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NTPDase and 5'-nucleotidase activities in physiological and disease conditions: New perspectives for human health

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Abstract. Extracellular nucleotides and nucleosides act as signaling molecules involved in a wide spectrum of biological effects. Their levels are controlled by a complex cell surface-located group of enzymes called ectonucleotidases. There are four major families of ectonucleotidases, nucleoside triphosphate diphosphohydrolases (NTPDases/CD39), ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs), alkaline phosphatases and ecto-5'-nucleotidase. In the last few years, substantial progress has been made toward the molecular identification of members of the ectonucleotidase families and their enzyme structures and functions. In this review, there is an emphasis on the involvement of NTPDase and 5'-nucleotidase activities in disease processes in several tissues and cell types. Brief background information is given about the general characteristics of these enzymes, followed by a discussion of their roles in thromboregulatory events in diabetes, hypertension, hypercholesterolemia and cancer, as well as in pathological conditions where platelets are less responsive, such as in chronic renal failure. In addition, immunomodulation and cell-cell interactions involving these enzymes are considered, as well as ATP and ADP hydrolysis under different clinical conditions related with alterations in the immune system, such as acute lymphoblastic leukemia (ALL), B-chronic lymphocytic leukemia (B-CLL) and infections associated with human immunodeficiency virus (HIV). Finally, changes in ATP, ADP and AMP hydrolysis induced by inborn errors of metabolism, seizures and epilepsy are discussed in order to highlight the importance of these enzymes in the control of neuronal activity in pathological conditions. Despite advances made toward understanding the molecular structure of ectonucleotidases, much more investigation will be necessary to entirely grasp their role in physiological and pathological conditions.

Keywords: NTPDases, ectonucleotidases, extracellular ATP, adenosine, purinergic system

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1. Introduction

Nucleotides and nucleosides are important biological molecules involved in many biological processes [3,107,145]. Nucleosides that participate in biological processes are formed by a sugar and heterocyclic nucleobase. Nucleotides, which are phosphate esters of nucleosides, occur as monomers and as building units of polymeric nucleic acids and contain an additional phosphoester group. These molecules are involved in the transmission of genetic information, provide currency units for biological energy transfer and also produce essential intermediates for intracellular signaling processes. In 1929, Drury and Szent-Györgyi [40] confirmed the essential action of ATP and adenosine on the heart and coronary blood vessels. Since then, many biological functions for ATP have been demonstrated. For example, ATP present in the extracellular compartment is accountable for the regulation of a multiplicity of biological processes, such as neurotransmission, cardiac function, bone metabolism, liver glycogen metabolism and inflammation [46,62]. Extracellular tri and diphosphate nucleosides such as ATP and ADP, together with adenosine, are well-known for controlling the vascular response to endothelial injury. ADP is the most important promoter of platelet aggregation, whereas adenosine is a powerful inhibitor of this process. In addition, ATP is also important in chloride transport, vasodilation, airway epithelia, muscle contraction, cartilage disease and renal function [20,145].

ATP is stored in the synaptic vesicle together with other transmitters and, after nerve stimulation, this molecule is released into the synaptic cleft [131]. In addition, ATP is released from non-neuronal cells stimulated by a paracrine mechanism [60]. While intracellular concentrations of ATP are very elevated (3–10 mM), its extracellular concentrations are considerably lower. ATP is an unstable molecule that cannot cross biological membranes by diffusion or active transport. Its breakdown is carried out by specific enzymes located on the outer surface of cells, called ecto-enzymes. Since the identification of ATP in 1929, many enzymes capable of its hydrolysis has been identified [102]. The name ecto-ATPase was used for the first time by Engelhardt in 1957 in his studies on nucleated erythrocytes [49]. In the same year, Wachstein and Meisel [138] demonstrated nucleoside phosphate activity in tissue samples by microscopy.

Ectonucleotidases are enzymes that hydrolyze extracellular nucleotides to their respective nucleosides. The molecular identification of enzymes that hydrolyze a variety of purine and pyrimidine nucleoside tri and diphosphates began eleven years ago when potato apyrase was cloned [58]. In the past decade, several ectonucleotidases have been discovered, cloned and characterized [107]. There are four major families of ectonucleotidases, namely, E-NTPDases (ATP → ADP → AMP), alkaline phosphatases (ATP → ADP → AMP → Adenosine), E-NPP-type ecto-nucleotide pyrophosphatase/phosphodiesterases (ATP → AMP), and Ecto-5'-nucleotidase (AMP → Adenosine) Fig. 1 [146–148]. In several tissues and cells, NTPDases operate jointly with other enzymes forming a "complex cell surface-located" nucleotide hydrolyses and conversion mechanism. The enzymes, adenylate kinase, nucleoside diphosphate kinase, and CD38/NADase make part of this machinery in addition to the enzymes cited above [107]. These specific ectonucleotidases are expressed by cells and control the ecto-nucleotide metabolism in their microenvironment. In this review, we will focus on two families of ectonucleotidases: the E-NTPDase family and the ecto-5'-nucleotidase family.

Many ecto-enzymes, such as NTPDase and 5'-nucleotidase, also have been given CD designations by immunologists and EC numbers assigned by enzymologists [110]. For example, NTPDase1 is CD39 (E.C. 3.6.1.5) and ecto-5'-nucleotidase is CD73 (E.C. 3.1.3.5). CD39's biochemical activity is the rate-limiting component of the ectoenzymatic chain that metabolizes extracellular nucleoside di- and triphosphate to its respective nucleoside, adenosine [30].

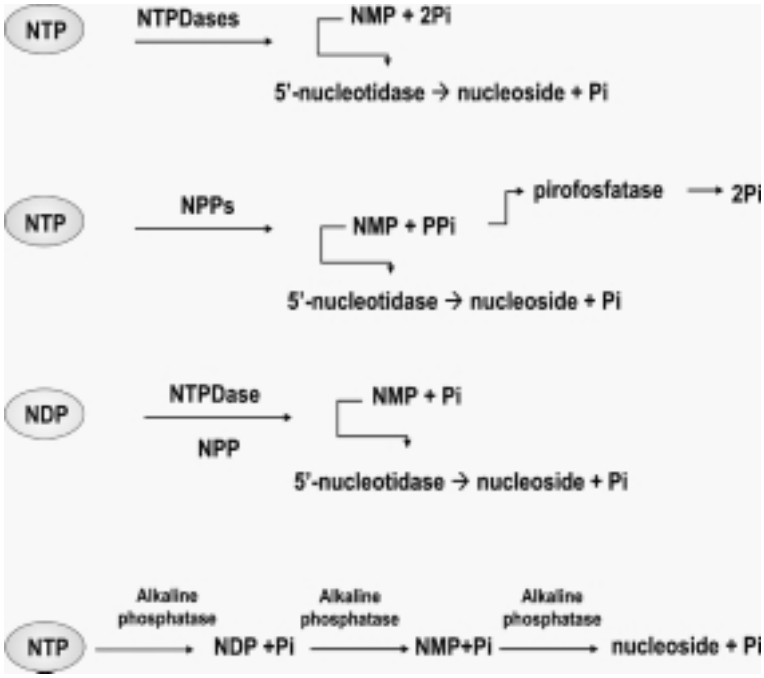

Fig. 1. Nucleotide hydrolysis promoted by different nucleotidase activities. NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; NTPDases, nucleoside triphosphate diphosphohydrolases; NPP, nucleotide pyrophosphatase phosphodiesterase; Pi, inorganic phosphate.

