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## Membrane lipid rafts coordinate estrogen-dependent signaling in human platelets<sup>☆</sup>

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### Abstract

The impact of estrogens on the viability of cardiovascular system and their ability to regulate platelet function is still an open and debated question. We have previously shown that estrogen is able to significantly potentiate the aggregation induced by low doses of thrombin and to initiate a rapid and reversible signaling pathway mediated by ER $\beta$ -directed activation of the tyrosine kinases Src and Pyk2 at the level of the plasma membrane. Lipid rafts are critical, cholesterol-enriched membrane domains, which play a major role in blood platelet activation processes. In this work, we investigated the role of lipid rafts in 17 $\beta$ -estradiol signaling in human platelets. We observed that membrane rafts were essential for both 17 $\beta$ -estradiol-dependent potentiation of platelet aggregation induced by subthreshold concentrations of thrombin and 17 $\beta$ -estradiol-induced phosphorylation of Src. 17 $\beta$ -estradiol caused the reversible translocation of ER $\beta$  to the raft fractions and promoted the rapid and transient recruitment to, and activation within the membrane raft domains of the tyrosine kinases Src and Pyk2. The raft integrity was essential with this respect, as these effects of 17 $\beta$ -estradiol were completely inhibited by cholesterol depletion. This paper provides evidence for the first time that membrane lipid rafts coordinate estrogen signaling in human platelets.

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**Keywords:** Platelets; Lipid rafts; Estrogen

### 1. Introduction

Steroid hormones bind to nuclear receptors and regulate a number of biological processes, including morphogenesis, cell proliferation, differentiation, and apoptosis [1,2]. Recently it has been reported that, in addition to these classical actions, steroid hormones can also induce rapid, non-genomic effects in different cell types [3]. These effects include regulation of adenylyl cyclase, stimulation of p21<sup>ras</sup> and mitogen-activated protein kinases, activation of phosphatidylinositol 3-kinase (PI3-K), and mobilization of Ca<sup>2+</sup> from intracellular stores [4–11]. Estrogens can also affect the cardiovascular system,

although conflicting effects have been reported. In fact, it has been shown that estrogens can, on one hand, improve vessel wall viability by acting as a vasodilator and hypotensive agents, but, on the other hand, they can also increase the thrombotic risk when used in contraceptive therapy or at acute doses. Sex steroid hormones have also been shown to affect the platelet functionality [12–18]. Recently, we have provided evidence that 17 $\beta$ -estradiol (17 $\beta$ -E2) exerts a priming effect on thrombin-dependent platelet aggregation. Moreover, we have demonstrated that in human platelets 17 $\beta$ -E2 promotes protein tyrosine phosphorylation, and orchestrates the assembly of a signaling complex, which includes the estrogen receptor, Src, Pyk2, and PI3-K. These events are dependent on the engagement of estrogen receptor beta (ER $\beta$ ), which is the predominant estrogen receptor in platelets, and is associated to the membranes [19].

It is very well known that individual lipids within the plasma membrane are unequally distributed and form distinct micro-

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domains with peculiar biological functions. Among these, regions characterized by amount of glycosphingolipids, saturated phospholipids and cholesterol higher than the rest of the membrane, and called lipid rafts, have been proposed to play a role in membrane signaling [20]. Some membrane-associated signaling proteins, including tyrosine kinases of the Src family, G $\alpha$  subunits of heterotrimeric G proteins, endothelial nitric oxide synthase, and the adaptor protein LAT, have been found to localize into lipid rafts [21]. Some of these proteins have a weak affinity for membrane rafts, which, however, may increase upon cell stimulation [22]. At this time, most findings support the notion that rapid estrogen signaling is mediated by ER associated with specialized lipid raft domains that serve to concentrate and provide a matrix for receptors and related signaling proteins [23–26].

Removal of cholesterol from lipid rafts disorganizes these structures, and has been shown to alter several cell responses to extracellular agonists, and to cause, for instance, inhibition of Ca<sup>2+</sup> mobilization following stimulation of T cells [27], reduction of tyrosine phosphorylation and association with Lyn of aggregated Fc $\epsilon$ RI in mast cells [28], and hyperactivation of ERK in stimulated fibroblasts [29].

Lipid rafts play also a role in platelet signaling [30]. Recent works have demonstrated that these membrane microdomains orchestrate the signaling through the collagen platelet receptor glycoprotein (GP) VI, which is constitutively associated with the Fc receptor  $\gamma$ -chain (FcR $\gamma$ -chain). The GPVI–FcR $\gamma$ -chain complex is recruited into lipid rafts upon the engagement of GPVI by its ligand, and this event is essential for the subsequent phosphorylation of FcR $\gamma$ -chain [31]. Similarly, the major platelet adhesion receptor, the GPIb-IX-V, strongly increases its association with lipid rafts upon stimulation with von Willebrand Factor [32], and, during platelet spreading, cholesterol-enriched microdomains accumulate at the extended tips of the formed filopodia, containing higher amount of c-Src and the tetraspanning CD63 [33]. Furthermore, it has been shown that platelet activation and aggregation induced by low doses of thrombin are dependent on lipid raft integrity [34].

