaction

After homogenization, total RNA was extracted from tissues with a classical phenol/chloroform extraction protocol. The derived cDNA was then submitted to real-time quantitative polymerase chain reaction analysis.

Statistical Analysis

To test the effect of treatments, groups were compared for statistical significance using Mann–Whitney *U* test. To test the respective roles of treatment and genotype (ER α deficiency), a 2-way ANOVA was performed. When an interaction was observed between the 2

factors, the effect of the treatment in each genotype was studied using a Bonferroni post-test. A value of $P < 0.05$ was considered as statistically significant.

Results

Chronic E2 Treatment Decreases Neointimal Hyperplasia After Mechanical Injury of the Femoral Artery

To assess the effect of E2 on the development of arterial neointimal hyperplasia, ovariectomized wild-type female mice were chronically treated with either vehicle or E2 (Figure 1B and 1C). In response to mechanical injury of the femoral artery, neointimal hyperplasia, expressed as the neointima/media ratio, was reduced by 62% after E2 treatment (Figure 2A). This reduction was solely because of the prevention of neointimal proliferation because E2 treatment did not elicit any medial remodeling (Figure 2A). Histological analysis of the injured arteries (Figure 2B) showed a large neointima in control mice, whereas it was reduced to few cellular layers in E2-treated animals (Figure 2B, top). The neointima from both control and E2-treated mice was mostly composed of SMA-positive cells (Figure 2B, middle). The periadventitial CD3⁺-T-cell content remained unchanged between E2-treated mice and controls (Figure 2B, bottom).

Simultaneous Endothelial and Hematopoietic ER α Deletion Does Not Affect the Action of E2 on Neointimal Hyperplasia but It Impairs E2-Induced Endothelial Healing

Given the importance of endothelial ER α in numerous vascular protective effects of E2, we investigated the potential effects of a deletion of ER α in this cell type on neointimal hyperplasia development. For this purpose, we used mice expressing the Cre recombinase under the control of the Tie2 promoter that carried a floxed ER α -encoding sequence (Figure 1A).²² In addition to the endothelial deletion of ER α , these mice also presented with an 80% decrease in ER α expression in the bone marrow.²² As expected, placebo-treated control *Tie2Cre⁻ER α ^{lox/lox}* mice displayed a large neointima, which was reduced by E2 treatment (Figure 3A). This beneficial effect of E2 was similar for *Tie2Cre⁺ER α ^{lox/lox}* mice, as indicated by the 2-way ANOVA results, which show the absence of any interaction ($P=0.86$) and a highly significant effect of E2 treatment ($P=0.0006$).

Mechanical injury of the femoral artery leads to a loss in the endothelial integrity of the injured portion. This result was confirmed by anti-CD31 immunostaining of endothelial cells in the injured femoral arteries of placebo-treated *Tie2Cre⁻ER α ^{lox/lox}* and *Tie2Cre⁺ER α ^{lox/lox}* mice (Figure 3B). E2 treatment accelerated endothelial coverage in *Tie2Cre⁻ER α ^{lox/lox}*,

