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Role of adenosine in inflammatory processes

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INTRODUCTION

1. ADENOSINE

Adenosine is an ubiquitous purine nucleoside, playing a fundamental role in many biological processes such as energy generation and proteins metabolism, but in the last two decades it has become clear that adenosine is a mediator involved in the pathogenesis of many inflammatory disorders.

1.1. Adenosine endogenous production and metabolic pathway

Adenosine consists of the purine base adenine in glycosidic linkage with ribose (Figure 1); endogenous adenosine derives from dephosphorylation of the nucleotide adenosine 5'-monophosphate (AMP) to adenosine by the enzyme ecto-5'-nucleotidase, that is present in the cell membrane and belongs to the ectonucleotidases family; the ectonucleotidases include ectonucleoside triphosphate diphosphohydrolases, ectonucleotide pyrophosphatase /phosphodiesterases, alkaline phosphatases and 5'-nucleotidases (Zimmermann et al., 2002). Under normal conditions most adenosine is derived from intracellular AMP that is at low levels in the cell and any AMP that accumulates, diffuses down its concentration gradient out of the cell and encounters the cell membrane ecto-5'-nucleotidase; intracellular AMP is derived from the cleavage of adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) during the cycle of energy generation and the dephosphorylation of AMP to adenosine is considered the last step of the enzymatic chain (Fredholm et al., 2001a), in fact this metabolic pathway lasts few hundred milliseconds and the dephosphorylation of AMP to adenosine seems to be the rate-limiting step (Dunwiddie et al., 1997). Adenosine

can also be formed in the intracellular environment due to the activity of intracellular 5'-nucleotidases of which two isoforms, cN-I and cN-II, have been cloned (Fredholm et al., 2001a).

During conditions of high-energy demand, 5'-nucleotidases metabolise intracellular AMP to adenosine to release energy (Polosa et al., 2002); at this stage, the requirement for more energy leads to the breakdown of adenosine to inosine and hypoxanthine by the enzyme adenosine deaminase (ADA) (Trams and Lauter, 1974). The enzyme adenosine kinase is responsible for converting adenosine to AMP (which in turn is reconverted to ADP and ATP, as a part of the energy cycle) and is largely responsible for the resulting low levels of intracellular adenosine (Polosa et al., 2002). Once formed, extracellular adenosine is transported inside the cell by nucleoside transporters which are able to maintain high level of adenosine against a concentration gradient (Fredholm et al., 2001a), and an increase in extracellular active levels of adenosine can be induced by drugs decreasing the activity of these transporters; adenosine transporters have been cloned and named as ENT1 and ENT2 (equilibrative transport) and CNT1 and CNT2 (concentrative transport).

Under conditions of high energy demand and/or hypoxia, intracellular AMP is metabolised to adenosine (Bodansky and Schwartz, 1968; Mentzer et al., 1975); an example of this high energy demand is an inflammatory environment where a large number of inflammatory cells compete for a limited oxygen supply. Ischemia, hypoxia and electrical stimulation can also increase the level of adenosine (Zetterstrom et al., 1982; Berne and Rubio, 1974) and the increase can be up to 100-fold during ischemia (Rudolphi et al., 1992);

adenosine release is also affected by neurotransmitters such as NMDA, dopamine and nitric oxide (Fredholm et al., 2001a).

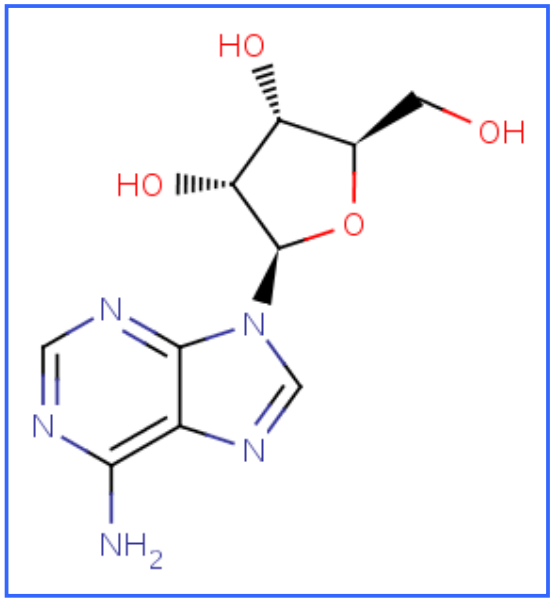
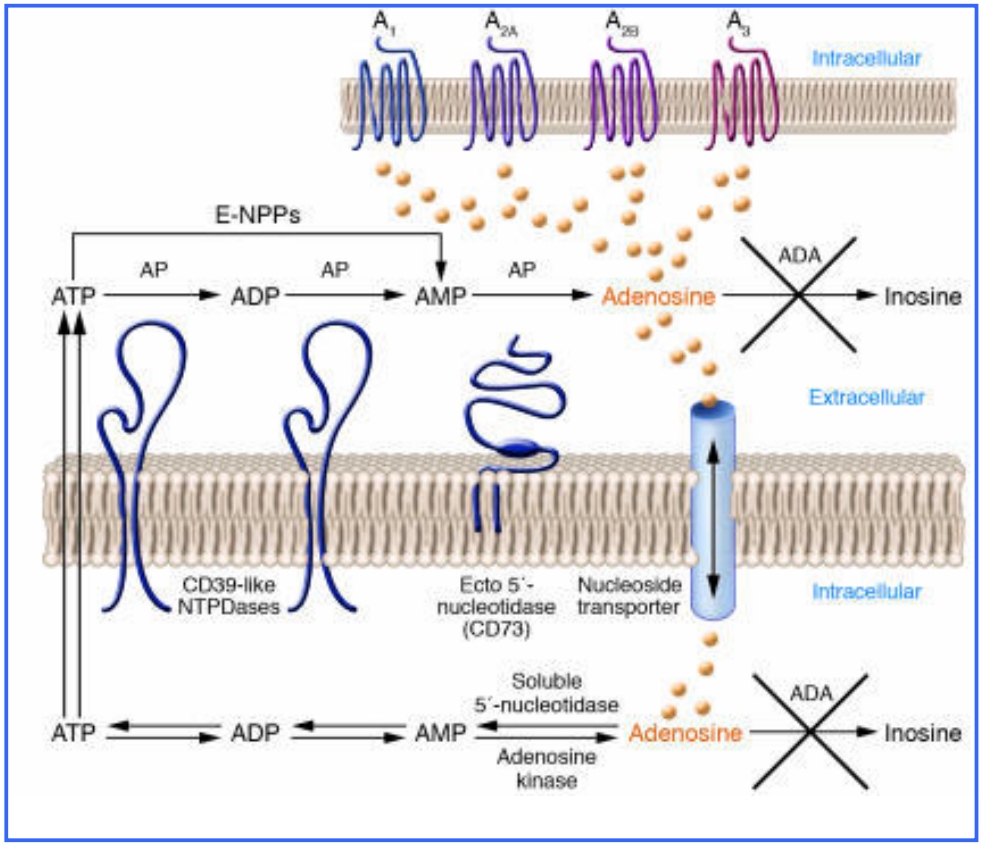


Figure 1: Adenosine structure



<u>Figure 2</u>: Adenosine metabolic pathway. Once into extracellular space, adenosine is able to activate adenosine receptors; for abbreviations, see the text.

1.2. Biological effects of adenosine

Adenosine is released into the extracellular fluid under physiological and pathological conditions (during hypoxia and ischaemia) and exerts a wide variety of effects on many different tissues and organ systems (Ramkumar et al., 1988; Feoktistov and Biaggioni, 1997; Pelleg and Porter, 1990), almost including: the heart (Belardinelli et al., 1989), with an antiarrhythmic/arrhythmogenic effect by the depression of pacemaker activities, by slowing A.V. conduction, modulating autonomic control and triggering synthesis and release of prostaglandins, although the antiarrhythmic effect is preponderant; the kidney (Churchill et al., 1982), where adenosine decreases/increases renal blood flow, decreases glomerular filtration rate, decreases/increases renin release, stimulates renal sympathetic nerves; blood and blood vessels, with coronary vasodilatation and reduction of peripheral vascular resistance; lungs (Spicuzza et al., 2006), with bronchoconstriction and increment of vascular resistance; nervous system (Fredholm et al., 1988), where adenosine depresses neurotransmission, reduces neuronal firing, induces spinal analgesia, and has pro- and anti-nociceptive properties, neuro-protective, anti-convulsive, anti-psychotic, anxiolytic effects and is a locomotor activity depressant; immune system (Huang et al., 1997), generally inhibiting function, for example inhibiting neutrophil and platelet function, although adenosine has both pro- and anti-inflammatory effects. These and other effects are summarised in Table 1.

Table 1: Biological effects of adenosine (from Livingston et al., 2004; adapted from Pelleg et al., 1990).

Organ	Effects
Heart	Depression of pacemaker activities, slows AV conduction, antiarrhythmic/arrhythmogenic effect, modulatesautonomic control, triggers synthesis and release of prostaglandins.
Kidney	Decreases/increases renal blood flow, decreases glomerular filtration rate, decreases/increases rennin release, stimulates renal sympathetic nerves.
Blood and Blood vessels	Coronary vasodilatation, reduces peripheral vascular resistance.
Lungs	Bronchoconstriction, increases vascular resistance.
Liver	Vasoconstriction, stimulates glycogenolysis.
Pancreas	Potentiates glycogen release.
Adrenals	Stimulates stereogenesis.
Central nervous system	Depresses neurotransmission, reduces neuronal firing, induces spinal analgesia, pro-and anti-nociceptive properties, neuro-protective effects, anti-convulsive effects, anti-psychotic effects, anxiolytic agent, locomotor depressant.
Immune function	Generally inhibits function e. g. inhibits neutrophil and platelet function, although has both pro- and anti-inflammatory effects.
Metabolism	Inhibits adipocyte lipolysis, stimulates adipocyte glucose oxidation, stimulates glucose uptake in the heart, regulation of intestinal tone and secretion.
Other	Induces breathing, induces hypothermia, sedative, activates cellular antioxidant enzymes, anti-diabetic, role in apoptosis, role in cell growth e.g. inhibits smooth muscle cell growth, modulates gene expression.

1.3. Adenosine receptors classification

Membrane-bound receptors mediate adenosine cell signalling and determine the variety of its effects; at present, there are four established adenosine receptors in humans that have been cloned, designed as A_1, A_{2A}, A_{2B} and A_3 receptors. These receptors are seven transmembrane-spanning proteins and members of the G protein-coupled receptors; they belong to the large family of the so-called purinergic receptors, which are subdivided in P_1 (activated by adenosine) and P_2 (activated by ATP). Adenosine and its agonists act via these receptors and are able to modulate the activity of adenylate cyclase, the enzyme responsible for increasing cyclic AMP (cAMP); the different receptor subtypes have differential stimulatory and inhibitory effects on this enzyme and bind extracellular adenosine with different affinities, as reported in Table 2 (Ralevic & Burnstock, 1998; Fredholm et al., 2001a; Bours M.J.L. et al., 2006). It must be noted that adenosine breakdown product inosine also exhibits agonistic action on A_1, A_{2A}, A_{2B} and A_3 receptors at micromolar concentrations (Jin et al., 1997; Fredholm et al., 2001b; Gomez & Sitkovsky, 2003; Hasko et al., 2004).

Table 2: P_1 and P_2 receptor subtypes with estimated affinity for physiologic ligands (from Bours M.J.L. et al., 2006).

Subtype	Physiologic ligand
P_1 receptors	
A_1	Adenosine (EC50: 0.18–0.53 μM) Inosine (EC50: 290 μM)
A_{2A}	Adenosine (EC50: 0.56–0.95 μM) Inosine (EC50: 50 μM)
A_{2B}	Adenosine (EC50: 16.2–64.1 μM)
A_3	Adenosine (EC50: 0.18–0.53 μM) Inosine (EC50: 0.03–2.5 μM)
P_2 receptors	
P_2X_1	ATP (EC50: 0.05–1 μM)
P_2X_2	ATP (EC50: 1–30 μM)
P_2X_3	ATP (EC50: 0.3–1 μM)
P_2X_4	ATP (EC50: 1–10 μM)
P_2X_5	ATP (EC50: 1–10 μM)
P_2X_6	ATP (EC50: 1–12 μM)
P_2X_7	ATP (EC50: 100–780 μM)
P_2Y_1	ADP (EC50: 8 μM)
P_2Y_2	UTP (EC50: 0.14 μM) = ATP (EC50: 0.23 μM)
P_2Y_4	UTP (EC50: 2.5–2.6 μM) » ATP, UDP
P_2Y_6	UDP (EC50: 0.3 μM) »UTP (EC50: 6 μM)
P_2Y_{11}	ATP (EC50: 17 μM)
P_2Y_{12}	ADP (EC50: 0.07 μM)
P_2Y_{13}	ADP (EC50: 0.06 μM) >ATP (EC50: 0.26 μM)
P_2Y_{14}	UDP-glucose (EC50: 0.1–0.5 μM)

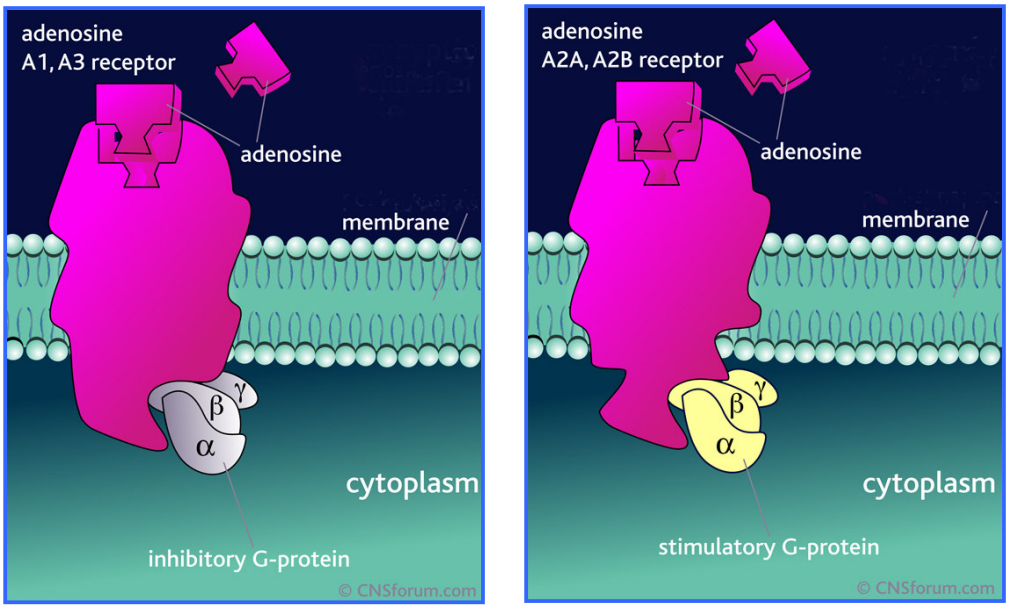


Figure 3: Adenosine receptor subtypes. A drawing.

