Platelets of Female Mice Are Intrinsically More Sensitive to Agonists Than Are Platelets of Males

Xing-Hong Leng, Song Yun Hong, Susana Larrucea, Wei Zhang, Tong-Tong Li, José A. López, Paul F. Bray

Objective—It has been reported that women fare worse after ischemic coronary events, but the mechanisms remain unclear. Because platelets play a central role in the formation of occlusive thrombi at sites of ruptured atherosclerotic plaques, we studied male/female paired mouse littermates for sex differences in platelet function.

Methods and Results—We compared platelet reactivity in male/female mouse littermates by monitoring agonist-induced fibrinogen (FGN) binding and platelet aggregation. Compared with the platelets from males, platelets from females bound more FGN in response to low concentrations of thrombin and collagen-related peptide. Female platelets also demonstrated greater aggregation in response to adenosine diphosphate and collagen-related peptide. Platelet protein tyrosine phosphorylation on activation also showed small differences between sexes. These differences are independent of platelet size and surface expression of \( \alpha_{IIb}\beta_3 \) and GPIb-IX-V, and they were not blocked by apyrase or aspirin. The sex differences we observed were intrinsic to platelets, because they were observed in washed platelets, but not when platelets were in plasma.

Conclusions—The platelets of female mice were more reactive than those of males in a manner independent of COX-1 and secreted ADP. (Arterioscler Thromb Vasc Biol. 2004;24:376-381.)

Key Words: platelet • thrombin • collagen-related peptide

Coronary heart disease is the most common cause of morbidity and mortality among American women. Although symptomatic coronary artery disease develops in women \( \approx 5 \) to 10 years later than in men,\(^1\) the prognosis is worse for women after myocardial infarction,\(^2\) coronary revascularization,\(^3\) or thrombolytic therapy.\(^4\) A number of social, anatomic, and physiological mechanisms have been postulated to explain this gender difference,\(^5\) but the mechanisms responsible remain largely unknown. Platelets play a central role in the formation of occlusive thrombi in atherosclerotic coronary arteries,\(^6\) and gender differences in platelet reactivity have been reported in a small number of studies. Female platelets have been shown to be hyperreactive compared with male platelets in some studies,\(^7,8\) but not in others.\(^9,10\) However, these studies have been rather small, and none considered confounding variables that affect platelet function (eg, cigarette use, plasma factors, and so forth).

Animal models of coronary heart disease are now commonplace, and some have addressed possible sex differences in thrombus formation. Intravenous injection of arachidonic acid or the stable thromboxane A\(_2\) analog U46619 have shown a greater mortality rate in male compared with female rats, mice, and rabbits.\(^11-13\) These in vivo systems are difficult to interpret mechanistically because none has addressed which tissues (platelets, endothelial cells, smooth muscle cells, etc) and systems (inflammation, coagulation, fibrinolysis, vasoconstriction) are influenced by sex. In addition, the thrombosis reported in these in vivo models occurs in the pulmonary and not coronary vasculature,\(^14\) raising concerns about their relevance to coronary heart disease. Importantly, these in vivo approaches have not specifically addressed whether platelets contribute to the sex difference in thrombosis.

Only a few studies have isolated animal platelets for in vitro assays of sex differences, and conflicting results have been obtained. Johnson et al observed greater in vitro aggregation of platelets from male than female rats.\(^15\) However, others have observed no sex difference\(^16\) or female hyperreactivity in rat platelets.\(^17\) Gender-specific differences in the porcine platelet content of vasoactive factors and in interactions with the vessel wall have consistently been observed.\(^18,19\) The only in vitro study using mouse platelets showed no sex difference to U46619, but rather a greater slope of aggregation in male platelets to high-dose adenosine diphosphate (ADP) and arachidonic acid.\(^13\) But these in vitro studies of mouse platelet function were performed in plasma, making it impossible to assess whether the sex difference is intrinsic to platelets or to plasma.

Our goal in this study was to test the hypothesis that there are sex differences intrinsic to murine platelets. Recognizing

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that the mouse system is not a perfect model for the human, it nevertheless offers certain advantages. There is less heterogeneity because laboratory mice are subject to fewer environmental alterations than humans, who encounter numerous variables affecting platelet function. Studying male/female paired littermates of inbred mice also minimizes genetic and environmental differences. We found that compared with those of males, platelets from female mice are hyperreactive to multiple agonists using multiple assays.

