Effect of 17β-Estradiol on Inhibition of Platelet Aggregation In Vitro Is Mediated by an Increase in NO Synthesis

Yukiko Nakano, Tetsuya Oshima, Hideo Matsuura, Goro Kajiyama, Masayuki Kambe

Abstract—The low prevalence of coronary heart disease in premenopausal women and its increase after menopause are well established. Although estrogen is thought to play a role in protecting the vasculature, the mechanism has not been fully clarified. The contribution of platelets to atherosclerotic cardiovascular diseases is well recognized. The present study focused on the still-controversial effect of estrogen on platelet function. We investigated the in vitro effects of estrogen on human platelets, including their aggregation, Ca2+ metabolism, the synthesis of cyclic nucleotides, and NO (nitrite/nitrate) synthesis after stimulation with thrombin or ADP. Pretreatment of platelets with 17β-estradiol reduced the platelet aggregation induced by thrombin or ADP, whereas 17α-estradiol had no effect. 17β-Estradiol accelerated the recovery of [Ca2+], after the agonist-induced peak and reduced the area under the curve of accumulated platelet [Ca2+], but did not alter the baseline [Ca2+]. Ca2+ influx induced by thrombin or ADP, the release of Ca2+ from internal stores, or the size of internal Cu2+ stores. Pretreatment of platelets with 17β-estradiol had no effect on the intracellular concentration of cAMP but increased that of cGMP in agonist-stimulated platelets. Additionally, 17β-estradiol increased the platelet concentration of nitrite/nitrate in a dose-dependent manner. These effects of 17β-estradiol on platelet aggregation, Ca2+ metabolism, and NO synthesis were abolished by exposure to Nω-monomethyl-L-arginine, an NO synthesis inhibitor. These results suggest that 17β-estradiol plays an important role in inhibiting platelet aggregation by promoting Ca2+ extrusion or reuptake activity that is dependent on the production of cGMP by increasing NO synthesis. (Arterioscler Thromb Vasc Biol. 1998;18:961-967.)

Key Words: 17β-estradiol ■ platelet aggregation ■ c GMP ■ nitric oxide ■ intracellular Ca2+

Estrogen is important in preventing atherosclerotic vascular diseases. For example, after menopause, women become as vulnerable to death due to cardiovascular disease as are men.1 Estrogen replacement therapy has been shown to reduce the risk of coronary atherosclerosis in postmenopausal women.2–4 Several mechanisms are postulated for this antiatherosclerotic effect; among them are the improvement of the lipid profile5,6 and a direct vasodilator effect of estrogen.7 Concerning the latter, in vitro studies have reported that human endothelial NO synthesis can be regulated by estrogen8–10 and that estrogen can inhibit the contraction of coronary smooth muscle mainly by the inhibition of Ca2+ influx11. Paradoxically, epidemiological investigations have shown that women who take oral contraceptives are at an increased risk for thromboembolic events, such as deep-vein thrombosis of the lower limbs or pulmonary embolism.12,13 The aggregating and secreting responses of platelets are intimately involved in the development of atherosclerotic plaque and the thromboembolic complications that result from plaque rupture.14,15 However, the effect of estrogen on platelet function remains controversial.16–22

Accordingly, the main purposes of the present study were to examine the effects of estrogen on platelet function and to clarify mechanism of the phenomenon. We investigated the effects of estrogen on the aggregation of human platelets stimulated in vitro by thrombin, a strong agonist, or by ADP, a weak physiological agonist. We also attempted to determine the effects of estrogen on the metabolism of such intracellular second messengers as Ca2+ and cyclic nucleotides in platelets. We analyzed the concentration of NO and the effects of inhibition of NO synthesis by Nω-monomethyl-L-arginine (L-NMMA) on platelets pretreated with estrogen to determine whether this phenomenon could be related to NO synthesis, in that estrogen intimately linked to NO synthesis in several other cell types and intrinsic NO synthesis have been demonstrated in human platelets.

