Adenosine and blood platelets

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Abstract Adenosine is an important regulatory metabolite and an inhibitor of platelet activation. Adenosine released from different cells or generated through the activity of cell-surface ectoenzymes exerts its effects through the binding of four different G-protein-coupled adenosine receptors. In platelets, binding of A₂ subtypes (A_{2A} or A_{2B}) leads to consequent elevation of intracellular cyclic adenosine monophosphate, an inhibitor of platelet activation. The significance of this ligand and its receptors for platelet activation is addressed in this review, including how adenosine metabolism and its A₂ subtype receptors impact the expression and activity of adenosine diphosphate receptors. The expression of A₂ adenosine receptors is induced by conditions such as oxidative stress, a hallmark of aging. The effect of adenosine receptors on platelet activation during aging is also discussed, as well as potential therapeutic applications.

Keywords Adenosine · A_{2B} adenosine receptor · A_{2A} adenosine receptor · ADP-mediated platelet activation and aggregation · Cyclic adenosine monophosphate (cAMP)

Sources of adenosine

Adenosine can be generated through the extracellular metabolism of adenosine 5'-triphosphate (ATP). Intracellu-

lar ATP concentration is in the millimolar range; thus, a compromised membrane integrity following injury, inflammation, or hypoxia will lead to a significant increase in extracellular ATP [1]. Adenosine is centrally involved in the signaling cascade of related events, including anti-inflammatory actions, angiogenesis, oxygen supply/demand ratio, and ischemic pre- and postconditioning [2, 3]. ATP can additionally be released from cells following mechanical stress [4], hypotonic stress [5], or nerve stimulation [6]. Dense granules of aggregating platelets are another important source of ATP and ADP [7]. Membrane-bound enzymes, including ectonucleoside triphosphate dephosphorylases (E-NTPDases) such as CD39, serve to dephosphorylate ATP to adenosine 5'-diphosphate (ADP) to adenosine 5'-monophosphate (AMP) [8, 9]. The apyrase CD39 is important for the initial phosphohydrolysis of ATP to AMP [10]. Ectopyrophosphatase/phosphodiesterases (NPPs) hydrolyse ATP directly to AMP [11]. CD73, an ecto-5'-nucleotidase, performs the conversion of extracellular AMP to adenosine [12]. CD39, A₁ AR, and A_{2B} AR associate with caveolae. Thus, adenosine-generating enzymes are thought to be localized within close proximity to the location of adenosine receptors on the cell surface, which facilitates rapid binding of newly generated adenosine to its receptors [13–18]. Adenosine kinase (AK) is inhibited by hypoxic conditions, resulting in an increase in extracellular adenosine [19]. Hypoxic conditions also involve anaerobic metabolism and intracellular degradation of ATP, increasing adenosine concentration [3, 20]. Two families of bidirectional transporters on the cell membrane can facilitate the transport of intracellular adenosine outside the cell: concentration-based transporters and equilibrium-dependent transporters. Concentrative transporters (CNTs) use a sodium gradient generated through a sodium pump to transport adenosine against its concentration gradient [21].

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The more abundant equilibrative transporters (ENTs) are able to move adenosine into extracellular space through facilitated diffusion (ENT1 and ENT2) or cation-dependent transport (ENT3 and ENT4) [22]. In addition, erythrocytes are able to uptake adenosine through ENT, which rapidly removes adenosine from the plasma [23, 24]. This uptake promotes platelet aggregation by prevention of inhibitory effects of adenosine on this process [24, 25].

The endothelial E-NTPDase1/CD39 has an important role in the vasculature system; this enzyme plays an important role in ending proinflammatory and thrombotic events mediated by ATP and ADP, which maintains hemostasis within the vasculature. The endothelial cells and endocardial cells express this enzyme on the luminal surface of vasculature, which facilitates the inhibition, and reversal of platelet aggregation initiated by the presence of both ATP and ADP [26–28]. NTPDase 2 is expressed mainly in the microvasculature of both pericytes and adventitial cells within vessels [29]. NTPDase 2 converts ATP to ADP, leading to the initiation of platelet aggregation [29]. Of importance, the NTPDase 1/CD39 has been localized on the surface of neutrophils, monocytes, and

selective subsets of T and B cells, but not on red blood cells or platelets [30]. Figure 1 depicts adenosine metabolism and its implications in platelet activation.