Methods

Materials

Prostaglandin E₁, aspirin, and indomethacin were purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated antibodies (anti-mouse CD41 and CD62) were obtained from BD Pharmingen (San Diego, CA). The blocking hamster mAb 1B5 (anti-murine α₉β₃), polyclonal anti-PAR3 IgG,²⁰ and thrombin were gifts from Dr. Barry S. Coller (The Rockefeller University, New York, NY), Dr. Shaun R. Coughlin (University of California, San Francisco) and Dr. John Fenton (New York State Department of Health, Albany), respectively. Collagen-related peptide was synthesized at Baylor College of Medicine and cross-linked with glutaraldehyde. PAR4 activation peptide (PAR4AP) AYPGKF was synthesized at Baylor College of Medicine as carboxyl-terminal amides, purified by high-pressure liquid chromatography, and characterized by mass spectroscopy. Human fibrinogen (FGN) was from Enzyme Research Laboratories (South Bend, IN). FITC-conjugated (Pierce, Rockford, IL) FGN was made as previously described.²¹

Animals and Platelet Preparation

All animal studies were performed in accordance with institutional guidelines. In all studies, 9 to 12-week-old C57BL/6J female mice were compared pairwise with male littermates. Blood (≈0.6 mL) was collected from the inferior vena cava of isolurane-anesthetized mice into 3.8% sodium-citrate. Whole blood was diluted with an equal volume of HEPES Modified Tyrode’s buffer (TH buffer; 12 mmol/L Na bicarbonate, 138 mmol/L NaCl, 5.5 mmol/L glucose, 2.9 mmol/L KCl, 10 mmol/L HEPES, pH 7.4) containing 0.38% citrate, and centrifuged at 50 g for 10 minutes at room temperature. The platelet-rich plasma was recovered and centrifuged at 350 g for 5 minutes. The supernatant was removed and the resulting platelet pellet was rinsed with TH/0.38% citrate buffer and resuspended in TH buffer at a concentration of 2.5 × 10⁷/mL.

Flow Cytometry Analysis

Platelets were defined by their forward- and side-scatter characteristics. Platelet FGN binding was performed essentially as previously described.²¹ Washed platelets were diluted with TD buffer (TH buffer with 0.4 mmol/L MgCl₂, 0.8 mmol/L CaCl₂) to 2.5 × 10¹⁰/mL. FITC-conjugated FGN (100 μg/mL) was added to diluted platelets before activation with agonists. After fixation with 1% paraformaldehyde, samples were analyzed by a FACSort flow cytometer (Becton-Dickinson). Mean fluorescence intensities (MFI) of platelets were recorded. Baseline (≈10 MFI) and non-specific binding (≈10 MFI and very close to baseline) were defined as binding obtained with no agonist and with agonist in the presence of 10 mmol/L EDTA, respectively. In some experiments, platelet-rich plasma was pretreated with 1 mmol/L acetylsalicylic acid (ASA) for 30 minutes at 37°C and then washed. Other inhibitors were added to platelets 2 to 3 minutes before the agonist was added. All experiments were performed in duplicate or triplicate. Results are presented either using the MFI or as a ratio of female-to-male FGN binding, as explained in Results. When MFI was used, the specific MFI (MFI sample−MFI non-specific) values were used.

Integrin α₉β₃ was quantified using washed platelets with and without activation by thrombin in the presence of FITC-conjugated anti-α₉ (anti-CD41) antibody. Surface expression of PAR3 was assessed using flow cytometry as described.²² Briefly, fixed platelets were incubated with anti-PAR3 IgG (10 μg/mL) for 1 hour, washed, incubated with FITC-conjugated goat anti-rabbit IgG, and analyzed by flow cytometry. Surface expression of P-selectin (CD62) was determined using FITC-conjugated anti-CD62, and the percent positive cells was determined as described.²³

Platelet Aggregation

Platelet aggregation was determined in a Bio/Data aggregometer as described.²⁴ When aggregations were performed on washed platelets, it was necessary to add FGN, which serves as a ligand to cross-link platelets and is the most important physiological ligand mediating platelet aggregation in plasma. Human FGN is considered a useful surrogate for mouse FGN, because it demonstrates the same receptor specificity.²⁵–²⁸ Briefly, washed platelets were resuspended at 2.5 × 10¹⁰/mL in TH buffer with 0.005 U/mL apyrase to prevent desensitization²⁴–³⁰ and allowed to rest for 1 hour. Platelet suspensions were activated by the indicated agonists in the presence of 50 μg/mL human FGN, 0.8 mmol/L calcium, and 0.4 mmol/L magnesium.²⁵–²⁸