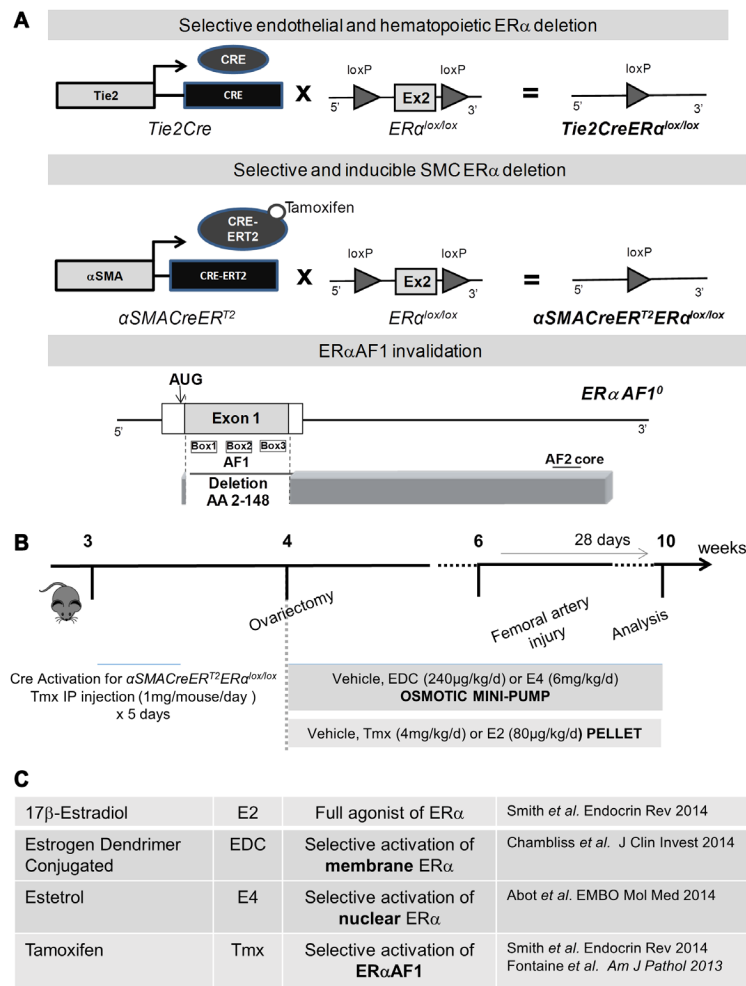


Figure 1. Estrogen ligands, mouse models, and intimal hyperplasia protocol used in the study. A, Schematic representation of the *Tie2Cre⁺ER α ^{lox/lox}*, *αSMACreERT2⁺ER α ^{lox/lox}* and *ER α AF1^{0/0}* mouse model and **(B)** of the protocol used to evaluate neointimal hyperplasia. **C**, Mechanism of estrogen receptor α (ER α) activation by 17 β -estradiol (E₂), estrol (E₄), tamoxifen (Tmx) and estrogen dendrimer conjugate (EDC). CRE indicates Cre recombinase.

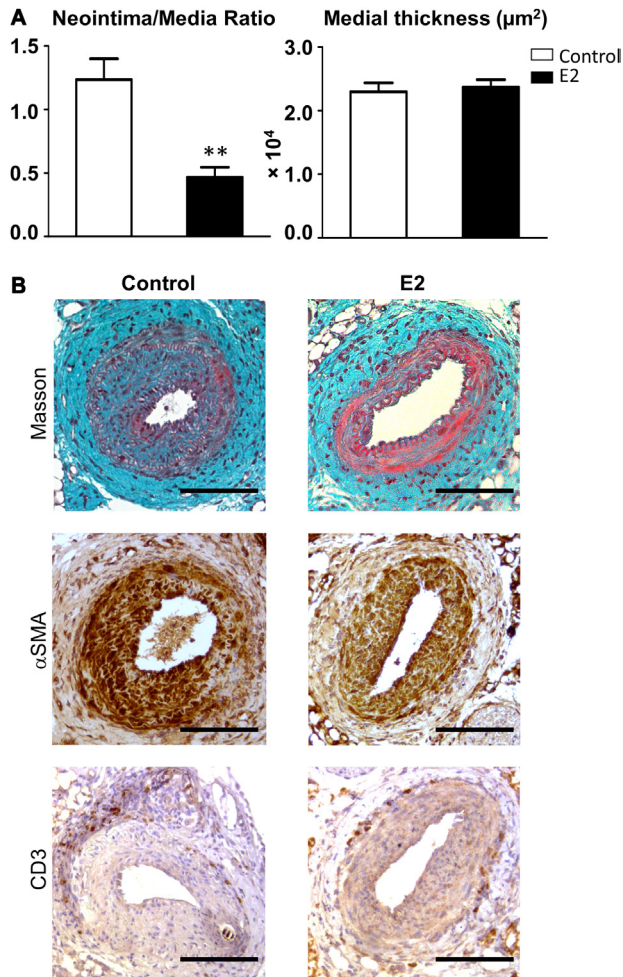


Figure 2. Chronic estradiol treatment decreases intimal hyperplasia after mechanical injury of the femoral artery without affecting medial thickness. Four-week-old wild-type female mice were ovariectomized and subcutaneously treated with placebo (control) or 17 β -estradiol (E2) until the end of the protocol. At 6 weeks, mice were submitted to mechanical injury of the femoral artery. Twenty-eight days later, mice were euthanized, and arteries were harvested for morphometric and immunohistological analysis. **A**, Quantitative analysis of neointima/media ratio (left) and medial thickness (right) of control (white bars) and E2 treated (black bars) ovariectomized mice. Values are presented as mean \pm SEM (n=7–15), and statistically compared with Mann–Whitney U test. ** $P < 0.01$. **B**, Representative images of injured femoral arteries cross sections of control (left) and 17 β -estradiol (E2, right) treated ovariectomized mice stained with Masson Trichrome (top), immunostained with anti- α smooth muscle actin (SMA) antibody to detect smooth muscle cells (middle) or with anti-CD3 antibody to detect T lymphocytes (bottom). Bars, 100 μm .

