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Platelet Characteristics Change With Aging: Role of Estrogen Receptor β

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Estrogen receptor beta (βER) is the predominant estrogen receptor in platelets. Experiments were designed to define phenotypic changes in platelets with aging following deletion of βER (βERKO). Blood was collected from wild-type and βERKO female mice at 4–7 (young) and 24–25 (aged) months of age. In young animals, total number of platelets, number of platelets containing RNA (reticulated platelets), aggregation, dense body adenosine triphosphate secretion, and alpha granular secretion were the same in both groups. With aging, total number of platelets decreased but reticulated platelets increased in βERKO mice; aggregation and dense granule adenosine triphosphate secretion decreased whereas basal expression of fibrinogen receptors increased with age in wild-type and βERKO mice. Basal expression of P-selectin and annexin V binding increased with aging only in βERKO mice; thrombin did not increase expression in these mice. Therefore, deletion of βER is associated with specific platelet functions, which are expressed only with age-associated reproductive senescence.

INCIDENCE of adverse thrombotic events such as stroke, myocardial infarction, deep venous thrombosis, and pulmonary embolism increases with age. These events are influenced by sex and estrogenic hormones (1–7). Increases in coagulation proteins such as fibrinogen and factors VIII and IX without concomitant increases in anticoagulants may contribute to changes in thrombosis (1). Platelets contribute to thrombosis by providing membrane surface for thrombin generation (8,9). The content of mitogenic and vasoactive factors in platelets, their ability to aggregate, and their activated secretion of adenosine triphosphate (ATP) from dense bodies changes with the hormonal transitions of puberty, differing between males and females and with surgical ovariectomy in females (10–12). However, little is known regarding how platelet functions change with natural transition of age-associated reproductive senescence.

Thrombogenic activity of platelets depends on the concentration of receptors and on receptor-activated signaling within platelets. Both estrogen receptor alpha (αER) and beta (βER) are found in bone marrow megakaryocytes and platelets (10,12–15). The two receptors are distinct proteins encoded by separate genes with differential tissue and cellular distribution (16). βER is the predominant estrogen receptor subtype in human and porcine platelets (17–19). The physiological ligand, 17β-estradiol, does not discriminate between αER and βER subtypes, which show high homology in the ligand-binding domain. Surgical menopause increases expression of estrogen receptors in pig platelets (10); platelets from these animals show increased aggregation and secretion (20). It is not known how estrogen receptors affect platelet activities in the settings of decreases in endogenous hormones as would occur with reproductive senescence of aging. Selective disruption of the predominant platelet estrogen receptor (βER) would provide a means of investigating the role of this receptor in platelet physiology with age-associated reproductive senescence. Therefore, experiments were designed to define the phenotype of platelets in wild-type (WT) and βER knockout mice prior to and following aging to reproductive senescence.

Methods

Animals

Female $\alpha ER^{+/+}/\beta ER^{+/+}$ (WT, $n = 10$) and $\alpha ER^{+/+}/\beta ER^{-/-}$ (βER knockout, $n = 10$) C57Bl/6 mice were obtained from Dr. Kenneth S. Korach, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Female mice were housed in stainless steel cages with groups of five animals in each, kept in 12-hour light/dark cycles with free access to water, and fed with normal laboratory mouse chow every morning. Experiments were approved by the Institutional Animal Care and Use Committee, Mayo Clinic College of Medicine, Rochester, Minnesota.

Materials

Phycoerythrin-conjugated hamster antimouse CD61-PE and rat antimouse CD62P-FITC monoclonal antibodies were purchased from PharMingen International (San Diego, CA). Fluorescein-conjugated chicken antihuman fibrinogen polyclonal antibody was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Collagen (equine tendon) was obtained from Helena Laboratories (Beaumont, TX). HEPES, Hanks' balanced salts, prostaglandin E_1, and

mouse thrombin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents used in this study were of analytical reagent grade.