In this work, we have investigated the role of lipid rafts in 17 $\beta$ -E2-dependent signaling in human platelets. We found that membrane rafts were essential both for 17 $\beta$ -E2-dependent potentiation of platelet aggregation induced by subthreshold concentrations of thrombin, and for 17 $\beta$ -E2-induced phosphorylation of Src. We found that ER $\beta$  reversibly translocated to the lipid raft fractions in a hormone-dependent manner, and promoted the rapid and transient recruitment and activation of the tyrosine kinases Src and Pyk2 within the membrane raft domains. In addition, we demonstrated for the first time that membrane lipid rafts coordinate estrogen signaling in human platelets.

## 2. Materials and methods

### 2.1. Materials

Polyclonal antibodies against ER $\beta$  and Pyk2, anti-goat horseradish-peroxidase-conjugated antibody, and a polyclonal antibody against c-Src

(SRC2) recognizing the Src family kinases Src (p60), Yes (p62) and Fyn (p59) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal antibodies against p85-PI3-K and LAT were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Polyclonal anti p44/42 MAP Kinase and phospho-specific polyclonal antibody anti-Pyk2 (Tyr402) were from Cell Signaling Technology Inc. (Beverly, MA, USA). Goat anti-mouse IgG horseradish-peroxidase-labeled antibodies, and enhanced chemiluminescence reagents were from Perkin Elmer Life Science Inc. (Boston, MA, USA) and goat anti-rabbit IgG horseradish-peroxidase-labeled antibody was from NEN<sup>TM</sup> Life Science Products (Brussels, Belgium). Phospho-specific polyclonal antibody anti-Src kinases (Tyr418), cholera toxin B subunit-peroxidase conjugated, 17 $\beta$ -E2, methyl- $\beta$ -cyclodextrin, protease inhibitor cocktail, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF) and Na<sub>3</sub>VO<sub>4</sub> were purchased from Sigma-Aldrich (San Luis, MO, USA). Brij58 was from Pierce (Rochford, IL, USA). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA, USA). Hybond-P PVDF transfer membranes and Hyperfilm-ECL were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other reagents were of analytical grade.

### 2.2. Platelet isolation, methyl- $\beta$ -cyclodextrin treatment and platelet aggregation

Blood was withdrawn from 20 to 40 years old healthy male volunteers, who had not taken any drugs for at least 2 weeks before venipuncture, using ACD (130 mM citric acid, 152 mM sodium citrate, 112 mM glucose), as anticoagulant, and platelet-rich plasma was prepared by centrifugation at 180 $\times$ g for 10 min at 20 °C. For membrane cholesterol depletion, platelet-rich plasma was incubated with 20 mM methyl- $\beta$ -cyclodextrin (M $\beta$ CD) for 30 min at 37 °C. Platelets were recovered, and washed twice with PIPES buffer (20 mM Pipes, 136 mM NaCl, pH 6.5). Platelet count was adjusted to 3  $\times$  10<sup>8</sup> cells/ml in HEPES buffer containing 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub> and 2 mM glucose. Samples of platelet suspension (0.4 ml) were pre-warmed at 37 °C in a Chrono-log lumi-aggregometer, treated with vehicle or with 17 $\beta$ -E2 (100 nM) for 30 s and then stimulated with low or high (1 U/ml) doses of thrombin. As low dose of thrombin was considered the concentration of the agonist able to induce normal shape change but negligible aggregation. Usually it ranged between 0.04 and 0.08 U/ml, depending on the specific reactivity of platelets from different donors. Platelet aggregation was monitored continuously for at least 5 min as increase in light transmission.

### 2.3. Platelet lysis and recovery of membrane lipid rafts

Washed platelets, untreated or treated with M $\beta$ CD as described above, were adjusted to a final concentration of 2.5  $\times$  10<sup>9</sup> cells/ml in HEPES buffer containing 0.1% glucose. Samples of platelet suspension were treated with vehicle or with 100 nM 17 $\beta$ -E2 for 30 and 60 s, and then lysed with ice-cold 0.1% Brij58-containing buffer (0.1% Brij58, 150 mM NaCl, 25 mM Mes, pH 6.5, and 2 mM PMSF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1X protease inhibitor cocktail) or with ice-cold 0.1% Triton X-100-containing buffer (0.1% Triton X-100, 150 mM NaCl, 25 mM Mes, pH 6.5, and 2 mM PMSF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1X protease inhibitor cocktail). Lysates were adjusted to 40% sucrose by the addition of an equal volume of 80% sucrose in Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, and 150 mM NaCl, 2 mM PMSF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1X protease inhibitor cocktail). A six steps discontinuous gradient of 10–30% sucrose in MBS was formed on the top of the 40% homogenate in an ultracentrifuge tube. Tubes were centrifuged at 160,000 $\times$ g at 4 °C for 16 h. Twelve fractions of equal volumes (1 ml) were then collected from the top of the tube. Fractions were analyzed by dot blot with peroxidase conjugated-cholera toxin subunit to identify the fractions enriched in the GM1 ganglioside. In some experiments aliquots of each fraction were analyzed by immunoblotting with antibodies to p44/42 ERK and LAT. Raft, and non-raft (soluble), fractions were typically pooled, and aliquots (200  $\mu$ l) were analyzed by immunoblotting.