but not in *Tie2Cre⁺ ER $\alpha^{\text{lox/lox}}$* animals, confirming the importance of E2 in endothelial healing through a direct effect on the endothelium.

E2 Decreases Neointimal Hyperplasia by Directly Targeting Arterial SMC

To determine whether VSMC ER α is involved in the decrease in neointimal hyperplasia in response to E2, we established a new mouse model containing a selective deletion of ER α in SMC. For this purpose, we crossed previously described mice expressing the Cre-ER^{T2} fusion gene under the control

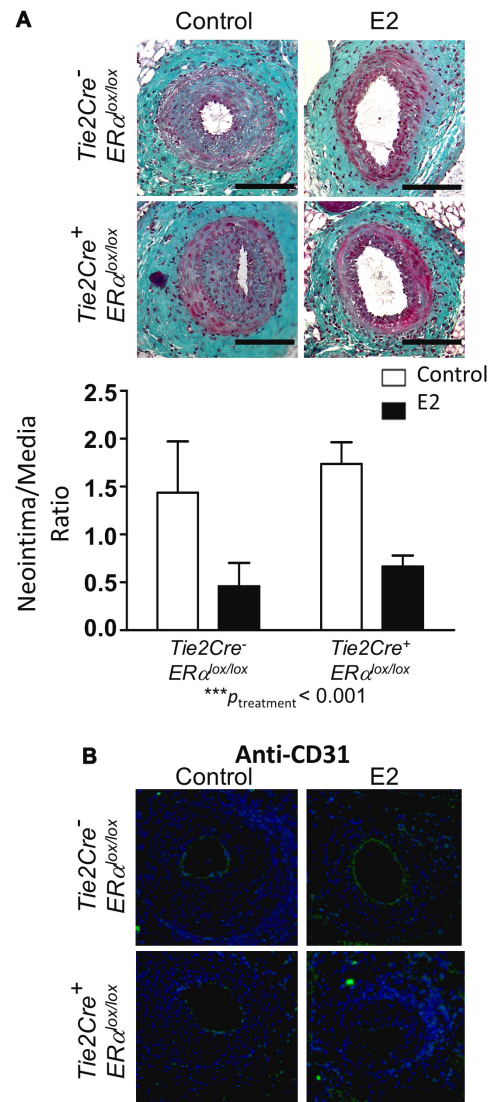


Figure 3. Loss of endothelial estrogen receptor α (ER α) expression does not affect the action of E2 on intimal hyperplasia. Four-week-old *Tie2Cre⁻ ER $\alpha^{\text{lox/lox}}$* and *Tie2Cre⁺ ER $\alpha^{\text{lox/lox}}$* female mice were ovariectomized and subcutaneously treated with placebo (control) or 17 β -estradiol (E2) until the end of the protocol. At 6 weeks, mice were submitted to mechanical injury of the femoral artery. Twenty-eight days later, mice were euthanized, and arteries were harvested for morphometry (**A**) and immunostaining (**B**). **A**, **Top**, Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Bars, 100 μm . **Bottom**, Quantitative analysis of neointima/media ratio of indicated mice. Values are presented as mean \pm SEM (n=8 mice per group). A statistical 2-way ANOVA test revealed no significant interaction. The overall effect of the treatment was *** $P_{\text{treatment}} < 0.001$. **B**, Representative images of injured femoral arteries cross sections of indicated mice, stained with anti-CD31 antibody and counterstained with DAPI (blue). Bars, 100 μm .

of the α SMA promoter²³ with *ER $\alpha^{\text{lox/lox}}$* mice (Figure 1A). We verified the specific deletion of ER α in the SMC compartment from *α SMACreER^{T2+} ER $\alpha^{\text{lox/lox}}$* mice in which the nuclear action of Cre recombinase had been induced by tamoxifen injection (Figure 4A). ER α mRNA expression was almost totally abrogated from the mediae dissected from the aortae of these mice, whereas its expression was preserved in the skeletal muscle tissue and in the cardiac muscle tissue (Figure 4A).

