Estrogenic regulation of tissue factor and tissue factor pathway inhibitor in platelets

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Jayachandran, Muthuvel, Antonio Sanzo, Whyte G. Owen, and Virginia M. Miller. Estrogenic regulation of tissue factor and tissue factor pathway inhibitor in platelets. Am J Physiol Heart Circ Physiol 289: H1908–H1916, 2005. First published June 17, 2005; doi:10.1152/ajpheart.01292.2004.—Oral estrogen treatment increases thrombotic risk. Tissue factor (TF), tissue factor pathway inhibitor (TFPI), and platelet interaction with leukocytes are important determinants of thrombogenesis. Therefore, the present study was designed to define and compare platelet TF and TFPI mRNA and adhesion protein expression in platelets derived from animals treated with different types of oral estrogens. Ovariectomized pigs were treated with 17β-estradiol (2 mg/day), conjugated equine estrogen (CEE; 0.625 mg/day), or raloxifene (60 mg/day) for 4 wk. Compared with intact animals, ovariectomy and treatment differentially affected populations of leukocytes: neutrophils decreased whereas lymphocytes increased significantly 4 wk after ovariectomy and with 17β-estradiol and CEE treatments; eosinophils increased only with 17β-estradiol treatment. Content of TF protein increased in platelets from 17β-estradiol- and raloxifene-treated pigs, whereas TF mRNA was detected only in platelets from 17β-estradiol- and CEE treated pigs. TFPI mRNA increased in platelets after ovariectomy and estrogen treatment. Only a trace of TFPI protein was detected, but a higher-molecular-mass protein was observed in all treatment groups. Expression of CD40 and CD40 ligand increased with ovariectomy and decreased with 17β-estradiol and CEE treatments more than with raloxifene. The ratio of activated to basal P-selectin expression decreased with ovariectomy and increased with raloxifene treatments. These results suggest that estrogenic formulations may affect individual thrombotic risk by different mechanisms that regulate TF and platelet-leukocytic interactions. These studies provide the rationale for evaluation of interactions among platelets and TF and TFPI expression on thrombin generation during estrogen treatment in humans.

conjugated equine estrogen; CD40; 17β-estradiol; leukocytes; raloxifene

EFFECTS OF ESTROGEN THERAPY on thrombosis are controversial. Although observational studies suggest that risk of cardiovascular disease decreases with estrogen treatment, prospective randomized studies report increases in arterial and venous thrombosis (21, 22, 24, 25, 49). Estrogen affects the coagulation system by several mechanisms including production of procoagulant factors (VII, X, XII, and XIII) in the liver and decreasing production of anticoagulant factors, e.g., protein S, antithrombin, and antifibrinolytic protein plasminogen-activator inhibitor type 1 (15, 19, 34, 35, 40, 41, 43, 56, 57).

Prothrombotic effects of estrogen therapy also may be due to changes in coagulation proteins produced in platelets. Platelets, which contribute to clinical syndromes like stroke, myocardial infarction, and acute limb ischemia, contain tissue factor (TF), the initiator of intravascular thrombin generation necessary for formation of fibrin clots (9, 14, 55, 61). TF is a 47-kDa glycoprotein that initiates the coagulation cascade through allosteric activation of plasma serine protease factor VIIa (FVIIa). The resulting TF-FVIIa complex activates coagulation factors IX and X needed for proteolytic generation of thrombin (11). This TF-mediated reaction is considered a major regulator of coagulation, hemostasis, and thrombogenesis (44, 46, 52, 53, 60). The enzymatic activity of TF-FVIIa is regulated by a tissue factor pathway inhibitor (TFPI). TFPI circulates in the blood with lipoproteins and is present in platelets. In addition, a large amount of TFPI is present in vascular endothelium (7). TFPI has three Kunitz-type inhibitor domains, and these domains inhibit factor Xa (FXa) and TF-FVIIa simultaneously (8). TF contributes to the pathogenesis of thrombosis and vascular and inflammatory disorders, whereas TFPI inhibits development of intimal hyperplasia, thrombotic occlusions in atherosclerotic vessels, unstable angina, and myocardial infarction (31). Therefore, the ratio between TF and TFPI expression and their activities are important determinants of hemostasis and thrombosis (10).