Methods

Materials

The ovarian steroids 17α-estradiol and 17β-estradiol (Sigma Chemical Co) were dissolved in 95% ethanol and diluted 100-fold to obtain a final concentration of 0.95% each. This concentration of ethanol was determined to have no affect on [Ca2+], or cyclic nucleotides in any experiments. Thrombin and ADP (Sigma) were dissolved in deionized water; ionomycin (Sigma) was dissolved in DMSO; Isobutyl methylxanthine (Sigma) was dissolved in DMSO; and L-NMMA (Sigma) was dissolved in deionized water. Fura 2 acetoxyethyl ester (fura 2-AM) (Molecular Probes) was dissolved in DMSO.

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Preparation of Platelets
Platelets were obtained from 37 healthy Japanese volunteers (14 men, 15 premenopausal women, and 8 postmenopausal women; mean age ± SD, 38 ± 16 years). None of the subjects had taken any medication for at least 2 weeks before the study. All gave their informed consent for participation in the study. The premenopausal women were within 1 week of onset of a menstrual period, a time of low levels of circulating plasma estradiol and progesterone. Their mean plasma concentration of 17β-estradiol was 35.6 ± 5.6 pg/mL, and progesterone was 0.46 ± 0.06 ng/mL. In the postmenopausal women, the plasma concentration of 17β-estradiol was 13.4 ± 3.5 pg/mL. Plasma hormone levels were measured by use of an enzyme immunoassay kit (Boehringer Mannheim). Venous blood was drawn from each subject into 1/10 volume of 3.8% sodium citrate by use of the two-syringe technique. Blood samples were centrifuged at 800 g for 5 minutes at room temperature to obtain platelet-rich plasma, which was gel filtered through a Sepharose 2B-CL column (Pharmacia LKB Biotechnology) that had been equilibrated with a medium that contained (in mmol/L) NaCl 145, KCl 5, MgSO4 1, HEPES 10, and glucose 5 (pH 7.4).

Measurement of Cytosolic Concentration of Free Ca2+

For the measurement of [Ca2+]i, the washed platelet solution was adjusted to a concentration of 10^8 cells/mL and incubated for 30 minutes at 37°C with fura 2-AM 2 μmol/L together with 0.02% Pluronic F127 (Molecular Probes). The fura 2–loaded platelet suspension was incubated for 20 minutes at 37°C with 17α-estradiol or 17β-estradiol, each at a final concentration of 10^{-9} to 10^{-4} mol/L, or with ethanol. This step was followed by a 10-minute incubation with L-NMMA 1 mmol/L or vehicle. The platelet suspension was resuspended with the extracellular fura 2-AM. Next, the suspension was adjusted to a concentration of 10^9 platelets per mL, and CaCl2 was added at a final concentration of 1 mmol/L. An aliquot of the cell suspension was stirred in a quartz cuvette at 37°C, and fluorescence was monitored with a spectrofluorometer (DM3000CM, SPEX Industries). The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm. [Ca2+]i was calculated by use of the general formula described by Grynkiewicz et al. as follows: [Ca2+]i = Kd ([R - Rmin]/[Rmax - R]) x (Sf/Sp), where Kd is 224 mmol/L for fura 2. R is the ratio of fluorescence at excitation wavelengths of 340 and 380 nm in an intact-cell suspension, Rmax is the ratio of absorbance of Ca2+-bound dye at 340 nm to that at 380 nm, Rmin is the absorbance ratio (340 nm/380 nm) of Ca2+-free dye, and Sf/Sp is the fluorescence ratio of Ca2+-bound dye to Ca2+-free dye at 380 nm. The intracellular concentration of fura 2 was determined by use of an in vitro fura 2 calibration curve prepared by measuring the fluorescence of known concentrations of fura 2. We measured the basal [Ca2+]i, thrombin-activated (final concentration, 0.1 U/mL), and ADP-evoked (final concentration, 40 μmol/L) changes in [Ca2+]i in the presence or absence of extracellular Ca2+. We also measured the increase in [Ca2+]i in response to 5 μmol/L ionomycin in the absence of extracellular Ca2+ as an index of intracellular Ca2+ discharge capacity. To measure the response of [Ca2+]i to agonists in the absence of extracellular Ca2+, after the fluorescence in the resting state had been recorded, EGTA 10 mmol/L was added to the buffer containing Ca2+ 1 mmol/L at pH 7.4. To estimate the recovery of [Ca2+]i, the Ca2+ transient was measured at 5-second intervals for 30 seconds after the peak response to thrombin 1.0 U/mL in the absence of extracellular Ca2+. The slope of the line and the area under the plotted line were calculated to determine Ca2+ extrusion and reuptake. Fluorescence was corrected for extracellular dye leakage with EGTA and for autofluorescence by subtracting the fluorescence of unloaded platelets.

