Chronic estradiol treatment reduces platelet responses and protects mice from thromboembolism through the hematopoietic estrogen receptor α

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Although estrogens are known to have a deleterious effect on the venous thrombosis risk and a preventive action on the development of arterial atheroma, their effect on platelet function in vivo remains unclear. Here, we demonstrate that a chronic high physiologic level of estradiol (E2) in mice leads to a marked decrease in platelet responsiveness ex vivo and in vivo compared with ovarectomized controls. E2 treatment led to increased bleeding time and a resistance to thromboembolism. Hematopoietic chimeric mice harboring a selective deletion of estrogen receptors (ERs) α or β were used to demonstrate that the effects of E2 were exclusively because of hematopoietic ERα. Within ERα the activation function-1 domain was not required for resistance to thromboembolism, as was previously shown for atheroprotection. This domain is mandatory for E2-mediated reproductive function and suggests that this role is controlled independently. Differential proteomics indicated that E2 treatment modulated the expression of platelet proteins including β1 tubulin and a few other proteins that may impact platelet production and activation. Overall, these data demonstrate a previously unrecognized role for E2 in regulating the platelet proteome and platelet function, and point to new potential antithrombotic and vasculoprotective therapeutic strategies. (Blood. 2012;120(8):1703-1712)

Introduction

Estradiol-17β (E2) and estrogen receptors (ERs) are well known for their pivotal role in sexual development and reproduction. However, they can also modulate cardiovascular and metabolic risk, autoimmune disease progression, and cancer growth. These pleiotropic effects are a consequence of both the widespread expression of ER in many cell populations within the body as well as possibly reflecting the ancestral status of ER in the steroid receptor family.1 Epidemiologic and experimental studies now support an atheroprotective effect of both endogenous and exogenous estrogens. The Women’s Health Initiative study did not show a coronary protective effect of estrogen in postmenopausal women,2 but subsequent studies have shown that this was because of both inappropriate timing (ie, administering the hormone therapy too late) and the identity of the associated progestin.3,4 The route of administration of estrogens (oral versus transdermal) has also been shown to have a major impact on the risk of venous thromboembolism.5 Together these studies indicate that the beneficial or deleterious action of estrogens is strongly influenced by the dose, route, and timing of the hormonal treatment alongside age, genetic, and environmental factors.

Although the impact of estrogens on coagulation and venous thromboembolism risk has received much attention, their effect on platelet function remains poorly characterized in vivo. After estrogen treatment, one study reported reduced platelet activation6 whereas 2 others found increased activation.7,8 Platelet activation markers were also found at higher levels in postmenopausal compared with premenopausal women.9 Furthermore, platelet aggregation and dense granule secretion were reportedly decreased after E2 treatment in animal models including rabbit and pig.10-13 Although these studies favor an inhibitory role for E2 on platelet activation, there is clearly a need for further investigation.

E2 effects are mediated by ERα and/or ERβ which are members of the nuclear receptor superfamily encoded by 2 distinct genes.14 ERs can be divided into 6 domains, labeled A to F, and harbors 2 transactivation functions (AF-1 and AF-2) which are located within regions A/B and E, respectively.2 Both ERα and β are expressed in megakaryocytes and circulating platelets,15,16 In contrast to the in vivo studies discussed in the previous paragraph,10-13 addition of E2 to washed platelets in vitro was shown to increase the activity of intracellular signaling molecules such as Src, Pyk2, and phosphoinositide 3-kinase through the extragenomic effects of ERβ, leading to a potentiation of platelet activation by subthreshold concentrations of thrombin.18 However, it should be emphasized that a major difference between acute in vitro E2 addition and chronic in vivo E2 treatment is that the latter can have an impact on platelets through genomic mechanisms and can also affect the differentiation of myeloid progenitors19 and megakaryocytes.20 Therefore, the effect of estrogens on platelet function in vivo remains currently unclear.