Activated platelets attach to the blood vessel wall and leukocytes (e.g., monocytes and neutrophils) through the surface expression of the platelet α-granular protein P-selectin and other membrane adhesion molecules [CD40 and CD40 ligand (CD40L)] expressed on surface membranes. Indeed, individual thrombotic propensity may be related to changes in populations of leukocytes, in particular lymphocytes (32). In addition, platelet-monocyte contact results in gene expression and de novo synthesis of TF (14). However, there is a paucity of information and conflicting results regarding the influence of estrogenic regulation of platelet membrane proteins, TF, and TFPI (3, 4, 58). Therefore, the present study was designed to define and compare how different types of clinically relevant estrogens affect TF and TFPI and expression of adhesion molecules in platelets.

MATERIALS AND METHODS

Antibodies and Chemicals

Anti-human TF monoclonal antibody and anti-human TFPI polyclonal antibody, recombinant TF, and TFPI protein were purchased from American Diagnostic (Greenwich, CT). Anti-human CD40 and CD40L rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tris, glycine, sodium orthovanadate, and lauryl sulfate (SDS) were purchased from Sigma (St. Louis, Missouri).

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MO). Human anti-rabbit polyclonal P-selectin antibody and annexin V-FITC were obtained from BD Biosciences (San Francisco, CA). Hanks’ balanced salt powder (without NaHCO₃), α-glucose, and bovine serum albumin (fatty acid free) were obtained from Gibco-Bio-Technology (Rockville, MD). Firefly (Pho tinus pyralis) luciferase and luciferin were purchased from Roche Diagnostics (Indianapolis, IN). TRIZol reagent was purchased from Invitrogen-Life Technologies (Carlsbad, CA). Ambion’s DNA-free Kit was purchased from Ambion (Austin, TX). Primers were synthesized in the Molecular Biology Core, Mayo Clinic College of Medicine. All other reagents and solvents used in this study were of analytical/reagent grade.

Animals and Experimental Design

Sexually mature female pigs (4 crossbred: Yorkshire, Hampshire, Duroc, and Landrace; 7 mo of age) were used in this study. External genitalia of pigs this age show enlargement and discharge associated with estrus. Ovaries were removed laparoscopically under anesthesia (2 mg/kg xylazine, 0.44 mg/kg glycopyrrolate, and 5 mg/kg telazol). Under anesthesia and before ovarioectomy (Pre-OVX) and 4 wk after ovarioectomy (4-wk OVX), blood samples were collected from femoral arteries of all pigs. At 4 wk after ovarioectomy, pigs were randomly assigned to one of four treatment groups: untreated (8-wk OVX), oral 17β-estradiol (2 mg/day), oral conjugated equine estrogen (CEE; 2 mg/kg xylazine, 0.44 mg/kg glycopyrrolate, and 5 mg/kg telazol). At 4 wk after ovarioectomy (untreated for 8 wk or untreated 4-wk OVX + 4-wk hormone treatment), pigs were anesthetized by intramuscular injection of xylazine (2 mg/kg), glycopyrrolate (0.44 mg/kg), telazol (5 mg/kg), and ketamine (4.5 mg/kg). Blood was collected from the carotid artery into 50-ml anticoagulated [5 ml anticoagulant citrate dextrose solution USP (ACD) Formula A: Baxter Healthcare] polypropylene centrifuge tubes for determination of TF and TFPI and into EDTA-anticoagulated tubes for determination of blood cell counts. Total and differential cells were counted with a three-part differential Coulter counter (model HL750, Coulter, Miami, FL) in the Mayo Clinic Hematology Laboratory. The Institutional Animal Care and Use Committee of Mayo Clinic approved this study. Platelets from these animals were used in another study, and uteri from these animals showed changes consistent with efficacy of treatment (26).