### 2.4. Analysis of ER $\beta$ localization within the platelet membranes

Aliquots of combined raft fractions and soluble fractions were subjected to protein precipitation with TCA. One volume of 100% TCA was added to 4

volumes of protein samples. After incubation for 30 min at 4 °C, samples were centrifuged at 13,000 rpm for 15 min. The supernatants were carefully removed, and the pellets were washed twice with 200  $\mu$ l cold acetone. The precipitated proteins were then dried by placing tubes in a fumehood for 10 min. Dried samples were solubilized in 3X Laemmly buffer (70  $\mu$ l) and analyzed by immunoblotting with antibodies to ER $\beta$ .

### 2.5. Electrophoresis and Immunoblotting analysis

Samples of washed platelets suspension, pre-treated or not with 20 mM M $\beta$ CD for 30 min at 37 °C, were further incubated with 100 nM 17 $\beta$ -E2 for times ranging from 30 to 180 s. Aliquots of each sample containing the same amount of proteins were analyzed by immunoblotting using anti-pSrc-Tyr418 and anti-Src antibodies. Aliquots of combined raft fractions and soluble fractions were analyzed by SDS-PAGE on a 10% polyacrylamide gel, transferred to PVDF membrane, and probed with antibodies specific to Pyk2, Src and p85-P13-K. The activation state of Src and Pyk2 was evaluated by phospho-specific polyclonal antibody anti-Pyk2 (Tyr402) and anti-Src kinases (Tyr418). Chemiluminescent signals were detected using the Versadoc Imaging System and analyzed using Quantity One software (Bio-Rad Laboratories). Only unsaturated images were considered.

## 3. Results

### 3.1. Lipid rafts mediate 17 $\beta$ -E2-dependent potentiation of thrombin-induced platelet aggregation

A growing number of evidence indicates that the cholesterol-rich membrane regions named lipid rafts, are implicated in the mechanisms of platelet activation by physiologic agonists. We first investigated the effect of lipid rafts disruption by membrane cholesterol depletion on platelet aggregation caused by high and low doses of thrombin. In agreement with previously reported data [34], we found that cholesterol depletion with methyl- $\beta$ -cyclodextrin (M $\beta$ CD) did not affect maximal platelet aggregation caused by high doses of thrombin (data not shown). By contrast, Fig. 1a–b shows

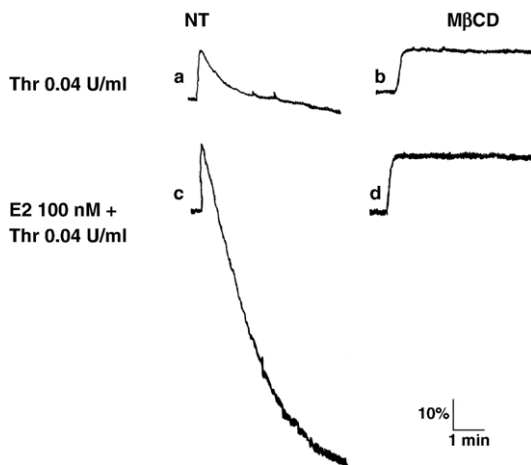


Fig. 1. Lipid rafts mediate the 17 $\beta$ -E2 potentiation of thrombin-induced platelet aggregation. Samples of washed platelets suspension, pre-treated (M $\beta$ CD) or not (NT) with 20 mM M $\beta$ CD for 30 min at 37 °C, were stimulated with 0.04 U/ml thrombin (Thr) alone (panels a and b), or in association with 100 nM 17 $\beta$ -E2, (panels c and d). Aggregation traces from a representative experiment out of 6 performed with platelets obtained from different donors are reported.