Assay of Cyclic Nucleotides and Determination of Intraplatelet cAMP and cGMP
Gel-filtered platelets, 10^7/mL, were diluted with HEPES buffer that contained 1 mmol/L CaCl2. Platelets sampled from 12 subjects were preincubated for 20 minutes at 37°C with 17α-estradiol, 17β-

### Table 1. Concentration of 17β-Estradiol in Male Platelets Preincubated With 17β-Estradiol

<table>
<thead>
<tr>
<th>Concentration of intraplatelet 17β-estradiol, pg/mL</th>
<th>0</th>
<th>10^{-8}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
<th>10^{-5}</th>
<th>10^{-4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubated with 17β-estradiol, mol/L</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

### Table 2. Effect of 17β-Estradiol on Platelet Aggregation

<table>
<thead>
<tr>
<th>17β-Estradiol Pretreatment</th>
<th>Control</th>
<th>10^{-8} mol/L</th>
<th>10^{-7} mol/L</th>
<th>10^{-6} mol/L</th>
<th>10^{-5} mol/L</th>
<th>10^{-4} mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum % aggregation (area) to agonists</td>
<td>85±4.6</td>
<td>85±4.6</td>
<td>85±4.6</td>
<td>85±4.6</td>
<td>85±4.6</td>
<td>85±4.6</td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>(6587±229)</td>
<td>(6250±342)</td>
<td>(6245±363)</td>
<td>(6226±342)</td>
<td>(6144±363)*</td>
<td>(5724±301)*</td>
</tr>
<tr>
<td>Thrombin 0.3 U/mL</td>
<td>82±4.3</td>
<td>82±4.3</td>
<td>82±4.3</td>
<td>82±4.3</td>
<td>82±4.3</td>
<td>82±4.3</td>
</tr>
<tr>
<td>ADP 40 μmol/L</td>
<td>64±4.0</td>
<td>64±4.0</td>
<td>64±4.0</td>
<td>64±4.0</td>
<td>64±4.0</td>
<td>64±4.0</td>
</tr>
<tr>
<td>ADP 12 μmol/L</td>
<td>58±3.7</td>
<td>58±3.7</td>
<td>58±3.7</td>
<td>58±3.7</td>
<td>58±3.7</td>
<td>58±3.7</td>
</tr>
</tbody>
</table>

*p<0.05 compared with control.
estradiol, or ethanol, followed by a 5-minute incubation with 0.1 mmol/L isobutyl methylxanthine; then 1.0 U/mL thrombin was added. After 30 seconds, the samples were treated with ice-cold 25% perchloric acid to stop the reaction and stored at −80°C. After the samples had been thawed at 4°C, the concentrations of cAMP and cGMP were measured by use of an enzyme immunoassay kit with acetylation (Amersham).

