

Loss of estrogen receptor β decreases mitochondrial energetic potential and increases thrombogenicity of platelets in aged female mice

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Abstract Platelets derived from aged (reproductively senescent) female mice with genetic deletion of estrogen receptor beta (β ER) are more thrombogenic than those from age-matched wild-type (WT) mice. Intracellular processes contributing to this increased thrombogenicity are not known. Experiments were

designed to identify subcellular localization of estrogen receptors and evaluate both glycolytic and mitochondrial energetic processes which might affect platelet activation. Platelets and blood from aged (22–24 months) WT and estrogen receptor β knock-out (β ERKO) female mice were used in this study. Body, spleen weight, and serum concentrations of follicle-stimulating hormone and 17β -estradiol were comparable between WT and β ERKO mice. Number of spontaneous deaths was greater in the β ERKO colony (50% compared to 30% in WT) over the course of 24 months. In resting (nonactivated) platelets, estrogen receptors did not appear to colocalize with mitochondria by immunostaining. Lactate production and mitochondrial membrane potential of intact platelets were similar in both groups of mice. However, activities of NADH dehydrogenase, cytochrome *bc₁* complex, and cytochrome *c* oxidase of the electron transport chain were reduced in mitochondria isolated from platelets from β ERKO compared to WT mice. There were a significantly higher number of phosphatidylserine-expressing platelet-derived microvesicles in the plasma and a greater thrombin-generating capacity in β ERKO compared to WT mice. These results suggest that deficiencies in β ER affect energy metabolism of platelets resulting in greater production of circulating thrombogenic microvesicles and could potentially explain increased predisposition to thromboembolism in some elderly females.

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Introduction

Incidence of cardiovascular disease, including thrombosis and associated events such as stroke, myocardial infarction, and pulmonary embolism, increases in women following the decline of circulating estrogen associated with menopause (Bushnell et al. 2006; Hu et al. 1999; Mari et al. 2007; Rexrode et al. 2007). The cellular basis for this increased risk is not entirely clear. Cellular responses to estrogen are initiated mostly through binding to one or both estrogen receptors α (α ER) and β (β ER). Platelets, cytoplasmic fragments of bone marrow megakaryocytes, are required for normal hemostasis and thrombosis. Although both estrogen receptors are present in bone marrow megakaryocytes and platelets, β ER is the predominant estrogen receptor subtype in anucleated platelets and appears to play a role in mediating aging-associated changes in platelet number and activity (Bracamonte et al. 2002; Jayachandran et al. 2003; Jayachandran and Miller 2002; Khetawat et al. 2000; Moro et al. 2005; Nagata et al. 2003). Genetic deletion of β ER did not affect platelet characteristics in young female mice, but in aged female mice, numbers of circulating platelets, platelet aggregation, and ATP secretion from dense granules decreased, whereas number of young (reticulated) platelets and platelet procoagulant surface expression of P-selectin and phosphatidylserine increased (Jayachandran et al. 2005).

Activation processes (aggregation and secretion) of platelets utilize energy provided by glycolysis and oxidative phosphorylation (Holmsen 1975; Merlo-Pich et al. 2004; Salganicoff and Fukami 1972). Therefore, an energetic defect within platelets associated with aging and ER deficiency may explain increased thrombogenicity of blood in the elderly female. Both α ER and β ER are associated with neuronal and cardiac mitochondria (Chen et al. 2004; Yang et al. 2004) and could regulate genes required for mitochondrial function (O'Lone et al. 2007). Activated platelets contribute to thrombotic complications by providing membrane phosphatidylserine required for thrombin generation (Bouchard and Tracy 2001; Butenas and Mann 2002). During

activation, platelets shed sealed submicron-sized plasma membrane vesicles called microvesicles (or microparticles), which express phosphatidylserine on their surface and are thus thrombogenic. The present study was designed to determine how the loss of β ER could affect energy metabolism in platelets and thus provide a link between the number and characteristics of circulating microvesicles in aged female mice. It was hypothesized that loss of β ER would affect platelet energy metabolism to increase shedding of thrombogenic microvesicles.

Methods

Animals Three- to 4-month-old female α ER^{+/+}/ β ER^{+/+} (wild-type (WT)) and α ER^{+/+}/ β ER^{-/-} (β ER knockout (β ERKO)), C57BL/6 mice were obtained from the colony at National Institutes of Environmental Health Sciences, Research Triangle Park, NC, USA. These mice have an insertion disruption in exon3 of the mouse β ER gene. The insert was designed to not encode any protein, and the mRNA analysis of these mice shows that all mRNA is out of frame. There is no evidence that measurable β ER protein is produced in these mice. Animals were housed from the time of arrival until used in experiments (23–24 months of age) in stainless steel cages with five animals per cage and kept in 12-hour light/dark cycles at Mayo Clinic, Rochester, MN, USA, with free access to food (laboratory mouse chow) and water. Experiments were approved by the Institutional Animal Care and Use Committee, Mayo Clinic, Rochester, MN, USA.

Blood collection Mice were anesthetized for less than 1 min with isoflurane in a closed chamber. Blood was collected from the retro-orbital sinus plexus through siliconized capillary tubes coated with hirudin and tick anticoagulant peptide into 1.5-mL polypropylene tubes containing 5 μ L of 100 μ M hirudin and 1 mM tick anticoagulant peptide. Tubes without anticoagulant were used for serum preparation. Plasma and serum were prepared by centrifugation for 15 min at 3,000 \times g and then stored at -20°C until analysis.