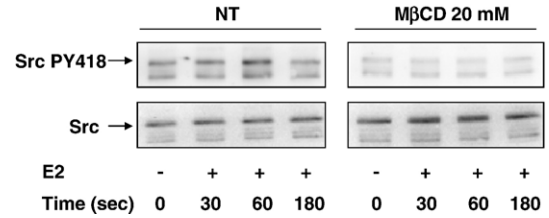


Fig. 2. Platelet lipid rafts play a role in 17 $\beta$ -E2-induced Src kinase activation. Samples of washed platelets, untreated (NT) or pre-treated with 20 mM M $\beta$ CD for 30 min at 37 °C, were incubated with 100 nM 17 $\beta$ -E2 for times ranging from 30 to 180 s, as indicated. Aliquots of each sample containing the same amount of proteins were simultaneously analyzed by immunoblotting using anti-pSrc-Tyr418 (top panels) and anti-Src antibodies (lower panels). The immunoblot analysis shown in the figure is from a representative experiment out of the 4 performed with platelets obtained from different donors.

that M $\beta$ CD-treated platelets stimulated with low doses of thrombin were still able to change their shape, but they did not aggregate at all. As shown in Fig. 1a–c, and as previously demonstrated [19], 17 $\beta$ -E2 induced a significant potentiation of platelet aggregation induced by a low dose of thrombin. However, Fig. 1d shows that 17 $\beta$ -E2 was unable to potentiate the effect of a low dose of thrombin in M $\beta$ CD-treated platelets. These results indicate that the integrity of membrane lipid rafts is crucial for 17 $\beta$ -E2-dependent signal transduction in platelets, and suggest a key role for these microdomains in the 17 $\beta$ -E2-dependent priming of platelet aggregation.

### 3.2. Lipid rafts are required for Src kinase activation in 17 $\beta$ -E2-treated platelets

The rapid, non-genomic effects of 17 $\beta$ -E2 in human platelets are mediated by ER $\beta$  receptors, which recruit and activate the tyrosine kinase Src [19]. To investigate the role of membrane lipid rafts in 17 $\beta$ -E2-induced signal transduction pathway, we analyzed the effect of cholesterol depletion by M $\beta$ CD on the hormone-dependent activation of Src. Fig. 2 shows that in untreated platelets 17 $\beta$ -E2 promoted an evident phosphorylation of the Src kinase, that reached a maximum within 1 min of incubation and then rapidly decreased to the basal level. However, no 17 $\beta$ -E2-dependent tyrosine phosphorylation of Src was detected in platelets treated with M $\beta$ CD. This implies that the integrity of membrane lipid rafts is essential for the 17 $\beta$ -E2-dependent activation of the tyrosine kinase Src in platelets.

### 3.3. ER $\beta$ translocates to the lipid rafts upon stimulation with 17 $\beta$ -E2

We next investigated whether ER $\beta$  was resident within the lipid rafts of the platelet membrane. Recent reports have described different experimental protocols as suitable to isolate lipid rafts from human platelets, using either 0.1% Brij58 or 0.1% Triton X-100 [20,35]. In preliminary experiments, upon platelet lysis with Brij58, we found that GM1 ganglioside, a positive marker for membrane lipid rafts, was recovered in less

dense fractions than upon platelet lysis with Triton X-100, in accordance with Wonerow et al. [36] (data not shown). In addition, more reproducible results were generally obtained by using Brij58-lysed platelets, and, therefore, this detergent was adopted for all the experiments described in this paper. Fig. 3A shows that GM1 ganglioside was mainly recovered in the fractions 4 to 7. LAT, a protein resident in membrane lipid rafts, was detected in the same fractions. By contrast ERK, known as negative marker of lipid rafts, was found exclusively in the fractions 11 and 12. The treatment of platelets with M $\beta$ CD resulted in GM1 ganglioside redistribution throughout the sucrose gradient, demonstrating that cholesterol is essential to segregate the GM1 ganglioside in less dense fractions of platelet membrane (Fig. 3B).

GM1-positive fractions of each sample were pooled as the lipid raft fraction. Moreover, fractions 11 and 12 were separately collected as the soluble fraction, while the pellet was discharged.

The combined fractions were then subjected to protein precipitation with TCA, and analyzed by immunoblotting with anti-ER $\beta$  specific antibody. A very small amount of ER $\beta$  was detected in the soluble fractions of resting platelets (Fig. 4). Upon stimulation with 17 $\beta$ -E2, a rapid and transient translocation of ER $\beta$  to the lipid rafts occurred, with a maximum at 30 s. Interestingly, despite the faintness of the ER $\beta$  bands, a concomitant decrease of ER $\beta$  could be detected in the soluble fractions obtained from the same samples (Fig. 4). Moreover, in M $\beta$ CD-cholesterol-depleted platelets ER $\beta$  was no longer detected in the lipid raft fractions. Accordingly with the evidence shown in Fig. 3, we assumed that M $\beta$ CD rendered ER $\beta$  undetectable by distributing the receptor overall the fractions. These results indicate that the engagement of ER $\beta$  by its specific ligand caused the rapid and transient recruitment of ER $\beta$  to the membrane lipid rafts.

