FEBS Letters 588 (2014) 3154–3159

journal homepage: www.FEBSLetters.org

The association of thromboxane A_2 receptor with lipid rafts is a determinant for platelet functional responses

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ARTICLE INFO

Article history:
Received 17 June 2014
Accepted 24 June 2014
Available online 1 July 2014

Edited by Veli-Pekka Lehto

Keywords:
Lipid raft
Platelet
Signal transduction
Thromboxane A_2
Thromboxane receptor

ABSTRACT

We have investigated the presence of thromboxane A_2 (TXA_2) receptor associated with lipid rafts in human platelets and the regulation of platelet function in response to TXA_2 receptor agonists when lipid rafts are disrupted by cholesterol extraction. Platelet aggregation with TXA_2 analogs U46619 and IBOP was almost blunted in cholesterol-depleted platelets, as well as $\alpha_{IIb}\beta_3$ integrin activation and P-selectin exposure. Raft disruption also inhibited TXA_2-induced cytosolic calcium increase and nucleotide release, ruling out an implication of $P2Y_{12}$ receptor. An important proportion of TXA_2 receptor (40%) was colocalized at lipid rafts. The presence of the TXA_2 receptor associated with lipid rafts in platelets is important for functional platelet responses to TXA_2.

1. Introduction

The inhibition of platelet thromboxane A_2 (TXA_2) synthesis by aspirin constitutes the first front of antiplatelet treatment in patients with acute cardiovascular disease and, in secondary prevention, to reduce the risk of a new event among patients at high risk of occlusive vascular events [1]. TXA_2 is produced by activated platelets and acts by reinforcing platelet activation and by inducing the recruitment of new platelets to the growing thrombus. In addition, TXA_2 is a powerful vasoconstrictor. Actions of TXA_2 on platelets are mediated by the activation of TXA_2 receptor in the membrane of cells. The TXA_2 receptor belongs to the superfamily of seven transmembrane-domains receptors. In platelets, two isoforms of TXA_2 receptor (TP_α and TP_β) have been identified [2]. Both the TP_α and TP_β subtypes mediate the stimulation of phospholipase C and an increase in intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol. The formation of inositol 1,4,5-triphosphate induces an increase in the cytosolic concentration of Ca^{2+}, whereas the release of diacylglycerol activates PKC [2]. These actions of the TXA_2 receptors in platelets are mediated by the association of the receptor to G_q and G_{13} proteins [3].

The cell membrane microdomains called lipid rafts are sphingolipid and cholesterol-based structures consisting of very small domains (20–50 nm) of tightly packed lipids displaying lateral mobility [4]. Lipid raft formation should therefore be facilitated in membranes rich in cholesterol and sphingolipids that promote the formation of liquid-ordered domains in the presence of cholesterol [5]. Interestingly, there is a relatively high amount of sphingomyelin in human platelets plasma membrane as compared to other cell types, suggesting a lipid composition in favor of lipid raft formation in these cells [6]. Several previous studies have suggested that lipid rafts are highly dynamic platelet membrane structures involved in critical signaling mechanisms [4,7]. It has been reported that the presence of different proteins associated with lipid rafts, including membrane receptors (CD36, GPIb, GPVI, P2Y12), signal transduction partners (LAT, src, G proteins) and enzymes (PI3K, PLCγ_2) [6]. However, to the best of our knowledge, the presence of TXA_2 receptor in lipid rafts in platelets has not been previously described, although in other cell types the localization of TXA_2 receptors in lipid rafts have already been demonstrated [8,9].

The aim of this study was to explore the association of the TXA_2 receptor with lipid rafts in human platelets and the functional implications of this location for the platelet responses to TXA_2. Our results demonstrate, for the first time, the importance of lipid rafts for TXA_2-induced platelet responses, a process related with the presence of a fraction of TXA_2 receptors associated with lipid rafts.

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http://dx.doi.org/10.1016/j.febslet.2014.06.057

2. Methods

2.1. Antibodies

TXA_2 receptor (Cayman Chemicals, Ann Harbor, MI); CD36 (Santa Cruz Biotechnology, Dallas, TX); CD62-FITC, CD61-PE, CD42-phycoerythrin (PE) (Beckman Coulter, Barcelona, Spain); PAC-1-fluorescein isothiocyanate (FITC) (Becton Dickinson, Madrid, Spain).

2.2. Blood collection and platelet processing

Venous blood was obtained from healthy fasting donors, drug-free for at least 15 days, after informed consent, as approved by the institutional review board of the Hospital La Fe. Platelet isolation and washing were performed as described [10]. After washing, platelets were re-suspended in HBSS buffer (in mmol/L): 0.8 $MgSO_4$, 5.36 KCl, 0.441 KH_2PO_4, 137 NaCl, 0.34 Na_2HPO_4, 5.55 Glucose, 20 HEPES, pH 7.4.

2.3. Depletion of platelet cholesterol

Platelet rich plasma (PRP) was incubated (15 min., 37 °C) with 5 mM methyl-β-cyclodextrin (MβCD) (Sigma Aldrich, Madrid, Spain) [11]. Platelets were then isolated and re-suspended as described [10].

2.4. Platelet aggregation

Platelet aggregation was assessed by optical aggregometry in 300 μl of washed platelets (2×10^8 platelets/ml) in HBSS buffer supplemented with 1 mM $CaCl_2$ (final concentration) at 37 °C with constant stirring (1000 rpm) in a Chrono-Log 490–2D platelet aggregometer (Chrono-Log Corporation, Havertown, PA). The amplitude (percentage) of the platelet aggregation response was monitored up to 3 min. in reference to a buffer blank.