Adenosine and its receptors

Adenosine signals through binding four distinct G-protein-coupled adenosine receptors, which are characterized by their inhibition or stimulation of adenylyl cyclase (AC) [31]. These receptors are distinguished by their different binding affinities for adenosine, G-protein coupling and subsequent signaling pathways, pharmacological profile, and sequence. The A_1 adenosine receptor (AR) is coupled to the inhibitory G proteins, G_o or G_i . The A_1 receptor activation leads to reduction of 3'-5'-cyclic adenosine monophosphate (cAMP) production or increased calcium levels, depending on effector pathways. The A_3 AR receptor is similarly coupled to the inhibitory G proteins, G_o or G_i , with a resulting inhibition of AC, and a consequent reduction in cAMP. Both A_2 adenosine receptors, A_{2A} and A_{2B} , are coupled to G_s , leading to stimulation

Fig. 1 Overview of adenosine metabolism and platelet activation: several receptor/ligand partners and hemodynamic parameters activate platelets. Following inflammation, ischemia, hypoxia, or cellular injury, adenosine 5'-triphosphate (ATP) is released from cells. Adenosine is generated extracellularly through metabolism of adenosine 5'-triphosphate (ATP) to adenosine 5'-diphosphate (ADP) to adenosine 5'-monophosphate (AMP). This is mediated by membrane-bound enzymes, including ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) such as CD39, ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP), and ecto-5'-nucleotidase (ecto-5'-NT), e. g., CD73. Binding of ADP to the P2Y₁ G_q-protein-coupled receptor leads to mobilization of calcium levels. ADP activation of the P2Y₁₂ G₁₂-protein-coupled receptor results in inhibition of adenylyl cyclase

(AC) with a consequent reduction in cAMP levels. Activation of the A_{2B} AR-coupled G_s protein leads to activation of AC and subsequent elevation of the cAMP levels; simultaneous activation of the G_q protein results in an increase of intracellular Ca^{+2} . The A_{2B} AR activation leads to an inhibition of the effect mediated through the P2Y₁ receptor. The activation of thrombin receptor, PAR-1, through G_q contributes to the generation of thromboxane A₂ (TxA₂), which increases intracellular Ca^{+2} levels and contributes to platelet secretion of ADP. Activation of the prostacyclin (PGI₂) receptor contributes to elevated cAMP levels. Concentration-based transporters (CNTs) and equilibrium-dependent transporters (ENTs) contribute to extracellular adenosine levels

of AC and consequent elevation of cAMP. The A_{2B} AR can also couple to G_q , which leads to subsequent modification of intracellular calcium levels. The A_1 AR, A_3 AR, and A_{2A} AR are high affinity adenosine receptors, whereas the A_{2B} AR is a low affinity adenosine receptor [31].

The different subtypes of adenosine receptors are localized in various tissues and cell types. The A_1 AR is significantly expressed in the brain, heart, adipose tissue, stomach, vas deferens testis, spleen, kidney, aorta, eye, liver, and bladder [32]. The A_{2A} AR is highly expressed in the olfactory tubercle, nucleus accumbens, and striatum [32]; this receptor is also expressed in immune cells [33, 34], platelets [35, 36], lung [32], heart [37], and the vasculature [32, 38]. The A_{2B} AR is expressed primarily in the vasculature, brain, and retina, with low levels of expression in various tissues and cell types, including platelets at baseline [32, 39]. The lung and liver highly express the A_3 AR, with moderate expression in the lung, testis, kidney, placenta, brain, heart, spleen, bladder, uterus, jejunum, proximal colon, aorta, and eyes [32]. The different tissue and cell type distribution of the four subtypes of adenosine receptors distinguishes the predominant roles of each receptor.

Platelet activation and inhibition: overview

Several platelet receptors and agonists are known to induce platelet activation, including binding of ADP to $P2Y_1$ or $P2Y_{12}$ receptors, binding of thromboxane A_2 (Tx A_2) to Tx A_2 receptor, binding of thrombin to PAR-1 and PAR-4 receptors, and binding of collagen to GPVI and GPIb α receptors (reviewed in [40–44]). Collagen and thrombin are available via the extracellular matrix and circulation, while ADP and Tx A_2 are synthesized by platelets (Tx A_2 via the aspirin-sensitive cyclooxygenase-1(COX-1)). Except for GPVI, the above receptors are all G-protein-coupled receptors, and they all signal through G_q to activate phospholipase C- β (PLC- β), except for $P2Y_{12}$ which signals via G_i to inhibit cAMP levels and activate Akt (reviewed in [45, 46]). Collagen too activates PLC, albeit PLC γ 2. PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3), which mediates the release of Ca^{+2} -sensitive protein, CalDAG-GEF which with other regulators induce talin translocation to membrane-bound $\alpha IIb\beta 3$ [47]. Consequent conformational changes in activated platelets expose binding sites for fibrinogen (reviewed in [48–50]). Ligand binding to GPIb α leads to a complex with filamin and changes in the platelet membrane cytoskeleton [51, 52].

