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Loss of Reelin protects mice against arterial thrombosis by impairing integrin activation and thrombus formation under high shear conditions

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ABSTRACT

Reelin is a secreted glycoprotein and essential for brain development and plasticity. Recent studies provide evidence that Reelin modifies platelet actin cytoskeletal dynamics. In this study we sought to dissect the contribution of Reelin in arterial thrombus formation. Here we analyzed the impact of Reelin in arterial thrombosis ex vivo and in vivo using Reelin deficient (reeler) and wildtype mice. We found that Reelin is secreted upon platelet activation and mediates signaling via glycoprotein (GP)Ib, the amyloid precursor protein (APP) and apolipoprotein E receptor 2 (ApoER2) to induce activation of Akt, extracellular signal-regulated kinase (Erk), SYK and Phospholipase Cγ2. Moreover, our data identifies Reelin as first physiological ligand for platelet APP. Platelets from reeler mice displayed attenuated platelet adhesion and significantly reduced thrombus formation under high shear conditions indicating an important role for Reelin in GPIb-dependent integrin $\alpha_{IIb}\beta_3$ activation. Accordingly, adhesion to immobilized vWF as well as integrin activation and the phosphorylation of Erk and Akt after GPIb engagement was reduced in Reelin deficient platelets. Defective Reelin signaling translated into protection from arterial thrombosis and cerebral ischemia/reperfusion injury beside normal hemostasis. Furthermore, treatment with an antagonistic antibody specific for Reelin protects wildtype mice from occlusive thrombus formation. Mechanistically, GPIb co-localizes to the major Reelin receptor APP in platelets suggesting that Reelin-induced effects on GPIb signaling are mediated by APP-GPIb interaction. These results indicate that Reelin is an important regulator of GPIb-mediated platelet activation and may represent a new therapeutic target for the prevention and treatment of cardio- and cerebrovascular diseases.

1. Introduction

Platelets are the key players in (patho-) physiological processes of hemostasis and thrombus formation upon tissue trauma. Vascular injury leads to exposure of extracellular matrix proteins important for adhesion and activation of circulating platelets. A first interaction is initiated by collagen-bound von Willebrand Factor (vWF), released from endothelial cells and platelet glycoprotein (GP)Ib-IX-V complex. Consequently, platelets come into close proximity of the injured vessel allowing the interaction of GPVI, the major collagen receptor on platelet membranes, and collagen [45]. GPVI binding to collagen leads to platelet activation such as degranulation of the second wave mediators' adenosine diphosphate (ADP) and thromboxane A2 (TxA2) and integrin $\alpha_{IIb}\beta_3$ activation. Fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ induces aggregate formation and thrombus formation resulting in vascular occlusion [28,33].

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While it is well known that collagen binding to GPVI induces robust platelet activation, the consequences of vWF binding to GPIb were discussed for years. Initially it was assumed that GPIb binding to vWF only induces platelet tethering to the extracellular matrix to allow the interaction of GPVI with collagen important for first intracellular activation signals. However, recent studies provide strong evidence that vWF-GPIb interaction generates intracellular signals e.g. tyrosine phosphorylation of SYK and phospholipase (PL) Cγ2 to induce integrin $\alpha_{IIb}\beta_3$ activation [1,10,29]. Moreover, association of GPIb and phosphatidylinositol-3 (PI3)-kinase has been described that involves Akt phosphorylation important for integrin activation [27].

Reelin is an extracellular matrix protein which is produced by Cajal-Retzius neurons and plays an important role in brain development, because it controls neuronal positioning and migration. Reelin is known to mediate the formation of laminated brain structures during development by binding to the lipoprotein receptors apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) [17]. The intracellular domains of these receptors interact with the adapter protein Disabled1 (Dab1) to induce signal transduction into the cell [39] including Src family kinases [3] and PI3-kinase activation [37]. Reelin influences cytoskeletal reorganization because Reelin appears to induce cytoskeletal stabilization by mediating cofilin phosphorylation [23]. Furthermore, Reelin mediates the formation of filopodia and lamellipodia via signaling through apolipoprotein E receptor 2 (ApoER2) and the Rho GTPases Cdc42 and Rac1 [25,41,42].

Reelin is also found in blood plasma, endothelial cells, liver, pituitary pars intermedia and adrenal chromaffin cells [31,35], but the role of Reelin in the circulation and peripheral tissues is widely unknown. Reelin is expressed in platelets where it co-localizes to F-actin and thus might influence the reorganization of the platelet cytoskeleton [41]. In a more recent study Tseng and colleagues provide evidence for Reelin to play a role in the coagulation activation by interacting with phospholipids at the platelet membrane [40]. Here we analyzed receptor mediated Reelin signaling in platelets and the impact of Reelin in platelet activation and thrombus formation in vitro and in vivo to understand the impact of Reelin in hemostasis and arterial thrombosis.

