Tamoxifen Elicits Atheroprotection through Estrogen Receptor \( \alpha \) AF-1 But Does Not Accelerate Reendothelialization

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Based on both experimental and clinical data, tamoxifen has been proposed to have cardiovascular benefits, although the mechanism(s) contributing to that protective effect are still poorly understood. In vitro experiments demonstrated that tamoxifen elicits its transcriptional effect through estrogen receptor (ER) \( \alpha \), but other targets can participate in its actions. However, although tamoxifen selectively activates the activating function (AF)-1 of ER\( \alpha \), we recently showed that this ER\( \alpha \) subfunction is dispensable for the atheroprotective action of \( 17\beta \)-estradiol (E2), the main ligand of ERs. The goal of the present work is to determine to which extent ER\( \alpha \) and its AF-1 mediate the vasculoprotective action of tamoxifen. Our data confirm that tamoxifen exerts an atheroprotective action on low density lipoprotein receptor (LDL-r/−) female mice, but, in contrast to E2, it fails to accelerate reendothelialization after carotid electric injury. Tamoxifen and E2 elicit differences in gene expression profiles in the mouse aorta. Finally, the atheroprotective action of tamoxifen is abrogated in ER\( \alpha \)/−/LDL-r/− mice and in LDL-r/− mice selectively deficient in ER\( \alpha \) AF-1 (ER\( \alpha \)AF-1/0/0 LDL-r/−). Our results demonstrate, for the first time to our knowledge, that tamoxifen mediates its actions in vivo through the selective activation of ER\( \alpha \) AF-1, which is sufficient to prevent atheroma, but not to accelerate endothelial healing. (Am J Pathol 2013, 183: 304–312; http://dx.doi.org/10.1016/j.ajpath.2013.03.010)

Tamoxifen is one of the major drugs used for hormonotherapy of estrogen receptor (ER)–positive breast cancers.\(^1,2\) In addition to the clear reduction in the risk of recurrence and of developing new breast cancer, tamoxifen has been proposed to exert cardiovascular beneficial effects for several years.\(^3\) Indeed, epidemiological studies\(^4,5\) suggest and experimental results clearly demonstrate a major atheroprotective action of tamoxifen. This drug strongly reduces the progression of atherosclerosis in monkey,\(^6\) rabbit,\(^7\) and mouse,\(^8–10\) at variance with the other selective estrogen receptor modulator, raloxifene.\(^3,11,12\) Consistent with these preclinical studies, numerous clinical trials report that tamoxifen significantly decreases myocardial infarction incidence and fatal outcome.\(^3,13,14\)

Tamoxifen is a ligand for estrogen receptors (ERs \( \alpha \) and \( \beta \)) and exerts estrogen agonist actions on some targets, such as uterus and bone, while acting as an estrogen antagonist on breast. ERs belong to the nuclear receptor subfamily whose members, based on structural and functional similarities, can be subdivided into six distinct regions, termed A to F. The two activation functions (AF-1 and AF-2), involved in the transcriptional activity of ERs, are located within regions B and E, respectively.\(^15,16\) ERs activate gene expression by two major ways. In the best understood mode of action, the so-called classic pathway, ERs bind to specific estrogen response elements (EREs), within or near promoters of target genes, and recruit co-activator complex, complex.
leading to gene expression by remodeling chromatin and by contacting the basal transcription machinery. In a second mode of action, ERs use unspecified protein–protein interactions that enhance the activity of heterologous transcription factors. For example, both ERs enhance the transcription of genes that contain activator protein 1 (AP1) sites, the cognate binding site for the Jun/Fos complex. Through its active metabolite, 4-hydroxytamoxifen (4-OHT), tamoxifen regulates ERα-mediated gene transcription in a cell-specific manner, depending at least in part, on the relative roles of ERαAF-1 and ERαAF-2. Indeed, in vitro experiments demonstrate that tamoxifen activates ERαAF-1–dependent gene expression, but fails to induce co-activator binding in ERαAF-2–mediated transcription. 