Hormone assays Serum follicle stimulating hormone (FSH) and plasma 17 β -estradiol were measured with rodent ELISA kits from Endocrine Technologies Inc., San Francisco, CA, USA.

Immunofluorescence Blood was diluted in an equal volume of modified Tyrode's solution (NaCl 137 mM, KCl 2.7 mM, NaHCO₃ 11.9 mM, NaH₂PO₄ 0.41 mM, MgCl₂ 1 mM, glucose 5.5 mM, HEPES 5 mM, pH 7.4) and centrifuged at 150×g for 15 min to obtain platelet-rich plasma (PRP). Samples (100 μL) of PRP adjusted with Tyrode's solution 1×10⁵ platelets/μl were stained simultaneously with Mitotracker Red (200 nM) and plated onto 25-mm poly-L-lysine-coated glass coverslips at room temperature for 20 min. The adherent platelets were rinsed briefly with Tyrode's solution and fixed in 2% paraformaldehyde for 10 min. After fixation, platelets were permeabilized with 0.2% Triton X-100 for 10 min and then rinsed three times with Tyrode's solution. Nonspecific antibody binding sites were blocked with blocking buffer (2% bovine serum albumin and 5% normal goat serum) for 1 h. Platelets were then incubated overnight at 4°C with primary αER or βER antibodies at 1:50 dilution in blocking buffer. Platelets were then rinsed three times for 10 min with blocking buffer and incubated at room temperature in darkness for 1 h in goat antirabbit IgG-conjugated Alexa Fluor-488 secondary antibody (1:200 dilution). After incubation, coverslips were rinsed three times and mounted on glass slides using ProLong Gold antifade reagent. For each experiment, controls, in which the primary antibody or Mitotracker Red were omitted, were also processed. Images were obtained using a Zeiss LSM 510 confocal laser scanning microscope equipped with a Zeiss 100×/1.4 numerical aperture oil objective and configured for dual-excitation and emission of laser signals simultaneously with DIC imaging. Optical slices, 0.8 μm in thickness, were obtained of discoid platelets.

Lactate assay PRP was prepared as described above and divided into equal volumes containing the same number of platelets into one of three tubes: Tube-1 for control (basal), tube-2 contained antimycin A (100 μM for 1 h) to inhibit mitochondrial respiratory chain, and tube-3 with collagen (6 μg/mL for 5 min) to induce platelet activation. After all treatments, PRP was centrifuged to pellet platelets at 2,600×g for 15 min. Platelet poor plasma was separated into another tube. Pelleted platelets were washed with tyrode buffer and then resuspended with lysis buffer and lysed by passing through 26-gauge needle for

8–10 times. Lactate concentration of platelet lysate and platelet poor plasma from control, Antimycin A, and collagen-treated samples was measured using lactate assay kit from Biovision, Mountain View, CA, USA.

Mitochondrial membrane potential in unstimulated platelets Membrane potential of mitochondria in intact platelets was determined in freshly prepared platelet-rich plasma by flow cytometry (FACSCanto™) using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; mitochondrial membrane potential detection kit from Cell Technology Inc., Mountain View, CA, USA). Platelets were gated by forward and size scatter. Mitochondrial membrane potential is expressed as a percentage of polarized (high-red fluorescence), partially polarized (high-red and green fluorescence), and depolarized (high green fluorescence) mitochondria.

Superoxide (O₂⁻) generation The rate of O₂⁻ generation from isolated mitochondria of platelets was measured as superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction assay using a modified method (Dzeja et al. 2003). The reaction mixture contains 0.1 M potassium phosphate (pH 7.4), 130 μM acetylated cytochrome *c*, 5 μM rotenone, 20 mM glutamate/malate, and 50–60 μg/mL mitochondrial protein. One hundred units of SOD/mL were added to the reference cuvette. Cytochrome *c* reduction was recorded at 37°C for 10 min by monitoring absorbance at 550–540 nm in the presence or absence of SOD. The production of O₂⁻ was estimated with the extinction coefficient of 19 mM⁻¹ cm⁻¹. Rate of superoxide generation is expressed as nanomoles (nmoles) per minute per milligram protein.

Activity of isolated mitochondrial enzymes Activities of electron transport chain (ETC) complexes were determined as described previously (Preston et al. 2008). In brief, platelets were disrupted with two cycles of 30 seconds sonication (Branson sonifier with microtip, at level 4) and centrifuged for 15 min at 1,000×g to pellet unbroken cells and debris. The isolated mitochondria were disrupted by three rapid freeze-thaw cycles and treated with 1 mM *n*-dodecyl-β-D-maltoside. Activity of complex I (NADH dehydrogenase) was measured by the rotenone-sensitive reduction of ubiquinone-1 (Darley-Usmar

Table 1 Baseline characteristics of mice

	Wild type ($n=8-10$)	β ERKO ($n=8-10$)
Age (months)	23–24	23–24
Body weight (g)	33 \pm 1.8	33 \pm 2.3
Spleen weight (mg)	189 \pm 37	322 \pm 103
Serum FSH (ng/mL)	0.7 \pm 0.1	0.4 \pm 0.1
Plasma 17 β -estradiol (pg/mL)	6 \pm 0.2	8 \pm 0.9
Number of deaths in house (from 4 months to 24 months)	6/20	10/20

