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Adenosine metabolism in the vascular system

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Abstract

The concept of extracellular purinergic signaling was first proposed by Geoffrey Burnstock in the early 1970s. Since then, extracellular ATP and its metabolites ADP and adenosine have attracted an enormous amount of attention in terms of their involvement in a wide range of immunomodulatory, thromboregulatory, angiogenic, vasoactive and other pathophysiological activities in different organs and tissues, including the vascular system. In addition to significant progress in understanding the properties of nucleotide- and adenosine-selective receptors, recent studies have begun to uncover the complexity of regulatory mechanisms governing the duration and magnitude of the purinergic signaling cascade. This knowledge has led to the development of new paradigms in understanding the entire purinome by taking into account the multitude of signaling and metabolic pathways involved in biological effects of ATP and adenosine and compartmentalization of the adenosine system. Along with the "canonical route" of ATP breakdown to adenosine via sequential ecto-nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39) and ecto-5'-nucleotidase/CD73 activities, it has now become clear that purine metabolism is the result of concerted effort between ATP release, its metabolism through redundant nucleotide-inactivating and counteracting ATP-regenerating ectoenzymatic pathways, as well as cellular nucleoside uptake and phosphorylation of adenosine to ATP through complex phosphotransfer reactions. In this review I provide an overview of key enzymes involved in adenosine metabolic network, with special emphasis on the emerging roles of purine-converting ectoenzymes as novel targets for cancer and vascular therapies.

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1. Introduction

ATP and other adenine nucleotides and adenosine (ADO) are found in all animal organ systems where they produce diverse effects both by intracellular and extracellular mechanisms. Intracellular ATP is the essential energy currency molecule of the cell, which is primarily utilized to drive energy-requiring processes such as active transport, cell motility and biosynthesis, whereas extracellular ATP acts as powerful signaling molecule. The concept of extracellular purinergic signaling was first proposed by Geoffrey Burnstock in the early 1970s [1]. Geoff also proposed a basis for distinguishing two types of purinergic receptors, one selective to ADO (called P1), which was antagonized by methylxanthines, and the other selective for ATP/ADP (called P2) [2, 3]. These first studies were met with skepticism, which only disappeared in the early 1990s when the plasma membrane P2 receptors for extracellular ATP were identified, cloned, and extensively characterized. At the same time, we have witnessed an explosion of information on the important role of ADO capable of mediating diverse signaling events via binding to its own nucleoside-specific P1 receptors [4]. Since then, extracellular ATP and ADO have emerged as important ubiquitous signaling molecules mediating signaling effects in virtually all organs and tissues, including the vascular system. Most models of purinergic signaling cascade depend on interactions between distinct processes including (i) release of ATP and other nucleotides and related compounds into the extracellular milieu, (ii) activation of nucleotide- and ADO-selective receptors, (iii) metabolism of nucleotides and nucleosides through ectoenzymes, and finally (iv) the re-uptake of nucleotide-derived ADO for replenishing the intracellular ATP pool through complex phosphotransfer reactions [5, 6].

Because extracellular ATP and ADO are involved in a variety of critical functions, it is not surprising that multiple human disorders (including cancer) have been related to CD39, CD73, and other ecto-nucleotidases coordinately controlling the equilibrium between these two purinergic agonists [7-9]. However, recent data point out the need for a more careful evaluation of the entire purinome by taking into account the redundancy of extracellular ADO-producing and -inactivating pathways, as well as intracellular ADO metabolism and compartmentalization of the ADO system [10] (**Fig. 1**). My research focus for the past 20 years has been to investigate the role of purine-converting ectoenzymes in inflammation, vascular remodeling, and cancer, and in this review I provide an overview of extracellular ADO metabolism in the vascular system and summarize recent advances in this evolving field. Special attention is also given to the emerging roles of purinergic enzymes as novel targets for cancer and vascular therapies. Since this article is dedicated to the memory of Geoffrey Burnstock (1929-2020), it also includes some personal recollections of Geoff.

2. Release of ATP and other purinergic agonists into the extracellular milieu

The appearance of ATP and other nucleotides and ADO in the extracellular milieu represents a crucial component for initiating a signaling cascade. While some studies have shown that extracellular ADO may originate from the secretion from intracellular stores [11, 12], it is now generally accepted that the release of endogenous ATP and its subsequent metabolism constitutes a primary source for generation of extracellular ADO [5, 13, 14]. The release of intracellular ATP might occur upon cell lysis during organ injury, necrosis and apoptosis, as well as via non-lytic mechanisms which include regulated vesicular exocytosis, pore-forming ion channels and transporters (**Fig. 2**) [5, 13, 15, 16]. Vesicular release, either alone or in conjunction with other extracellular mediators and neurotransmitters, occurs in nerve terminals, endocrine cells, platelets, and other excitatory/secretory tissues. Cellular mechanisms of cargo-vesicle trafficking include nucleotide secretion from specialized exocytotic granules (synaptic vesicles, chromaffin granules or dense core granules) or through a member of the solute carrier (SLC) family of ion transporters, the vesicular nucleotide transporter (VNUT/SLC17A9) [13, 14]. Another type of conductive nucleotide release occurs from epithelial and endothelial cells, lymphocytes, astrocytes, hepatocytes and

other non-excitatory cells, and includes electro-diffusional movement or facilitated diffusion through ATP-conducting Cl^- channels and nucleotide transporters, such as maxi anion channels, pore-forming connexin (specifically, Cx43, Cx30 and Cx26) and pannexin 1 (Panx1) hemichannels, volume-regulated anion channels (VRAC, also known as volume-sensitive outwardly rectifying (VSOR) channel), P2X7 receptors [13, 15, 16], as well as the newly identified voltage-gated calcium homeostasis modulator-1 (CALHM1) channel [16] and mechanosensing Ca^{2+}-permeable cation channel Piezo1 [17] (**Fig. 2**).

Along with stimuli-mediated ATP release, the cells are able to maintain ATP and its metabolites (ADP, AMP, ADO, inosine, and PP_i) at certain characteristic steady-state nanomolar levels, thereby contributing to the constitutive activation and/or desensitization of purinergic receptors [18-20]. While the exact mechanisms responsible for maintenance of ATP and other purines at basal levels remain unknown, one interesting hypothesis is that extracellular ATP levels might reflect a minor fraction (so-called "purinergic tone") of the actual mass of pericellular "ATP halo", which is localized in the immediate cell vicinity and exchanged with bulk phase via dissociation-association mechanisms [18] (**Fig.2**). In fact, quantification of extracellular ATP by using cell surface-targeted luciferase [21-23], fluorescent ATP sensors [24, 25], and other biosensor-based [5, 12, 26] and computational [27] approaches provides independent lines of evidence that ATP is constitutively retained in the pericellular space of lymphocytes, epithelial and cancer cells at relatively high (micromolar) levels, without significant nucleotide convection into the bulk milieu.

3. Biological effects of ATP, ADO and other purinergic agonists

Extracellular ATP and its dephosphorylated derivatives ADP and ADO have attracted an enormous amount of attention in terms of their involvement in a wide range of pathophysiological activities, including potent neurotransmission in the central nervous system, non-adrenergic and non-cholinergic smooth muscle contractility, [4, 11, 28], brain injury and acute and chronic neuroinflammation [12, 29, 30], inotropic, chronotropic, and arrhythmogenic effects in the myocardium [31], angiogenesis and vascular remodeling [32-34], blood flow distribution and oxygen delivery [35, 36], immunomodulation and control of leukocyte trafficking between the blood and tissues [37-41], activation and aggregation of platelets at sites of vascular injury [42-44], pulmonary disorders [45], retinal function and neurovascular coupling in the eye [46-48], renal function [49], and tumorigenesis [9, 39, 50]. In terms of the vascular system, intravascular ATP generally functions as a "danger sensor" and "find-me" signal guiding phagocytic cells to the site of inflammation and alerting the immune system to the presence of pathogen-associated molecular patterns, and in addition mediates diverse vasoactive and pro-angiogenic effects [37, 40, 50-52] (**Fig. 3**). These effects are mediated via two major nucleotide-selective receptor subfamilies, P2X and P2Y. P2X receptors are ligand-gated ion channels that comprise of seven receptors (P2X1 through P2X7), whereas G-protein-coupled P2Y receptors are categorized into a subfamily of receptors that predominantly couple to G_q and activate phospholipase C-β ($P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$), and G_i-coupled receptors that inhibit adenylyl cyclase and regulate ion channels ($P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) [4, 37, 38, 53]. Unlike ATP-specific P2X receptors, different P2Y receptor subtypes selectively recognize ATP and/or other purinergic agonists, including ADP, UTP, UDP, UDP-glucose, and UDP-galactose. ATP-derived ADO, in turn, attenuates the inflammation and tissue damage and also maintains endothelial barrier function via binding to its own G-protein coupled ADO receptors (AR) that function by activating ($A_{2A}R$ and $A_{2B}R$) or inhibiting (A_1R and A_3R) adenylyl cyclase [4, 32, 37-39, 53] (**Fig. 3**).