Tamoxifen also binds to a high-affinity binding site different from ERs. This binding site, named microsomal anti-estrogen binding site, is a hetero-oligomeric complex composed of 3β-hydroxysterol-DΔ7-Δ7-isomerase and 3β-hydroxysterol-DΔ7-reductase and has no affinity for estrogens. In addition, tamoxifen exerts ER-independent effects through its actions on estrogen-related receptors and G-protein–coupled estrogen receptor (also called GPR30). We have recently shown that ERαAF-1 is dispensable for the atheroprotective action of 17β-estradiol (E2), the major endogenous ligand of ERα. The goal of the present work was to determine to which extent ERα and its AF-1 mediate the vasculoprotective action of tamoxifen using a mouse model deficient for ERα (ERα+/−LDL-r−/−) or selectively deficient for its AF-1 (ERαAF-1−/−LDL-r−/−).

Materials and Methods

Mice

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research (Institut National de la Santé et de la Recherche Médicale) and were approved by the local Animal Care and Use Committee. The investigation conforms with the directive 2010/63/EU of the European parliament. ERα+/− and ERαAF-1−/− mice were generated as previously described on a C57Bl/6J genetic background. Briefly, ERαAF-1−/− was generated through a targeted deletion using a knock-in strategy, through which 441 nucleotides of exon 1 were deleted. The truncated protein lacks the A domain, and all three motifs constituting ERαAF-1 (AF-1 boxes 1 to 3) in the B domain, thus yielding a 451–amino acid long 49-kDa protein. To generate the double-deficient mice, LDL-r−/− female mice, purchased from Charles River (L’Arbresle, France), were crossed with ERα+/− or ERαAF-1−/− mice. Heterozygous ERα+/−LDL-r−/− and ERαAF-1−/−LDL-r−/− mice were generated and used as the parental genotypes. Before surgical and euthanasia procedures, mice were anesthetized with a combination of 100 mg/kg ketamine hydrochloride (Panpharma, Fougères, France) and 5 mg/kg xylazine (Sigma-Aldrich, Isle d’Abeau Chesnes, France) via i.p. injection.

Mouse Carotid Injury and Quantification of Reendothelialization

The carotid electric injury was performed as previously described. Bilateral ovariectomy was performed at 4 weeks of age, and mice concomitantly received s.c. pellets releasing placebo, E2 [17β-estradiol, 0.1 mg, 60-day release (ie, 80 μg/kg per day); Innovative Research of America, Sarasota, FL], or tamoxifen [5 mg, 60-day release (ie, 4 mg/kg per day); Innovative Research of America]. Two weeks later, carotid injury was performed and reendothelialization was evaluated after 3 days. Briefly, surgery was performed with a stereomicroscope (SMZ800; Nikon, Champigny Sur Marne, France), and the left common carotid artery was exposed via an anterior incision in the neck. The electric injury was applied to the distal part (4 mm precisely) of the common carotid artery with a bipolar microregulator. En face confocal microscopy was used to visualize the endothelial monolayer and to determine the reendothelialization area, as previously described.

Analyses of Atherosclerosis Lesions

Bilateral ovariectomy was performed at 4 weeks of age, and mice received pellets of E2 or tamoxifen (similar doses as previously described), both at weeks 6 and 14. At 6 weeks of age, mice were switched to a hypercholesterolemic atherogenic diet (1.25% cholesterol, 6% fat, and no cholate; TD96335; Harlan Teklad, Madison, WI). At 18 weeks, mice that fasted overnight were anesthetized and blood was collected from the retro-orbital venous plexus. Total and high-density lipoprotein (HDL) plasma cholesterol concentrations were determined, as described later. Lipid deposition size was estimated at the aortic sinus, as previously described. Each heart was frozen on a cryostat mount with optimal cutting temperature compound (Tissue-Tek, Torrance, CA). One hundred sections (10 μm thick) were prepared from the top of the left ventricle, where the aortic valves were first visible, up to a position in the aorta where the valve cusps were just disappearing from the field. After drying for 2 hours, the sections were stained with oil red O and counterstained with Mayer’s hematoxylin. Of the 100 sections, each separated by 90 μm, 10 were used for specific morphometric evaluation of intimal lesions using a computerized Biocom (Köln, Germany) morphometry system. The first and most proximal section to the heart was taken 90 μm distal to the point where the aorta first becomes rounded. The mean lesion size (expressed in μm²) in these 10 sections was used to evaluate the lesion size of each animal. Other sections were dedicated to lesion composition analysis, including collagen detection and immunohistochemical (IHC) staining. Collagen fibers were stained with Sirius red. For IHC staining of macrophages, a rat monoclonal anti-CD68 antibody (clone FA-11; AbCys, Paris, France) was used. At least four sections per animal were analyzed for each immunostaining.
Determination of Plasma Lipids

Total cholesterol was assayed using the CHOD-PAP kit (Horiba ABX, Montpellier, France). The HDL fraction was isolated from 10 \( \mu L \) of serum and assayed using the C-HDL + Third generation kit (Roche, Lyon, France).