Statistical Analysis

Statistical analyses were performed using StatView software (SAS Institute, Cary, NC). The collagen-related peptide-induced FGN binding, and collagen-related peptide and ADP-induced platelet aggregation data were analyzed by ANOVA for the main effect of sex. Because of the day-to-day variation in absolute MFI in the thrombin-stimulated platelet FGN binding assays, these data were nonparametric and the Wilcoxon signed rank test was used to test for differences between the groups. Results are reported as the mean±SEM; P<0.05 was considered significant.

Results

Sex Differences in Platelet Reactivity to Thrombin

To determine whether mice exhibit intrinsic sex differences in platelet function, washed platelets from male/female paired littermates were tested for their reactivity to thrombin. Because of the inherent day-to-day variability in many parameters of the thrombin-induced platelet FGN binding assay, the values for a particular day could be compared only with controls performed simultaneously. We designed our experiments so that paired female/male littermates were sacrificed at the same time and platelets were processed and activated side-by-side to focus on the comparison (the relative reactivity, not the absolute binding) between sexes. In this case, ratios of FGN bindings (female/male) for each thrombin concentration were obtained daily, allowing us to compare ratios obtained on different days. We found that platelets from female mice on average bound 50% more and 21% more FGN than platelets from their male littermates at 0.005 U/mL.

### TABLE 1. Gender Difference in Thrombin-Stimulated Platelet Fibrinogen Binding

<table>
<thead>
<tr>
<th>Thrombin (U/mL)</th>
<th>Female/Male</th>
<th>No. Pairs* (F/M/E)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>1.50±0.10</td>
<td>24/3/0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0.01</td>
<td>1.21±0.06</td>
<td>18/8/1</td>
<td>0.02</td>
</tr>
<tr>
<td>0.02</td>
<td>1.04±0.03</td>
<td>15/12/0</td>
<td>0.88</td>
</tr>
</tbody>
</table>

*Total of 27 pairs were tested.
†Wilcoxon signed rank test for female vs male binding.
Platelets were activated with thrombin, and using an anti-mouse PAR3 antisera. Data are means from 8 female/male paired littermates. MFI indicates mean fluorescence intensity. Differences were observed in either PAR3 surface expression. PAR3 levels were assessed using flow cytometric analysis of platelets from female/male paired littermates. A, Effect of αIIbβ3 blockade on platelet FGN binding. Washed platelets were activated with 0.01 U/mL thrombin for 20 minutes at 37°C in the presence of FITC-labeled FGN and increasing concentrations of the αIIbβ3 blocking antibody, 1B5. Data shown are representative of 3 separate experiments, each performed in triplicate. B, Platelet surface expression of αIIbβ3 in male/female littermate mouse pairs. Washed platelets were activated with thrombin, and αIIb levels assessed using an anti-mouse αIIb (CD41) antibody. Representative result of 5 female/male littermate pairs, each performed in triplicate. C, PAR3 surface expression. PAR3 levels were assessed using anti-PAR3 antisera. Data are means from 8 female/male paired littersmates. MFI indicates mean fluorescence intensity.

Table 2. Gender Difference in PAR4AP-Stimulated Platelet Fibrinogen Binding

<table>
<thead>
<tr>
<th>PAR4AP (μM)</th>
<th>Female/Male (± SE)</th>
<th>No. Pairs* (F/M)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.08±0.33</td>
<td>14/14</td>
<td>0.43</td>
</tr>
<tr>
<td>100</td>
<td>1.00±0.20</td>
<td>14/14</td>
<td>0.78</td>
</tr>
<tr>
<td>200</td>
<td>0.97±0.13</td>
<td>13/12</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Total of 28 pairs were tested. Three fewer pairs were studied at 200 μM PAR4AP.
†Wilcoxon signed rank test for female vs male binding.

Sex Difference in FGN Binding to Collagen-Related Peptide-Activated Platelets

We considered whether the sex difference in platelet responsiveness was observed with other agonists, because specificity (or no specificity) to thrombin would have important implications regarding potential mechanisms responsible for the sex difference. Cross-linked collagen-related peptide potently activates platelets through the GPVI-Fcγ complex. Collagen-related-peptide induced greater FGN binding to female platelets than to platelets from males (Figure 2A). Thus, a second agonist produced similar findings in the same FITC-FGN binding assay.