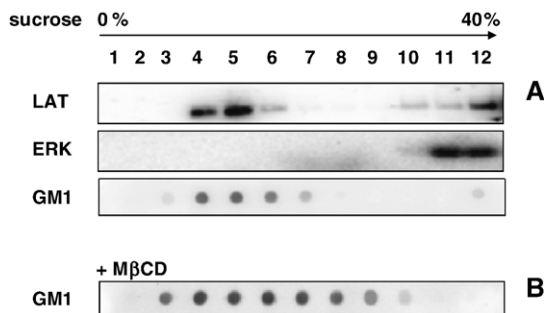


Fig. 3. Platelet lysis and recovery of membrane lipid rafts by sucrose density gradient. Samples of platelet suspension were lysed with ice-cold 0.1% Brij58-containing buffer and centrifuged in sucrose density gradient as described in Materials and methods. 12 fractions of equal volumes (1 ml) were collected and analyzed by dot blot with peroxidase-conjugated-cholera toxin subunit to identify the fractions containing the GM1 ganglioside and by immunoblot with anti-LAT and anti-p44/42 ERK antibodies (A). Samples of platelet suspension treated with 20 mM M $\beta$ CD for 30 min at 37 °C were fractionated and analyzed by dot blot with peroxidase-conjugated-cholera toxin in the same manner (B). The analysis reported in the figure are from a representative experiment out of the 4 performed with platelets obtained from different donors.

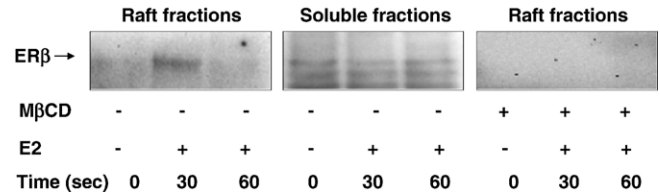


Fig. 4. ER $\beta$  translocates to the lipid rafts upon stimulation of platelets with 17 $\beta$ -E2. Samples of platelet suspension, treated or not with 20 mM M $\beta$ CD for 30 min at 37 °C, were incubated with vehicle or with 100 nM 17 $\beta$ -E2 for 30 or 60 s, as indicated, and then lysed in ice-cold 0.1% Brij58-containing buffer. Cell lysates were fractionated as described in Materials and methods. GM1-containing fractions (raft fractions) and more dense soluble fractions were combined, proteins were precipitated with TCA, and then analyzed by immunoblotting with anti-ER $\beta$  specific antibody. The immunoblot analysis shown in the figure is from a representative experiment out of the 8 performed with platelets obtained from different donors.

### 3.4. Lipid rafts mediate the 17 $\beta$ -E2 signaling in human platelets

We have previously shown that Src, Pyk2 and p85-PI3-K associated to ER $\beta$  upon treatment of human platelets with 17 $\beta$ -E2 [19]. To further characterize the role of lipid rafts in 17 $\beta$ -E2 signaling, we investigated the presence of these signal transducers in the lipid rafts isolated from 17 $\beta$ -E2-treated cells. Samples of resting and 17 $\beta$ -E2-treated platelets were lysed with Brij58 and fractionated by sucrose gradient centrifugation as described. Aliquots of combined raft and soluble fractions were analyzed by immunoblotting with antibodies specific for the Tyr418-phosphorylated form of Src (pSrc-Tyr418), Src, the Tyr402-phosphorylated form of Pyk2 (pPyk2-Tyr402), Pyk2 and p85-PI3-K. Fig. 5 shows that in resting platelets the signal transducers considered were unequally distributed between raft and soluble fractions. In fact,

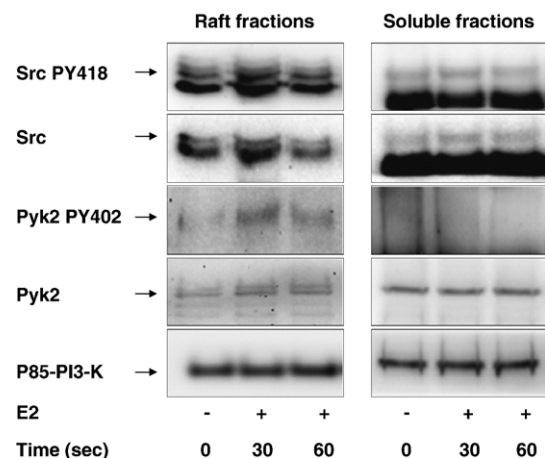


Fig. 5. Lipid rafts mediate the 17 $\beta$ -E2-dependent signaling in human platelets. Samples of platelet suspension were treated with vehicle or with 100 nM 17 $\beta$ -E2 for 30 or 60 s, and then lysed in ice-cold 0.1% Brij58-containing buffer. Raft fractions and soluble fractions obtained as described in Fig. 4 were analyzed by immunoblotting with antibodies specific for the Tyr418-phosphorylated form of Src (pSrc-Tyr418), Src, the Tyr402-phosphorylated form of Pyk2 (pPyk2-Tyr402), Pyk2 and p85-PI3-K. The immunoblot analysis shown in the figure is from a representative experiment out of the 6 performed with platelets obtained from different donors.