In this study, we examined the effect of E2 on platelets using a mouse model. Although endogenous estrogens had no significant influence on platelet function, a chronic high physiologic dose of
E2 (200 μg/kg/d of E2 injected subcutaneously) decreased platelet responsiveness ex vivo, increased tail-bleeding time, and protected animals against thromboembolism. Using mice in which either ERα or ERβ was inactivated, we found that the hematopoietic ERα alone was responsible for the observed E2 effects. Although ERα-AF-1 is mandatory for its reproductive actions, it is dispensable in mediating the atheroprotective properties of E2.21 We found here that AF-1 is also not essential for the effects of E2 on thromboembolism. In addition, we provided evidence that E2 treatment can modulate the platelet proteome. These novel effects of E2 treatment on mouse platelets in vivo suggest its potential for strategies for antplatelet and vasculoprotective therapies.

Methods

Mice and surgical procedures

Female C57BL/6J mice were purchased from Charles River. ERα-deficient mice (ERα+/−) were maintained at our animal facility and screened by polymerase chain reaction (PCR) genotyping, as previously described.22 ERα−/−, ERβ−/−, and ERα-AF-1−/− mouse founders were generously provided by P. Chambon and A. Krust (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). Mice were anesthetized by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg). Tail-bleeding time was monitored as described previously.24,25

Bone marrow transplantation

Two weeks after ovariectomy, recipient mice were lethally irradiated (9.2 Gy, γ source) then intravenously reconstituted with bone marrow cells from either ERα−/−, ERα−/−, ERβ−/−, or ERβ−/−;AF-1−/−;AF-1−/− mice. Two weeks later, mice were implanted or not with an E2 pellet. Bactrim from either ERα−/−, ERα−/−, ERβ−/−, or ERβ−/−;AF-1−/−;AF-1−/− mice was inactivated, we found that the hematopoietic ERα alone was responsible for the observed E2 effects. Although ERα-AF-1 is mandatory for its reproductive actions, it is dispensable in mediating the atheroprotective properties of E2.21 We found here that AF-1 is also not essential for the effects of E2 on thromboembolism. In addition, we provided evidence that E2 treatment can modulate the platelet proteome. These novel effects of E2 treatment on mouse platelets in vivo suggest its potential for strategies for antplatelet and vasculoprotective therapies.

Materials

Collagen was from Nycomed, U46619 was from QBiogene Inc, PPACK was from Calbiochem, and FITC-labeled anti-β-selectin Ab was from BD Biosciences/BD Pharmingen. DiOC6, and Oregon Green 488–conjugated fibrinogen were from Invitrogen. FITC-labeled anti-β2 GPI and FITC-labeled anti-integrin α2 chain (CD49b) Abs were from Emfret Analytics. Anti-phospho-MLC (Ser19) and anti-total MLC Abs were from Santa Cruz Biotechnology Inc. All other reagents were purchased from Sigma-Aldrich. Convulxin, kindly provided by Dr M. Jandrot-Perrus (Inserm 698, Paris, France), was purified from the venom of Crotalus durissus terrificus.23 The β1 tubulin ELISA kit was from Antibodies-Online and ICI 182780 from Tocris Bioscience.

Tail-bleeding time

Mice were anesthetized and a 3-mm segment of tail tip was cut off and the bleeding time was monitored as described previously.24,25

Preparation of platelets and in vitro aggregation studies

Platelets were prepared as previously described and resuspended in modified HEPES-Tyrode buffer containing 2 mM CaCl2 (pH 7.38) at a density of 5 × 10^8 platelets/mL in the presence of the ADP scavenger apyrase (adenosine-5′-triphosphate diphosphohydrolase) then incubated for 1 hour at 37°C before stimulation. Platelets were counted by microscopy using a Malassez chamber. Optical platelet aggregation experiments were monitored by a turbidimetric method using a dual-channel aggregometer (Payton Associates) with continuous stirring at 900 rev/min, 37°C.

Fibrinogen-binding assays

Washed platelets (1 × 10^8 platelets/mL) were incubated simultaneously with 0.1 or 0.3 IU/mL thrombin, Oregon Green 488–conjugated fibrinogen (150 μg/mL in final volume) and Tyrode buffer in a final volume of 100 μL. After 10 minutes at 37°C without shaking, samples were fixed by the addition of formalin (1% in final volume) and then diluted 5 times with HEPES-Tyrode buffer. Samples were analyzed by flow cytometry using a FACScan and Win MDI software.