Inhibition of platelet activation is achieved by blocking $\alpha IIb\beta 3$ (e.g., abciximab) or by receptor antagonists, including clopidogrel and prasugrel for $P2Y_{12}$ or by

inhibition of ligand production, such as aspirin blockade of COX-1, or by stimulating cAMP levels (which affects Ca^{2+} release), e.g., including dipyridamole, a phosphodiesterase inhibitor. Dipyridamole has also been demonstrated to be an adenosine transport inhibitor, leading to a subsequent elevation of adenosine levels and consequent antithrombotic effect [53, 54]. Additionally, prostacyclin (PGI2) mediates inhibition of platelet activation through activation of adenylate cyclase and elevation of cAMP levels [55]. In this regard, A_2 adenosine receptors, which elevate cAMP, merit further attention.

A_2 -type adenosine receptors and platelet activation

A_{2A} AR and platelet function

Early studies identified the A_{2A} AR to be an important receptor expressed on platelets and a mediator of adenosine inhibition of platelet aggregation [56–58]. This is achieved through inhibition of mobilization of internal calcium stores and influx of external calcium, both associated with activation of adenylate cyclase [59]. Dionisotti et al. confirmed the expression of A_{2A} AR on human platelet membranes using the antagonist radioligand [3H]SCH 58261 [60]. A_{2A} AR knockout (KO) mice demonstrated the following hemodynamic properties: increase in blood pressure and heart rate, and an increase in platelet aggregation [36]. It has been shown that adenosine and analogues inhibit the aggregation of platelets through the stimulation of cAMP generation. Count of blood cell populations, including platelets, was similar between the A_{2A} AR KO mice and wild-type mice. Platelet aggregation post-ADP stimulation and subsequent changes in aggregation following treatment with nonselective AR agonist, 5'-*N*-ethyl-carboxamido-adenosine (NECA), were explored. In the KO model, aggregation of platelets was deemed to be more efficient. NECA treatment led to inhibition of platelet aggregation in wild-type mice, but demonstrated no effect in A_{2A} AR-null mice. The lack of the effect of NECA and the A_{2A} AR agonist, 4-[2-[[6-Amino-9-(*N*-ethyl- β -D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene-propanoic acid hydrochloride (CGS 21680), on A_{2A} AR-null mice suggested that the A_{2A} AR mediates the antiaggregatory effects of adenosine and analogs in platelets. In wild-type mice, the administration of the A_{2A} AR agonist CGS 21680 resulted in the induction of hypotension and reactive tachycardia, which represents A_{2A} AR mediation of the vascular vasodilatory effects of adenosine. Deficiency in the A_{2A} AR resulted in a significant elevation in blood pressure, demonstrative of the role endogenous adenosine in maintaining vascular tone via tonic vasodilation [36]. This feature (blood pressure), however, is

dependent on the mouse strain on which the A_{2A} AR KO was analyzed [61]. Cooper et al. [60, 62] and other groups [63] have demonstrated that in human platelets, the A_{2A} AR mediates a NECA-elicited elevation in platelet cAMP levels, a pertinent component of platelet activation.

An additional study by Linden et al. [64] explored if the antithrombotic effects of A_2 agonism is due to the inhibition of platelet aggregation, using canine and human models. Brief treatment with the A_2 AR agonist CGS 21680 improved the patency of the coronary arteries in a recurrent thrombosis model in canine. This observation was not associated with a reduction of platelet reactivity; i.e., there was not a downregulation in flow cytometric indicators of activation and aggregation of platelets. Using canine platelets in vitro, the authors demonstrated similar results with no reduction of P-selectin platelet expression, and no inhibition of monocyte–platelet aggregates upon treatment with A_{2A} AR agonist. Thus, platelet responsiveness to A_2 AR agonists is species-dependent; in a human recurrent thrombosis model, the A_{2A} AR agonist significantly inhibited platelet activation. The in vitro aggregation response to collagen in human blood samples was drastically inhibited following treatment with the A_{2A} AR agonist CGS 21680. The treatment inhibited an increase in platelet surface expression of P-selectin and the aggregation of monocytes and platelets following stimulation with ADP and U46619 (thromboxane agonist). This study supports the potential for A_2 AR activation to achieve inhibition of platelet function in the clinic [64].