Extracellular nucleotides may be related with the development of several pathologies including disorders of the immune system and neurodegenerative and vascular diseases [13,107,117]. This review focuses on NTPDase activity in the vascular, immune and central nervous systems. We have attempted to emphasize disease conditions that can cause changes in enzyme activity, mainly in platelets, lymphocytes, blood serum and synaptosomes. In regard to platelets, we will discuss NTPDase and 5'-nucleotidase activities in diabetes, hypertension, pregnancy, chronic renal failure, breast cancer, prostate cancer, uterine cervix neoplasia, myocardial infarction, hypercholesterolemia, and acute stroke. In regard to lymphocytes, we will discuss NTPDase and 5'-nucleotidase in acute and chronic lymphocytic leukemia and AIDS. We will also mention the influence of epilepsy and seizures and the inborn errors of metabolism on these enzymes in synaptosomes and blood serum. Although we will discuss 5'-nucleotidase, the main focus of this review will be on NTPDase activity.

2. NTPDases: General characteristics

NTPDases (ecto-nucleoside triphosphate diphosphohydrolase) are a group of glycosylated extracellular enzymes that hydrolyze extracellular adenosine tri- and diphosphates to adenosine monophosphate.

NTPDases are activated by millimolar concentrations of either Ca^{2+} or Mg^{2+} and when these cations are absent these enzymes show no activity. NTPDases have an alkaline optimum pH. They are not inhibited by known inhibitors of various intracellular ATPases, such as P-, F- and V-type ATPases or alkaline phosphatase [102,144].

At least eight different members of the E-NTPDase family (NTPDases 1–8) have been discovered, cloned and studied over the last few years. Scientists present at the Second International Workshop on

Ecto-ATPases specified that all E-NTPDase family components should be termed as NTPDase proteins and ordered according to their discovery and characterization [107,146,147]. Each NTPDase member possesses different enzymatic properties and a separate location.

NTPDases 1, 2, 3 and 8 are cell surface-located enzymes with a catalytic site facing extracellularly, whereas NTPDases 4–7 demonstrate evidence of intracellular localization [136]. There is evidence that the difference in substrate preference among the members of the E-NTPDase family may result from small differences in protein structure that have an effect on substrate binding [147]. NTPDase1 hydrolyses ATP and ADP equally well (NTP $\approx$ NDP) while NTPDases3 and 8 prefer ATP over ADP (NTP $>$ NDP). NTPDase 2 shows great preference for hydrolyzing nucleoside triphosphates (NTP $>>>>$ NDP) and for this reason it was designated an ecto-ATPase years ago [74,147]. NTPDases4 and 5 prefer to hydrolyze nucleotides in this order: UDP $>$ GDP $>$ CDP, while NTPDase 6 has a preference for the order GDP $>$ IDP $>$ UDP and NTPDase7 hydrolyzes uridine, guanosine and cytosine tri-phosphates (UTP, GTP, CTP) [107,136].

NTPDases are involved in a series of physiological and disease processes in a multiplicity of tissues. Generally speaking, ectonucleotidases limit the effect of ATP on P2 receptors after being released from purinergic nerves. Another significant role of ectonucleotidases is their involvement in adenosine synthesis. Adenosine produced by ectonucleotidases either acts on the P1 receptor or may be taken up by cells and employed in the re-synthesis of nucleotides [16]. The distribution of ectonucleotidases seems to be as ubiquitous as that of nucleotide receptors. Purinoceptors are divided into P2 (P2X and P2Y subtypes) and P1 receptors. Type P1 is responsive to adenosine and type P2 responds to ATP, ADP, UTP, UDP and not to AMP [18]. Many studies have reported that responses mediated by P2X receptors are more efficiently evoked by stable analogues of ATP such as α, β-methylene ATP than by ATP alone [28, 64]. While ATP can activate both P2X and P2Y receptors, ADP interacts with the P2Y subtype receptor. Adenosine produced by 5'-nucleotidase can act on four different types of the P1 receptor (A_1, A_{2A}, A_{2B} and A_3). It is important to emphasize that uracil nucleotides are also substrates of NTPDases and agonists of P2 receptors.

3. 5'-Nucleotidase: General characteristics

5'-Nucleotidase, an enzyme that promotes the hydrolysis of adenosine 5'-monophosphate (AMP) to adenosine (5'-nucleotidases), was reported for the first time 70 years ago for its activity in heart and skeletal muscle [143]. This enzyme has been found in bacteria, vertebrate tissues and plants cells and it exhibits large dissimilarity in the variety of substrates hydrolyzed, as well as, in substrate specificity [126,143]. Its activity is not only found in the cytosolic membrane-bound form but also in the extracelullar membrane-bound form. While soluble 5'-nucleotidase controls intracellular levels of nucleoside 5'-monophosphate, the extracellular surface-located 5'-nucleotidase plays a part in the cascade that hydrolyzes ATP to adenosine [65]. Ecto 5'-nucleotidase (CD 73) is a glycosilphosphatidylinositol (GPI)-linked, which is found on the outside of a variety of cell types and hydrolyzes extracellular nucleoside monophosphates to nucleoside intermediates [71]. Previously, 5'-nucleotidase was shown to have a cosignalling role in T lymphocyte proliferation. Airas et al. [2] concluded that L-VAP-2 (lymphocyte-vascular adhesion protein 2 and CD73 are the same glycoprotein, thus proving that CD73 is involved in lymphocyte binding to the endothelium.

The central function of 5'-nucleotidase is the extracellular production of nucleoside [27]. Other ecto-ATPases together with this enzyme form the cascade to finish the action of nucleotides such as ATP and extracellular signaling molecules acting on P2X and P2Y receptors. 5'-nucleotidase is present

in basically all tissues and its primary structure has been determined in bovine and rat liver, electric ray brain, mouse kidney and human placenta, showing that the enzyme consists of 548 amino acids with a molecular mass varying between 62 and 74 kDa [90,105,128,135,144]. The protein occurs as a homodimer with interchain disulfide bridges and hydrolyzes a variety of nucleoside 5'-nucleoside monophosphates such as AMP, CMP, UMP, IMP and GMP. Normally AMP is the most successfully hydrolyzed nucleotide. The final product of 5'-nucleotidase is adenosine which acts on P1 receptors. Multiple biological processes, as for example, the control of cell proliferation, differentiation, apoptosis, neurotransmission and platelet aggregation, involve purinergic receptor signaling [53].

4. Role of NTPDase1 in thromboregulation

Thromboregulation is defined as a process or group of processes by which circulating blood cells and cells of the vessel wall interact to regulate or inhibit thrombus formation [82,84,85].