4. Metabolism of ADO and related compounds in the vascular system

Current findings support the presence of an extensive network of ectoenzymes that are co-expressed to a variable extent among the mammalian tissues and share similarities in substrate specificity (**Table 1** and **Fig. 1**). Because of the large body of literature covering different aspects of cellular purine homeostasis,

here I will focus on the recent progress toward understanding the complexity, redundancy, and biological and clinical relevance of ectoenzymatic pathways controlling ATP and ADO levels in the vascular system. More detailed information on the structure, molecular properties, and cellular functions of key purinergic ectoenzymes can be found in seminal publications, in particular those focused on the enzymes of ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) [6, 54-56], ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) [6, 55, 57, 58], alkaline phosphatase [5, 55, 56, 59], adenylate kinase [60, 61] and nucleoside diphosphate kinase (NDPK/NME/NM23) [5, 62, 63] families, ecto-5'-nucleotidase/CD73 [29, 55, 64-66], and adenosine deaminase [6, 67, 68].

4.1. Breakdown of ATP to ADO via the CD39-CD73 axis

The breakdown of ATP is largely mediated by the enzymes of the NTPDase family comprising of four cell-surface members (NTPDase1, 2, 3 and 8), which display broad substrate specificity towards different purine and pyrimidine nucleoside tri- and diphosphates with K_m values in the 50-200 μmol/L range (**Table 1**) [6, 54, 55, 69]. NTPDase1 (also known as CD39) is the most extensively characterized member of this family, which is abundantly expressed on vascular endothelial, smooth muscle cells, T- and B-lymphocytes and other hematopoietic cells [8, 54, 70-73] (also **Figs 4** and **5**). Other members of NTPDase family also contribute to the metabolism of extracellular ATP and other nucleotides. This is particularly important for the physiology of microvascular pericytes and adventitial cells (NTPDase2) [71], the human neuroretina and optic nerve head (NTPDase2) [74], portal fibroblasts of the liver (NTPDase2) [75], pancreatic β-cells (NTPDase3) [76], salivary glands, gastrointestinal tract, and other components of the digestive system (NTPDase2 and 3) [77], the progenitor cells in the mouse postnatal neurogenic zones and also astrocytes in the adult brain (NTPDase2) [78, 79], as well as liver canaliculi and brush border membranes of the kidney (NTPDase8) [54, 75].

The subsequent hydrolysis of ATP/ADP-derived AMP is mediated by ecto-5'-nucleotidase/CD73, which is anchored to the plasma membrane at their C-termini through glycophosphatidylinositol (GPI) and hydrolyzes extracellular AMP to ADO with K_m values in the low micromolar range (10-40 μmol/L) (**Table 1**) [5, 55, 64-66, 70, 80]. The enzyme is expressed to a variable extent in different tissues, with high expression in the draining lymph nodes, blood and lymphatic vascular endothelial cells [81-83], brain [29], kidney, liver, heart and lung [64-66], as well as in a range of circulating myeloid cells, natural killer cells and lymphocytes [73, 84]. CD73 is also expressed specifically in photoreceptor precursors and continues to be expressed in differentiated photoreceptors [74, 85, 86] (**Fig. 4**) and therefore, it has been employed as the cell surface marker for the enrichment of pluripotent stem cell-derived photoreceptor populations and the isolation of photoreceptors from retinal organoids [85, 87]. Besides CD73, yet another nucleotidase, prostatic acid phosphatase (PAP), is expressed in salivary glands, prostate lobes, kidney, liver, dorsal spinal cord, lymphoid organs and other cells and tissues where it can also hydrolyze extracellular AMP within a wide range of pH from ~4 to 8 (**Table 1**) [6, 56, 82]. Overall, in view of diverse, pro-inflammatory (ATP), pro-thrombotic (ADP), anti-inflammatory (ADO), and vasodilatory (ATP, ADO) effects of intravascular purines (**Fig. 3**), a major role of the CD39-CD73 axis in the vascular system is to implement a switch from an immune-stimulating, inflammatory environment to an immunosuppressive environment, thus keeping the hemostatic process tightly regulated to prevent excessive clot formation and vessel occlusion.

4.2. Non-canonical ADO-generating ectoenzymatic pathways

4.2.1. ATP inactivation via the ENPP-CD73 axis

In contrast to traditional paradigms that focus almost exclusively on the CD39-CD73 axis, it has become clear now that other ectoenzymes may contribute to the tuned regulation of ATP and ADO levels in certain synergistic or compensatory manners [6, 10, 55]. In particular, two members of ENPP family, ENPP1 and ENPP3, are relevant in the context of the purinergic signaling cascade due to their ability of hydrolyzing ATP and other (di)nucleotides at fairly comparable rates with apparent K_m values of ~40-200 µmol/L (**Table 1**) [6, 55, 88, 89]. In bone tissue, ENPP1 activity is mainly associated with osteoblast- and chondrocyte-derived membrane structures termed "matrix vesicles", where it has been identified as the key PP_i-generating enzyme ensuring bone matrix mineralization [55, 90, 91]. Several data also provide evidence for the involvement of ENPP isozymes in inhibition of insulin signaling and glucose homeostasis (ENPP1) [91], generalized arterial calcification of infancy and pseudoxanthoma elasticum (ENPP1) [92, 93], synapse formation during brain development (ENPP1) [79], tumorigenesis (ENPP1 and ENPP3) [94, 95], vascular smooth muscle cell calcification (ENPP1 and ENPP3) [96], intestinal epithelial barrier function and host defense during microbial infection (ENPP1) [97, 98], as well as preventing the ATP-dependent chronic allergic responses by basophils and mast cells (ENPP3) [99]. Thus, the ENPP-CD73 axis may act as an alternative CD39-independent adenosinergic loop in the vascular system and other tissues, which allows bypassing the generation of ADP as the principal platelet-recruiting agent and at the same time, generating another important by-product of ENPP reaction, PP_i (**Fig. 1**).

4.2.2. Tissue-nonspecific alkaline phosphatase

Yet another enzyme potentially implicated in extracellular nucleotide metabolism is the member of alkaline phosphatase family, tissue-nonspecific alkaline phosphatase (TNAP) (**Table 1**). The enzyme is attached to the plasma membrane via a GPI-anchor and is capable of hydrolyzing adenine nucleotides, inorganic polyphosphates (polyP), diphosphate (PP_i), and other phosphate-containing compounds, with release of inorganic phosphate (P_i) and with a pH optimum for this catalytic reaction lying in the alkaline range from 8 to 11 [55, 59, 100]. TNAP is highly expressed on mineral-depositing matrix vesicles of osteoblasts and osteocytes, where it controls, in concert with ENPP1 and phosphate transporters, normal bone matrix mineralization, soft tissue calcification and calcium phosphate homeostasis via directional hydrolysis of mineralization inhibitor PP_i [59, 101]. TNAP is also selectively located at exchange surfaces, such as vascular endothelial and smooth muscle cells, the apical brush border of enterocytes, kidney tubules, the mucosal surfaces of airways, and blood and lymphatic vessels and capillaries [55, 59, 78, 79, 81, 96].

Taking into account surprisingly broad substrate-specificity of TNAP and other members of this family towards different phosphomonoesthers and also their implication in other cellular functions (e.g. immunoglobulin G transport, metabolism of vitamin B_6, and absorption of lipids) [6, 59], further studies are required to validate the relevance of TNAP in the context of the purinergic signaling cascade. For example, PolyP comprise another group of metabolic and signaling compounds in eukaryotes, which consist of unbranched chains of three up to 1000 inorganic phosphate (P_i) residues linked by high-energy phospho-anhydride bonds, and are capable of storing metabolic energy both intracellularly and within the extracellular matrix [100, 102, 103]. While our understanding of the physiological relevance of these polyanions in mammalian cells is hampered by a lack of knowledge concerning the enzymes involved in polyP metabolism [102, 103], recent data suggest that extracellular polyP acts as a major phosphate source for generation of ADP and ATP via a concerted action of TNAP and ecto-adenylate kinase and in this way, may control astroglial signal transmission in the mammalian brain [104], and also blood clotting, bone mineralization and cartilage formation [100].

