Effect of sex difference on platelet adhesion, spreading and aggregate formation under flow

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Summary
There are clear but poorly understood differences in the etiology and prognosis of thrombotic diseases in men and women. Due to the fact that platelets play a central role in the formation of occlusive thrombi in atherosclerotic coronary arteries, previous studies have examined whether sex differences exist for platelets, and have obtained conflicting results. Additionally, due to the increased use of genetically modified mouse models to explore the molecular mechanisms underlying platelet activation and thrombotic disorders, it is critical to determine if sex is a confounding variable. Our study of the role of sex differences in platelet function was designed to utilise purified platelets from inbred paired female/male littermates in order to minimise genetic and environmental variability. In the current study, we demonstrate that platelet adhesion to and spreading on immobilised fibrinogen, thrombin or collagen was equivalent for both female and male mouse platelets. The ability of the soluble agonist thrombin or convulxin to potentiate platelet P-selectin exposure, fibrinogen binding, or adhesion and spreading on immobilised fibrinogen was equivalent for both female and male mouse platelets. Our data show that an equivalent degree of platelet adhesion and aggregation on collagen or fibrinogen under shear flow was observed for both female and male mouse platelets. In conclusion, our data would argue against an intrinsic difference for female mouse platelets in regulating the major functional platelet responses: platelet adhesion, spreading, or aggregation under flow.

Keywords
Platelet activation, platelet spreading, flow adhesion, sex differences

Introduction
Coronary heart disease is the most common cause of morbidity among American women. Although symptomatic coronary artery disease (CAD) develops in women ~5 to 10 years later than it does in men (1), the prognosis is far worse for women after myocardial infarction, coronary revascularisation, or thrombolytic therapy (2–4). A number of physiological, social, and anatomical causes for this gender disparity in CAD prognosis have been postulated (5); however, the underlying mechanisms remain unclear. Due to the fact that platelet reactivity is intimately involved in thrombus formation, inflammation, and atherosclerosis progression, it is vital to determine the role of sex differences in platelet behavior.

Evidence that sex differences play a pro-aggregatory role in female human platelets was first demonstrated over 30 years ago (6). It has been shown that, although there are no differences in the total number of integrin αIIbβ3 expressed on platelets between men and women, agonist stimulation of platelets from women leads to the conversion of a greater proportion of available αIIbβ3 receptors into the active confirmation (7). A large study of over 1,200 patients demonstrated that female platelets were significantly more reactive to 10 out of the 12 platelet agonists tested (8). In contrast, a number of smaller studies failed to detect any differences in the reactivity of female human platelets as compared to male human platelets (9–12), although these studies were not designed to account for confounding natural and environmental variables (i.e. age, cigarette use, body mass index) that exist within the human population.

Previous studies have examined whether sex differences exist for murine platelets. Although studies involving inbred paired female/male littermates minimise genetic and environmental variability, conflicting results have been obtained. Reports have been published demonstrating hyperreactivity in fe-
Figure 1: Female and male mouse platelet adhesion and spreading on fibrinogen. A) Washed mouse platelets (2 × 10^7/ml) from female/male paired littersmates were placed on 100 µg/ml fibrinogen-coated coverslips for 45 min at 37°C and imaged using DIC microscopy. B) The surface area of 300 female platelets (open boxes) and 300 male platelets (closed boxes) on fibrinogen was quantified using Image J. Surface areas are plotted as a frequency distribution. C) Female (open boxes) or male (closed boxes) mouse platelets were exposed to coverslips coated with 5, 10, or 100 µg/ml fibrinogen, and the number of adherent platelets was quantified using Image J. Results are means ± SEM of 5–8 experiments.