Blood Collection

Mice were anesthetized for less than 1 minute with anesthetic ether in a closed chamber. Blood was collected from the retro-orbital sinus through heparin-coated capillary tubes into tubes containing 50 μL of acid citrate dextrose (ACD) solution formula A (Baxter Healthcare Corp., Deerfield, IL). Using this procedure, blood was collected from the same mice at 6–7 months of age (400–500 μL/mouse) and at 24 months of age (800–1100 μL/mouse). Platelets were counted in whole blood diluted in physiological saline (1:10 dilution) using a three-part differential Coulter counter (model T660; Coulter Corp., Miami, FL). All experiments were carried out on diluted blood, therefore, concentrations of endogenous hormones would be present but the concentration of endogenous hormones varied depending on the different preparations required for each assay.

Analysis of Reticulated Platelets

Reticulated platelets (those containing RNA) were counted by flow cytometry (11,20). The numbers of circulating reticulated platelets in WT and βER knockout mice are presented as percentage of total platelet number.

Platelet Aggregation

Aggregation was performed using an impedance method. Whole blood containing a defined number of platelets was diluted with physiological saline. Aggregation was induced with either collagen (6 μg/mL) or ATP (10 μM) in a whole blood aggregometer (model 560-VS; Chrono-Log; Havertown, PA). Due to the small volume of blood which could be obtained from young mice, aggregation was determined using a single concentration of collagen (6 μg/ml). However, in preliminary experiments, different concentrations of collagen (1, 3, 6, and 9 μg/mL) were used to establish a dose-response curve. It was determined, based on these experiments, that 6 μg/ml collagen was the effective dose giving 50% of the maximal response (ED_{50}). Gain for the impedance was set at 20 ohms, and maximal platelet aggregation (plateau response) results are presented as ohms (Ω).

Platelet Dense Granule Secretion

ATP secretion from dense granules in blood diluted 1:1000 in sterile Hanks' balanced salt solution was measured by luciferin bioluminescence in response to mouse thrombin (21). In preliminary experiments, different concentrations of mouse thrombin (0.02, 0.1, 0.2, 0.4, and 2 U) were used to obtain a dose-response curve for secretion. It was determined, based on these experiments, that 0.2 U of thrombin was the effective dose giving 50% of the maximal response (ED_{50}), so 0.2 U was used for all secretion experiments. Data were acquired for 2–5 min at 1-second intervals, and rate of release is expressed as nanomoles per platelet per minute, whereas total release (plateau response) is expressed as nanomoles per platelet.

Expression of Membrane Proteins in Activated Platelets

Expression of the adhesion molecules, P-selectin, and fibrinogen binding was determined with use of rat monoclonal antimouse P-selectin FITC antibody and chicken antihuman fibrinogen FITC antibody, respectively (11). To measure membrane phosphatidylserine, platelets were incubated with annexin V-FITC in the dark for 30 minutes, fixed with 1% formaldehyde for 30 minutes at room temperature, and then centrifuged at 2300 *g* for 10 minutes. Supernatants were discarded. Fixed platelets were suspended in 1 mL of 1X phosphate-buffered saline, and annexin-V-positive platelets were measured by flow cytometry. 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 (11). For activated expression of P-selectin, fibrinogen binding, and annexin V binding, platelets were incubated with mouse thrombin (0.2 U) and collagen (6 μg/ml) for 10 minutes.

Statistical Analysis

All values are presented as mean ± standard error of the mean. Statistical significance was evaluated by one-way analysis of variance followed by Bonferroni's multiple comparison test and Student's *t* test unpaired observations; differences at a level of $p < .05$ are considered significant. All experiments were carried out independently using 4–10 individual mice from WT and βERKO colonies.

Results

Neither numbers of circulating platelets nor percentage of platelets containing messenger RNA (mRNA, reticulated) differed between young WT and βERKO mice. Platelet number and percentage of reticulated platelets did not change with aging in WT mice, whereas platelet number decreased and percentage of reticulated platelets increased significantly with aging in βERKO mice (Figure 1). Mean platelet volumes were similar among young and aged WT and βERKO mice (data not shown).

Platelet aggregation was not significantly different between young WT and βERKO mice (Figure 2C). There were no statistically significant differences in lag time to maximal aggregation between groups. However, aggregation, both the rate at which it proceeded and the extent (maximal aggregation), decreased significantly with aging in βERKO mice (Figure 2).