ADP is a major promoter of platelet aggregation, clearly establishing the physiological and pathological importance of this nucleotide in platelet function. Agents that modify or block the action of ADP on platelets are important for the treatment of thrombosis [5,29]. The presence of enzymes that hydrolyze ADP in the circulation is very important for limiting platelet aggregation and thrombus formation [99]. It is known that most NTPDase activity occurs in the endothelial cells. However, the importance of enzymes located on the platelet surface cannot be disregarded, as platelets are numerous and mobile. These characteristics give platelets some advantages in the transfer of information to and from different places in the organism during their lifespan. Platelets have a 7–9 day lifespan in the circulation and, obviously, during this time they can interact with each other and with other blood cells in their natural environment. In fact, it is well known that cell-cell interactions and interactions between blood cells and the vessel wall are critical components of the hemostatic and thrombotic processes [83]. In this context, ecto-proteins such as NTPDase1 are very important because they are continuously in contact with the extracellular environment. Additionally, the presence of receptors on the platelet surface to ATP and ADP reinforces the importance of platelet NTPDase (Fig. 2).

Studies have shown that CD39 inhibits platelet aggregation via three mechanisms: 1) by hydrolysis of ATP and/or ADP; 2) by participation in the enzymatic chain with 5'-nucleotidase to promote the formation of the anti-aggregatory metabolite adenosine [106] and 3) by blocking the binding of fibrinogen or the von Willebrand factor to platelets by inhibiting the activation of platelet glycoprotein (GP) IIB/IIIA adhesion receptors [80]. The first mechanism represents the blockade of platelet responsiveness to the prothrombotic agonist ADP, demonstrating the possibility of using CD39 as a potential antithrombotic agent [51,80,83,106].

Catalytic differences between NTPDase1 and NTPDase2 are associated with differences in their ability to regulate platelet function. Sévigny et al. [109] observed distinct localizations of these ecto-enzymes. They postulated that NTPDase1 may abrogate platelet aggregation and recruitment in intact vessels by converting ADP to adenosine monophosphates, while NTPDase2 expression most likely promote platelet microthrombus formation at sites of extravasation following vessel injury. In fact, Matsuno et al. [86] observed that $P2Y_{12}$ receptors played a significant role in the development of platelet microaggregation in patients with diabetes. They suggested that the measurement of microaggregation could be a useful marker to estimate thrombogenesis.

The CD39$-/-$ mice model has confirmed the importance of purinergic signaling in both hemostasis and thromboregulation. CD39 and purinergic mediators may act in concert to modulate blood fluidity and platelet activation. Enjyoji et al. [50], using knockout mice by targeting the ATG start site and

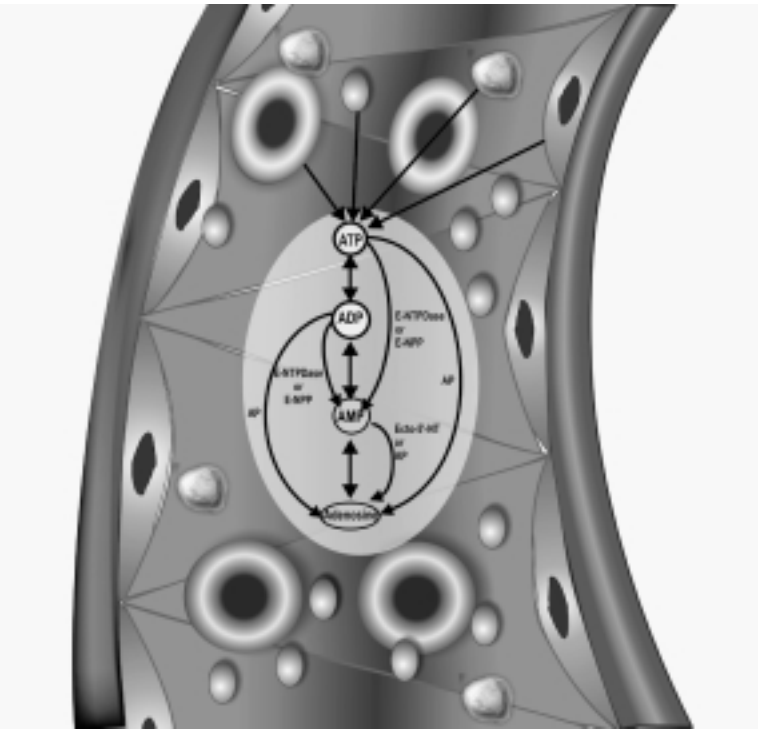

Fig. 2. Integration of nucleotide hydrolysis promoted by different cellular ectonucleotidase activities in the blood.

a portion of the 5'UTR (5' Untranslated Region), observed that CD39-deficient mice had prolonged bleeding times with minimally perturbed coagulation parameters. Platelet interactions with injured mesenteric vasculature were reduced *in vivo* and platelets failed to aggregate to agonists in vitro. This hypofunction in platelets was reversible and associated with type $P2Y_1$ receptor desensitization. In fact, the authors suggested that deficiency of vascular NTPDase activity was compensated, at least to some extent, by a reversible desensitization response from platelet receptors. However, in that study, treatment of CD39$-/-$ platelets with a soluble potato apyrase (solCD39) restored the aggregatory potential in platelets tested with ADP, collagen and thrombin [50].

In another study, Pinsky et al. [100] used the knockout strategy in the elimination of the enzymatically active extracellular domain (specifically ACR 2–4) of the CD39 gene and observed that baseline hematological and hemostatic parameters as well as bleeding time were normal, while a prothrombotic phenotype was observed after the induction of stroke. An increase in cerebral infarct volume and a reduction in postischemic perfusion were observed in the CD39$-/-$ mice.

However, in both experiments, the use of solCD39 was beneficial and reversed the alteration observed in platelet function [50,100] as well as reducing cerebral infarct volume, even when administered 3h after the induction of stroke [100]. This fact emphasizes the role of CD39 in the thromboregulatory process. In 1994, Schetinger et al. [111] suggested that the modulation of NTPDase might be involved in molecular events that follow both cerebral ischemia and reperfusion. Later, they observed that preconditioning caused a delayed enhancement in NTPDase and 5'-nucleotidase activities that would conceivably lead to increased adenosine production and cytoprotection [112]. Recently, Grenz et al. [57] demonstrated the contribution of E-NTPDase 1 (CD39) to renal protection from ischemia-reperfusion injury. An induction of CD39 by ischemic preconditioning (IP) was observed. In CD39$-/-$ mice, they observed

that IP protection was abolished and adenosine production was attenuated. The authors suggested apyrase treatment as a novel pharmacological approach to renal diseases precipitated by limited oxygen availability.