while appreciable amount of Src was already present in the membrane lipid rafts of resting cells, Pyk2 was mainly located in the soluble fractions, and p85-PI3-K seemed to be equally distributed in raft and soluble fractions. A mild Src kinase activation was detected already in the rafts of resting cells. However, upon 17 $\beta$ -E2 platelet stimulation, a consistent increase in Src Tyr418 phosphorylation occurred, which was paralleled by a concomitant increase of Src associated with the lipid rafts. As reported by the antibody datasheets, we found that antibodies to Src and pSrc-Tyr418 recognized at least a couple of proteins that were presumably two members of the Src kinase family. We did not identify which members of the Src kinase family were modulated by 17 $\beta$ -E2, however Fig. 5 shows that they behaved in the same manner with this respect. Moreover, 17 $\beta$ -E2-dependent Src kinases activation and translocation to raft fractions were rapid and reversible events, indicating that they were presumably related to each other.

Besides this, in 17 $\beta$ -E2-stimulated cells a discrete amount of Pyk2 was recruited to the lipid rafts with a kinetic similar to that of Src (Fig. 5). However, whilst the 17 $\beta$ -E2-induced association with the raft fractions and activation of Src seemed to be concomitant events, the tyrosine-phosphorylation of Pyk2 persisted up to 60 s of treatment with 17 $\beta$ -E2. Moreover, we found that 17 $\beta$ -E2 did not modulate the p85-PI3-K distribution within platelet membrane fractions (Fig. 5).

Finally, the integrity of the lipid rafts was found to be crucial for 17 $\beta$ -E2 signaling, as neither Src nor Pyk2 were detected in these microdomains upon M $\beta$ CD treatment (data not shown).

#### 4. Discussion

It has been previously shown that estrogen can significantly potentiate the aggregation induced by low doses of thrombin, by initiating a rapid and reversible signaling pathway mediated by ER $\beta$ -directed activation of the tyrosine kinases Src and Pyk2 at the level of the plasma membrane [19].

In this work we have demonstrated that membrane lipid rafts play an important role in these events, as they mediate the estrogen-dependent outside-in signaling in platelets. The involvement of lipid rafts in estrogen-dependent signaling in these cells was indicated by studies with the cholesterol-binding agent M $\beta$ CD. We found that in platelets in which the integrity of lipid rafts had been disrupted through cholesterol depletion by M $\beta$ CD, treatment with 17 $\beta$ -E2 failed to induce tyrosine phosphorylation of Src, and did not cause any potentiation of thrombin-dependent platelet aggregation. In agreement with previous works [35], we found that lipid raft integrity was not required for the aggregation induced by high doses of thrombin. Our results imply that membrane lipid rafts exert a specific and fundamental role in the signaling pathways activated by estrogens in human platelets.

In human platelets the main estrogen receptor is a glycosylated form of ER $\beta$  that is localized in the membranes [19,37]. In this work, we have shown that estrogen induced a rapid but transient translocation of the membrane-associated ER $\beta$  into hydrophobic microdomains of the platelet membrane.

The 17 $\beta$ -E2-dependent translocation of ER $\beta$  to these hydrophobic microdomains required the presence of cholesterol in the membrane, thus it is reasonable to assume that the hormone drives ER $\beta$  to membrane lipid rafts. Although additional experiments will be required to completely clarify the mechanism of this translocation, it is likely that binding of 17 $\beta$ -E2 may cause a conformational change of ER $\beta$  promoting its selective relocation within rafts.

We found that the treatment of platelets with 17 $\beta$ -E2 resulted in the recruitment of the tyrosine kinases Src and Pyk2 to lipid rafts, which was concomitant with their rapid and reversible tyrosine-phosphorylation. In agreement with previous works [38], we have found that at least two members of the Src kinase family were constitutively associated to the membrane rafts also in human platelets. However, we have also provided evidence that estrogens caused an evident increase of the association of Src proteins with these microdomains, and promoted the tyrosine phosphorylation of rafts-associated Src kinases. In the light of our results it is reasonable to suppose that 17 $\beta$ -E2-engaged ER $\beta$  is responsible for the recruitment to the rafts of the signal transducers involved in 17 $\beta$ -E2-dependent signaling, in particular Src kinases and Pyk2. This hypothesis is confirmed by the inhibition of both Src activation and 17 $\beta$ -E2-dependent translocation of ER $\beta$ , Src and Pyk2 to lipid rafts in M $\beta$ CD-treated platelets. Moreover, in a previous work, we have shown that in 17 $\beta$ -E2-treated platelets Src, Pyk2 and PI3-K associated to ER $\beta$  to form a signal molecular complex [19]. However, in contrast to what was reported for other cells, PI3-K did not play any active role in 17 $\beta$ -E2-dependent signaling in human platelets. Here we confirmed and extended these findings, by showing that distribution of PI3-K between raft and soluble fractions, was not modulated by 17 $\beta$ -E2.