Flow Cytometry Analysis

P-selectin-positive platelets. Blood collected in citrate tubes was allowed to stand for 15–20 min to separate platelet-rich plasma (PRP). Twenty microliters of citrated blood PRP was diluted into 2 ml (diluted 1:100) of Hanks’ balanced salts solution, without NaHCO₃, buffered (pH 7.4) with 20 mM HEPES, supplemented with 1 mg/ml bovine serum albumin, 0.5% glucose, 1 μM tick anticoagulant peptide, and 25 nM hirudin (H/H⁺ medium). Diluted samples were centrifuged at 1,100 g for 10 min to separate fibrinogen and other plasma proteins and to wash the platelets. Washed platelets were reconstituted in 1 ml of H/H⁺ medium. One hundred microliters of reconstituted platelets was pipetted into different flow cytometric tubes for activation with different agonists. Both control (nonactivated in the absence of agonist) and activated (exposed to 50 nM pig thrombin, 10 μM ADP, 6 μg collagen, and 5 μM calcium ionophore A-23187 for 10 min) platelets were incubated with rabbit polyclonal anti-human P-selectin antibody (mouse monoclonal anti-human P-selectin antibody did not work in porcine platelets) for 30 min. Platelets incubated with the P-selectin antibody were fixed with 1% formaldehyde for 30 min at room temperature and centrifuged at 2,300 g for 10 min to remove unbound antibody by discarding the supernatant. Fixed platelets were incubated with secondary goat anti-rabbit FITC antibody in the dark for 30 min. All incubations were carried out at room temperature. Two milliliters of dilution buffer (1× PBS) was added to each tube, followed by centrifugation at 22°C and 2,300 g for 15 min. Supernatants were discarded, and pellets were resuspended with 1 ml of 1× PBS. Platelets in the resuspended samples were analyzed by flow cytometry (FACSCalibur; Becton Dickinson) within 2 h. Log forward scatter (for size characteristic) and log side scatter (for granularity) were used to identify platelets. The platelet cloud was gated electronically to exclude red and white blood cells (27).

Annexin V-positive platelets. To determine annexin V-positive platelets, PRP was prepared, diluted, washed, and activated for determination of P-selectin. Nonactivated and activated platelets were incubated with annexin V-FITC in the dark for 30 min, fixed with 1% formaldehyde for 30 min at room temperature, and centrifuged at 2,300 g for 10 min. The supernatant was discarded. Fixed platelets were diluted, and annexin V-positive platelets were measured by flow cytometry similar to P-selectin.

Platelet Preparation for Immunoblotting and RNA Isolation

Platelets were separated from the ACD-anticoagulated whole blood and washed as described previously (25, 27, 28). The purity of washed platelets was validated with a three-part differential Coulter counter (model HL750, Coulter) in the Mayo Clinic Hematology Laboratory. Washed platelets were resuspended in lysis buffer (1% SDS, 1 mM sodium orthovanadate, 10 mM Tris·HCl pH 7.4) and stored at −70°C for immunoblot analysis, and platelets were resuspended in TRIZol for RNA isolation.

RT-PCR for TF and TFPI mRNA Determination

RNA was extracted from porcine platelets with TRIZol reagent according to the manufacturer’s protocol (Invitrogen-Life Technologies) (23). DNA contamination in isolated total RNA was eliminated by DNase treatment with an Ambion DNA-free kit. Total RNA of all samples was quantified by measuring optical density at 260 nm, and purity was determined from the 260 nm-to-280 nm ratio. Isolated RNA was then converted to first-strand cDNA with Super-Script II RT (Invitrogen) in the presence of random primers (25–250 ng). platelet RNA (5 μg), dNTP mix (10 mM), sterile water, 5× first-strand buffer (4 μl), and 0.1 M DTT (2 μl). The synthesized cDNA was removed from RNA complementary with Escherichia coli RNase H. Two microliters of cDNA was used for amplification of TF, TFPI, and β-actin. The following primers were used for mRNA determination in porcine platelets: TF: forward primer 5′-AAC-AAA-CCACTCAACCGACAC-GC3′, reverse primer 5′-GTCG-AGGAAATGTTGCGGATCTCCTGAC3′; TFPI: forward primer 5′-GTGCGAAGAATTTATATATGGG-3′, reverse primer 5′-CCACT-GTACCTAAAATGGGGC3′; β-actin: forward primer 5′-TGGCATT-GTCAATTGACAGCATCG-3′, reverse primer 5′-CGCATTTCATGATCAGGTTG-3′.