1.4. A_1 adenosine receptors

The human A_1 receptor gene has been localised to the chromosome 1q32 and codes for a 326 amino acid protein with a molecular weight of ~ 36.7 KDa (Olah and Stiles, 1995; Townsend-Nicholson et al., 1995); these receptors have a high affinity for adenosine and for adenosine analogues substituted at the N6 position, for example L-N6-phenylisopropyladenosine (L-PIA) (Feostikov and Biaggioni, 1997). A_1 receptors mediate the inhibition of adenylate cyclase, but can also inhibit G protein-coupled activation of voltage dependent Ca^{2+} channels and induce phospholipase C activation (Stiles, 1992); this adenosine receptor subtype is found in adipose tissue, heart muscle, central nervous system, airways and inflammatory cells such as neutrophils (Polosa, 2002).

1.5. A_2 adenosine receptors

On the contrary to the A_1 receptor, A_2 receptor stimulation leads to the activation of adenylate cyclase resulting in the elevation of intracellular cAMP (Olah and Stiles, 1995; Moreau and Huber, 1999). These receptors bind adenosine with less affinity than A_1 receptors and are preferentially stimulated by adenosine analogues substituted at the 5'-N position, for example 5'-N-ethylcarboxamideadenosine (NECA) (Livingston, 2004). A_2 receptors are more widely distributed than A_1 receptors, and are found in pre- and post-synaptic nerve terminals, mast cells, airway smooth muscle and circulating leukocytes (Polosa, 2002).

A_2 receptors are subdivided into the A_{2A} and A_{2B} receptors, based on high and low affinity for adenosine, respectively. In most cell types the A_{2A} subtype inhibits intracellular calcium levels whereas the A_{2B} potentiates them (Feostikov and Biaggioni, 1997). The human A_{2A} receptor gene has been localised to the chromosome 22q11.2 and codes for a 337 amino acid protein with a molecular weight of 45 KDa (Le, 1996; Moreau and Huber, 1999); A_{2A} receptors are expressed in the central nervous system, vascular smooth muscle, endothelium and on neutrophils, platelets, mast cells and T cells (Polosa, 2002).

The A_{2B} receptor, although structurally closely related to the A_{2A} receptor and able to activate adenylate cyclase, is functionally very different. It has been postulated that this subtype may utilise signal transduction systems other than adenylate cyclase because of these functional differences (Polosa, 2002). The human A_{2B} receptor gene has been localised to the chromosome 17p11.2-p12 and

codes for a 332 amino acid protein with a molecular weight of ~ 37.0 KDa (Olah and Stiles, 1995; Townsend-Nicholson et al., 1995). It has been identified widely including in the brain, human bronchial epithelium, endothelial cells, muscle cells, neurons, glial cells, fibroblasts and mast cells (Polosa, 2002).

1.6. A_3 adenosine receptors

Similarly to the A_1 receptor, stimulation of the A_3 adenosine receptor leads to inhibition of adenylate cyclase (Olah and Stiles, 1995). It has also been shown to stimulate directly phospholipases C and D and experiments in HL-60 promyeloid leukaemia cells have shown that A_3 receptor activation results in the influx of calcium and its release from intracellular stores (Jacobson, 1998). The human A_3 receptor gene has been localised to the chromosome 1p13.3 and consists of a 337 amino acid protein with a molecular weight of 36.0 - 37.0 KDa (Olah and Stiles, 1995; Moreau and Huber, 1999; Atkinson et al., 1997). In comparison with the other adenosine receptors, the A_3 receptor exhibits large differences in structure, tissue distribution and its functional and pharmacological properties among species (Linden, 1994); furthermore, the possibility of two potential sub-types of A_3 receptors has been raised (Jacobson, 1998). The A_3 receptor is widely distributed, being found in the kidney, testis, lung, mast cells, eosinophils, neutrophils, the heart and brain cortex (Polosa, 2002).

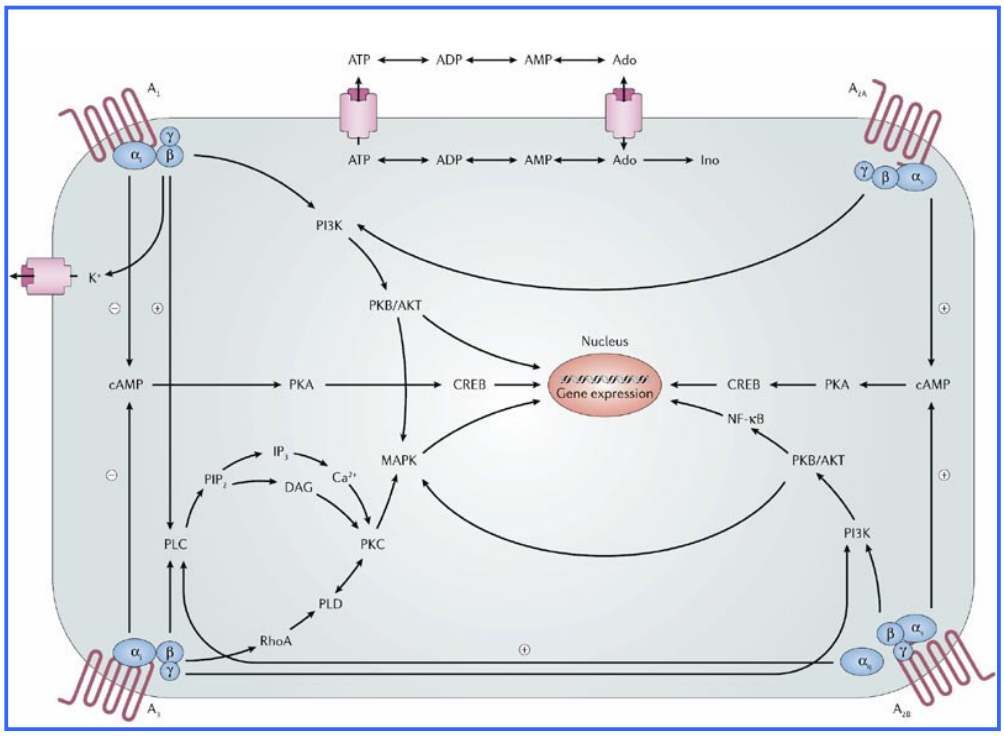


Figure 4: **Adenosine receptor signalling pathways. The major signal transduction mechanisms; PLC, phospholipase C; MAPK, mitogen-activated protein kinase; CREB, cAMP response element binding; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PI_3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PK, protein kinase; PLD, phospholipase D; NF-kB, nuclear factor-kB.**

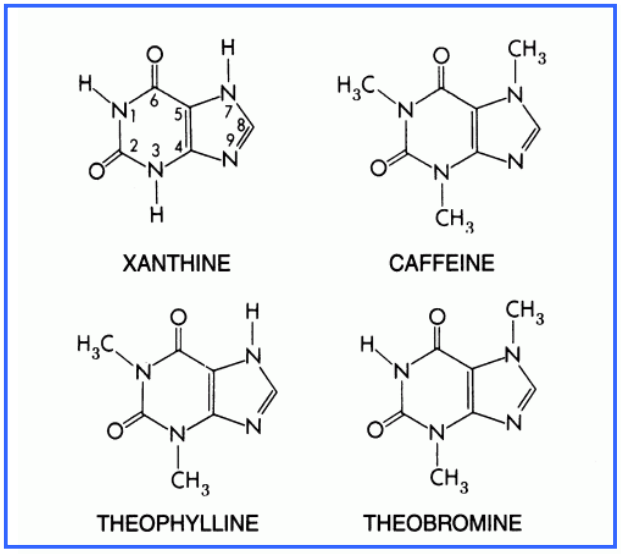


Figure 5: **Xanthines: structure. These natural products act blocking adenosine receptors.**

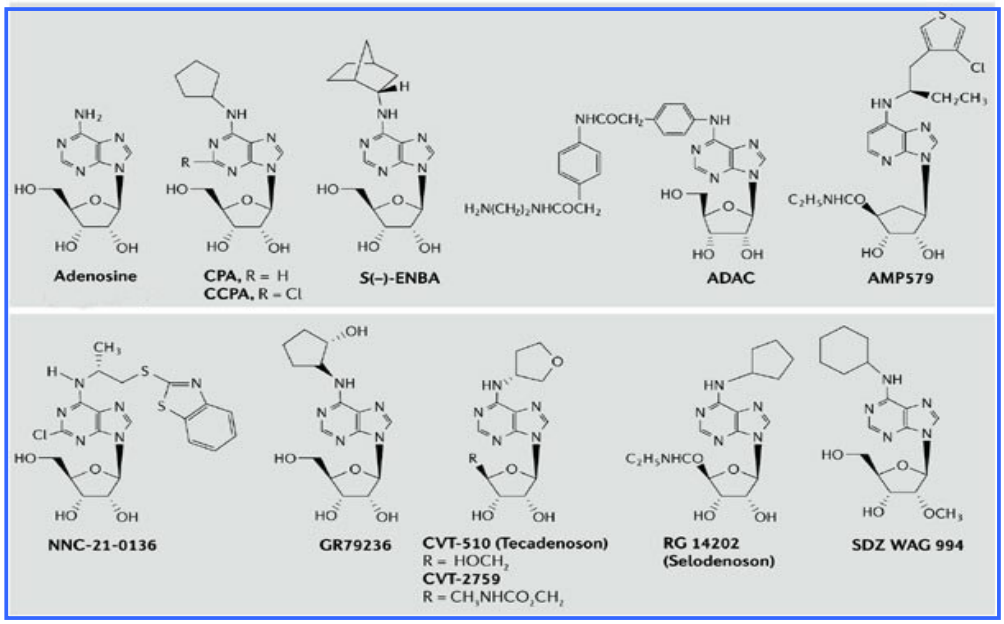


Figure 6: **Adenosine A_1 receptor agonists: structures**

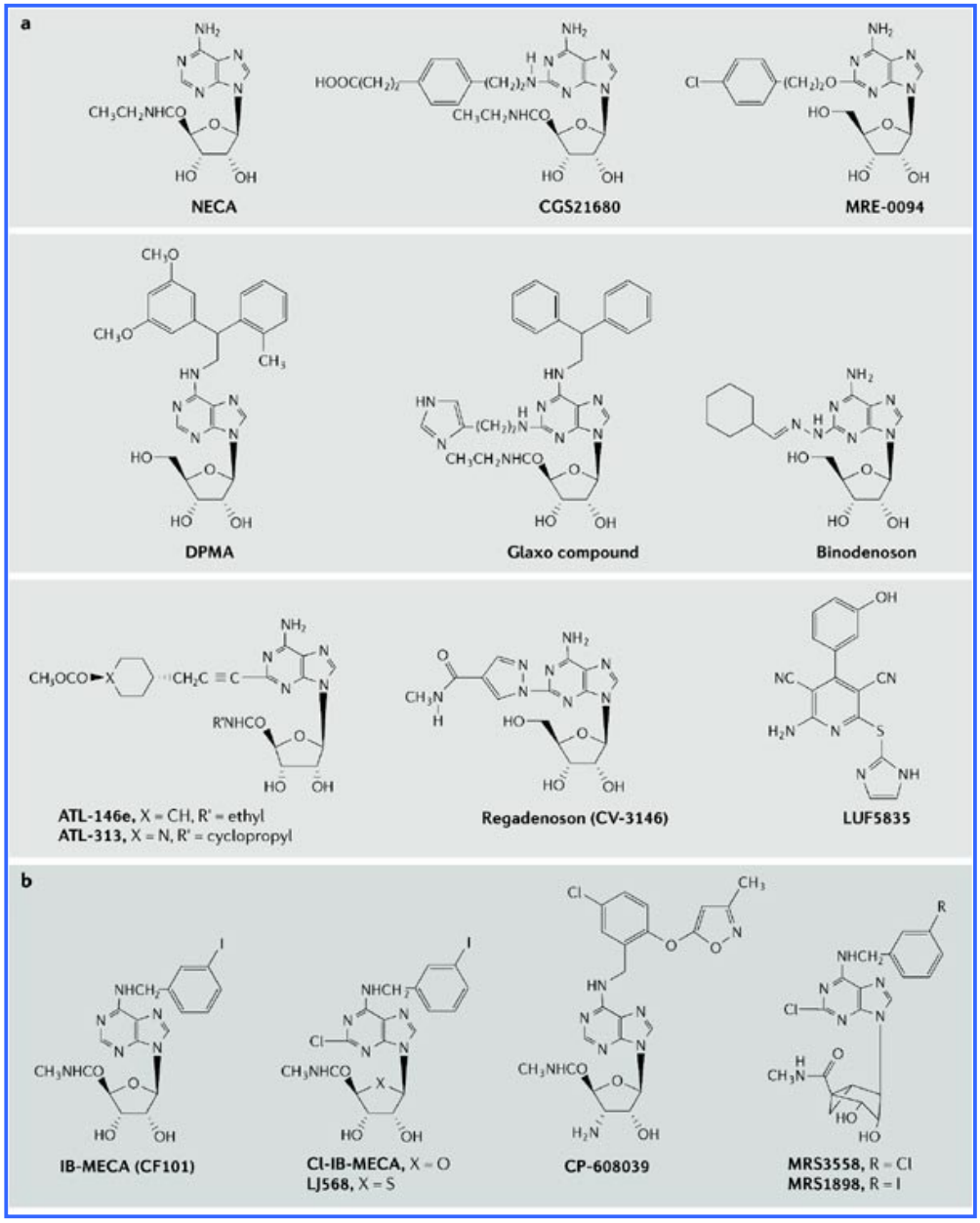


Figure 7: Adenosine A_2 (a) and A_3 (b) receptor agonists: structures.

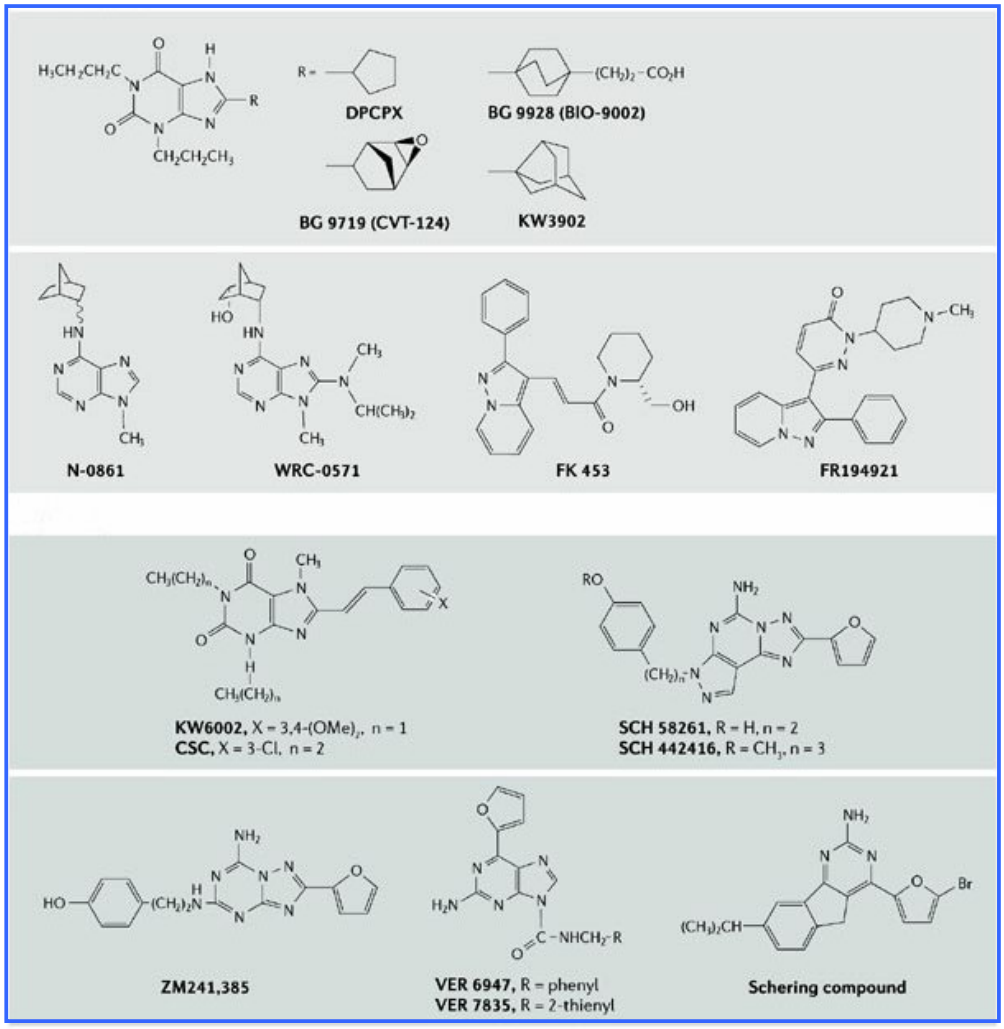


Figure 8: Adenosine A_1 (a) and A_{2A} (b) receptor antagonists: structures.