Clot retraction experiments

Platelet-rich plasma (PRP) was obtained from pooled blood samples from several mice by centrifugation for 4 minutes at 250g at 37°C. Platelets were then washed with modified HEPES-Tyrode buffer (pH 6.7) containing 2 mM EGTA and 0.35% BSA, and resuspended (3 × 10^8 platelets/mL) without heparin in their autologous platelet-poor plasma containing 2 mM MgCl2 and 2 mM EGTA. Thrombin and atroxin were added at a final concentration of 2 IU/mL and 0.1 μg/mL, respectively, and the reaction mixtures were left unstirred at 37°C, as previously described.24,25

Gel electrophoresis and immunoblotting

Proteins were analyzed as previously described using the relevant Abs.

Ex vivo flow-based adhesion studies

Glass micropipillaries (Ibidii) were coated with 500 μg/mL type I collagen from equine tendon for 1 hour at 37°C. Bioflux plates (Bioflux 200 from Labtech) were coated with 100 μg/mL fibrinogen. Blood was drawn into lepirudin (200 IU/mL), and DiOC6 (2μM, 30 minutes at 37°C) was used to label platelets in whole blood. Labeled blood was then perfused through collagen-coated glass micropipillaries at a wall shear rate of 1500 seconds⁻¹, followed by washing for 2 minutes at the same shear rate with PBS. For fibrinogen-coated microcapillaries, PPACK-treated whole blood (80μM) was perfused through bioflux plates at a wall shear rate of 1500 seconds⁻¹ or 4000 seconds⁻¹. Thrombus formation was visualized with a 40× long-working-distance objective in real time (acquisition rate: 1 frame every 5 seconds) for both fluorescent and transmitted light microscopy and the analysis was performed as previously described.24,25

Carotid artery thrombosis

The right and left carotids were dissected free from surrounding tissues. Two flow probes were connected to a Transonic model T403 flow meter (Transonic System; Emka Technologies) to record the blood flow (millili-seconds) for both fluorescent and transmitted light microscopy and the analysis was performed as previously described.24,25

Thromboembolism

Acute systemic vascular thromboembolism was induced by injecting a mixture of collagen (0.4 mg/kg) and epinephrine (60 μg/kg) into the right jugular vein of anesthetized mice.24

Histochemical analysis of lung mouse

To visualize thrombi in the pulmonary vasculature, anesthetized mice were euthanized 10 minutes after injection of the collagen (0.4 mg/kg) and epinephrine (60 μg/kg) mixture. Lungs were excised and formalin-fixed. Paraffin sections (5-μm thick) were stained with hematoxylin-eosin and analyzed. Platelets were identified with a rabbit anti-α1b integrin Ab.
Nano-LC-MS/MS and database search analysis

Proteins were digested by incubating each spot excised from 2-dimensional (2D) gels with modified sequencing grade trypsin overnight at 37°C. The resulting peptides were extracted from the gel and resuspended in 5% acetonitrile, 0.05% trifluoroacetic acid. The peptide mixtures were analyzed by nano-LC-MS/MS using an Ultimate 3000 system (Dionex) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The Mascot Daemon software (Version 2.2.0.3; Matrix Science) was used to perform searches in the SwissProt-Trembl database. Mascot results were parsed with the in-house developed software Mascot File Parsing and Quantification (MFPaQ; Version 4.0) and an identified protein was considered as a hit if it was identified with at least 2 peptides with a score greater than the significance threshold score for a probability of $P < .05$ or at least 1 peptide with a score greater than the significance threshold score of $P < .001$, as determined by the Mascot Search program.

Electron microscopy

Platelets were prepared as previously described, then examined using a transmission electron microscope at an accelerating voltage of 5 kV.