There are several pathological conditions in which platelets do not react normally. We studied NTPDase and 5'-nucleotidase activities in human pathological conditions where platelets are more reactive, such as diabetes, hypertension, hypercholesterolemia and cancer, as well as in pathological conditions where platelets are less responsive, such as in chronic renal failure. The results confirmed that the diabetic, hypertensive and diabetic/hypertensive subjects presented diminished platelet aggregation and bleeding and coagulation times [79]. However, they presented increased ATP and ADP hydrolysis by NTPDase. There was also elevated AMP hydrolysis only in diabetic subjects. We concluded that these findings were related to a compensatory organic response. The organism could be avoiding coagulation processes by increasing ADP depletion and increasing adenosine production. Recently, we measured the CD39 expression in these groups and found that it was increased by around 25% in diabetic, hypertensive and diabetic/hypertensive subjects (Lunkes et al., unpublished data). Additionally, using an experimental model of type 1 diabetes induced by alloxan in rats, we observed that NTPDase and 5'-nucleotidase activities were increased in platelets [78,89] and synaptosomes [78]. Taken together, these results may indicate that, in diabetic rats, both NTPDase and 5'-nucleotidase from the central nervous system (CNS) and platelets respond similarly with increased activity [78].

In another study, Leal et al. [75] investigated NTPDase and 5'-nucleotidase activities in pregnancies with complications (hypertension and gestational diabetes mellitus) and without complications. An increase was observed in NTPDase and 5'-nucleotidase in all pregnant groups. As a consequence, enhanced ATP, ADP and AMP hydrolysis was ascribed to the pregnancy itself, regardless of a normal or high risk for thrombosis. The enhanced NTPDase and 5'-nucleotidase activities in platelets suggest that these enzymes are involved in the thromboregulation process during pregnancy.

In all the situations cited in this section, there is high platelet responsiveness. In contrast, in chronic renal failure (CRF) a delayed bleeding time is observed. We measured NTPDase and 5'-nucleotidase activities in platelets from patients both undergoing hemodialysis (HD) treatment and not undergoing hemodialysis (ND) [118]. The results showed an increase in platelet NTPDase activity with ATP as substrate in CRF patients on HD treatment, but a decrease in ADP hydrolysis in both HD and ND patients. In addition, 5'- nucleotidase activity was elevated in the HD and ND groups. A significant correlation was found between ATP, ADP and AMP hydrolysis and plasma creatinine and urea levels. Our results suggest the existence of alterations in nucleotide hydrolysis in platelets of CRF patients. Possibly, this altered nucleotide hydrolysis could contribute to abnormalities in hemostasis found in CRF.

5. Ectonucleotidase activity in atherosclerosis and myocardial infarction

Atherosclerosis is the main cause of ischemic stroke and cardiovascular disease and is now considered to be an inflammatory disease [59]. NTPDase exerts a cardioprotective action by reducing ATP-mediated NE (norepinephrine) release [115]. In this context, solCD39 may exert cardioprotection [82,84,85]. In fact, recently it was demonstrated by Köhler et al. [67] that CD39 (E-NTPDase1) provides myocardial protection during cardiac ischemia/reperfusion injury. The authors suggest the use of apyrase in the treatment of myocardial ischemia.

Platelets are an important source of ATP since they store near molar amounts of this nucleotide inside their dense granules. Di Virgilio [36] cited that although nucleotide release from platelets is generally viewed in the context of clot formation, we should not forget that platelets are also one of the most

important sources of inflammatory mediators. Thus, ATP release from activated platelets is certainly proof of ATP release during inflammation, which is important in typical inflammatory lesions where platelets play a key role, such as in atheromatous plaque. Furthermore, there is evidence that ATP is released from endothelial cells and sympathetic nerves as well as from damaged cells in atherosclerosis, hypertension, restenosis, cancer, and ischemia. Apparently, in these pathological conditions, vascular smooth muscle and endothelial cells proliferate (neovascularization) [19,54,116]. Normal serum ATP levels are in the low micromolar range, but transiently they can reach high values [54].

In this context, we studied ATP and ADP hydrolysis in platelets from patients with high cholesterol levels. A possible association between cholesterol levels and inflammatory markers, such as oxidized low density lipoprotein (OxLDL), highly sensitive C-reactive protein (hsCRP) and OxLDL autoantibodies was also investigated. Hypercholesterolemia was associated with enhanced inflammatory response, oxidative stress and ATP and ADP hydrolysis. The increased ATP and ADP hydrolysis was confirmed by an increase in CD39 expression, which is possibly related to a compensatory response to the inflammatory and pro-oxidative state associated with hypercholesterolemia [41].

While studying circulating lymphocytes as prototypical for NTPDase activities from endothelial cells in patients with coronary artery disease (CAD), El-Omar et al. [48] observed a decreased ratio of ADPase to ATPase activities. They suggested that this altered ADPase/ATPase activity ratio in patients may represent a reduction in the endogenous defense system against platelet-driven thrombotic events. These data may pinpoint a population of patients with excessive platelet reactivity in their circulation. NTPDase was also measured in platelets from patients with acute and chronic heart failure and no changes in ATP and ADP hydrolysis were observed [108].

6. Ectonucleotidase activity in cancer

Tumorogenesis may be promoted by several changes in cellular components, including proteins exposed on the cell surface, such as ecto-enzymes. NTPDase and 5'-nucleotidase activities were analyzed in platelets from breast cancer patients. Initially, patients were compared in terms of length (in years) of tamoxifen use. Results demonstrated that ATP hydrolysis was enhanced and ADP hydrolysis was reduced as a function of tamoxifen use, while AMP hydrolysis was unchanged [4]. The expression of ecto-5'-nucleotidase is negatively regulated by the estrogen receptor (ER) in breast cancer cells; as ER expression decreases, ecto-5'-nucleotidase increases, leading to increased generation of adenosine, which may be related to breast cancer progression [123].

It is known that adenosine accumulates in solid tumors at high concentrations, and has been shown to stimulate tumor growth and angiogenesis and to inhibit cytokine synthesis, adhesion of immune cells to the endothelial wall, and the function of T-cells, macrophages, and natural killer cells [122].

In fact, Dzhandzhugazyan et al. [45] observed an association between the overexpression of CD39 in differentiated human melanomas and the escape of tumor cells from immunological effector mechanisms at early stages of tumor progression, showing the importance of the purinergic system in tumor differentiation. Recently, it was demonstrated by Buffon et al. [17] that the presence of NTPDase and 5'-nucleotidase enzymes in Walker 256 tumor cells may be important for regulation of the extracellular adenine nucleotide/adenine nucleoside ratio, therefore leading to tumor growth. Using quantitative real-time PCR, Entpd1 (CD39), Entpd2 (CD39L1) and CD73 were identified as the dominant genes expressed by the Walker 256 tumor.

Acute lymphoblastic leukemia (ALL) is a disease characterized both by uncontrolled proliferation and by maturation arrest of lymphoid progenitor cells in the bone marrow, resulting in an excess of malignant

cells with possible infiltration in the blood, central nervous system (CNS) and other organs [101,134]. ALL is more prevalent, and has a better prognosis, in children (5–9 years old) and accounts for more than 50% of the hematopoietic malignances in childhood [39]. In general, ALL standard treatment protocols consist of induction and maintenance remission with chemotherapeutic drugs. We studied ALL subjects divided into four groups: remission induction (RI), remission maintenance (RM) and out-of-treatment (OT), and a control group of 33 healthy subjects. ATP and ADP hydrolysis were reduced in the RI and RM groups, but in the OT group ATP hydrolysis was enhanced and ADP remained normal (Morsch et al., unpublished data).