Data are presented as mean \pm SEM

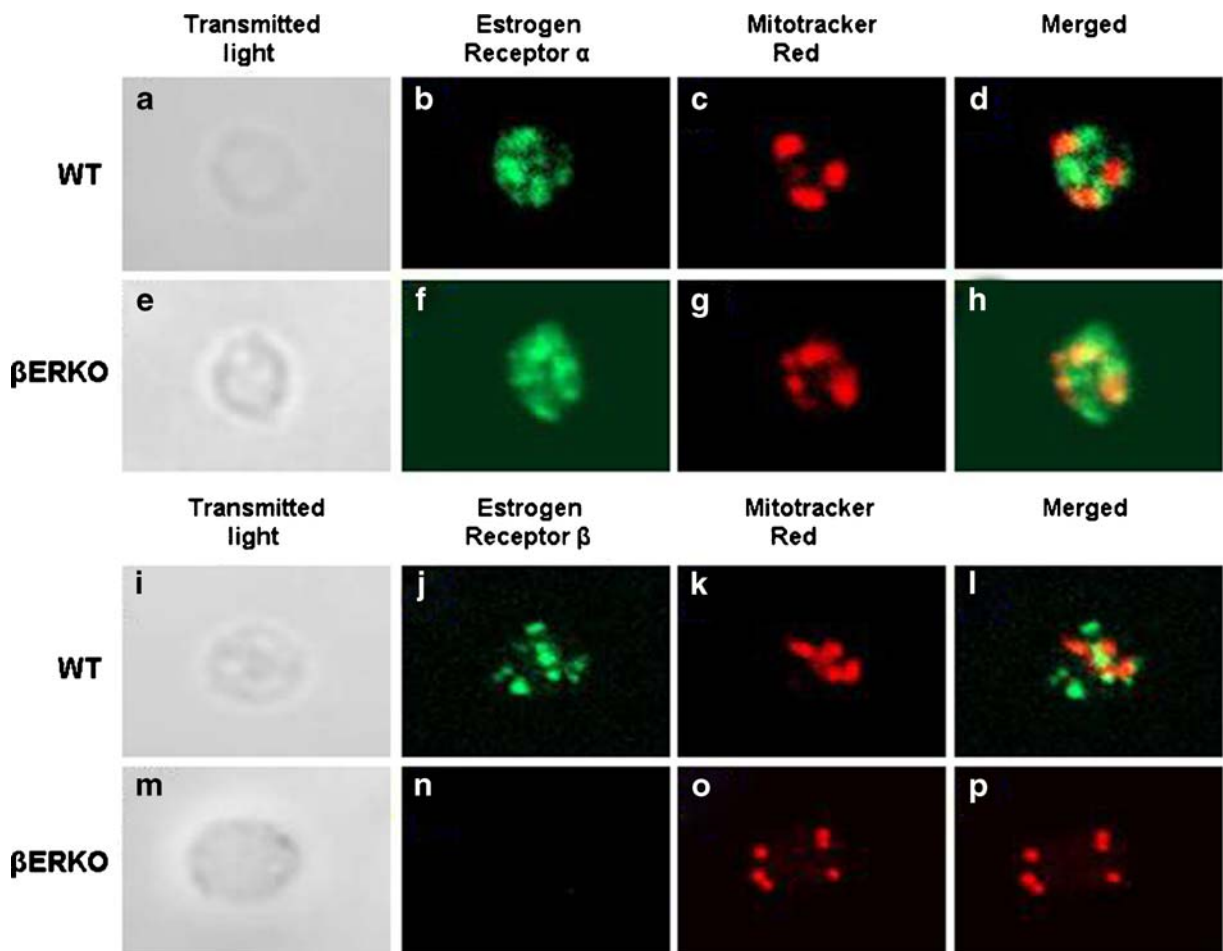


Fig. 1 Confocal laser-scanning microscopic images of immunolocalization of α ER and β ER in resting mouse platelets. Shown are individual nonactivated platelets from wild type ($a-d$ for α ER, $i-l$ for β ER) and β ERKO mice ($e-h$ for α ER, $m-p$ for β ER). *Left panels* show differential interference-contrast

images of each platelet (a, e, i, m). Estrogen receptor staining is shown in *green*. All were costained with Mitotracker Red to identify mitochondria (c, g, k, o), shown in *red*. Merged images showing signals from both corresponding fluorescent channels are shown in d, h, l , and p

Table 2 Lactate production and mitochondrial membrane potential in platelets from aged female wild type (WT) and estrogen receptor β knockout (β ERKO) mice (data are shown as mean \pm SEM)

	WT (n=8)	β ERKO (n=8)
Lactate production (nmol/ μ g protein)		
Control	0.06 \pm 0.01	0.05 \pm 0.01
Antimycin A	0.12 \pm 0.03	0.10 \pm 0.02
Collagen	0.13 \pm 0.02*	0.12 \pm 0.02*
Mitochondrial membrane potential (% number of mitochondria)		
Polarized	19 \pm 9	32 \pm 11
Partially polarized	8 \pm 2	6 \pm 2
Depolarized	56 \pm 7	45 \pm 10

