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REVIEW

P1 receptors and cytokine secretion

Maria P. Abbracchio · Stefania Ceruti

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Abstract Evidence has accumulated in the last three decades to suggest tissue protection and regeneration by adenosine in multiple different cell types. Adenosine produced in hypoxic or inflamed environments reduces tissue injury and promotes repair by receptor-mediated mechanisms. Among other actions, regulation of cytokine production and secretion by immune cells, astrocytes and microglia (the brain immunocytes) has emerged as a main mechanism at the basis of adenosine effects in diseases characterized by a marked inflammatory component. Many recent studies have highlighted that signalling through A_1 and A_{2A} adenosine receptors can powerfully prevent the release of pro-inflammatory cytokines, thus inhibiting inflammation and reperfusion injury. However, the activation of adenosine receptors is not invariably protective of tissues, as signalling through the A_{2B} adenosine receptor has been linked to pro-inflammatory actions which are, at least in part, mediated by increased release of pro-inflammatory cytokines from epithelial cells, astrocytes and fibroblasts. Here, we discuss the multiple actions of P1 receptors on cytokine secretion, by analyzing, in particular, the role of the various adenosine receptor subtypes, the complex reciprocal interplay between the adenosine and the cytokine systems, their pathophysiological significance and the potential of adenosine receptor ligands as new anti-inflammatory agents.

Key words adenosine · asthma · central nervous system · chronic heart failure · cytokines · immune cells · inflammation

M. P. Abbracchio (✉) · S. Ceruti
Laboratory of Molecular and Cellular Pharmacology of Purinergic Transmission, Department of Pharmacological Sciences, University of Milan, Via Balzaretti, 9, 20133 Milan, Italy
e-mail: mariapia.abbracchio@unimi.it

Abbreviations

ACTH	adrenocorticotropic hormone
ADA	adenosine deaminase
CGS15493	9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c] quinazolin-5-amine
CHF	chronic heart failure
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
GH	growth hormone
IFNγ	interferon gamma
IL	interleukin
$Ins(1,4,5)P_3$	inositol(1,4,5)-trisphosphate
IPDX	3-isobutyl-8-pyrrolidinoxanthine
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
NOS	nitric oxide synthase
PBMC	peripheral blood mononuclear cells
PG	prostaglandin
PRL	prolactin
SCH58261	5-amino-7-(2-phenylethyl)-2-(2-furyl) pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine
Th1/2	type 1/2 helper lymphocytes
TNFα	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor
ZM241385	4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo (2,3-a)(1,3,5)triazin-5-yl amino]ethyl)phenol

Introduction

Adenosine acts as a local mediator with generally cytoprotective functions in mammalian organisms. These actions are mediated by the activation of four distinct adenosine re-

ceptor subtypes—referred to as the A_1, A_{2A}, A_{2B} and A_3 receptors—which are all members of the G protein-coupled receptor (GPCR) superfamily and each of which has a unique tissue distribution, pharmacological profile and effector coupling (not reviewed here; for a recent seminal review, the reader is referred to Jacobson and Gao [1]).

As elegantly reviewed by Linden [2], adenosine promotes tissue protection and repair through four general modes of action: increased oxygen supply/demand ratio, preconditioning, anti-inflammatory effects and stimulation of angiogenesis. Thus, adenosine downregulation of inflammatory and immune responses in injured tissues plays a crucial role in the beneficial effects induced by this nucleoside. In recent years, the elucidation of the molecular mechanisms at the basis of these actions has revealed that they occur, at least in part, through modulation of the production and release of pro-inflammatory cytokines. Here, we review some of the most compelling evidence on the roles of the various adenosine receptor subtypes in cytokine secretion, with major focus on the direct effects of adenosine on cytokine production and release by immune cells, by astrocytes and by microglia, the brain immunocytes. Due to the obvious involvement of immune cells in inflammatory diseases of the respiratory and cardiovascular systems, we also discuss modulation of cytokine secretion by adenosine in asthma and chronic heart failure.

Adenosine modulation of cytokine secretion in immune cells: is adenosine the danger sensor that stops the immune response?

The first demonstrations of the immunosuppressive effects mediated by intracellular cyclic adenosine monophosphate (cAMP) and extracellular adenosine date back to the 1970s [3, 4]. Since then, detailed studies on the pharmacological effects of adenosine [5, 6] and on the involvement of specific adenosine receptor subtypes ([7, 8]; see also below) have expanded those original descriptions to cells of both the innate and adaptive immune systems, highlighting adenosine as a critical player in the physiological mechanisms that downregulate activated immune cells and protect tissues from inflammatory damage (for seminal and comprehensive reviews, see Sitkovsky et al. [9] and Sitkovsky and Ohta [10]).

It is known that immune cell-mediated destruction of pathogens may result in excessive collateral damage to normal tissue and that the failure to control activated immune cells and to downregulate acute inflammatory responses may cause immunopathologies and chronic inflammatory diseases. It is believed that regulation of the immune system requires at least two ‘danger’ signals (elegantly reviewed in Sitkovsky and Ohta [10]). The first danger signal indicates the presence of pathogens, injury or mutations resulting in the death or scavenging of cells (reviewed by Matzinger [11]). As a result of this danger signal, immune cells are activated to initiate immune responses: they kill pathogens by cytokines, reactive oxygen and nitrogen species and cellular cytotoxicity, and expand inflammation by attracting and activating many other effector cells through the release of pro-inflammatory cytokines and chemokines. However, uncontrolled expansion of inflammatory responses might cause tissue damage and loss of function: this is at the basis of the critical dichotomy of inflammation, which is often described as a ‘double-edged sword’ [12]. Inflammation and immune reactions are indeed believed to start as time- and site-specific defense mechanisms. Failure to resolve an acute beneficial response could later lead to a vicious and anarchic state of chronic activation, which causes healthy tissue damage. To avoid excessive collateral tissue damage, therefore, the tissue may release a second danger signal that can evoke anti-inflammatory responses. This second danger signal indicates the danger from overactive immune cells and would trigger the downregulation of the pro-inflammatory activities of the immune system to prevent destruction of healthy tissues. As underlined by Sitkovsky and Ohta [10], the final outcome will be determined by a balance between the first pro-inflammatory danger signal and the second anti-inflammatory signal. The mechanisms that are triggered by the second danger signal are related to the extent of change to tissue microenvironment and are timed with a high level of precision, so that immune cells keep the ability to destroy remaining pathogens but with much less damage to healthy cells. Sitkovsky and Ohta [10] observed that the least tolerated damage during an immune response is damage to the structures of the microcirculation, which would cause an interruption of the local blood supply. On this basis, they proposed local tissue hypoxia as the primary event indicating the need to stop overactive immune cells and adenosine as a key “OFF” signal mediating downregulation of immune cell activity. Even short periods of hypoxia are indeed known to strongly elevate adenosine levels as a result, on the one side, of rapid breakdown of ATP, and, on the other side, of hypoxia-induced inhibition of adenosine kinase, which, under normal conditions, rephosphorylates the nucleoside to AMP. Hypoxia, such as that associated with brain ischaemia, also results in upregulation of adenine nucleotide metabolizing ectoenzymes, which may lead to an even more enhanced production of adenosine. Interestingly, such an upregulation has been demonstrated to occur in vivo upon induction of middle cerebral artery occlusion in rodents as a result of brain ischaemia [13].