**NO (Nitrite/Nitrate) Analysis**

Platelet counts were adjusted to 10⁸/mL by diluting the gel-filtered platelets with HEPES buffer. CaCl₂ was added at a final concentration of 1 mmol/L. The platelet samples were incubated for 20 minutes at 37°C with 1 mmol/L L-NMMA or vehicle. The reaction was stopped by adding ice-cold 25% perchloric acid 30 seconds after thrombin (1.0 U/mL) had been added, and the samples were stored at −80°C. Platelet levels of NO were determined with a kit for assaying nitrite/nitrate (Cayman Chemical Co) by reacting each sample with Griess reagent. Absorbance was measured at 540 nm.

**Statistical Analysis**

Values are expressed as mean±SEM. Comparisons between groups were made by one-way ANOVA. Changes in each variable over time were compared among the three groups of subjects by use of a two-factor (group and time) repeated-measures ANOVA and were analyzed by Scheffe’s F test. A level of P<0.05 was accepted as statistically significant.

**Results**

**Concentration of 17β-Estradiol in Platelets Preincubated With 17β-Estradiol**

We measured the concentration of 17β-estradiol in platelets from 10 of the male subjects. The number of platelets was adjusted to 10⁹/mL, and they were then preincubated with 17β-estradiol at a final concentration of 10⁻⁸ to 10⁻⁶ mol/L, or with ethanol. The refiltered samples were treated with 25% perchloric acid and the concentration of 17β-estradiol was measured. The results are shown in Table 1. The plasma concentration of 17β-estradiol in the male subjects was 17.5±3.6 pg/mL. We also measured the concentration of 17β-estradiol in 6 premenopausal women whose plasma concentration of 17β-estradiol was 105.9±16.8 pg/mL. The intraplatelet concentration of 17β-estradiol in the 6 premenopausal women was 1352±205 pg/mL.

**Effect of Estradiol on Platelet Aggregation**

Concentrations of 17β-estradiol of 10⁻⁸ to 10⁻⁶ mol/L had no effect on platelet aggregation. At concentrations >10⁻⁵ mol/L, 17β-estradiol dose-dependently reduced the platelet aggregation induced by thrombin as well as that by ADP according to the percent maximal aggregation and the area under the aggregation curve (Table 2). The inhibitory effects of 17β-estradiol on platelet aggregation were blunted by L-NMMA. The inhibitory effects of 17β-estradiol on platelet aggregation were greater after ADP induction than after thrombin induction. At 10⁻⁸ to 10⁻⁶ mol/L, 17α-estradiol had no effect on platelet aggregation (data not shown).

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**Table 3. Effect of 17β-Estradiol on Platelet [Ca²⁺], in the Nonstimulated Condition and the Response of [Ca²⁺], Induced by Agonists**

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Control</th>
<th>10⁻⁸ mol/L</th>
<th>10⁻⁷ mol/L</th>
<th>10⁻⁶ mol/L 17β-Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular Ca²⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>16.9±0.7</td>
<td>16.0±0.6</td>
<td>16.2±0.6</td>
<td>24.5±0.9</td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>59.3±5.7</td>
<td>63.0±5.7</td>
<td>60.4±5.7</td>
<td>61.3±6.4</td>
</tr>
<tr>
<td>Extracellular Ca²⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>241±21.6</td>
<td>234±21.1</td>
<td>230±20.9</td>
<td>246±24.1</td>
</tr>
<tr>
<td>Thrombin 1.0 U/mL</td>
<td>46.0±5.9</td>
<td>51.7±6.0</td>
<td>45.2±5.5</td>
<td>47.1±6.9</td>
</tr>
<tr>
<td>L-NMMA 1.0 U/mL</td>
<td>586±58.0</td>
<td>570±58.9</td>
<td>610±64.2</td>
<td>602±66.7</td>
</tr>
</tbody>
</table>

