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Original Paper

Functional Estrogen Receptors of Red Blood Cells. Do They Influence Intracellular Signaling?

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Key Words
Estrogen receptors • Red blood cells • Cell signaling • Redox changes

Abstract
Background/Aims: Estrogen could play a key role in the mechanisms underlying sex-related disparity in the incidence of thrombotic events. We investigated whether estrogen receptors (ERs) were expressed in human red blood cells (RBCs), and if they affected cell signaling of erythrocyte constitutive isoform of endothelial NO-synthase (eNOS) and nitric oxide (NO) release. ***Methods:*** RBCs from 29 non-smoker volunteers (15 males and 14 females) aged between 20 and 40 years were analyzed by cytometry and western blot. In particular, content and distribution of ER-α and ER-β, tyrosine kinases and eNOS phosphorylation and NO release were analyzed. ***Results:*** We demonstrated that: i) both ER-α and ER-β were expressed by RBCs; ii) they were both functionally active; and iii) ERs distribution and function were different in males and females. In particular, ERs modulated eNOS phosphorylation and NO release in RBCs from both sexes, but they induced the phosphorylation of specific tyrosine residues of kinases linked to eNOS activation and NO release in the RBCs from females only. ***Conclusion:*** Collectively, these data suggest that ERs could play a critical role in RBC intracellular signaling. The possible implication of this signaling in sex-linked risk disparity in human cardiovascular diseases, e.g. in thrombotic events, may not be ruled out.

Introduction

Red blood cells (RBCs) have been proposed as real-time biomarkers and pathogenetic determinants in several human cardiovascular diseases (CVD) [1, 2]. Changes in RBC morphological and functional characteristics such as adhesivity, aggregability, and

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deformability have been detected in a number of human pathologic conditions, mostly those displaying systemic oxidative stress as a hallmark. For instance, changes in RBCs adhesiveness/aggregation and morphology have been detected in inflammatory conditions, plaque instability and atheroma progression in patients with coronary artery disease [1-3]. Redox change of RBCs is believed to be a potent atherogenic stimulus, contributing to the deposition of cholesterol at the atherosclerotic plaque [4] and playing a role in the pathogenesis of hypertension [5] and stroke [6].

Besides the well-known activity of oxygen transport to tissues, RBCs serve the important function of circulating scavengers towards reactive oxygen and nitrogen species (RONS). Under non-inflammatory conditions, the low levels of RONS generated in the vasculature can easily be handled by the RBC antioxidant machinery. These reactive species include those generated by hemoglobin inside the RBCs, i.e. the superoxide radical produced by partially oxygenated hemoglobin and nitric oxide (NO) generated by deoxyhemoglobin [7] and/or, as recently reported, by an active and functional endothelial NO-synthase (eNOS) isoform localized in the plasma membrane and in the cytoplasm [8-10].

As a potent vasodilator and an anti-inflammatory molecule [11], NO is a central regulator of the vascular function. The decreased production and/or bioactivity of NO may dramatically impair endothelial and vascular homeostasis, a key event in the development and progression of atherosclerosis. Both endothelial cells (ECs) and RBCs substantially contribute to the circulating NO pool. eNOS activation in ECs has been demonstrated to result from the interaction of 17β estradiol (E2: the active, natural form of estrogen) with estrogen receptor (ER)-α. ER-α is a transcription factor that, when bound by E2, moves to the nucleus, binds to specific sites on chromatin, and activates or represses target gene transcription (the classical genomic pathway). In addition to its genomic pathway, a fraction of cellular ER-α is palmitoylated and forms signaling complexes in caveolae on the plasma membrane. One of the best characterized functions of the membrane-bound ER-α non-genomic pathway is the phosphorylation of several important cellular kinases, including, AKT, ERK1/2 and P38 [12-14]. In particular, by phosphorylating specific tyrosine and threonine residues of target proteins, the mitogen-activated protein kinase (MAPK) ERK1/2 regulates cellular activities, e.g., cell adhesion and cell survival [15]. AKT, also known as protein kinase B, is an important regulator of various cellular processes, comprising glucose metabolism and cell survival [16]. AKT stimulates eNOS by directly phosphorylating the enzyme at Ser-1177 [17]. MAPK P38 pathway, regulates cell proliferation and apoptosis [18-19].

In ECs, both ER-α and ER-β, the other described isoform of ER, are found in the plasma membrane. However in ECs only ER-α seems able to interact with PI3K and active eNOS [20].

As for endothelial NOS, RBC eNOS activity depends on the phosphorylation at Ser-1177, which is in turn regulated by AKT-PI3K pathway [8, 21]. eNOS-derived NO has been reported to regulate RBC membrane deformability and inhibit platelet functions. eNOS content and activity in RBCs have been observed compromised in patients with coronary artery disease [9-8]. RBCs possess ER-α and ER-β and RBC deformability was also affected by estradiol concentrations, but little is known about the estrogen/ER signaling pathway in these cells [22-24].

On these bases, also considering that pre-menopausal females are less prone to experience coronary atherosclerosis than males of the same age [25], the present study was aimed at investigating sex differences in ER-α and ER-β content in RBCs also evaluating if and how their activation might affect RBC intracellular signaling, with particular regards to eNOS activation.