TF mRNA determination. Each PCR cycle (total 30 cycles) was performed for 30 s to denature at 94°C, followed by 15 s for annealing at 65°C and extension at 72°C for 1 min 15 s.

TFPI and β-actin mRNA determination. Each PCR cycle was performed for 45 s to denature at 94°C, followed by 30 s for annealing at 55°C and extension at 72°C for 1 min 30 s. A total of 35 PCR cycles were performed for porcine TFPI and β-actin mRNA.

Ten microliters of PCR products were mixed with two microliters of 6× loading dye and analyzed for expression of TF, TFPI, and β-actin mRNA by electrophoresis using 1.5% agarose gel containing ethidium bromide. Expression of TF and TFPI mRNA was normalized to β-actin expression.

Immunoblotting of TF, TFPI, CD40, and CD40L

Platelet lysate was prepared from washed platelets as described previously (25, 27, 28). Total protein in platelet lysate was determined with a biocinchoninic acid protein assay kit (Pierce, Rockford, IL). For
Western blotting, platelet lysate was mixed with an equal volume of 2X electrophoresis sample buffer (1X = 125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol) and heated at 95°C for 5 min. Equal amounts of each sample (100 μg protein) were loaded in each lane and separated by SDS-PAGE using 7.5% and 12% SDS polyacrylamide gel (Bio-Rad) for TF, TFPI, CD40, CD40L, and β-actin. After electrophoretic separation, proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) for colorimetric detection and/or Hybond-C extra nitrocellulose membrane for enhanced chemiluminescence (ECL) detection with Trans-Blot SD semidry transfer cell (Bio-Rad). An equal amount of protein transfer and load was verified from β-actin expression. Membranes containing transferred protein were blocked with 5% nonfat dry milk (Bio-Rad) dissolved in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) for 1 h and were incubated at 4°C with the following specific primary antibodies with appropriate dilution in transfer buffer overnight.

Membranes incubated with rabbit anti-human polyclonal TF IgG (5–10 μg/ml), mouse anti-human monoclonal TF IgG1 (5–10 μg/ml), rabbit anti-human TFPI polyclonal IgG (5–10 μg/ml), anti-human TF protein was present in platelet lysate of all groups of pigs and was detected similarly with polyclonal or monoclonal (not shown) antibodies, with Western blotting (shown), or with an enhanced chemiluminescence method (not shown). However, TF mRNA was not expressed before or after ovariectomy, and only a trace of TF mRNA was expressed in platelets from 17β-estradiol- and conjugated equine estrogen (CEE)-treated pigs. Membranes incubated with rabbit anti-human polyclonal TF IgG (5–10 μg/ml), mouse anti-human monoclonal TF IgG1 (5–10 μg/ml), rabbit anti-human TFPI polyclonal IgG (5–10 μg/ml), anti-human TF mRNA expression did not change with ovariectomy or hormone treatment. Similar results were obtained in 4 or 5 experiments with platelets from 5 different pigs in each treatment group. Recombinant TF protein was used as positive control for TF protein detection. As positive control for TF mRNA expression, mRNA was extracted from porcine endothelial cells treated with interleukin-1α (10 ng/ml). OVX, ovariectomized; 17β-E2, 17β-estradiol; Ralox, raloxifene.