* P <0.05, statistically significant from control

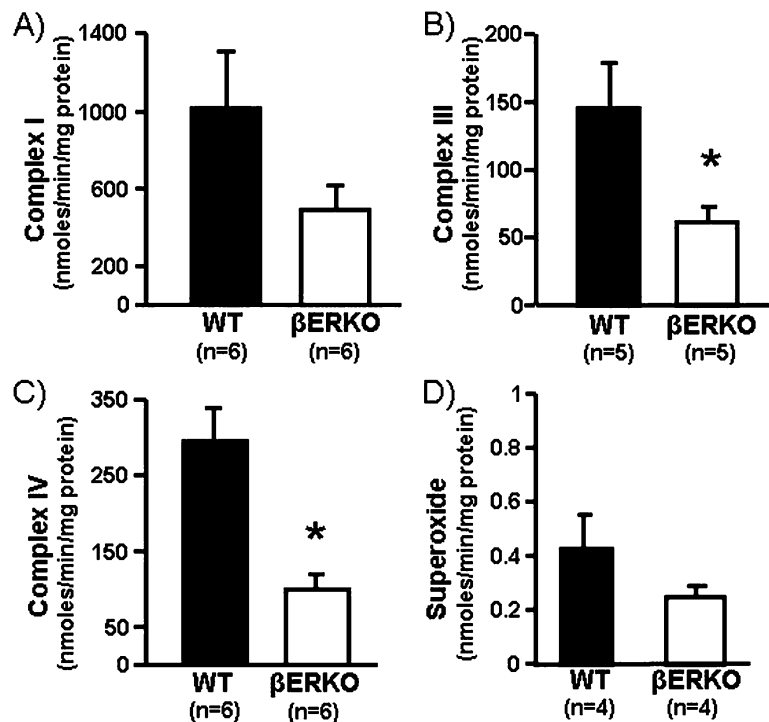
1987). Activity of complex III (cytochrome bc_1 complex) was determined by measuring reduction of decylubiquinol–cytochrome c (Kwong and Sohal 2000; Trumpower and Edwards 1979). Activity of complex IV (cytochrome c oxidase) activity was measured by a colorimetric assay kit (Sigma-Aldrich Inc., Saint Louis, MO). Activities of ETC complexes are expressed as nanomoles per minute per milligram protein.

Isolation of plasma microvesicles (microparticles)

Platelet-free plasma was prepared by double centrifugation of anticoagulated blood at 3,000 \times g for 15 min.

After the platelet count ≤ 1 was verified by Coulter counter and flow cytometry, the plasma sample (0.2 mL) was centrifuged at 60,000 \times g for 30 min. Plasma supernatants were stored at -80°C for other analysis. Pelleted microvesicles were resuspended with 0.8 mL of twice-filtered (0.2 μm) Hanks'/HEPES buffer pH 7.4, vortexed for 2 min, and centrifuged again at 60,000 \times g for 30 min to remove plasma contaminants. After centrifugation, the supernatant was discarded, and the pellet containing the microvesicles was suspended in the above buffer and vortexed for 1–2 min prior to analysis by electron microscopy and flow cytometry.

Fig. 2 Cumulative data of activity of complex I (a; NADH dehydrogenase), complex III (b; cytochrome bc_1 complex), and complex IV (c; cytochrome c oxidase) of the electron transport chain and rate of superoxide generation (d) in mitochondria isolated from platelets of aged WT and β ERKO mice. * P <0.05 denotes statistical significance between aged WT and β ERKO mice