Materials and Methods

Blood sampling

Twenty nine non-smoker volunteers (15 males and 14 pre-menopausal females aged between 20 and 40 years) were recruited for this study at the Institute of Hematology, University "La Sapienza" of Rome

(Italy). Eight post-menopausal females aged between 58-65 years who did not take hormone replacement therapy were also enrolled for selected experiments. Following the rules of good medical practice, the nature and purpose of the study were explained to all participants who then gave their informed consent. The investigation conformes to the principles outlined in the Declaration of Helsinki. All participants had stopped taking aspirin or NSAIDs at least one week before the beginning of the study. To exclude the interference of sex hormones with RBC aggregation, only females in follicular phase after menstruation were enrolled. The levels of estradiol and progesterone measured in this phase of the menstrual cycle were 48 ± 14 pg/mL and 1.3 ± 1.2 ng/mL, respectively. To have comparable results, blood samples were taken at the same time point (between 9 and 10 am).

Isolation and treatment of RBCs

Unless otherwise indicated, all chemicals were from Sigma-Aldrich (Milan, Italy). Fresh human blood from healthy donors was drawn into heparinized tubes. For RBCs isolation, whole blood was centrifuged for 10min at 1.500g. The plasma and buffy coat were removed. RBCs were washed twice in isotonic PBS, pH 7.4, and suspended in the same buffer to the initial hematocrit concentration. No appreciable cell lysis was observed during the RBC preparation procedure. ERs were stimulated for 15 minutes at 37°C with 10nM propyl pyrazoletriol (PPT) and 10nM diarylpropionitrile (DPN).

In selected experiments, RBCs were treated for 5 min with 10 or 30nM of 17-β estradiol (E2, Sigma-Aldrich) in presence or absence of selected ERs antagonists, i.e., ER-α antagonist methyl-piperidino-pyrazole (MPP; Sigma-Aldrich) and ER-β antagonist Tetrahydrocannabinol 10nM (THC; Tocris Bioscience, Bristol, UK). Ghosts were prepared from RBCs lysed in 10 volumes ice-cold 5mM phosphate buffer, pH 8.0, containing 0.15mM phenylmethylsulfonyl fluoride, 10mg/ml leupeptin, 10mg/ml aprotinin (lysis buffer). Hb-free membranes were prepared by centrifuging cell lysates at 40, 000g for 10min at 4°C, removing hemolysate and washing several times with lysis buffer.

Analytical cytology

RBCs were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, washed in the same buffer and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After washing with cold PBS, samples were incubated for 30 minutes at 37°C with monoclonal or polyclonal antibodies. Monoclonal antibodies: anti-ER-α, anti-ER-β, anti-ERK 1/2, anti-P38, anti-phosphorylated P38 (all Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phosphorylated ERK 1/2 (BD PharMingen, San Diego, CA). Polyclonal antibodies: anti-AKT, anti-phosphorylated AKT (Thr 308)-R (all Santa Cruz Biotechnology), and anti-eNOS (phospho S-1177) (Cambridge, MA). As negative control we used mouse or rabbit IgG1 immunoglobulin isotype (Sigma). Samples were washed thrice in PBS to be then incubated with secondary antibody FITC-conjugated: anti-mouse (Invitrogen, Carlsbad, CA) or anti-rabbit (Invitrogen, Carlsbad, CA). For NO detection, samples were incubated with 4, 5-Diaminofluorescein diacetate (Enzo Life Sciences, Lausen, Switzerland) for 30 min at 37°C and washed three times in PBS. All the samples were recorded with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) equipped with a 488nm argon laser. At least 20, 000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis.

SDS-PAGE and Western blotting

Protein content was determined by the Bradford assay (Bio-Rad Laboratories Inc., CA, USA). Lysed RBCs were subjected to 12-15% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE), and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with 5% defatted dried milk in TBS, containing 0.05% Tween 20, and probed with monoclonal or polyclonal antibodies. To remove antibodies, nitrocellulose filters were incubated with stripping buffer (Thermo Scientific) for 30 min, rinsed with washing buffer, blocked with 3% bovine serum albumin for 4 h, rinsed again, and blotted with the following monoclonal antibodies: anti-ER-α, anti-ER-β, anti-ERK 1/2, anti-P38, anti-phosphorylated P38 (all Santa Cruz Biotechnology), anti-phosphorylated ERK 1/2 (BD PharMingen, San Diego, CA) and anti-eNOS (Cambridge, MA, USA). Polyclonal antibodies: anti-AKT, anti-phosphorylated AKT (Thr 308)-R, and anti-eNOS (phospho S-1177) (Cambridge). Bound antibodies were visualized with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (all Jackson ImmunoResearch Inc. West Grove, PA, USA) and immunoreactivity assessed by chemiluminescence reaction, using the ECL

Western Chemiluminescent HRP Substrate (Millipore, Mussachusset, USA). Densitometric scanning analysis was performed by ChemiDoc MP system (Bio-Rad). To ensure the presence of equal amounts of protein, the membranes were reprobed with an anti-GAPDH (Santa Cruz).

Statistical analyses

Cytofluorimetric results were statistically analyzed by using the nonparametric Kolmogorov–Smirnov test using Cell Quest Software. At least 20, 000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semiquantitative analysis. Student's t-test was used for the statistical analysis of the collected data.