Noteworthy, ER $\beta$  is a membrane protein poorly expressed in platelets. It can be inferred that lipid rafts may represent a structural–functional link between the engaged estrogen receptor and its specific intracellular interactors. Moreover, lipid rafts could bring some hierarchy to the multitude of signaling pathways contributing to platelet activation, based on the concentration of interactive signaling complexes in well-defined morphological membrane compartments.

In conclusion, we propose that membrane lipid rafts play a key role in 17 $\beta$ -E2-dependent signaling in human platelets and are directly involved in the molecular mechanism by which estrogen exerts a priming effect on platelet aggregation induced by low doses of thrombin.

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#### References

- [1] M. Beato, P. Herrlich, G. Schutz, Steroid hormone receptors: many actors in search of a plot, *Cell* 83 (1995) 851–885.

- [2] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, *Cell* 83 (1995) 835–839.
- [3] E. Falkenstein, H.C. Tillmann, M. Christ, M. Feuring, M. Wehling, Multiple actions of steroid hormones: a focus on rapid, nongenomic effects, *Pharmacol. Rev.* 52 (2000) 513–556.
- [4] C.M. Szego, J.S. Davis, Adenosina 3',5'-monophosphate in rat uterus: acute elevation by estrogen, *Proc. Natl. Acad. Sci. U. S. A.* 58 (1967) 1711–1718.
- [5] R.X. Song, A. McPherson, L. Adam, Y. Bao, M. Shupnik, R. Kumar, R.J. Santen, Linkage of rapid estrogen action to MAPK activation by ER $\alpha$ -Shc association and Shc pathway activation, *Mol. Endocrinol.* 16 (2002) 116–127.
- [6] T. Simoncini, A. Hafezi-Moghadam, D.P. Brazil, K. Ley, W. Chin, J.K. Liao, Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase, *Nature* 407 (2000) 538–541.
- [7] P. Morley, J.F. Whitfield, B.C. Vanderhyden, B.K. Tsang, J.L. Schwartz, A new, nongenomic estrogen action: the rapid release of intracellular calcium, *Endocrinology* 131 (1992) 1305–1312.
- [8] A. Migliaccio, M. Di Domenico, G. Castoria, A. de Falco, P. Contempo, E. Nola, F. Auricchio, Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol–receptor complex in MCF-7 cells, *EMBO J.* 15 (1996) 1292–1300.
- [9] S. Valera, M. Ballivet, D. Bertrand, Progesterone modulates a neuronal nicotinic acetylcholine receptor, *Proc. Natl. Acad. Sci. U. S. A.* 99 (1992) 9949–9953.
- [10] K. Honda, H. Sawada, T. Kihara, M. Urushitani, T. Nakamizo, A. Akaike, S. Shimohama, Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons, *J. Neurosci. Res.* 60 (2000) 321–327.
- [11] K.S. Russell, M.P. Hynes, D. Sinha, E. Cleriarne, J.R. Bender, Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 5930–5935.
- [12] R.S. Elkeles, J.R. Hampton, J.R. Mitchell, Effect of oestrogens on human platelet behaviour, *Lancet* 2 (1968) 315–318.
- [13] R. Ross, J.A. Glomset, The pathogenesis of atherosclerosis: Part 1, *N. Engl. J. Med.* 295 (1976) 369–377.
- [14] V. Fuster, L. Badimon, J.J. Badimon, J.H. Chesebro, The pathogenesis of coronary artery disease and the acute coronary syndromes, *N. Engl. J. Med.* 326 (1992) 310–318.
- [15] J. Bar, J. Lahav, M. Hod, Z. Ben-Rafael, I. Weinberger, J. Brosens, Regulation of platelet aggregation and adenosine triphosphate release in vitro by 17 $\beta$ -estradiol and medroxyprogesterone acetate in postmenopausal women, *Thromb. Haemost.* 84 (2000) 695–700.
- [16] K.D. Bouboulas, G.E. Cooke, C.M. Roos, P.F. Bray, P.J. Goldschmidt-Clermont, The PIA polymorphism of glycoprotein IIIa functions as a modifier for the effect of estrogen on platelet aggregation, *Arch. Pathol. Lab. Med.* 125 (2001) 112–115.
- [17] M.E. Miller, S.L. Thorpe, G.M. Dores, Influence of hormones on platelet intracellular calcium, *Thromb. Res.* 77 (1995) 515–530.
- [18] I. Anwaar, M. Rendell, A. Gottsater, F. Lindgarde, U.L. Hulthen, I. Mattiasson, Hormone replacement therapy in healthy postmenopausal women. Effects on intraplatelet cyclic guanosine monophosphate, plasma endothelin-1 and neopterin, *J. Intern. Med.* 247 (2000) 463–470.