Platelets from young WT and βERKO mice showed nearly equal dense body secretory rates and yields in response to thrombin. In both groups of mice, the rate of ATP secretion decreased significantly with aging (Figure 3). In addition, the rate of secretion was significantly lower in platelets from aged βERKO mice compared to aged WT mice (Figure 3). However, the total content of ATP decreased similarly with age in both WT and βERKO mice, such that with aging there were no statistically significant differences in the ATP content between platelets from young or aged WT and βERKO mice (Figure 3).

Basal and thrombin-stimulated expression of P-selectin, fibrinogen binding, and phosphatidylserine (annexin V binding) was similar between young WT and βERKO mice (Figure 4). However, basal percentage of platelets positive for P-selectin and annexin V increased significantly in βERKO mice with aging (Figure 4, A and C). Basal fibrinogen-positive platelets increased significantly in both WT and

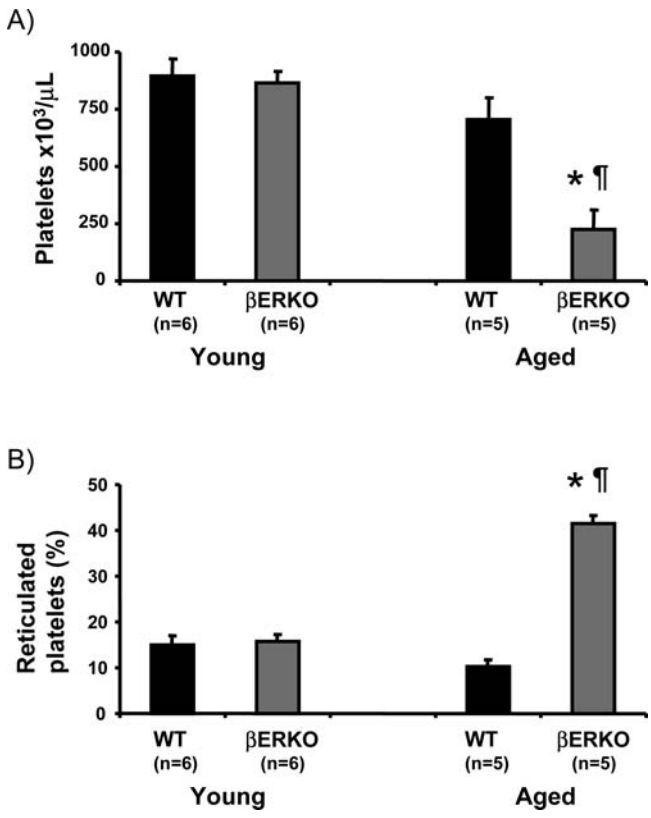

Figure 1. Total number of platelets (**A**) and percentage of total platelets containing RNA (reticulated platelets, **B**) in young and aged wild-type (WT) and βERKO mice. Total number of neither platelets nor reticulated platelets changed with age in WT mice. However, the number of platelets decreased but the percentage of reticulated platelets increased ($p < .05$) significantly with age in βERKO mice. Data are presented as mean ± standard error of the mean. *$p <$ .05, between aged WT and βERKO mice; ¶$p < .05$, between young and aged βERKO mice.