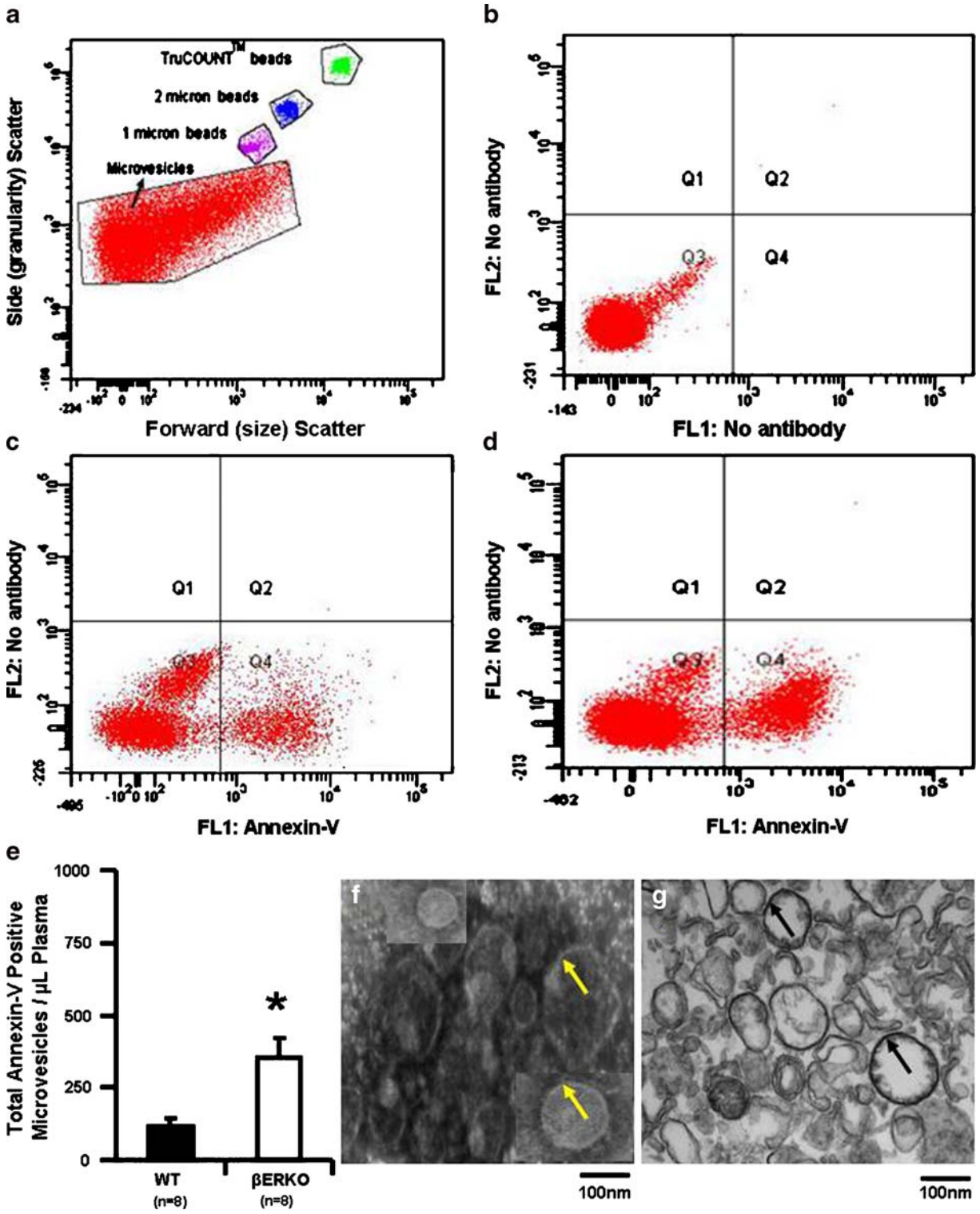


Fig. 3 Representative scatter plot of microvesicles obtained by FACSCanto™ flow cytometry. **a** Gates of microvesicles with and without fluorescein-conjugated antibodies and calibration (size and TruCount™ Beads) beads. **b, c, d** Representative quadrants derived from the microvesicle gate shown in **a**. Microvesicles only shown in **b**; Annexin-V-FITC labeled microvesicles from wild type (**c**) and β ERKO (**d**) mice. Phosphatidylserine (annexin-V)-negative ($Q3$) and positive ($Q4$) microvesicles from WT and β ERKO mice. **e** Cumulative data of total number of phosphatidylserine positive microvesicles from aged WT and β ERKO mice. Representative images of scanning (**f**) and transmission (**g**) electron microscopy of isolated microvesicles from mouse blood. Arrowheads indicate membranes. * $P < 0.05$ denotes significant difference between aged WT and β ERKO mice

Electron microscopy Isolated microvesicles were identified by scanning and transmission electron microscopy as described previously for microvesicles from human (Jayachandran et al. 2008).

Flow cytometry Identification and characterization of isolated microvesicles from blood was described previously (Jayachandran et al. 2008). In brief, a FACSCanto™ (BD Biosciences, San Jose, CA) was used to define microvesicles by size and positive fluorescence. Gates to define size were set with an internal standard of 1- and 2- μ m beads (Sigma-Aldrich) and TruCOUNT™ beads (BD Biosciences, San Jose, CA). All buffers and antibodies were filtered twice through 0.2- μ m filter to eliminate chemical particles and reduce instrument noise. Isolated microvesicles (50 μ L) were incubated with 4 μ L of fluorescein conjugated annexin-V and/or combination of annexin-V fluorescein with phycoerythrin (PE)-conjugated platelet-specific antibodies (i.e., CD61 or CD41) and/or isotype control for 30 min. Once stained, microvesicles were fixed with 400 μ L of 1% paraformaldehyde for 15 min, and 50 μ L of TruCOUNT™ beads were added to enable intensities of isotype control and absolute vesicle/particle counts. The threshold for positively stained microvesicles was set using isotype control antibodies conjugated with PE. In addition, microvesicles prepared and stained in phosphate-buffered saline or HEPES buffered saline pH 7.4 without calcium were used as negative controls for annexin-V positivity. Flow cytometric acquisition of microvesicles was stopped automatically after acquiring 30,000 events or after 3 min. Outer leaflet phosphatidylserine and platelet-derived microvesicles were identified with

annexin-V-Fluorescein isothiocyanate (FITC) and hamster antimouse CD61 (integrin β_3)-phycoerythrin (CD61-PE) and/or rat antimouse CD41 (α IIb)-PE, respectively (Jayachandran et al. 2008). The absolute number of microvesicles was calculated from the number of events in the region containing microvesicles divided by the number of events in the calibration bead region times the number of calibration beads per test volume (Jayachandran et al. 2008). Phosphatidylserine and platelet-derived microvesicles are expressed as number per microliter plasma.

Procoagulant activity Microvesicles (20,000 in Hanks'/HEPES) were incubated in Tyrode's solution containing 5 nM human Factor Xa and 10 nM human Factor Va at 37°C for 3 min. Then, 2- μ M human prothrombin and 50- μ M fluorogenic substrate (D-VPR-ANSNH-C₄H₉ • 2 HCl) were added, and the change in fluorescence was measured immediately with $\lambda_{ex}=355$ and $\lambda_{em}=450$ nm for 10 min. Thrombin generation is expressed as relative fluorescence vs time.