Table 1. Total white blood cells, differential leukocytes, and platelets in blood from gonadally intact, ovariectomized, and hormone-treated pigs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pre-OVX (n = 23 or 24)</th>
<th>4-wk OVX (n = 20–24)</th>
<th>8-wk OVX (n = 6)</th>
<th>17β-Estradiol (n = 6)</th>
<th>CEE (n = 6)</th>
<th>Raloxifene (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC × 10⁹/l</td>
<td>16.58 ± 0.85</td>
<td>13.32 ± 0.62*</td>
<td>13.02 ± 1.06</td>
<td>10.88 ± 0.84*</td>
<td>12.13 ± 0.24*</td>
<td>12.62 ± 1.22*</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>51.65 ± 2.50</td>
<td>32.35 ± 2.28*</td>
<td>40.83 ± 4.70</td>
<td>33.17 ± 2.71*</td>
<td>37.83 ± 4.70*</td>
<td>41.33 ± 4.46</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>42.74 ± 2.32</td>
<td>61.96 ± 2.59*</td>
<td>51.83 ± 4.87</td>
<td>60.33 ± 3.04*</td>
<td>58.17 ± 4.69*</td>
<td>52.17 ± 4.05</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td>5.04 ± 0.69</td>
<td>4.57 ± 0.52</td>
<td>5.33 ± 1.36</td>
<td>4.83 ± 0.79</td>
<td>2.33 ± 0.61</td>
<td>5.33 ± 1.43</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>0.09 ± 0.06</td>
<td>0.45 ± 0.17</td>
<td>0.33 ± 0.21</td>
<td>1.17 ± 0.65*</td>
<td>0.50 ± 0.22</td>
<td>0.33 ± 0.21</td>
</tr>
<tr>
<td>Basophils, %</td>
<td>0.65 ± 0.15</td>
<td>1.30 ± 0.30</td>
<td>0.50 ± 0.34</td>
<td>1.00 ± 0.45</td>
<td>0.83 ± 0.31</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE. OVX, ovariectomy; CEE, conjugated equine estrogen; WBC, white blood cells. *P < 0.05, significant differences between Pre-OVX and other respective groups.
CD40 (1:300 dilution), CD40L (1:300 dilution), and β-actin mouse monoclonal IgG1 (1:1,000 dilution) primary antibodies were washed twice (5 min each) in 1× Tris-buffered saline (Bio-Rad). Membranes treated with primary monoclonal antibodies were washed and treated with secondary goat anti-mouse IgG-HRP-conjugated antibody (50 μl in 10 ml of 1× Tris buffered saline), and membranes treated with primary polyclonal antibodies were treated with secondary goat anti-rabbit IgG-HRP-conjugated antibody (50 ml in 10 ml of 1× Tris buffered saline) for 2 h at room temperature. Membranes incubated with the secondary antibody were washed twice (5 min each) in 1× Tris-buffered saline (Bio-Rad) and treated Opti-4CN (Bio-Rad) substrate (freshly prepared according to the manufacturer’s instructions) for 2–5 min for colorimetric determination of specific protein expression. Membranes treated with colorimetric substrate showed specific protein expression and were washed two or three times (5–10 min each) with reverse-osmosis water to clear off the background. Intensity of specific proteins was determined with UN-SCAN-IT positive segment analysis.

The same primary monoclonal and polyclonal antibodies (less antibody concentration than colorimetric) also were used for ECL detection of TF, TFPI, CD40, CD40L, and β-actin in platelet lysates as described previously (27). Peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies (1:5,000 or 1:10,000 dilutions) were used. Similar expression of proteins was obtained with colorimetric and ECL methods.

**Statistical Analysis**

Data are presented as means ± SE. Data were analyzed by one-way ANOVA followed by Dunnett’s test to identify differences among groups. Student’s t-test was also used to identify significant differences between two groups. Statistical significance was accepted as P < 0.05.

**RESULTS**

Total numbers of white blood cells decreased after ovariectomy and were not increased by estrogen treatments. However, not all leukocytes were affected the same by ovariectomy and treatments. Neutrophils decreased significantly with ovariectomy and hormone treatment, whereas lymphocytes increased significantly with ovariectomy, 17β-estradiol, and CEE treatments (Table 1). Eosinophils increased significantly with 17β-estradiol treatment. The cytokine TNF-α decreased significantly with ovariectomy in serum and adipose tissue. Neutrophils decreased significantly with ovariectomy and hormone treatments.