B-chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of monoclonal mature B-lymphocytes in peripheral blood, bone marrow and lymphoid tissues and is associated with immunological disorders [21,72]. The variability in the prognosis, as well as the selection of different treatment options, depends on individual risk. The most common methods to determine the progress of the disease are the two clinical staging systems proposed by Binet and Rai, which define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stages [117]. We observed that ATP and ADP hydrolysis were increased in all stages, with the highest levels in the advanced group (Zanin et al., unpublished data).

The altered activity in lymphocytes from patients with ALL and B-CLL gives evidence that NTPDase may play an important role in the modulation of the disease, via ATP and ADP hydrolysis. Currently, we are studying the probable mechanisms for such alterations.

7. Immunomodulation and cell-cell interactions

Purinergic signaling seems to be even more complex in inflammatory conditions during which signaling is subject not only to changing levels of extracellular nucleotides/nucleosides, but also to additional modulating factors, such as dynamic changes in the expression of purinergic receptors and ecto-enzymes in response to various immunomediators that are synthetized during the course of inflammatory and immune responses (Fig. 1) [13,68]. Adenosine acts as an immunosuppressive agent, which may have relevance for both cell-mediated and inflammatory immune responses [122]. In addition to activated platelets, which release a physiologically significant amount of ATP, many other cell types may also actively release ATP [122]. There may be a high concentration of ATP at the site of inflammation as a consequence of its active or passive release from sympathetic neural terminals, mast cells, lymphocytes, macrophages, the endothelium and necrotic cells [37,142]. There is evidence that released ATP exerts a proinflammatory role and induces cytokine release by acting at P2X7 receptors [37]. In contrast, ATP was shown to possess anti-inflammatory properties in a model of lipopolysaccharide (LPS)-phyrohemagglutinin (PHA)-stimulated and in radiation-induced inflammation in human blood, by acting on P2Y11 receptors, inhibiting TNF-α release and increasing IL10 release [129,130]. Recently, Vuaden et al. [137] demonstrated that lipopolysaccharides alter nucleotidase activities from lymphocytes and serum of rats. The authors suggested that the changes in enzyme activities act in the regulation of extracellular nucleosides and nucleotides in a model able to trigger inflammatory process [137].

There are molecules whose role is to alert the body of an impending danger and to initiate and shape the subsequent immune response. Nucleotides are perfectly suited for this task as they are easily released upon damage of the cell membrane, rapidly diffuse in the extracellular environment and ligate specific plasma membrane receptors expressed by dendritic cells (DCs) and other mononuclear phagocytes [122]. For these reasons, nucleotides can be considered "danger signals" (an excellent designation for the term danger signal is given by Di Virgilio) [122]. Extracellular Ado appears to be an

important immunosuppressive and tissue-healing factor. Like ATP, Ado can also be considered a danger molecule because its extracellular levels rise markedly in response to tissue damage [13,66,122].

Normally, purinergic signaling may preferentially trigger and mediate short-term (acute) processes that affect cellular metabolism, adhesion, activation or migration [44]. However, purinergic signaling may also have a profound impact on other more protracted reactions observed in several chronic inflammatory states, such as cell proliferation, differentiation, and apoptosis [13,20,37,123].

Conceptually, the idea of ectoenzymatic control of leukocyte trafficking allows new insights into adhesive events and offers multiple novel targets for manipulating the movement of immune cells [110]. ATP-generation and ATP-consuming pathways coexist on the surface of leukocytes and endothelial cells and their dynamic balance regulates local ATP and adenosine levels in this microenvironment [110].

CD39 activity and expression are present to varying degrees on all leukocyte types. Pulte et al. [104] observed that CD39 is expressed on neutrophils, lymphocytes and monocytes. The enzyme was found in $>$ 90% monocytes, neutrophils and B-lymphocytes and in 6% of T-lymphocytes and natural killer cells. The authors suggested that differences between leukocyte types should be considered when examining CD39 in a disease state. Koziak et al. [73] observed a correlation between the levels of CD39 expression and NTPDase activity in human NK cells. Furthermore, E-NTPDases may also exert extra-enzymatic functions in that CD39 has been proposed to be a cell-surface signaling molecule playing a role in the regulation of effector functions of activated lymphocytes [38].

The classical activation of monocytes/macrophages (Mo/Mø) during early inflammatory events may involve purinergic signaling by extracellular ATP, while Ado mediated purinergic signaling might be involved in the switch to an alternative activated (Mo/Mø) phenotype, which is a prerequisite for the resolution of inflammation and tissue healing. Thus, it seems that ATP and Ado are endogenous signaling molecules that may regulate (Mo/Mø) function and may contribute to phenotype switching of macrophages during inflammatory and immune responses [13].

DCs from CD39 knockout mice appear to have an impaired T cell-stimulatory function, and it was hypothesized that the apparent role of CD39 in ATP-mediated signaling in murine DCs might be two-fold [56]. Firstly, CD39 could prevent desensitization of certain P2 receptors by hydrolyzing ATP and, secondly, simultaneously prevent over activation of other P2 receptors by lowering ATP levels.

In addition, in another study Mizumoto et al. [91] observed that exposure to ATP concentrations of up to 10 mM in CD39$-/-$ mice caused no significant death of CD39$-/-$ dendritic cells (DCs), whereas 2.5 mM ATP was sufficient to kill wild-type DCs. The pretreatment of CD39$-/-$ DCs with soluble apyrase restored ATP responsiveness, demonstrating P2-receptor desensitization in CD39$-/-$ DCs.

Furthermore, CD39-null mice spontaneously developed autoimmune alopecia. Fifteen percent developed skin lesions characterized by extensive and well-delineated hair loss. The skin lesions were characterized by the presence of a population of CD4+ and CD8+ lymphocytes within the damaged hair follicles [44].

Deaglio et al. [30] reported the identification of CD39 together with CD73 as novel cell surface markers of CD4+ Treg cells. The authors suggested that CD39 and CD73 are surface markers of T reg cells, which impart a specific biochemical signature characterized by adenosine generation, which has functional relevance for cellular immunoregulation.