2.5. Dense granules release

Dense granules release was monitored by platelet nucleotide release as previously described [12]. Briefly, after 3 min. of platelet aggregation, samples were transferred to an eppendorf tube and centrifuged (1 min., 13 000×g), and the supernatant was collected. Perchloric acid (1:10 v/v, final concentration 0.3 N) was added to supernatants, kept in ice for 1 h, and the precipitated proteins were pelleted by centrifugation (8 min., 13 000×g, 4 °C). Supernatants were carefully neutralized with KOH, centrifuged (8 min., 13 000×g, 4 °C), and kept at −80 °C until further processing. Thawed samples were filtrated and injected in a Waters 600E HPLC system equipped with a C18 column (Teknokroma, Barcelona, Spain) and a UV detector Waters 486. Isocratic elution (0.4 ml/min., 30 °C) was performed with a mobile phase consisting of 200 mmol/L KH_2PO_4 adjusted to pH 6 with NH_4OH. Nucleotide elution was monitored at 254 nm (Lambda-Max Model 480, Waters–Waters Cromatografía SA, Barcelona, Spain). Concentration of nucleotides was calculated using a standard calibration curve of adenine nucleotides.

2.6. Flow cytometry

MβCD-free or MβCD-treated washed platelets (2×10^8 platelets/ml) in HBSS buffer plus 1 mmol/L $CaCl_2$ were incubated without stirring (10 min., 37 °C). Agonists were added, and incubation was continued for 5 min. without stirring. Duplicate 10-μL aliquots of stimulated platelets were transferred to polypropylene tubes that contained 100 μL HBSS buffer without calcium. To each sample, saturating concentrations of PAC-1-FITC or CD62-FITC plus a general platelet marker (CD42-PE for PAC-1 or CD61-PE for CD62 analysis) were added, kept undisturbed (30 min., 20 °C, dark), quench-diluted with 1 ml ice-cold HBSS, and maintained at 4 °C in the dark [10]. Platelets were gated based on size and CD42/CD61 fluorescence. Results are reported as percentages of platelets expressing PAC-1 or CD62 in a total of 5000 platelets per sample analyzed in an EPICS XL-MCL flow cytometer (Beckman Coulter, Madrid, Spain).

2.7. Measurement of cytosolic free Ca^{2+} concentration

PRP (treated or not with MβCD) was incubated with 1.5 μM FURA 2/AM (37 °C, 45 min.), washed and resuspended in HBSS buffer (75×10^5 platelet/ml) containing 1 mM calcium. Changes in FURA 2/AM fluorescence were continuously monitored after agonist addition by dual excitation fluorimetry at 340 and 380 nm, at 37 °C with stirring in an RF-1501 spectrofluorophotometer (Shimadzu, Duisburg, Germany), and the calcium concentration was calculated as described [13].

Isolation of lipid rafts fractions and identification of associated proteins was performed as previously described [14]. Washed platelets (4×10^8 platelets/ml) were lysed on ice with lysis buffer (final concentration (in mmol/L): 50 Tris–HCl pH 7.4, 100 NaCl, 5 EDTA, 50 NaF, 10 sodium pyrophosphate, 1 Na_3VO_4, 1% CHAPS supplemented with 1× protease inhibitor cocktail III (Merck Chemicals Ltd, Nottingham, UK). After complete rupture of platelets by aspirating repeatedly with a Hamilton syringe, lysates were mixed 1:1 with MNE buffer (MES (2-(N-morpholino) ethanesulfonic acid) 25 mmol/L pH 6.5, EDTA 5 mmol/L, NaCl 150 mmol/L) containing 80% sucrose. 1.5 ml of this mixture was laid on the bottom of an ultracentrifuge tube and sequencially overlaid carefully with 1.5 ml of 30% and 750 μl of 5% sucrose in MNE buffer. Samples were centrifuged (200 000×g, 18 h, 4 °C). Aliquots of 300 μl were carefully collected sequentially from the upper surface, mixed 1:1 with Laemmli sample buffer, and boiled 5 min. Equal volumes of sample were loaded on 4–12% polyacrylamide preformed gels (Life Technologies, Madrid, Spain), and the separated proteins were transferred to nitrocellulose membranes [10]. Immunodetection of CD36 and TXA_2 receptor were performed as previously described [10]. Images were scanned and quantified using the freely available public domain software ImageJ 1.45e (NIH, http:// rsbweb.nih.gov/ij/).

2.8. Statistical analysis

Significance was determined by Student's *t*-test. Results were expressed as mean ± S.E.M. of at least three different experiments.

3. Results

3.1. Regulation of thromboxane-induced platelet activation by the lipid rafts

To determine whether lipid rafts played a role in thromboxane-induced platelet activation, we investigated the effect of membrane cholesterol depletion on platelet aggregation, P-selectin exposure, and $\alpha_{IIb}\beta_3$ activation. We used two stable, structurally different analogs of thromboxane: U46619 (1 μM) and IBOP (10 nM) (Cayman Chemicals, Ann Arbor, MI). After cholesterol depletion with MβCD, U46119- and IBOP-induced aggregation were significantly inhibited (Fig. 1A). The different patterns of inhibition could be attributed to the different concentrations of the two thromboxane analogs. Interestingly, platelet aggregation induced