**Fig. 2.** Representative membranes and gels of tissue factor pathway inhibitor (TFPI) protein (A) and mRNA (B) and β-actin mRNA (C) expression in platelet lysate from gonadally intact, OVX, and hormone-treated OVX pigs. TFPI mRNA was expressed in platelets from all groups of pigs, whereas only a trace of TFPI protein was expressed in platelets from CEE- and raloxifene-treated pigs. However, anti-human TFPI polyclonal antibody identified significant amounts of a higher-molecular-mass protein in all groups (?). The higher-molecular-mass protein may be a complex of TFPI with TF, TF-FVIIa, or FXa, or a variant of TFPI. Detection of this protein is not related to secondary antibody reaction, because membranes treated only with secondary antibody did not show any protein expression (data not shown). Recombinant TFPI protein was used as a positive control for TFPI protein detection. Expression of TFPI mRNA (B) increased with ovariectomy and hormone treatments. β-Actin mRNA expression did not change with ovariectomy or hormone treatment. Similar results were obtained in 4 or 5 experiments with platelets from 5 different pigs in each treatment group. Recombinant TFPI protein was used as positive control for detection of TFPI protein.
estradiol treatment, but monocytes and basophils were not changed after ovariectomy or with any treatment (Table 1).

The number of platelets and mean platelet volume did not change with ovariectomy and hormone treatment, whereas the percentage of reticulated platelets increased at 4-wk OVX and with 17β-estradiol treatment (26). Red blood cells, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentrations did not change with ovariectomy or treatment (data not shown).

Expression of TF protein was greater in platelet lysate from pigs treated with 17β-estradiol and raloxifene compared with 8-wk OVX (Fig. 1, A and B). Similar changes were observed with either the polyclonal or monoclonal antibodies for TF and with evaluation by either colorimetric or ECL detection methods. Trace amounts of TF mRNA were detected only in platelets from 17β-estradiol- and CEE-treated pigs (Fig. 1C). Expression of β-actin, used as an internal control for RT-PCR, did not show variability among treatment groups (Fig. 1D).

Unlike TF, only a trace of TFPI protein was detected in platelet lysate from hormone-treated OVX pigs (Fig. 2A). However, the anti-human TFPI polyclonal antibody detected a 38-kDa TFPI protein and also another higher-molecular-mass (~76-kDa) protein in the platelet lysate (Fig. 2A). This higher-molecular-mass protein was not related to a secondary antibody reaction because the membrane, exposed only to a secondary antibody, did not show any protein expression (data not shown).

Expression of TFPI mRNA increased after OVX and estrogen treatments (Fig. 2B). There was no change in control (β-actin) mRNA after OVX or estrogen treatments (Fig. 2C).

Expression of the adhesion receptor CD40 (Fig. 3, A and B) increased 2.5-fold after ovariectomy. CD40L (Fig. 3, A and C) increased after ovariectomy by 0.5-fold over preovariectomy expression. Treatment with 17β-estradiol consistently reduced expression of CD40L to levels observed before ovariectomy.

Membrane expression of platelet α-granule P-selectin was determined with and without agonist activation. Pig thrombin (50 nM), ADP (10 μM), and calcium ionophore (A-23187: 5 μM), but not collagen (6 μg/ml), stimulated P-selectin expression (Fig. 4A). Membrane expression of phosphatidylserine (PS) was measured by annexin V binding. PS expression was induced with collagen (6 μg/ml) and calcium ionophore (5 μM), but not with thrombin (50 nM) or ADP (10 μM) (Fig. 4B). Similar patterns of agonist-induced activation were observed in platelets from all treatment groups.

Although the selectivity of agonist-induced expression (maximal %) of P-selectin did not change with ovariectomy or treatment, the ratio between basal and activated expression of

\[ \text{Relative Intensity Ratio} = \frac{\text{Activated expression}}{\text{Basal expression}} \]

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and probably reflect differences in duration and mode of estrogen treatment (12, 16, 33, 38, 39, 42, 48). The number of lymphocytes correlates with size of thrombus formation to vascular injury; therefore, increased lymphocytes and decreased neutrophils after ovariectomy and estrogen treatment in this study may reflect increased individual thrombogenic propensity (32).