Figure 2: Real time imaging of mouse platelet spreading on fibrinogen. A) Purified mouse platelets (2 × 10^7/ml) from female/male paired littersmates were exposed to a fibrinogen-coated surface and observed in real time using DIC microscopy. Images depict representative morphology of a single female and male mouse platelet spreading on fibrinogen. B) The mean surface area (µm²) of female (open boxes) and male platelets (closed boxes) was quantified at the indicated time points using Image J. Values are mean ± SEM of at least 20 platelets. C) Platelets were allowed to adhere to immobilised fibrinogen for 10, 20 or 45 min prior to imaging. D) The number of adherent female and male platelets on fibrinogen is reported as the mean ± SEM at the indicated time points. Images are representative of 4–8 experiments.
male rat platelets (13). In contrast, studies have shown that male rat platelets are more sensitive to agonists than female rat platelets (14), while others have not detected any sex differences in murine platelet function (15). A recent study by Leng et al. using purified mouse platelets found that compared to males, female mouse platelets demonstrated increased fibrinogen binding and aggregation following stimulation with either thrombin or the glycoprotein (GP)VI-agonist, collagen-related peptide (16). Although genetically modified mice are increasingly used as a model to explore the molecular mechanisms underlying haemorrhagic and thrombotic disorders, sex has not been fully explored as a potential confounding variable. Therefore, this study was designed to determine if sex differences affect mouse platelet adhesion, spreading, and aggregation under shear flow.

Materials and methods

Reagents
Fibrillar type I collagen (Horm) from equine tendon was purchased from Chrono-log Corporation (Havertown, PA, USA). Human fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Convulxin was purchased from Pentapharm (Basel, Switzerland). Oregon Green 488-conjugated fibrinogen (OG-fibrinogen) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were from Sigma (St. Louis, MO, USA) or previously named sources (17, 18).

Preparation of murine washed platelets and red blood cells
All animals were maintained using housing and husbandry in accordance with institutional regulations. In all studies, 9- to 12-week-old C57BL/6J female mice were compared pairwise with male littersmates. Murine blood was drawn from CO2 terminally anesthetised mice into acid/citrate/dextrose as previously described (19). Platelet-rich plasma (PRP) was prepared via centrifugation of whole blood at 200×g for 6 minutes (min). Washed platelets were prepared via centrifugation of PRP at 1,000×g in the presence of prostacyclin (0.1 μg/ml) for 10 min. The pellet was resuspended in modified HEPES-Tyrode buffer (in mM: 129 NaCl, 0.34 Na2HPO4, 2.9 KCl, 12 NaHCO3, 20 HEPES, 5 glucose, 1 MgCl2; pH 7.3) to the desired concentration. Autologous red blood cells (RBC) were isolated following the initial centrifugation of whole blood. Following PRP removal, the RBCs were pelleted by further centrifugation (2,000×g for 10 min), followed by washing (3×) with HEPES buffer (in mM: 10 HEPES, 140 NaCl, 5 glucose; pH 7.3). Washed platelets were then reconstituted with 50% (v/v) autologous packed red blood cells. For whole blood studies, murine blood was taken into 40 μm PP ACK and 10 U/ml heparin.

Platelet adhesion assays
Glass coverslips were incubated with a suspension of fibrinogen (5–100 μg/ml), thrombin (1–50 μg/ml), or soluble collagen (1–50 μg/ml) for 1 hour (h) at room temperature (RT). Surfaces were then blocked with denatured bovine serum albumin (BSA) (5 mg/ml) for 1 h at RT followed by subsequent washing with phosphate-buffered saline (PBS) before use in spreading assays. Quiescent platelets failed to bind or spread on surfaces coated with denatured BSA (data not shown). For spreading experiments, washed platelets (2 × 107/ml) were incubated on protein-coated coverslips at 37°C for 10, 20, or 45 min as previously described (20). Subsequently, coverslips were gently washed with PBS to remove unbound cells. Platelet

Figure 3: Agonist-induced mouse platelet adhesion and spreading on fibrinogen. A) Washed mouse platelets (2 × 10^7/ml) from female/male paired littersmates were placed on 100 μg/ml fibrinogen-coated coverslips in the presence of vehicle (–), the GPCR agonist, thrombin (0.1 U/ml), the GPVI-agonist, convulxin (0.1 μg/ml), or 1 mM Ca2+ for 45 min at 37°C and imaged using DIC microscopy. B) Female (open symbols) and male (closed symbols) mouse platelets (2 × 10^7/ml) were stimulated with a range of thrombin concentrations (0.001, 0.005, 0.01, 0.1 U/ml) for 10 min prior to exposure to coverslips that had been coated with either 5, 10, or 100 μg/ml fibrinogen. Results are means ± SEM of 5–8 experiments.
spreading was imaged using Köhler illuminated Nomarski differential interference contrast (DIC) optics with a Zeiss 63× oil immersion 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200M microscope and recorded using Stallion 4.0 (Intelligent Imaging Innovations, Inc., Denver, CO, USA). To compute the degree of adhesion and surface area of spreading platelets, images were manually outlined and quantified by determining the number of pixels within each outline using a java plug-in for Image J software as previously described (21). Imaging a graticule under the same conditions allowed the conversion of pixel size to microns.