Analysis of mRNA Levels by qPCR

Bilateral ovariectomy was performed at 4 weeks of age. Two weeks after surgery, mice were chronically treated and sacrificed, as previously indicated, or mice were injected with a single dose of placebo, 8 \( \mu g/kg \) E2, or 4 mg/kg tamoxifen and were euthanized 6 hours later. Dissected thoracic aortas were homogenized using a Precellys tissue homogenizer (Bertin Technology, Cedex, France), and total RNA from tissues was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA). A total of 1 \( \mu g \) was reverse transcribed for 10 minutes at 25\( ^\circ \)C and for 2 hours at 37\( ^\circ \)C in a 20-\( \mu L \) final volume using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Villebon sur Yvette, France). Quantitative real-time PCRs (qPCRs) were performed on the StepOne instrument (Applied Biosystems). Primers were validated by testing PCR efficiency using standard curves (95\% ≤ efficiency ≤ 105\%). Gene expression was quantified using the comparative \( C_T \) method; hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) was used as a reference.

Transfection Assays

HepG2 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich), supplemented with 10\% fetal calf serum (Biowest, Nuaille, France) and antibiotics (Sigma-Aldrich) at 37\( ^\circ \)C in 5\% CO\(_2\). Transfections were performed using jetPEI reagent, according to the manufacturer’s instructions (Polyplus; Ozyme, Saint Quentin en Yvelines, France). One day before transfection, cells were plated in 24-well plates at 50\% confluence. One hour before transfection, the medium was replaced with phenol red-free Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) containing 2.5\% charcoal-stripped fetal calf serum (Biowest). Transfection was performed with 100 ng of complement (C3) containing (ERE) promoter or AP1 site cloned upstream of the thymidine kinase promoter-driven renilla luciferase (luc) reporter, 100 ng of cytomegalovirus (CMV)–\( \beta \)-galactosidase internal control, and 50 ng of pSG5, pSG5-ER\(_{\alpha}\), or pSG5-ER\(_{\alpha}\)AF-1\(^9\) expression vectors. After an overnight incubation, cells were treated for 24 hours with 10 nmol/L E2, 2 \( \mu \)mol/L 4-hydroxytamoxifen, or ethanol (vehicle control). Cells were then harvested, and luc and \( \beta \)-galactosidase assays were performed as previously described.\(^{34,35}\)

Statistical Analysis

To test the significance of different treatments, a one-way analysis of variance, followed by a Bonferroni post test, was performed. To test the respective roles of tamoxifen treatment and genotype (ER\(_{\alpha}\) or ER\(_{\alpha}\)AF-1 deficiency), a two-way analysis of variance was realized. When an interaction was observed between the two factors, the effect of tamoxifen treatment was studied in each genotype using a Bonferroni post test. \( P < 0.05 \) was considered statistically significant.

Results

Tamoxifen Prevents Atheroma But Fails to Accelerate Endothelial Healing

Previous studies have reported that tamoxifen prevents lesion formation in different mouse models when their diet was supplemented with tamoxifen.\(^8-10\) We confirmed these results using the well-known model of atheroma, the low density lipoprotein receptor (\( LDL-r^{-/-} \)) mouse under a high-cholesterol diet. As expected, s.c. tamoxifen treatment (4 mg/kg per day) for 12 weeks protected ovariectomized \( LDL-r^{-/-} \) in terms of lipid deposition at the aortic sinus compared with placebo-treated mice (Figure 1, A and B, and Supplemental Table S1). Tamoxifen also decreased total and HDL plasma cholesterol, but no trend toward a change was observed on total cholesterol/HDL cholesterol ratio (Supplemental Table S1). We and others reported that E2 down-regulates vascular cell adhesion molecule (VCAM)-1, a key molecular actor of monocyte recruitment in atheroma.\(^34,35\)