Over the last several years, we have studied ATP and ADP hydrolysis in different clinical conditions related with alterations in the immune system, such as in human immunodeficiency virus (HIV) infection. Human immunodeficiency virus (HIV) infection results in alterations in immune cells, such as the increase or decrease of cytokine secretion and immunodeficiency. HIV causes a state of chronic cellular activation that can induce apoptosis in lymphocyte T-helpers, making the patient susceptible to opportunistic

infections. We have carried out research on the biochemical mechanisms involved in this immune response to HIV. We observed that ATP and ADP hydrolysis are essential for the immune response to HIV [76]. Our results clearly indicated an increase of NTPDase1 (EC 3.6.1.5) activity in lymphocytes of HIV-positive patients, confirmed by an enhanced CD39 expression on its surface. These results suggest that NTPDase 1 may play an important role in maintaining an adequate balance between the generation and consumption of ATP and preserving cellular integrity and the immune response to the HIV infection [76]. Recently, Barat et al. [7] demonstrated that NTPDase1/CD39 is incorporated into human immunodeficiency type 1 particles, where it remains biologically active. They observed that both laboratory-adapted and clinical isolates of HIV-1 can incorporate an active NTPDase1/CD39 in their envelope. Such CD39-bearing viral particles could act as circulating enzymes, affecting several physiological processes by influencing local levels of ATP, ADP and adenosine [7].

Kas-Deelen et al. [70] reported that cytomegalovirus infection induced increased expression and activity of ecto-NTPDase (CD39) and ecto-5'-nucleotidase (CD73) in endothelial cells. In this context, the production of adenosine is probably enhanced. Besides its effect as an anti-aggregant, adenosine has a modulatory role in the inflammatory response.

Recently, a study using CD39-null mice demonstrated the importance of CD39 and purinergic signaling, potentially acting by P2X7, impacting fibrosis and playing an important role in modulating extracellular matrix remodeling in inflammatory diseases of the pancreas [69].

8. Neutrotransmission and neuronal activity

8.1. Inborn errors of metabolism and ectonucleotidases

Inborn errors of metabolism (IEM) encompass a large number of genetic diseases involving disorders of the metabolism. The term IEM was coined by the British physician, Archibald Garrod, in the early 20th century. He is known for the "one gene, one enzyme" hypothesis, which arose from his studies on the nature and inheritance of alkaptonuria. The majority of IEM are due to defects of single genes which code for enzymes that facilitate the conversion of substrates into others products. In most of the disorders, problems arise due to accumulation of substances which are toxic or interfere with normal function, or due to reduced ability to synthesize essential compounds. Individually, these diseases are rare, but collectively they are common [24]. Presentation can occur at any time, even in adulthood [114].

We have studied the effect of various substances known to accumulate in IEM on ectonucleotidases in brain and serum of rats. Wyse et al. [140] showed that phenylalanine, which is increased in tissue of patients with phenylketonuria (PKU), altered ATP and ADP hydrolysis. Phenylalanine inhibited NTPDase at 2.0 mM, whereas activated it at 5.0 mM. Phenylpyruvate, a phenylalanine metabolite, showed the opposite effect, suggesting that these substances bind to two different sites on the enzyme. This study also showed that rats subjected to chemically hyperphenylalaninemia induced by alpha-metylphenylalanine increased ATP diphosphohydrolase activity in synaptosomes from rat cerebral cortex. In addition, it was shown that rats subjected to acute or chronic hyperphenylalaninemia-induced by p-chrophenylalanine and decapited 1 week after the last injection displayed a reduction of NTPDase activity in synaptosomes from the cerebral cortex [139]. Another study demonstrated that phenylalanine and phenylpyruvate *in vitro* inhibited NTPDase activity acting at the same binding site [8]. Tissue accumulation of arginine, N-acetylarginine, argininic acid and homoarginine occurs in hypergininemia, an inborn error of the urea cycle. Balz and colleagues [6] showed that these guanidine compounds (1.5 to 3.0 mM) enhanced NTPDase 1 activity, but not 5'-nucleotidase, in synaptosomes from rat cerebral cortex.

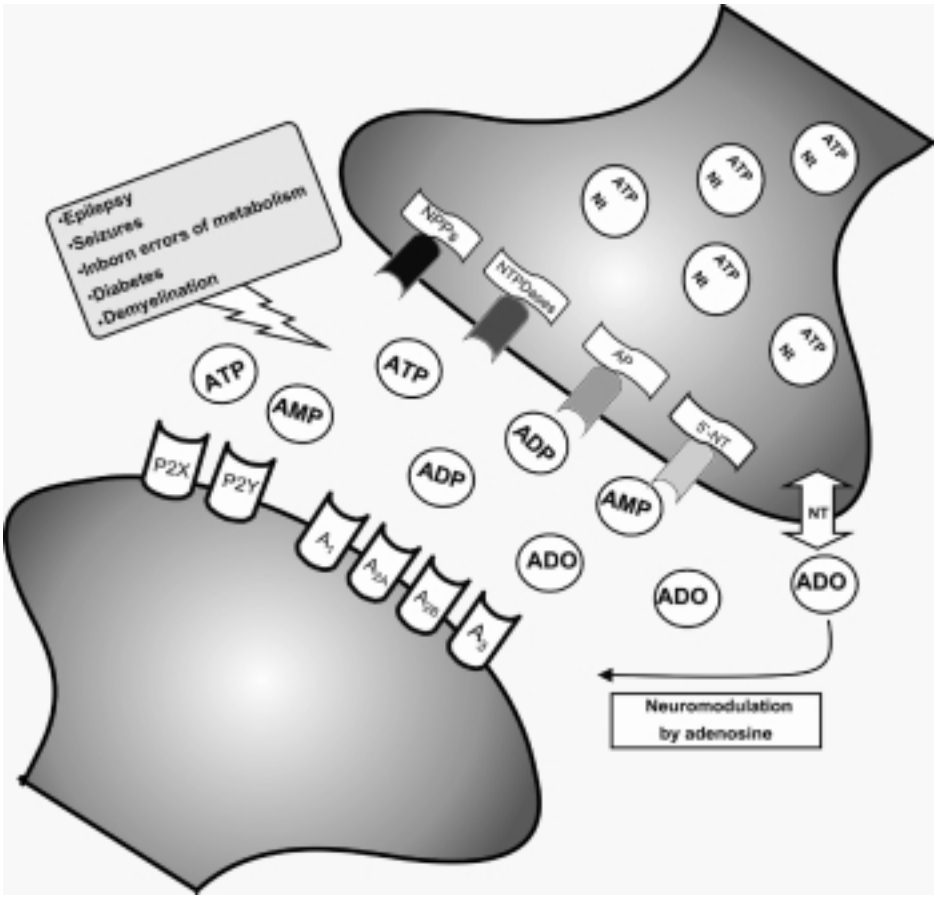

Fig. 3. Pathological conditions alter nucleotide and nucleoside levels in the synaptic cleft. Nt, neurotransmitter; NTPDases, nucleoside triphosphate diphosphohydrolases; NPP, nucleotide pyrophosphatase phosphodiesterase; AP, alkaline phosphatase; 5'-NT, 5'-nucleotidase; NT, nucleoside transporter; ADO, adenosine.