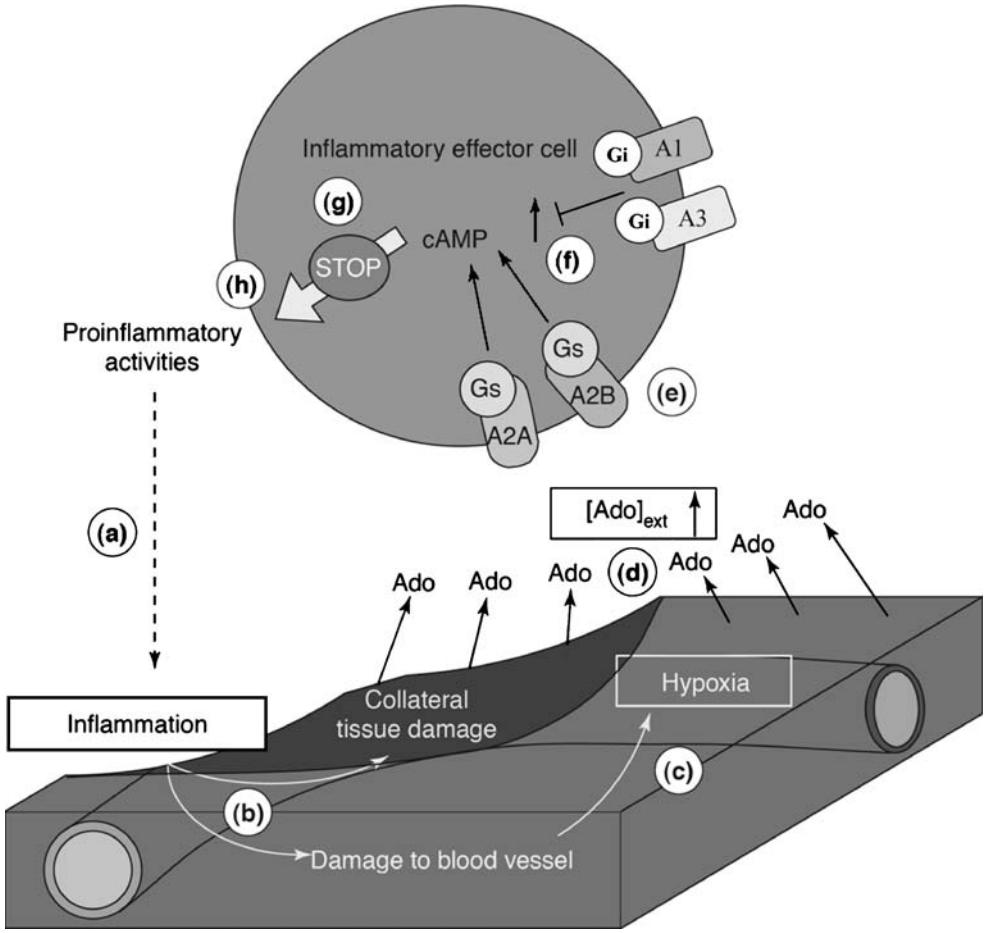

Fig. 1 Role of A_2 adenosine receptors in delayed negative feedback downregulation of activated immune cells in inflammation. **a** Pathogens, virus-infected, mutated or otherwise injured cells activate resident, recruited or tissue-surveying immune cells inducing them to produce pro-inflammatory molecules (e.g. cytokines, reactive oxygen species). **b** This leads to pathogen destruction and, as a result of strong activation, some non-infected, 'innocent' bystander cells in collateral tissue can also be damaged. **c** According to the hypothesis raised by Sitkovsky and Ohta [10], it is the collateral damage to the microvasculature owing to the continuing production of inflammatory molecules that is of crucial significance in signalling the extent of tissue damage. In particular, increased damage to microvasculature may result in the interruption of blood supply and low oxygen tension (hypoxia) in the most damaged microenvironment. **d** Local tissue hypoxia leads to the accumulation of extracellular adenosine (*Ado*), as a result of both hypoxia-associated decrease in intracellular levels of ATP and inhibition of adenosine kinase (see text). **e** As the oxygen tension falls further, the concentrations of extracellular adenosine (*[Ado]ext*) will increase and this, in turn, will determine the intensity of signalling through sequential recruitment of high-affinity (A_{2A}) and low-affinity (A_{2B}) adenosine receptors. **f** The sufficiently high extracellular adenosine levels will trigger the maximal activation of Gs protein-coupled A_{2A} and A_{2B} adenosine receptors and the accumulation of intracellular cAMP, which has strong immunosuppressive properties. **g** The increased cAMP then strongly inhibits ongoing effector functions and prevents their triggering in the newly activated immune cells that have just arrived in the inflamed area. Immune cells also express Gi protein-coupled A_1 and A_3 receptors, which inhibit adenylyl cyclase and cAMP formation, which, in turn, would provide another level of control to prevent the premature inhibition of immune cells by A_2 receptors. **h** This delayed negative feedback mechanism might provide immune cells sufficient time to destroy the pathogen but also prevents additional collateral tissue damage by inhibiting the production of pro-inflammatory cytokines and cytotoxic molecules. Reprinted and modified from Sitkovsky and Ohta [10], copyright 2005 with permission from Elsevier

Among known adenosine receptors, A_2 receptors have properties that make them particularly well suited to serve as sensors of pro-inflammatory activities and to act as stop signals of overstimulated immune cells. First of all, both A_{2A} and A_{2B} receptors are coupled to Gs proteins and to increases of intracellular cAMP, which is in line with the prime importance of cAMP elevation in inhibition of immune cell activity (in this respect, see also Raskovalova et al. [14]) (Fig. 1). Interestingly, the A_{2A} receptor has high affinity and the A_{2B} receptor has low affinity for adenosine, suggesting that they could be recruited sequentially, depending upon the extent of adenosine accumulation at the site of inflammation, and, thus, upon the degree of hypoxia. This would allow a graded escalation of the