Results

ERs content and localization in RBCs from males and females

ER content and localization were evaluated in RBCs from blood samples of healthy age-matched pre-menopausal females and males, by using different techniques. By flow cytometry analysis, we found that both ER-α and ER-β were expressed in RBCs, but differently in males and females (Fig. 1A, left panel). In particular, ER-α content was significantly ($p < 0.001$) higher in RBCs from males, while ER-β content in RBCs was comparable between the two sexes. Typical flow cytometric profiles of ER-α and ER-β content in RBCs from a representative male and a representative female were shown in Fig. 1A (right panels). The analysis of ER-α content by western blotting confirmed that ER-α was significantly higher in RBCs from males than from females ($p < 0.05$, Fig. 1B, left panels) whereas no significant differences were observed regarding ER-β content in RBCs from both sexes (Fig. 1B, right panels). We further investigated ER-α and ER-β distribution in RBCs by immunofluorescence analysis. The representative images of Fig. 1C show that ER-α (left panel) and ER-β (right panel) were differently distributed in RBCs from males and females. In RBCs from the males, ER-α was mainly localized in the cytoplasm, while in the female RBCs it was mainly localized in the subcortical region. By contrast, no sex difference was detectable in ER-β distribution with a predominance in cytoplasm localization (Fig. 1C, right panels). The different localization of ER-α and ER-β in RBCs in male and female was then evaluated by western blotting analysis on purified RBC membranes. The obtained results confirmed that: i) ER-α membrane content was significantly higher in RBCs from females than in those from males (left panels) and ii) ER-β (right panels) expression in cell membrane was similar in RBCs from both sexes (Fig. 1D). Hence, to evaluate whether ERs could be recruited to the membrane after activation by 17β estradiol (E2), RBCs of 8 pre-menopausal females, 8 post-menopausal females and 8 males were treated for 5 min with 10 and 30 nM E2 (Fig. 2). E2 treatment significantly increased ER-α (Fig. 2A and B) and ER-β (Fig. 2C and D) recruitment at plasma membrane in RBCs from pre-menopausal females, post-menopausal females, and males. These data supported a role for estrogen in the recruitment of ER-α, at membrane level in RBCs, potentially explaining its higher membrane content in RBCs from pre- menopausal females in comparison to those from males.

ERK1/2, AKT/PI3K, P38 content and phosphorylation signaling pathway in RBCs from males and females

As reported by Nakaya and co-workers [13], in ECs, ERs-mediated signaling was linked to the phosphorylation of some key kinases, such as ERK1/2, AKT/PI3K and P38. To investigate the likely existence of a similar pathway in RBCs, and to evaluate potential sex differences in these signaling, we compared the content and the phosphorylation levels of these kinases in RBCs from healthy age-matched pre-menopausal females and males by flow cytometry and western blotting analysis (Fig. 3). We found that ERK1/2 (Fig. 3A and B) and AKT (Fig. 3C and D) were contained in RBCs in a sex-independent manner. Conversely, P38 content was significantly higher in RBCs from males than in those from females ($p < 0.05$) by flow cytometry (Fig. 3E) and western blotting (Fig. 3F). Moreover, we found that in RBCs of both