Megakaryocyte purification and culture

Mice were ovariectomized and treated or not with E2 under the same protocol as described in “Mice and surgical procedures.” Bone marrow cells were obtained from femora and tibiae of mice by flushing. Cells expressing 1 or more of the following surface proteins, CD16/CD32, Gr1, B220, CD11b were depleted using immunomagnetic beads (sheep anti-rat IgG Dynabeads; Invitrogen). The remaining population was cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (containing estrogens), 2mM L-glutamine, penicillin/streptomycin, and 20 ng/mL murine stem cell factor (SCF) at 37°C under 5% CO2 for 2 days in the presence or absence of the anti-estrogen ICI 182780 (10^{-6}M). Cells were then cultured for an additional 4 to 5 days in the presence of 20 ng/mL murine SCF and 100 ng/mL murine thrombopoietin. The cell population was then enriched in mature megakaryocytes using a 1.5%/3% bovine serum albumin (BSA) gradient under gravity (1 g) for 45 minutes at room temperature. Megakaryocytes were lysed and their β1 tubulin content was analyzed using the ELISA test.

Statistics

Results are expressed as mean ± SEM. Statistical analyses were performed using Excel software (Student t test). $P$ values less than .05 were considered statistically significant.
Results

Increased bleeding time and platelet aggregation defects after chronic E2 treatment in mice

Ovariectomized mice were treated or not with an E2 pellet for 3 weeks. This type of treatment was previously reported by our team to induce a stable E2 plasma level of approximately 0.3nM (80 pg/mL). This level is in the high range of pregnant mice. The average plasma concentration in E2 is below the threshold of detection in untreated ovariectomized mice.

Platelet hemostatic function was analyzed in vivo by measuring tail-bleeding time (Figure 1A). In both control and untreated ovariectomized mice producing very low levels of estrogens and sham-operated mice, a normal bleeding time was observed (324 ± 53 seconds and 366 ± 64 seconds, respectively; mean ± SEM), indicating that endogenous E2 levels had no impact on primary hemostasis. Conversely, bleeding times were increased in E2-treated mice: of 23 mice tested, bleeding was weakly prolonged in 9 cases and considerably increased in 14 with 3 of these displaying a bleeding time greater than 30 minutes.

To investigate intrinsic platelet functional defects, aggregation of washed platelets was monitored after stimulation by thrombin and the thromboxane A2 analog (U46619), which both signal via G protein–coupled receptors (Figure 1B). In contrast to ovariectomized mice platelets, which aggregated in a dose-dependent manner to both agonists, platelets from E2-treated mice failed to aggregate in response to low concentrations of thrombin or U46619. At higher concentrations of these agonists, platelets aggregated with only mild defects in intensity and/or velocity. The expression of P-selectin in E2-treated mouse platelets was not modified at high or low doses of thrombin or U46619 (not shown), indicating that platelet shape change was also not modified by these agonists but, using scanning electron microscopy, we consistently observed an increase in the length of filopodia in platelets from E2-treated mice stimulated by thrombin or U46619 in suspension and under nonaggregating conditions (Figure 1C).

At low doses of collagen, platelets from E2-treated mice aggregated slower and less efficiently than control platelets. Collagen binds to both the GPVI and integrin α2β1 receptors, while the snake venom toxin convulxin (Cvx) is a selective GPVI agonist. Platelets from E2-treated mice were much less responsive to 5nM Cvx compared with control platelets and did not aggregate at all at low doses of Cvx (Figure 1B). It is noteworthy that expression of GPVI and α2β1 was not modified by E2 treatment as confirmed by flow cytometry (supplemental Figure 1; available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Flow cytometry was also used to assess fibrinogen binding to the αIIbβ3 integrin downstream of thrombin stimulation. In accordance with the aggregation responses, E2 treatment induced a reduction of fibrinogen binding at low thrombin concentrations but not at high concentrations (Figure 1D). However, despite normal fibrinogen binding and aggregation traces in response to high concentrations of agonists, microscopy analysis (Figure 1E) indicated that, compared with controls, platelets from E2-treated mice formed smaller aggregates in response to all agonists tested even at high concentrations. This suggests a defect in αIIbβ3 outside-in signaling. Consistent with this, retraction of the fibrin clot by washed platelets, which also requires functional αIIbβ3 alongside tight membrane-cytoskeleton interactions and the contractile actomyosin system, was strongly reduced on E2 treatment (Figure 2A). In addition, thrombin-induced myosin light chain (MLC) phosphorylation, mandatory for actomyosin cross-bridge formation and contractility, was significantly decreased in platelets from E2-treated mice (Figure 2B). Together, these data suggest a defect in αIIbβ3-mediated platelet responses after E2 treatment. This was confirmed under physiologic arterial flow conditions using a flow-based adhesion assay where whole blood was perfused over a fibrinogen matrix. E2 treatment reduced platelet binding (supplemental Figure 2). We then perfused whole blood over a collagen matrix and analyzed the effects of E2 treatment. After 2 minutes, control mouse platelets formed numerous densely packed thrombi while E2 treatment reduced both the surface coverage and the volume of the thrombi (Figure 3A-C). In addition, platelets from E2-treated mice only formed a single platelet layer in many areas of the collagen matrix. The reduced thrombus volume seen after 2 minutes in E2-treated mice is not thought to be because of the 25% reduction in platelet count observed after E2 (Table 1) because such a reduction did not affect the final thrombus size in control blood under these conditions (M.-P.G., unpublished observation, January 2011).