inhibitory signal and would also allow, under some circumstances, inhibition of immune cells only partially without completely stopping pathogen destruction. In this context, site- and time-specific actions of adenosine would also be favoured by its short half-life in vivo, which make this nucleoside act in an autocrine or paracrine manner as a local "metabokine" (see Sitkovsky and Ohta [10] and references therein). Globally, this would assure the local inhibition of immune cells in the most injured and therefore adenosine-rich areas, while permitting ongoing pathogen destruction in neighbouring areas. It is interesting to underline that adenosine signalling via Gs protein-coupled A_{2A} and A_{2B} receptors can be counteracted by Gi protein-coupled A_1 and A_3 receptors. Virtually all immune cells express both A_1 and A_2 receptors, which would on the one side assure that no immune cell can escape inhibitory signalling by adenosine, but, on the other side, would provide another level of control to prevent the premature inhibition of immune cells by A_2 receptors. In line with this hypothesis, Gi protein-coupled versus Gs protein-coupled adenosine receptors do have opposite effects on the same neutrophil function (ibidem). Moreover, it seems that, besides inhibiting inflammatory processes, A_2 receptors also have the capacity to redirect the effector class of the immune response and change the patterns of cytokine and chemokine secretion by activated immune cells [15]. By activating A_2 receptors, adenosine can inhibit tumour necrosis factor alpha (TNFα), interleukin-12 (IL-12) and chemokine CXCL10 production, enhance the secretion of IL-10 and augment the release of the chemokine CCL17. Thus, activation of adenosine A_2 receptors might diminish the capacity of dendritic antigen-presenting cells to initiate and amplify type 1 T-helper (Th1) lymphocyte immune responses and therefore shift the Th1 versus the type 2 T-helper (Th2) lymphocyte balance (ibidem).

The generation of adenosine receptor gene-deficient mouse models has firmly established a unique role of A_2 adenosine receptors in several models of acute inflammation and of tissue injury [9, 10, 16, 17]. A_{2A} adenosine receptor-deficient mice showed dramatically increased local tissue damage and prolonged presence of pro-inflammatory cytokines, such as TNFα, interferon gamma (IFNγ) and IL-12, with respect to wild-type animals. Subthreshold doses of inflammatory stimuli, which do not cause liver injury in wild-type mice, induced extensive liver damage and elevated levels of cytokines in knockout mice. In line with these data, similar effects were observed upon injection of the A_{2A} receptor antagonist ZM241385 into wild-type mice, which developed severe tissue damage in response to subthreshold doses of inflammatory stimuli. These data strongly suggest an in vivo role of A_{2A} adenosine receptors in the regulation of inflammation and support the hypothesis that this mechanism is non-redundant because of the failure of other anti-inflammatory mechanisms to compensate for the absence of A_{2A} adenosine receptors.

Adenosine modulation of cytokine release in the lung: a key role in asthma

Inflammation plays a major role in both the development and progression of lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD). Following a chemical, environmental, mechanical or antigenic injury to the lung tissue, the release of various cytokines and chemokines is initially aimed at sustaining the repair process, but its deregulation and persistence later contribute to tissue destruction and remodelling [18].

Multiple cell types are recruited at the site of lung inflammation, including eosinophils, neutrophils, lymphocytes, macrophages and mast cells, and they represent the major sources of inflammatory mediators. In allergic asthma, a prominent role is played by Th2 lymphocytes which release various cytokines (e.g. IL-4, IL-5, IL-13 and IL-9; [19]) contributing to eosinophilia, IgE production, airway hyperreactivity and mucus hypersecretion. In all asthmatic conditions, pulmonary mast cells represent major players of the inflammatory reaction, since, following acute exposure to allergens, pollutants or other chemical stimuli, they degranulate releasing a huge variety of autacoids, including prostaglandin D_2, cytokines, cysteinyl leukotrienes, enzymes and histamine [20]. These chemical entities contribute to the development of the acute asthmatic attack, modulating bronchoconstriction, but also play an important role in the chronic development of the disease. In fact, mast cells have been implicated in airway remodelling [20] and are currently believed to be primarily responsible for the development of airway hyperreactivity (a characteristic feature of chronic asthma in which airways react in an exaggerated way to exposure to mild insults; [21]). During the chronic phase of asthma, lung fibroblasts, epithelial cells and smooth muscle cells further contribute both to the evolution of the disease and to airway remodelling [18].

We have previously mentioned that adenosine concentrations rapidly increase in hypoxic and inflamed tissues, due to ATP breakdown. Thus, it was anticipated that high adenosine concentrations could be found also in the lung of asthmatic patients and that the nucleoside might contribute to the disease. The first indication of a possible role for adenosine in mediating bronchoconstriction dates back to the 1980s when Cushley and co-workers demonstrated that inhaled adenosine was ineffective on normal, but was a potent bronchoconstrictor of asthmatic airways [22]. These results were confirmed a few years later also in patients with chronic obstructive pulmonary disease, by utilizing

AMP (which is rapidly broken down to adenosine) as a bronchoconstrictant [23].

From the very beginning, it was hypothesized that adenosine was not acting directly on bronchial smooth muscle cells, but exerted its bronchoconstrictant activity through an indirect mechanism, involving mast cell degranulation. This hypothesis was later confirmed by the ability of histamine H1 and cysteinyl leukotriene receptor 1-selective antagonists as well as cyclooxygenase 1 and 2 inhibitors and mast cell stabilizing drugs (i.e. sodium cromoglicate) to effectively counteract the effects of inhaled AMP [24]. Indeed, high histamine plasma concentrations were detected in asthmatic patients after AMP inhalation, due to mast cell degranulation accompanied by a rapid airway narrowing [25]. The selective effect of adenosine in asthmatic subjects has recently opened up the opportunity to develop a diagnostic test based on adenosine or AMP inhalation challenge [26] which could effectively discriminate between asthmatic and patients affected by COPD. In fact, adenosine-mediated bronchoconstriction is highly enhanced when the allergic component is relevant, like in asthma, but is lower in the case of COPD [27]. AMP inhalation might also be used to monitor the efficacy of the corticosteroid therapy [27]. Anyway, despite the importance of such a diagnostic test, its use should be carefully evaluated due to the development of an inflammatory response after AMP inhalation that could have problematic outcomes [28]. The important role played by adenosine in modulating allergic response has been very recently further highlighted in ragweed-sensitized mice where adenosine inhalation increased infiltration of inflammatory cells and the appearance of markers of inflammation (such as eotaxin) in the bronchoalveolar lavage [29].

