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Mini Review

Purinergic control of inflammation and thrombosis: Role of P2X1 receptors

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ABSTRACT

Inflammation shifts the hemostatic mechanisms in favor of thrombosis. Upon tissue damage or infection, a sudden increase of extracellular ATP occurs, that might contribute to the crosstalk between inflammation and thrombosis. On platelets, P2X1 receptors act to amplify platelet activation and aggregation induced by other platelet agonists. These receptors critically contribute to thrombus stability in small arteries. Besides platelets, studies by our group indicate that these receptors are expressed by neutrophils. They promote neutrophil chemotaxis, both *in vitro* and *in vivo*. In a laser-induced injury mouse model of thrombosis, it appears that neutrophils are required to initiate thrombus formation and coagulation activation on inflamed arteriolar endothelia. In this model, by using P2X1−/− mice, we recently showed that P2X1 receptors, expressed on platelets and neutrophils, play a key role in thrombus growth and fibrin generation. Intriguingly, in a model of endotoxemia, P2X1−/− mice exhibited aggravated oxidative tissue damage, along with exacerbated thrombocytopenia and increased activation of coagulation, which translated into higher susceptibility to septic shock. Thus, besides its ability to recruit neutrophils and platelets on inflamed endothelia, the P2X1 receptor also contributes to limit the activation of circulating neutrophils under systemic inflammatory conditions. Taken together, these data suggest that P2X1 receptors are involved in the interplay between platelets, neutrophils and thrombosis. We propose that activation of these receptors by ATP on neutrophils and platelets represents a new mechanism that regulates thrombo-inflammation.

Contents

1. Introduction: hemostasis and thrombosis

Hemostasis is the process that maintains the integrity of a closed circulatory system after vascular damage. Vessel wall injury and the extravasation of blood from the circulation rapidly initiate events in the vessel wall and in the blood that seal the breach. Circulating platelets are recruited to the site of injury, where they become a major

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component of the developing thrombus; blood coagulation is initiated by endothelium-expressed tissue factor and leads to the generation of thrombin and fibrin. Under normal conditions, regulatory mechanisms restrain thrombus formation both temporally and spatially [1]. When pathologic processes overwhelm the regulatory mechanisms of hemostasis, excessive quantities of thrombin form, initiating thrombosis. Thrombosis is a critical event in the arterial disease progression and is associated with myocardial infarction and stroke, accounting for considerable morbidity and mortality [2].

2. Platelet P2 receptors

Adenosine diphosphate (ADP) plays crucial roles in the physiological process of hemostasis and in the development and extension of arterial thrombosis. By itself ADP is a weak agonist of platelet aggregation inducing only reversible responses as compared to strong agonists such as thrombin or collagen. However, due to its presence in large amounts in the platelet dense granules and its release upon activation at sites of vascular injury, ADP is an important so-called secondary agonist amplifying most of the platelet responses, which contributes to the stabilization of the thrombus [3–5]. More recent studies indicate that ATP, co-released with ADP, should be considered alongside ADP and thromboxane A_2 as a significant secondary platelet agonist [6,7].

The receptors for extracellular nucleotides belong to the P2 family which consists of two classes of membrane receptors: P2X ligand-gated cation channels (P2X1–7) and G protein-coupled P2Y receptors (P2Y1,2,4,6,11,12,13,14) [8]. Starting from the concept of a unique P2T receptor (T for thrombocyte) originally postulated on the basis of pharmacological data, a model of three platelet P2 receptors progressively emerged. These are the P2X1 cation channel activated by ATP and two G protein-coupled receptors, P2Y1 and P2Y12, both activated by ADP [4,9]. Each of these receptors has a specific function during platelet activation and aggregation, which logically has implications for their involvement in thrombosis.

Large-scale clinical trials have demonstrated the beneficial effects of thienopyridines, targeting P2Y12 receptors, in the prevention of major cardiac events after coronary artery stenting and in the secondary prevention of major vascular events in patients with a history of cerebrovascular, coronary or peripheral artery disease. More recently, new classes of P2Y12 inhibitors have been developed in order to circumvent clopidogrel limitations (*i.e.* variability of platelet inhibitory effect) for the management of ischemic coronary syndromes [10–12].