In contrast, studies demonstrated that arginine in vitro decreased NTPDase and 5'-nucleotidase activities in rat hippocampus. Rats subjected to intracerebroventricular arginine administration showed a reduction in ATP, ADP and AMP hydrolysis in rat hippocampus and serum and L-NAME, an inhibitor of nitric oxide synthase, prevented such effects [31,34]. Proline, an amino acid accumulated in hyperprolinemia, did not alter nucleotide hydrolysis when added to enzyme assays, but when administered acutely or chronically, it decreased ATP hydrolysis in rat cerebral cortex synaptosomes; ADP and AMP hydrolysis were not altered by proline administration [33]. Chronic administration of proline did not alter nucleotide hydrolysis when the rats were killed 12 h after the last injection, but decreased ATP and ADP hydrolysis when they were sacrificed 3 h after the last injection. 1.0 mM Proline (*in vitro*) significantly increased ATP, ADP and AMP hydrolysis in rat serum [32]. The acute administration of homocysteine, an amino acid considered to be a risk factor for neurodegenerative and heart diseases, significantly increased ATP, ADP and AMP hydrolysis in young rat serum [9]. In conclusion, based on these results, it seems reasonable to postulate that these substances accumulated in different IEM alter the hydrolysis of nucleotides and could alter, at least in part, the responses mediated by adenine nucleotides in the central nervous and peripheral systems of patients affected by these disorders (Fig. 3). However, it is also possible that these responses are associated with some of the symptoms of these metabolic diseases.

8.2. Epilepsy, seizures and ectonucleotidases

Neurological disorders, such as epilepsy, represent yet another set of conditions in which the involvement of ectonucleotidases has been demonstrated. A significant decrease in ecto-ATPase activity in the posterior part of epileptic hippocampus was seen in cultured glia cells raised from neonatal, seizure-prone mice [93,132]. It is interesting to observe that the chromosomal position of human CD39/ecto-apyrase (10g23.1 to q24.1) [81] is collocated with the gene involved in partial human epilepsy with audiogenic symptoms (10q.22 to 24) [98]. The localization of these genes led to the hypothesis that CD39 is probably related to epilepsy. Moreover, studies have shown a reduction in ecto-ATPase activity in rat cerebral cortex during prolonged status epilepticus (SE) induced by the sequential administration of lithium and pilocarpine [94].

Patients with temporal lobe epilepsy have shown an increased distribution of 5'-nucleotidase in dentate gyrus and in mossy fiber endings in CA4 and CA3 areas, when compared with activity in normal human hippocampus [77]. The presence of 5'-nucleotidase was observed in mossy fibers of rat dentate gyrus after systemic kainate injection and induction of kindling, being less detected in normal hippocampus [113]. The activity of 5'-nucleotidase in cerebellum subcellular fractions was verified after the administration of the convulsant 3-mercaptopropionic acid. The membrane enzyme presented an increase in its activity in certain fractions containing nerve endings and microsomes during seizure and postseizure periods [52].

Several findings have shown that ectonucleotidases are altered after the induction of chronic animal models of epilepsy, such as pilocarpine, kainic acid and kindling models. ATP diphosphohydrolase and 5'-nucleotidase from rat hippocampus and cerebral cortex synaptosomes significantly increased at 48–52 h, 7–9 days and 45–50 days after induction of status epilepticus (SE) by pilocarpine or kainic acid models [11]. However, only 5'-nucleotidase activity remained elevated at 100–110 days after treatment with kainic acid [11]. In addition, an increase in ecto-5'-nucleotidase staining in the hippocampus during silent and chronic phases has also been shown [133]. These findings suggest that the increase of ectonucleotidase activities could be involved in modulation of seizure activity within a time window (48 h–110 days) after SE, contributing to the production of extracellular adenosine, a known endogenous neuromodulator [42]. If ATP is released in large amounts for a long time, it may promote a dramatic increase in intracellular calcium levels mediated by P2X receptors, which could represent significant damage, such as that induced by an excess of glutamate [47]. If all members of the ectonucleotidase pathway work at an elevated rate, an efficient removal of extracellular ATP and adenosine production could occur in this condition. Thus, adenosine could modulate the release of a variety of neurotransmitters, including glutamate, acetylcholine, noradrenaline and dopamine [35]. In summary, after SE, an important adaptive plasticity of the ectonucleotidase pathway could occur to decrease levels of ATP, an excitatory neurotransmitter, and to increase levels of adenosine, a neuroprotective compound [10].

In addition, rats showing greater resistance to pentylenetetrazol (PTZ) kindling presented an increase in ATP hydrolysis in hippocampus and cerebral cortex synaptosomes [10]. To examine whether the altered ATP hydrolysis was due to chronic, long-lasting changes induced by kindling or by the drug, we investigated enzyme activities after a single acute seizure induced by the convulsant PTZ. Changes in ectonucleotidase activities were not seen after a single injection of PTZ at any time (immediately, 1 h, 24 h and 5 days) [10]. Such alterations seem to be related to the chronic, long-lasting synaptic activity induced by kindling, because these changes were not seen in acute seizures, which are probably insufficient to activate these mechanisms. Taken together, these results support the hypothesis that changes in nucleotide hydrolysis may represent an important mechanism in the modulation of epileptogenesis [12].

There is evidence that conventional antiepileptic drugs (AEDs) have some influence on purinergic transmission in the CNS [63]. It has been shown that carbamazepine can inhibit the in vitro activity of ecto-ATPase in rat brain synaptosomal plasma membranes [25]. However, it has also been shown that carbamazepine treatment was not able to change ATP, ADP, and AMP hydrolysis in naive rat brain synaptosomes [26]. Phenytoin and carbamazepine were able to prevent an increase in ectonucleotidase activities elicited by pilocarpine in brain synaptosomes, but sodium valproate was only able to avert the increase of ATP and ADP hydrolysis in hippocampal synaptosomes. Pilocarpine did not affect the gene expression of NTPDase1, NTPDase2, NTPDase3, and ecto-5'-nucleotidase in the cerebral cortex, but led to increased levels in hippocampus. Therefore, anticonvulsant drugs may modulate plastic events related to the increase of nucleotidase expression and activities in pilocarpine-treated rats [25,26].

Although it is well established that the breakdown of ATP is mediated by membrane-bound ectonucleotidases, studies have indicated that soluble nucleotidases, probably released from sympathetic nerves, are also involved in ATP breakdown to adenosine [131,141]. Studies have shown that a single PTZ injection led to significantly increased ATP, ADP and AMP hydrolysis in rat blood serum (40–50%) for up to 24 h [15]. Changes in nucleotide hydrolysis observed after a single administration of PTZ could not be attributed to phosphodiesterase activity since PTZ-treated rats did not demonstrate significant differences in the hydrolysis of the substrate marker of this enzyme when compared with control rats [15]. Likewise, animals subjected to PTZ-kindling (30 mg/kg PTZ, i.p., once every 48 h, totaling 10 stimulations) demonstrated increased ATP, ADP and AMP hydrolysis (42, 40, and 45%, respectively), while phosphodiesterase activity was unchanged [14]. These results suggest once more that an increase in ectonucleotidase activities and, possibly, in adenosine levels, could represent an important compensatory mechanism in the development of chronic epilepsy. The fact that this increase can also be measured in serum could mean that these enzymes might be useful as plasma markers of seizures in epilepsy [14]. In addition, soluble nucleotidase activities from cerebrospinal fluid increased 10 min after pentylenetetrazol-induced seizures and returned to control levels after 240 min, whereas guanosine and inosine levels increased only after 30 min [96]. The S100B and neuron-specific enolase (NSE) levels were also altered after PTZ-induced seizures. Taken together, we can hypothesize that such events may modulate seizure expression, and are therefore promising biochemical brain markers to evaluate neural injury after acute seizures [96]. In addition, it has been shown that pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid [97]. A significant decrease (50%) in the expression of NTPDase1 was observed, but there were no changes in the transcript mRNA levels for the enzymes NTPDase2, 3, 5, 6 and 5'-nucleotidase in hippocampus from PTZ-kindled rats. Such alterations indicate that the modulatory role of purines in the CNS could be affected by PTZ-kindling [95].