4.2.3. Extracellular ADO formation from NAD, diadenosine polyphosphates and other related compounds

Along with the canonical routes of ATP breakdown into ADO, extracellular ADO can be generated from other related compounds, including cAMP, nicotinamide adenine dinucleotide (NAD) [94, 105, 106], dinucleoside polyphosphates (Ap_nA) [5, 97, 107], 2'3' cyclic GMP-AMP (cGAMP) and other cyclic dinucleotides [41, 98]. Recent years have witnessed a resurgence of interest in NAD^+ metabolism, which has been driven by the discoveries of the important role of NAD^+ and its degradation products in various biological activities, including mitochondrial function, cellular bioenergetics, DNA repair, intercellular communication, immunity and inflammation [105, 106, 108]. Accumulating evidence suggests that NAD^+ levels decline with age at a systemic level in diverse organisms, including rodents and humans, thereby affecting the longevity and contributing to the development of age-associated pathologies. In turn, preventing NAD^+ degradation via inhibition of CD38 activity was shown to serve as a promising 'NAD^+ boosting' therapy for different metabolic diseases [109]. The great majority (>90%) of this multifunctional enzyme has a type II membrane orientation and functions as an ecto-NADase, with a small fraction of CD38 being also expressed as a type III plasma membrane protein with the catalytic site facing the inside of the cell [110]. In contrast to ATP mediating its signaling events via a series of nucleotide-specific receptors, NAD^+ mainly regulates cellular functions through CD38 and another functionally related enzyme CD157, thereby giving rise to the generation of biologically active second messengers like cyclic ADP-ribose, nicotinic acid adenine dinucleotide phosphate (NAADP) and ADO [105, 110]. The later metabolite is particularly relevant in terms of the purinergic signaling, and multiple studies have been focused on the CD38-NPP1-CD73 axis as an alternative ADO producing pathway in human lymphocytes, cancer cells and other cell types [10, 94, 106] (**Table 1** and **Fig.1**).

Ap_nA are a group of naturally occurring molecules consisting of two ADO molecules bridged by a variable number of phosphates (n=2-6), which are produced and released from adrenal glands, myocardium, platelets, neutrophils and chromaffin cells [31, 111] and, after degradation by the enzymes of ENPP family, may serve as a potential source of extracellular nucleotides and ADO [5, 55]. For example, the release of diadenosine triphosphate (Ap_3A) from activated human neutrophils and its subsequent breakdown to ADO via concerted action of ENPP1 and CD73 expressed on surface of intestinal epithelial cells may represent a novel leukocyte-epithelial cross-talk mechanism playing an important role in the metabolic control of innate inflammatory pathways [97]. Recent data on the ability of epithelial ENPP1 to hydrolyze extracellular cGAMP highlighted yet another ATP-independent adenosinergic pathway by which microbial- and host-produced cyclic dinucleotides may control the innate immune response via generation of extracellular ADO and activation of $A_{2B}R$ on basolateral surface of epithelial cells lining mucosal surfaces [98]. Another member of ENPP family, ENPP4, may also regulate purinergic signaling via conversion of platelet-derived Ap_3A into ADP and AMP, thereby augmenting platelet aggregation at the site of vascular injury [107].

4.3. Extracellular ATP re-synthesis via reverse phosphotransfer reactions

In contrast to traditional paradigms that focus on nucleotide-inactivating/ADO-producing pathways, it has now become clear that "classical" intracellular ATP-regenerating enzymes of adenylate kinase (AK) and NDPK/NME/NM23 families can also be co-expressed on the cell surface (**Table 1**). The most prominent member of AK family, AK1, is expressed in well-differentiated tissues with a high-energy demand, like brain, heart and skeletal muscle, where it catalyzes the reversible phosphoryl transfers ATP + AMP $\leftrightarrow$ 2ADP [60, 61]. The enzymes of NDPK family catalyze the exchange of a γ-phosphate between nucleoside tri- and diphosphates and have a broad substrate specificity towards different ribo- and deoxyribonucleotides of purines and pyrimidines [112]. The first two members of this family, NME1 and NME2 (previously designated as NDPK-A and NDPK-B, respectively), are ubiquitously expressed in different tissues, with the highest expression in the brain, kidney, liver, pancreas and heart [62, 63]. The expression of ecto-nucleotide kinases on human and mouse lymphocytes [26, 73, 113], epithelial cells

[114], vascular and lymphatic endothelial cells [70, 83, 100, 115], and their ability to transphosphorylate extracellular ATP, ADP and other nucleotides provide evidence for the co-existence of spatially arranged phosphotransfer networks coordinately maintaining balanced nucleotide levels in the cell vicinity.

In principle, the duration and magnitude of purinergic signaling can be coordinated via two opposite, ATP-consuming and ATP-regenerating, pathways, where sequential ATP breakdown to ADO and further to inosine/hypoxanthine is counterbalanced by re-synthesis of high-energy phosphoryls through phosphotransfer reactions [5, 6]. The release of a particular nucleotide alone would cause its rapid inactivation through sequential ecto-nucleotidase reactions, which presumably occurs via accumulation of extracellular ATP and other nucleotide substrates on the cell surface followed by preferential "hand-to-hand" delivery of the generated product for the succeeding phosphatase reactions [116]. More recent computation analysis confirmed co-localization of CD39 and CD73 within confined membrane domains, and further demonstrated the significant role of electrostatics and intermediate channeling in facilitating sequential nucleotide hydrolysis catalyzed by these coupled ecto-nucleotidase reactions within junctional interfaces between the cells [27]. Under normal physiological conditions, the ecto-nucleotide kinases seem not to compete with ecto-nucleotidases for a limited pool of the released nucleotide substrates. Indeed, the nucleotide-binding centers of AK are assembled with large domain movements upon simultaneous binding of both substrates, whereas the existence of enzyme either in ATP- or AMP-binding forms does not cause sufficient conformational changes [60]. However, acute changes in the specific ratios of nucleoside tri-, di- and monophosphates (usually occurring in the settings of inflammation, hypoxia and oxidative stress [40, 50]), in conjunction with feed-forward inhibition of CD73 activity by precursor ATP/ADP [70], will determine the directional shift from a nucleotide-inactivating pathways towards reverse ATP re-synthesis (**Fig.1**). Nonetheless, the interplay between extracellular ATP-consuming/ADO-generating and reverse ATP-regenerating pathways has been rather neglected so far.

4.4. Extracellular and intracellular metabolism of ADO and other nucleosides

Extracellular ADO appears in the interstitial milieu only transiently as an intermediate metabolite of the nucleotide-hydrolyzing enzymatic chain to act as a short-term signaling molecule that needs to be removed in a coordinated fashion, either through adenosine deaminase (ADA) activity [5, 67, 117] or transport into the cell via the equilibrative and/or concentrative nucleoside transporters [118]. Thus, the termination of extracellular ADO signaling is the result of a concerted effort between nucleoside transport systems and extracellular and intracellular ADO metabolism. Two distinct ADA isoforms, ADA1 and ADA2, are found in humans (**Table 1**) [6, 119, 120]. The most relevant in terms of a purinergic signaling isozyme, ADA1 (usually quoted as ADA), is mainly localized in the cytoplasm but is also co-expressed as an ectoenzyme on the surfaces of lymphocytes, dendritic cells and other lymphoid and non-lymphoid tissues [73, 113, 121]. ADA1 and especially, ADA2 possess rather low affinities towards ADO (K_m values in the range of 40-60 µM and ~2 mM, respectively) [117, 121] and therefore, might be particularly pertinent for the clearance of extracellular ADO during inflammation when ADO levels become excessively high. Several other key enzymes contribute to the regulation of intracellular and extracellular ADO levels, including S-adenosylhomocysteine hydrolase, cytosolic 5'-nucleotidase, purine nucleoside phosphorylase, and adenosine kinase (ADK) [11, 65]. The high affinity, low capacity enzyme ADK (especially, its cytosolic isoform ADK-S) provides the major metabolic route of ADO clearance under physiological conditions by phosphorylation of ADO into AMP [10, 122] and in addition, may act as dihydronicotinamide riboside (NRH) kinase playing a key role in NAD^+ biosynthesis pathways in mammalian cells [123].