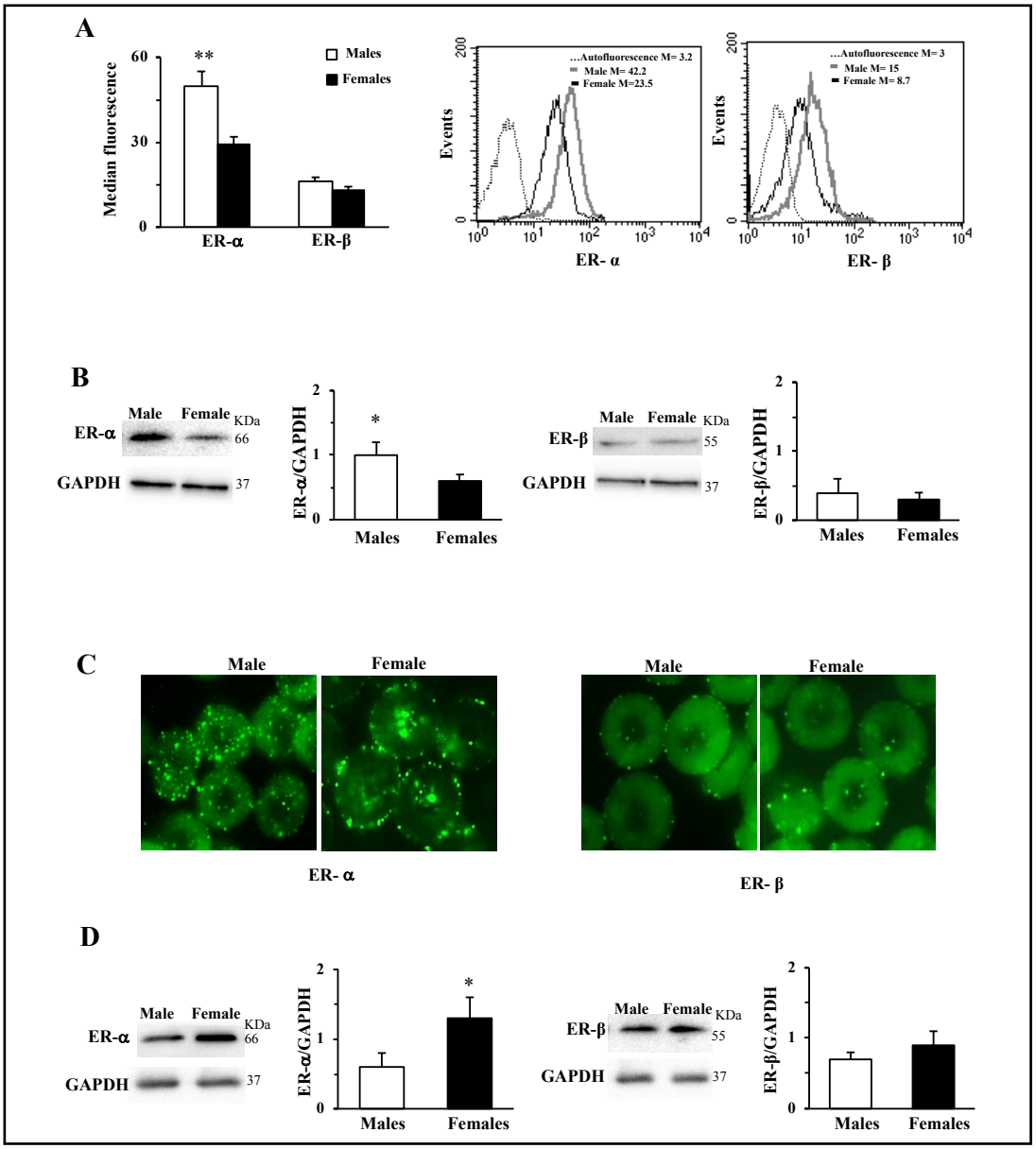

Fig. 1. Estrogen receptors (ERs) exist in human red blood cells and are differently present in males and females. (A, left panel) Cytometric analysis of ER-α and ER-β content in RBCs from males and females. ER-α content was significantly ($p < 0.001$) higher in RBCs from males and ER-β was poorly content in RBCs without any significant difference between the two sexes. Numbers are the median values of fluorescence intensity. (A, right panels) Evaluation of ERs in RBCs from a representative male and a representative female. (B) Representative blotting of ER-α (left panels) and ER-β (right panels), normalized for the GAPDH content, and histograms showing densitometric analysis of three different experiments. The western blotting analysis confirmed the cytometric data ($p < 0.05$). (C, left panels) Micrographs obtained by static cytometry showing ER-α distribution in RBCs from representative male and representative female. (C, right panels) Micrographs obtained by static cytometry showing ER-β distribution in RBCs from a representative male and a representative female. In RBCs from the males, ER-α was mainly localized in the cytoplasm, while in RBCs from the females it was mainly localized in the cell membrane. Conversely, no sex difference was detectable in ER-β distribution. (D) Representative blotting of ER-α and ER-β membrane levels, and densitometry analysis of three different experiments. Membrane ER-α content was higher in RBCs from females with respect to males ($p < 0.05$) (D, left panels), and membrane ER-β content was comparable in RBCs from both sexes (D, right panels). * $p < 0.05$; ** $p < 0.001$.