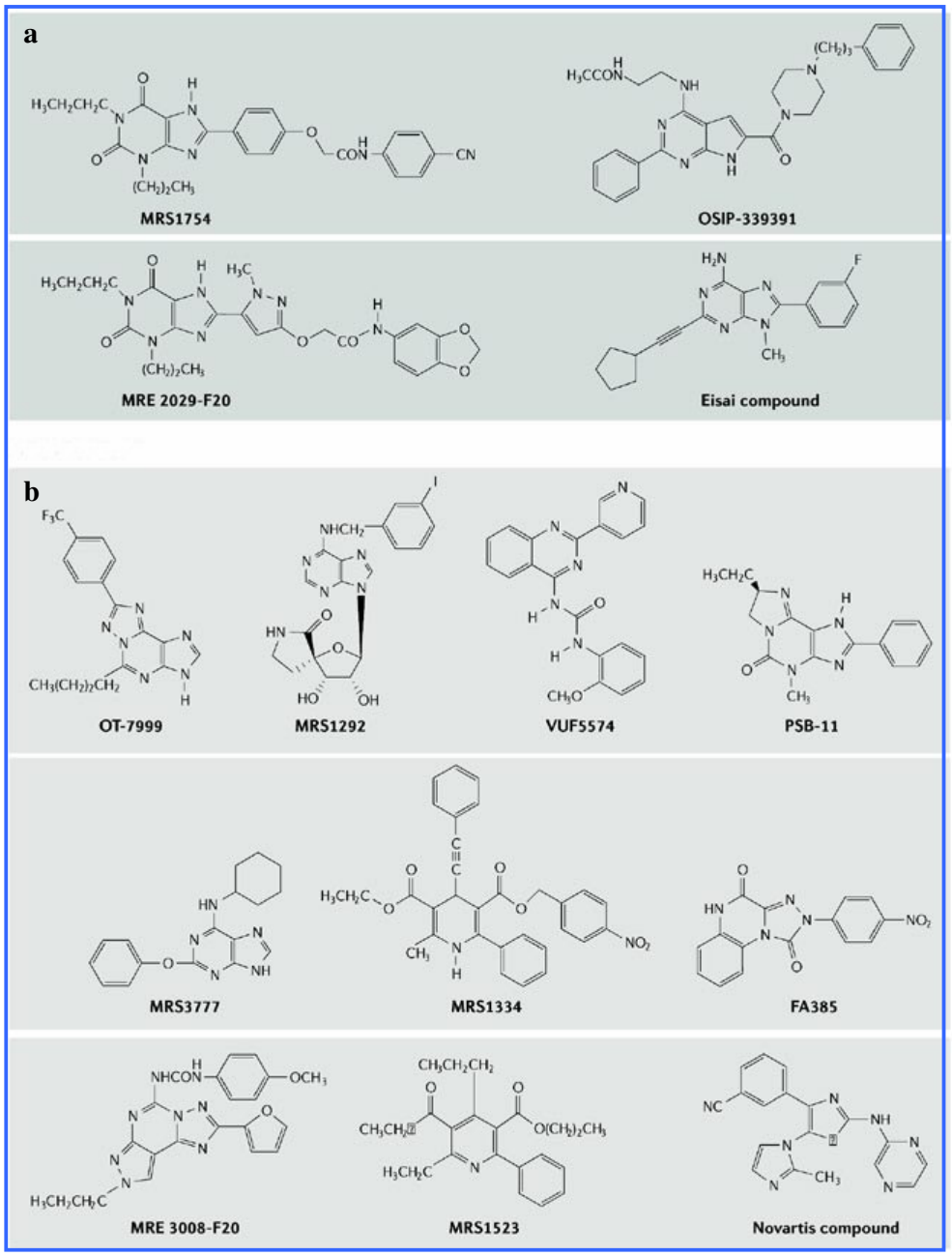


Figure 9: Adenosine A_{2B} (a) and A_3 (b) receptor antagonists: structures.

2. ADENOSINE AND INFLAMMATION

It has been known for a long time that inflammatory tissue damage is accompanied by accumulation of extracellular adenosine in inflamed areas due to its release from non-immune and immune cells (Sitkowsky, 2003); local tissue hypoxia in inflamed areas represents one of the most important conditions leading to adenosine release and accumulation (Winn et al., 1981; Van Belle et al., 1987; Rudolphi and Shubert, 1995), and also contributing to the accumulation of adenosine is the release of rapidly metabolized ADP and ATP from various cells including platelets, mast cells, and endothelial cells (Linden, 2001) (Figure 10). Investigations during more than three decades on the effects of adenosine on inflammatory processes and of the immunosuppressive properties of adenosine receptor-mediated signalling can be divided into two main periods: the first two decades provided the first demonstrations of the influence of extracellular adenosine on cells involved in the inflammatory response; the next two decades provided superior investigative tools and inquiries through the synthesis of novel ligands and molecular cloning of adenosine receptor subtypes. It was shown that adenosine has both pro- and anti-inflammatory effects, and different adenosine receptors may have opposite effects on the same function; for example, neutrophil adherence to endothelium could be inhibited by A_{2A} receptors and enhanced by A_1 receptors (Cronstein et al., 1992), and similarly mast cell degranulation could be inhibited by A_{2A} receptors and enhanced by A_{2B} receptors (Polosa, 2002). In contrast, A_3 receptors could inhibit eosinophil migration and trigger degranulation of rodent mast cells; of special interest are the documented effects of adenosine on

secretion of pro-inflammatory cytokines, like IL-12 and TNF-α, by monocytes and macrophages (Hasko et al., 1996; Eigler et al., 1997).

In conclusion, the current stage of inflammation and adenosine studies is characterized by utilization of molecules able to manipulate adenosine receptors and adenosine receptors signalling in different disease models, and also by the need to better understand the dual role of adenosine in the inflammatory responses.

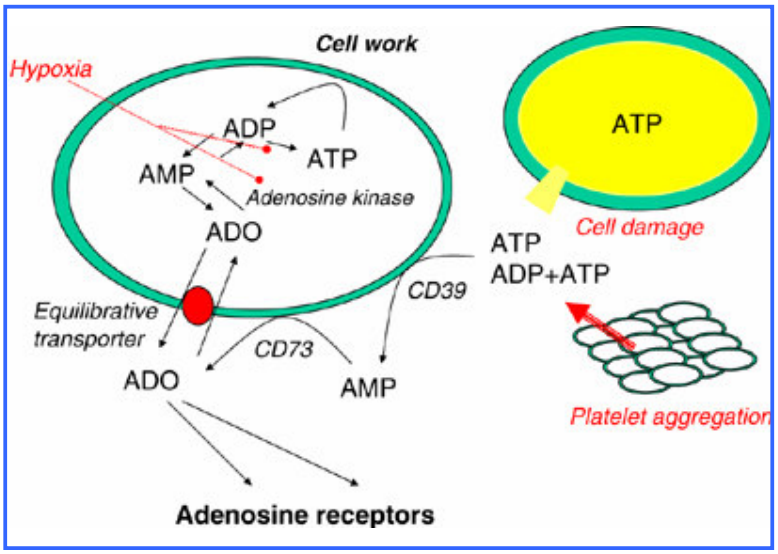


Figure 10: Adenosine, also a mediator of inflammation

2.1. The bases of inflammation

Inflammation is characterized by five hallmarks: *rubor* (redness), *tumor* (swelling), *calor* (heat), *dolor* (pain) and *functio lesa* (loss of functionality); these symptoms result from recruitment, activation and interaction with tissues of immune cells including monocytes/macrophages, lymphocytes, mast cells, basophils, neutrophils, eosinophils and T-cells; the cellular component normally resides in blood and move into the damaged area via diapedesis; acute inflammatory response is mediated by granulocytes, while chronic inflammation is mediated by mononuclear cells, such as monocytes and lymphocytes. Inflammation occurs at a site of infection or may be caused by non-infectious tissue insults, such as hypoxia, allergy, mechanical or thermal trauma. Usually, it is a very localized response that serves to refrain and eliminate the damaging agent and to repair the injured tissue; however, the normally beneficial immune arsenal is a cause of disease when it results in inappropriate and/or extensive injury to host tissues, in fact abnormalities associated with persistent inflammation comprise a large group of disorders which underly a variety of human diseases, including asthma, ischemic heart disease, autoimmune diseases, cancer.

2.2. The effects of adenosine on inflammatory cell function

2.2.1. Neutrophils and adenosine

Neutrophils are the body's first line of defence against pathogens and the destruction of pathogens mediated by these cells plays a crucial role in the early stages of the inflammatory and immune response; neutrophils are capable of releasing both adenosine and ATP (Cronstein et al., 1983); thus, their function is subject to autocrine and paracrine control by exogenous adenosine. All four adenosine receptor subtypes are expressed on neutrophils, and their expression further increases under inflammatory stimuli. Changes in A_2 receptor expression on human neutrophils were found in some rheumatic diseases (Martini et al., 1991). The effects of adenosine on neutrophil recruitment from the circulation were studied in many works; at submicromolar concentrations, adenosine enhances neutrophil adhesion to the vascular endothelium by stimulation of A_1 receptors on both neutrophils (Cronstein et al., 1992; Felsch et al., 1995) and endothelial cells (Zahler et al., 1994; Zahler and Becker, 1998), while at micromolar levels adenosine inhibits adhesion of neutrophils to vascular endothelial cells through A_{2A} and A_{2B} receptors expressed by neutrophils (Eltzschig et al., 2004; Sullivan et al., 2004); endothelial A_3 receptors may also contribute to the adenosine-mediated inhibition of adhesion (Jordan et al., 1999). Neutrophil phagocytosis could be enhanced via A_1 receptors and inhibited via A_2 receptors (Salmon and Cronstein, 1990; Zalavary et al., 1994; Zalavary and Bengtsson, 1998); moreover, it was recently demonstrated that A_{2A} activation up-regulates neutrophil cycloxygenase-2 (COX-2) expression and increases PGE_2

generation, a prostaglandin with anti-inflammatory properties (Pouliot et al., 2002; Cadieux et al., 2005).

2.2.2. Monocytes/macrofages and adenosine

Mononuclear phagocytes are innate immune cells that reside in the bloodstream, as monocytes, or in various tissues, as macrophages; in contrast to neutrophils, which principally contribute to acute inflammatory responses, monocytes/macrophages are the major component of chronic inflammatory responses. Monocyte-like precursors colonize extravascular sites early during embryogenesis to become resident macrophages and acquire morphological and functional properties that are characteristic for the tissue in which they reside (for example, Kupffer cells in the liver, microglial cells in the brain). During postnatal life, circulating monocytes are capable of migrating into various tissues in response to damage or infection, where they transform into macrophages, which are the major source of inflammatory mediators during inflammatory and immune responses. All four adenosine receptor subtypes are expressed by both monocytes (Thiele et al., 2004) and macrophages (Nemeth et al., 2005), but receptor expression appear to change during maturation; whereas expression of the A_1, A_2 and A_3 receptor subtypes is relatively low in quiescent monocytes (Sajjadi et al., 1996; Fossetta et al., 2003), the expression of these receptors increases during differentiation into macrophages (Sajjadi et al., 1996; Thiele et al., 2004). Extracellular adenosine may affect monocyte adhesion during inflammation modulating adhesion molecule expression on endothelium, and several *in vivo* findings support a regulatory role for extracellular adenosine in adhesive

interactions between monocytes and the vascular endothelium (Bours et al, 2006). Many studies investigated the effects of adenosine on cytokine production by monocytes/macrophages; it has been shown that adenosine inhibits production of the potent pro-inflammatory cytokines IL-12 and TNF-α via multiple receptors, like A_{2A}, A_{2B} and A_3 receptors, and as shown in murine macrophages, adenosine inhibits LPS-mediated production of IFN-γ via A_3 receptors (Hasko et al., 2000). Macrophages phagocytosis is enhanced through A_1 receptors activation and inhibited through A_2 receptors (Salmon et al., 1993); furthermore, extracellular adenosine affects both RNS (reactive nitrogen species) and ROS (reactive oxygen species) production by monocytes/macrophages.

2.2.3. Lymphocytes and adenosine

Lymphocytes are central to humoral and cellular immune responses; B lymphocytes, originating from the bone marrow, mediate humoral immunity by producing antibodies that neutralize and eliminate extracellular pathogens; T lymphocytes are mediators of cell-mediated immunity directed against intracellular pathogens. Thymus-derived T lymphocytes are usually subclassified in CD4 expressing Th cells, which orchestrate immune responses by secreting multiple cytokines, and CD8 expressing cytotoxic T cells (CTLs), which are able to recognize and kill infected cells through either exocytosis of cytotoxic granule proteins or apoptosis-inducing membrane-bound proteins and with functions resembling those of natural killer (NK) cells, which are an additional class of lymphocytes participating in innate defence mechanisms. Adenosine seems to play an important role on modulation of T lymphocytes function; both peripheral

CTLs and Th cells express A_{2A}, A_{2B} and A_3 receptors, but A_{2A} receptors are proposed to be the predominantly expressed in peripheral T lymphocytes (Huang et al., 1997; Koshiba et al., 1999); probably, extracellular adenosine effect on these cells is mediated through A_{2A} receptor (Bours et al, 2006). It has been shown that adenosine at micromolar levels (50 μM) promotes vascular barrier function and inhibits lymphocytes transmigration (Henttinen et al., 2003); moreover, cytokine production by activated Th cells is modulated by extracellular adenosine through A_{2A} receptors (Koshiba et al., 1999), and it was demonstrated that TCR (T-cell receptor)-mediated activation of murine T cells induce a rapid up-regulation of functional A_{2A} receptors and the signalling of the up-regulated A_{2A} receptors inhibit IFN-γ release by these cells (Lappas et al., 2005). On the contrary, little is known about the influence of adenosine receptors on the function of B lymphocytes and NK cells, but it is known that B lymphocytes express A_1 and A_{2A} receptors, while NK cells express A_1, A_{2A} and A_3 receptors, and NK cell cytolytic function is enhanced by extremely low concentrations of A_1 receptor agonists and inhibited by A_{2A} receptor agonists (Priebe et al., 1990).

