Supplemental Material

Methods

Ovariectomy and treatments

Bilateral ovariectomy was performed at 4 weeks of age following anesthesia with a mixture of xylazine and ketamine, and mice concomitantly received estrogen or SERM treatment:

- For chronic E2 and tamoxifen treatments, mice were implanted with *s.c.* pellets releasing either placebo or E2 (17β-estradiol, 0.1 mg, 60-day release (*i.e.*, 80 µg/kg/day); Innovative Research of America, Sarasota, FL). We systematically checked that placebo-treated ovariectomized mice had an atrophied uterus (<10 mg), non-detectable circulating levels of E2 (<5 pg/ml i.e. <20 pmol/L), and that those implanted with an E2-releasing pellet had a significant increase in uterine weight and serum E2 concentrations (100–150 pg/mL). Previous work allowed to determine that a high physiological dose of E2 allow an optimal action on the various cells of the vessel wall, in particular endothelial cells [1, 2] as well as smooth muscle cells [3] or prevention of the atheromatous process [4, 5]. Thus, in the present work, we decided to choose a dose of E2 plasma level in the nM range [4], i.e. similar the the level reached during the estrous cycle in women, although it should be reminded that Sex Hormone Binding Globulin (SHBG) remains expressed in adult women, but not in adult mice, making the species comparison somewhat arbitrary [6].
- For chronic tamoxifen treatments, mice were implanted with *s.c.* pellets releasing either placebo or tamoxifen (5 mg, 60-day release (*i.e.*, 4 mg/kg/day); Innovative Research of America, Sarasota, FL).
- For chronic estetrol treatment [7], mice received *s.c.* mini-osmotic pumps (Alzet®, model 2004, 0.25 µL/h, 28 days) releasing either vehicle or E4 (6 mg/kg/day).
- For chronic Estradiol-Conjugated Dendrimer treatment [8], mice received *s.c.* mini-osmotic pumps (Alzet®, model 2004, 0.25 µL/h, 28 days) releasing either Empty Dendrimer or Estrogen-Conjugated Dendrimer (EDC, 240 µg/kg/day).

Mice were submitted to a femoral artery wire injury 2 weeks after the start of the treatment (Figures 1C and Supplemental Figure 1).

Femoral artery wire injury in mice

The femoral artery wire injury was performed as previously described [9]. Briefly, general anesthesia was achieved with 2% isoflurane, delivered through a mask. After incision of the skin, the femoral artery was carefully isolated from the surrounding vein and nerve, and an incision was made under a surgical microscope (Carl Zeiss). A 0.35 mm diameter angioplasty guidewire with a 0.25 mm tip (gift from Abbott Vascular, Rungis, France) was advanced into the artery through a 6 mm-long portion, then pulled back and advanced three times, in order to achieve deendothelialization. After removal of the wire, the arteriotomy site was ligated with 8-0 sutures (Prolene, Ethicon), and blood flow was restored. After skin closure, the mice were allowed to recover on a heating pad for 15 minutes, and placed back in clean cage. For subsequent neointimal hyperplasia analysis, mice were sacrificed 28 days later.

Femoral artery processing and morphometry

28 days after the femoral artery wire injury, mice were injected *i.p.* with pentobarbital and blood was removed from the circulation by perfusion through an open intracardiac circuit with 5 mL PBS. The injured femoral arteries were harvested and fixed for 4 h in 4% paraformaldehyde at pH 8 for 12 hours. The arteries were then embedded in paraffin and 4 µm-thick cross-sections were obtained. 4 slides per artery (situated at distance = 0.5, 2.0, 3.5 and 5.0 mm of the arterial incision) were analyzed. Briefly, slides were stained with Masson's trichrome, then the areas delimited by the EEL (A_{EEL}), the IEL (A_{IEL})

and the lumen (A_{lum}) were measured using LAS software (Leica). The medial area (A_{med}, μm^2) was calculated as: $A_{med} = A_{EEL} - A_{IEL}$. The intimal area ($A_{NI}$, μm^2) was calculated as: $A_{NI} = A_{IEL} - A_{lum}$. Intimal hyperplasia was expressed as a ratio of A_{NI}/A_{med} (referred to as the neointima/media ratio). Each A_{NI}/A_{med} is the mean value of the 4 analyzed sections.

Immunohistochemistry

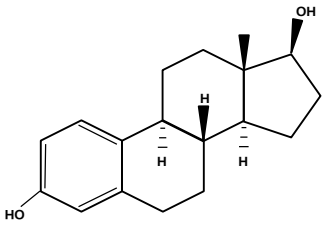
For αSM-actin and CD3, paraffin-embedded artery cross-sections were stained using a standard ABC-peroxidase/DAB immunostaining protocol (all the detection reagents were from Dako, France). Primary antibodies were used to detect αSM-actin-positive SMCs (actin, smooth muscle, rabbit polyclonal antibody, 1:25; Thermo Fischer Scientific) or CD3-positive T lymphocytes (rabbit anti-CD3, 1:100, Zytomed Systems). The detection was achieved with a biotinylated secondary antibody (Goat Polyclonal anti-Rabbit Immunoglobulins, Biotinylated, Dako, France) For CD31-positive endothelial cell detection, paraffin-embedded artery cross-sections were stained with an immunofluorescence protocol. Sections were stained with an anti-CD31 antibody (rat anti-mouse PECAM-1, 1:100, CliniSciences). The secondary antibody was coupled to a fluorophore for fluorescence-based detection (Alexa Fluor-488 coupled, goat anti-rat IgG, Thermo Fisher Scientific, France). All images were acquired using a microscope at 320x magnification (Leica).