A_{2B} AR and platelet function

Platelets have a significantly large density of A_{2A} ARs, compared to A_{2B} AR, and previous studies have postulated that this was the sole receptor involved in platelet activation following stimulation with adenosine [62, 63, 65]. A_{2B} AR-null mice demonstrated a higher platelet aggregation response following ADP stimulation [66]. Recent expression studies demonstrated comparative mRNA expression levels of A_{2A} and A_{2B} AR expression in the platelet [67]. The study by Yang et al. [66] presented a new role for the A_{2B} AR in adenosine-mediated effects on platelet aggregation, particularly under stress. The presence of A_{2B} AR in platelets was demonstrated using cAMP assays and selective pharmacological ligands for specific adenosine receptor subtypes. However, this was mainly demonstrated under conditions of induced expression of the A_{2B} AR, i.e., vascular injury. Stimulation with NECA, the A_2 AR agonist, led to increased levels of cAMP in platelets; costimulation with the A_{2B} AR antagonist, *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide (MRS 1754), led to partial reversal of the observed NECA-induced effect,

evidence that platelets express a functional A_{2B} AR. The remaining cAMP activity after NECA and MRS 1754 was only slightly higher than that at baseline, making it difficult to identify A_{2A} AR solely based on activity profile. The presence of an active and functional A_{2A} AR on the platelet of A_{2B} AR KO mice was confirmed through the use of an A_{2A} AR agonist, CGS 21680, and the measurement of elevated cAMP levels. Platelets from A_{2B} AR KO cells demonstrated a lower basal level of cAMP levels comparative to wild type, and a similar decrease in the protein kinase A (PKA) substrate and vasodilator-stimulated phosphoprotein (VASP) phosphorylation [66].

Using agonist stimulation and platelets from KO mice and control mice, the role of the receptor in platelet aggregation was further elucidated. Under the above-described conditions of vascular injury, the A_{2B} AR KO mice demonstrated a significantly higher percentage of platelet aggregation compared to the wild-type samples, in both ADP-stimulated platelets and collagen-stimulated platelets. The use of the A_{2A} AR agonist CGS 21680 also resulted in a decrease of ADP-mediated platelet aggregation in both A_{2B} AR KO and wild-type mice. This suggests that the difference in platelet response between the wild-type and knockout mice is not due to an alteration of the A_{2A} AR. Considering the inducibility of expression of the A_{2B} AR under stresses, such as injury, elevated reactive oxygen species, or TNF- α [68, 69], this receptor's new role in mediating inhibition of platelet aggregation reveals a new target for controlling thrombosis.

Newly identified regulation of P2Y receptors by A_{2B} AR or cAMP

Basal A_{2B} AR expression in megakaryocytes and platelets is relatively low. As mentioned above, upon systemic inflammation or injury, this receptor is highly upregulated [68, 69]. In the case of elevated expression, the A_{2B} AR-mediated inhibition of the platelet aggregatory response is strongly present. Intriguingly, the absence of the A_{2B} AR leads to a consequent upregulation of the P2Y₁ ADP receptor expression; the presence of the A_{2B} AR and activation lead to an elevation of cAMP and a downregulation of mRNA expression of the P2Y₁ ADP receptor. Elevation of cAMP by forskolin treatment mimics the effect of A_2 AR activation on P2Y₁ receptor expression (about twofold upregulation) [61]. It is well established that the presence and level of expression of the P2Y₁ receptor are important for the initiation of platelet aggregation induced by ADP and is important in thrombotic states [70–72]. An earlier report showed that transgenic elevation of the P2Y₁ receptor in platelets (in the range of twofold) affects platelet aggregation response [73]. The above studies suggest an

inverse relationship between the expression of A_{2B} AR and the $P2Y_1$ ADP receptor, which could explain the difference in platelet aggregation response between wild-type mice and the A_{2B} AR-null mice. Electron microscopy demonstrated that the frequency of platelet shape change induced by ADP is much greater in the KO model relative to the wild-type control. The induction of the A_{2B} AR following a stressful event positions it as a significant inhibitor of platelet aggregation through alterations in cAMP and regulation of the ADP receptor through A_{2B} AR-mediated alterations in cAMP levels [66].

Adenosine receptors and platelets during aging

As mentioned above, the expression of the A_{2B} AR is induced by oxidative stress [69], and the latter is a hallmark of aging and vascular disease, among other conditions (discussed in [74–80]). In both cerebral infarctions and coronary heart disease, activated platelets are present [81–84], defined by structural characteristics, including pseudopods, folds, centralization, and vacuoles [85, 86]. Platelet activation can contribute to atherosclerotic plaque rupture or infarction (reviewed in [87, 88]). Across studies in the elderly population, there is an increased concentration of clotting factors and a reduction in fibrinolytic activity in elderly populations [89, 90]. Other factors that are reported to be altered with aging include: increased platelet aggregability, decreased concentration of cGMP, asymmetric phospholipid structure, reduced membrane potential of mitochondria, elevated concentrations of immunoglobulin G and fibrinogen, and impaired reactivity of platelets to thrombin [91–94]. In elderly populations, the concomitant presence of atherosclerosis complicates the distinction between age-related or vascular disease-associated changes in platelets and the clotting cascade.