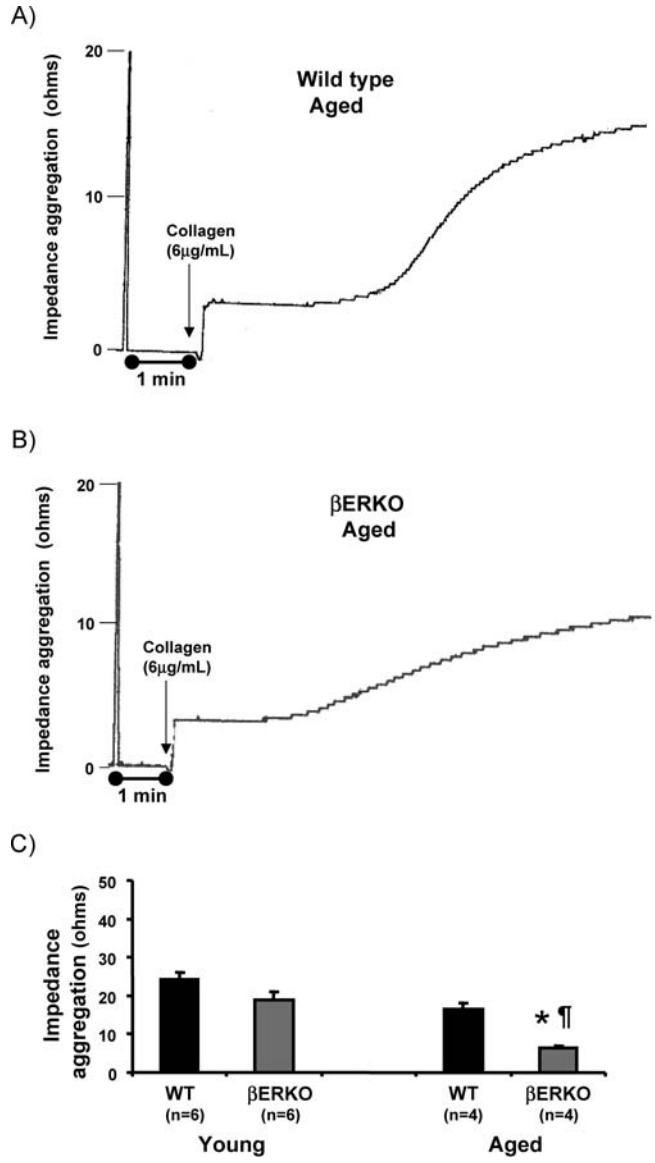

Figure 2. Representative traces of platelet aggregation in whole blood in response to collagen (6 μg/mL) in aged wild-type (WT, **A**) and βERKO (**B**) mice. Cumulative data of platelet aggregation measured as impedance (ohms) from young and aged WT and βERKO mice are shown as a bar diagram (**C**). Maximal aggregation was similar between platelets derived from young WT and βERKO mice (C, left bars), whereas maximal aggregation decreased significantly ($p < .05$) in βERKO mice with aging (C, right bars). Data are presented as mean ± standard error of the mean. *$p < .05$, between aged WT and βERKO mice; ¶$p < .05$, between aggregation in young and aged βERKO mice.

βERKO mice with aging (Figure 4B), and percentage of fibrinogen-positive platelets increased comparably in response to thrombin stimulation in both groups (Figure 4). Even though collagen induced platelet aggregation, it did not activate expression of P-selectin, fibrinogen binding, or annexin V binding (data not shown).

Discussion

Results of this study demonstrate the phenotypic consequences of disruption of βER on platelet characteristics and functions, and indicate that the phenotype may not be expressed until reproductive senescence with natural aging. This phenotype includes changes in platelet number, reticulated platelets, aggregation, and secretion from both dense and alpha granules with aging in βERKO compared to WT mice. These changes represent a consequence of loss of βER that may only express as a phenotype with age-associated loss of ovarian hormones.

Decreases in the total number of platelets with simultaneous increases in the number of reticulated platelets with age in βERKO mice is characteristic of what is observed in destructive platelet disorders such as immune thrombocytopenic purpura (22). Phosphatidylserine expression on platelets is implicated in platelet clearance. Therefore, increased surface expression of phosphatidylserine under basal, i.e., unstimulated conditions, in platelets derived from aged βERKO mice, is consistent with shortened platelet survival (23). Platelets are released into the circulation as a consequence of megakaryocytic fragmentation. Although estrogen treatment enhances megakaryocytes and proplatelet formation in vitro (14), the increase in percentage of reticulated platelets in aged animals probably reflects a control mechanism independent of estrogenic regulation, as the animals were reproductively senescent and lacked βER.

