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LETTER

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Identification of apolipoprotein A-I as a target of platelet tyrosine kinases

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The antiplatelet properties of apolipoprotein A-I (apoA-I) account at least in part for its well-established antiatherogenic character. Nevertheless, the underlying signaling pathways are poorly understood.[1,2] Therefore, we read with great interest the article by Jones and colleagues,[3] who demonstrate that the inhibitory effects of apoA-I on platelet function are accompanied by alterations in Akt signaling. In addition to Akt, numerous protein kinases and phosphatases are involved in platelet activation catalyzing reversible protein phosphorylation, an important regulatory mechanism for almost every aspect of platelet function.[4] Here, we identify apoA-I as a substrate for a platelet tyrosine kinase upon platelet activation, revealing the reciprocal interaction of apoA-I with platelets.

Upon platelet activation, several proteins undergo changes in tyrosine phosphorylation, a phenomenon first described by Ferrell and Martin in 1988.[5] We have previously reported that a protein with the apparent molecular mass of 27 kDa (p27), which is not tyrosine phosphorylated in resting platelets, becomes transiently tyrosine phosphorylated in response to stimulation with various agonists (e.g. thrombin, collagen, U46619, 2-MeS-ADP).[6] As shown here (Figure 1A), after stimulation with thrombin (0.1 U/ml) maximal p27 tyrosine phosphorylation occured after 30 s, followed by a rapid dephosphorylation within 5 min. At the time point of maximal phosphorylation (30 s), the analysis of our data revealed no significant differences in the extent of p27 tyrosine phosphorylation in response to 0.1 U/ml thrombin compared to 3 µg/ml collagen (Adj. Vol. [OD*mm^2] 2.36 ± 1.09 vs. 2.51 ± 0.91, $n = 6$) (Figure 1B).

Unaware of its identity, we already extensively studied key signaling pathways for their contribution to p27 tyrosine phosphorylation in our laboratory. Complete p27 tyrosine phosphorylation requires signal amplification by secreted ADP via $P2Y_{12}$-Gαi-signaling. Reinforcing effects of thromboxane A_2 are indispensable for collagen-elicited p27 tyrosine phosphorylation, but not mandatory after thrombin-stimulation. Furthermore, outside-in-signaling via integrin $\alpha_{IIb}\beta_3$ mediates phosphatase activity.[6]

To determine the identity of p27, we isolated platelets from recently outdated platelet concentrates from the University blood bank in Halle. Preformed platelet aggregates were removed by centrifugation (275 g, 10 min). After a second centrifugation step (700 g, 10 min), the pellet was suspended in sample buffer (137 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 362 µM NaH_2PO_4*H_2O, 10 mM HEPES, 5.6 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02 U/ml apyrase, pH 7.4). Platelet suspensions ($3.75 \pm 1.25 \times 10^{11}$ platelets/ml) were stimulated with 1 U/ml thrombin (Sigma, Munich, Germany) for 30 s prior to addition of lysis buffer (10 mM Tris-HCl pH 7.4, 1% Triton X-100, 5 mM EDTA pH 7.4, 10 µM leupeptin, 1 mM pefabloc, 15 µM aprotinin, 25 µM benzamidine, 10 µM ε-aminocaproic acid, 10 µM pepstatin A, 1 mM sodium orthovanadate, 5 µg/ml ciclosporin A, 1 µM calpeptin). Platelet lysates were homogenized (275 g, 2 min) and sonificated twice (4°C, 30 s). After incubation (20 min, 4°C), the lysates were centrifuged (30 min 49 000 g, 4°C). The supernatant was applied to a DEAE-sepharose column (Deutsche Pharmacia GmbH, Frankfurt, Germany) equilibrated with buffer A pH 7.4 (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 0.05% Triton X-100, 2 mM EDTA, 25 mM NaF, 25 mM $Na_4P_2O_7$, 25 mM ß-glycerophosphate, 1 mM sodium orthovanadate). Elution was performed using buffer B pH 6.9 (100 mM NaH_2PO_4, 100 mM Na_2HPO_4, 0.05% Triton X-100, 2 mM EDTA, 25 mM NaF, 25 mM $Na_4P_2O_7$, 25 mM ß-glycerophosphate, 1 mM sodium chloride). Fractions containing phosphorylated p27, as confirmed by immunoblotting, were pooled and loaded onto a sulfopropyl sepharose column (Deutsche Pharmacia GmbH, Frankfurt, Germany) equilibrated with buffer A pH 6.8. Samples were eluted with buffer B pH 6.5. To remove immunoglobulins, the fractions tested positive for phosphorylated p27 were pooled, adjusted to pH 7.0 and applied to a protein G sepharose column equilibrated with buffer A pH 7.0. Elution was performed with 100 mM glycine-HCl pH 3.0 and 0.5 ml