Although the agonists used in these assays signal via different intracellular pathways, a critical common negative regulator of platelet function is cAMP; therefore cAMP levels were tested in E2-treated platelets. E2 treatment was found to have no effect on cAMP levels neither under resting conditions nor after incubation with PGE1 (supplemental Figure 3).

Overall, our results point to a decrease in platelet responsiveness after long-term E2 treatment in mice. This was observed...
downstream of numerous agonists that act via different signaling pathways and was consistent across various platelet function assays.

**Chronic E2 treatment protects mice from thromboembolism**

To evaluate the effect of E2 treatment on intravascular platelet aggregation, an injury to the carotid artery was induced by FeCl₃ and the velocity of blood flow and time to occlusion were determined. While 100% of control animals presented a complete occlusion within 10 minutes, E2 treatment reduced the formation of a stable occlusive thrombus to 40% of cases (Figure 4). Unstable occlusions were observed in 50% of cases and 10% of the treated mice exhibited a complete resistance to occlusion. The potential of E2 in preventing occlusive thrombus formation in vivo was tested using another model of acute vascular occlusion that was induced by intrajugular injection of a mixture of collagen (0.4 mg/kg) and epinephrine (60 μg/kg). As shown in Figure 5A, 100% (12 of 12) of control mice and 91.6% (11 of 12) of sham-operated mice died within 5 minutes of injection. In sharp contrast, 100% (10 of 10) of E2-treated mice survived. Interestingly, these mice were still protected from thromboembolism when a dose of 1 mg/kg collagen was injected (not shown). Histologic examination of lung tissue from control mice revealed large occlusive platelet thrombi throughout the pulmonary vasculature, particularly in large vessels (Figure 5B). Occlusive pulmonary thrombi in E2-treated mice were only observed in small vessels. These thrombi contained significantly less platelets compared with untreated mice, as shown by the intensity of the integrin staining (Figure 5C). Interestingly, E2 treatment did not modify standard coagulation tests (prothrombin time and activated partial thromboplastin time), although it increased levels of plasma fibrinogen and factors VIII, IX, and XI (supplemental Table 1). Overall, these results clearly indicate that, after E2 treatment, mice are protected from intravascular thrombosis independent of functional deficiencies in coagulation.

**ERα in hematopoietic cells supports the increased bleeding time and protection from thromboembolism in E2-treated mice**