**Measurement of P-selectin expression and fibrinogen-binding by flow cytometry**

Suspensions of washed murine platelets (2 × 10^7/ml) in the presence of 1 mM Ca^{2+}, were treated with thrombin (0–0.1 U/ml) or convulxin (0–0.5 μg/ml) in the presence of a fluorescein isothiocyanate (FITC)-conjugated anti-CD62P mAb (10 μg/ml) or OG-fibrinogen (100 μg/ml) for 15 min. Samples were diluted with modified HEPES-Tyrode buffer, followed by flow cytometric analysis on a Becton Dickinson FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Platelets were identified by logarithmic signal amplification for forward and side scatter, and the geometric mean fluorescence of each sample was recorded.

**Flow adhesion studies**

Microcapillary tubes were coated with fibrillar collagen (100 μg/ml) or fibrinogen (100 μg/ml) as described above, and mounted on the stage of an inverted microscope (Zeiss Axiovert 200M). In separate experiments, PPP/HPACK/heparin-anticoagulated whole blood or reconstituted blood was perfused through the chamber at a wall shear rate of 300 s^{-1}, followed by washing.
for 4 min at the same shear rate with HEPES-Tyrode buffer and imaged using DIC microscopy as previously described (22). In selected experiments, whole blood was fluorescently labelled with DiOC₆ (2 μM, 10 min at 37°C), and the accumulation of DiOC₆-labeled platelets was monitored in real time using fluorescence microscopy.

**Analysis of data**
Experiments were carried out on at least three occasions, and images shown are representative data from one experiment. Where applicable, results are shown as mean ± standard error of the mean (SEM). Statistical significance was determined using Student’s t-test, with values of p < 0.05 selected to be statistically significant.

**Results**

**Sex difference in murine platelet adhesion and spreading**
To determine if sex differences affect murine platelet binding to fibrinogen, platelets were simultaneously purified from female/male paired littermate mice and layered over surface-immobilised fibrinogen for 45 min. Our results demonstrate that both male and female mouse platelets underwent partial spreading on fibrinogen, with formation of filopodia and limited lamellipodia-like structures (Fig. 1A). The surface area of each set of platelets was measured and plotted as a frequency distribution (Fig. 1B). Overlapping frequency distribution curves were observed for male and female mouse platelets. Additionally, our results demonstrate that equivalent levels of female or male platelets bound to surfaces coated with 5, 10, or 100 μg/ml fibrinogen (Fig. 1D).

To further investigate the kinetics of platelet spreading, we examined spreading of female and male murine platelets on fibrinogen in real time using DIC microscopy. An equivalent time course of spreading, consisting of the generation of short, dynamic filopodia and limited formation of lamellae-like structures resulting in a 40–50% increase in surface area, was observed for both female and male platelets (Fig. 2A, B). Furthermore, no statistical difference was observed for the number of adherent platelets on 100 μg/ml fibrinogen at 10 min, 20 min, and 45 min time points for both female and male platelets (Fig. 2C, D).

A further set of experiments was designed to investigate the role of sex differences in regulating agonist-induced platelet spreading. As shown in Figure 3A, both female and male platelets generated similar degrees of circumferential lamellae on fibrinogen in the presence of the G-protein-coupled receptor (GPCR) agonist, thrombin, or the GPVI agonist, convulxin. In order to determine if sex differences play a role in agonist-induced platelet adhesion, female and male mouse platelets were stimulated with a range of thrombin concentrations (0.001, 0.005, 0.01, 0.1 U/ml) prior to exposure to coverslips that had been coated with a solution of either 5, 10, or 100 μg/ml fibrinogen. Following thrombin stimulation, the number of mouse platelets that bound to fibrinogen was equivalent for both female and male platelets for all thrombin concentrations tested (Fig. 3B). Similar levels of platelet adhesion and changes in morphology were observed for male and female platelets on fibrinogen in the presence of 1 mM Ca²⁺ (Fig. 3A).