Figure 1 Tamoxifen has an atheroprotective effect but fails to accelerate reendothelialization after carotid electric injury. A and B: Four-week-old ovariectomized \( LDL-r^{-/-} \) mice were given either placebo or 4 mg/kg per day tamoxifen (Tmx) and switched to an atherogenic diet from the age of 6 to 18 weeks. A: Representative micrographs of oil red 0 (OR0) lipid-stained cyescences of the aortic sinus. B: Quantification of lipid deposition. C and D: Four-week-old ovariectomized mice were given placebo, 80 \( \mu g/kg \) per day estradiol (E2), or 4 mg/kg per day Tmx for 2 weeks. Electric injury was applied to the distal part (4 mm precisely) of the common carotid artery, and the endothelial regeneration process was evaluated 3 days after injury. C: Representative \( en face \) confocal IHC analysis of the intima tunica of the carotid artery. Nuclei, stained with propidium iodide, appear in dark blue. D: Quantification of the reendothelialized area. The effect of tamoxifen treatment was studied using a one-way ANOVA and Bonferroni post test. \( **P < 0.01, ***P < 0.001. \)
We show that this down-regulation of VCAM-1 is also observed in tamoxifen-treated LDL-r/C0/C0 mice (Supplemental Figure S1A). In addition, expression of the inflammatory mediators of atherosclerosis (monocyte chemoattractant protein-1 and macrophage migration inhibitory factor) (Supplemental Figure 1, B and C) and expression of the macrophage marker genes (F4/80 and CD68) (Supplemental Figure 1, D and E) on whole aorta were similarly decreased by E2 or tamoxifen compared with placebo-treated mice. Taken together, these results show that E2 and tamoxifen s.c. treatments both confer mice atheroprotection.

In addition to its atheroprotective effect, we and others previously demonstrated that E2 also favors another major vasculoprotective action (namely, the acceleration of endothelial healing).28,29,36 To ascertain if tamoxifen was able to elicit this beneficial action, we studied reendothelialization after electric carotid injury in placebo-, E2-, and tamoxifen-treated mice. At day 3 after injury, E2 induced a significant enlargement of the reendothelialized area compared with placebo-treated mice, whereas tamoxifen had no accelerative effect on endothelial healing (Figure 1, C and D).

Acute E2 and Tamoxifen Treatments Elicit Different Gene Expression Changes in the Aorta

Then, to evaluate transcriptional change in response to E2 and tamoxifen in the vessel, we assessed the expression of several key genes in the aorta from mice exposed to acute E2 or tamoxifen treatment. Mendelsohn and colleagues37 reported and we32 recently confirmed that Gremlin 2 and Uridine-diphospho-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase like 2 (Galnt12; B) and VCAM-1 (C) mRNA levels from aortas were quantified by qPCR and normalized to HPRT mRNA levels. Results are expressed according to the level in aortas from placebo, set as 1. The significance of the observed effects was evaluated using one-way analysis of variance, followed by Bonferroni's post hoc test. ***P < 0.001.

**Figure 2** Estradiol and tamoxifen treatments cause differential gene expression in the aorta. Four-week-old ovariectomized LDL-r/C0/C0 mice received a single s.c. injection with placebo, 8 μg/kg E2, or 4 mg/kg tamoxifen (Tmx) and were euthanized 6 hours later. Gremlin 2 (A), UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase like 2 (Galnt12; B), and VCAM-1 (C) mRNA levels from aortas were quantified by qPCR and normalized to HPRT mRNA levels. Results are expressed according to the level in aortas from placebo, set as 1. The significance of the observed effects was evaluated using one-way analysis of variance, followed by Bonferroni’s post hoc test. ***P < 0.001.

Figure 3 ERα is necessary for the atheroprotective action of tamoxifen

Because ERα was shown to be necessary for the E2-mediated vasculoprotection,31 we then explored the involvement of this receptor in the atheroprotective effect of tamoxifen using ERα−/− LDL-r−/− mice. Lesion sizes at the aortic sinus were not by tamoxifen, after short-term treatment (Figure 2C). These data underline that E2 and tamoxifen elicit different gene expression changes in the mouse aorta.