The molecular basis of these E2 effects were investigated by grafting lethally irradiated wild-type ovariectomized mice with bone marrow harvested from ERα+/+, ERβ+/+, ERα-/-, or ERβ-/- mice. PCR was used to confirm the loss of expression of hematopoietic ERα or ERβ in spleen cell lysates from these chimera mice (not shown). Platelet count was not significantly affected by the graft (Table 1). As expected, the tail-bleeding time of control mice and 91.6% (11 of 12) of sham-operated mice died within 5 minutes of injection. In sharp contrast, 100% (10 of 10) of E2-treated mice survived. Interestingly, these mice were still protected from thromboembolism when a dose of 1 mg/kg collagen was injected (not shown). Histologic examination of lung tissue from control mice revealed large occlusive platelet thrombi throughout the pulmonary vasculature, particularly in large vessels (Figure 5B). Occlusive pulmonary thrombi in E2-treated mice were only observed in small vessels. These thrombi contained significantly less platelets compared with untreated mice, as shown by the intensity of the integrin staining (Figure 5C). Interestingly, E2 treatment did not modify standard coagulation tests (prothrombin time and activated partial thromboplastin time), although it increased levels of plasma fibrinogen and factors VIII, IX, and XI (supplemental Table 1). Overall, these results clearly indicate that, after E2 treatment, mice are protected from intravascular thrombosis independent of functional deficiencies in coagulation.
of most control mice engrafted with wild-type bone marrow was prolonged after E2 treatment (Figure 6A). Interestingly, ERα<sup>-/-</sup> bone marrow chimeras treated with E2 had a normal bleeding time, whereas that of E2-treated ERβ<sup>-/-</sup> bone marrow chimeras was similar to control animals, with 7 of 13 mice bleeding for more than 30 minutes (Figure 6B). Thus, hematopoietic ERα, but not ERβ, is required for the prolongation of bleeding time induced by chronic E2 treatment.

Furthermore, hematopoietic ERα was found to be essential for the E2-mediated protection of thromboembolism observed after injection of a collagen/epinephrine mixture in vivo. Most E2-treated mice reconstituted with ERα<sup>-/-</sup>/ERβ<sup>-/-</sup> bone marrow chimeras died within 5 minutes (Figure 6C-D) while the protective effect of E2 was present in ERα<sup>+/+</sup>, ERβ<sup>+/+</sup>, and ERβ<sup>-/-</sup> bone marrow chimeras. Histologic analysis of lung tissue from these mice confirmed occlusive platelet-rich thrombi localized in both small and large vessels (data not shown).

To study the role of the AF-1 domain of ERα in vivo, we used a mouse model lacking the AF-1 region (ERαAF-1<sup>-</sup>) because of a deletion of the first exon encoding the A/B domain. This resulted in the 66-kDa ERα protein being replaced by a 49-kDa ERα isoform.21 As shown by the generation of hematopoietic chimera, we found that E2-mediated resistance to thromboembolism still occurred in the absence of ERαAF-1 (Figure 6E). Tail-bleeding time also increased on E2 treatment (510 ± 80 seconds vs 250 ± 90 seconds, P < .01, n = 9 and n = 8, respectively), although this effect was not as pronounced as in control E2-treated mice, suggesting a partial contribution of the AF-1 domain.

**E2 treatment impacts on the platelet proteome: the example of β1 tubulin**

Because E2 treatment could affect megakaryocyte gene expression, we performed differential proteome analysis using 2D-DIGE coupled to mass spectrometry on platelets from mice treated or not with E2. Results were consistent over 3 independent series (each containing 3 or 4 mice per group): 4 protein spots were found to be down-regulated (1.66-, 1.59-, 1.74-, and 1.73-fold) and 1 was up-regulated (1.99-fold) in E2-treated mice compared with control animals (supplemental Figure 4A-B). Proteins present in these spots were identified by mass spectrometry (Figure 7A) as cytoskeleton proteins (β1 tubulin, α4-A tubulin, and pacsin 2), secreted proteins (transforming growth factor β-1, thrombospondin-1, and leukocyte elastase inhibitor), a protein involved in the regulation of....

