

INCOMPLETE THROMBIN INHIBITION: BIOCHEMICAL AND FUNCTIONAL EVIDENCE OF ADP P2Y1 RECEPTOR DESENSITIZATION IN HUMAN PLATELETS

E. Schiera, S. De Corci, A. Di Battista, F.M. Pulcinelli

Experimental Medicine Sapienza University of Rome, Italy

Background and Aims. Adenosine diphosphate (ADP) is a key agonist of platelet activation and aggregation through the stimulation of purinergic receptors $P2Y_1$ and $P2Y_{12}$. Tight regulation of ADP-mediated signaling is essential during platelet plug formation. It has recently been demonstrated that the $P2Y_1$ receptor, upon ADP stimulation, can be downregulated through a protein kinase C (PKC)-dependent phosphorylation mechanism, leading to receptor desensitization. First we demonstrated that a prior platelet thrombin activation reduces PLC activation and Calcium mobilization induced by ADP, suggesting a capability of thrombin to densitize P2Y1 receptor. When pre-analytical procedures are not properly followed, ADP responses is lacking. The aim of this study was to investigate whether residual thrombin activity, under such conditions, induces desensitization of platelet $P2Y_1$ signaling pathways in a PKC-dependent manner, thereby to verify if limits ADP induced platelet response.
Methods. Human platelets obtained from sodium citrate-anticoagulated blood were prepared under different agitation conditions (3 times manual inversions vs mild shaking; in presence or absence of hirudin, a thrombin inhibitor). Platelets were stimulated with ADP (0.8, 2, and 4 μM) to assess both platelet aggregation and in washed, aspirin-treated platelets, intracellular calcium mobilization was measured using FURA-2 as a probe. It was also evaluated PKC activity by analyzing the phosphorylation of PKC substrates at baseline (T=0) and after 2 hours (T=2h) by Western blot analysis.
Results. Platelets analyzed 2 hours after blood collection, subjected to mild shaking and not treated with hirudin, showed reduced ADP-induced aggregation, accompanied by a marked attenuation of intracellular calcium mobilization, indicating decreased responsiveness of the $P2Y_1$ receptor. Normal response were observed both in the shaking hirudin treated sample and in samples correctly agitate by inversion. In contrast, PKC shows a slightly activation immediately after the blood sampling that become higher after two hours, in shaking treated platelets. Hirudin treatment showed no PKC activation either immediately or after 2 hours, as well as agitate sample and no treated with hirudin.
Conclusions. Our findings demonstrate that insufficient inhibition of thrombin during platelet preparation induces desensitization of the $P2Y_1$ receptor, leading to reduced platelet responsiveness to ADP. This artefact can be the cause of variability in platelet aggregation results across different laboratories. A thrombin-dependent, PKC-mediated mechanism may represent an additional regulatory pathway limiting platelet activation during sustained stimulation. To the best of our knowledge, this is the first evidence that a thrombin contributing to the prevention of excessive platelet aggregate, similar to the limitation of platelet plug formation endothelial dependent.