**Figure 1. Recovery from peak intracellular Ca²⁺ responses in a Ca²⁺-free medium induced by 1.0 U/mL thrombin in human platelets. The rate of decay of [Ca²⁺] after the peak was greater in the 17β-estradiol-pretreated platelets than in controls. This effect was blunted by L-NMMA. Open circles indicate without 17β-estradiol; filled circles, with 10⁻⁸ mol/L 17β-estradiol, ×, with 10⁻⁷ mol/L 17β-estradiol; and filled diamonds, with 10⁻⁶ mol/L 17β-estradiol and L-NMMA. *Overall curves for platelets pretreated with 10⁻⁸ or 10⁻⁷ mol/L 17β-estradiol were significantly different from control.
Effect of 17β-Estradiol on Platelet [Ca^{2+}],

Estrogen pretreatment had no effect on the intracellular concentration of fura 2, leakage of this dye, or $R_{\text{max}}$, the index of hydrolysis of the fluorescence indicator. We investigated the metabolism of Ca^{2+} in platelets that had been pretreated with 17β-estradiol at concentration $>10^{-5}$ mol/L (Table 3), because the concentrations of 17β-estradiol showed a significant inhibitory effect on platelet aggregation. Exposure to 17β-estradiol did not alter baseline [Ca^{2+}],. The platelet levels of the [Ca^{2+}], response induced by 1.0 U/mL thrombin averaged 595.7±22.2 nmol/L in the presence of extracellular Ca^{2+} and 235.0±21.2 nmol/L in the absence of extracellular Ca^{2+}. In contrast to thrombin, the platelet [Ca^{2+}], response induced by 40 μmol/L ADP showed no great difference between measurements made in the presence and absence of extracellular Ca^{2+} (mean of 60.9±5.6 and 47.6±5.8 nmol/L, respectively). Additionally, ADP evoked an increase in platelet [Ca^{2+}], without measurable delay, unlike the effect of thrombin. 17β-Estradiol did not affect the maximal response of platelet [Ca^{2+}], induced by thrombin or by ADP in the presence or absence of extracellular Ca^{2+}, indicating that the Ca^{2+} influx or release of Ca^{2+} from internal stores in stimulated platelets was unaffected by estrogen. This steroid hormone had no effect on intracellular Ca^{2+} discharge capacity or on the response of platelets [Ca^{2+}], to 5 μmol/L ionomycin in the absence of extracellular Ca^{2+} (Table 3). However, the rate of decay of platelet [Ca^{2+}], after the peak response to 1.0 U/mL thrombin in the absence of extracellular Ca^{2+} was significantly accelerated ($P<0.05$, Figure 1), and the area under the declining curve of [Ca^{2+}], was decreased ($P<0.05$ by 17β-estradiol $10^{-5}$ to $10^{-4}$ mol/L) in a concentration-dependent manner, although the peak [Ca^{2+}], was unaffected. The effect of 17β-estradiol on the rate of decay of platelet [Ca^{2+}], was blunted by L-NMMA. Figure 2 shows the relationship between these variables among the three groups with respect to the area under the platelet aggregation curve and the area of accumulated [Ca^{2+}],, after the peak had been reached by stimulation with thrombin. A highly significant correlation was observed between these two areas ($r=0.857, \ P<0.0001$). The area of platelet [Ca^{2+}], rather than the peak [Ca^{2+}], level appeared to be important in determining platelet aggregation.
The intracellular levels of cGMP in platelets stimulated by thrombin increased by pretreating platelets with 17β-estradiol had no effect on the level of cAMP but significantly increased cGMP in a concentration-dependent manner at a concentration >10^{-5} mol/L. Filled circles indicate cGMP; open circles, cAMP. *P<0.05 vs corresponding control values.

**Figure 3.** Concentration-response curve for the effects of 17β-estradiol on cAMP and cGMP. Pretreatment of platelets with 17β-estradiol had no effect on the level of cAMP but significantly increased cGMP in a concentration-dependent manner at a concentration >10^{-5} mol/L. Filled circles indicate cGMP; open circles, cAMP. *P<0.05 vs corresponding control values.