2.2.4. Mast cells and adenosine

Mast cells are necessary during inflammation for the development of allergic reactions, through cross-linking of their surface receptors for IgE (FcεRI), leading to degranulation and the release of vasoactive, pro-inflammatory and nociceptive mediators that include histamine, cytokines and proteolytic enzymes, while these cells are rarely seen to degranulate during auto-immune or non-immune inflammatory processes. Adenosine receptors are expressed on mast cells

but the exact pattern of receptor subtype expression depends on the source of the mast cells (human, murine, rat, etc.), so it is not unsurprising that adenosine effects on mast cells differ among species depending on the receptor involved, on the receptor expression level and on the concentration of adenosine in the tissue. An early work demonstrated that adenosine potentiates histamine release induced by antigen and other non-immunological stimuli, including compound 48/80 and calcium ionophore, from rat peritoneal mast cells (Marquardt et al., 1978); it was also shown that adenosine enhanced the histamine release and prolonged the cAMP response in purified rat peritoneal mast cells following immunological challenge suggesting an A_2 receptor mechanism (Church et al., 1985; Church and Hughes, 1985), but also A_3 receptors are probably responsible for enhancing the release of mast cell mediators (Linden et al., 1993; Salvatore et al., 1993). Several more recent studies in animal models (Tigani et al., 2000; Tilley et al., 2000) suggest that adenosine can directly activate mast cells *in vivo* in the absence of additional stimuli. It has been supposed that adenosine acts bi-modally on human lung mast cells suppressing histamine release at low concentration via A_{2A} receptors and enhancing histamine release at high concentration via A_{2B} receptors (Polosa, 2002).

3. ADENOSINE AND ASTHMA

Evidence for adenosine's role in bronchial asthma was first demonstrated more than twenty years ago when a group of asthma patients exhibited bronchoconstriction in response to aerosolized adenosine while normal individuals didn't display response (Cushley et al., 1983). Adenosine levels are increased in the bronchoalveolar lavage (BAL) fluid of asthmatics compared to normal subjects, suggesting there is an imbalance in the homeostatic mechanisms involved in purine metabolism (Driver et al., 1993); furthermore, adenosine levels are increased in the lung lavage fluid after specific allergen challenge in sensitised rabbits (Ali et al., 1991). Inhalation of allergen in atopic asthmatics increases adenosine plasma levels up to 3-fold (Mann et al., 1986); in asthmatic and non-asthmatic subjects adenosine plasma levels increase in response to exercise, but the response is significantly greater in atopic asthmatics (Vizi et al., 2002); elevated concentrations of adenosine in the lungs of adenosine deaminase-deficient mouse models have produced a lung phenotype with features of asthma (Blackburn et al., 2000; Chunn et al, 2001; Zhong et al., 2001).

The specific cellular source of adenosine in bronchoalveolar lavage fluid is unknown, but is likely to include mast cells along with epithelial cells, neutrophils and platelets (Livingston et al., 2004).

3.1. Bronchial asthma physiopathology

Asthma is a chronic pulmonary disease characterized by acute exacerbations of coughing, dyspnoea, wheezing and chest tightness; patients

usually have reduced forced expiratory volume in one second (FEV_1) as well as reduced airflow. Characteristic features of asthma are airway inflammation and bronchial hyperresponsiveness (Bousquet et al., 2000). Heightened airway responsiveness to a wide variety of pharmacological and physical agents is typical of asthma, and the degree of airway responsiveness usually correlates with the severity of the disease (Hargreave et al., 1980). A listing of clinical findings in asthma is given in Table 3; severity of asthma is based on the frequency and intensity of symptoms. The mildest grade is characterized by acute attacks upon allergen exposure, and with symptoms reversed by β-adrenergic agonists (Linssen, 1991); lung function is normal between acute attacks. More severe grades of asthma are characterized by sustained increase in airway resistance (the late-phase response), impaired basal lung function and heightened airways responsiveness to non-specific irritants (hyperreactivity); cellular inflammation is common and comprised of increased number of mast cells and eosinophils in the bronchial mucosa (Dunnill, 1960); the inflammation is believed to contribute both to the late-phase response and to the heightened airway reactivity.

Table 3: Clinical findings in bronchial asthma (adapted from Karol M.H. et al., 1994).

Coughing, wheezing, dyspnoea
Chest tightness
Airflow disturbance, occurring either immediately following or hours after exposure to allergen
Impaired basal lung function (in severe cases)
Bronchial hyperreactivity to pharmacological agents
Eosinophilic airway inflammation
Increased mast cells in the bronchial mucosa
Activated T-cells in the bronchial mucosa
Elevation of serum IgE, both antigen-specific and total

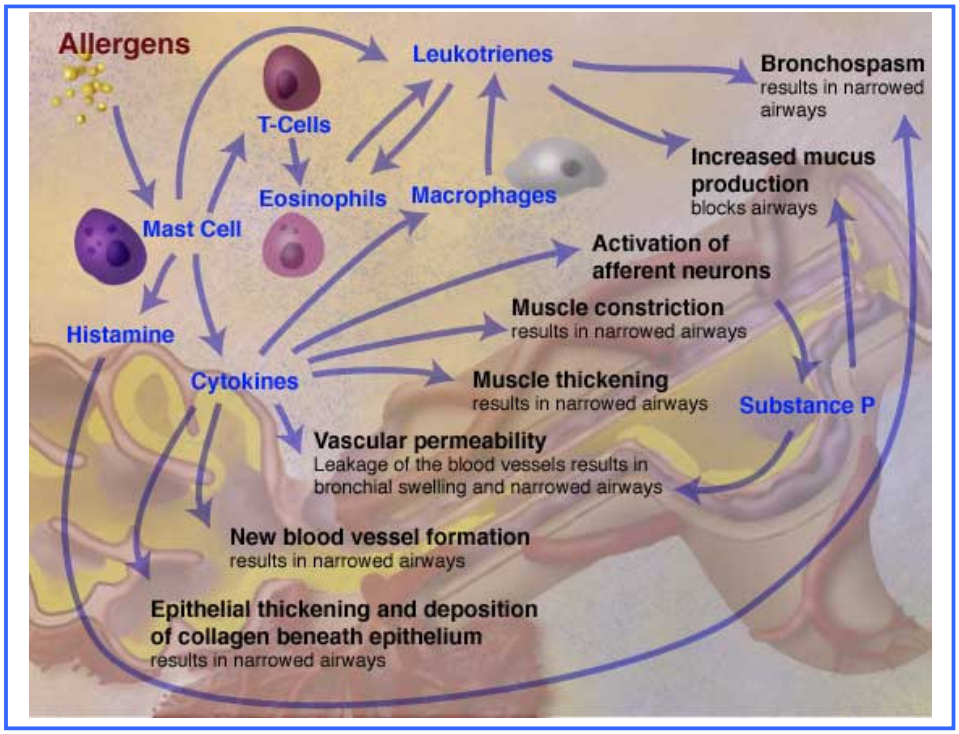


Figure 11: Asthma physiopathology. A scheme of stimuli, cells and molecules involved and their effects.

Asthma appears to be under the influence of both genetic and environmental factors. Based on the relationship between airway hyperreactivity and serum immunoglobulin E (IgE) (Burrows et al., 1989; Sears et al., 1991), it has been suggested that asthma has always an atopic component, where atopy is defined as the presence of elevated IgE (two-thirds of patients with asthma result atopic by skin tests) (Smith, 1988). Familial studies have provided evidence for a genetic component in development of allergy, and inheritance of atopy appears to be dominant and associated with a specific gene on the short arm of chromosome 11, and linkage is expressed predominantly through the mother; genes derived from the father appear not to be expressed (Young et al., 1992). Environmental exposure to the offending allergen is necessary for the expression of genes to the allergen and the level of allergen exposure governs the expression of the gene(s) (Holgate, 1993). Sensitization appears to be favoured when exposure to allergen occurs in presence of irritants, such as pollutants, or with upper respiratory viral infections, but the mechanism of this environmentally contributed adjuvant activity is unclear, it has been hypothesized to occur through inflammation and enhanced penetration of allergen through mucosal barriers (Holt, 1989).

Estabilished the pivotal role of environmental and genetic factors in the disease process, the pathogenic mechanisms of bronchial asthma remain unclear, although it is currently the most prevalent respiratory disease. Convincing evidence has been presented for humoral immunological aetiology of the early-onset response; this response is believed to result from mediators released by activated granulated cells, such as mast cells (Holgate, 1993). The rapid response is reversed by β_2-agonists, and probably results from contraction of airway smooth

muscles, which present receptors for the released mediators; the crosslinking of mast cell-bound IgE is followed by cytokine release (including tumour necrosis factor-α (TNF-α) and interleukin-4 (IL-4) from cells. Mast cells are also believed to have a central role in the subsequent inflammatory process: they are effector cells containing ready-to-release granules, and mediators released from this cell type can cause bronchial smooth muscle contraction, and induce migration of neutrophils and eosinophils to the site of degranulation; compounds preventing mast cell degranulation, like sodium cromoglycate and nedocromil sodium, have long been in use as asthma treatments (Kelly et al., 2001).

The late-phase response is characterized by inflammation; increased numbers of inflammatory cells are found among the epithelial cells and these include intact and degranulated eosinophils, lymphocytes, activated macrophages and partly degranulated mast cells (Laitinen et al., 1985; Gibson et al., 1993; Poston et al., 1992; Pesci et al., 1993). T-cells appear to have a fundamental role in the inflammation: many features of the late-phase response, including eosinophilia, mast cell activation and IgE production, appear to be regulated by T-lymphocytes that probably regulate inflammation through production of cytokines, such as IL-4 and IL-5. Atopic asthmatics have been found to possess activated T-cells in their airways (Robinson et al., 1992); additional evidence of T-cell regulation of the inflammatory response has been obtained from animal studies (Iwamoto et al., 1993). Recently, it has been demonstrated that goblet cell hyperplasia precedes the inflammatory infiltrate and persists even after the number of inflammatory cells decreases, indicating that some of the phenotypic changes in airway epithelium are not caused by inflammation (Blyth et al., 1996).

In asthma, remodelling is almost always present in biopsies, as shown by collagen deposition on the reticular basement membrane, but is not always clinically demonstrated, while the destruction and subsequent remodelling of the normal bronchial architecture are manifested by an accelerated decline in FEV_1; this irreversible component of the airway obstruction is more prominent in severe patients and even persists after an aggressive treatment (Bousquet et al., 2000).

Animal models are typically developed for either elucidating pathogenic mechanisms of bronchial asthma and/or testing new antiasthmatic drugs, in fact they are necessary to permit prediction of the efficacy of new chemicals before the latter reach large scale production; animal models have been able to reproduce several symptoms and features (airway hyperresponsiveness, elevated IgE or other anaphylactic antibody class, pulmonary eosinophilia, mast cell and/or eosinophil derived products in the bronchial mucosa or bronchoalveolar lavage, diaphragmatic contractions, cyanosis, lung resistance, microvascular leakage of proteins, elevated ratio of interleukin-4 and/or interferon-γ, increased airway resistance, abnormal airflow, airway mucus and airway smooth muscle hypertrophy), reproducing both the acute airway spastic response associated with asthma, as well as the late-onset inflammatory reactions. Animal models can be particularly effective in elucidating factors associated with development of airway sensitivity and expression of responses, enabling identification of the importance of antigen dose (or extent of antigen exposure) to the processes of sensitization and elicitation of responses (Karol et al., 1987; Karol, 1983), and they are ideally suited to investigation of the role of environmental factors and of genetic predisposition in development of asthma. With all the benefits to be derived from

the development and use of animal models for study of asthma, there exist caveats: attention must always be given to identify differences between animal and human systems; anatomical, physiological and biochemical differences exist between species. Characteristics, such as airway morphometry, breathing patterns, innervation of respiratory tract tissue, and circulation to the lung, are critical to sensitization and to asthma; these factors, along with immunological components, must be considered in the evaluation of an animal model.

3.2. Adenosine-induced bronchoconstricition

Bronchoconstrictor responses to adenosine have been studied in humans, guinea pigs, rabbits and rats. In general, normal guinea pigs and rabbits do not respond to inhaled adenosine and only sensitised guinea pigs and allergic rabbits show bronchoconstrictor responses; the responses of rat airways to adenosine in vivo are very much dependent on the strain of rat but are usually markedly increased hours after sensitisation of the airways with allergen (Fozard and Hannon, 2000). Inhalation of aerosolised adenosine (or the more soluble AMP) results in bronchoconstriction in allergic and non allergic asthmatic human subjects. This airway response, confirmed also following intravenous adenosine administration (Drake et al., 1994), is not seen in non atopic non asthmatic subjects, but atopic non asthmatic subjects show a variable response with some demonstrating bronchoconstriction (Cushley et al., 1983; Cushley et al., 1985).

Guanosine (a structurally related molecule) and inosine (an adenosine metabolite) have no effect on airway calibre suggesting that the response is specific to adenosine; ADP and AMP are equipotent in terms of

bronchoconstriction (Mann J.S. et al., 1986), and instillation of AMP directly into the airway segment of asthmatics via the bronchoscope has been shown to cause a rapid reduction of airway calibre, which is paralleled by the release of histamine, tryptase, and PGD_2 (Finney et al., 1985).

Interestingly, significant (2–3-fold) increases in circulating adenosine levels (but not inosine) were reported after inhaled allergen and methacholine challenge in atopic asthmatics (Mann et al., 1986); the kinetics of the observed increases in plasma adenosine after challenge were similar for allergen and methacholine, despite dissimilar time courses for airflow obstruction. Allergen induced increase in plasma adenosine (2–5 min after provocation) continued to rise in parallel with the onset of bronchoconstriction; with methacholine, plasma adenosine concentrations continued to rise up to 30 min after provocation, long after peak bronchoconstriction (5 min) and when the airway was starting to recover.

Dipyridamole, an adenosine uptake inhibitor, has been shown to enhance adenosine-induced bronchoconstriction in asthmatic subjects suggesting a cell surface receptor-mediated mechanism (Crimi et al., 1988). Further evidence came from work showing that theophylline (an adenosine receptor antagonist) provided protection against adenosine-induced bronchoconstriction; this protection was 2–5 times greater than that produced against histamine-induced bronchoconstriction (Cushley et al., 1984; Mann et al., 1985).

Mechanisms underlying bronchoconstriction have been explored by several *in vitro* and *in vivo* studies but results were often conflicting due mainly to differences in bronchial response among species and to differences in the basal

tone of airway smooth muscle (Polosa et al., 2002). *In vitro*, adenosine weakly contracts human and guinea-pig airways maintained at basal tone but relaxes guinea pig airways pre-contracted with carbachol (Advenier et al., 1982; Finney et al., 1985), and it has been observed that contraction induced by adenosine is more marked in isolated airway from asthmatics than non asthmatics (Bjorck et al., 1992).