Due to the existence of ubiquitous nucleoside transport systems, the intracellular metabolism of ADO and compartmentalization of the ADO system are expected to play an equally important yet understudied

role for the regulation of biological effects of ADO. While the detailed characterization of these pathways lies beyond the scope of this review, it is pertinent to note that, along with “classical” AR-mediated pathways, ADO exerts cytotoxic and other biological effects via receptor-independent mechanisms [124-128]. The intrinsic mechanisms of ADO action remain unknown and can fall into two categories: (*a*) role that is linked to the intracellular uptake and metabolism of ADO, with subsequent deregulation of phosphorylated AMP-activated protein kinase (AMPK) and related signaling pathways [126, 129], and (*b*) a specific emerging role of ADK-L isoform, which is expressed in the cell nucleus and acts as epigenetic regulator [125, 127].

4.5. ADO metabolism by soluble and microvesicle-wrapped enzymes

The ability of cell-free human plasma to dephosphorylate [130] or transphosphorylate [131] ATP and other adenine nucleotides was first demonstrated over half century ago. Subsequent chromatographic and colorimetric assays allowed the identification of several ATPase, ADPase, and AMPase (CD73) activities in the plasma and serum from humans and other species [132-134], whereas the reverse ATP regeneration is mediated through the concerted action of soluble AK (mainly comprising of the AK1 isoform) and NDPK activities [134, 135]. While the initial step of ATP breakdown in cell-free plasma or serum is thought to be mainly attributable to ENPP1 activity [57], recent studies elicited the co-existence of other soluble enzymes, CD39 and AK1, which account for 10-15% of the total ATP-inactivating pool in human serum and in addition, may utilize ADP as another preferred substrate [135, 136]. The presence of a broad spectrum of soluble nucleotide hydrolases and kinases was also demonstrated in other biological fluids such as human lymph [83], the liquid covering airway surfaces [114], pancreatic juice [137], and vitreous fluids [74, 138]. Furthermore, both ADA1 [67, 134] and ADA2 [120] constitutively circulate as soluble enzymes in the human blood, with the former isozyme being also identified in the human vitreous fluids [74]. The nature, origin and exact mechanisms underlying the appearance of soluble purinergic enzymes in the bloodstream and other biological fluids remain largely unknown. Interestingly, certain portions of cellular AK and/or NDPK was shown to be released from the platelets and red blood cells [131], airway epithelial cells [114], and breast cancer cells [139], whereas the potential sources for secreted CD39 and CD73 might include nerve terminals [140], pancreatic acini [137], vascular endothelium [54, 141], and other cell types. Alternatively, CD39 and CD73 can also be incorporated into exosomes (a subclass of membrane-derived extracellular vesicles secreted into the extracellular milieu by most cell types), where they impact endothelial activation and inhibit angiogenesis [142, 143], mediate immunosuppressive effects in the tumor microenvironment [8, 144], modulate vascular thrombosis by preventing platelet aggregations [145, 146], and control pancreatic secretion [147], and gastrointestinal and hepatic functions [77]. Further studies are required to understand the relevance of soluble and exosome-wrapped purinergic activities contributing, in conjunction with the larger framework of membrane-bound ectoenzymes, to the spatial propagation or termination of purinergic signaling responses in the vascular system.

5. Dysregulation of extracellular ADO metabolism in the vascular system

5.1. The role of ADO metabolism in inflammation and tumorigenesis

CD39 and CD73 have traditionally been considered to be the key enzymes involved in the control of inflammation and immune function. According to this scenario, apoptotic or necrotic cells release ATP, which binds to excitatory ATP-specific receptors and subsequently triggers inflammatory responses by amplifying T cell receptor signaling in lymphocytes and by promoting inflammasome activation in macrophages and dendritic cells. High nucleotide-inactivating activities in turn contribute to the clearance

of pro-inflammatory ATP, simultaneously leading to $A_{2A}R$-mediated inhibition of effector immune cells [9, 41, 50, 148]. The current view of CD39 and CD73 as an important anti-inflammatory target is largely based on the premises that the expression levels of these two ecto-nucleotidases are up-regulated on surfaces of vascular endothelial, epithelial, lymphoid and tumor cells as a result of tissue hypoxia, inflammation and oxidative stress [40, 128, 148]. New insights have been gained in the past two decades into the implication of the CD39-CD73-$A_{2A}R$ axis in various immune-mediated inflammatory diseases, including rheumatoid arthritis, psoriasis, and bacterial infection [38, 73, 149], renal [49] and intestinal inflammation [40, 77], and cancer [7, 9]. The role of ADO-inactivating enzyme ADA in immunodeficiency is also well characterized. Inherited ADA deficiency results in severe combined immunodeficiency (SCID), which was the first genetic disorder treated by gene therapy. ADA–SCID is a rare metabolic disorder accounting for approximately 10–15% of all SCID cases and characterized by impaired T, B, and natural killer cell development and function, complete absence of cellular and humoral immunity, and recurrent infections [68]. Genetic deficiencies in ADA in humans result in a severe lymphopenia and immunodeficiency due to abnormal thymocyte development and peripheral T-cell activation and in addition, exhibit phenotypes in other physiological systems including the renal, hepatic, skeletal and pulmonary alterations, neurological abnormalities and behavioral impairments [67, 121]. Moreover, human ADA2 deficiency (DADA2), due to autosomal-recessive loss-of-function mutations in the ADA2 gene (previously known as CECR1), is the first described monogenic type of small- and medium-size vessel vasculitis and other forms of vasculopathy [150]. Recent data also demonstrated the enhanced ADO-mediated formation of neutrophil extracellular traps (NETs) and subsequent TNF-α production by activated macrophages in patients with DADA2 [151]. Data on selective binding of soluble ADA1 and ADA2 isozymes to different immune cell subsets provide promise for the efficient delivery of these ADO removing enzymes and directional manipulation of local ADO levels in areas of tumor growth and at sites of inflammation [120].

5.2. The role of ADO metabolism in vascular thrombosis and related diseases

Platelet activation and recruitment, followed by haemostatic plug formation, is generally initiated either via formation of thromboxane-A_2 (TXA_2) by cyclooxygenase-1 (COX1, also known as PTGS1) or secretion of ADP from dense granules with subsequent activation of platelet ADP-selective $P2Y_1$/$P2Y_{12}$ receptors. Inhibition of $P2Y_{12}R$, in combination with other antiplatelet agents (including COX1 inhibitor aspirin and antagonists of TXA_2, protease-activated receptor 1, and glycoprotein IIb/IIIa), has proved to be successful in reducing the morbidity and mortality associated with arterial thrombosis manifesting as myocardial infarction and ischemic stroke [43, 44]. Currently available $P2Y_{12}R$ antagonists comprise two classes of drugs: the thienopyridines (clopidogrel, prasugrel, and ticlopidine) and the nucleoside-nucleotide derivatives (cangrelor and ticagrelor) [44]. In turn, vascular endothelium controls platelet reactivity and prevents thrombus formation via three pathways, including nitric oxide and prostaglandin-I_2 synthesis and ADP scavenging by CD39 [42, 152]. Data from mutant mice lacking or overexpressing CD39 [146, 153, 154] and CD73 [155, 156] further confirmed the critical regulatory role for these ectoenzymes in platelet reactivity and thromboregulation.

Along with regulation of blood clotting and coagulation, CD39 and CD73 can directly drive local and systemic inflammatory responses associated with the onset of cardiovascular events. Therefore, many studies have investigated the role of this nucleotide phosphohydrolysis pathway in different diseases associated with vascular thrombosis and inflammation, such as thrombus formation at atherosclerotic plaque disruption sites [157, 158], arteriogenesis [159], acute kidney injury and chronic kidney disease [49, 160], deep vein thrombosis [52], bacterial infection [73], post-infarction myocardial repair and rupture [161, 162], pulmonary arterial hypertension [163], acute lung injury [40], sepsis [164], and stroke [42, 146]. Recent data also provide a novel insight into the important neuroprotective role of the CD39-

CD73 axis in the brain, where it provides a negative feedback control of neuronal activity by microglia via termination of ATP-mediated activation of microglial $P2Y_{12}R$ and concurrent suppression of neuronal activity via production of ADO and activation of A_1R [30]. Noteworthy, while CD39 and CD73 are traditionally viewed as central players in the termination of proinflammatory and prothrombotic effects of circulating ATP and ADP, roles of alternative purine-converting ectoenzymes (such as ENPP1, TNAP, adenylate kinase, and NDPK) in the context of vascular thrombosis and inflammation is now emerging [6, 55, 161, 165, 166].