![Figure 4. Thrombotic response of mice to ferric chloride injury of the carotid artery. Flow rates were measured in the carotid artery after exposure to 7% FeCl<sub>3</sub> for 2 minutes. The experiment was stopped after 30 minutes. (A) For each genotype, the number of mice forming a stable occlusion is shown in black. The number of mice that formed an unstable or partial occlusion is shown in white. The number of mice that did not form an occlusion is shown in gray. (B) Representative flow traces for each case (stable occlusion, no occlusion, and unstable occlusion).](image-url)
oxidative stress (thioredoxin-like protein), heat shock protein 60, and the elongation factor 18. It is noteworthy that a discrepancy between the position on the 2D-DIGE protein spot patterns and the molecular weight or pl was observed for transforming growth factor β-1, thrombospondin-1, and transgelin-2, suggesting that they were in fact fragments of the native proteins. We focused our attention on β1 tubulin which is specifically expressed in platelets and mature megakaryocyes and plays a role in platelet biogenesis, structure, and function.33,34 The role of the other identified proteins is unknown or poorly characterized in platelets. The decreased expression of β1 tubulin in platelets from E2-treated mice observed by 2D-DIGE was confirmed using a specific ELISA test (Figure 7B). Interestingly, the changes observed in platelet proteins appear to originate from initial effects on hematopoietic progenitors. Indeed, the effect of in vivo E2 treatment, through the ERα receptors, was critical for the modulation of β1 tubulin expression in megakaryocytes (Figure 7C). The presence of the anti-estrogen ICI 182780 during the in vitro differentiation did not impact these changes, showing the importance of the in vivo conditioning of the cells. However, as shown by transmission electron microscopy (Figure 7D), the decrease in β1 tubulin had no significant effect on the number of microtubule coils in the marginal band (10.25 ± 0.40 per control platelet vs 9.47 ± 0.54 in E2-treated platelets; n = 27 and n = 17, respectively) nor on the discoid shape and size of the resting platelets (not shown). As shown in Figure 1C, after thrombin or U46619 stimulation in suspension and under nonaggregating conditions, E2-treated platelets extended their filopodia significantly more than control platelets while their hyalomer surface area was higher (2.21 μm² ± 0.06 vs 1.9 μm² ± 0.05; P < .01, n = 90 and n = 73, respectively, on thrombin stimulation), suggesting a decrease in internal platelet contraction, a mechanism linked to microtubule reorganization.35

Discussion

Using a mouse model alongside ex vivo and in vivo approaches, we found that a chronic high physiologic level of estrogen equivalent to that observed in pregnant mice had a significant inhibitory effect on platelet aggregation. This effect was most obvious when washed platelet aggregation was monitored in response to low concentrations of physiologic agonists but was also detected in response to high concentrations because platelets still formed smaller aggregates. The inhibitory effect of E2 treatment on platelet responsiveness was observed under different conditions and with various agonists known to activate distinct signaling pathways (via either ITAM/tyrosine kinase-linked receptors, heterotrimeric G protein-coupled receptors and/or integrin-linked focal adhesion structures), suggesting that E2 affects a central mechanism controlling the global sensitivity of platelets. The defect in aggregation at low doses of agonists correlated with a decrease in fibrinogen binding to αIIbβ3. At high concentrations of agonists, fibrinogen binding was virtually normal despite a reduction in the size of platelet aggregates. This was because of a decrease in αIIbβ3-mediated functions, as shown by the reduction in platelet-induced fibrin clot retraction and adhesion of platelets to a fibrinogen-coated surface under flow conditions. In vitro thrombus formation assays conducted under physiologic flow conditions on a collagen matrix confirmed that platelets from E2-treated mice are less efficient at forming thrombi compared with control platelets.

Importantly, E2 treatment also has a marked inhibitory effect on platelet activation in vivo. Besides increasing the tail-bleeding time, it induced an impressive resistance to thromboembolism after injection of an epinephrine-collagen mixture and a significant protection to occlusive arterial thrombosis induced by an FeCl3 injury of the carotid. Histologic analysis of mouse lungs after injection of an epinephrine-collagen mixture indicated that both small and large vessels were totally occluded in untreated mice while their hyalomer surface area was higher in E2-treated mice extended their filopodia significantly more than control platelets while their hyalomer surface area was higher (2.21 μm² ± 0.06 vs 1.9 μm² ± 0.05; P < .01, n = 90 and n = 73, respectively, on thrombin stimulation), suggesting a decrease in internal platelet contraction, a mechanism linked to microtubule reorganization.35
To delineate the role of AF1, we recently generated mice selectively deficient in ERα AF-1. Using these ERα AF-1<sup>−/−</sup> mice, we found that ERα AF-1 is indeed dispensable for the major vasculo-protective actions of E2 such as the prevention of atheroma, acceleration of endothelial healing, and increased endothelial nitric oxide production. Here, we show that AF-1 is not required for E2-induced resistance to thromboembolism.