Under basal conditions, P-selectin and glycoprotein IIb/IIIa are present in α-granules and the plasma membrane of

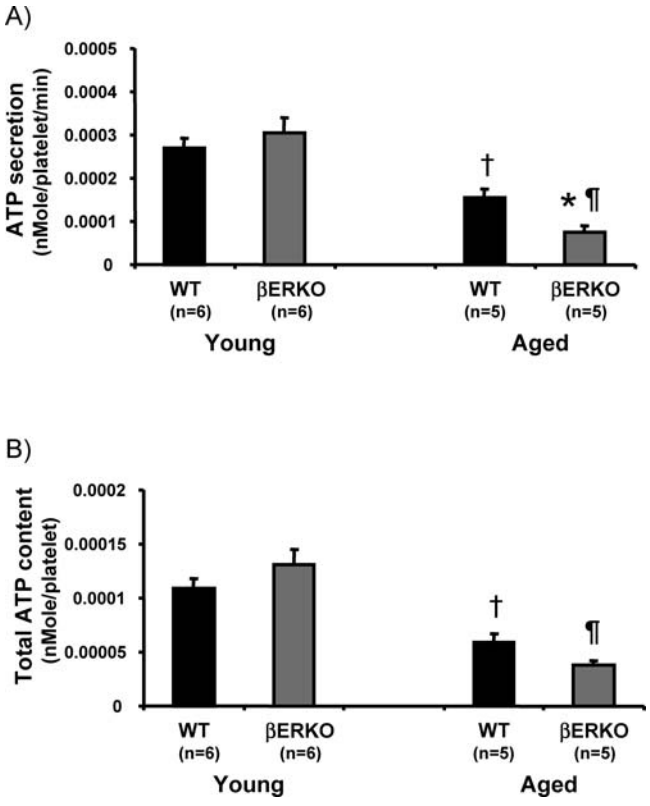

Figure 3. Cumulative rate (**A**) and maximum (**B**) dense body adenosine triphosphate secretion in response to thrombin (0.2 U) in platelets derived from young and aged wild-type (WT) and βERKO mice. The rate and maximum secretion were similar in young WT and βERKO mice, whereas adenosine triphosphate secretion decreased significantly ($p < .05$) in both groups of mice with aging. Data are presented as mean ± standard error of the mean. *$p < .05$, between aged WT and βERKO mice; ¶$p < .05$, between young and aged βERKO mice; †$p < .05$, between young and aged WT mice.