Several studies have demonstrated that adenosine effects are mediated by activation of its surface receptors, but important differences among species have been observed. In fact, with the discovery and cloning of the A_3 adenosine receptor subtype, its prominent role in modulating mast cell degranulation was described in rodents. The situation appears to be different in human mast cells, where the A_{2B} adenosine receptor is the likely candidate for mediation of the pro-inflammatory and bronchoconstrictant effects of the nucleoside in asthmatic patients [18]. For instance, data from HMC-1 cells (a human mast cell line) have shown that activation of A_{2B} receptors leads to IL-8 generation, which can be attenuated by rather selective antagonists [30], and to enhanced IL-4 and IL-13 secretion when cells are co-cultured with human B cells [31]. Both the A_3 and the A_{2B} subtypes are low-affinity receptors for adenosine and can therefore be activated only when high levels of the nucleoside are present, such as in chronic inflammatory situations. The involvement of the low-affinity A_{2B} (coupled to both adenylyl cyclase and phospholipase C) in the pro-inflammatory actions of adenosine could also explain its selective action on asthmatic subjects. In fact, under physiological conditions, low adenosine concentrations activate the high-affinity A_{2A} receptor subtype localized on mast cells and coupled to increases in intracellular cAMP concentrations which, in turn, inhibit histamine release [27] (Fig. 2). Conversely, upon chronic inflammation, high adenosine levels are reached leading to activation of the A_{2B} subtype which may, in turn, promote histamine release by raising inositol(1,4,5)-trisphosphate [$Ins(1,4,5)P_3$] concentrations [27] (Fig. 2).

The discovery of the role of the A_{2B} adenosine receptor in promoting lung inflammation has also helped to clarify the mechanism of action of a well-known anti-asthmatic agent, such as theophylline, which has been used in therapy for over 50 years [32]. Theophylline is known as both a phosphodiesterase inhibitor and an antagonist at adenosine receptors, but its anti-asthmatic actions were mainly ascribed to the former activity based on the fact that enprofylline, another xanthine derivative, is as effective as theophylline in treating asthma but bears a much lower affinity for adenosine receptors [33]. The discovery that the affinity of these two molecules at the A_{2B} receptor is similar and lies below the range of plasma concentrations that are observed in therapy has further clarified the important role played by this receptor subtype in both the onset and development of lung inflammatory diseases [32].

Activation of the A_{2B} adenosine receptor has been also demonstrated to play an important role in the development of airway hyperresponsiveness, contributing to the chronic evolution of the disease, by modulating cytokine secretion from various cell types. As previously mentioned, stimulation of the A_{2B} receptor on human mast cells results in the engagement of Th2 lymphocytes which in turn release IL-4 and IL-13 [31]. IL-13 is involved in IgE synthesis from B cells, leading to chronic inflammation, alveolar remodelling, pulmonary fibrosis and mucus production [19]. Indeed, A_{2B} stimulation on human lung fibroblasts induces IL-6 release which autocrinally promotes their pathological differentiation into myofibroblasts [34]. Upon adenosine stimulation also bronchial smooth muscle cells release IL-6 and monocyte chemotactic protein-1 (MCP-1, also known as CCL-2; [35]), another important mediator of disease progression. Finally, it has been also demonstrated that adenosine can upregulate mucin gene expression in human airway epithelial cells [36]. Based on these observations, selective A_{2B} antagonists (such as IPDX, CGS15493 and CVT 6883; [37, 38]) have been proposed as powerful and effective anti-asthmatic drugs; some of them are currently in clinical trials for the long-term treatment of lung diseases [32].

In very recent years, elegant studies from Blackburn and co-workers have further highlighted the importance of high adenosine concentrations in the development of lung

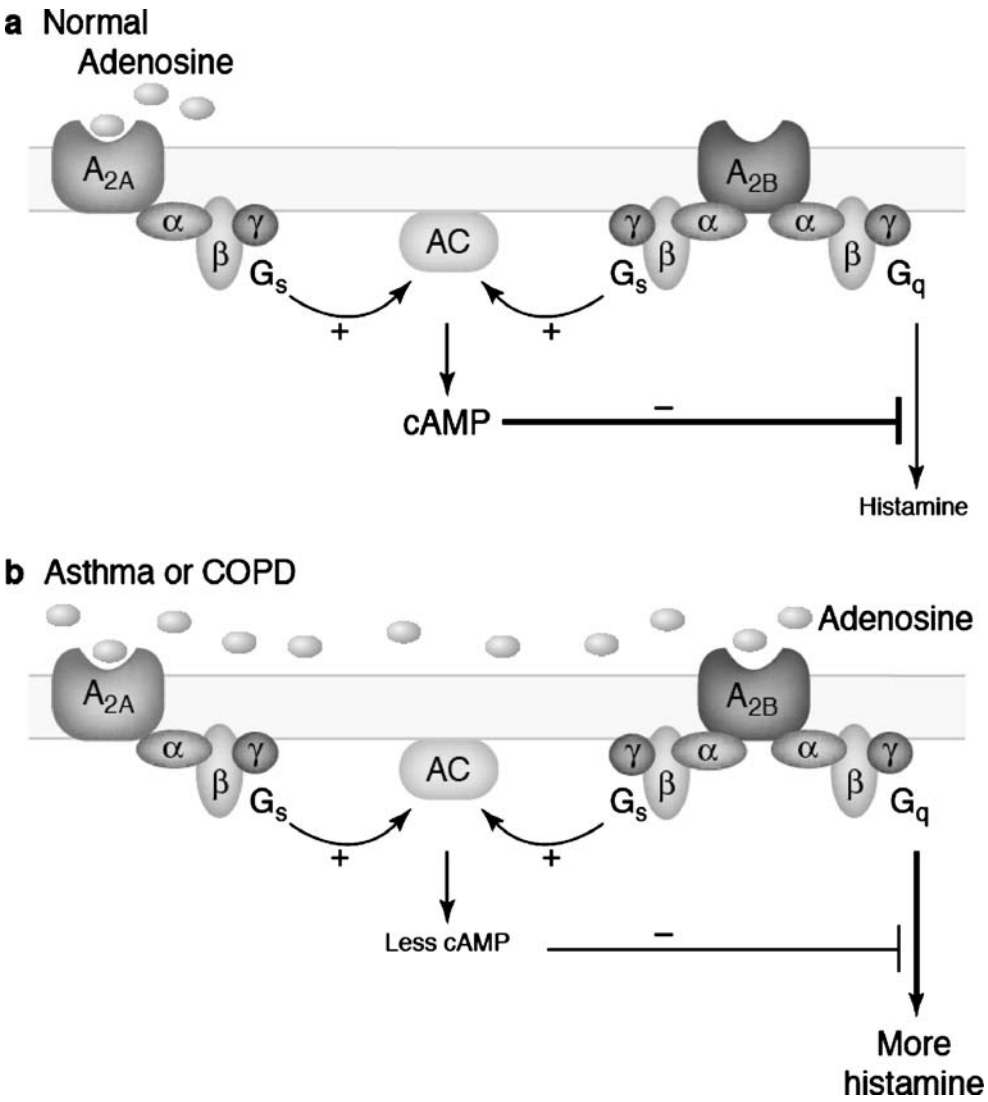

Fig. 2 Role of A_2 adenosine receptors in the modulation of the release of histamine in the lung under physiological or pathological conditions. Both high-affinity A_{2A} and low-affinity A_{2B} adenosine receptors are positively coupled to cAMP production through Gs. In addition, the A_{2B} subtype can also promote Ins(1,4,5)P_3 production via Gq activation. Under normal conditions **a**, low extracellular adenosine concentrations activate the A_{2A} receptor subtype, leading to the increase of intracellular cAMP concentrations, which are known to inhibit histamine release. In asthma and COPD, high extracellular adenosine concentrations are reached **b**. This in turn might lead to the downregulation of high-affinity A_{2A} receptors and might therefore increase the relative importance of the low-affinity A_{2B} subtype. The concomitant reduction in cAMP concentrations, paralleled by overproduction of Ins(1,4,5)P_3 through Gq activation, will promote histamine release as the final outcome, thus contributing to the development and exacerbation of the disease. Reprinted from Spicuzza et al. [27], copyright 2003 with permission from Elsevier