**Discussion**

The present study has demonstrated the ability of 17β-estradiol to reduce platelet aggregation, which plays a key role in the formation of atherosclerotic plaque. The suppressive effect of estrogen on platelet activity may be one of the mechanisms responsible for its beneficial effects in reducing the incidence of cardiovascular events in postmenopausal women. We also investigated the mechanism of the antiplatelet effect of estrogen. Because [Ca^{2+}], is one of the most important cellular second messengers that determine platelet function, we studied the effect of pretreatment with estradiol on Ca^{2+} handling in platelets. 17β-Estradiol did not alter baseline [Ca^{2+}], thrombin- or ADP-induced Ca^{2+} influx, release of Ca^{2+} from internal stores, or the size of internal Ca^{2+} stores. Although 17β-estradiol did not alter the peak amplitude of thrombin response in Ca^{2+}-free medium, this steroid did potentiate return of the Ca^{2+} transient to resting levels after stimulation by thrombin due to an increase in Ca^{2+} reuptake or its extrusion. In addition, the area under the curve of platelet aggregation was strongly correlated with the area under the declining line of postpeak [Ca^{2+}], rather than with peak [Ca^{2+}]. A prolonged Ca^{2+} response evokes a significant increase in the secretion of dense granules of platelets (degranulation); thus, an early Ca^{2+} decay due to pretreatment with 17β-estradiol would be expected to reduce such secretion. There is no conflict between the ability of 17β-estradiol to reduce platelet aggregation and the acceleration of [Ca^{2+}] recovery after the agonist-stimulated peak.

Methodological issues are important in the assessment of Ca^{2+} handling in fura 2–loaded cells. We attempted to minimize the methodological problems that might have obscured the effects of estrogen. First, we demonstrated that estrogen pretreatment lacked an effect on the intracellular metabolism of fura 2, such as the intracellular concentration of fura 2 and R_{max}, which is the index of dye ester hydrolysis. We were able to confidently compare [Ca^{2+}], values determined in fura 2–loaded platelets from the different groups. Second, we corrected for fura 2 leakage, which, if uncorrected, leads to errors in calculating [Ca^{2+}]. Third, to minimize cell activation, we separated the platelets by gel filtration instead of centrifugation. Basal [Ca^{2+}], in the present study was low compared with previously reported values from studies that used a similar method.

It has been suggested that increased levels of cAMP and cGMP activate the resequestration of Ca^{2+} into internal stores and the extrusion of Ca^{2+} across the plasma membrane. In a study of the effect of estrogen on the metabolism of arachidonic acid by aortas and platelets in rats, 17β-estradiol was found to significantly stimulate the production of prostaglandin I_2 in the aorta but lacked a significant effect on

**Effect of 17β-Estradiol on NO in Platelets**

The effects of estrogen on the NO content of platelets are unaffected by pretreatment with 17α-estradiol. Estradiol did not alter their intracellular level of NO. Filled circles indicate 17β-estradiol pretreatment; filled squares, L-NMMA and 17β-estradiol pretreatment; and open circles, 17α-estradiol pretreatment. *P<0.05 vs corresponding control values.

**Figure 4.** Concentration-response curve for the effects of 17α/β-estradiol on nitrite/nitrate concentration. Nitrite/nitrate concentration was significantly increased by pretreating platelets with 17β-estradiol at a concentration >10^{-5} mol/L. This effect was blunted by L-NMMA. Pretreatment of platelets with 17α-estradiol did not alter their intracellular level of NO. Filled circles indicate 17β-estradiol pretreatment; filled squares, L-NMMA and 17β-estradiol pretreatment; and open circles, 17α-estradiol pretreatment. *P<0.05 vs corresponding control values.
Antiaggregating Effect of Estrogen and NO

Figure 5. Parameter relationships in the three groups of subjects regarding the concentration of nitrite/nitrate and the area of accumulated [Ca\(^{2+}\)] after stimulation by thrombin. Open circles indicate without 17β-estradiol; filled circles, with 10\(^{-5}\) mol/L 17β-estradiol; and ×, with 10\(^{-4}\) mol/L 17β-estradiol. A highly significant correlation between these two areas was observed (r=0.954, P<0.0001).