The question remains as to the mechanism by which E2 mediates its hematopoietic ERα-dependent antiaggregative action. ERα AF-1<sup>−/−</sup> is still able to modulate genomic signaling through its other activation function AF-2, as well as membrane-initiated/nongenomic signaling. Interestingly, platelets might represent a model to dissociate the acute, membrane-initiated, nongenomic effects of E2 from the genomic, long-term effects. Indeed, anucleated platelets can only respond through nongenomic means after addition of E2 in vitro. Moro et al reported an acute proaggregative effect of E2 on washed human platelets that was driven by ERβ, although this acute effect of E2 appears to be modest in isolated mouse platelets (M.-P.G., unpublished data, January 2011). In vivo, E2 can act on megakaryocytes through genomic and nongenomic mechanisms. Addition of E2 to megakaryocytes increases their differentiation in vitro and modulates the expression of the 2 ERs. The contrasting effects of E2 seen here in the short- versus long-term are reminiscent of the effect of E2 on macrophages. Acute E2 elicits a weak anti-inflammatory action on isolated peritoneal macrophages whereas chronic E2 has a strong proinflammatory action in vivo. Here we show that long-term E2 treatment modulates the platelet proteome. Very reproducibly, in 3 independent series of control and E2-treated mice, we found a decreased expression of several platelet proteins including β1 tubulin, a major constituent of microtubules which modulate platelet production and function. While this decrease, confirmed by a specific ELISA test, was significant (39%) it did not impact the number of microtubule coils in the marginal band or the size and discoid shape of resting platelets. In humans, heterozygous carriers of the Q43P β1 tubulin variant show a reduced expression of the protein associated with protection against arterial thrombosis. The decrease in β1 tubulin expression observed in E2-treated platelets may thus contribute to the platelet functional defects or production and the resistance to thromboembolism. However, it is likely a chance in a cluster of proteins that might be responsible for the observed phenotype. In E2-treated mice, the decrease in β1 tubulin expression is accompanied by a reduced expression of other proteins including pacsin2, a protein involved in remodeling of the actin cytoskeleton, and the thioredoxin-like protein, a regulator of oxidative stress. Changes in membrane protein expression cannot be excluded because the 2D-DIGE approach is powerful for comparative cytosolic protein profiling but more limited in analyzing extreme isoelectric points or molecular weights and hydrophobic proteins. Our data strongly suggest that the change in expression of different proteins is because of ERα stimulation affecting hematopoietic progenitors or megakaryocytes, and the combined effects of these changes may in turn affect platelet responsiveness. It will be interesting to investigate the mechanisms by which E2 affects protein expression in these cells. Two transcription factors are possible targets because they are already known to regulate β1 tubulin expression in mice: the erythromegakaryocytic factor NF-E2 and GATA1. Importantly, transcriptional activity of GATA1 has been shown to be repressed by estrogens in a model of erythroid progenitor cells. Overall, these results strongly suggest that E2 treatment can modulate gene expression during megakaryocyte
differentiation through ERα leading to modification in platelet responsiveness.

The data reported herein show promise for developing new pharmacologic strategies to prevent thrombosis. ERαAF-1 is mandatory for mediating the sexual effects of E2 in vivo as well as the proliferative action of E2 on breast cancer cell lines. However, in striking contrast, both the major vasculoprotective actions of E2 and, as shown here, the effects of E2 on the resistance to thromboembolism do not require AF-1. Thus, our results provide the rationale for developing selective modulators of ERα which cause minimal activation of AF-1. These would retain their beneficial cardiovascular actions but not affect sexual effects or have any proliferative action on breast cancer cells. Deciphering the molecular mechanisms elicited by E2 treatment at the level of the megakaryocyte/platelet may open new perspectives in the field of thromboprotection.

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Authorship

Contribution: M.-C.V., M.-P.G., J.-F.A., and B.P. designed the study and wrote the manuscript; M.-C.V., M.-P.G., C.C., C.E.T., M.M., and N.S.L. performed research and collected and analyzed data; and M.-C.V., M.-P.G., F.L., P.S., M.S., J.-F.A., and B.P. critically reviewed the manuscript.

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Chronic estradiol treatment reduces platelet responses and protects mice from thromboembolism through the hematopoietic estrogen receptor α