diseases by utilizing adenosine deaminase (ADA)-deficient mice [39]. The most relevant phenomenon observed in these animals is the development of an "asthmatic" inflammatory phenotype, accompanied by all the classic symptoms and cellular changes observed in patients [40]. Indeed, the genetic removal of the A_1 adenosine receptor subtype further strengthened the asthmatic phenotype, highlighting a protective or modulatory role for this receptor subtype against the development of lung inflammation in ADA-deficient mice which might have a functional counterpart also in humans [41].

Finally, a protective role has been also demonstrated for the A_{2A} subtype (see also above). Its activation, in fact, suppresses activation and degranulation of neutrophils, mast cells, monocytes and T lymphocytes [27, 32, 42], thus envisaging the possible use of selective A_{2A} agonists as therapeutic agents.

Adenosine modulation of cytokine release in the brain: beyond its role as a retaliatory neuroprotective metabolite

More than 20 years ago, a seminal paper from Newby introduced the concept of "retaliatory metabolite" to recapitulate the protective adenosine functions in brain and heart ischaemic tissues [43]. An increasing amount of details on adenosine functions have subsequently come from the work of several groups in this field, and very recent observations suggest the situation not to be so well-defined as it seemed at the beginning.

It was already known that following ischaemic and/or traumatic injury or under inflammatory situations extracellular adenosine concentrations increase several fold over basal levels due to the rapid breakdown of ATP [44]. This is particularly true in the central nervous system, where ATP

is massively co-released from synaptic terminals together with excitatory neurotransmitters [45], and adenine nucleotides derive from damaged or dying cells undergoing nucleic acid degradation. Ectonucleotides rapidly degrade nucleotides to adenosine, whose concentration rises from the nanomolar range under basal condition to 10-50 μM following ischaemia [46]. The neuroprotective actions of adenosine have been known for several years and have been mainly associated with activation of the presynaptic A_1 receptor subtype leading to decreases of neuronal firing and of excitatory neurotransmitter release [47]. Acting on postsynaptic A_1 receptors, adenosine is also able to hyperpolarize plasma membranes, thus reducing the propagation of excitatory stimuli. On the contrary, the role of the A_{2A} adenosine receptor subtype is controversial. Under some experimental paradigms, activation of A_{2A} receptors leads to neuroprotection; however, induction of neuronal death has also been proposed, since A_{2A} receptor antagonists are neuroprotective in several experimental models of neurodegeneration [48].

Not only neurons, but also glial cells (i.e. astrocytes and microglia) express all the four cloned adenosine receptor subtypes, with the exception of the A_{2B} receptors that have not been found in microglia. In recent years, the discovery of the important role of glial cells in controlling brain response to traumatic injury has opened up the possibility that some of the effects mediated by adenosine in the brain may also depend on its ability to modulate glial cell functions.

Astrocytes and microglia react rapidly to noxious stimuli by increasing their proliferation rate, leading to the formation of an astrocytic scar isolating damaged neurons (the so-called reactive astrogliosis) and to the recruitment of microglial cells at the site of injury [49]. Adenosine exerts a double action on the proliferation of glial cells, with the A_1 and the A_{2A} receptor subtype reducing and enhancing astrocyte proliferation, respectively [46]. The effect on microglial cell proliferation is less clear and depends upon the cellular environment and the receptor subtypes that are expressed in any given experimental model [50, 51].

In the long term, glial cells contribute to brain remodelling and to the final outcome of the traumatic event through their ability to release both pro- and anti-inflammatory cytokines [46]. Adenosine receptors have been demonstrated to modulate cytokine release from glial cells in different ways. In astrocytes, the overall result of adenosine receptor engagement is the development of an anti-inflammatory phenotype. In fact, A_1 receptor activation induced nerve growth factor (NGF) release from astrocytes [52], whereas A_{2B} and the A_3 receptor subtypes mediated increases of the cytokines IL-6 and CCL2, respectively [53, 54]. Moreover, A_{2A} receptors play an important role in downregulating nitric oxide synthase (NOS) following pro-inflammatory stimuli, such lipopolysaccharide (LPS), IFNγ or TNFα plus IL-1β [55].

The role of adenosine in controlling cytokine release from microglial cells appears to be more contradictory. So far, no role for A_1 receptors has been demonstrated, whereas activation of the A_{2A} receptor subtype seems to drive the appearance of a pro-inflammatory phenotype, with an upregulation of cyclooxygenase-2 (COX-2) expression, followed by increased prostaglandin E $(PGE)_2$ synthesis [56], together with an augmented NO production [57]. Nevertheless, an anti-inflammatory role for some COX-2 products, such as PGD_2 and PGJ_2 has been recently demonstrated [46]. Indeed, the recruitment of the microglial A_{2A} receptor has been also associated with an increase in NGF production [58], thus suggesting that the final outcome of adenosine receptor activation is probably influenced by the cellular environment, as hypothesized for microglial cell proliferation (see above). Studies on human microglial cells have suggested that the A_{2A} receptor subtype is preferentially expressed by activated microglia [59]. If confirmed, these observations might contribute to depicting a scenario where the A_{2A} receptor subtype plays a neurodegenerative role upon pathologic conditions. Finally, the role of the A_3 adenosine receptor in controlling microglial functions was unclear until very recently, when its activation was demonstrated to promote ERK1/2 phosphorylation [60] and to reduce LPS-induced TNFα production through inhibition of the PI3-kinase/AKT pathway [61]. The apparent contradiction in adenosine modulation of microglial cell functions, with the development of either a pro- or an anti-inflammatory phenotype, is in agreement with the double-edged sword role exerted by the inflammatory process, where failure to resolve an initial beneficial inflammatory reaction leads to a delayed and chronic detrimental situation ([12]; see also above). Thus, different receptor subtypes might be recruited at different times after the initial traumatic/inflammatory trigger, also depending upon changes in adenosine concentrations over time, contributing to the plasticity of brain response to traumatic and ischaemic events.