Sex Difference in Platelet Aggregation

We next assessed whether a different assay would also show female platelet hyperreactivity. We observed greater ADP-induced platelet aggregation in washed platelets from the female littersmates (Figure 2B). Platelets from females also demonstrated greater aggregation relative to that of males in response to collagen-related peptide in the absence of plasma (Figure 2C).

Influence of Plasma on Sex Difference in Platelet Function

Because other animal studies tested platelet aggregation only in the presence of plasma, we felt it important to compare platelet reactivities using washed platelets and platelets in plasma from the same mouse. Figure 2C shows the aggregation results of platelets in either buffer or plasma. The collagen-related peptide-induced aggregation showed a sex difference in the absence (P=0.03), but not the presence (P=0.86), of plasma.

Potential for Sex Differences in Platelet Signaling

Platelet activation leads to redistribution of P-selectin to the platelet plasma membrane, secretion of ADP, and generation...
of thromboxane A₂ (the latter two feed-back to enhance platelet activation and FGN binding). When stimulated with low concentrations of thrombin, more female platelets demonstrated surface P-selectin than male platelets (data not shown). This correlated with the FGN binding data shown in Table 1. The sex difference in platelet FGN binding persisted in the presence of the ADP scavenger apyrase, although activation was suppressed in both sexes (Figure 3). In addition, the sex difference in platelet activation was not blocked by aspirin (ASA), indomethacin, or the combination of ASA and apyrase (Figure 3, indomethacin data not shown).

We also examined protein tyrosine phosphorylation patterns on platelet stimulation and observed small differences between sexes in response to thrombin (Figure 4) and collagen-related peptide stimulation (data not shown), although the patterns of differentially phosphorylated proteins were not identical in response to different agonists. The differential phosphorylation between sexes was detected at earlier time points but lost with longer incubation times (not shown). We found the greatest difference between sexes was at 3 minutes of 0.005 U/mL thrombin stimulation.

**Discussion**

Mouse models have become integral reagents in the armamentarium for research on the pathophysiology of vascular diseases. Nearly 30 years have passed since the initial reports suggesting a gender difference in platelet thrombus formation, but this has remained a poorly studied area. Investigators have generally ignored sex as a confounding variable when analyzing their mouse models of vascular diseases. Because platelet reactivity is intimately involved in thrombus formation, inflammation, and atherosclerosis progression, it is crucial to understand the factors that affect platelet behavior. The major findings of this study were that platelets from females demonstrated greater reactivity to agonists compared with the platelets from males and that this sex effect was intrinsic to the platelets. The similarity between mouse and human with respect to this gender difference in platelet reactivity supports the use of mouse models of arterial thrombosis and stresses the importance of...
considering gender in animal and human studies of these phenotypes.

Female platelets showed a lower threshold for reactivity to thrombin than did male platelets. The relatively greater reactivity of female platelets shown as a ratio in Table 1 was not caused by a lack of reactivity of male platelets, because the latter showed an appropriate dose–response to agonists (eg, Figure 2A and B). There were no sex differences in the surface expression of platelet GP

b and PAR3 that could account for the observed functional differences to thrombin (Figure 1B and C). In addition to the PARs, GP

a serves as a high-affinity binding site for thrombin and is involved in platelet activation. However, in data not shown, we observed no difference in GP

a surface expression between 11 female and 11 male C57BL/6J mice. We cannot exclude a sex difference in PAR4 levels, but this seems unlikely because the PAR4 peptide elicited no sex difference in FGN binding. Different responses to thrombin and the thrombin receptor activating peptide have been repeatedly observed in human platelets. Our FGN binding studies were performed in a very dilute platelet suspension, and low concentrations of agonists were used such that no aggregation occurred (confirmed by flow cytometry). Thus, the sex differences in platelet reactivity may not, by itself, lead to a greater tendency toward in vivo thrombosis in females than in males. However, this could be true in some cases and it is important to understand the sex influence of each physiological component contributing to hemostasis. Although the specific mechanisms involved in greater reactivity of female platelets remain to be determined, future studies will involve examination of the potential roles of various candidate hormones (eg, estrogen, progesterone, testosterone, prolactin, and so forth) and their corresponding hormone receptors. A better understanding of the sex differences in platelet reactivity may lead to more rational preventative and therapeutic approaches to managing disorders of arterial thrombus.

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References

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