We next examined the role of sex differences on platelet adhesion to immobilised thrombin or collagen, which potentiate platelet activation and spreading downstream of protease activated receptors (PARs) or GPVI, respectively (18). Both female and male plates bound to and spread on surface-immobilised thrombin or soluble collagen (Figs. 4A and 5A). Platelet spreading on either thrombin or collagen was equivalent for both female and male platelets, as demonstrated by the indistinguishable frequency distribution curves of platelet surface area on either surface (Figs. 4B and 5B). Our results demonstrate that equivalent levels of female or male platelets bound to surfaces coated with either 1, 5, or 50 μg/ml thrombin (Fig. 4C) or collagen (Fig. 5C).

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**Figure 6: Effect of sex on platelet P-selectin exposure and fibrinogen binding.** A) Washed platelets (2 × 10⁷/ml) from male and female mice were stimulated with either thrombin or convulxin. The degree of FITC-conjugated anti-P-selectin mAb or OG-fibrinogen binding was recorded via FACS analysis. Results are geometric mean fluorescence (mean ± SEM) from 4–5 experiments.
Sex difference in agonist-induced platelet secretion and fibrinogen binding

The role of sex differences in agonist-induced platelet secretion and fibrinogen binding was investigated by determining mouse platelet P-selectin exposure and fluorescently-labelled fibrinogen binding in response to stimulation with either the GPCR-agonist thrombin or the GPVI-agonist convulxin. Our results show that both fibrinogen binding and P-selectin exposure induced by either thrombin (0.06 – 0.005 U/ml) or convulxin (0.5 – 0.05 μg/ml) was equivalent for male and female mouse platelets (Fig. 6).

Sex difference in platelet aggregate formation on collagen or fibrinogen under shear flow

We next wished to investigate the role of sex in platelet accumulation and aggregation under shear flow conditions using an in vitro flow-based assay. Whole blood from mice was perfused over collagen- or fibrinogen-coated surfaces at a shear rate of 300 s⁻¹. Blood from female and male mice exhibited robust formation of densely packed platelet aggregates on collagen, while a single layer of spread platelets formed on fibrinogen (Fig. 7A). We utilised fluorescent microscopy to characterise DiOC₆ labelled mouse platelet accumulation on collagen or fibrinogen during perfusion of whole blood. Our results in Figure 7B demonstrate that an equivalent degree of female and male platelets bound to collagen or fibrinogen after 1, 2, or 3 min of flow.

A further set of experiments was designed to eliminate the potential effects of plasma proteins in this assay. Washed mouse platelets were reconstituted at a concentration of 3×10⁹/ml with washed red blood cells to a haematocrit of 50% before perfusion over a collagen-coated surface for 3 min at a shear rate of 300 s⁻¹. Our data demonstrate that an equivalent degree of platelet surface area coverage was observed on collagen under flow in the absence of plasma proteins for both female and male mouse platelets (Fig. 7C, D).

Discussion

Sex differences in the epidemiology of thromboembolic diseases have been described in a number of clinical settings. It has been established that the risk of CAD in women increases after menopause (26), although the pathophysiologic mechanisms explaining these findings have not been fully elucidated. There is both indirect and direct evidence that the sex steroid hormone 17β-estradiol (E₂) directly regulates platelet function, although there exists a degree of controversy surrounding this hypothesis as conflicting results have been reported (27–33). Alternatively, there is evidence that a level of hyperreactivity is intrinsic to female platelets (6, 7, 13, 16). Due to the fact that platelet reactivity is intimately involved in thrombus formation, inflammation, and atherosclerosis progression, it is vital to determine if sex differences, whether hormonal or intrinsic, play a role in platelet physiology. With regards to platelet adhesion, spreading, or aggregate formation under flow, our data would suggest that mouse platelet function is independent of sex.