An important contribution to the production of cytokines and chemokines in the brain during ischaemia comes from infiltrating blood immune cells. A specific section of this review is dedicated to a detailed analysis of the role of adenosine in modulating immune cell function. Concerning the role of immune cell adenosine receptors in brain pathologies, a potent protective role for the A_1 receptor subtype has been demonstrated in experimental allergic encephalomyelitis, an animal model of multiple sclerosis [62]. In fact, A_1 adenosine receptor null mice develop a severe form of the disease with respect to wild-type litter mates, characterized by demyelination and oligodendrocyte cytotoxicity evoked by pro-inflammatory molecules (mainly IL-1β and matrix metalloproteinase-12) produced by macrophages [62]. This scenario recapitulates findings in

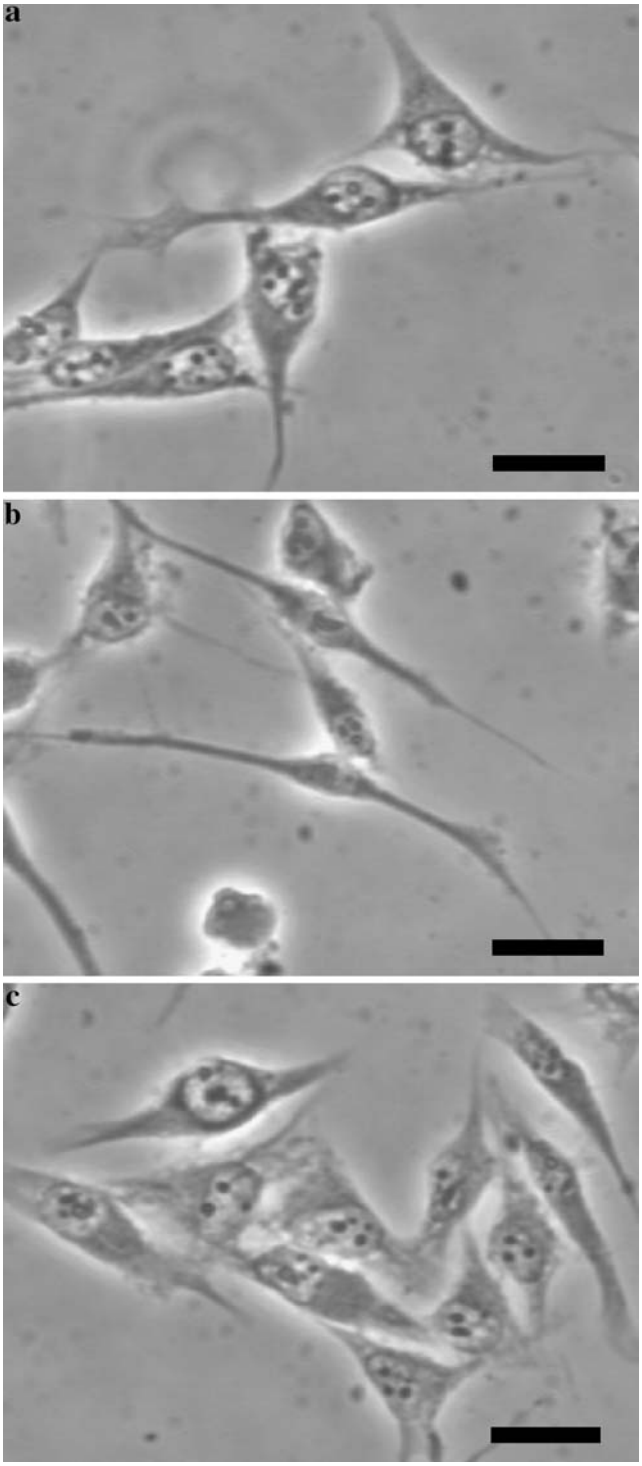

Fig. 3 In vitro induction of reactive astrogliosis by the A_{2B} adenosine receptor in TNFα-treated cells. Exposure of human astrocytoma cells to TNFα increases A_{2B} receptor signalling and G protein coupling by reducing agonist-dependent receptor phosphorylation and desensitization, without affecting receptor protein and mRNA levels (not shown; see Trincavelli et al. [68] for details). From a functional point of view, these biochemical changes translate into the ability of the A_{2B} receptor to induce elongation of astrocytic processes, a typical hallmark of reactive astrogliosis. In fact, marked morphological changes can be observed in cells preincubated with 1,000 U/ml TNFα for 24 h, and subsequently exposed to 1 μM NECA for 30 min, followed by an additional 72 h in drug-free medium **b**, with respect to cultures exposed to TNFα alone, which induced no effect "per se" **a**. Quantification of results indicates a 25-30% elongation of cell processes by NECA in the presence of TNFα, with respect to TNFα alone. No effect on process elongation was detected when cells were exposed to NECA without TNFα pretreatment (see Trincavelli et al. [68] for details). NECA-induced astrocytic elongation can be completely inhibited by the concomitant exposure to MRS 1706 10 nM, **c**, a selective A_{2B} antagonist, thus confirming a specific involvement of this receptor subtype in the observed effects. Magnification: ×32. Scale bar: 30 μM. Reprinted from Trincavelli et al. [68], copyright 2004 with permission from Blackwell Publishing

multiple sclerosis patients, thus suggesting that modulation of the A_1 adenosine receptor subtype might represent a novel pharmacological approach to currently incurable demyelinating diseases.

Again, the role of the A_{2A} receptor subtype expressed by infiltrating cells in modulating brain damage appears contradictory. In fact, inactivation of the A_{2A} receptor of bone marrow-derived cells has been demonstrated to protect brain tissue from middle cerebral arterial occlusion injury; this effect was accompanied by a parallel reduction in the production of pro-inflammatory cytokines from infiltrating macrophages [46], suggesting a detrimental role for this receptor subtype during ischaemic brain damage (see also above). On the contrary, the A_{2A} receptor plays a

neuroprotective and anti-inflammatory role in a rat model of endotoxin-induced meningitis [46] and has been recently demonstrated to prevent human immunodeficiency virus (HIV)-1 Tat-induced production of TNFα by macrophages [63]. Taken together, these observations further confirm an anti-inflammatory and neuroprotective role played by the A_1 adenosine receptor subtype, whilst the role of the A_{2A} receptor in neurodegeneration might depend upon the timing of the disease and the peculiar mechanisms at the basis of its aetiopathology.

A key role for adenosine A_1 and A_3 receptor subtypes has been also described in ischaemic preconditioning, where a mild and transient ischaemic attack reduces the susceptibility of brain tissue to a subsequent and prolonged ischaemic episode. A role for adenosine in protection against release of cytokines and cytotoxic molecules from residential macrophages in ischaemic preconditioning of the heart has been clearly demonstrated (for review, see Picano and Abbracchio [64] and references therein). Given the recent observation of an altered pattern of main cytokine expression (in particular IL-1 and IL-6) in ischaemic animals previously subjected to preconditioning with respect to non-conditioned animals [65], a possible role for adenosine receptors in modulating cytokine release during the induction of cerebral ischaemic preconditioning can also be foreseen.