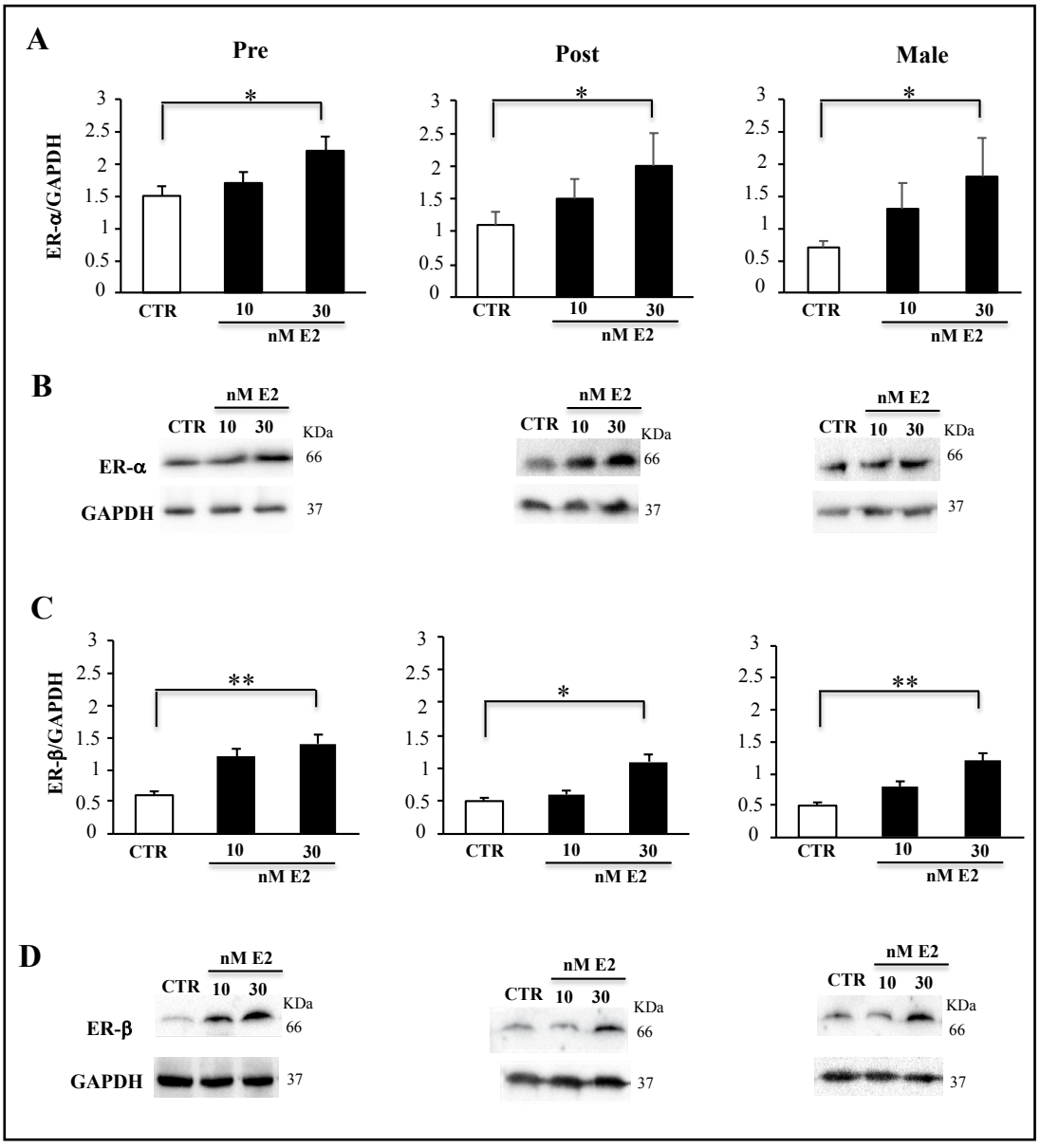

Fig. 2. Effect of E2 on ER-α and ER-β present in membrane of RBCs from pre-menopausal and post-menopausal females as well as from males. To evaluate whether ERs are constitutively present in the RBC membrane, or if they are recruited to the membrane after activation of the E2- ligand, experiment were carried out by western blot in RBCs membranes, treated for 5 min with 10 and 30nM of E2. Representative blotting of ER-α (B) and ER-β (D) membrane levels, and densitometry analysis of eight different experiments are shown. (A and C, respectively). After treatment with E2, the presence of ER-α in membrane significantly increases in RBCs from pre-menopausal and post-menopausal females and from males (A). Similar trend is also observed for ER-β membrane content (C). * $p < 0.05$; ** $p < 0.001$.

sexes, ERK1/2, AKT, and P38 were present in their phosphorylated states and that the levels of p-ERK1/2 (Fig. 3A and B, right panels), p-AKT (Fig. 3C and D, right panels), and p-P38 (Fig. 3E and F, right panels) were significantly higher in RBCs from females than in those from males ($p < 0.05$ for p-ERK1/2 and $p < 0.01$ for p-AKT and p-P38).

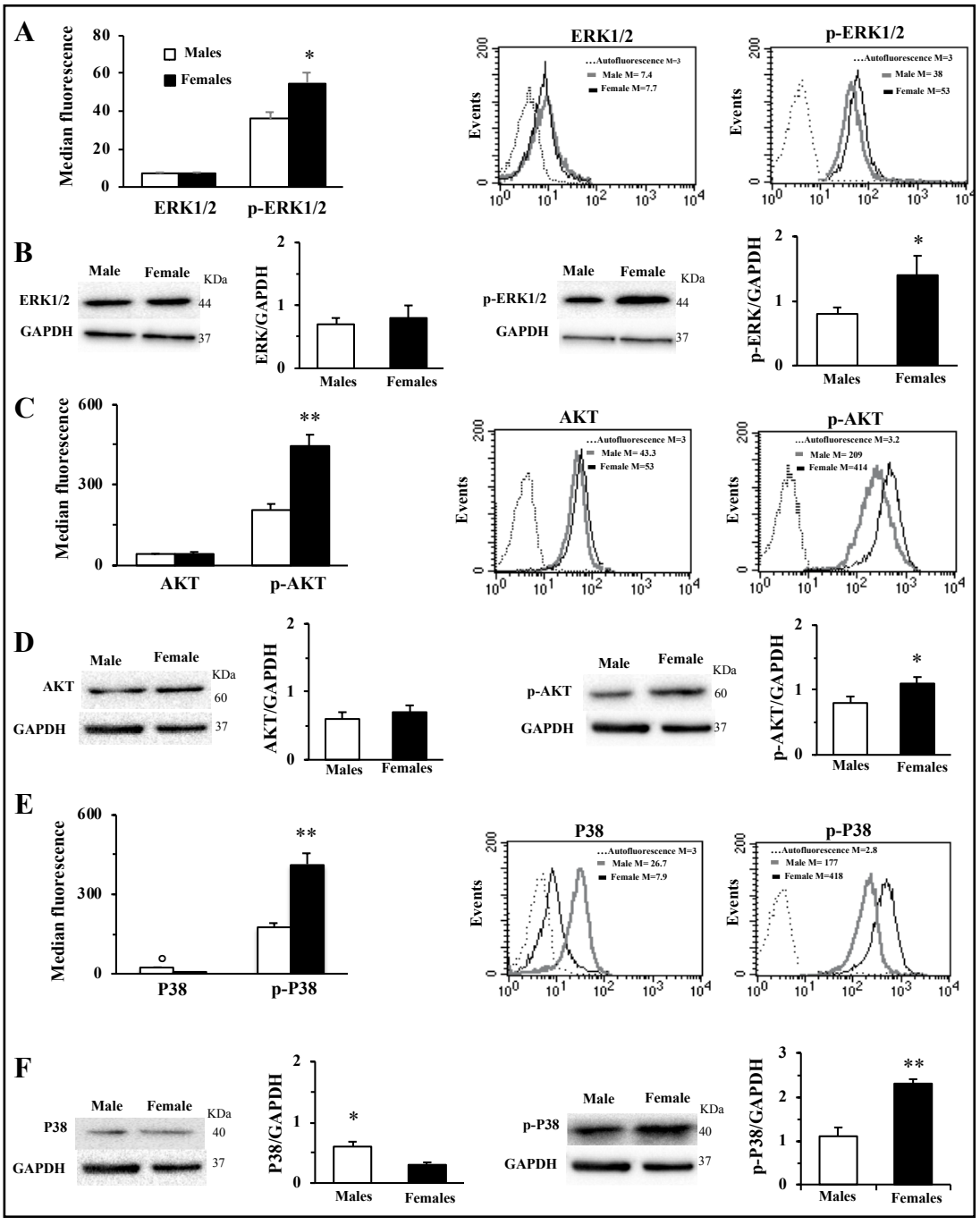

Fig. 3. ERs are linked to phosphorylation of ERK1/2, AKT and P38 kinases. Cytometric analysis of ERK1/2 (A), AKT (C) and P38 kinases (E), and their phosphorylated forms. These kinases were expressed in RBCs in a sex-independent (ERK, AKT) or dependent (P38, more expressed in males) manner, and were constitutively phosphorylated in RBCs from both sexes. Numbers are the median values of fluorescence intensity. The dot plots show data of p-ERK1/2, p-AKT and p-P38 in RBCs from a representative male and a representative female (A, C, E, right panels). The same samples were used for western blotting analysis of ERK1/2 (B), AKT (D) and P38 kinases (F) (left panels) and their phosphorylated form (right panels). For this purpose, nitrocellulose filters were stripped and incubated with the different antibodies. Representative blotting normalized for the GAPDH content, and histogram showing densitometry analysis of three different experiments. The levels of these phosphorylated kinases were significantly higher in females than in males. * $p < 0.05$; ** $p < 0.001$.