Prior studies have examined the role of sex differences in mediating mouse platelet function in response to thrombin (16). Leng et al. found that female mouse platelets exhibited a lower threshold for reactivity to thrombin, as measured by the ability of exceedingly low doses of thrombin (0.005–0.01 U/ml) to preferentially enhance female versus male platelet binding to fibrinogen in solution (16). No differences in fibrinogen binding were observed at slightly higher doses of thrombin (0.02 U/ml). As PAR4 is the only murine PAR known to mediate signalling...

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Figure 7: Female and male mouse platelet aggregate formation on collagen under flow. A) Mouse blood anticoagulated with 40 μM PPACK and 10 U/ml heparin was perfused through a fibrillar-collagen-coated or fibrinogen-coated capillary at a shear rate of 300 s⁻¹ for 5 min. B) The degree of DiOC₆ labelled platelet deposition on collagen or fibrinogen was recorded during perfusion. C) Washed mouse platelets (3×10⁹/ml) from female/male paired littermates were reconstituted with autologous RBCs (50% v/v) prior to perfusion through fibrillar collagen-coated capillaries at a shear rate of 300 s⁻¹ for 3 min. D) Platelet surface coverage was quantified using Image J. Images are representative of 4–9 experiments; values are reported as mean ± SEM.
downstream of thrombin in mouse platelets, Leng et al. then went on to monitor fibrinogen binding to platelets that had been activated with a PAR4 activation peptide, yet failed to detect any sex difference for PAR4 agonist-induced fibrinogen binding. This data would suggest that female mouse platelets show a lower threshold for reactivity to thrombin in a PAR4-independent manner, although a possible mechanism by which thrombin acts independent of PAR4 in a sex-specific manner is currently unknown. We therefore designed our study to investigate if the sex differences observed by Leng et al. translated to functional differences in mouse platelet function by monitoring the ability of thrombin to potentiate female and male platelet binding to immobilised fibrinogen. Our results demonstrated that stimulation of platelets with thrombin potentiated platelet binding to fibrinogen, even at doses as low as 0.005 U/ml thrombin. However, we failed to detect a difference in the sexes with regards to platelet binding to either soluble or immobilised fibrinogen following stimulation with 0.005, 0.05 or 0.1 U/ml thrombin. These results suggest that the reported differences in female/male platelet fibrinogen binding in response to thrombin do not translate to a functional difference in the ability of thrombin to potentiate mouse platelet adhesion or spreading on immobilised fibrinogen.

Leng et al. then went on to show that female platelets exhibited a lower threshold for reactivity to the GPVI agonist collagen-related peptide (CRP), as measured by the ability of CRP to preferentially enhance female versus male platelet binding to fibrinogen in solution (16). Our data demonstrate that these reported sex differences do not translate to a detectable role in GPVI-mediated platelet P-selectin expression, platelet adhesion and spreading, or platelet binding and aggregation under shear flow. It is noteworthy that our shear flow studies utilised platelets both in isolation from plasma proteins as well as in whole blood, and demonstrated equivalent levels of adhesion and aggregation on collagen under shear flow for both female and male mouse platelets under both conditions.

Mouse platelet adhesion, spreading and flow assays are routinely used by researchers to delineate platelet signal transduction pathways. Our findings would suggest that sex differences play a negligible role in regulating these functional platelet responses. Whether sex differences can be detected in more subtle assays, such as calcium mobilisation or tyrosine phosphorylation of the effector enzyme phospholipase Cγ2, remains to be determined.

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What is known about this topic?

- The prognosis for coronary heart disease is far worse for women after myocardial infarction, coronary revascularisation, or thrombolytic therapy.
- Conflicting results have been published regarding the role of sex differences in platelet physiology.
- Sex has generally been ignored as a confounding variable in knockout mouse models of platelet activation and thrombotic disorders.

What does this paper add?

- Sex differences do not play a role in mouse platelet adhesion or spreading on fibrinogen, thrombin, or collagen.
- Sex differences do not play a role in the ability of the GPCR-agonist thrombin or GPVI-agonist convulxin to potentiate mouse platelet adhesion and spreading on fibrinogen.
- Sex differences do not play a role in mouse platelet aggregate formation on collagen or fibrinogen under shear.

References