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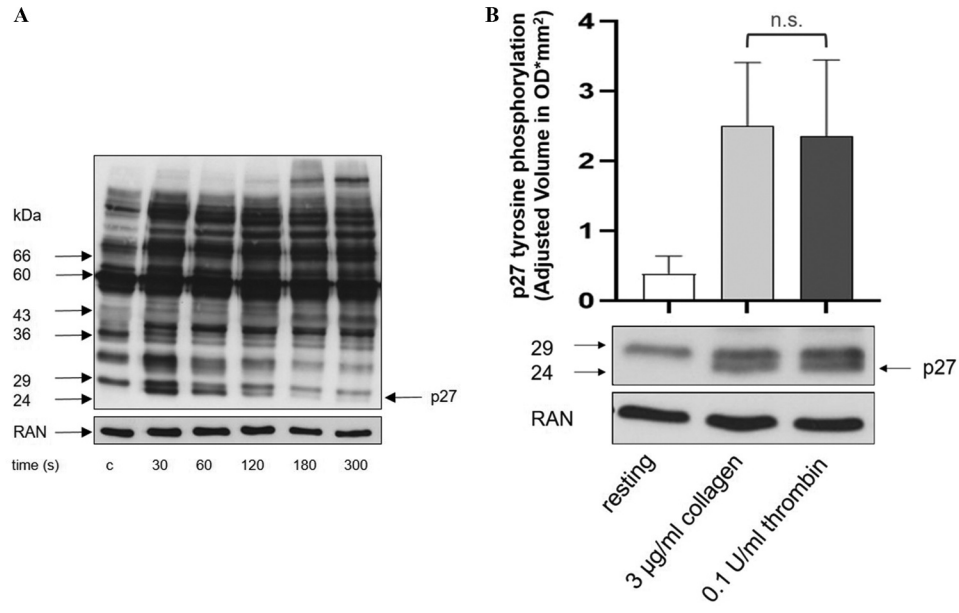

Figure 1. Washed human platelets were stimulated as previously reported.[6] Samples were analyzed by immunoblotting using anti-phosphotyrosine antibodies (4G10). Loading controls were performed with antibodies recognizing RAN. Unstimulated platelets (c, resting) were incubated with sample buffer for 60 s. The index on the left represents molecular mass standards. A: washed platelets were stimulated with thrombin (0.1 U/ml; from Sigma, Munich, Germany) for the indicated times. The immunoblot shown is representative for three similar experiments. B: washed human platelets were stimulated with thrombin (0.1 U/ml) or collagen (3 µg/ml; from Nycomed, Ismaning, Germany) for 30 seconds. Samples were analyzed as described above and the tyrosine phosphorylation of p27 was densitometrically quantified. Results are given as mean ± S.D. of six similar experiments. Statistical analysis was performed by one-way ANOVA and post-hoc Tukey´s test. p-values were defined as $p > .05$ (ns; not significant).

fractions were collected in tubes loaded with 50 µl 1 M Tris-HCl pH 8.0. All samples were compacted with a rotary evaporator and subsequently suspended in 100 µl 1 M Tris-HCl pH 8.0, each. Samples containing phosphorylated p27 were pooled and after tryptic protein digest analyzed by MALDI-TOF mass spectrometry peptide mass fingerprint using MASCOT database search.[7] p27 was identified as apolipoprotein A-I, with a phosphorylation detected at Tyr42, a previously uncharacterized phosphorylation site.