Several studies suggest that the bronchial response to adenosine is not due to a direct effect, but most likely to mast cell degranulation; a first correlation between adenosine receptors and mast cells was already observed in 1978 when Marquardt reported that adenosine, although ineffective alone, potentiated histamine release induced by anti-immunoglobulin E, concanavalin A, compound 40/80 and by the calcium ionophore A23187 in isolated rat mast cells (Marquardt et al., 1978). Given the role of mast cells in asthma, the involvement of adenosine receptors in mast cell degranulation has raised great interest and has been widely explored. *In vitro* studies indicate that adenosine enhances the release of histamine and other preformed mediators from immunologically primed rodents mast cells (Marquardt and Wasserman, 1982); the increase of histamine release from rat peritoneal mast cells is insensitive to methylxanthines thus suggesting that this process might be mediated by a receptor with low affinity for xanthines, such as the adenosine A_3 receptor (Zhou et al., 1992). It is important that biochemical characteristics of mast cells and response to pharmacological agents can vary among species and among anatomical sites and there is recent evidence that adenosine A_{2B} receptors mediate the release of inflammatory mediators. Adenosine is able to increase mediator release from human mast cells obtained

with mechanical dispersion or enzymatic dispersion of lung, with leukotriene C_4 that is one of these mediators (Peachell et al., 1988), and same results have been shown on mast cells obtained from bronchoalveolar lavage (Forsythe et al., 1999). In parenchymal human lung mast cells obtained from surgical specimens, adenosine does not induce the release of histamine directly, but only when mast cells are immunologically activated (Peachell et al., 1991). Evidence for a role of mast cells in bronchoconstriction induced by adenosine is also available from *in vivo* studies; inhalation of AMP in asthmatics increases circulating levels of histamine (Phillips et al., 1990). Agents blocking mast cells degranulation, such as sodium cromoglycate and nedocromil sodium, inhibit bronchoconstriction induced by AMP in asthmatics and, in addition, pre-medication with the H_1 histamine receptor antagonists, terfenadine and astemizole, inhibits bronchoconstriction induced by AMP in the asthmatic subjects (Rafferty et al., 1987; Richards et al., 1988; Phillips et al., 1989a; Phillips et al., 1989b). Instillation of AMP in asthmatic bronchi or in the nose of patients with allergic rhinitis increases histamine and tryptase concentrations in the lavage fluid (Polosa et al., 1995; Polosa et al., 1999), whereas inhaled heparin, by means of inhibition of mast cells activation, attenuates airway response to AMP (Polosa and Holgate, 1997) and the response induced by nasal provocation with AMP (Zeng et al., 2004). Adenosine also stimulates the release of other mediators from mast cells, including prostanoids, since bronchoconstriction induced by AMP is attenuated by indomethacin, flurbiprofen and lysine acetylsalicilate (Phillips et al., 1989a; Phillips et al., 1989b; Crimi et al., 1995). Increased concentration of prostaglandin D_2 has also been found in bronchoalveolar lavage after instillation of adenosine

(Polosa et al., 1995); furthermore, a role for cysteinyl-leukotrienes in AMP-induced bronchoconstriction has also been suggested since montelukast, a leukotriene receptor antagonist, attenuates acute AMP-induced bronchoconstriction (Rorke et al., 2002).

Activation of neural pathways (cholinergic and peptidergic) is suspected to contribute to the contractile airway response to adenosine; the involvement of cholinergic reflexes has been supposed following the observation that in rat and in man inhaled ipratropium bromide attenuates bronchoconstriction induced by AMP (Polosa et al., 1991). The neural contribution to AMP-induced bronchoconstriction is also suggested by the observation that inhaled furosemide and bumetanide, loop diuretics modulating sensory nerve responses in the airways, inhibit bronchial response to AMP (O'Connor et al., 1991; Polosa et al., 1993). Furthermore, in guinea-pigs *in vivo* bronchoconstriction induced by adenosine is attenuated by treatment with capsaicin, a sensory nerves depleting agent (Manzini and Ballati, 1990). The role of neuropeptides in adenosine-induced bronchoconstriction is also supported by the observation that repeated challenges with inhaled bradykinin (a model of neuropeptides depletion in human airways) attenuate the bronchial response to adenosine (Polosa et al., 1992), and it was proved that in rat pulmonary circulation adenosine induces vasoconstriction by activation of neuropeptides-producing nerves (Meade et al., 1996). Recently it has been speculated that, since both bradykinin B_2 and adenosine receptors have been identified on mast cells and on peptidergic nerves, adenosine and bradykinin may share a common activation pathway through the release of neuropeptides known to activate mast cells (Rajakulasingam et al., 1994; Holgate, 2005).

However, inhibition of neutral endopeptidase (NEP) induced by inhaled phosphoramidon failed to elicit any significant enhancement of the bronchial response to AMP, suggesting that release of endogenous neuropeptides has little importance in the airway response to adenosine (Polosa et al., 1997).

After all, it was speculated that in asthma adenosine stimulates mast cells to degranulate and to release histamine, which causes an additive effect through vagal nerve stimulation (Polosa, 2002); but although adenosine-induced mast cell degranulation is an important component of the airway response to adenosine, it is likely not the complete mechanism, and so further investigation is required.

More recent publications have also used AMP as a bronchoprovocant (Lee et al., 2004; Luijk et al., 2004; Zeng et al., 2004), as it is rapidly broken down to adenosine and is more soluble than the parent nucleoside alone but with the same effect (Mann et al., 1986); this selectivity makes it an attractive target for diagnostics, in fact measuring bronchoconstriction with inhaled AMP provides a useful and non invasive means of diagnosis (Van den Berge et al., 2004a). In addition, recent evidence suggest that bronchial challenge with inhaled AMP may be an useful tool to asses and monitor airway inflammation; bronchial hyperresponsiveness to AMP correlates better than bronchial hyperresponsiveness to histamine or methacholine (the two most commonly used agents to assess bronchial hyperresponsiveness) with markers of airway inflammation such as sputum, blood and bronchial tissue eosinophilia and exhaled nitric oxide (eNO) (van den Toorn et al., 2001; De Meer et al., 2002; Van den Berge et al., 2004b). Control of airway inflammation is a major target of the pharmacological treatment in asthma, and if changes in bronchial response to AMP reflect fine changes in

airway inflammation, bronchial challenge with AMP would provide an excellent tool to follow these changes and to assess the efficacy of the anti-inflammatory treatment. In fact, early studies have shown that regular treatment with inhaled steroids reduces airway response to AMP as compared to methacholine (O'Connor et al., 1992; Wilson and Lipworth, 2000), while more recently it was shown that in asthmatics bronchial response to inhaled AMP is sensitive to inhaled steroid budesonide after one week treatment; whereas, changes in bronchial response to methacholine together with changes in percentage eosinophil and epithelial cell counts in the sputum can be observed only by the fourth week of treatment (Prosperini et al., 2002).

3.3. Adenosine and airway inflammation

A great amount of works has been done in the last decade to understand the functional role of adenosine in airway inflammation and the involvement of this nucleoside in several aspects of inflammation, such as recruitment of inflammatory cells, release of inflammatory mediators, airway remodelling and mucus secretion. Above all, endogenous released adenosine is a potent regulator of airway inflammation, particularly of allergic inflammation; human lung *in vitro* releases adenosine upon stimulation with allergens in presence of inhibitors of adenosine deaminase (Konnaris et al., 1996). In atopic asthmatics bronchial challenge with allergens increases blood levels of adenosine (Mann et al., 1986) and exercise-induced bronchoconstriction is also followed by an increase in circulating adenosine levels (Vizi et al., 2002); increase in adenosine concentration was also found in the bronchoalveolar lavage of patients with

asthma. In addition to increase mast cells activation, adenosine promotes release of inflammatory cytokines from human monocytes (Le Vraux et al., 1993) and smooth muscles, leukocytes chemotaxis via adenosine A_{2B} receptors and eosinophils recruitment and activation via adenosine A_3 receptors (Young et al., 2004). The development of non-invasive techniques to assess airway inflammation, such as evaluation of soluble mediators in exhaled breath, has further confirmed the involvement of adenosine in airway inflammation. In fact, increased levels of adenosine have been found in exhaled breath condensate of steroids-naive asthmatics as compared to healthy subjects and steroids treated patients (Huszar et al., 2002); adenosine levels also increase in exhaled breath condensate during exercise-induced bronchoconstriction in asthmatics, but not in healthy subjects (Csoma et al., 2005).

Although the precise source of adenosine release (mast cells, smooth muscle, epithelial cells) remains uncertain, it is likely that adenosine may contribute to the bronchoconstriction induced by other bronchoprovocants such as allergens (Huszar et al., 1998); it is well known that adenosine potentiates the release of inflammatory mediators when human mast cells are immunologically primed (Peachell et al., 1991) and therefore it has been recently emphasized the link between airway response to adenosine and the state of atopy; in fact, a number of studies indicate that atopic asthmatics are significantly more responsive to inhaled AMP than non atopic asthmatic (Spicuzza et al., 2003).

The notion that inflammatory cytokines can regulate adenosine receptors expression (Khoa et al., 2001; Xaus et al., 1999) further suggests a role for adenosine in the inflammatory environment; furthermore, in transgenic animals

the severity of airway inflammation and lung remodelling is amplified by the release of adenosine (Blackburn et al., 2003), confirming that this mediator might serve a regulatory role in lung inflammatory diseases. It was recently investigated on adenosine receptors and cell types mediating the pro-inflammatory and anti-inflammatory effect of adenosine in the lung by the means of an amino deaminase deficient model of mice (Tilley and Boucher, 2005); in this model, inflammatory effects of adenosine are mediated by the A_3 receptors involved in mast-cell dependent increase in vascular permeability, adenosine-induced mast cells degranulation, antigen-induced mast cells degranulation, mucus secretion and recruitment of inflammatory cells in the lung. A_{2B} receptors also contribute to mast cells degranulation, while A_1 and A_3 receptors present on macrophages mediate the antiinflammatory effects of adenosine through an increase in the release of anti-inflammatory mediators (IL-10 and PGE_2) and inhibition in the release of pro-inflammatory mediators (TFN-α and matrix metalloproteinase).

There is opinion that adenosine accumulation in the lung is not only a product of lung inflammation and damage, but can directly affect signalling pathway that lead to features of chronic lung disease (Blackburn, 2003). Some authors have speculated that the pro- and anti-inflammatory properties of adenosine may be dictated by its level in the lung: in the initial stage, low levels of adenosine would activate high affinity receptors, such as adenosine A_{2A} receptors, triggering a protective pathway, but when lung inflammation is severe, high levels of adenosine release would activate the low affinity adenosine A_{2B} receptors triggering injurious signaling pathways that further exacerbate inflammation (Polosa et al., 2000; Blackburn, 2003).

Following evidence on the role of adenosine in inflammation, it has been suggested that targeting adenosine receptors might be a valuable approach for the development of antiinflammatory treatments in diseases characterized by chronic airway inflammation, such as asthma. Although some selective agonist/antagonist are currently under development, one major problem is to establish which receptor/s should be targeted; indeed, distinct pro- and anti-inflammatory functions of adenosine are likely to be dependent on the dynamic regulation of specific adenosine receptors on specific cell types in a given inflammatory environment; moreover, as yet said, adenosine receptors are widely spread in human body, therefore a selective targeting of the site of action should be granted.

3.4. Adenosine receptors in airways

In the respiratory system all four adenosine receptors are expressed, and these are present on inflammatory/immune cells (mast cells, macrophages, lymphocytes, fibroblasts, eosinophils and neutrophils) recruited into the site of inflammation as well as in some structural components of lungs, such as airway smooth muscle, epithelium and secretive cells (Polosa et al., 2002) (Figure 12).

It is now clear that the variety of cellular responses induced by adenosine in airways are mostly mediated through interaction with its receptors on the surface of target cells (Olah and Stiles, 1995). The ability of the adenosine uptake inhibitor, dipyridamole, to enhance adenosine-induced effects leads to the suggestion that its actions are likely to be mediated through stimulation of specific cell surface located receptors (Stafford, 1966; Crimi et al., 1988); in addition, theophylline, a drug that is frequently used for resolution of airway obstruction in

asthma, is known to attenuate adenosine-induced bronchoconstriction through an adenosine receptor antagonistic activity at therapeutic plasma levels (20-120 mM) (Polosa, 2002). In the early '80s, understanding of adenosine receptors was simply based on the activity of "specific" agonists/antagonists and limited to a division between A_1 receptors (which decreased intracellular cyclic AMP (cAMP) levels) and A_2 receptors (which increased intracellular cAMP) (Van Calker et al., 1979; Londos et al., 1980). Suggestion that the adenosine A_1 receptor antagonistic activity of theophylline might explain its clinical potency fell down when it was discovered that another xanthine, enprofylline, was clinically effective as theophylline although devoid of adenosine A_1 receptor antagonistic activity. Enprofylline was also able to block adenosine-induced bronchospasm in asthmatic subjects and, despite its lack of A_1 receptor antagonistic activity, was more potent in this respect than theophylline; this paradox, named “enprofylline paradox”, was at that time considered to be evidence against adenosine receptor antagonism as an explanation of the clinical efficacy of xanthines. In the ’90s, information about new adenosine receptor subtypes provided a possible explanation for the enprofylline paradox, in fact the application of molecular cloning techniques has expanded the range of known adenosine receptors to include the A_3 receptor (Salvatore et al., 1993) and the A_{2B} receptor, whereas the originally described A_2 receptor now was designated A_{2A} (Pierce et al., 1992). Enprofylline (as well as theophylline) inhibits ligand binding to the human recombinant adenosine A_{2B} receptor with a Ki of ~ 7 mM (Robeva et al, 1996; Linden et al., 1999), a value which lies within the typical plasma levels of enprofylline (5–25 mM) after therapeutic dosage. However, the fact that enprofylline has little affinity with the

A_1 receptor does not mean that the A_1 receptor has not any role in asthma; indeed, an antisense oligonucleotide against the adenosine A_1 receptor was under investigation for asthma therapy (Metzger and Nyce, 1999).

Description of adenosine A_1 receptor expression and distribution has been widely carried out in mammals; binding data indicate that A_1 receptor is not particularly abundant in normal human lung, but its presence, possibly associated with nerves, is supported by functional studies; this subtype is also present on human neutrophils, its activation promotes chemotaxis (Cronstein et al., 1990) and increases adherence to endothelial cells (Cronstein et al., 1990; Felsch et al., 1995). With specific regard to asthma, it was shown that rabbits immunised at birth with antigen develop airways hyperreactivity to adenosine by a mechanism involving upregulation of A_1 receptor (Ali et al., 1994; el-Hashim et al., 1996). Successively, in an elegant extension of these studies, using rabbits engineered with an antisense oligo-deoxynucleotide (ODN) targeted against adenosine A_1 receptors of the lung to reduce their numbers, it was confirmed that adenosine-mediated bronchoconstriction is mediated by this receptor subtype (Nyce and Metzger, 1997). An early study showed that in the airways of non asthmatic subjects there was not evidence of A_1 adenosine receptors (Joad and Kott, 1993); conversely, another study shown that adenosine–induced contraction of isolated bronchi from asthmatic patients was mediated, via adenosine A_1 receptors, by histamine and leukotrienes (Bjorck et al., 1992). More recently, studies performed with radiobinding ligands have shown the presence of A_1 adenosine receptors on cultured human airway smooth muscle cells (Zhong et al., 2004); moreover, elevated transcript levels for adenosine A_1 receptor, particularly in alveolar

macrophages, were found in a model of amino deaminase-deficient mice, and genetic removal of these receptors resulted in an increased lung inflammation and injury with mucus metaplasia and alveolar destruction (Sun et al., 2005). According to this evidence, adenosine A_1 receptor seems to play a dual role in the lung probably due to the pattern of its distribution; however, its role in asthma is still unclear.