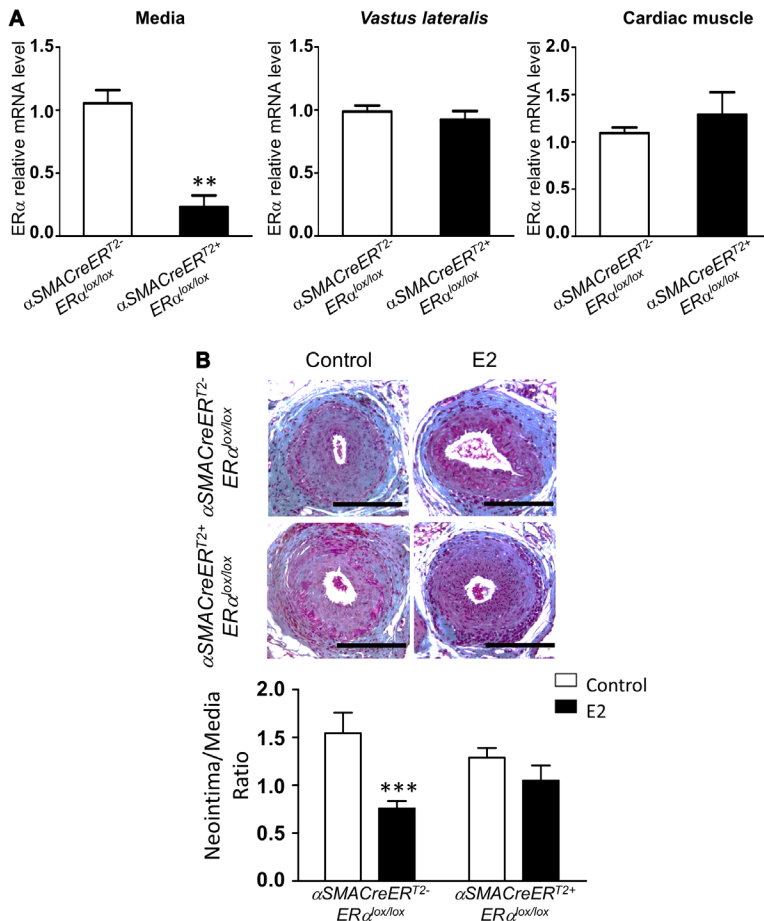


Figure 4. 17 β -Estradiol (E2) decreases intimal hyperplasia through its specific action on smooth muscle cells. **A**, mRNA levels from media, vastus lateralis muscle and heart from ovariectomized tamoxifen-injected α SMACreER^{T2} ER α ^{lox/lox} vs α SMACreER^{T2} ER α ^{lox/lox} were measured by quantitative polymerase chain reaction and normalized to *TPT1* expression. Results are expressed as mean \pm SEM. **B**, Four-week-old tamoxifen-injected α SMACreER^{T2} ER α ^{lox/lox} and α SMACreER^{T2} ER α ^{lox/lox} female mice were ovariectomized and subcutaneously treated with placebo (control) or E2 until the end of the protocol. At 6 weeks, mice were submitted to mechanical injury of the femoral artery. Twenty-eight days later, mice were euthanized, and arteries were harvested for morphometric analysis. Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Bars, 100 μ m. Quantitative analysis of neointima/media ratio of indicated mice. Values are mean \pm SEM (n=10–16 mice per group). As a statistical 2-way ANOVA revealed a significant interaction ($P=0.04$), it was followed by a Bonferroni post-test ($***P<0.001$).

We then addressed the effects of specific ER α deletion in SMC on injury-induced neointimal hyperplasia (Figure 1B). In both genotypes, placebo-treated mice displayed a large neointimal hyperplasia (Figure 4B). E2 treatment decreased neointimal hyperplasia in α SMACreER^{T2} ER α ^{lox/lox} control mice but failed to have such an effect in α SMACreER^{T2} ER α ^{lox/lox} mice (Figure 4B).