Not only can adenosine influence cytokine expression and release, but a tight cross-talk between cytokine and growth factor networks and adenosine receptors can be envisaged based on recent observations. In fact, exposure of rat cortical astrocytes to IL-6 upregulates adenosine A_1 receptor expression [66], and inhibition of the A_{2A} receptor subtype by the selective antagonist SCH58261 completely prevented basic fibroblast growth factor (bFGF)-induced reactive astrogliosis [67]. Moreover, TNFα increased A_{2B} adenosine receptor functional response and G-protein coupling in astrocytes in vitro, without any changes in receptor levels but by inhibiting receptor phosphorylation and downregulation. The functional outcome was that activation of this receptor subtype in human astrocytoma cells induced reactive astrogliosis only in the presence of the pro-inflammatory cytokine ([68]; Fig. 3). Taken together, these results suggest that a highly complex interconnection among adenosine signalling pathways, cytokines and growth factors is involved in the generation of the final outcome in response to ischaemic, traumatic and inflammatory events. The in-depth knowledge of the various regulatory pathways might open up new therapeutic strategies to both acute and chronic neurodegenerative disorders.

Finally, increases in cytokine concentrations (in particular IL-6) are known to be potent signals for hormone release by the pituitary gland [i.e. adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL) and gonadotrophin] [69]. Adenosine A_{2B} receptors are highly expressed by the folliculostellate cells of the anterior pituitary, and their activation stimulates IL-6 and vascular endothelial growth factor (VEGF) release [69]. Since folliculostellate cells provide a cellular network integrating both locally generated and systemic signals to modulate hormone secretion, activation of the adenosine A_{2B} receptor may represent an important regulatory pathway in the neuroimmune system [69]. A_{2B}-mediated IL-6 and VEGF production might also play an important role during the development and growth of pituitary tumours, since it can be envisaged that high adenosine concentrations are achieved in the hypoxic core of the tumoural mass due to ATP breakdown [69].

Adenosine modulation of cytokine production in the heart: an efficient, although incomplete, mechanism of protection against heart failure progression

Since the sentinel description by Levine and colleagues of inflammatory cytokines in patients with heart failure in 1990 [70], there has been a growing interest in understanding the role of these molecules in regulating cardiovascular function under both physiological and pathological conditions. In particular, data have been accumulated to suggest that many aspects of heart failure can be explained by the known biological effects of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 [71]. When expressed at sufficiently high concentrations, such as those found in patients with chronic heart failure (CHF), cytokines are sufficient to mimic several aspects of the so-called heart failure phenotype, including progressive left ventricular dysfunction, pulmonary oedema, left ventricular remodelling, foetal gene expression and cardiomyopathy [72, 73, 74]. In CHF patients, both cardiac myocytes, heart residential macrophages and peripheral blood mononuclear cells (PBMC) are able to produce great amounts of TNFα and IL-6. Of these, TNFα represents a serious candidate as a mediator of the myocardial dysfunction progression and remodelling that are part of the natural history of CHF, since it induces hypotension, decreases myocardial contractility and ejection fraction and also exerts a direct cytotoxic effect on cardiac myocytes [75, 76, 77]. All this evidence supports the "cytokine hypothesis" [78] that heart failure progresses, at least in part, as a result of the toxic effects exerted on the heart and the peripheral circulation by endogenous cytokine cascades. Of course, this does not imply that cytokines cause heart failure "per se", but rather that the overexpression of cytokine cascades or, alternatively, a dysfunction of the mechanisms and factors regulating their secretion (see also below), contributes to disease progression.

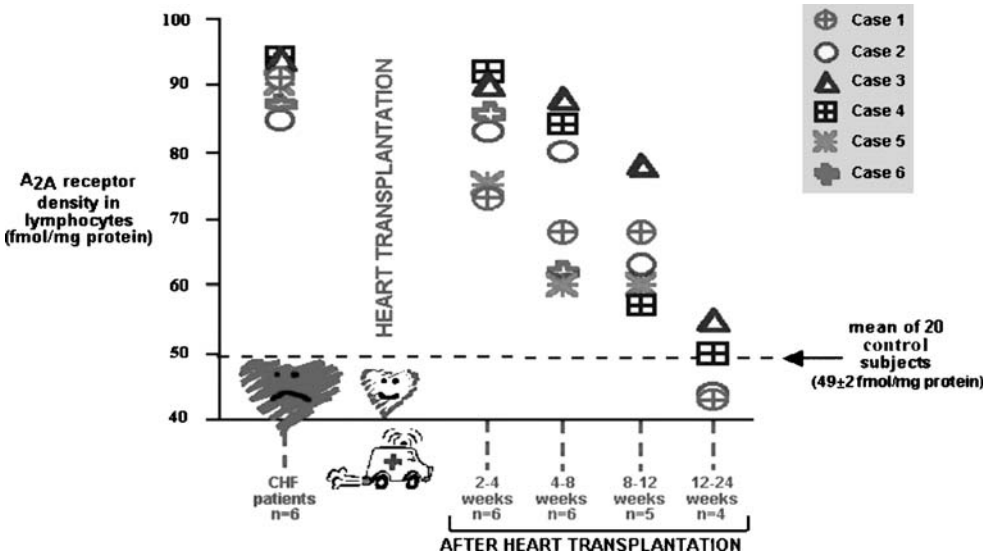

Fig. 4 Alterations of peripheral A_{2A} receptors in CHF patients progressively normalize after heart transplantation in parallel with the normalization of haemodynamic conditions and of plasma cytokine concentrations. In CHF patients, plasma levels of adenosine, TNFα and its soluble receptor are elevated, whereas in peripheral circulating cells A_{2A} adenosine receptors are upregulated (see Varani et al. [80] for more details). In these patients, the latter change mirrors the A_{2A} receptor changes occurring in the heart, the disease target organ. In a cohort of these patients, A_{2A} receptors in peripheral blood circulating cells have been studied longitudinally as a function of time before and after heart transplantation. The graph shows the longitudinal analysis of A_{2A} receptor density (Bmax) as determined with [^{3}H]-ZM 241385 binding in the lymphocytes of six patients, before and at different times after heart transplantation (see *inset* for legend to individual cases). *Dotted horizontal line* indicates the mean value of A_{2A} receptor density in control healthy subjects. Receptor density gradually returned to normal values within 6 months after transplant. Similarly, the K_D value of [^{3}H]-ZM 241385 binding gradually and progressively decreased to control values within the same time period (data not shown). This trend was consistently evident for all evaluated patients and was also detected in the neutrophils of the same subjects, showing a progressive normalization of binding parameters to control values as a function of time. In transplanted patients, plasma adenosine TNFα and IL-6 levels also showed a trend to a decrease to values within 3-6 months after transplant (see Capecchi et al. [82]). Therefore, the cytokine milieu may regulate the function and the expression of A_{2A} adenosine receptors, thus contributing to establishing a negative feedback mechanism against the progressive loop between pro-inflammatory cytokines and heart failure (see also Capecchi et al. [82]). Transplantation results in normalization of haemodynamics, reduction of inflammation and normalization of the number and function of A_{2A} adenosine receptors. Modified from Varani et al. [80], copyright 2003 with permission from The Federation of American Society for Experimental Biology