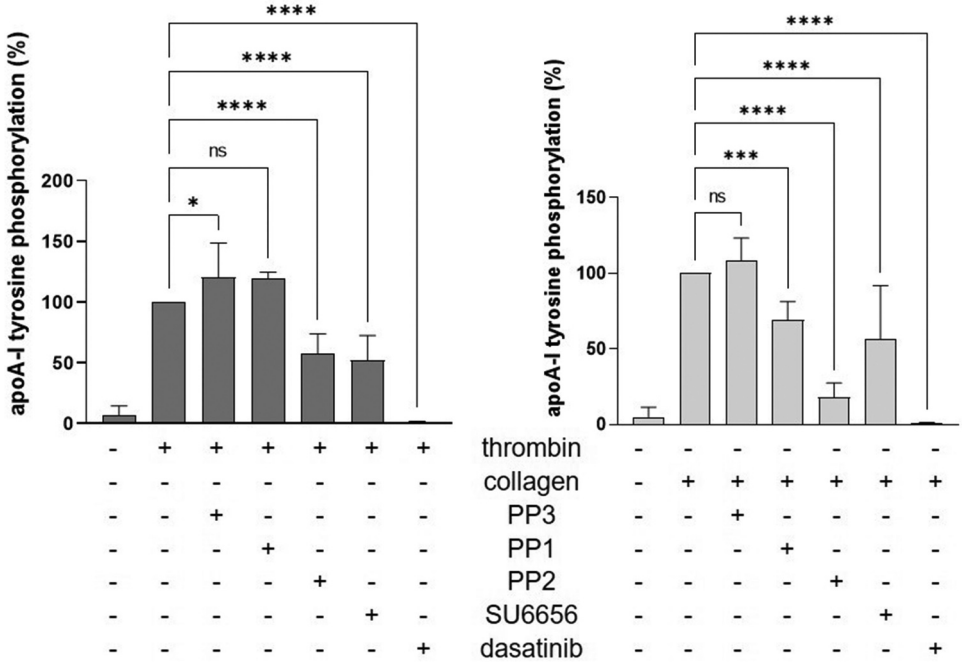

Figure 2. Washed human platelets (5×10^8/ml) were preincubated for 5 min with buffer, the negative control agent PP3 (10 µM; from Merck, Darmstadt, Germany) or various SFK-inhibitors (10 µM each, purchased from Sigma, Munich, Germany) prior to stimulation with thrombin (0.1 U/ml; left; from Sigma, Munich, Germany) or collagen (3 µg/ml; right; from Nycomed, Ismaning, Germany). Reactions were stopped after 30 s by addition of SDS-sample buffer. Samples were analyzed with anti-phosphotyrosine antibody (4G10). The tyrosine phosphorylation of p27 was densitometrically quantified.[6] There were no significant differences in p27 tyrosine phosphorylation under these conditions of stimulation (Figure 1B). Therefore, data were transformed by setting the intensity of phospho-protein band of the sample stimulated with thrombin or collagen alone as 100%. Results are given as mean ± S.D. of at least three similar experiments. Statistical analysis was performed by one-way ANOVA and post-hoc Dunnett´s test. p-values were defined as $p > .05$ (ns; not significant), $p < .05$ (*), $p < .001$ (***), $p < .0001$ (****).

By Scansite database search a weak match between the peptide sequence surrounding Tyr42 and the binding motifs of PDGF receptor β tyrosine kinase, EGF receptor tyrosine kinase, ITK and the Src family kinases (SFKs) Lck, Src, Fgr and Fyn was obtained.[8]

The sheer abundance of SFKs in human platelets makes them promising candidates in the search for the tyrosine kinase that phosphorylates apoA-I. To investigate the role of SFKs, washed human platelets were prepared, stimulated and analyzed as described previously.[6] Preincubation with PP2 (10 μM), SU6656 (10 μM) and dasatinib (10 μM) decreased thrombin-mediated apoA-I tyrosine phosphorylation to 57 ± 16, 52 ± 20 and 1 ± 1%, respectively. In contrast, PP1 (10 μM) increased apoA-I tyrosine phosphorylation to 119 ± 6%. After collagen-stimulation (3 μg/ml), the strongest effect was again observed after preincubation with dasatinib (1 ± 1%). PP1, SU6656, PP2 reduced collagen-mediated apoA-I tyrosine phosphorylation to 69 ± 12, 57 ± 35 and 18 ± 9%, respectively (Figure 2). Consistent with literature, none of the SFK-inhibitors used in our study significantly impaired thrombin-induced platelet aggregation, while SFK-inhibition apparently affected aggregation in response to collagen or CRP (own data not shown).[9–11] Despite convincing evidence for the involvement of SFKs in apoA-I tyrosine phosphorylation, the present results do not allow us to reliably determine whether any and which of the four SFKs mentioned above directly phosphorylates apoA-I.

Finally, there remains the crucial question of the significance of apoA-I Tyr42 phosphorylation. The N-terminal 43 residues of apoA-I are considered to stabilize the structure of lipid-free apoA-I, facilitate homodimerization and support lipid binding.[12] However, whether the phosphorylation of Tyr42 enhances or impedes lipid uptake is unclear. Increased lipid efflux from platelets to apoA-I would interfere with efficient signal transduction by lipid raft associated receptors (e.g. GPIbIXV and GPVI-FcRγ).[13] This may provide an explanation for how apoA-I reduces convulxin-induced Akt-activation.[3] Because apoA-I is released from thrombin-stimulated platelets,[14] apoA-I may even act as part of a negative feedback loop upon platelet activation. Given the relatively small amount of apoA-I in platelets,[15] it remains questionable whether its release would be relevant. However, if Tyr42-phosphorylation enhances apoA-I effects (e.g. increases the affinity to its platelet receptor SR-BI[3] or its capability to bind lipid cargo) this would be conceivable.

Taken together, apoA-I not only modulates protein kinase Akt activity,[3] but itself serves as a substrate for a platelet tyrosine kinase upon platelet activation. Our data reveal a previously uncharacterized phosphorylation site at Tyr42 and demonstrate that SFKs are involved in apoA-I Tyr42 phosphorylation underscoring the complex embedment of apoA-I in platelet signaling. Further investigations are required to clarify which kinase directly phosphorylates apoA-I and to unravel the functional consequences of apoA-I tyrosine phosphorylation in the context of platelet activation.

Our study was approved by the Ethics Committee of Martin-Luther-University Halle-Wittenberg (on 17 May 2001) and performed according to the Declaration of Helsinki. All healthy volunteers gave written informed consent prior to blood donation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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