5.3. The role of ADO metabolism in angiogenesis and vascular remodeling

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, occurs during normal embryonic development and in various pathological conditions where hypoxia, ischemia, or inflammation are prominent features [167]. Accumulating evidence indicates that both ATP [45, 168] and ADO [32, 33, 39, 169] act as important regulators of fibrosis, wound healing, tissue repair and neovascularization in different organs and tissues. The angiogenic effects of extracellular ATP are mediated through activation of endothelial $P2Y_1$ and $P2Y_{13}$ receptors and PI3K/mTOR and ERK1/2 pathways [168], whereas ADO triggers release of interleukin-8, basic fibroblast growth factor (bFGF), and VEGF from endothelial cells via $A_{2B}R$ and at the same time, inhibits the production of the anti-angiogenic factor thrombospondin-1 via activation of $A_{2A}R$ [33, 169]. Numerous studies, including our own, have demonstrated the important regulatory role of CD39 and/or CD73 in modulation of a number of steps involved in angiogenesis and vasculogenesis, including the promotion of endothelial cell proliferation, migration and tube formation, as well as maintenance of endothelial and epithelial barrier integrities [83, 97, 115, 170]. Studies with CD39-null mice further demonstrated an important role of CD39 in activation of integrin-associated signaling pathways and abrogation of tumor angiogenesis and metastasis [171], whereas overexpression of CD39 decreases vascular smooth muscle cell proliferation and prevents neointima formation after angioplasty [172]. In addition to the classical strategies aimed to control angiogenesis by direct targeting of the endothelium, recent studies are also focused on the ability of stromal cells and pericytes to regulate angiogenesis, vascular modeling, and inflammation through the release of soluble factors or direct crosstalk with endothelial and immune cells [173, 174]. CD73-generated ADO produced locally by perivascular fibroblasts/pericytes may act on different AR subtypes in an autocrine and/or paracrine fashion, thereby attenuating macrophage infiltration in the kidney interstitial microenvironment and preventing progressive fibrosis [160]. Recent study has also demonstrated that extracellular vesicles released by bone marrow-derived mesenchymal stromal cells express high CD39 and CD73 activities and in this way, inhibit extracellular matrix remodeling, retinal neovascularization and tumor associated angiogenesis through activation of adenosinergic signaling pathways [143].

Other purinergic enzymes may also play a critical role in diseases characterized by excessive angiogenesis, including cancer [7, 10] and ocular diseases [47, 74]. Data on the presence of a broad spectrum of membrane-bound and soluble nucleotide-inactivating and ATP-regenerating enzymes in the human [74] and mouse [47, 86] (**Fig. 4**) sensory neuroretina, ocular blood vessels, and vitreous fluid further extend our knowledge on the regulatory mechanisms of ADO metabolism and signaling in the eye. Moreover, specific shifts in purine homeostasis in diabetic retinopathy eyes from the generation of anti-inflammatory ADO towards a pro-inflammatory and pro-angiogenic ATP-regenerating phenotype provide a novel insight into the role of ocular ADO homeostasis in the pathogenesis of diabetes-induced vascular leakage, retinal fibrosis and neovascularization [74, 138]. Recent data also provide evidence for complex implication of both extrinsic and intrinsic mechanisms into a tuned control of ADO metabolism in endothelial cells in the settings of hypoxia and angiogenesis, which may include down-regulation of ADK activity with respective increase in intracellular ADO levels and epigenetic DNA hypomethylation in the

promoter regions of several pro-angiogenic genes [175], and also activation of cellular purine turnover and phosphotransfer networks [72].

5.4. The role of ADO metabolism in lymphangiogenesis

Lymphatic endothelial cells of lymphatic vessels and capillaries represent a distinct endothelial cells lineage, frequently distinguished from blood vascular endothelial cells based on their expression of the transcription factor prospero homeobox-1 (Prox1), transmembrane O-glycoprotein podoplanin, vascular endothelial growth factor receptor-3 (VEGFR3), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1), and neuropilin-2 [176, 177]. Lymphatic vasculature shows remarkable plasticity and heterogeneity and now emerges as active tissue-specific player in diverse physiological and pathological processes in skin, lymph nodes, small intestine, meninges, brain, and eye [177]. The enzyme CD73 has long been known as a useful lymphatic endothelial cell-specific marker for identifying lymphatic capillaries and collecting vessels [81]. More recent histochemical analyses of human and murine lymph nodes and other lymphoid tissues further demonstrated selective CD73 distribution in the germinal centers, connective tissues, high endothelial venules and afferent lymphatic vessels [6, 83, 178], and also in human lymph [83]. Studies with $CD73^{-/-}$ mice provided evidence that CD73-generated ADO may restrict the migration of lymphocytes across high endothelial venules into draining lymph nodes after an inflammatory stimulus [178]. This contrasts with another report from the same authors showing promoting effects of CD73 on the entrance of pathogenic T cells into the central nervous system in experimental autoimmune encephalomyelitis, a model for multiple sclerosis [179]. Interestingly, human dermal microvascular lymphatic endothelial cells were shown to display higher CD73 activity than blood endothelial cells isolated from the same donor [83]. Though, in sharp contrast to blood endothelium, lymphatic endothelial cells were unresponsive to adenosinergic signaling during the regulation of endothelial sprouting and permeability control [83]. ADO also inhibited the proliferation and migration of lymphatic endothelial cells *in vitro* but stimulated lymphangiogenesis *in vivo* [180]. Overall, the function of lymphatic CD73 still remains enigmatic and controversial. Further investigation is warranted to understand the role of CD73 in lymphangiogenesis.

5.5. Purinergic mechanisms implicated in the integrated control of blood flow and vascular tone

In addition to modulating immune responses, thrombosis, and angiogenesis, ADO and its precursor nucleotide ATP may affect vessel function by increasing coronary and microvascular blood flow. Intravascular ATP is released from endothelial cells in response to the increased shear stress, hypoxia, ischemia, and other inflammatory stimuli [40, 141, 181]. Erythrocytes represent another important source of ATP, which can be released during hypoxia, acidosis, strenuous exercise and mechanical deformation [36]. Non-invasive transthoracic Doppler echocardiography imaging analysis revealed that infusion of different concentrations of ATP and ADO, as well as a non-hydrolysable ATP analogue ATPγS into the tail vein of C57Bl/6 mice triggered substantial increases in coronary blood flow velocity, which can be prevented by pretreatment of the animals with the non-selective P2 antagonists PPADS and suramin [157] (**Fig. 5A**). These data, when analyzed together with our earlier findings on different vasodilatory and sympatholytic effects of exogenous ATP and other nucleotides and ADO in the human skeletal muscle vasculature [182], indicate that ATP exerts vasodilatation via a dual mechanism. These hyperemic effects of ATP include (i) direct binding to endothelial $P2Y_1$, $P2Y_2$ and/or $P2X_4$ receptors, and (ii) further conversion to ADO and activation of $A_{2A}R$ and, to some extent, $A_{2B}R$ on vascular endothelial and smooth muscle cells [32, 34, 35, 181]. Activation of endothelial nucleotide- and ADO-selective receptors in turn leads to the release of endothelium-derived relaxant factors (nitric oxide, endothelium-derived hyperpolarizing factor and prostacyclin), which inhibit platelet aggregation and thus ensures patent blood flow [35, 181]. CD39, CD73, and other ecto-nucleotidases are selectively co-expressed on vascular

endothelial and smooth muscle cells in murine aorta [157, 183, 184] (**Fig. 5B**), and other arterial, venous, microvascular and cerebral blood vessels [54, 71, 72].

In contrast to the enhanced nucleotide phosphohydrolysis following the exposure of endothelial cells to short-term hypoxia [40, 72, 148], CD39 and CD73 activities may be down-regulated or even lost on vascular and microvascular endothelium and other cell types at sites of vascular injury and atherogenesis [145, 157], during chronic oxygen deprivation [115], organ transplantation [152], and also arterial [185] and pulmonary [163] hypertension. Significant down-regulation of CD39 activity has also been demonstrated in aortas from apolipoprotein E-deficient ($ApoE^{-/-}$) mice prone to atherosclerosis [157, 184], and also in blood vessels from abdominal aortas of patients with atherosclerosis and atherosclerosis-like diseases [186]. Therefore, it is tempting to speculate that the directional manipulation of CD39 and other intravascular nucleotidase activities is a promising therapeutic strategy for the treatment of atherosclerosis with the desired outcome to prevent disturbed blood flow, sustained triggering of chronic inflammatory responses and atheroma progression via local inactivation of ATP and ADP at sites of atherogenesis.