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**Figure 3** ERα is necessary to atheroprotective action of tamoxifen in LDL-r−/− deficient mice. Four-week-old ovariectomized ERα+/− LDL-r−/− and ERα−/− LDL-r−/− female mice received s.c. either a placebo or tamoxifen (Tmx; 4 mg/kg per day) pellet and were switched to a hypercholesterolemic diet from the age of 6 to 18 weeks. A: Representative micrographs of oil red O (ORO) lipid-stained cryosections of the aortic sinus. B: Quantification of lipid deposition at the aortic sinus. A two-way analysis of variance indicated an interaction between tamoxifen treatment and ERα genotype. P = 0.01. The effect of tamoxifen treatment was studied in each genotype using Bonferroni’s post test. **P < 0.01.
similar in both placebo-treated \(ER\alpha^{+/+}\) LDL\(^{-/-}\) and \(ER\alpha^{-/-}\) LDL\(^{-/-}\) mice (Figure 3, A and B), as previously reported.\(^{31}\) As expected, tamoxifen prevented lipid deposition in ovariec-tomized control \(ER\alpha^{+/+}\) LDL\(^{-/-}\) mice exposed to a high-cholesterol diet, whereas this effect was completely abolished in \(ER\alpha^{-/-}\) LDL\(^{-/-}\) mice. This result clearly demonstrates that the atheroprotective effect of tamoxifen is entirely \(ER\alpha\)-dependent. In addition, \(ER\alpha\) was also found to be necessary for the tamoxifen effects on body weight and uterine hypertrophy (Supplemental Table S2). Conversely, a significant decrease in total and HDL plasma cholesterol was still observed in \(ER\alpha^{-/-}\) LDL\(^{-/-}\) tamoxifen-treated mice compared with control, suggesting an \(ER\alpha\)-independent effect.

**ER\(\alpha\)AF-1 Is Necessary to the Atheroprotective Action of Tamoxifen**

Both tamoxifen (Figure 3) and E2\(^{28}\) induced atheroprotection in an \(ER\alpha\)-dependent manner. We recently established that the \(ER\alpha\)AF-1 is dispensable for the E2 vasculoprotective effects.\(^{28}\) However, previous in vitro experiments demonstrated that tamoxifen exerts \(ER\alpha\)AF-1 agonist and \(ER\alpha\)AF-2 antagonist actions.\(^{38,39}\) To further delineate the role of \(ER\alpha\)AF-1 in tamoxifen atheroprotective actions, we did the following.

We reconsidered the role of \(ER\alpha\)AF-1 on classic and AP-1-mediated transcriptional response to tamoxifen using transfection assays, using the small \(ER\alpha\) isoform lacking the N-terminal portion (domains A/B) and thereby AF-1 (\(ER\alpha\)AF-1\(^{0}\)). Both \(ER\alpha\) AF-1 and AF-2 have been shown to exert their transcriptional activity in a cell-specific manner. Accordingly, cell contexts can be defined as AF-1 or AF-2 permissive, depending on which AF is principally involved in \(ER\alpha\) activity.\(^{40}\) We examined the effect of 4-OHT on its ability to stimulate complement C3 containing \(ER\alpha\)-AF-1 and AP1 (Figure 4, C and D) promoters in transient transfection assays in two cell lines, HepG2 (AF-1 permissive) and HeLa (AF-2 permissive), previously characterized as devoid of endogenous \(ER\alpha\).\(^{40}\) In the AF-1–permissive HepG2 cells,\(^{40}\) although 4-OHT induced C3 (ERE) and AP1-promoted transcriptional activity using a wild-type \(ER\alpha\) construct, this action was entirely (Figure 4A) or dramatically (Figure 4C) reduced using the \(ER\alpha\)AF-1\(^{10}\) mutant. In addition, by using HeLa cells that are mainly \(ER\alpha\)AF-2 permissive,\(^{40}\) 4-OHT was unable to induce gene transcription, through either ERE or AP-1 pathways, at variance with E2. Altogether, these results clearly confirm that \(ER\alpha\)AF-1 is crucial to induce gene expression changes in response to tamoxifen, but not to E2, and that response to tamoxifen is dependent on the cellular context.