3. Platelet P2X1 receptors

The P2X1 receptor belongs to a family of ATP-gated ion channels, comprising seven mammalian receptor subunits (P2X1–7) that assemble to form a variety of homotrimeric and heterotrimeric receptors widely expressed in the body. Each P2X subunit contains two transmembrane domains, intracellular amino and carboxy termini and a large extracellular ligand-binding loop. P2X receptors vary in their kinetics of desensitization and pharmacology, although all are activated by the physiological ligand ATP [13]. The function of P2X1 receptors in neurogenic smooth muscle contraction, and in thrombosis has been well documented [14–17]. Mutagenesis studies identified residues important in agonist action, the inter-subunit nature of the binding site, the location of the channel gate, and interactions between the transmembrane regions [18–21]. The crystallization of a zebrafish P2X4 receptor in both resting and ATP-bound open states [22,23] demonstrated extensive conformational changes in the receptor associated with agonist binding and channel gating. Individual P2X receptor subunits have been described by analogy to a dolphin, with the ATP binding site formed predominantly from residues in the upper and lower body regions of adjacent subunits. Agonist binding induces movement of the dorsal fin, left flipper, and the cysteine-rich head regions closing the ATP binding pocket. This movement is translated through the body region to the transmembrane regions and results in opening of the channel gate.

The P2X1 receptor plays an important role in thrombus formation especially under high-shear conditions. P2X1-deficient mice have no prolongation of bleeding time as compared to the wild-type mice, indicating that they conserve normal hemostasis [24]. In contrast, they display resistance to the systemic thromboembolism induced by the injection of a mixture of collagen and adrenaline and to localized laser-induced injury of the vessel wall of mesenteric arteries. Conversely, increased arterial thrombosis has been reported in the microcirculation of mice overexpressing the human P2X1 receptor [25]. The P2X1 antagonist NF449 [4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis-(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid octasodium salt] has an inhibitory effect on platelet activation *ex vivo* and on thrombosis *in vivo* [26,27]. Platelet P2X1 receptor function can also be inhibited by using heat shock protein 90 inhibitors, which may be as effective as selective antagonists in regulating thrombosis [28].

About 10% of current flow through the P2X1 receptor is mediated by Ca^{2+} [29]. These ion channels can therefore provide a significant source of direct Ca^{2+} influx into the cell following activation, as well as causing membrane depolarization. The time course of ATP-evoked P2X1 receptor-mediated currents is concentration-dependent with low concentrations taking several seconds to reach a peak response, which can be sustained for >30 s. In contrast, at maximal agonist concentrations, P2X1 receptor currents peak within tens of milliseconds and desensitize completely within seconds [30]. In platelets, P2X1-mediated increase in intracellular Ca^{2+} leads to the activation of ERK1/2 MAPK and MLCK that phosphorylates myosin light chain (MLC), a process accompanying platelet shape change and degranulation [31]. P2X1 receptor signaling represents a significant pathway for early Ca^{2+}-mobilization following activation of a variety of major platelet receptors through both G-proteins and tyrosine kinases [6,32]. Furthermore, P2X1 receptors seem to play a pivotal role in the activation of aspirin-treated platelets by thrombin and epinephrine [33]. Since aspirin is used extensively to manage cardiovascular diseases and since, in clinical research, much attention has been focused on “aspirin resistance” (meaning treatment failure), the finding that P2X1 receptors can circumvent the action of aspirin on platelet stimulation by thrombin is of major importance. P2X1-mediated Ca2 + mobilization has been involved in platelet responses to microbial pathogen-associated molecular patterns acting through Toll-like receptor 2 [34], suggesting a role for P2X1 in platelet-dependent sensing of bacterial components. Moreover, such P2X1 signals would be resistant to endogenous platelet inhibiting agents, such as prostacyclin, which may be particularly important during early thrombotic or immune-dependent platelet activation [35].

These results clearly indicate that the P2X1 receptor might be considered as a potential target for antiplatelet strategies, with the interesting feature that P2X1 antagonists should be effective only at sites of severe stenosis where shear forces are very high, without having a deleterious effect on normal hemostasis.

4. Neutrophil P2X1 receptors

We recently showed that P2X1 receptors are also expressed on neutrophils [36]. P2X1 activation causes ROCK-dependent MLC phosphorylation, promoting cytoskeletal reorganization and neutrophil deformation during chemotaxis. Intriguingly, we found that P2X1 deficiency increases neutrophil NADPH oxidase activity [37]. Indeed, *ex vivo* stimulation of P2X1−/− neutrophils with various stimuli, including bacterial formylated peptides, phorbol esters, and opsonized zymosan particles resulted in increased production of reactive oxygen species as compared to neutrophils isolated from wild-type mice. These results indicated that P2X1 would act to limit systemic neutrophil activation through a negative feedback loop, allowing them to migrate