The effects of 17β-estradiol on platelet aggregation and [Ca\(^{2+}\)], decay were blunted by L-NMMA. We also observed a highly significant correlation between the area of accumulated [Ca\(^{2+}\)] after stimulation by thrombin and the level of NO synthesis. This correlation suggests that the effect of 17β-estradiol on human platelet aggregation is a consequence of interactions between NO synthesis, cyclic nucleotides, and [Ca\(^{2+}\)]. The concentration of 17β-estradiol at which effects were observed (10\(^{-4}\) to 4 mol/L) was higher than its normal circulating levels (typically 10\(^{-8}\) to 10\(^{-10}\) mol/L). The fact that such supraphysiological levels of 17β-estradiol were required for the inhibition of platelet function suggests several possibilities. Higher concentrations of hormones are required for some short-term membrane effects in vitro. There is a possibility that chronic exposure of circulating platelets to physiological levels of 17β-estradiol in vivo may have the same effect as the supraphysiological levels of 17β-estradiol in vitro. We measured the intraplatelet concentration of 17β-estradiol (preincubation with 10\(^{-8}\) to 10\(^{-4}\) mol/L 17β-estradiol) in men. The intraplatelet concentration of 17β-estradiol in the male subjects did not reach physiological levels as in premenopausal women until the concentration of 17β-estradiol for preincubation exceeded 10\(^{-3}\) mol/L. The mechanism by which 17β-estradiol is taken into platelets has not been clarified. Because there is a significant difference in the concentration of 17β-estradiol between the inside and outside of platelets, we suggest that some transport mechanism exists in platelets and that 17β-estradiol may partition into the platelet membrane pool. Therefore, in some acute events, such mechanisms may abruptly increase the concentration of 17β-estradiol within platelets.

Estrogen receptors are present in the nucleus, where they stimulate the synthesis of mRNA and protein. Given that platelets are anuclear and that the time course of estrogen action is very rapid, it is possible that a high concentration of this steroid exerts nonspecific effects on platelet membrane fluidity. However, because 17α-estradiol did not affect platelet function, we suggest that the observed effects of 17β-estradiol were specific to this hormone. Previous studies have shown that several kinds of cells in some mammalian species may bear nongenomic cell surface receptors for 17β-estradiol. As a result, 17β-estradiol may act on an as-yet- unidentified platelet membrane receptor.

In conclusion, 17β-estradiol appears to play an important role in inhibiting the aggregation of human platelets in vitro.

Recent studies have confirmed a relationship between estrogen and NO. Several studies have shown that estrogen directly modifies the L-arginine/NO pathway in vascular endothelial cells. Studies in vivo have found that estrogen potentiates endothelium-dependent vasodilation. Other studies have found that estrogen increases NO synthesis in human aortic endothelial cells. Because 17β-estradiol is intimately linked to NO synthesis in endothelial cells and human platelets also have an intrinsic L-arginine/NO pathway, it is possible that 17β-estradiol influences the intraplatelet synthesis of NO. We analyzed the nitrite/nitrate concentration in platelets and found that pretreatment with 17β-estradiol increased the platelet concentration of nitrite/nitrate and that this effect was blunted by L-NMMA. These findings indicate that 17β-estradiol increases cGMP via the stimulation of intrinsic NO synthesis in human platelets.