Platelets are affected by acute preconditioning ischemia in events such as unstable angina and acute myocardial infarction through the attenuation of platelet adhesion and subsequent aggregation. This antiplatelet effect is thought to be mediated through release of adenosine from myocardium post-ischemia–reperfusion and activation of the platelet A_2 receptors [95, 96]. An *in vitro* model of platelet aggregation and the use of an A_2 agonist investigated the effect of A_2 agonism on platelet activity and responsiveness, and how these parameters change with age. Young adult rabbits (6 months) post- A_2 AR agonist treatment demonstrated a reduced platelet aggregatory response relative to vehicle treatment. Older rabbits (4 years) demonstrated a refractory response to the A_2 AR agonist, CGS 21680, with no alteration in platelet aggregation response. A rat model demonstrated similar results. These studies point to an age-related loss of platelet responsive-

ness to preconditioning mediated through adenosine. This study is limited in that it addresses short-term treatment, and effect on A_2 agonism; future studies should address chronic stimulation of the A_2 receptors and the effect of platelet responsiveness [97].

Applications of antiplatelet therapies and potential prospects for adenosine analogs

In inflammatory disease states, the activity of NTPDase in the vasculature is diminished [98], which contributes to the changes in vascular permeability and to localized clotting cascade events [98, 99]. Thus, potential therapies to improve vascular function in states of inflammation include inducing expression of NTPDase by injured vasculature or administering soluble forms of the enzyme for anticoagulation treatment. Both are currently being tested; soluble forms of human NTPDase/CD39 are currently being evaluated for antithrombotic therapy; this potential therapy has been demonstrated to reduce both infarct size and provide cardioprotection in wild-type and CD39-null mice in cases of acute myocardial infarction [100]. Soluble nucleotides are thus thought to be of importance in areas of inflammation and injury to assist with inactivation of sudden elevation of nucleotides. Soluble human apyrases have been redesigned to bind ADP with high affinity; this altered substrate specificity was found to be sufficient to inhibit ADP-mediated platelet aggregation; this has the potential for future antithrombotic therapeutics [101]. Recently, it has been demonstrated by Hart et al. [102] that treatment of both murine and human blood with a 5'-nucleotidase (5'-NT) results in the inhibition of ADP- and collagen-mediated platelet aggregation; the platelet aggregation was restored upon administration of a specific 5'-NT inhibitor. This observation has two implications: if one chooses to administer 5'-NT for clinical use, for indications such as prevention of vascular leakage, protection for ischemic events, or resolution of inflammation in acute lung injury, one must carefully consider the adverse influence of antiaggregatory effects for individual patients. Additionally, the side effect of antiaggregation could serve as a secondary therapeutic approach for cases involving an excess of aggregation or thrombosis [102].

Rapid activation of platelets at sites of vascular injury implicates them as potential targets for maintaining hemostasis and in the event of arterial thrombus formation.

Following atherosclerotic plaque rupture, subsequent platelet adhesion and aggregation contribute to the formation of a thrombus which can culminate in acute coronary syndromes, peripheral artery disease, and stroke [103]. Platelet activation is implicated in early development of atherosclerosis [104, 105]. Currently available antiplatelet

therapies are not optimal. Aspirin and clopidogrel have been demonstrated to prevent cardiovascular disease and are well-tolerated. With these drugs, the risk of serious vascular disease and manifestations, including myocardial infarction and/or stroke, is reduced by 25% in high-risk patients [106].

To date, adenosine has not been used as antiplatelet therapy because of its short half-life and ability to target its four different receptors in various tissues. Also, there are no human studies with adenosine analogs that target the A_2 AR, likely because it partially inhibits ADP-induced platelet activation, and there are direct inhibitors of the ADP receptor. However, considering that the above ADP receptor inhibitors are not well tolerated or effective in all individuals, one might consider receptor-specific adenosine analogs that alter the levels of cAMP and Ca^{2+} to affect platelet aggregation, as well as control the expression of $P2Y_1$ receptors. Ligands of the A_{2B} AR would fit under this category. Additionally, dipyridamole could be considered to target indirectly adenosine receptors, given its ability to potentiate levels of cAMP through its inhibition of phosphodiesterase [54].

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