Although total numbers of blood platelets did not change with ovariectomy and hormone treatment, reticulated platelets increased after ovariectomy and 17β-estradiol treatment, suggesting increased production of platelets from bone marrow megakaryocytes or increased mRNA stability in circulating platelets (26). As platelets do not have nuclei, the mRNA retained in circulating platelets is derived from genomic regulation of transcription in bone marrow megakaryocytes.

Increased expression of TF protein was observed with 17β-estradiol and raloxifene treatments. The fact that the antibodies also detected recombinant TF at the same molecular mass suggests that the binding was specific for TF. However, with increased expression of TF protein, TF mRNA was not detected. Resting human platelets contain TF protein and significant amounts of TF mRNA, which might potentially contribute in protein synthesis (9). The half-life of TF mRNA in human platelets varies from ~1 to 4 h (6, 17). However, the half-life of TF mRNA in porcine platelets is not known. Therefore, from this study, it is not possible to rule out a genomic effect of the estrogen treatments on TF transcription in the megakaryocytes. In addition to transcriptional and translational regulation of TF within platelets, functionally active TF might be derived from leukocytes through particle transfer or be taken up from plasma by the platelets (47). It is unlikely that the TF measured in the lysate is derived from leukocyte contaminants of the preparation, as differential Coulter count yielded <0.1–0 leukocyte contamination in the washed platelet preparation. An increase of TF in plasma and monocytes has been reported with some clinical conditions such as acute coronary syndrome and sepsis (13, 36, 54). Further study is needed to determine the source of TF in platelets. However, platelet deposition and TF staining are higher in lipid-rich plaques, indicating that TF, perhaps of platelet origin, may be an important determinant of the thrombogenicity of atheromatous lesions (60).

TFPI is a potent endogenous early inhibitor of TF-dependent procoagulant activity (30, 45). Changes in TFPI mRNA did not correspond with increases in protein of the exact molecular mass for TFPI, suggesting that the mRNA may not be translated. However, expression of a higher-molecular-mass protein may reflect TFPI complexed to other proteins such as TF or FXa or may represent a variant of TFPI. Until a monoclonal antibody is available against porcine TFPI, further study is needed to determine the nature of this higher-molecular-mass protein detected by the polyclonal antibody. Although the antibodies were directed against human TFPI, it is unlikely that this higher-molecular-mass protein represents only nonspecific binding as the antibody only detected in lysate from pig endothelial cells a single 38-kDa protein corresponding to human recombinant TFPI (unpublished observation).

Estrogen treatment reduces TFPI secretion from cultured endothelial cells, and oral contraceptive use and estrogen treatment reduce plasma circulating TFPI antigen and activity in women (5, 18, 37). However, it is not known whether P-selectin tended to decrease with ovariectomy and increased significantly only with raloxifene treatment (Fig. 5). Expression of P-selectin in response to thrombin (Fig. 5A) and ADP (Fig. 5B) showed the same pattern. Neither basal nor agonist (thrombin and collagen)-activated PS (annexin V binding) expression was altered by ovariectomy or hormone treatment (data not shown).

**DISCUSSION**

The results of this study demonstrate that estrogenic treatments differentially regulate circulating leukocytes and expression of TF and TFPI and membrane expression of P-selectin in porcine platelets. Neutrophils decreased, whereas lymphocytes increased, with ovariectomy. Because these changes were not restored by estrogen treatment, ovarian hormones other than estrogen regulate leukocyte production. The effects of estrogen on total and differential leukocytes show species variability and probably reflect differences in duration and mode of

![Diagram](http://ajpheart.physiology.org/)