Activation of Nuclear and Not Membrane ER α Is Sufficient for the Suppression of Neointimal Hyperplasia

Once we had identified SMC as the main target cell for the action of E2 in modulating neointimal hyperplasia, we sought to dissect the molecular mechanisms involved. Thus, we adopted a pharmacological approach using an estrogen dendrimer conjugate (EDC) that selectively activates MISS,²¹ and estetrol that selectively activates nuclear ER α (Figure 1C; Online Figure I).²⁴ Whereas chronic EDC treatment failed to decrease neointimal hyperplasia (Figure 5A), estetrol prevented neointimal hyperplasia formation to the same extent as E2 (Figure 5B). These results strongly suggest that the nuclear effects of ER α , but not membrane ER α , are sufficient to decrease neointimal hyperplasia.

AF1 Is Both Necessary and Sufficient for the Reduction of Neointimal Hyperplasia

ER α AF1 has previously been found necessary for the proliferative effects of E2 on the endometrium, but it does not play a role in the accelerative effect of E2 on

re-endothelialization.¹⁶ Therefore, we sought to evaluate the role of this key transcriptional function of ER α on the prevention of neointimal hyperplasia. Ovariectomized ER α AF1^{+/+} and ER α AF1^{0/0} mice (Figure 1A), treated with E2 or vehicle control, were submitted to mechanical injury of the femoral artery. As expected, E2 decreased neointimal hyperplasia in control ER α AF1^{+/+} mice (Figure 6A). In striking contrast, we found that the antiproliferative effect of E2 on neointimal hyperplasia was not observed in mice genetically deficient in ER α AF1 function (Figure 6A), demonstrating that ER α AF1 is necessary for this effect. Second, we treated ovariectomized C57Bl/6J mice with tamoxifen (Figure 1C; Online Figure I), a selective ER modulator characterized as a selective agonist of AF1 function but an antagonist of AF2 function.^{13,25} The pronounced antiproliferative effect of tamoxifen highlighted that ER α AF1 activation is sufficient for preventing the development of neointimal hyperplasia (Figure 6B). At the same time, we verified that tamoxifen also elicited the growth of the uterus (Figure 6C), confirming the proliferative action of ER α AF1 activation that has previously been demonstrated in this tissue.¹⁵

Discussion

Our results show that E2 is able to widely prevent neointimal hyperplasia within the vascular wall using a mouse model of endovascular mechanical injury of the femoral artery. Because