6. Purine-converting ectoenzymes: emerging targets in cancer and vascular therapies

The above findings provide a solid background for the directional manipulation of the ATP-ADO axis as a novel strategy for tissue-specific therapeutic interventions. Selective targeting of this pathway in cancer has evolved into novel immunotherapeutic approaches, which are particularly focused on combined therapies with anti-CD39 antibodies [8, 187], anti-CD73 antibodies [7, 9, 84], and small-molecule enzyme inhibitors [86, 188, 189], either alone or in combination with $A_{2A}R$ antagonists and other immune checkpoint inhibitors [7, 9, 148]. However, the realization that ADO metabolism is more complex than previously recognized justifies an armamentarium of other strategies, which could potentially improve the efficacy of ongoing clinical trials. For example, the identification of the ENPP1-CD73 axis on epithelial-derived cancer cells as an alternative CD39-independent adenosinergic loop implies that tumors might have the capability to bypass CD39-based cancer therapeutics [91, 128]. A broad spectrum of non-nucleotide-derived inhibitors of ENPP1 and ENPP3 comprising polysulfonates, polysaccharides, polyoxometalates, small heterocyclic compounds [58] and aryl pyrazole derivatives [190] have been synthesized recently and tested for their therapeutic potential as anti-cancer drugs. Recent studies also identified ENPP3 as a potential cancer-specific antigen [95], with anti-ENPP3 antibody (AGS-16M8F)-drug conjugate being currently tested in Phase I (NCT01114230) and Phase II (NCT02639182) clinical trials in patients with advanced refractory renal cell carcinomas. Moreover, CD38 has been identified as an additional immune checkpoint, and multiple clinical trials have been initiated recently for evaluating the therapeutic potential of daratumumab, isatuximab, and other anti-CD38 antibodies in cancer patients [10, 105]. Selective targeting cytosolic (ADK-S) and nuclear (ADK-L) isoforms of ADK in tumor cells using gene therapy or small molecule-based pharmacological approaches might also permit more tuned regulation of extracellular and intracellular ADO levels in a compartment-specific manner [10, 125].

As outlined above, CD39 activity may be markedly down-regulated on vascular endothelium and other cell types at sites of vascular injury and chronic inflammation. In turn, rapid inactivation of ATP and other circulating nucleotides via transiently up-regulated serum ecto-nucleotidase activities may serve as another important auxiliary effector system counteracting acute pro-thrombotic state during vigorous exercise in humans [136], and contributing to the maintenance of adenosinergic signaling pathway in human and mouse blood during adaptation to high altitude and hypoxia [129]. It is pertinent to note that soluble NTPDase (apyrase) is considered a promising drug, which prevents sustained ADP-induced activation of the major platelet adhesion receptor GPIIb/IIIa and inhibits thrombus formation during

sepsis, vascular injury, ischemia-reperfusion and other pro-thrombotic conditions [145, 191, 192]. Other strategies to offset the reduced effectiveness of endothelial nucleotidases and prevent excessive vascular thrombosis include CD39 expression in the injured vessels [193, 194], up-regulation of endogenous CD39 through the use of phosphodiesterase3-inhibiting drugs [195], and targeting of soluble CD39 (targ-CD39) recombinantly fused to activated platelet GPIIb/IIIa-specific single-chain antibody [164, 196]. CD73, ADA and other enzymes of intravascular ADO metabolism have also been explored and translated as tractable therapeutic targets for preventing arterial calcification, sickle cell anemia, sepsis, ischemic injury, and other cardiovascular disorders (**Table 2**).

Changes in soluble serum CD73 activities may also serve as important biomarkers in predicting the severity of hepatobiliary diseases [133], acute pancreatitis [197], and arterial calcification [93, 165], while soluble CD39 can be associated with several key aspects of peripheral atherosclerosis and thrombosis [198]. Both ENPP1 and TNAP also circulate as soluble enzymes in the human bloodstream [5, 57, 59], and have been widely used as important biomarkers for different diseases, including rickets, osteomalacia, osteoporosis, vascular calcification, chronic kidney disease, prostate cancer, bone metastasis [55, 59, 101, 199]. Clearly, identifying the exact mechanisms by which the extensive network of membrane-associated and secreted purinergic activities coordinately regulates intravascular and intratumoral ATP and ADO concentrations and the further translation of soluble and microparticle-associated enzymes as potential circulating biomarkers and anti-metastatic drugs will be an area of intense interest in the future.

7. My encounters with Geoffrey Burnstock

This Invited Review was prepared for special issue of this Journal entitled “A Tribute to Geoffrey Burnstock”. I started working on ecto-5’-nucleotidase already in my home country Belarus in the early 1990s. At that time, this ectoenzyme was mainly considered a general marker for plasma membranes of eukaryotic cells showing the enrichment of plasma membrane preparation during subcellular fractionation of tissue homogenates. We were also going through very difficult times trying to survive the collapse of the Soviet Union, while at the same time doing science. I desperately wrote multiple requests to various research centers trying to find suitable work. Geoff enthusiastically welcomed my ideas and took a risk by inviting me to join his lab in London, which I did in 1997–98. I have co-authored four publications with Geoff. One of these papers described the effect of shear stress on the release of soluble ectoenzymes by vascular endothelial cells and it has been well accepted and widely cited by the international community [141]. After moving to Finland, I continued to maintain friendly and business relations with Geoff. We regularly saw each other at many International Congresses and scientific events in several countries. I even had the opportunity and privilege to host Geoff and his wife Nomi in Finland, where, along with a scientific program, they were able to explore the beauty of the short Scandinavian summer, visit the islands and, of course, get to know the local mosquitoes and taste grilled sausages. I have always admired Geoff's great curiosity, openness and passion for science. Looking back at my encounters with Geoff, I must conclude that he has had a huge impact on both my academic career and my personal life. Geoff, my family and I will miss you.

8. Conclusions and outlook

In addition to significant progress in understanding the properties of nucleotide- and ADO-selective receptors, recent studies have begun to uncover the complexity of regulatory mechanisms that are involved in biological effects of ADO. This knowledge has led to the development of new paradigms to understand the entire purinome as a complex and integrated network by taking into account the multitude of purinergic signaling pathways, redundancy of purine-converting ectoenzymatic pathways, as well as intracellular ADO metabolism and compartmentalization of the ADO system. The presence of an

extensive network of ectoenzymes regulating extracellular purine levels via two opposite, nucleotide-inactivating/ADO-generating and ATP-regenerating, pathways together with a specific repertoire of purinergic receptors provides great versatility in triggering and/or terminating signaling responses that are most appropriate for a particular cell or tissue. Several open questions remain to be answered. Given the very short half-life of ADO as an intermediate metabolite in the interstitial spaces between cells [11, 12], and its rapid cellular uptake via nucleoside transport system [118], more thorough research is required to assess intravascular ADO levels directly. Intrinsic AR-independent mechanisms of action of ADO and intracellular ADO metabolism also remain understudied, but likely have an equally important role [10].

It should be taken into account that vascular endothelial cells display remarkable heterogeneity in structure and function [200], and there are also significant variations in the purinergic regulatory mechanisms in different blood vessels and in different species related to the specific physiologic roles of the particular vessel [34]. The future of ADO-based interventions will therefore rely on better understanding of the commonalities and differences in the purinergic signature among vascular beds in different organs, and on this basis, guide in the future development of organ-specific and personalized vascular therapies. Furthermore, it is now becoming increasingly evident that endothelial cells require different metabolic adaptations, presumably because they differ from most other cell types in various biological functions and demands [167], thus rendering therapeutic targeting of intravascular ADO metabolism an even greater challenge. In conclusion, directional manipulation of ADO metabolism and signaling has the potential to offer new strategies to control vascular thrombosis, inflammation, tumor growth, local blood supply and other events in the cardiovascular system. However, the role of purinergic ectoenzymes as novel targets in emerging vascular and cancer therapies is just beginning to be unraveled.

Declaration of competing interest

The author declares that he has no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure Legends

Fig.1. Redundancy of ectoenzymatic pathways controlling extracellular ADO levels. Along with the "classical" ATP-inactivating/ADO-generating chain mediated via stepwise CD39-CD73 reactions (upper left), additional ectoenzymatic pathways can contribute to the metabolism of ADO. Those include the breakdown of ATP through the ENPP1 reaction and subsequent metabolism of ATP-derived metabolites PP_i and AMP by other ectoenzymes TNAP, CD73 and ADA (upper right), as well as an alternative ADO-producing route from extracellular NAD^+ via the CD38-ENPP1-CD73 axis (lower left). Acute changes in the specific ratios of nucleoside tri- and diphosphates (usually occurring in the settings of inflammation), in conjunction with feed-forward inhibition of CD73 activity by precursor ATP/ADP, can also determine the directional shift from ADO-producing pathway towards reverse ATP re-synthesis via ecto-adenylate kinase and NDPK reactions (lower right). The extracellularly generated ADO can be taken up by the cells through equilibrative or concentrative nucleoside transporters (NT) and subsequently phosphorylated into ATP via ADK and other intracellular nucleotide kinase reactions.

