Effects of Estrogen Replacement Therapies on Mouse Platelet Function and Glycoprotein VI Levels

Xing-Hong Leng, Wei Zhang, Bernhard Nieswandt, Paul F. Bray

Clinical trials have shown estrogen replacement therapy (ERT) is associated with adverse arterial vascular events. Arterial thrombosis is initiated by platelet activation, but the in vivo effects of estrogens on platelet function are not well understood. We used a murine model of menopause to examine 3 major ERT regimes and test the hypothesis that ERT affects the intrinsic platelet response to agonists. The 3 ERT regimes studied were: (1) oral conjugated equine estrogen (CEE), (2) oral 17-β estradiol (E2), and (3) subcutaneously implanted E2 (SQ E2). Paired ovariectomized littermates were treated with these regimes or placebo for 21 days. Two platelet agonists, thrombin and the GPVI-specific agonist collagen-related peptide (COL-RP), were used to evaluate platelet reactivity. Among the 3 regimens, (1) oral CEE enhanced platelet reactivity to COL-RP, (2) oral E2 had no effect on platelet reactivity to COL-RP and (3) SQ E2 increased platelet sensitivity to thrombin but lowered reactivity to COL-RP. Thus, the in vivo effects of estrogen on platelet function are agonist specific and dependent on hormone formulation and mode of delivery. The GPVI collagen receptor likely mediated some of these effects, because the ERT regimens induced changes in platelet surface GPVI expression corresponding to the observed platelet activation.

The results of randomized clinical trials of estrogen replacement therapy (ERT) in postmenopausal women have failed to demonstrate protection from cardiovascular events.1 ERT may adversely affect thrombosis, although the effects of ERT on platelet function are poorly understood and few studies have tested the effects of estrogens on intrinsic properties of platelets. The most commonly used ERT preparation in the United States is oral conjugated equine estrogen (CEE), which contains at least 10 estrogens, <10% of which is 17-β estradiol (E2).2 Considering the disappointing results of clinical trials with oral CEE, the systemic effects of oral and transdermal E2 have become increasingly relevant. To test the hypothesis that different ERT regimes alter platelet reactivity, 3 ERT regimes, oral CEE, oral E2, and subcutaneously (SQ) implanted E2 were tested in a murine model of menopause and the effects of these hormone treatments on platelet function were compared. Because of the increased appreciation of the role of the platelet collagen signaling receptor, GPVI, in coronary thrombosis,3 we used the GPVI-specific agonist collagen-related peptide (COL-RP), as well as thrombin, to evaluate platelet function in these mice. To our knowledge this is the first head-to-head comparison of different estrogen formulations and routes of administration on in vivo platelet function.

Materials and Methods

Animal Protocol

All animal studies were performed in accordance with institutional guidelines. C57BL/6 female mice, ages of 6 to 9 weeks, were bilaterally ovariectomized (OVX). OVX littermates were randomly assigned to either estrogen or placebo 3 d after the surgery. Endometrial thickness is an accepted biomarker of estrogen status,4 so our estrogen dosing was based on restoring the uterus size and appearance to a physiologic range, an effect also observed with human ERT. One hundred µg/kg of E2 (Sigma, St. Louis, Mo) or pulverized CEE (Premarin, Wyeth Ayerst, Pa) or the vehicle alone (1% carboxymethyl cellulose with 0.25% Tween 80) were administered by gavage daily. SQ E2 was administered as a 1-µg 21-day slow-release pellet (Innovative Research of America, Sarasota, Fla). Mice were euthanized after 21 days of ERT.

Assessment of Estrogen Activity

The biologic effect of estrogen was assessed by changes in mouse uter,5 because plasma E2 levels do not represent the biologic effect of estrogen activity in the oral ERT study and because the oral delivery follows an absorption metabolic decay curve. The doses used in this study produced an effect on the uterus similar to that observed with human ERT dosing. For completeness, plasma E2 was measured (Diagnostic Systems Laboratories) and was below the lower limit of detection (<1.5 pg/mL) after oral administration of CEE and E2 (probably attributable to the timing of plasma collection, which was ~18 hours after last gavage) and was 49.1±33.1 pg/mL after SQ administration (normal E2 is 31±13 to 58±27 pg/mL).6

Platelet Preparation and Analysis

Preparation of washed platelets, flow cytometric analysis of platelet size and fibrinogen binding, and platelet aggregation were performed as previously described.7 To maximize quality control, only 1 pair of mice (1 hormone-treated and 1 placebo-treated) were studied per day. COL-RP was synthesized and crosslinked with glutaraldehyde. Submaximal concentrations of COL-RP were used to assess platelet reactivity: 0.02 µg/mL for flow cytometry and 0.01 µg/mL for aggregation. Surface expression of platelet adhesive receptor glycoproteins (GPs) were quantified using PE-conjugated anti-GPIbα (Xia.G5-PE, Emfret Analytics, Würzburg, Germany), FITC-conjugated anti-GPIIb-IIIa (anti-CD41, BD Pharmingen), and anti-GPVI antibodies.8 Effects of ERT are presented as mean±SE percent change from the placebo value and analyzed with a 1-sample t test using StatView software (SAS Institute).
Results and Discussion