The effects of 17β-estradiol on platelet aggregation and [Ca\(^{2+}\)], decay were blunted by L-NMMA. We also observed a highly significant correlation between the area of accumulated [Ca\(^{2+}\)], after stimulation by thrombin and the level of NO synthesis. This correlation suggests that the effect of 17β-estradiol on human platelet aggregation is a consequence of interactions between NO synthesis, cyclic nucleotides, and [Ca\(^{2+}\)]. The concentration of 17β-estradiol at which effects were observed (10\(^{-4}\) to 4 mol/L) was higher than its normal circulating levels (typically 10\(^{-8}\) to 10\(^{-10}\) mol/L). The fact that such supraphysiological levels of 17β-estradiol were required for the inhibition of platelet function suggests several possibilities. Higher concentrations of hormones are required for some short-term membrane effects in vitro. There is a possibility that chronic exposure of circulating platelets to physiological levels of 17β-estradiol in vivo may have the same effect as the supraphysiological levels of 17β-estradiol in vitro. We measured the intraplatelet concentration of 17β-estradiol (preincubation with 10\(^{-8}\) to 10\(^{-4}\) mol/L 17β-estradiol) in men. The intraplatelet concentration of 17β-estradiol in the male subjects did not reach physiological levels as in premenopausal women until the concentration of 17β-estradiol for preincubation exceeded 10\(^{-3}\) mol/L. The mechanism by which 17β-estradiol is taken into platelets has not been clarified. Because there is a significant difference in the concentration of 17β-estradiol between the inside and outside of platelets, we suggest that some transport mechanism exists in platelets and that 17β-estradiol may partition into the platelet membrane pool. Therefore, in some acute events, such mechanisms may abruptly increase the concentration of 17β-estradiol within platelets.

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In conclusion, 17β-estradiol appears to play an important role in inhibiting the aggregation of human platelets in vitro.

thromboxane biosynthesis in platelets. To clarify the relevance of the altered Ca\(^{2+}\) handling caused by 17β-estradiol and cyclic nucleotides, we measured the platelet levels of cAMP and cGMP. Pretreating the platelets with 17β-estradiol had no effect on the levels of cAMP. Thus, the effect of 17β-estradiol appeared not to be mediated by cAMP metabolism. The levels of cGMP, however, were increased by concentrations of 17β-estradiol >10\(^{-3}\) mol/L. The ability of estrogen to accelerate Ca\(^{2+}\) decay may be mediated, at least in part, by an increase in intracellular cGMP.

Several cell types possess an L-arginine/NO pathway. Use of the reverse transcription–polymerase chain reaction has demonstrated expression of mRNA for the constitutive, endothelial form of NO synthase in human platelets. Gel electrophoresis identified a protein band of \(\approx 130\) kDa with NO synthase activity in the platelet cytosol. This direct evidence for the presence of a constitutive form of NO synthase is supported by other findings; this elevation of intraplatelet levels of cGMP by the NO-like compound S-nitrosocysteine has been correlated with the inhibition of human platelet secretion. The effect of N'-nitro-L-arginine methyl ester on platelet P-selectin expression, which is used as a marker of α-degranulation, has been shown to be mediated primarily by NO and cGMP.

Recent studies have confirmed a relationship between estrogen and NO. Several studies have shown that estrogen directly modifies the L-arginine/NO pathway in vascular endothelial cells. Studies in vivo have found that estrogen potentiates endothelium-dependent vasodilation. Other studies have found that estrogen increases NO synthesis in human aortic endothelial cells. Because 17β-estradiol is intimately linked to NO synthesis in endothelial cells and human platelets also have an intrinsic L-arginine/NO pathway, it is possible that 17β-estradiol influences the intraplatelet synthesis of NO. We analyzed the nitrite/nitrate concentration in platelets and found that pretreatment with 17β-estradiol increased the platelet concentration of nitrite/nitrate and that this effect was blunted by L-NMMA. These findings indicate that 17β-estradiol increases cGMP via the stimulation of intrinsic NO synthesis in human platelets.
The probable mechanism is the reduction of $[Ca^{2+}]$, by the increased production of cGMP that is dependent on NO.

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**References**

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