**Fig. 4.** Agonist-activated expression of P-selectin (A) and annexin V (B) on platelets from gonadally intact, sexually mature female pigs. Basal P-selectin (<10%) was obtained without centrifuged and washed platelets from gonadally intact pigs (data not shown). Centrifugation and washing activates platelets to express P-selectin (higher than basal level) in porcine platelets. Annexin V was not changed with centrifugation and washing. Data (n = 5–8) are shown as means ± SE. *Statistical difference between nonactivated and agonist-activated platelets (P < 0.05). Collagen, which did not activate α-granule P-selectin release, activated membrane phosphatidylserine (PS) translocation from inner to outer membrane. Thrombin (IIa) and ADP did not activate membrane PS translocation from inner membrane to outer membrane in pigs. Increases in the number of platelets to >75,000 platelets/activation did not change the results, suggesting that these agonists (thrombin and ADP) activate porcine α-granules by different mechanisms rather than membrane PS.

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plasma TFPI is taken up to form complexes with TF-FVIIa or FXa within platelets. Such complexes might induce signaling for TFPI transcription in megakaryocytes during platelet formation. A reduction of TFPI by 50% has been shown to cause an increase of TF-FVIIa catalytic activity (1).

Expression of the proinflammatory and procoagulant protein CD40L and its receptor CD40 increased in platelets after ovariectomy, an effect that tended to be reversed with natural estrogen (17βestradiol and CEE) more than with the synthetic compound raloxifene. Platelets express CD40L within seconds of activation in vitro and in the process of thrombus formation in vivo (20). Basic platelet activation induces CD40L expression on membrane surface and release of soluble mediators from intracellular granules (20). CD40L expression also induces expression of chemokines, TF, and adhesion molecules in human vascular endothelial cells (51). CD40-CD40L interactions participate in proinflammatory processes in several cell types (50). Therefore, increased expression of CD40L and receptor CD40 in platelets after depletion of ovarian hormones may indicate increased inflammatory reactions. If similar regulatory pathways exist in humans, they could contribute to an increased rate of atherosclerosis in women after menopause.

P-selectin is a cellular adhesion molecule released from α-granules of activated platelets. Platelet membrane-bound P-selectin interacts with P-selectin glycoprotein ligand-1 or glycoprotein Ib or sialyl Lewis X carbohydrate expressed on leukocytes, platelets, macrophages, and endothelial cells. Although platelet content of P-selectin did not vary with ovariectomy in sexually mature pigs (28), P-selectin expression tended to increase with ovariectomy and decrease with hormone treatment, indicating changes in activation of the platelets. These results are consistent with observations in humans, in whom estradiol injection reduces plasma P-selectin (29). P-selectin expression may be regulated by estrogen and progesterone and may depend on the formulation of treatment products. Estradiol and combined hormone therapy (2 mg estradiol and 0.5 mg/day trimestogeston or 10 mg/day dydrogesterone) increased the number of circulating activated P-selectin and glycoprotein 53-positive platelets in postmenopausal women, whereas transdermal 17β-estradiol (50 μg/24 h) combined with medroxyprogesterone acetate (10 mg) and oral 17β-estradiol (2 mg) combined with norethisterone acetate (1 mg) reduced platelet activation in women (2, 59).

In conclusion, ovariectomy decreased neutrophils but increased lymphocytes; these changes were not restored by oral estrogen treatment. TF protein is present in porcine platelets and increased with 17β-estradiol and raloxifene treatments. In contrast, TFPI protein was detected in trace amounts in platelets from estrogen-treated ovariectomized pigs, but TFPI protein complexes and TFPI mRNA increased after ovariectomy and estrogen treatments. Expression of CD40L and CD40 increased in platelets after ovariectomy and decreased with
17β-estradiol. Changes in content, production, and complexing of TF with TFPI and changes in CD40, CD40L, and P-selectin membrane protein may affect platelet-lymphocyte interaction contributing to thrombosis. Differences in efficacy among treatments most likely are due to efficacy of ligand-receptor interactions in megakaryocytes and to differences in the biological half-life of, for example, 17β-estradiol and raloxifene. These observations provide the basis for the design of studies to examine how TF, TFPI, and platelet-lymphocyte complexes affect thrombotic propensity in menopausal women taking hormone therapy and whether these changes in platelet characteristics alone are sufficient to increase thrombotic risk for an individual.

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