Fig.2. Nucleotide-releasing pathways. Along with massive nucleotide leakage upon cell damage, ATP and other nucleotides and ADO may appear in the extracellular milieu via various non-lytic pathways, including electrodiffusional movement through ATP release channels, facilitated diffusion by nucleotide-specific transporters, and vesicular exocytosis. Furthermore, preferential accumulation of micromolar concentrations of "ATP halo" in the pericellular space and its exchange with bulk extracellular nanomolar ATP pool via certain dissociation-association mechanisms has been proposed recently.

Fig.3. Biological effects of ADO and other purinergic agonists in the vasculature. Extracellular ATP, ADP and ADO mediate diverse signaling effects in virtually all components of the vascular wall. Intravascular ATP generally functions as a "danger sensor" and "find-me" signal guiding phagocytic cells to the site of inflammation and alerting the immune system to the presence of pathogen-associated molecular patterns. In addition, ATP mediates diverse vasoactive and pro-angiogenic effects, whereas platelet-derived ADP is considered a powerful pro-thrombotic molecule. ATP-derived ADO, in turn, attenuates the inflammation and tissue damage and also maintains vascular blood flow and endothelial barrier function.

Fig. 4. Selective distribution of CD73 and CD39 in mouse photoreceptor cells and retinal blood vessels. Free-floating vibratome-cut sections of the mouse eye were stained with antibodies against CD73 and CD39, together with molecular marker of Müller glial cells vimentin, as indicated. Maximum intensity projections of a confocal z-stack for each channel are shown, with the right-hand panel showing the merged image with nuclei counterstained with 4,6-diamidino-2-phenylindole (DAPI). Analysis of the distribution of key ecto-nucleotidases revealed high CD39 expression in the central artery and vein branching throughout the laminar region of the optic nerve head, whereas CD73 is ubiquitously distributed throughout the entire photoreceptor layer, with the highest enzyme expression detected in the outer segment discs. Scale bar, 50 µm. For experimental details refer: Schmies et al., 2020 [86].

Fig.5. Purinergic mechanisms of coronary blood flow and distribution of ecto-nucleotidase activities in mouse aorta. (A) Coronary blood velocity was determined in C57Bl/6 mice by using noninvasive transthoracic Doppler echocardiography imaging. Left-hand images show representative coronary flow velocity profiles obtained with pulsed-wave Doppler from the middle left coronary artery at rest (baseline) and during infusion of 40 µg/ml of ADO, ATP, and non-hydrolysable ATP analogue ATPγS. The indicated doses of different purinergic agents were administered into the tail veins of anesthetized animals (for 2 min at flow rate of 0.6 ml/hour), and their vasoactive potency was determined by

measuring the mean diastolic flow velocity (mean ± SEM, n=3-6). In certain assays, the mice were pretreated for 3 min with P2 antagonist suramin before infusion of ATP. *P<0.05 versus basal blood flow. (B) Histochemical analysis of the distribution of CD39 and CD73 activities in murine thoracic aorta was assayed by incubating tissue cryosections with ADP (300 μM) and AMP (1 mM) as respective enzyme substrates in the presence of $Pb(NO_3)_2$, followed by microscopic detection of nucleotide-derived P_i as a brown precipitate. In blank specimen, nucleotide substrate was omitted from the incubation solution. Tissue sections were also stained with hematoxylin and eosin (H&E). L, lumen; EC, endothelial cells; SMC, smooth muscle cells; Ad, adventitia. Scale bar, 200 μm. Reproduced and modified from Mercier et al., 2012 [157], with Elsevier permission.

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Table 1 – Major enzymes of adenosine metabolism

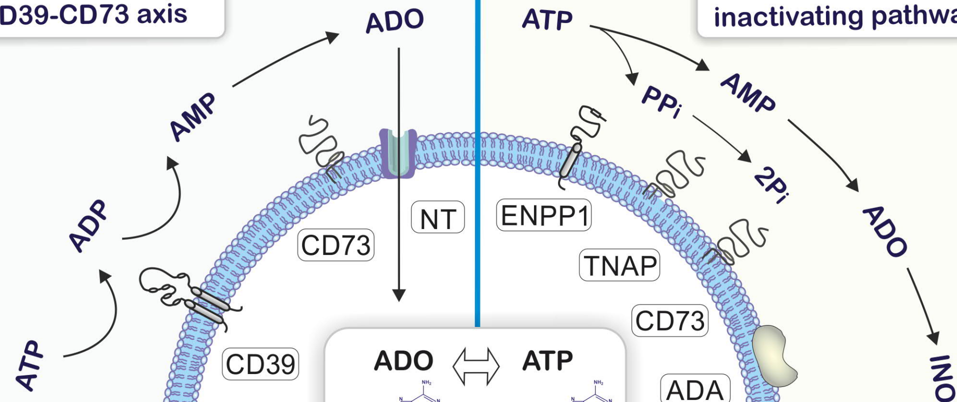
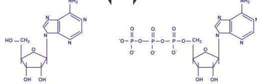
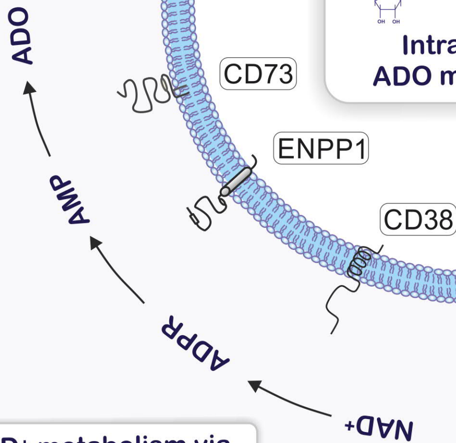
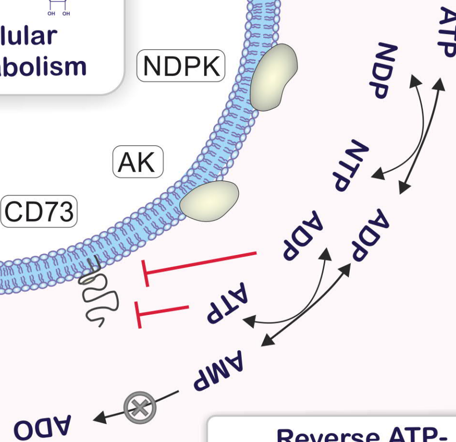
Substrate		Products	Enzyme or enzyme family	Alternative names and aliases	EC number	Isozyme	Gene name	Compartmentalization
ATP ADP	→ →	ADP + P_i AMP + P_i	NTPDase	CD39, apyrase, ATP-diphosphohydrolase, ecto-ATPase, nucleoside diphosphatase	3.6.1.5	NTPDase1 NTPDase2 NTPDase3 NTPDase8	*ENTPD1* *ENTPD2* *ENTPD3* *ENTPD8*	extracellular, soluble, microvesicles
ATP ADPR Ap_4A cGAMP	→ → → →	AMP + PP_i AMP + PP_i ATP + AMP AMP + GMP	ENPP	PC-1, CD203a, Phosphodiesterase-1β CD203c, $gp130^{RB13-6}$, B10	3.6.1.9 3.1.4.1	ENPP1 ENPP3	*ENPP1* *ENPP3*	extracellular, soluble
AMP IMP	→ →	ADO + P_i Inosine + P_i	Ecto-5'-nucleotidase	CD73	3.1.3.5		*NT5E*	extracellular, soluble, microvesicles
ATP PP_i $PolyP_n$	→ → →	ADO + $3P_i$ $2P_i$ $PolyP_{n-1}$ + P_i	ALP		3.1.3.1	TNAP	*ALPL*	extracellular, soluble, microvesicles
AMP TMP	→ →	ADO + P_i Thiamine + P_i	PAP	Fluoride-resistant acid phosphatase, TMPase	3.1.3.2		*ACP3*	extracellular, soluble
NAD^+ cADPR	→ →	ADPR + NA ADPR	CD38	NAD nucleosidase, NADase, cADPR hydrolase	3.2.2.5 3.2.2.6		*CD38*	extracellular, soluble, intracellular
ATP + AMP	↔	2ADP	AK	Myokinase	2.7.4.10	AK1	*AK1*	extracellular, soluble, intracellular
ATP + NDP	↔	ADP + NTP	NDPK	Nm23-H1, NME1 Nm23-H2, NME2	2.7.4.6	NDPK-A NDPK-B	*NME1* *NME2*	extracellular, soluble, intracellular
ADO	→	Inosine	ADA		3.1.3.1	ADA1 ADA2	*ADA1* *ADA2*	extracellular, soluble, intracellular