Materials Antibodies were purchased as follows: FITC- and PE-conjugated purified recombinant annexin-V, PE-conjugated hamster antimouse CD61 (CD61-PE), and rat antimouse CD41 (CD41-PE) monoclonal antibodies were from BD PharMingen International, San Diego, CA, USA. Rabbit antihuman estrogen receptor α (H184) and β (H150; recognizes 150 amino acids of the N terminus of the β -receptor) polyclonal antibodies were from Santacruz Biotechnology, Santa Cruz, CA, USA. Rabbit antihuman estrogen receptor β (ERb11-A; recognizes 16 amino acids on the C terminus) was from Alpha Diagnostic International, San Antonio, TX, USA. Mitotracker Red CMXRos, ProLong Gold antifade reagent, Alexa Fluor-conjugated goat antirabbit IgG, and normal goat serum were from Molecular Probes, Eugene, OR. Collagen (equine tendon) was from Helena Laboratories, Beaumont, TX, USA. HEPES, Hanks' balanced salts, mouse thrombin, bovine serum albumin, and poly-L-lysine were purchased from Sigma Chemical Co., St. Louis, MO, USA. Lactate assay kit was from Biovision, Mountain View, CA, USA. Paraformaldehyde (16% solution, EM grade) purchased from Electron Microscopy Sciences, Hatfield, PA, USA. All other reagents and solvents used in this study were of analytical/reagents grade.