Analysis of mRNA levels by RT-qPCR

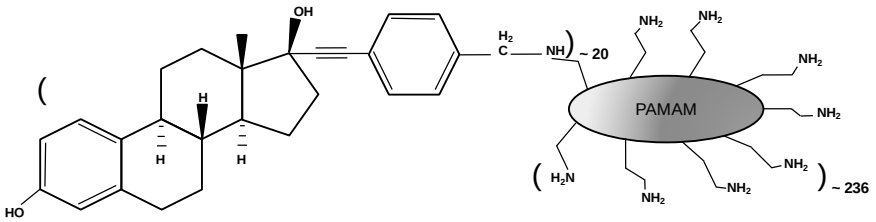
Dissected tissues were homogenized using a Precellys tissue homogenizer (Bertin Technol, France) and total RNA from tissues was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA) and a classical phenol/chloroform extraction protocol. One microgram of RNA was reverse transcribed (RT) at 25°C for 10 min and then at 37°C for 2 h in a final volume of 20 µL using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems) and further analyzed by quantitative-PCR.

Real-time quantitative PCRs (q-PCRs) were performed on the StepOne instrument (Applied Biosystems). Primers were validated by testing PCR efficiency using standard curves (95% ≤efficiency≤ 105%): ERα (NM_007956.4): TGATGCCAGGAGAGGCCAATGC (forward) and TGTCGCCCAGAGACTGCCTTCTT (reverse). Gene expression was quantified using the comparative Ct (threshold cycle) method, TPT1 (NM_009429.2), CCGGGAGATCGCGGAC (forward)/ TTCCACCGATGAGCGAGTC (reverse).

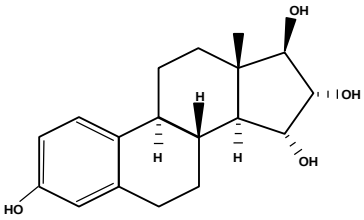
E2 ,17β –Estradiol:



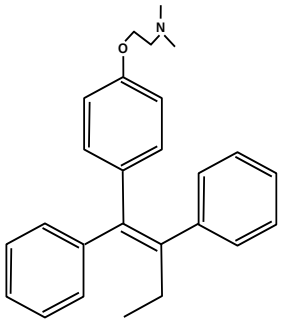
EDC , Estrogen dendrimer conjugated:



E4 , Estetrol:



Tmx, Tamoxifen:



Online Figure I

Chemical structures of 17β-estradiol (E_2), Estrogen Dendrimer Conjugate (EDC), estetrol (E_4) and tamoxifen (Tmx).

References

1. Brouchet L, Krust A, Dupont S, Chambon P, Bayard F, Arnal JF. Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor-alpha but not estrogen receptor-beta. *Circulation*. 2001;103:423-428
2. Darblade B, Pendaries C, Krust A, Dupont S, Fouque MJ, Rami J, Chambon P, Bayard F, Arnal JF. Estradiol alters nitric oxide production in the mouse aorta through the alpha-, but not beta-, estrogen receptor. *Circulation research*. 2002;90:413-419
3. Sullivan TR, Jr., Karas RH, Aronovitz M, Faller GT, Ziar JP, Smith JJ, O'Donnell TF, Jr., Mendelsohn ME. Estrogen inhibits the response-to-injury in a mouse carotid artery model. *The Journal of clinical investigation*. 1995;96:2482-2488
4. Elhage R, Arnal JF, Pieraggi MT, Duverger N, Fievet C, Faye JC, Bayard F. 17 beta-estradiol prevents fatty streak formation in apolipoprotein e-deficient mice. *Arteriosclerosis, thrombosis, and vascular biology*. 1997;17:2679-2684
5. Billon-Gales A, Fontaine C, Douin-Echinard V, Delpy L, Berges H, Calippe B, Lenfant F, Laurell H, Guery JC, Gourdy P, Arnal JF. Endothelial estrogen receptor-alpha plays a crucial role in the atheroprotective action of 17beta-estradiol in low-density lipoprotein receptor-deficient mice. *Circulation*. 2009;120:2567-2576

6. Janne M, Hogeveen KN, Deol HK, Hammond GL. Expression and regulation of human sex hormone-binding globulin transgenes in mice during development. *Endocrinology*. 1999;140:4166-4174
7. Abot A, Fontaine C, Buscato M, Solinhac R, Flouriot G, Fabre A, Drougard A, Rajan S, Laine M, Milon A, Muller I, Henrion D, Adlanmerini M, Valera MC, Gompel A, Gerard C, Pequeux C, Mestdagt M, Raymond-Letron I, Knauf C, Ferriere F, Valet P, Gourdy P, Katzenellenbogen BS, Katzenellenbogen JA, Lenfant F, Greene GL, Foidart JM, Arnal JF. The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation. *EMBO Mol Med*. 2014;6:1328-1346
8. Chambliss KL, Wu Q, Oltmann S, Konaniah ES, Umetani M, Korach KS, Thomas GD, Mineo C, Yuhanna IS, Kim SH, Madak-Erdogan Z, Maggi A, Dineen SP, Roland CL, Hui DY, Brekken RA, Katzenellenbogen JA, Katzenellenbogen BS, Shaul PW. Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. *The Journal of clinical investigation*. 2010;120:2319-2330
9. Smirnova NF, Gayral S, Pedros C, Loirand G, Vaillant N, Malet N, Kassem S, Calise D, Goudouneche D, Wymann MP, Hirsch E, Gadeau AP, Martinez LO, Saoudi A, Laffargue M. Targeting pi3kgamma activity decreases vascular trauma-induced intimal hyperplasia through modulation of the th1 response. *J Exp Med*. 2014;211:1779-1792