2. Materials and methods

2.1. Animal models

Reelin-deficient mice (spontaneous mutation, reeler, B6C3Fe *a/a-Reln^{rl}*/J, this mouse line involves C3HeB/FeJ*C57BL/6J), gene-targeted mice lacking Amyloid precursor protein (*App*$^{-/-}$, this mouse line involves 129/Sv/C57BL/6J), ApoER2 (*Lrp8*$^{-/-}$, B6;129S6-*Lrp8^{tm1Her}*/J, this mouse line involves 129S6/SvEvTac*C57BL/6J) and the extracytoplasmic domain of GPIbα (mGP Ibα$^{-/-}$, IL-4R/Ibα^{Tg}), (all on a C57Bl/6J background), respectively, and the corresponding wild-type littermates were bred from breeder pairs. Experiments were performed with male and female mice aged 1–6 months. Mice were anesthetized with Ketamin (Ketavet®, Pfizer, 100 mg/kg) and Xylazin (2% Bernburg, medistar, 5 mg/kg) by intraperitoneal (i.p.) injection before surgery. Euthanasia was performed by cervical dislocation. All animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The protocol was approved by Heinrich-Heine-University Animal Care Committee and by the district government of North Rhine-Westphalia (LANUV, NRW; Permit Number: 84-02.04.2013.A210; 84-02.05.20.12.284).

2.2. Murine platelet preparation

Murine heparin-anticoagulated blood from retro-orbital plexus was collected and centrifuged at 1800 rpm for 5 min at room temperature. To obtain platelet-rich plasma (PRP), the supernatant was centrifuged at 800 rpm for 6 min. PRP was washed twice at 2800 rpm for 5 min at room temperature and pellet was resuspended in Tyrode's buffer [136 mM NaCl, 0.4 mM Na_2HPO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 7.4] supplemented with prostacyclin (0.5 μM) and apyrase (0.02 U/ml). Before use, platelets were resuspended in the same buffer and incubated at 37 °C.

2.3. Human platelet preparation

Fresh citrate-anticoagulated blood (BD-Vacutainer®; Becton, Dickinson and Company) was obtained from healthy volunteers and blood was centrifuged at 200 g for 12 min. The PRP was separated and added to PBS [pH 6.5, 2.5 U/ml apyrase (Sigma), 1 μM PGI_2] in 1:1 volumetric ratio and centrifuged at 1000*g* for 6 min. The platelet pellet was resuspended in Tyrode buffer [137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 5.5 mM Glucose, 0.1% HIBSA, pH 7.4] and platelet counts were adjusted as required for the applied functional assay. The collection of blood samples was performed conform the declaration of Helsinki and approved by the Ethics Committee of the Heinrich-Heine-University based on the donors' consent.

2.4. Western blotting

Stimulated and lysed platelet and brain samples were separated via SDS-polyacrylamide gel and transferred onto nitrocellulose blotting membrane (GE Healthcare Life Sciences). Subsequently, the membrane was blocked using 5% milk powder in TBST (Tis-Buffered Saline with 10% Tween 20) and probed with antibodies against Reelin (20E12), PAK/p-PAK1/2 (Cell Signaling, #2602/#2601, 1:500), ApoER2 (Abcam, #ab108208, 1:1000), APP [Y188] from Abcam (ab32136)/(Biomol/Epitomics, #1565, 1:1000), phospho-ERK1/2 (Cell Signaling, #4370, 1:2000), phospho-Akt (Cell Signaling, #4060, 1:1000), phospho-PLCγ2 (Cell Signaling, #3874, 1:500) and phospho-SYK (Cell Signaling, #2711, 1:500), GPVI (emfret Analytics, JAQ1 # M011-1, 1:1000), Integrin $\alpha_{IIb}\beta_3$ (emfret Analytics, Leo.H4, # M021-0, 1:1000) and Integrin α5 chain (emfret Analytics, Tap.A12, # M080-1).

2.5. Flow chamber

Coverslips (24 × 60 mm) were coated with 200 μg/ml fibrillar type I collagen (HORM®, Nycomed) overnight and then blocked with 1% BSA solution for at least 60 min. Tyrode buffer was pre-warmed at 37 °C. Mice were anesthetized with isoflurane (Forene®, abbvie) and blood was taken from the retro-orbital plexus of each mouse and collected in a tube containing 300 μl heparin (20 U/ml in PBS). As indicated whole blood was labelled with Dylight X488 (emfret Analytics, #X488, 0.1 μg/ml) and perfused through the flow chamber at a shear rate of 1000 s^{-1} and 1700 s^{-1}; platelet adhesion and thrombus formation were evaluated. In another set of experiments coverslips (24 × 60 mm) were coated with rabbit antibody against human vWF (1:500; DakoCytomation, A0082) for 1 h at 37 °C and blocked with 1% bovine serum albumin (BSA). To allow binding of vWF to immobilized antibody against VWF, blocked coverslips were incubated with murine plasma for 2 h at 37 °C. Platelet adhesion to the VWF matrix was counted per visual field.

2.6. Statistical analysis

Data are given as mean ± s.e.m. (standard error of mean) from at least three individual experiments (n represents the number of experiments). All data were tested for significance using one-way ANOVA or Student's paired *t*-test, where applicable. P < values < 0.05 were considered to be statistically significant.