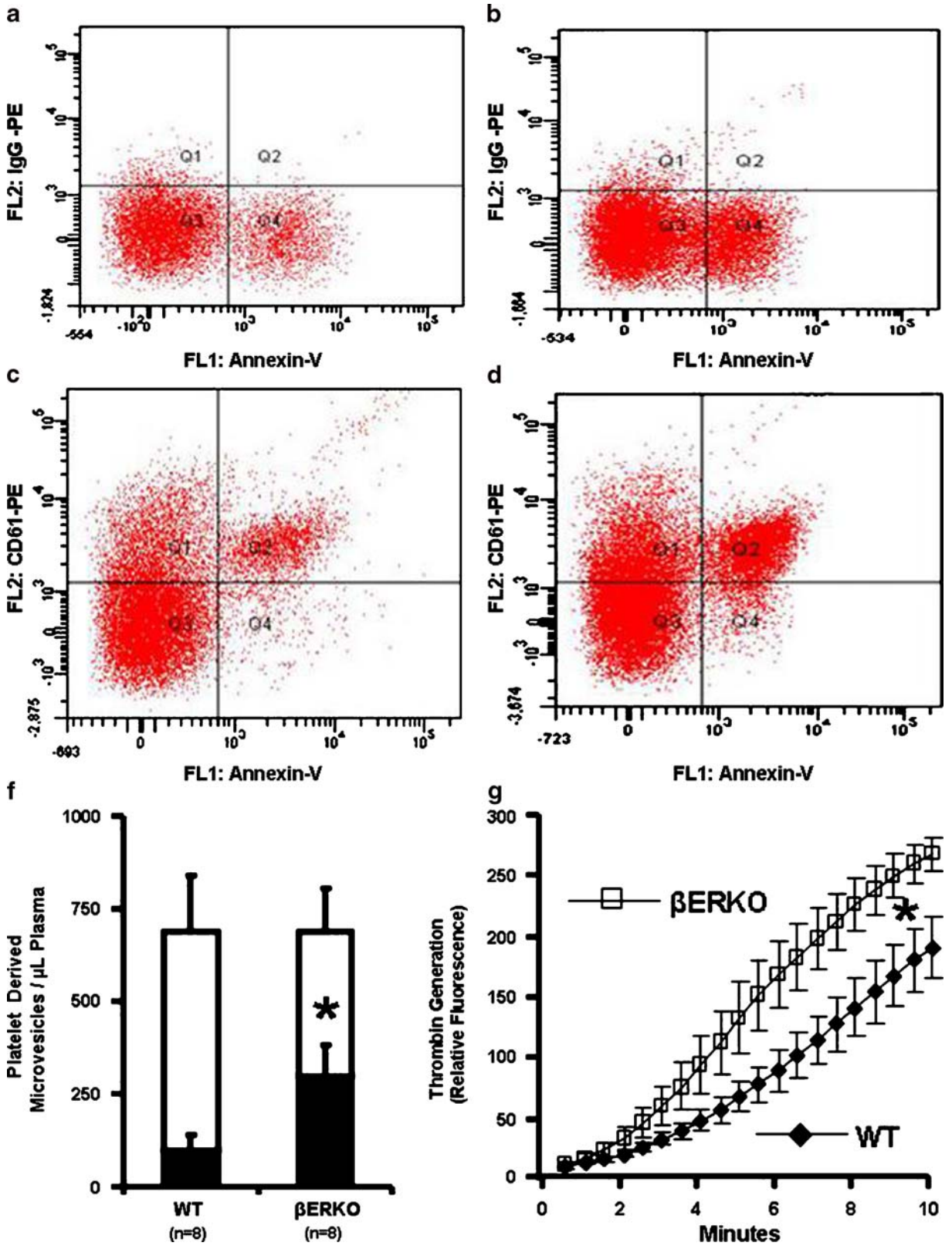


Fig. 4 Representative quadrants of PE-conjugated isotype IgG (**a** WT and **b** β ERKO) and PE-conjugated CD61 antibody (**c** WT and **d** β ERKO) in combination with fluorescein-conjugated annexin-V derived from scatter plots of microvesicles obtained by FACSCanto™ flow cytometry (see Fig. 3a). **e** Cumulative data of number of platelet-derived (Q1 + Q2, *unfilled*) microvesicles from aged WT and β ERKO mice, and proportion of those which are stained-positive for phosphatidylserine (Q2, *filled*). **f** Thrombin generation by microvesicles (20,000) derived from wild type ($n=8$; *filled diamond*) and β ERKO ($n=8$; *open square*) mice. * $P<0.05$ denotes significant difference between aged WT and β ERKO mice

Statistical analysis Statistical significance was evaluated by two-tailed unpaired Student's *t* test. Statistical significance was accepted at $P<0.05$. All values are presented as mean \pm SEM. All experiments were carried out independently; *n* equals the number of individual mice from wild-type and β ERKO colonies.

Results

Body and spleen weights were similar between wild type and β ERKO mice; concentrations of serum FSH and plasma estradiol were also comparable between groups (Table 1). Of the 20 mice in each group, six (30%) WT and ten (50%) β ERKO died during the course of 24 months. Autopsies were not performed on these animals; cause of death was not determined. Among the ten β ERKO mice surviving until sacrifice, two had ovarian tumors, whereas none of surviving WT animals had similar tumors ($n=14$). Mice with tumors were excluded from this study.

Both estrogen receptors α and β were identified in platelets of WT mice by immunofluorescence. Most platelets had three to seven mitochondria. In both mouse types, α ER staining was easily identified as diffuse labeling throughout each platelet with additional small distinct regions of higher concentration. The antibody which recognized the C terminus of the β ER gave inconsistent staining, and in some cases, scant positive staining was observed in platelets from β ERKO mice. On the contrary, the antibody which recognized the N terminus of β ER was punctuated in platelets from WT mice and absent in platelets from β ERKO mice (Fig. 1). There was little to no colocalization of ERs with mitochondria (Fig. 1).

Lactate concentrations in plasma containing fixed number of platelets did not change significantly

following either inhibition of mitochondrial respiratory chain with antimycin A or activation with collagen and did not differ in plasma from WT and β ERKO mice (data not shown). Lactate concentrations in platelet lysates increased by 50% following inhibition of mitochondrial respiration with antimycin A and by 60–70% following collagen activation in both groups of mice (Table 2). The mitochondrial membrane potentials of intact platelets from β ERKO and WT mice were not significantly different (Table 2). Activities of electron transport chain complexes I, III, and IV and the generation of superoxide were about 45–50% lower in mitochondria from β ERKO compared to WT mice reaching statistical significance at $P<0.05$ for complex III and IV (Fig. 2). Activities of complex II (succinate dehydrogenase) and complex V (ATP-synthase) were not detectable (below the detection limit of our assay) in isolated mitochondria of platelets from either group of mice (data not shown).

Isolated microvesicles from both β ERKO and WT mice were heterogeneous in size (Fig. 3). The total number of annexin-V positive microvesicles was significantly greater in β ERKO mice (Fig. 3). Although, the overall concentration of microvesicles derived from platelets (CD61 or CD41 positive) was similar between WT and β ERKO, the proportion of those which were positive for annexin-V was significantly higher in β ERKO mice (Fig. 4). Thrombin generating capacity of microvesicles was associated with annexin-V (surface phosphatidylserine) positivity and was significantly greater in β ERKO mice compared to WT (Fig. 4).

Discussion

Platelets generate energy during activation and granular secretion by both glycolysis and oxidative phosphorylation. Results of the present study demonstrate that loss of β ER did not alter anaerobic energy metabolism of intact platelets as determined by lactate production at baseline and following inhibition of mitochondrial respiration or platelet activation by collagen. However, the capacity for oxidative metabolism was diminished in platelets from aged female mice lacking β ER as evidenced by reduced activities of enzymes of the electron transport chain enzymes and generation of superoxide in isolated mitochondria when compared to age-matched WT mice. These observations

are consistent with decreases in energy requiring functions (aggregation and granular secretion) of platelets from aged β ERKO mice (Jayachandran et al. 2005). The overall decrease in oxidative capacity of these platelets in the absence of β ER may result in decreased viability of platelets and increased release of activated membrane microvesicles.

In other cells, estrogen can modulate mitochondrial functions including ATP synthesis and mitochondrial membrane potential, but it is unclear whether these actions are estrogen receptor-dependent mechanisms (Irwin et al. 2007; Nilsen and Brinton 2004; Nilsen et al. 2007). The plasma levels of 17β -estradiol in the present study were less than those reported by others and likely reflect their reproductive senescence at 24 months of age (Couse et al. 2003, 2004). The presence of ovarian tumors in the β ERKO mice is similar to what has been described in women with ovarian cancers associated with low expression of β ER (Bardin et al. 2004). It was not possible to determine if these cancers were responsible for the increased mortality of the aged β ERKO mice.