Both A_{2A} and A_{2B} receptors have been identified by reverse-transcriptase polymerase chain reaction (RT-PCR) on human bronchoalveolar lavage mast cells (Feoktistov and Biaggioni, 1998; Suzuki et al., 1998); the subunits of G protein (Gs) coupled adenosine A_{2A} and A_{2B} receptors are distinguished by their high and low affinity, respectively, for adenosine (Feoktistov et al., 1998). The A_{2A} receptors most relevant to human lung disease are those expressed on mast cells (Feoktistov and Biaggioni, 1998; Suzuki et al., 1998), neutrophils (Varani et al., 1998) and T-cells (Varani et al., 1997). In contrast to A_{2B} receptors, A_{2A} activation results in suppression of histamine and tryptase release from human mast cells (Hughes et al., 1984; Peachell et al., 1991; Suzuki et al., 1998), and this could provide a balanced control mechanism, because it is possible that at low concentrations of adenosine, only the cellular signalling provided by engagement of the high affinity A_{2A} receptors prevails, thus down-regulating mast cell mediator release, and conversely, in situations in which high concentrations of adenosine are reached, such as in asthma (Driver et al., 1993), the relative importance of the low-affinity A_{2B} receptor becomes greater with significant mast cell degranulation. In addition, it is well known that stimulation of A_{2A} receptors reduces neutrophil adherence to the endothelium (Cronstein et al., 1992), prevents

upregulation of integrin expression on FMLP (N-formyl-methionyl-leucyl-phenylalanine) stimulated neutrophils (Wollner et al., 1993) and inhibits degranulation of activated neutrophils and monocytes (Bouma et al., 1994; Fredholm et al., 1996; Hannon et al., 1998). A first evidence of the potential antiinflammatory role of adenosine A_{2A} receptors in airways derives from a study in an *in vivo* model of airway inflammation in which inhibition of leukocytes accumulation induced by methrotrexate is blocked by the selective adenosine A_2 receptor antagonist DMPX (Cronstein et al., 1993). More recently, it has been shown that, in sensitised Brown Norway rats, the inflammation induced by ovalbumin, consisting of an increase in leukocytes, protein content and eosinophils peroxidase activity in bronchoalveolar fluid, was dose-dependently inhibited by the adenosine A_{2A} receptor agonist CGS21680, and this effect was similar to that obtained with the glucocorticoid budesonide (Fozard and McCarthy, 2002). Administration of an A_{2A} receptor agonist therefore exhibited antiinflammatory potential in a disease such as asthma, where inflammation is strongly implicated, but unfortunately these receptors have a broad anatomical distribution with many effects through the body, including inhibition of platelet aggregation, vasodilatation and a variety of effects on the central nervous system (Ledent et al., 1997); these side effects represent the major limitation for the introduction of similar compounds in clinical trials, and so a selective topical action in the lung is required.

Adenosine A_{2B} receptors, as yet shown, display a lower affinity for adenosine and agonists, as compared to adenosine A_{2A} receptors. Although similarities in their structure and ability to increase intracellular cAMP have been

shown, the functional role of the two receptors is different, probably due to the fact that adenosine A_{2B} receptors can activate other intracellular pathways in addition to cAMP; actually, in contrast to A_{2A} receptors, stimulation of A_{2B} receptors can activate phospholipase C in human mast cells and in mouse bone marrow-derived mast cells (Marquardt et al., 1994; Feoktistov and Biaggioni, 1995). The discovery of adenosine A_{2B} receptors in the airways has raised great interest, and the presence of these receptors has now helped to explain some of adenosine physiological effects that had remained previously unexplained (Polosa et al., 2002; Holgate, 2005). Expression of these receptors was found in bronchial epithelium (Clancy et al., 1999), in cultured human airway smooth muscle (Mundell et al., 2001), in human mast cells (Marquardt et al., 1994), monocytes (Zhang et al., 2005) and fibroblasts (Zhong et al., 2005). Increasing evidence suggest that in rodents and man activation of adenosine A_{2B} receptors modulates mast cell function; early studies on mast cells from mouse bone marrow showed that the ability of adenosine to cause mast cell degranulation was not affected by the selective adenosine A_{2A} receptor agonist CGS 21680 (Marquardt et al., 1994). It was later shown that adenosine A_{2B} receptors have a pro-inflammatory role, participating in the remodelling process occurring in chronic inflammatory lung diseases (Feoktistov et al., 2001; Zhong et al., 2004; Zhong et al., 2005), as confirmed by a study showing that activation of these receptors up-regulates several cytokines (IL-3, IL-4, IL-8 and IL-13) in mast cells and promotes IgE synthesis by lymphocytes B (Ryzhov et al., 2004a; Ryzhov et al., 2004b). This evidence suggests that adenosine A_{2B} receptors are deeply involved in the

mechanisms underlying mediator release by mast cells and A_{2B} antagonists with high selectivity hold a strong therapeutic potential.

A_3 adenosine receptors are the most recently discovered among adenosine receptors; both *in vitro* (Ramkumar et al., 1993; Thorne et al., 1996) and *in vivo* (Fozard et al., 1996; Shepherd et al., 1996) studies have established that activation of A_3 receptors results in mast-cell degranulation and/or enhancement of degranulation in response to allergen in a variety of rodent species, but unfortunately the distribution of these receptors in rodents does not reflects distribution in humans; in fact, A_3 receptor protein was not found in human mast cells, while it was found high density of this adenosine receptor in eosinophils both in the blood (Kohno et al., 1996; Knight et al., 1997) and in the airways (Walker et al., 1997), and activation of A_3 receptors on human eosinophils mediates inhibition of degranulation and superoxide anion release (Ezeamuzie and Philips, 1999). Adenosine A_3 receptors are also expressed in human neutrophils (Bouma et al., 1994) and their activation inhibits neutrophil degranulation induced by endotoxin (Gessi et al., 2002). Moreover, human lymphocytes express adenosine receptors with pharmacological and biochemical profile typical of the human A_3 receptor subtype (Gessi et al., 2004). It was shown that mRNA and protein of adenosine A_3 receptor are increased in lung from asthmatics (Walker et al., 1997). However, the functional role of these receptors in the asthmatic lung is unclear; in fact, it has been speculated that specific adenosine A_3 receptor agonists might be useful in eosinophil-dependent allergic diseases such as asthma and rhinitis (Walker et al., 1997); conversely, it has been more recently shown that A_3

receptor signalling contributes to airways inflammation and lung eosinophilia in adenosine deaminase-deficient mice (Young et al., 2004).

In conclusion, a detailed characterisation of adenosine receptor subtypes has been conducted in human and rodents lung tissues and isolated bronchoalveolar lavage cells to define their pharmacological role in adenosine-induced responses in airways; however, the limited specificity of available adenosine receptor agonists and antagonists (Collins and Hourani, 1993; Olah and Stiles, 1995; Klotz et al., 1998) and conflicting studies results make difficult to define the adenosine receptor subtype responsible for adenosine-induced bronchoconstriction and inflammatory responses, but likely more than one receptor subtype is involved.

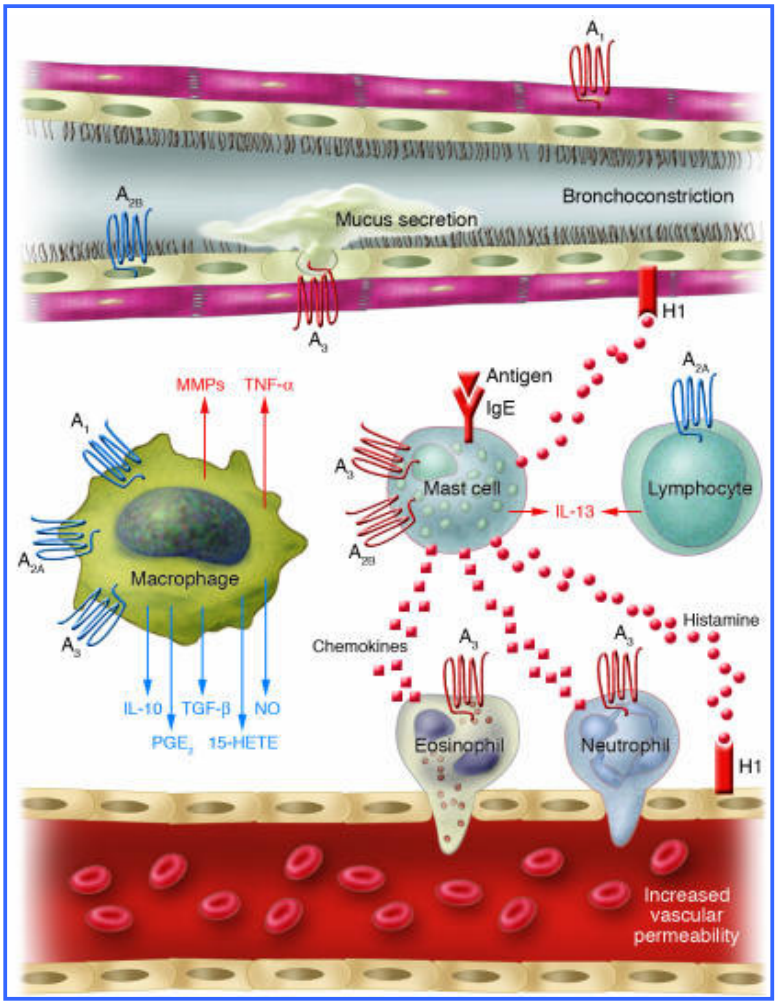


Figure 12: Adenosine receptors in the respiratory system.

4. ADENOSINE AND CARDIOPROTECTION

In previous years, a protective effect on the injury caused by ischemia and reperfusion in the heart was attributed to adenosine, and these effects were documented in different experimental *in vivo* and *in vitro* models (Donato and Gelpi, 2003). Moreover, adenosine or adenosine receptor agonist administration prior to ischemia reduces the size of the infarction, improves the recovery of the ventricular function during reperfusion and prolongs the time period to the ischemic contracture (Donato and Gelpi, 2003). However, focusing on a potential therapeutic application it is important to understand the mechanisms involved in adenosine cardioprotection.

4.1. Ischemic and reperfusion injury of the myocardium

Acute myocardial infarction resulting from coronary artery occlusion is a common cause of morbidity and mortality worldwide; the therapeutic strategy is, therefore, to rapidly restore coronary flow using thrombolysis or percutaneous angioplasty to salvage as much ischemic myocardium as possible (Tissier et al., 2008).

Recent developments in immunology and cell biology have demonstrated the importance of inflammation in the pathogenesis of post-ischemic organ dysfunction. Whereas prolonged ischemia causes anoxic cell death, recent evidence suggests that sublethal injury may be amplified by inflammatory and cytotoxic injury cascades activated during the reperfusion period. Ischemia is a state of tissue oxygen deprivation accompanied by a reduced washout of the

resulting metabolites (Star, 1998); reperfusion is the restoration of blood flow to the ischemic tissue. Despite the unequivocal benefit of reperfusion of blood to an ischemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue (Bonventre, 1993); in fact, reperfusion injury has been well described in the literature to cause organ damage in the brain, heart, lungs, liver, kidneys and skeletal muscle. The susceptibility of tissue to ischemia reperfusion injury (IRI) is a major obstacle to both reperfusion after an infarct and successful organ transplantation.

A growing body of evidence, primarily from animal models and preliminary human studies, has revealed that inflammatory mechanisms play a major role in the pathogenesis of ischemia/reperfusion injury, leading to the identification of multiple inflammatory mediators, including cytokines. The pathophysiology of the IRI is complex because the inflammatory aspect includes both cellular and humoral components, and mechanisms may be organ-dependent, with similar but distinct pathways involved in different organs. It was supposed that blood flow interruption to the tissue produces the accumulation of anaerobic metabolites and free radicals, but oxygen available with reperfusion generates reactive oxygen species (ROS) with oxidative cell damage, subsequent synthesis of cytokines and leukocyte-mediated tissue injury (Chamoun et al., 2000).

4.2. Adenosine and cardiac ischemia/reperfusion injury

Several biochemical substances released from the ischemic myocardium are capable of protecting the heart against ischemic insult (Parratt, 1993), and adenosine has cardioprotective effects in myocardial ischemia (Ely and Berne,

1992; Lasley and Mentzer, 1992; Thornton et al., 1992). It is known that adenosine concentration in the coronary effluent is markedly increased after ischemia (Kitakaze et al., 1993), and this nucleoside acts as an effector for myocardial cells, sympathetic nerve cells, endothelial cells, leukocytes and platelets, dysfunction of which may result in cellular injury (Hori and Kitakaze, 1991). At the present, the exact source of extracellular adenosine in the injured tissue is not well defined, but likely results from a combination of intracellular metabolism and amplified extracellular phosphohydrolysis of adenine nucleotides via ecto-nucleotidases (Berne, 1980; Obata, 2002). There is evidence that myocardial ischemia also increases catecholamine levels (Dart et al., 1984; Rona, 1985; Schomig, 1989; Strasser et al., 1992; Obata et al., 1994), and the stimulation of α_1-adrenoreceptors by endogenous norepinephrine with subsequent activation of protein kinase C (PKC) can increase adenosine production via activation of ecto-5'-nucleotidase; furthermore, increased nitric oxide (NO) facilitates the production of adenosine via cGMP-mediated activation of ecto - 5'-nucleotidases (Obata, 2002).

Adenosine has been implicated to protect against lethal reperfusion injury; in fact, it has been demonstrated that treatment with adenosine during reperfusion reduce infarct size in pre-clinical experiments and clinical trials (Gross and Auchampach, 2007). Adenosine, has also been shown to be involved in the cardioprotection due to ischemic pre-condictioning (Lankford et al., 2006) .

4.3. Adenosine receptors and cardioprotection

All four adenosine receptors are expressed in different cell types of the heart and vessels, and thus the effects of adenosine and adenosine receptors on

myocardial responses to ischemia and reperfusion may not necessarily reflect direct myocyte responses but indirect actions at other cell types, including inflammatory cells; it was noted that A_1 and A_{2A} receptors are expressed in adult ventricular myocytes (Marala and Mustafa, 1998; Kilpatrick et al., 2002), while presently there is no definitive evidence that functional A_3 or A_{2B} proteins are expressed in adult mammalian myocytes. A_1 receptor is the most extensively studied adenosine receptor subtype within the context of cardiac protection, but the A_3 receptor was recently found to trigger protective responses similar to those for A_1 receptor (though via distinct signaling); more recently, potential protective roles of A_{2A} and A_{2B} receptors attracted attention (Peart and Headrick, 2007).

Protection with A_1 receptor agonism was observed in all species examined, and recently a non-redundant role for endogenous A_1 receptor activation in dictating intrinsic ischemic tolerance (the heart ability to withstand cellular injury and death during ischemic insult) was exposed through analysis of A_1 receptor knockout mice (Reichelt et al., 2005; Morrison et al., 2006); it was shown that ischemia inhibits myocardial A_1 receptor trascription in mice (Ashton et al., 2003a; Ashton et al., 2003b), but further evidence is required.