ER-α and ER-β activation and ERK1/2, AKT/PI3K and P38 phosphorylation in RBCs from males and females

To evaluate ER function in kinase phosphorylation, we stimulated the receptors with the ER-α agonist PPT or with the ER-β agonist DPN. Western blotting analysis showed that PPT and DPN treatments affected the content of the phosphorylated kinase fractions in a sex-dependent manner (Fig. 4). In particular, a decreased content of p-ERK1/2, p-AKT and p-P38 was measured in males (Fig. 4A-C, left panels). Conversely, an increased content of these phosphorylated proteins was measured in treated RBCs from females (Fig. 4A-C, middle panels). Again, these data were confirmed by cytofluorimetric analysis (Fig. 4A-C, right panels). In fact, a decreased content of p-ERK1/2 (-16% and -35% for PPT and DPN, respectively), p-AKT (-27% and -39% for PPT and DPN, respectively) and p-P38 (-7 % and -26%, respectively) was measured in males, whereas an increase in p-ERK1/2 (+16% and +28% for PPT and DPN, respectively), p-AKT (+69% and +65% for PPT and DPN, respectively) and p-P38 (+13 % and +20%, respectively) was detected in females. To confirm that both ER-α and ER-β activation could be involved in these events, we treated RBCs with E2 in presence of MPP and THC, selective antagonists of ER-α and ER-β respectively. In RBCs from females both the ER antagonists significantly reduced AKT/PI3K phosphorylation induced by E2, whereas in RBCs from males no significant effect was observed (Supplementary Fig. 1 - for all supplemental material see www.cellphysiolbiochem.com). These results highlight the sex-dependent activation of ER, which can affect the kinase-mediated intracellular signaling in RBCs from females.

RBC eNOS phosphorylation and NO production

The evidence of sex differences in ER-mediated phosphorylation of kinases, process known to be involved in eNOS activation, led us to hypothesize that eNOS activity could be subject to sex-related control in RBCs. To address the issue, we looked for the presence of a functionally active eNOS in RBCs. Considering that eNOS phosphorylation at Ser-1177 residue is the pre-requisite for the activation of the enzyme and NO production [26-27], we measured the amount of phosphorylated eNOS (p-eNOS) by both flow cytometry (Fig. 5A, left panels) and western blotting analysis (Fig. 5A, right panels). Data showed that p-eNOS content was significantly ($p < 0.05$) higher in RBCs from females than in RBCs from males (Fig. 5A). To further dissect the connection between ERs-linked signaling and NO pathway, we measured p-eNOS content in RBCs treated with ER agonists (Fig. 5B). Western blotting analysis showed that treatment with PPT and DPN increased significantly p-eNOS levels in RBCs from both males and females (Fig. 5B, left and middle panels, respectively). Flow cytometry analysis confirmed that the treatment of RBCs with PPT and DPN increased the phosphorylated form of p-eNOS by 24% and 38% in RBCs from males, and by 46% and 21% in RBCs from females, respectively (Fig. 5B, right panel). Since p-eNOS is a prerequisite for NO production, constitutive levels of NO were measured by flow cytometry (Fig. 5C, left and middle panels). Interestingly, a significant ($p < 0.05$) sex difference in NO content was found as RBCs from females released more NO than those from males. Using ER agonists, we found that in RBCs from males NO release increased by about 15% and 16% after PPT and DPN treatment, respectively. In RBCs from females, NO release increased by about 20% after PPT treatment and, surprisingly, decreased by about 16% after DPN treatment (Fig. 5C, right panel). These data highlight that eNOS phosphorylation and NO production are under the control of ERs in RBCs from both males and females.