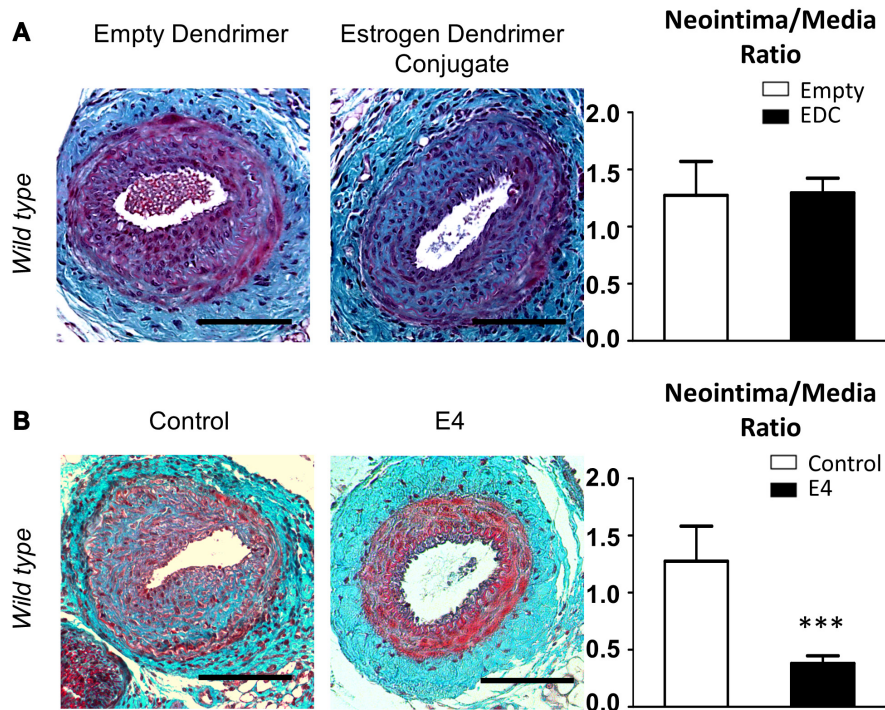


Figure 5. Genomic functions of estrogen receptor α (ER α) mediate protection from intimal hyperplasia. **A**, Four-week-old wild-type female mice were ovariectomized and subcutaneously implanted with estrogen dendrimer conjugate or empty dendrimer-eluting osmotic minipumps. Two weeks later, animals were submitted to mechanical injury of the femoral artery. Arteries were harvested 28 days after the injury for morphometric analysis. **Left**, Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Bars, 100 μ m. **Right**, Quantitative analysis of neointima/media ratio of indicated mice. Values are presented as mean \pm SEM (n=10–12 mice per group), and statistically compared with Mann–Whitney *U* test. **B**, Four-week-old wild-type female mice were ovariectomized and subcutaneously implanted with control of estretol (E4)-eluting osmotic minipumps. Two weeks later, animals were submitted to mechanical injury of the femoral artery. Arteries were harvested 28 days after the injury for morphometric analysis. **Left**, Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Bars, 100 μ m. **Right**, Quantitative analysis of neointima/media ratio of indicated mice. Values are presented as mean \pm SEM (n=7–12 mice per group), and statistically compared with Mann–Whitney *U* test. ****P*<0.001.

of the central role of the endothelium in the vascular wall, in particular, in the control of VSMC proliferation,^{4,26} it is commonly thought that triggering endothelial healing can have a beneficial action on neointimal hyperplasia. Using a Cre-Lox strategy, we have demonstrated here that ER α in the endothelium is not necessary for the suppression of SMC proliferation in response to E2, in contrast to the effects of E2 on the acceleration of endothelial healing and the prevention of atheroma.²² In addition, the preservation of the suppressive effects of E2 on neointimal hyperplasia in *Tie2Cre⁺ ER α ^{lox/lox}* mice suggests that ER α is also dispensable in bone marrow–derived cells, because in this model, medullar ER α expression is also largely abrogated.²² Consistent with this observation, immunostaining analysis of injured femoral arteries suggested the presence of similar numbers of CD3-positive T lymphocytes in E2- and placebo-treated mice. E2 has been described to also have direct inhibitory effect on SMC proliferation and migration in vitro.²⁷ Accordingly, we have shown that ER α in SMC is essential for the prevention of femoral artery neointimal hyperplasia in vivo through the generation of a SMC-specific conditional knockout of ER α . Finally, using a combination of genetic and pharmacological approaches, we have demonstrated that nuclear activation involving ER α AF1 is both necessary and sufficient to prevent neointimal hyperplasia. This result contrasts with the dispensable role of ER α AF1 in

mediating the effect of E2 on endothelial healing¹⁶ and the lack of tamoxifen activity on re-endothelialization.²⁵

Taken together, our results confirm the crucial role of ER α in neointimal hyperplasia in response to E2 and show for the first time the direct action of E2 on SMC ER α . The role of ER β is less clear, with several studies having demonstrated that the selective activation of ER β is sufficient for inhibiting neointima formation.^{28–30} However, in ER β ^{-/-} mice, the effects of E2 on vascular media area in injured carotids were found to be similar to those in control mice.³¹ This observation fits with the results obtained in the present study, which suggests that the expression of ER β is not sufficient to mediate the effect of E2 in the absence of ER α . Overall, ER β involvement in postinjury VSMC proliferation seems to be dependent on sex, age, extent of vascular injury and anatomic site (ie, carotid versus femoral arteries). In addition, the estrogen response could involve or even be mediated by the G-protein–coupled receptor GPR30, the activation of which has also been proposed to inhibit SMC proliferation.³² The aim of the present study was to focus on the cellular and molecular mechanisms of ER α , but it will be interesting in future studies to assess the possible role of ER β and GPR30 in this model of neointimal hyperplasia. This was unfortunately beyond the scope of the present study because of the complexity of their inter-relationship and the controversy over the available animal models targeting both ER β and GPR30.^{33,34}