Although both α ER and β ER are present in platelets, β ER is the predominant ER in platelets of humans, pigs, and mice (Jayachandran and Miller 2002, 2003; Jorgensen et al. 1972; Khetawat et al. 2000). However, a consistent subcellular localization of β ER has not been established, as the receptor has been identified in mitochondria of primary cultures of rat neurons and cardiomyocytes, a murine hippocampal cell line, MCF-10F and trMCF cells, and human heart (Yang et al. 2004) but not in mitochondria of mouse liver and human T leukemia cells (Rezaul et al. 2005; Schwend and Gustafsson 2006). A high-quality antibody with high specificity for β ER is lacking and may account for these inconsistent findings. The immunofluorescence analysis in this study showed that β ER did not appear to colocalize with mitochondria of nonactivated (resting) platelets from aged female mice. The antibody used to detect β ER was produced against the N-terminal amino acid 1 to 150 of the protein. A potential limitation of this study was that other antibodies developed against other epitopes were not evaluated. In addition, direct analysis of β ER in isolated mitochondria was not feasible because of limited amounts of mitochondria in platelets. In the future, alternative techniques need to be applied to address this question.

Measures of platelet mitochondrial functions have been proposed to be used as peripheral biomarkers for diseases associated with aging (Biagini et al. 1998; Lenaz et al. 1998; Merlo Pich et al. 1996). Mitochondrial oxidative phosphorylation and ATP synthesis decline in platelets with aging, which is accompanied by increases in mitochondrial DNA damage and free radical production (Cortopassi and Wong 1999; Lee and Wei 2000; Xu et al. 2007). A simple biochemical phenomenon, Pasteur effect (a decreased mitochondrial function induce glycolysis in order to maintain a constant ATP synthesis in a cell) can be used to determine both glycolytic and mitochondrial ATP generation (D'Aurelio et al. 2001). Lactate production after inhibition of mitochondrial respiration differentiates relative glycolytic and mitochondrial ATP production. Lactate production and the ratio of oxidative ATP over glycolytic ATP are decreased in platelets from aged individuals suggesting that there is a decrease in ATP utilization with aging (Lenaz et al. 2000). In the present study, lactate production after inhibition of mitochondrial respiratory chain with Antimycin A or platelet activation with collagen was similar in platelets from aged WT and β ERKO mice. Likewise, in intact unstimulated platelets, mitochondrial membrane potential, a sensitive measure of mitochondrial function used as indicator of cell survival and platelet quality (Bertino et al. 2003; Leaver et al. 2006; Verhoeven et al. 2005; Wadhawan et al. 2004), was not different between WT and β ERKO mice. However, it is not clear how mitochondrial ATP production might vary between groups when the platelets would be stimulated with an agonist, for example, thrombin, thromboxane, or ATP. Additional studies are needed to clarify these issues.

Mitochondrial electron transport, ATP synthesis, and generation of reactive oxygen species are linked. Enzyme complexes of the electron transport chain are composed of subunits encoded by nuclear as well as mitochondrial DNA. With aging, there are decreases in the gene transcripts and functional activities of oxidative phosphorylation complexes in the rat heart and mitochondrial proteins in the mouse heart (Chakravarti et al. 2008; Preston et al. 2008). In mitochondria isolated from platelets of aged β ERKO mice, activities of oxidative phosphorylation complexes and superoxide production were lower compared to WT mice. As estrogen receptors regulate genes of the electron transport chain and reactive oxygen species generating

pathways (O'Lone et al. 2007), effect of β ER on regulation of these enzymes at the transcription and translational level warrants further investigation.

In a previous study of aged female mice, loss of β ER was associated with increased numbers of circulating activated platelets (Jayachandran et al. 2005). Consistent with this finding is the observation from the present study of increased number of platelet-derived phosphatidylserine (annexin-V)-positive microvesicles (marker of in vivo platelet activation) and their thrombogenic capacity (procoagulant activity) in β ERKO mice. Platelet-derived microvesicles are associated with thrombosis and thrombosis-associated events (Barry and FitzGerald 1999; Barry et al. 1998; Berckmans et al. 2001; Forlow et al. 2000; Heijnen et al. 1999; Horstman and Ahn 1999; Jy et al. 1995, 1999; Merten et al. 1999; Morel et al. 2006; VanWijk et al. 2003; Zwaal and Schroit 1997). Thus, this activated state of platelets may account for lower total number of circulating platelets and increased number of new (reticulated) platelets, reflecting increased production of platelets in aged β ERKO compared to WT mice (Jayachandran et al. 2005). Increased procoagulant state in the β ERKO mice may be responsible for the greater number of spontaneous deaths in this group over the 2 years of aging, which was also observed in mice with experimentally induced myocardial infarction (Pelzer et al. 2005).

In conclusion, the thrombin-generating capacity and number of platelet-derived phosphatidylserine-positive microvesicles increase with age in female β ERKO mice. Increased shedding platelet microvesicles may result from decreases in activity of enzymes of the mitochondrial electron transport chain. In women, polymorphisms in β ER are associated with thrombotic events including myocardial infarction, venous ulceration, and deep vein thrombosis (Alessio et al. 2007; Ashworth et al. 2008; Rexrode and Manson 2007). Results of present study provide one possible mechanism of how loss of β ER may increase thrombotic events in aged women by increasing activation of platelets and shedding of thrombogenic microvesicles in the circulation.

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