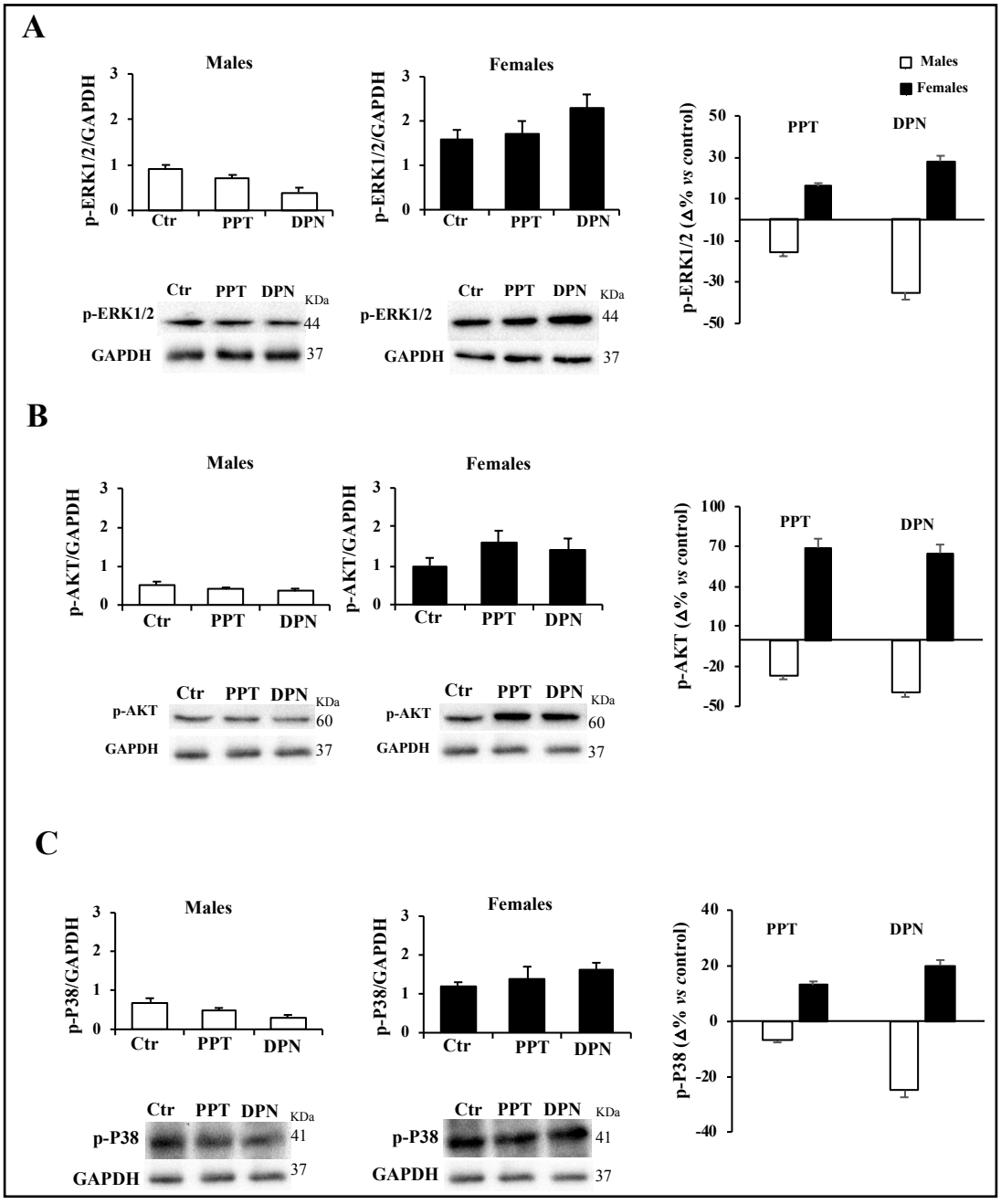

Fig. 4. ER-α agonist (PPT) and ER-β agonist (DPN) affect phosphorylation of kinases. (A-C, left and middle panels) Western blotting and densitometry analysis of p-ERK, p-AKT and p-P38, after treatment with PPT and DPN in RBCs from males and females. Representative blotting normalized for the GAPDH content, and histogram showing densitometry analysis of three different experiments. The agonists markedly affected the phosphorylation of these kinases in a sex-dependent manner. (A-C, right panels) Cytometric analysis. After treatment with ER agonists, the content of phosphorylated ERK, AKT and P38 decreased in RBCs from males and increased in RBCs from females. The data are the average of △% (treated vs controls).