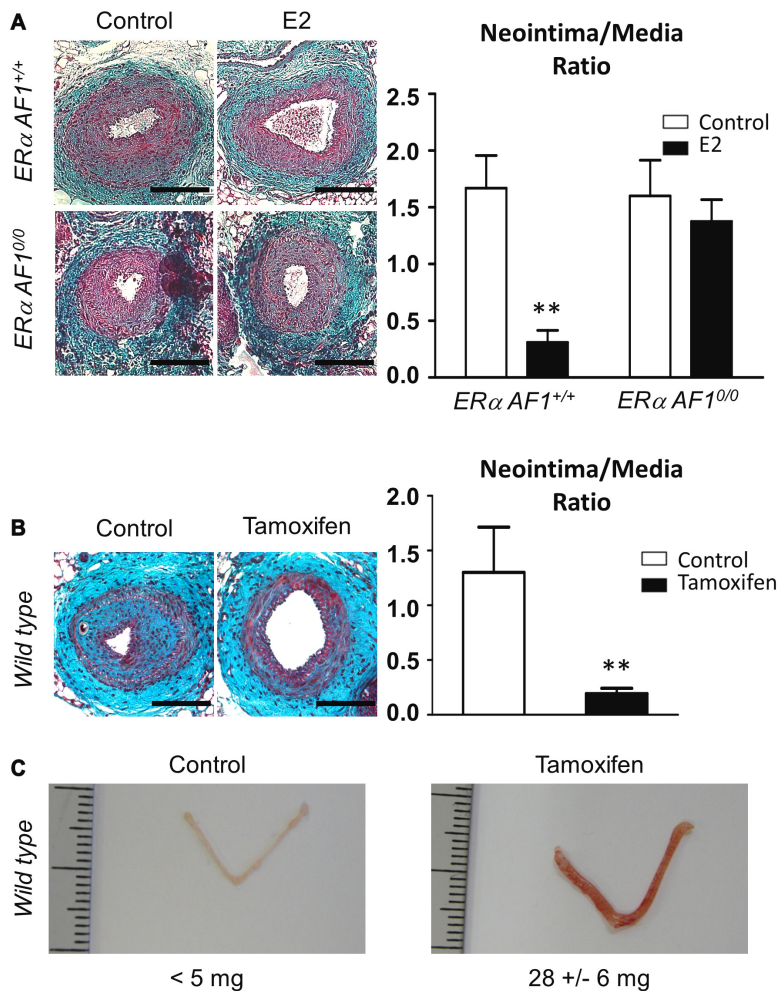


Figure 6. Activation function (AF) 1 of estrogen receptor α ($ER\alpha$) is necessary for the prevention of intimal hyperplasia by 17 β -estradiol (E2). **A, Four-week-old $ER\alpha AF1^{+/+}$ and $ER\alpha AF1^{0/0}$ female mice were ovariectomized and subcutaneously treated with placebo (control) or E2 until the end of the protocol. At 6 weeks, mice were submitted to mechanical injury of the femoral artery. Twenty-eight days later, mice were euthanized, arteries were harvested for morphometric analysis. **Left**, Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Bars, 100 μ m. **Right**, Quantitative analysis of neointima/media tissue ratio of indicated mice. Values are presented as mean \pm SEM (n=8–15 mice per group). As a statistical 2-way ANOVA revealed a significant interaction ($P=0.04$), it was followed by a Bonferroni post-test (** $P<0.01$). **B** and **C**, Four-week-old wild-type female mice were ovariectomized and subcutaneously implanted placebo or tamoxifen (4 mg/kg per day) pellets. Two weeks later, animals were submitted to mechanical injury of the femoral artery. Arteries were harvested 28 days after the injury for morphometric analysis. **B**, **Left**, Representative images of cross sections of femoral arteries of indicated mice stained with Masson Trichrome. Bars, 100 μ m. **Right**, Quantitative analysis of neointima/media ratio of indicated mice. Values are presented as mean \pm SEM (n=7–12 mice per group), and statistically compared with Mann–Whitney test. ** $P<0.01$. **C**, Representative images of uterus from indicated mice.**

In an attempt to dissect the molecular mechanisms of E2 action on neointimal hyperplasia, we used EDC to selectively activate $ER\alpha$ MISS. EDC failed to prevent neointimal hyperplasia, but estetrol, a natural selective estrogen receptor modulator selectively activating nuclear $ER\alpha$,²⁴ was able to prevent neointimal hyperplasia to a similar extent as E2. Taken together, these results demonstrate that nuclear activation is necessary and sufficient to prevent neointimal hyperplasia after endovascular mechanical injury of the femoral artery in a normocholesterolemic context. It was previously shown that E2 failed to decrease injury-induced proliferation of medial SMC in the carotid artery in mice expressing a peptide that inhibits $ER\alpha$ MISS.³⁵ In addition, Chambliss et al²¹ demonstrated that the formation of an atheromatous neointima could be prevented not only by E2 but also, at least in part, by EDC in a more complex model of carotid injury (as a consequence of hypercholesterolemia caused by ApoE deficiency). The discrepancies between our present findings and these studies may be attributed to differences in the models used because wire injury of the carotid artery results in a medial remodeling with little or no neointima formation.^{36,37} In contrast, femoral artery wire injury in the mouse leads to a large neointima formation, with massive proliferation of neointimal SMC.^{38,39} It is therefore likely that the observed differences in the molecular targets of E2 are attributable to the phenotype of the VSMC