- [19] L. Moro, S. Reineri, D. Piranda, D. Pietrapiana, P. Lova, A. Bertoni, A. Graziani, P. Defilippi, I. Canobbio, M. Torti, F. Sinigaglia, Non-genomic effects of 17 $\beta$ -estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor  $\beta$  and Src kinase, *Blood* 105 (2005) 115–121.
- [20] S. Bodin, H. Tronchere, B. Payrastra, Lipid rafts are critical membrane domain in blood platelet activation processes, *Biochim. Biophys. Acta* 1610 (2003) 247–257.
- [21] K. Simons, D. Toomre, Lipid rafts and signal transduction, *Nat. Rev., Mol. Cell Biol.* 1 (2000) 31–39.
- [22] T. Harder, P. Scheiffele, P. Verkade, K. Simons, Lipid domain structure of the plasma membrane revealed by patching of membrane components, *J. Cell Biol.* 141 (1998) 929–942.
- [23] K.L. Chambliss, I.S. Yuhanna, C. Mineo, P. Liu, Z. German, T.S. Sherman, M.E. Mendelsohn, R.G. Anderson, P.W. Shaul, Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae, *Circ. Res.* 87 (2000) E44–E52.
- [24] M.J. Kelly, E.R. Levin, Rapid actions of plasma membrane estrogen receptors, *Trends Endocrinol. Metab.* 12 (2001) 152–156.
- [25] M. Razandi, P. Oh, A. Pedram, J. Schnitzer, E.R. Levin, ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions, *Mol. Endocrinol.* 16 (2002) 100–115.
- [26] L. Li, M.P. Haynes, J.R. Bender, Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 4807–4812.
- [27] T.M. Stulnig, M. Berger, T. Sigmund, H. Stockinger, V. Horejsi, W. Waldhausl, Signal transduction via glycosyl phosphatidylinositol-anchored proteins in T cells is inhibited by lowering cholesterol, *J. Biol. Chem.* 272 (1997) 19242–19247.
- [28] E.D. Sheets, D. Holowka, B. Baird, Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of Fc $\epsilon$ RI and their association with detergent-resistant membranes, *J. Cell Biol.* 145 (1999) 877–887.
- [29] T. Furuchi, R.G.W. Anderson, Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase (ERK), *J. Biol. Chem.* 273 (1998) 21099–21104.
- [30] K. Gousset, W.F. Wolkers, N.M. Tsvetkova, A.E. Oliver, C.L. Filed, N.J. Walker, J.H. Crowe, F. Tablin, Evidence for a physiological role for membrane rafts in human platelets, *J. Cell. Physiol.* 190 (2002) 117–128.
- [31] D. Locke, H. Chen, Y. Liu, C. Liu, M.L. Kahn, Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI, *J. Biol. Chem.* 277 (2002) 18801–18809.
- [32] C.N. Shrimpton, G. Borthakur, S. Larrucea, M.A. Cruz, J.F. Dong, J.A. Lopez, Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation, *J. Exp. Med.* 196 (2002) 1057–1066.
- [33] H.F. Heijnen, M. van Lier, S. Waaijenborg, Y. Ohno-Iwashita, A.A. Waheed, M. Inomata, G. Gorter, W. Mobius, J.W. Akkerman, J.W. Slot, Concentration of rafts in platelet filopodia correlates with recruitment of c-Src and CD63 to these domains, *J. Thromb. Haemost.* 1 (2003) 1161–1173.
- [34] S. Bodin, C. Soulet, H. Tronchère, P. Sié, C. Gachet, M. Plantavid, B. Payrastra, Integrin-dependent interaction of lipid rafts with the actin cytoskeleton in activated human platelets, *J. Cell Sci.* 118 (2005) 759–769.
- [35] T.M. Quinton, S. Kim, J. Jin, S.P. Kunapuli, Lipid rafts are required in Galpha(i) signaling downstream of the P2Y1 receptor during ADP-mediated platelet activation, *J. Thromb. Haemost.* 3 (2005) 1036–1041.
- [36] P. Wonerow, A. Obergfell, J.I. Wilde, R. Bobe, N. Asazuma, T. Brdicka, A. Leo, B. Schraven, V. Horejsi, S.J. Shattil, S.P. Watson, Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase C $\gamma$ 2 regulation in platelets, *Biochem. J.* 364 (2002) 755–765.
- [37] M.L. Nealen, K.V. Vijayan, E. Bolton, P.F. Bray, Human platelets contain a glycosylated estrogen receptor beta, *Circ. Res.* 88 (2001) 438–442.
- [38] D.J. Dorahy, L.F. Lincz, C.J. Meldrum, G.F. Burns, Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36, *Biochem. J.* 319 (1996) 67–72.