The two A_2 subtypes (A_{2A} and A_{2B} receptors) are poorly understood in the context of myocardial ischemia/reperfusion, but are recently emerging as modulators of cardiovascular stress responses and, particularly, of inflammatory processes. In fact, both A_2 subtypes possess important immunomodulatory functions and inflammation is important in both early and late aspects of injury and remodelling following ischemia/reperfusion; thus, these receptors can regulate the impact of inflammatory processes on ischemic and post-ischemic

damage. A_{2A}-dependent cardioprotection includes inhibition of leukocyte-dependent inflammatory processes (Visser et al., 2000; Sullivan et al., 2001), and possibly a direct inotropic action (Dobson and Fenton, 1997) which appears selective for post-ischemic tissue (Lasley et al., 2001); enhanced vasodilatation during reperfusion may also provide benefit with A_{2A} agonism in experimental settings (Maddock et al., 2001; Peart et al., 2002), although with species differences (Peart and Headrick, 2007).

Proposed protective or beneficial effects of the A_{2B} receptor in ischemic/reperfused hearts could stem from responses mediated in other cell types; in fact, the A_{2B} receptor is known to activate angiogenic factors (Feoktistov et al., 2002; Feoktistov et al., 2003) and trigger coronary endothelial growth (Dubey et al., 2002), while molecular targeting of this receptor impairs neo-vascularization in non-cardiac tissues (Afzal et al., 2003); thus, the A_{2B} receptor may play a key role in modulation of vascular growth and tissue remodelling. Acute genetic manipulation of this subtype expression in cardiac fibroblasts supported the hypothesis for a role as anti-proliferative and antifibrotic (Chen et al., 2004), congruent with effects of A_{2B} receptor agonism in cardiac fibroblasts (Dubey et al., 2001). Thus, the adenosine A_{2B} receptor may play a role in dictating the progression of post-ischemic changes in myocardial phenotype and post-ischemic remodelling, limiting potential fibrosis and facilitating angiogenic growth (Wakeno et al., 2006).

Throughout the 10 years since cloning and characterization of A_3 receptor, this subtype has been shown to mediate cardioprotective effects in multiple species and models, but pronounced heterogeneity exists in the pharmacology of

A_3 agonists and antagonists across species (Muller, 2003), complicating interpretation and extrapolation of experimental findings; however, protection via this subtype appears to share some similarities with the A_1 receptor but precisely where and how it mediates its protective actions remains unclear (Peart and Headrick, 2007).

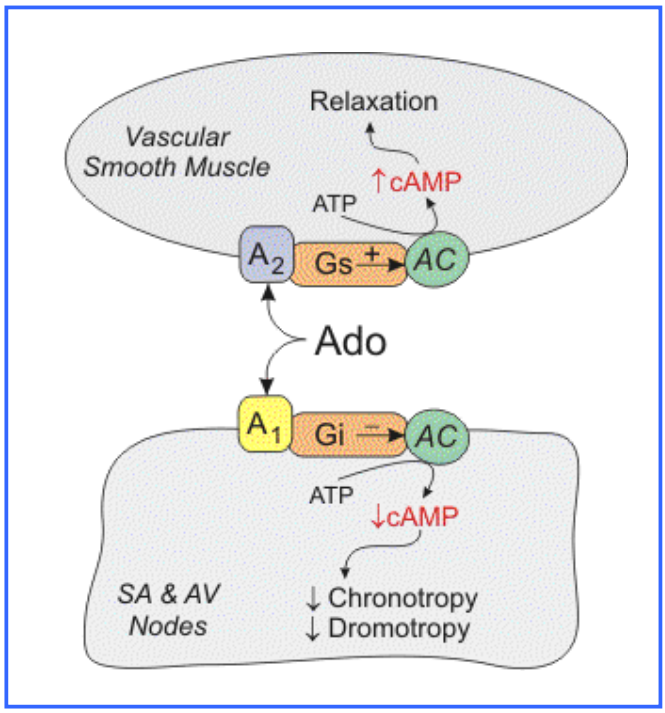


Figure 13: Adenosine receptors in the cardiovascular system. The two most important subtypes (cardiac A_1 and vascular A_{2A} receptors).

EXPERIMENTAL PART

1. ADENOSINE AND ACUTE INFLAMMATION

1.1. Materials and Methods

1.1.1. Materials

Aprotinin, bovine serum albumin (BSA), carrageenan, dimethyl sulfoxide (DMSO), o-dianisidine dihydrocloride, hexadecyltrimethylammonium bromide were purchased from Sigma (Italy). The adenosine A_{2A} receptor agonist, 2-[*p*(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxa-midoadenosine hydrocloride (CGS 21680), and the adenosine A_{2A} receptor antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), were purchased from Tocris Bioscience (U.K.). Dithiothreitol (DTT), phenyl-methylsulphonyl fluoride (PMSF) leupeptine were purchased from ICN Pharmaceuticals (Italy). Bradford reagent was purchased from Bio-Rad Laboratories (Italy). Polyclonal goat antibody to A_{2A} receptor was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-goat IgG conjugated to horseradish peroxidase was purchased from Dako (Denmark). ECL system was purchased from Amersham Pharmacia Biotech (Milan, Italy). All salts were purchased from Carlo Erba (Italy).

1.1.2. Carrageenan oedema

Male Wistar rats (120-150 g; Charles River, Italy) were slightly anaesthetized with enflurane and oedema was induced by injecting in the left hind paw carrageenan 1 % w/v (dissolved in distilled water; 0.1 ml); paw volume was

measured before carrageenan injection (time zero) and each hour for 6 hours, by a hydropletismometer (Ugo Basile, Comerio VA). Animals were divided in groups of 5 each and treated, just before oedema induction, with the selective adenosine A_{2A} receptor agonist, CGS 21680 (0.02, 0.2 and 2 mg/kg intraperitoneally); with the selective adenosine A_{2A} receptor antagonist, ZM 241385 (3 mg/kg ip.); with CGS 21680 (2 mg/kg ip.) *plus* ZM 241385 (3 mg/kg ip.) or with the vehicle (DMSO; 0.5 ml/kg ip.); an other group of rats was treated with CGS 21680 (30, 100 and 300 µg/paw) or with the vehicle (DMSO; 0.1 ml/paw) injected locally with carrageenan.

All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associate guidelines in the European Community Council Directive of 24 November 1986 (86/609/ECC).

1.1.3. Western Blot analysis

From different treated and control animals, at different times following oedema induction, inflamed paws were excised and the soft tissue was removed, frozen in liquid nitrogen and stored; the same tissue samples were also removed from non-inflamed animals. Tissue samples were then defrosted, weighed and homogenized with a Polytron homogenizer (3 cycles of 10 seconds at the maximum speed) in a lysis buffer containing: $MgCl_2$, 2 mM; sodium ortovanadate, 100 µM; β-glycerophosphate, 50 mM; EGTA, 1 mM; DTT, 1 mM; PMSF, 1 mM; leupeptin, 10 µg/ml and aprotinin, 10 µg/ml. After centrifugation at 2500 rpm for 10 minutes at 4°C, the supernatants were collected and centrifuged at 12000 rpm for 30 minutes at 4°C; the protein concentration was measured by the Bradford assay using BSA as standard. Membrane proteins (30 µg) were

briefly boiled and separated by 12 % sodium dodecyl sulphate (SDS)-polyacrilamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes for 45 min at 250 mA; non-specific antibody binding to the membrane was blocked with 5 % (w/v) non-fat milk and 0.1 % (w/v) BSA in PBS-Tween 20 0.1 % for 2 hour at room temperature. Membranes were then incubated overnight at 4°C on a shaker with a polyclonal goat antibody to A_{2A} receptor (1 : 500 in PBS-Tween 20 0.1 % containing 5 % non-fat milk and 0.1 % BSA); detection blots were washed with PBS-Tween 20 0.1% at 5 min intervals for 30 minutes and incubated with a secondary anti-goat antibody (1 : 5000) conjugated with horseradish peroxidase (HRP) for 2 hours at 4 °C. After 5 washes as described above, the immunoreactive bands were visualised using an enhanced chemiluminescence (ECL) system, as described by the manufacturer.

1.1.4. Myeloperoxidase (MPO) activity assay

Myeloperoxidase activity was measured as previously described (Mullane et al., 1985); from animals treated with the selective adenosine A_{2A} receptor agonist, CGS 21680 (0.02, 0.2 and 2 mg/kg ip.) or with the vehicle (DMSO; 0.5 ml/kg ip.), inflamed paws were excised after 3 hours from oedema induction and the soft tissue was removed, frozen in liquid nitrogen and stored. Tissue samples were then defrosted, weighed and homogenized in a solution containing 0.5 % (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged at 12000 rpm for 30 minutes at 4°C; an aliquot of the supernatant was then allowed to react with 0.167 mg/ml o-dianisidine dihydrocloride and 0.001 % H_2O_2. The rate of change in absorbance

was measured spectrophotometrically at 650 nm; MPO activity was defined as the quantity of enzyme degrading 1 μmol/min of peroxide at 37°C and was expressed in milliunits per g of wet tissue (mU/g tissue).

1.1.5. Statistical analysis

All data are expressed as mean ± S.E.M. and analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test. A value of $p < 0.05$ was considered significant.

1.2. Results

1.2.1. Effect of CGS 21680 on carrageenan oedema

Injection of carrageenan in the rat hind paw caused an oedema peaking between 3 and 4 hours; intraperitoneal treatment with the selective adenosine A_{2A} receptor agonist, CGS 21680, inhibited, in a dose-related manner (Figure 14), oedema development and this effect was reverted by co-administration with the selective adenosine A_{2A} receptor antagonist, ZM 241385, while ZM 241385 alone did not modify oedema development (Figure 15). CGS 21680 administered locally with carrageenan also reduced oedema in a dose-related manner (Figure 16).

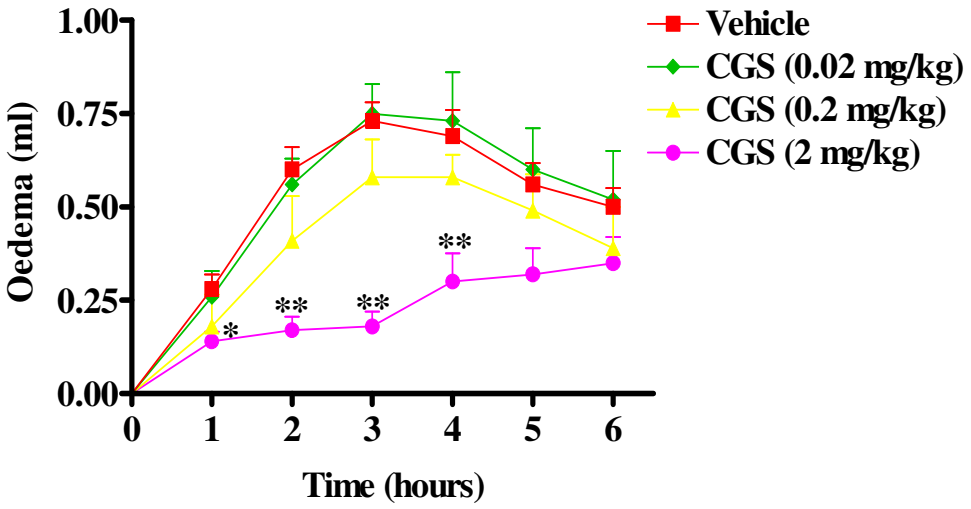


Figure 14: Effect of the selective adenosine A_{2A} receptor agonist, CGS 21680 (0.02, 0.2 and 2 mg/kg ip.), on carrageenan oedema; *p < 0.05, **p < 0.01 vs. vehicle. Dunnett's test (n = 10).

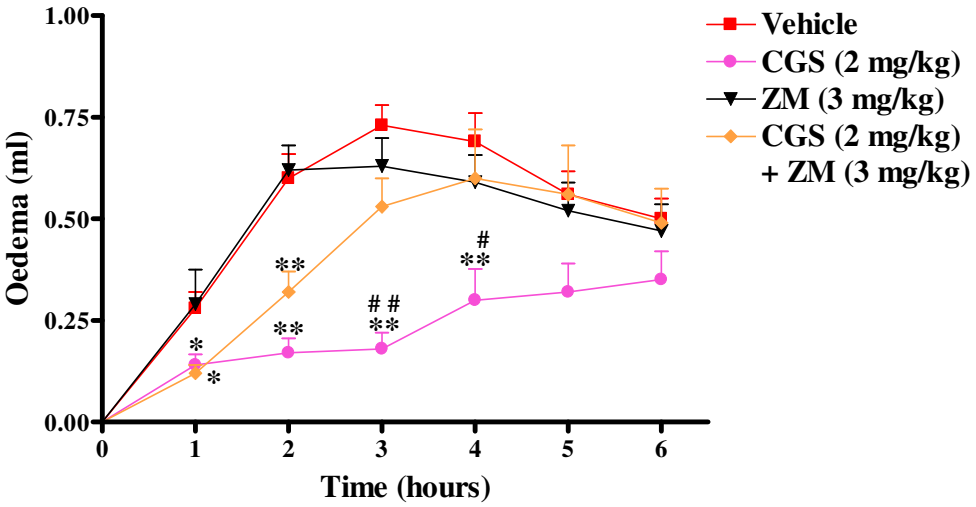


Figure 15: Effect of the selective A_{2A} receptor agonist, CGS 21680 (2 mg/kg ip.), of the selective A_{2A} receptor antagonist, ZM 241385 (3 mg/kg ip.), and of CGS 21680 *plus* ZM 241685 on carrageenan oedema; *p < 0.05, **p < 0.01 vs. vehicle; #p < 0.05, ## p < 0.01 vs. CGS 21680 + ZM 241385. Dunnett's test (n = 10).

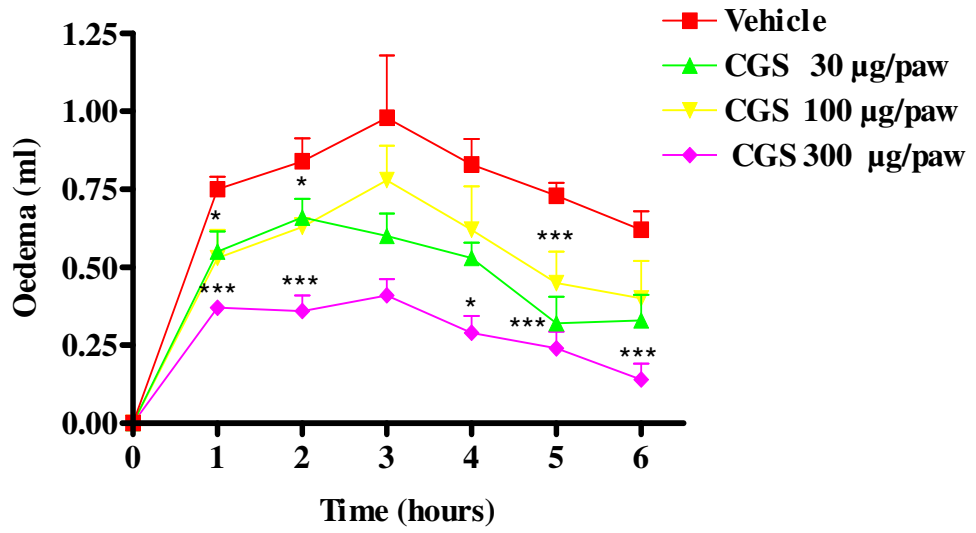


Figure 16: Effect of the selective A_{2A} receptor agonist, CGS 21680 (30, 100 and 300 µg/paw) administered locally, on carrageenan oedema; *p < 0.05, ***p < 0.001 vs. vehicle. Dunnett's test (n = 10).