We tested the involvement of \(ER\alpha\)AF-1 in the atheroprotective effect of tamoxifen in vivo. To this end, we used mice deleted for the A/B region of \(ER\alpha\) and, thereby, deficient in AF-1,\(^{28,38}\) bred with LDL\(^{-/-}\) mice (\(ER\alpha\)AF-1\(^{10}\)/LDL\(^{-/-}\)). As expected, analysis of the lesion size (Figure 5, A and B) as well as macrophage (CD68-positive cell) (Figure 5C) and collagen (Figure 5D) contents indicated that tamoxifen prevents fatty streak deposits at the aortic sinus from ovariec-tomized \(ER\alpha\)AF-1\(^{10}\)/LDL\(^{-/-}\) mice. The atheroprotective effect of tamoxifen was totally abolished in \(ER\alpha\)AF-1\(^{10}\)/LDL\(^{-/-}\) (Figure 5), demonstrating that \(ER\alpha\)AF-1 is absolutely required for the atheroprotective effect of tamoxifen. \(ER\alpha\)AF-1 was also found to be required for tamoxifen effect on body weight and uterine hypertrophy (Supplemental Table S3). Furthermore, as described using \(ER\alpha^{-/-}\)/LDL\(^{-/-}\) mice (Supplemental Table S2), tamoxifen’s effect on HDL and total cholesterol still remained efficient in \(ER\alpha\)AF-1\(^{10}\)/LDL\(^{-/-}\) mice (Supplemental Table S3).

**Discussion**

In agreement with previous studies in monkey,\(^6\) rabbit,\(^7\) and mice,\(^8–10\) the atheroprotective effect of tamoxifen was extended to another major model of atheromatous mice (ie, LDL\(^{-/-}\)/LDL\(^{-/-}\) mice). However, at variance with E2,\(^29\) tamoxifen was not able to accelerate reendothelialization in an electric model of carotid injury (Figure 1). Along with these different actions on vascular pathophysiological models, E2 and tamoxifen elicited striking differences in gene expression levels in the aorta (Figure 2). Different effects of E2 and tamoxifen were also found in terms of weight gain under a high-cholesterol diet. Indeed, consistent with previous
studies, tamoxifen significantly and importantly reduced body weight gain (Supplemental Tables S1–S3), contrary to E2. By using an apoE−/− mouse model, the sevenfold decrease in plasma cholesterol in response to tamoxifen was sufficient to explain the decrease of lesion development. Induction of LDL-r expression and activity by tamoxifen has been proposed to contribute to its hypolipidemic effect. Because the hypocholesterolemic effect of tamoxifen was lower in LDL-r−/− mice (less than twofold) (Supplemental Tables S1–S3), our results also suggest a role for LDL-r in its action on lipid metabolism. The effect of tamoxifen on cholesterol metabolism was shown to involve the binding to anti-estrogen binding site, leading to inhibition of 3β-hydroxysterol-Δ5-Δ7-isomerase, 3β-hydroxysterol-Δ7-reductase, and to perform the cholesterol-5,6-epoxide hydrolase activity. The persistence of the hypocholesterolemic effect of tamoxifen in ERα−/− LDL-r−/− mice, observed herein, supports the idea of an ERα-independent mechanism. Previous studies performed in cholesterol-fed ovariectomized rabbit, treated or untreated with tamoxifen, did not reveal any correlation between the circulating cholesterol and aortic atherosclerosis lesions. Several lines of evidence from the present work support the notion that the influence of tamoxifen on the cholesterol profile does not significantly contribute to its atheroprotective effect in mice. The tamoxifen-induced changes in total cholesterol concentrations were similar in ERα+/+ LDL-r−/− and in ERαAF-1+/+ LDL-r−/− compared with ERα−/− LDL-r−/− and ERαAF-1−/− LDL-r−/− mice, respectively, despite an atheroprotective effect in the former group (wild-type ERα), but not in the latter group (targeted ERα). However, because this effect still occurs in the presence of