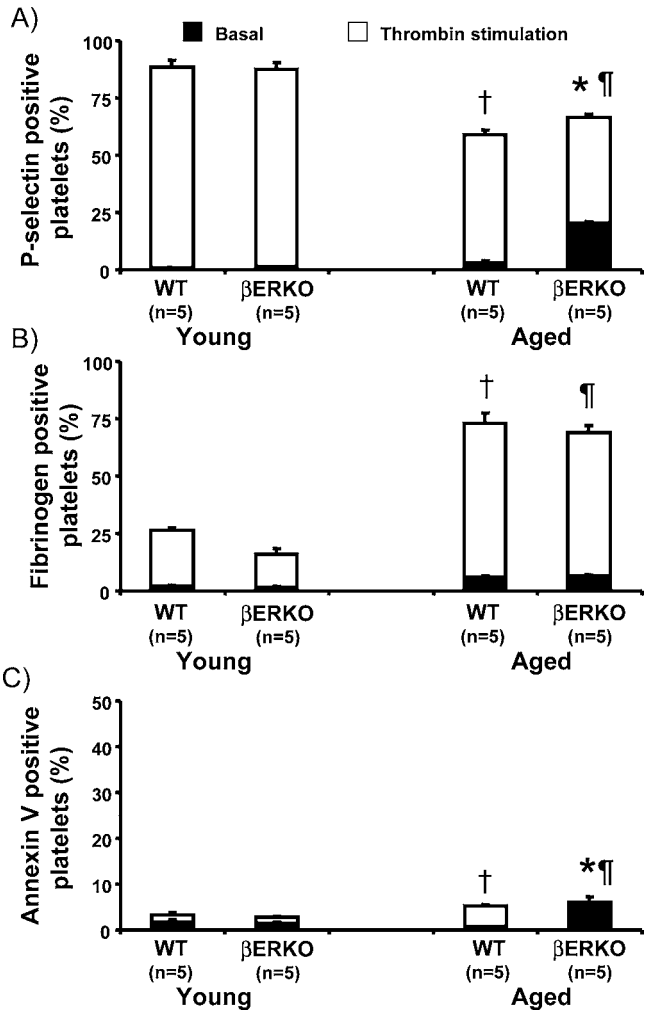

Figure 4. Expression of P-selectin (**A**), fibrinogen binding (**B**) and phosphatidylserine (annexin V binding, **C**) in platelets from young and aged wild-type (WT) and βERKO mice. Basal expression is shown as filled bars; thrombin (0.2 U)-stimulated expression is shown as open bars. Percentage of platelets expressing P-selectin and phosphatidylserine under basal conditions increased only ($p < .05$) in βERKO mice with aging. Fibrinogen binding increased significantly in both groups of animals with aging. Thrombin stimulation resulted in comparable expression of P-selectin and annexin V binding in aged WT and βERKO mice. However, the increase above basal expression was lower in βERKO than in WT mice. Data are presented as mean ± standard error of the mean. †$p < .05$, between basal and stimulated expression in young and aged WT mice; ¶$p < .05$, between basal and stimulated expression in young and aged βERKO mice; *$p < .05$, between basal and stimulated expression in aged WT and βERKO mice.

platelets. These proteins are rapidly expressed in the outer membrane of activated platelets (24,25). Platelet aggregation is induced by the activation of soluble fibrinogen to its platelet receptor (glycoprotein IIb/IIIa or integrin αIIb/β3). The significant increase in fibrinogen binding in both WT and βERKO mice would reflect increased platelet activation with age. However, because P-selectin expression increased only in βERKO mice with aging suggests that: a) control of alpha granule secretion of P-selectin and fibrinogen receptors (as measured indirectly by fibrinogen binding) is not the same, and b) these processes are modulated by βER-associated processes. It is not possible at this time to distinguish between a direct effect on the megakaryocytes and indirect effects on either megakaryocytes of blood platelets from cytokines released from vascular endothelium or other blood elements.

Thrombin activates expression of P-selectin and membrane phosphatidylserine. Therefore, the significant increase of thrombin induced P-selectin and annexin V binding in aged WT mice may be due to less basal activation in vivo. Thrombin-activated expression of these proteins may be decreased in aged βERKO mice because of an already activated state under basal conditions, suggesting a maximal capacity of expression per platelet. A recent study demonstrated that 17β-estradiol enhances thrombin-induced platelet aggregation by activating integrin αIIb/β3 through βER and Src kinase in humans (15), thus implicating an association of βER with changes in platelet aggregability through a specific intracellular pathway. The fact that, in response to thrombin, expression of P-selectin decreased whereas fibrinogen binding increased, further supports the concept that control of α-granule secretion and membrane receptor (integrin αIIb/β3) regulation may proceed through different cellular signaling pathways.

Platelet aggregation and exocytosis of secretory granules are energy-dependent processes (26,27). Deficiencies in mitochondrial bioenergetics are reported in platelets from

aged individuals (27). Decreased aggregation and dense granular ATP secretion with aging are consistent with observations in human platelets (28). Because age-associated decreases in platelet aggregation and maximal and rate of ATP secretion were highly significant in βERKO mice suggests that ER-mediated signaling is required to maintain these energy-dependent functions.

The present study was conducted with platelets from female animals only. Previous work from our group demonstrates sexual dimorphism in platelet characteristics with hormonal changes of puberty. Therefore, it might be expected that, with changes in the hormonal profile with aging, platelets from males may develop more ''female'' characteristics. Development of cardiovascular disease in males exceeds that of females, and polymorphisms in αER and methylation of this receptor with aging are associated with accelerated atherosclerotic processes in males (29–31). The impact of βERKO on platelet functions in males remains to be determined.

In conclusion, loss of βER is associated with decreases in platelet number, increases in reticulated platelets and basal surface adhesion molecules, and reduced activated secretion of ATP from dense and P-selectin from alpha granules with aging. These results demonstrate the phenotypic consequences of deletion of βER, the predominant estrogen receptor on platelets, longitudinally with natural aging to reproductive senescence. Platelets are important determinants of propensity for thrombosis because activated platelets accelerate thrombin generation (32). Results of this study suggest that a genetic disruption of an estrogen steroid receptor could affect an individual's propensity to thrombose with changes in hormonal status characteristic of reproductive senescence of aging.

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