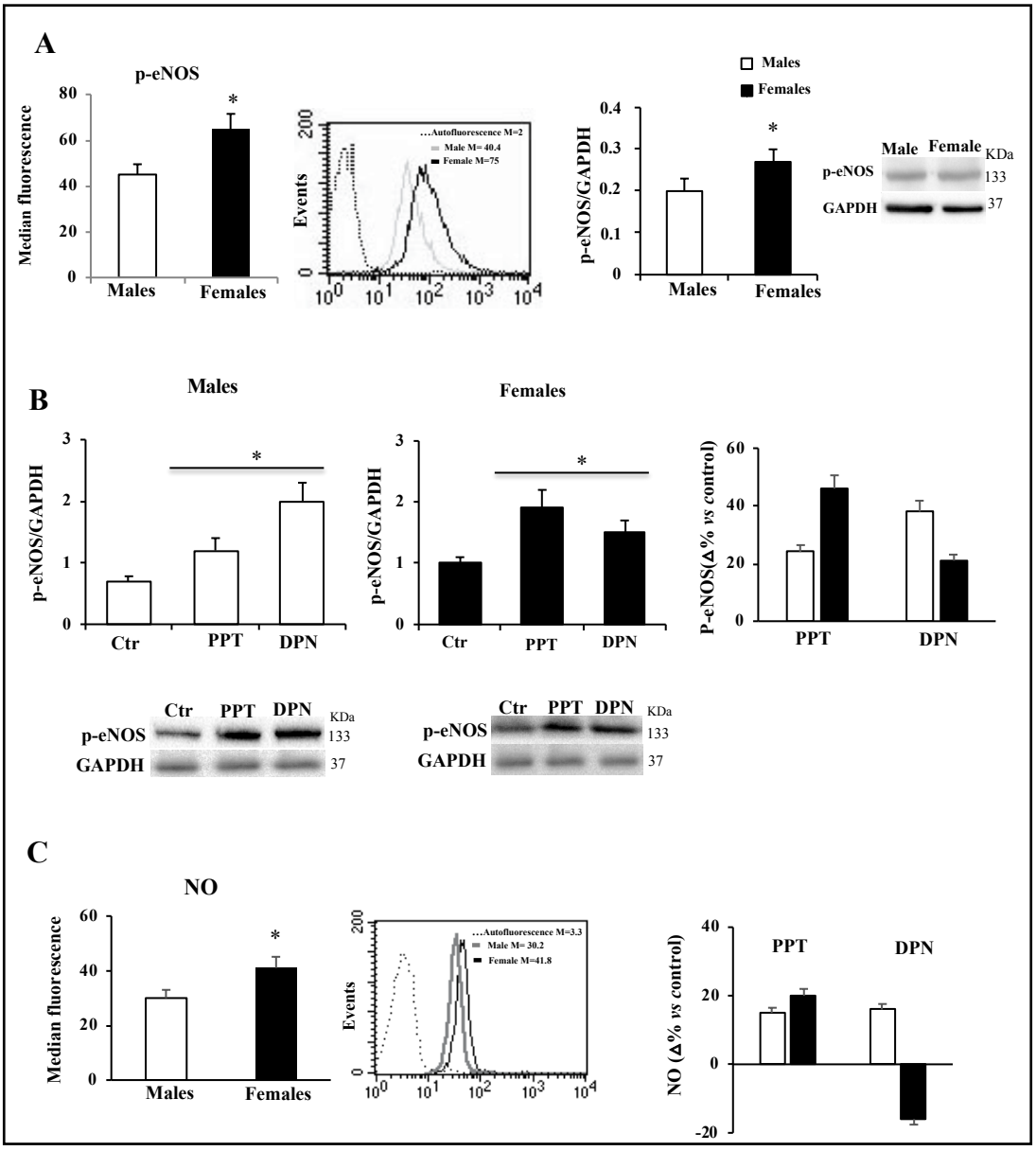

Fig. 5. RBC eNOS phosphorylation and NO production. (A, left panel) Cytometric analysis of phosphorylated eNOS (p-eNOS). (A, middle panel) Dot plot showing p-eNOS in RBCs from a representative male and a representative female. (A, right panels) Western blotting analysis of p-eNOS. The content of p-eNOS were significantly ($p < 0.05$) higher in RBCs from females than in those from males. (B, left and middle panels) Western blotting and densitometry analysis of p-eNOS. Representative blotting normalized for the GAPDH. Histograms show the densitometry analysis of three different experiments. Note that p-eNOS content significantly increased after treatment with ER-α agonist (PPT) and ER-β agonist (DPN) in both male and female RBCs. (B, right panel) Cytometric analysis of p-eNOS after treatment with PPT and DPN. The cytometric data are the average of △% (treated vs controls). (C, left panel) Cytometric analysis of NO levels. The histogram numbers are the median values of fluorescence intensity. (C, middle panel) Dot plot showing NO levels in RBC from a representative male and a representative female. (C, right panel) Cytometric analysis of NO levels after treatment with ER agonists. The cytometric data are the average of △% (treated vs controls). Note: i) a significant ($p < 0.05$) gender difference in NO levels; ii) both ER-α and ER-β were involved in NO production in RBCs from males; iii) only ER-α was involved in NO production in RBCs from females. * $p < 0.05$.

Discussion

In this study, we observed that: i) both ER-α and ER-β were expressed by RBCs; ii) they were both functionally active; and iii) ERs distribution and function were different in healthy age-matched pre-menopausal females and males. In RBCs from pre-menopausal females, ER-α was mainly localized at the plasma membrane, whereas in RBCs from the males, it was mainly localized in the cytoplasm. Interestingly, estrogen was able to recruit ER-α and ER-β at membrane level not only in pre-menopausal, but also in post-menopausal females and in males. To note that ER-α and ER-β localized at the plasma membrane are well-known to have the ability of initiating membrane delimited signaling, which enhances kinase signaling pathways. In this study we observed a higher level of the active, phosphorylated kinases in RBCs from female than in those from males. When ER-α or ER-β were activated by PPT (ER-α agonist) or DPN (ER-β agonist), phosphorylated kinase level increased further only in RBCs from females supporting a role for ER in this process and partially explaining the basal higher levels of phosphorylated kinases in pre-menopausal females, naturally exposed to estrogen.

Membrane-associated ER-α and/or ER-β have been demonstrated to mediate eNOS phosphorylation via activation of the PI-3 kinase/AKT pathway [28-30], but sex differences in this process has not been investigated yet. In this study, the content of phosphorylated eNOS and NO appeared more elevated in female RBCs in comparison with male RBCs. We observed that the stimulation of ERs induced eNOS phosphorylation and NO production in both males and females. Again, this results suggest that the natural exposure of RBCs to estrogen in pre-menopausal females could induce a higher basal levels of phosphorylated eNOS and NO in comparison to males. Moreover, our data suggest that in RBCs from females, the ER-mediated induction of eNOS phosphorylation and NO production could be triggered by kinase phosphorylation, whereas in RBCs from males other pathways may be involved. In fact, estrogen has been shown modulate NO production by a variety of alternative mechanisms [31-35]. Interestingly, confirming our results, estrogen supplementation has been observed to improve eNOS dependent vascular function in male transsexuals suggesting that effects of this steroid are present in both males and females [36-37]. Additionally, other sex associated factors, such as progesterone, could have a role in sex-related differences in RBC physiology modulating eNOS phosphorylation and NO production [38-39] and further studies are ongoing in our laboratories to better clarify the role of progesterone /progesterone receptors in RBC physiopathology.

Conclusion

Our study provides new lines of evidence supporting that ERs play a role in the modulation of RBCs intracellular signaling in males and females.

Estrogen regulation of eNOS in RBCs and bioavailability of NO may contribute to variability in risk factors, presentation of, and treatment for cardiovascular disease associated with aging, pregnancy, stress, and metabolic disorders in males and females [40]. Interestingly, Grau et al. [23] showed that RBC deformability were gender dependent and related to different estradiol levels that affect NO synthesizing pathways. A better understanding of the effects of ER-α and ER-β in this context could open new paths in therapeutic strategies based on the use of ER selective agonists such as phytoestrogens, e.g., genistein or silibinin.

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Disclosure Statement

No conflicts of interests exists.

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