Quinolinic acid, an endogenous convulsant compound, overstimulates the glutamatergic system by stimulating N-methyl-D-aspartate receptors, which enhances glutamate release and inhibits glutamate uptake. Glutamate releases adenosine, which is able to reduce glutamate release and depresses neuronal activity. The adenine nucleotide hydrolysis in hippocampal slices of adult rats was altered for up to 24 hours after seizures induced by QUIN [95]. Considering that slice preparations maintain tissue integrity, this study reinforces the idea that extracellular production of the neuroprotector adenosine may be involved in brain responses to seizures.

Studies have suggested that the immature brain is less vulnerable to morphologic and physiologic alterations after SE [61,121,124]. Indeed, there is a distinct sensitivity in developing rats to the pilocarpine model of temporal lobe epilepsy when compared to that of adult rats [23]. A previous study demonstrated that none of a group of 7- to 17-day-old rats subjected to pilocarpine-induced SE developed spontaneous

recurrent seizures in adulthood [103] and the susceptibility to spontaneous recurrent seizures in the chronic period of the pilocarpine model increased with age [103]. It has been shown that ATP and ADP hydrolysis in the hippocampus and cerebral cortex were not altered by pilocarpine treatment in female and male rats at 7–9, 14–16 and 27–30 days [26]. In addition, there were no changes in AMP hydrolysis in female and male rats subjected to this model at different ages, but a significant increase in AMP hydrolysis (71%) was observed in cerebral cortex synaptosomes from male rats at 27–30 days [26]. The different sensitivity of developing rats may be related to the immaturity of neuronal networks in the brain engaged in the generation and spread of seizure activity and these findings highlight differences between the purinergic system of young and adult rats submitted to the pilocarpine model of epilepsy. Therefore, the regulation of the nucleotidase pathway may play a modulatory role during the evolution of behavioral and pathophysiological changes related to temporal lobe epilepsy.

8.3. Ectonucleotidases and demyelination

Multiple sclerosis (MS) is a major demyelinating disease with an unknown etiology affecting the white matter of the central nervous system [125]. Although the pathological hallmark of this disease is primarily demyelination, other pathological events have been noted, such as changes in vascular permeability, which are considered crucial since they precede the development of MS lesions [127].

It is known that ATP and adenosine play a significant role in the pathophysiology of numerous acute and chronic disorders, including events related with CNS demyelination and remyelination [1]. Moreover, studies have reported that platelets are involved in thrombi development in the demyelinating plaques, suggesting that these cells may play a role in the demyelination of white matter [22]. Furthermore, platelets have been recognized for decades as key pathological components of the processes associated with vascular inflammation and thrombosis [5]. Our group studied NTPDase and 5'-nucleotidase activities in synaptosomes and platelets of rats submitted to demyelination by ethidium bromide (EB). We observed that EB selectively destroyed glial cells (oligodendrocytes and astrocytes), which control the processes of demyelination and remyelination [55,87,88]. The results demonstrated that NTPDase and 5'nucleotidase activities were increased in synaptosomes [120] and reduced in platelets [87,119, 120], indicating that there is an interaction between these enzymes and demyelinating events.

9. Concluding remarks

In diseases such as carcinogenesis, degenerative disorders, and ischemia/reperfusion injury, there is an imbalance between cell division and cell death. There is growing interest in long-term trophic actions of extracellular nucleotides and nucleosides in vascular cell proliferation and death [19]. Recent advances have enabled allowed researchers to define the physiological and pathophysiological implications of NTPDases in a considerable variety of tissues [5,82,107].

Enzymes whose catalytic site faces the extracellular space offer a great therapeutic opportunity (Figs 2 and 3) [38]. In this context, ectoenzymes are attractive candidates for designing new approaches to interfere with pathological events, such as leukocyte trafficking [110]. Marcus et al. [83] suggested that the use of aspirin with solCD39 could be a future therapeutic regimen for the prevention and treatment of stroke, coronary artery and peripheral vascular disease.

The dual nature of ectoenzymes also needs to be rigorously tested, because some of their functions seem to perform independently of their enzymatic activity [106,110]. It is feasible that the large extracellular domains of ectoenzymes and their association with other membrane proteins or components can mediate

responses without involvement of their catalytic activity [110]. In fact, this has been well observed for enzymes such as cholinesterases and 5'-nucleotidase, for example. In addition, the physical and functional association of ecto-nucleotidases with partners specialized in signal transduction is particularly relevant [30].

Furthermore, the failure to develop specific inhibitors remains a major impediment to ongoing new discoveries on NTPDase activity [106]. Recently, it was demonstrated that P2 receptor antagonists inhibited human and plasma membrane bound NTPDases. The inhibitors, reactive blue 2, suramin, NF279, NF449 and MRS2179, were tested on recombinant human and mouse NTPDases 1, 2, 3 and 8 [92].

Marcus et al. [82] suggested that CD39 represented the end results of an evolutionary process directed toward the metabolism of pro-thrombotic platelet-derived nucleotides by surface molecules on the endothelial cell. This serves as an elegant heterologous control system for excessive platelet accumulation and also for the maintenance of blood fluidity. From an evolutionary point of view, we can observe that this condition is also important in other animals, such as blood-feeding insects that need to avoid the development of both platelet aggregation and immune responses by the host, and that release soluble NTPDase to achieve this. In fact, Dwyer [43], discussing the salutary roles of CD39 in transplantation, said that inflammatory and coagulation systems are intimately associated and extracellular nucleotides and nucleoside modulates a variety of cellular processes that are implicated in vascular inflammation and thrombosis (Fig. 2).

We hope this review has provided new insights regarding NTPDase and 5'-nucleotidase activities mainly in coagulation and inflammation conditions. Undeniably, these enzyme activities are extremely important for maintaining physiological levels of ATP, ADP and adenosine in almost all tissues and cells. However, their activities during the course of some pathological conditions do not correspond exactly to the expected profile. Thus, further research will be necessary to entirely comprehend their functions operating coordinately.

Dedication

This work is dedicated to our dear professor, João José Freitas Sarkis, who taught us the importance of advancing research associated with ectonucleotidases. Professor Dr. Sarkis was an ectonucleotidase "enthusiast" and this has motivated us to write something in this subject of research and dedicate it to his memory.

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