These littermate- and placebo-controlled studies were designed to test the in vivo effects of different clinically relevant estrogen preparations and routes of estrogen administration on intrinsic platelet function. Orally administered CEE and E2 restored the uteri to a normal physiologic size (online Table, available at http://circres.ahajournals.org), appearance and weight. CEE increased COL-RP–induced platelet fibrinogen binding (Figure 1A) and aggregation (Figure 1B), but oral E2 had no such effects (Figure 1). These results suggest that a non-E2 component of CEE may be responsible for the enhanced platelet reactivity to COL-RP. SQ E2 restored the uterus size to high-normal size (online Table) in the presence of physiologically normal levels of E2 (Methods), but caused a reduction in platelet reactivity to COL-RP. Tail bleeding times were only measured in the SQ study, where E2 treated mice showed a 344±98% mean increase in bleeding time compared with placebo (P=0.006), consistent with the reduced response to CRP after SQ E2. These differential effects of ERT on platelet function attributable to the route of estrogen administration are strikingly similar to the ERT effects on plasma triglycerides levels, which are increased by oral E2 and decreased by transdermal E2.9

In an effort to address the possible mechanisms by which estrogens might affect platelet function, we measured levels of adhesive membrane GPs. No difference between placebo and any estrogen therapy was observed for GPIbα and GPIIb-IIIa (integrin αIIbβ3). However, the 3 ERT regimens altered platelet surface GPVI levels in a pattern similar to COL-RP–induced platelet activation (Figure 2A). A strong correlation between GPVI expression and COL-RP–induced platelet fibrinogen binding (n=11 pairs). MFI indicates mean fluorescence intensity; probability values, treatment vs placebo.

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Figure 1. Effects of estrogen regimens on COL-RP–induced platelet fibrinogen binding (A) and aggregation (B). Pairs of OVX female littermates were treated with each estrogen or placebo for 21 days, and washed platelets were isolated and stimulated with the GPVI-specific agonist, COL-RP. Percent change from placebo values is shown for 4 to 14 estrogen/placebo pairs. Aggregation was not performed on the oral E2 study mice. Probability values indicate treatment vs placebo.

Figure 2. Effects of estrogen regimens on platelet adhesive receptor expression. A. Surface receptor levels were determined on resting platelets by flow cytometry using fluorescently-labeled antibodies. Percent change from placebo is shown for 3 to 8 estrogen/placebo pairs. No receptor level in any regimen was different from placebo (P>0.05) except those indicated. B, correlation between GPVI expression and COL-RP–induced platelet fibrinogen binding (n=11 pairs). MFI indicates mean fluorescence intensity; probability values, treatment vs placebo.

This is the first report of ERT regulating GPVI expression and the lack of an ERT effect on GPIbα or GPIIb-IIIa expression (Figure 2A) suggests hormone-specific effects on GPVI expression. The pivotal role of the platelet GPVI collagen receptor in platelet activation and thrombus formation has been demonstrated using selective agonists and GPVI-deficient platelets.8 Modest reductions in GPVI density (∼12% less than normal platelet levels) can have a major effect on cell adhesion under high shear conditions.10 Estrogens could act directly on megakaryocytes to regulate GPVI expression (eg, GPVI transcription or altering the expression of a metalloproteinase that induces platelet GPVI shedding). Because our assays assessed the in vivo effects of estrogens,
we cannot exclude an indirect effect whereby estrogen could induce nonmegakaryocyte cells to secrete a factor that could regulate platelet GPVI expression.

Additional platelet activation in vivo likely occurs after platelet adhesion, and we also examined the effect of thrombin on platelets from the ERT-treated mice. Neither oral regimen had a significant influence on fibrinogen binding (Figure 3), whereas SQ E2 increased platelet sensitivity to thrombin. Other investigators have also found that transdermal E2 was more active than oral E2 in increasing platelet activation to thrombin. Interestingly, the responses to COL-RP and thrombin were not concordant, indicating estrogens influence platelet function in an agonist specific fashion.

It is important to note that any mechanism whereby estrogen affects platelet function could be influenced by at least 3 variables: non-E2 estrogens in CEE, higher plasma levels of E2 after SQ E2, or the differences attributable to peaks and troughs of oral estrogens compared with the steady levels of SQ estrogens. In addition, because oral estrogen results in high portal vein estrogen levels, at least some of the estrogen effects could be mediated by first-pass metabolism in the liver and altered hepatic gene expression.

In summary, we have shown that in vivo estrogens affect platelet reactivity in an agonist-specific fashion and depend on the hormone formulation and mode of delivery. Altered GPVI expression likely mediated some of these effects. Cautiously extrapolating to postmenopausal women using ERT, these estrogen-induced platelet functional changes could contribute to cardiovascular complications. More human studies are needed to assess specific aspects of vascular complications of hormone therapy; specifically, does oral E2 lack the reported risks of CEE? And is there risks related to daily fluctuations of estrogen levels?

Acknowledgments

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References

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Key Words: estrogen | glycoprotein VI | hormone replacement therapy | platelets

Figure 3. Effects of estrogen regimens on thrombin-induced platelet fibrinogen binding. Thrombin (0.005U/mL) induced fibrinogen (FGN) binding to washed platelets is shown. Percent change from placebo is shown for 4 to 13 estrogen/placebo pairs. Probability values indicates treatment vs placebo.
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**Online Table.** Characteristics of ovariectomized mice receiving estrogen therapies.

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<tr>
<td>Platelet size</td>
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<td>+1.7 ± 1.4</td>
<td>.237</td>
</tr>
</tbody>
</table>

*Percent change from the placebo value.

† Uteri after oral ERT were in normal size range of the diestrum cycle; uteri after SQ E2 was in the normal size range of late metoestrum cycle.