The possibility of exploiting endogenously generated factors that are capable of attenuating cytokine cascade in CHF is beginning to be explored. In this respect, an ideal system is represented by adenosine. This nucleoside has well-known homeostatic activities in regulating myocardial blood flow, release of catecholamines and, as more recently demonstrated, of cytokines from inflammatory cells and has been shown to reduce myocardial injury resulting from periods of ischaemia (for review, see Villarreal et al. [79]). The A_{2A} receptor seems to be particularly important in mediating these beneficial effects. Activation of this receptor subtype on immune cells (e.g. monocytes and lymphocytes) has long been known to mediate anti-inflammatory responses, including inhibition of TNFα release (see also above; for review see Sitkovsky et al. [9]), which may have interesting implications for the development of heart disease. In line with this hypothesis, an increase of A_{2A} adenosine receptor expression, density and activity has been reported in the PBMC of end-stage CHF patients compared to control subjects, in parallel with significant increases of the plasma levels of TNFα and soluble TNF receptors [80]. In these patients, upregulation of A_{2A} adenosine receptors in circulating cells progressively normalized after cardiac transplantation, in parallel with the normalization of haemodynamic conditions and with the reduction of plasma TNFα and soluble TNF receptors towards normal values (ibidem; Fig. 4). These data indicate a correlation between A_{2A} receptors and cytokine production in CHF and suggest that A_{2A} receptors are upregulated in an attempt to potentiate adenosine-mediated cytokine inhibition. This hypothesis is consistent with the demonstration that both adenosine and adenosine-interfering agents (i.e. dipyridamole and iodotubercidin, which inhibit adenosine uptake and intracellular phosphorylation, respectively) potently inhibit LPS-induced TNFα production and release [81]. These effects could be blocked by A_2 but not by A_1 or A_3 receptor antagonists (ibidem). The specific involvement of the A_{2A} receptor in these actions was confirmed in a later study. When stimulated ex vivo with LPS, the PBMC from CHF patients produce greater

amounts of TNFα in comparison with cells from healthy subjects [82]. However, despite increased TNFα production, activation of A_{2A} receptors (which are upregulated in CHF, see above) by the selective A_{2A} receptor agonist CGS21680 induced a comparable inhibition of TNFα release in both control and CHF patients. This effect was blocked by ZM241385, a specific A_{2A} antagonist. These results suggest that upregulated A_{2A} receptors in CHF patients are efficiently coupled to their transduction system. The inhibitory effect of A_{2A} adenosine receptors on LPS-induced TNFα production in monocytes could be attributed to the increase of intracellular cAMP, which has been shown to attenuate nuclear factor (NF)-κB-mediated transcriptional activity (see also below). In line with previous data suggesting the existence of a regulatory cross-talk between TNFα and A_{2A} adenosine receptor levels [83, 84], the ex vivo incubation of PBMC from control subjects with TNFα increased the expression of this adenosine receptor subtype [82]. In contrast, under the same experimental condition, TNFα did not further increase the expression of the A_{2A} receptor in the PBMC from CHF subjects, suggesting that, in these patients, maximal induction had already occurred in vivo.

On this basis, the following pathophysiological loop acting in chronic heart failure may be suggested: in CHF patients a high plasma level of endotoxin primes inflammatory cells to produce great amounts of cytokines; at the same time, high concentrations of TNFα may induce upregulation of the A_{2A} adenosine receptor, in an attempt to potentiate adenosine-mediated cytokine inhibition. Upregulation of the A_{2A} adenosine receptor in inflammatory cells from CHF patients may thus represent an efficient, although incomplete, mechanism of protection against inappropriate cytokine production in the diseased heart. These findings also suggest the A_{2A} adenosine receptor as a pharmacological target for novel therapeutic interventions aimed at slowing down heart failure progression even after activation of inflammatory cells has occurred.

Concluding remarks

The evidence reviewed above supports a crucial role for specific adenosine receptors (mainly the A_1 and A_{2A} receptor subtypes) in inhibition of pro-inflammatory cytokine release, which has obvious important implications for human pathophysiology. In principle, adenosine signalling through these receptors is aimed at protecting tissues against excessive inflammatory damage. Compelling evidence points to a crucial role for the A_{2A} adenosine receptor in limitation and termination of inflammation. As reviewed above and in Sitkovski et al. [9], no other factor could compensate fully for the loss of A_{2A} receptors on immune cells, suggesting that this mechanism is non-redundant and may have important implications in human diseases characterized by excessive inflammation and/or overactivation of immune cells. In line with this hypothesis, in the PBMC of patients with chronic heart failure, A_{2A} receptors were upregulated [80, 82], likely in an attempt to potentiate adenosine inhibition of cytokine secretion. However, in the end, this could not prevent heart disease progression, suggesting that this mechanism is not able to fully protect the heart against inappropriate cytokine production. Nevertheless, these data globally highlight the A_{2A} receptor as an interesting target for the development of new pharmacological strategies aimed at potentiating adenosine cytoprotection. In contrast, activation of the A_{2B} receptor seems to be responsible for pro-inflammatory actions, likely through activation of Gq proteins, calcium mobilization and stimulation of phospholipase C and mitogen-activated protein kinase (reviewed in Linden [2]). As an example of the deleterious consequences of this cascade, activation of A_{2B} receptors contributes to exacerbating histamine release and disease progression in asthma and COPD (see above). In line with this evidence, A_{2B} blockers have been hypothesized to be useful as anti-inflammatory agents.

The evidence summarized above also points to the existence of a highly complex interplay among adenosine receptors and cytokines. Not only can adenosine influence cytokine expression and release, but cytokines can themselves modify adenosine receptor function by either influencing their expression (e.g. [82]) or by inhibiting adenosine receptor phosphorylation and downregulation [68]. In some cases (for example in reactive astrogliosis) this may contribute to developing cytoprotective mechanisms in the first stages of the disease, but, when inflammation becomes chronic, a dysregulation of these same mechanisms may contribute to disease exacerbation. On this basis, it is clear that a more in-depth knowledge of the various regulatory pathways between adenosine and cytokines might open up new therapeutic strategies to fully exploit adenosine cytoprotection in acute and chronic degenerative diseases.

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