This table highlights the converting pathways for adenine nucleotides, adenosine (ADO), and related compounds. Abbreviations: ADA, adenosine deaminase; AK, adenylate kinase; ALP, alkaline phosphatase; cADPR, cyclic ADP-ribose; cGAMP, 2'3' cyclic GMP-AMP; EC number, Enzyme Commission number; ENPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; NA, nicotinamide; NAD^+, nicotinamide adenine dinucleotide; NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; NDPK, nucleoside diphosphate kinase; PolyP, inorganic polyphosphate; TMPase, thiamine monophosphatase; TNAP, tissue-nonspecific alkaline phosphatase

Table 2 – Ongoing clinical trials targeting adenosine metabolism in vascular therapies

Target	Drug +/- combination therapy	Company	Conditions	ClinicalTrials. gov identifier	Study phase
CD73 TNAP	Lowering TNAP activity in CD73$^{-/-}$ patients by using etidronate	NHLBI	ACDC	NCT01585402	II
CD73	Small-molecule CD73 inhibitor (AB680)	Arcus Biosciences	Healthy volunteers	NCT04575311	I
CD73	Observational study	NHGRI	ACDC	NCT00369421	
CD73	Observational study	Helsinki University Central Hospital	Circulatory failure	NCT03814564	
ALP	Intravenous infusion of bIAP during and after cardiac surgery	Alloksys Life Sciences B.V.	Ischemic injury and systemic inflammation	NCT03050476	II/III
ALP	Intravenous infusion of recombinant human ALP	AM-Pharma	Acute kidney injury due to sepsis	NCT04411472	III
ADA	ADA inhibitor pentostatin +/- GVHD prophylaxis and allogeneic blood or marrow transplantation	NCI	Primary immunodeficiency and lymphoproliferative disorders	NCT02579967 NCT03663933	II II
ADA	ADA inhibitor pentostatin +/- chemotherapy and immunotherapy	City of Hope Medical Center	Sickle cell disease	NCT03249831	I
ADA	ADA inhibitor pentostatin +/- radiotherapy and immunotherapy	NHLBI	Sickle cell disease, stem cell transplantation and GVHD	NCT02105766	II
ADA	ADA inhibitor pentostatin +/- immunotherapy	NHLBI	Sickle cell disease	NCT03077542	I/II
CD38	Anti-CD38 mAb (isatuximab) +/- immunotherapy	Sanofi	Kidney transplantation and immune system disorders	NCT04294459	I/II

Abbreviations: ACDC, arterial calcification due to CD73-deficiency; bIAP, bovine intestinal alkaline phosphatase, also known as bRESCAP; GVHD, graft-versus-host disease; Etidronate (the disodium salt of 1-hydroxyethylidene diphosphonic acid) is a nitrogen-containing bisphosphonate, also known as EHDP and Didronel; NCI, National Cancer Institute; NHGRI, National Human Genome Research Institute; NHLBI, National Heart, Lung, and Blood Institute.

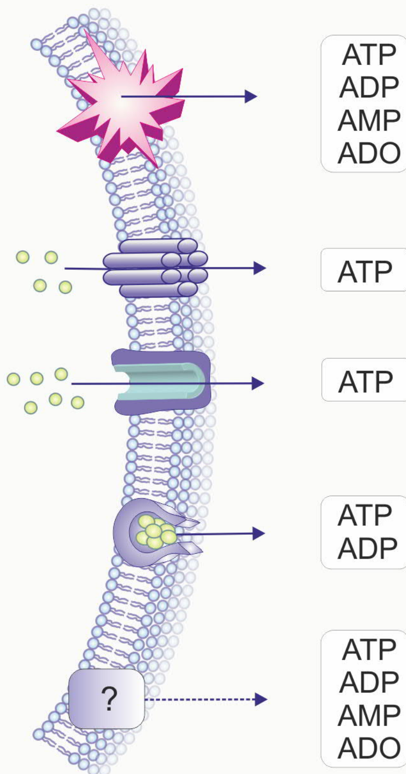




ATP inactivation via CD39-CD73 axis
Non-canonical purine-inactivating pathways
ADO
AMP
ADP
ATP
NT
CD73
CD39
ATP
AMP
PPi
2Pi
ADO
INO
ENPP1
TNAP
CD73
ADA
ADO
ATP
Intracellular ADO metabolism
CD73
ENPP1
CD38
NDPK
AK
CD73
ADO
AMP
ADPR
NAD+
ATP
NDP
NTP
ADP
ADP
ATP
AMP
ADO
NAD+ metabolism via CD38-ENPP1-CD73 axis
Reverse ATP-regenerating pathway

Cell damage during organ injury, traumatic shock, ischemia, or inflammation

Conductive ATP release via Cx hemichannels, PANX1, Piezo1, VRAC, CALHM1, and maxi anion channels

Cargo-vesicle trafficking, exocytotic granule secretion, SLC17A9/VNUT

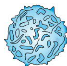
Basal release of ATP and other purinergic agonists



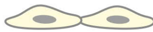
Cellular ATP pools (mol/L)	iATP (~10^{-3})	"ATP halo" (10^{-6}-10^{-4})	eATP (~10^{-8})

Platelets: Prothrombotic effects (ADP); inhibition of platelet activation (ADO)

Red blood cells: sensing and controlling blood flow via ATP release

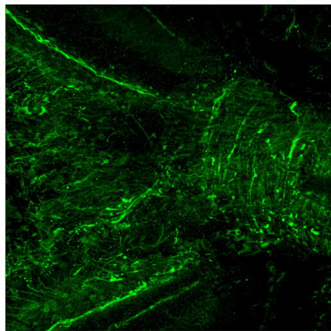

T-cells/B-cells: “Danger signal” (ATP); anti-inflammatory effects (ADO)

Monocytes/Macrophages: “Find me” signal (ATP), secretion of TNFα and other cytokines (ATP, ADO)

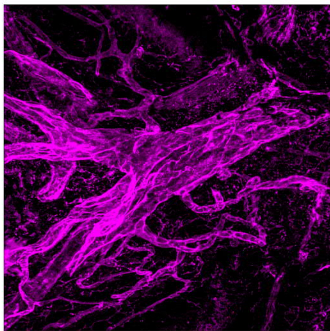

EC: vasculogenesis and angiogenesis (ATP, ADP); vasodilation (ATP, ADO); maintenance of barrier function (ADO)

Perivascular cells: mitogenic and vasoconstrictive effects (ATP)

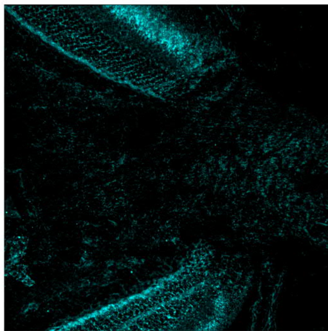
Adventitial fibroblasts: vascular remodeling, secretion of cytokines (ATP)



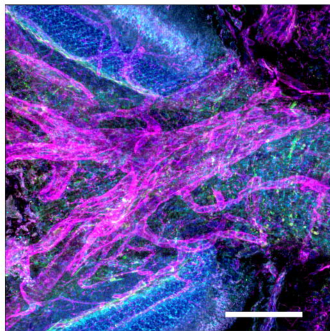
Vimentin (green)



CD39 (magenta)



CD73 (cyan)



Merge +DAPI (blue)

A

Mean diastolic velocity (m/s)

0.9
0.6
0.3
0

Baseline

ADO (40)

ATP (40)

ATPγS (40)

0 150 300 0 150 300

Time (ms)

Treatment (μg/ml)

Baseline
ADO (40) *
ADO (40) *
ATP (40) *
ATP (400) *
UTP (400) *
ATPγS (40) *
Suramin (4)
ATP (40)

0.3 0.6 0.9

Flow velocity (m/s)

B

H&E

L

EC

SMC

Ad

ADPase

AMPase

EC

Blank