involved in the 2 models. The results of both studies demonstrate that the proliferation of neointimal VSMC is inhibited via nuclear $ER\alpha AF1$ activation by E2, whereas $ER\alpha$ MISS controls medial SMC remodeling in the elastic carotid artery. Altogether, these findings seem complementary and enable us to discriminate between the molecular targets of E2 depending on the phenotype of VSMC (medial versus neointimal, ie, synthetic) and their anatomic origin (elastic versus muscular artery). It is also important to note that carotid injury in ApoE-deficient mice leads to the formation of a complex lesion with predominantly inflammatory cells and poor SMA-positive cells.⁴⁰ Altogether, these studies emphasize that the roles of the $ER\alpha$ subfunctions (here MISS versus AF1) seem to vary according to the differentiation state of the SMC.

Thus, the results presented here show that whereas the effects of E2 on endothelial cells are essentially dependent on $ER\alpha$ MISS, the effects of E2 on SMC proliferation require the nuclear effect of $ER\alpha$ to prevent neointimal hyperplasia, particularly the $ER\alpha AF1$ subfunction. We previously demonstrated that $ER\alpha AF1$ is both necessary and sufficient for the proliferative effect of $ER\alpha$ on the epithelium of the uterus,¹⁵ and show here that this same function is able to mediate the antiproliferative effect of estrogens in another cell type and tissue. Importantly, in breast cancer, $ER\alpha AF1$ is recognized as a convergence point for growth factor and hormonal

activation.⁴¹ Thus, our *in vivo* data highlight ER α AF1 as a major functional element of ER α , one that appears to contribute to the integration of various signals that control cell proliferation in a strictly cell- and tissue-specific manner.

Altogether, these varied mouse models permit the dissection of the mechanisms of action of both estrogens and selective ER modulators. These findings also begin to highlight how ER α might best be modulated to optimize the expected benefit/risk ratio of its multitude of activities that could represent a novel facet of personalized medicine.

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Disclosures

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Novelty and Significance

What Is Known?

- 17 β -estradiol (E2) prevents neointimal hyperplasia in response to endovascular injury in various animal models and species, including rats, pigs, and sheep.
- Estrogen receptor alpha (ER α) mediates the action of E2 through regulation of gene transcription via 2 activation functions (AF), AF1 and AF2, and through activation of rapid signaling at the plasma membrane.
- ER α AF1 and ER α AF2, but not membrane ER α , mediate uterine endometrial proliferation in response to E2.
- Membrane ER α , but not ER α AF1 or ER α AF2, mediates the beneficial action of E2 on the endothelium.

What New Information Does This Article Contribute?

- E2 prevents neointimal hyperplasia in mice.
- Smooth muscle cell, but not endothelial, ER α is necessary to prevent neointimal hyperplasia in response to E2 in mice.
- ER α AF1 is necessary and sufficient to prevent neointimal hyperplasia.

This study, using a mouse model of endovascular mechanical injury of the femoral artery, shows that E2 is able to prevent neointimal hyperplasia. Our results further establish the crucial role of ER α in the prevention of neointimal hyperplasia by E2 and show for the first time the key role of smooth muscle cell ER α . Although the effects of E2 on endothelial cells are essentially dependent on membrane ER α , the effects of E2 on smooth muscle cell proliferation require the nuclear effect of ER α to prevent neointimal hyperplasia, in particular the ER α AF1 subfunction. We previously demonstrated that ER α AF1 is both necessary and sufficient for the proliferative effect of ER α on the epithelium of the uterus and show here that this same function is able to mediate the antiproliferative effect of E2 on arterial smooth muscle cell. This approach highlights the exquisite cell/tissue-specific actions of the ER α subfunctions and will help to delineate the spectrum of action of selective ER modulators, as that of the selective ER α AF1 activator tamoxifen used in the present study.