Figure 5 ERαAF-1 is necessary to the atheroprotective action of tamoxifen on LDL-r−/− deficient mice. Four-week-old ovariectomized ERαAF1+/+ LDL-r−/− and ERαAF1−/− LDL-r−/− female mice received s.c. either a placebo or tamoxifen (Tmx; 4 mg/kg per day) pellet and were switched to a hypercholesterolemic diet from the age of 6 to 18 weeks. A: Representative micrographs of oil red 0 lipid-stained cryosections of the aortic sinus. B: Quantification of lipid deposition at the aortic sinus. A two-way analysis of variance indicated an interaction between tamoxifen treatment and ERαAF genotype. P = 0.01. The effect of tamoxifen treatment was studied in each genotype using Bonferroni’s post test. **P < 0.001. Representative micrographs of CD68 (C) and Sirius red (D) staining of the aortic sinus.

ACCELERATION OF REENDOTHELIALIZATION

Figure 6 Respective role of ERα AF-1 and AF-2 in the vascular actions of E2 and tamoxifen.
that the atheroprotective effect of tamoxifen appears to be essentially mediated through a direct action on the arterial wall through an ERα and, more specifically, an ERzAF-1—dependent mechanism.

In vitro experiments have demonstrated a key role of ERzAF-1 in the transcriptional activity of tamoxifen (Figure 4). Different association of co-activator proteins with AF-1 versus AF-2 could determine the affinity of tamoxifen- and estrogen-ligated ERs for different gene promoters and could explain the major differences in the regulation of aortic gene expression (Figure 2). To determine the role of ERzAF-1 in tamoxifen actions in vivo, and more particularly in the atheroprotective one, we used ERzAF-1 knockout mice in which the sequence coding for the main part of the A/B region, including AF-1, is deleted. We previously showed that ERzAF-1 is dispensable for the E2 atheroprotective action and demonstrate the requirement of this ERα function to mediate this beneficial effect in response to tamoxifen (Figure 5). We also show herein that tamoxifen actions on body weight and uterine hypertrophy entirely rely on ERzAF-1. These results highlight, for the first time, to our knowledge, in vivo, the crucial role of ERzAF-1 for several effects of tamoxifen. In addition, in contrast to the dispensable role of AF-1, ERzAF-2 is absolutely required to induce atheroprotection in response to E2. Thus, ERα mediates both tamoxifen and E2 atheroprotective effects, but the subfunctions of ERα involved in this action are different (Figure 6).

Finally, another major difference between E2 and tamoxifen is related to their action on endothelial healing after carotid electric injury, because tamoxifen fails to accelerate reendothelialization in vivo (Figure 1). This is consistent with in vitro studies that reported that E2, but not 4-OHT, stimulates endothelial cell migration and proliferation. Tamoxifen is known to induce transforming growth factor-β activity, which inhibits both endothelial proliferation and migration. Such a mechanism could contribute to the lack of accelerative effect in response to tamoxifen. These differences between E2 and tamoxifen actions could also be because of nongenomic ERα-mediated actions. Indeed, in addition to the classic transcriptional actions mediated through ERzAF1 and/or AF2, E2 induces rapid nongenomic actions involving a pool of ERs localized at the plasma membrane. In particular, E2, but not 4-OHT, rapidly activates ERs, resulting in mitogen-activated protein kinase and endothelial nitric oxide synthase activation in cultured endothelial cells. We previously reported that ERα, but neither AF1 nor AF-2, is necessary to induce the accelerative effect of E2 in vivo. Chambliss et al recently provided evidence supporting the physiological relevance of the membrane initiated steroid signaling pathway in vascular pathophysiological features using an estrogen-dendrimer conjugate, a compound that selectively activates nonnuclear ERs in vivo; this conjugate is able to accelerate reendothelialization in the same model of carotid electric injury used in the present study. Altogether, these data suggest that tamoxifen does not elicit the non-genomic action required to accelerate reendothelialization.

To conclude, the present study demonstrates that, although tamoxifen and E2 exert a similar atheroprotective action through an ERα-dependent mechanism, molecular targets accounting for this beneficial action appear to be different. Indeed, although ERzAF-1 is dispensable for E2-mediated protective effects on arteries, tamoxifen action entirely relies on ERzAF-1. Furthermore, tamoxifen elicits only partial vasculoprotection compared with E2, because the selective activation of ERzAF-1 is sufficient to prevent atheroma, but not to accelerate endothelial healing.

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Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.03.010.

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