1.2.2. Western Blot of A_{2A} receptor

Western Blot analysis performed on paws excised at different times after oedema induction showed an increased A_{2A} protein expression starting 1 hour following carrageenan injection, and peaking between the third and the fourth hour (Figure 17); the increased A_{2A} protein expression observed at 3 hours after oedema induction was reduced to control values by treatment with CGS 21680 2 mg/kg ip. (Figure 18).

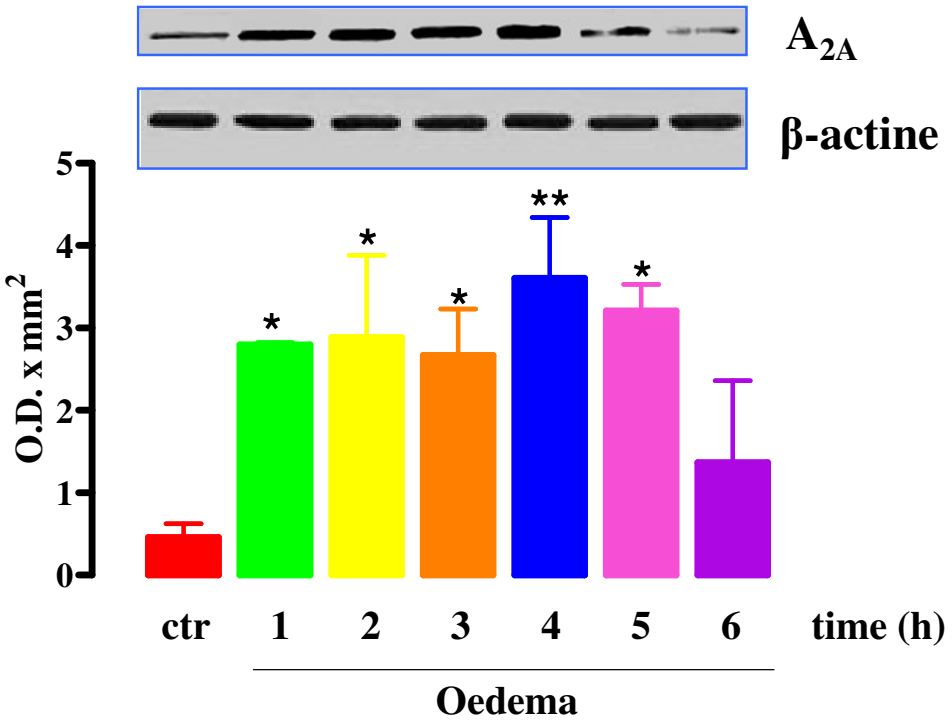


Figure 17: Detection of adenosine A_{2A} receptor and optical density analysis in inflamed paws; *p < 0.05, **p< 0.01 vs. control. Dunnett's test (n = 3).

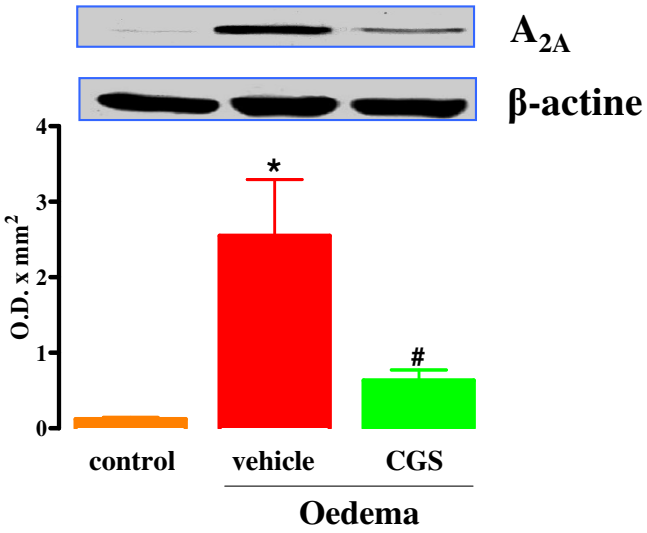


Figure 18: Effect of CGS 21680 (2 mg/kg ip.) on the proteic expression of the adenosine A_{2A} receptor in inflamed paws (3 hours after carrageenan injection); *p < 0.05 vs. control; #p < 0.05 vs. vehicle. Dunnett's test (n = 3).

1.2.3. MPO analysis

MPO activity measured in inflamed paws excised 3 hours after carrageenan injection was reduced after treatment with CGS 21680, compared with control values, and 2 mg/kg ip. (Figure 19).

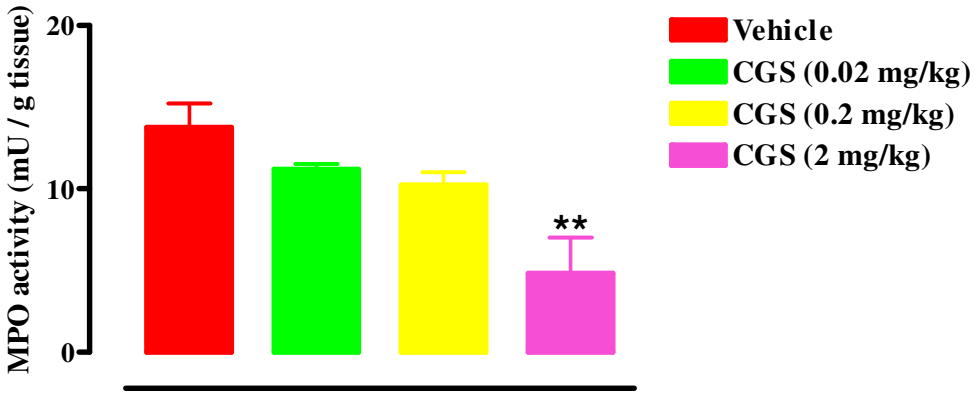


Figure 19: Effect of CGS 21680 (0.02, 0.2 and 2 mg/kg ip.) on myeloperoxidase (MPO) activity of inflamed paws (3 hours after carrageenan injection); **p < 0.01 vs. vehicle. Dunnett's test (n = 3).

2. ADENOSINE AND BRONCHIAL HYPERREACTIVITY

2.1. Materials and methods

2.1.1. Materials

Acetylcholine, adenosine, aluminium hydroxide gel, carbachol, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), ovalbumin, pentobarbital, salbutamol and urethane were purchased from Sigma (Italy). Haematoxylin was purchased from J.T. Baker (Holland). Eosin was purchased from Kaltek (Italy). Avidin and biotin were purchased from Signet Inc. (Dedham, MA). 3,3'-diaminobenzidine (DAB), protein block serum free, anti-goat and anti-rabbit IgG biotinylated were purchased from Dako (Denmark). Polyclonal goat antibodies to A_1, to A_{2A} , A_{2B}, A_3 receptors and polyclonal rabbit antibody to β_2-adrenergic receptor were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All salts and formalin were purchased from Carlo Erba (Italy).

2.1.2. Sensitization and challenge with allergen

Male Wistar rats (200-250g; Harlan, Italy) and male Brown Norway rats (200-250g; Charles River, Italy) were briefly anaesthetized with 4% isofluran in an anaesthetic chamber and were injected with ovalbumin mixed with aluminium hydroxide gel (50 mg/ml) 100 mg/kg ip. and 100 mg/kg sc. Starting from the 21st day after sensitization, rats were challenged with an aerosol of ovalbumin (5 mg/ml; 2 ml *per* animal) or saline under sodium pentobarbital anaesthesia (60 mg/kg ip); 24 hours thereafter, rats were used for the experiments.

All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associate guidelines in the European Community Council Directive of 24 November 1986 (86/609/ECC).

2.1.3. Measurement of bronchoconstriction *in vivo*

Animals were anaesthetized with urethane (10 ml/kg ip.; sol. 10 % w/v); the jugular vein and the carotid artery were cannulated respectively for drug administration and blood pressure monitoring. Rats were artificially ventilated (60 breaths/min.; 1 ml/100 g tidal volume) via a tracheal cannula and connected to a bronchospasm transducer (Ugo Basile, Italy) to monitor change in airway resistance. All data were acquired by a coputerized system (McLab).

After a stabilization period of 15 minutes, bronchoconstrictor responses to ovalbumin (1 mg/kg i.v.), adenosine (3 mg/kg i.v.) and carbachol (10 µg/kg i.v.) were established sequentially in groups of sensitized animals challenged with ovalbumin or saline and in control naïve rats.

2.1.4. Drug treatment

To investigate on the role of adenosine A_1 receptor on airway hyperreactivity, different groups of sensitized ovalbumin challenged rats were pre-treated with the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 100 µg/kg iv; - 5 minutes), before the administration of spasmogen agents.

2.1.5. Bronchial tissue study in vitro

Animals were sacrificed by cervical dislocation, exsanguinated and lungs were removed and placed into a Petri dish containing Krebs solution of the following composition (nM): NaCl, 115.3; KCl, 4.9; $CaCl_2$, 1.46, $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0 and glucose, 11.1; termosted at 37 °C and oxygenated (CO_2 5% *plus* O_2 95%); main bronchi were dissected free of parenchyma and mounted in 2.5 ml isolated organ baths containing Krebs solution, at 37 °C, oxygenated (CO_2 5% *plus* O_2 95%), and connected to an isometric force transducer (Ugo Basile, Italy) under a resting tension of 0.5 g. After about 60 minutes equilibration period, tissue reactivity was checked by evaluating the response to a single concentration of acetylcholine (Ach, 3 μM); after washing, a cumulative concentration response curve to salbutamol (0.001-0.4 μM) was performed on tissue pre-contracted with carbachol (1 μM). It was also evaluated the response to ovalbumin (10 μg) and to adenosine (300 μM) added before and after ovalbumin administration. All *in vitro* experiments were performed on tissues obtained from sensitized animals challenged with ovalbumin or with saline, and from control naïve rats.

2.1.6. Western Blot analysis

From different groups of sensitized and control animals, lungs were removed and homogenized as described above; protein samples (30 μg) were briefly boiled and subjects to electrophoresis on an SDS 10% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane using standard procedure (described above). The membranes were placed in 5 % (w/v) non-fat milk and 0.1 % (w/v) BSA in PBS-Tween 20 0.1 % for 1 hour at room temperature and then

incubated with a polyclonal goat antibody to A_1 receptor (1 : 500), to A_{2A} receptor (1 : 500), to A_{2B} receptor (1 : 250), to A_3 receptor (1 : 250) or with a polyclonal rabbit antibody to β_2-adrenergic receptor (1 : 500), all diluted in in PBS-Tween 20 0.1 % containing 5 % non-fat milk and 0.1 % BSA, overnight at 4 °C. Detection blots were then washed with PBS-Tween 20 0.1% at 5 min intervals for 30 minutes and incubated with a secondary anti-goat (1 : 5000) or anti-rabbit (1 : 10000) antibody, both conjugated with horseradish peroxidase (HRP) for 2 hours at 4 °C. After 5 washes, the immunoreactive bands were visualised using an enhanced chemiluminescence (ECL) system, as described by the manufacturer.

2.1.7. Histological analysis

Lungs were fixed in 10% (v/v) buffered formalin via a tracheal cannula, then removed and immersed in formalin for 24 hours; sections were cut (8 μm thick) and stained with haematoxylin and eosin to demarcate cell types. The sections were analyzed by using a standard light microscope (original magnification: x 40) and photographed by a Leica DFC320 video camera (Leica, Italy) connected to the microscope using the Leica Application Suite software V2.4.0. Histological examination was performed on tissue samples from naive and sensitized challenged with ovalbumin or saline animals, to evaluate the effect of sensitization and ovalbumin challenge on lung morphology.

2.1.8. Immunohistochemical analysis

Main bronchi were removed, embedded in OCT (optimal cutting temperature) medium and frozen in liquid nitrogen; 8 μm cryostat sections were

prepared and fixed with acetone for 10 minutes, air dried and rehydrated with PBS. For staining, sections were incubated with 0.3 % H_2O_2 in methanol for 10 minutes and protein block serum free was added for 30 minutes; endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. Sections were then incubated with a polyclonal goat antibody to A_1 receptor (1 : 300), to A_{2A} receptor (1 : 75), to A_{2B} receptor (1 : 75), to A_3 receptor (1 : 75) or with a polyclonal rabbit antibody to β_2-adrenergic receptor (1 : 300), all diluted in PBS containing 1 % BSA, for 1 hour; sections not incubated with primary antibody were used as negative control. Subsequently, sections were incubated with biotinylated anti-goat or anti-rabbit secondary antibody (both 1 : 500, for 15 minutes); enzymatic activity was detected with 3,3'-diaminobenzidine (DAB) substrate (brown colour), while hematoxylin was used to counterstain (blue background). Sections were then dehydrated and mounted in Entellan® medium; images were taken by the Leica DFC320 video camera (Leica, Italy) connected to the microscope (original magnification: x 40) using the Leica Application Suite software V2.4.0.

2.1.9. Statistical analysis

All data are expressed as mean ± S.E.M. and analyzed by Student's *t*-test for unpaired data, one way or two way analysis of variance (ANOVA) followed by Dunnett's test, as appropriate. A value of $p < 0.05$ was considered significant.

2.2. Results

2.2.1. Bronchoconstrictor responses to spasmogen agents in Wistar and Brown Norway rats

In control naïve rats ovalbumin (1 mg/kg iv.) caused a bronchoconstriction of 16.65 ± 3.54 % (n=17), that was significantly augmented in Wistar sensitized rats challenged with ovalbumin (36.65 ± 5.47 %, n=17; $p < 0.01$), but not in Brown Norway sensitized rats challenged with ovalbumin (20.36 ± 4.29 %, n=11; $p > 0.05$) (Figure 20). Similarly, in control naïve rats adenosine (3 mg/kg iv.) caused a bronchoconstriction of 13.82 ± 3.09 % (n=17), that was significantly augmented in Wistar sensitized rats challenged with ovalbumin (30.06 ± 4.92 %, n=16; $p < 0.01$), but not in Brown Norway sensitized rats challenged with ovalbumin (16.70 ± 3.95 %, n=10; $p > 0.05$) (Figure 21). On the contrary, there was no significant difference in the bronchial response to carbachol (10 µg/kg iv.) among groups (Figure 22).

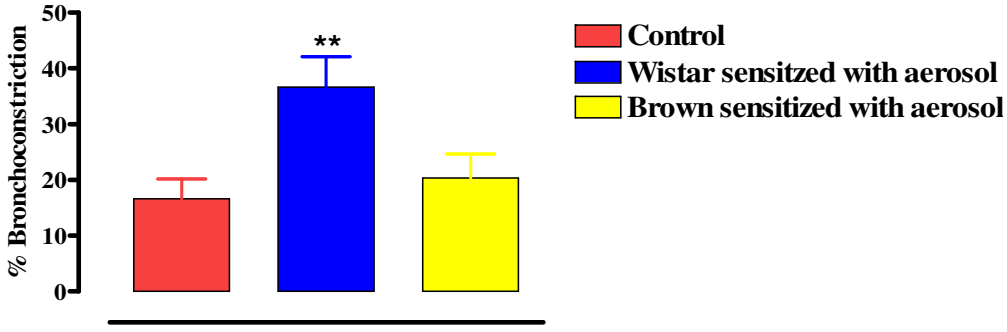


Figure 20: Bronchoconstrictor effect of ovalbumin (1 mg/kg iv.) in Wistar and Brown Norway rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA; **p < 0.01 vs. control. Dunnett's test (n = 11-17).

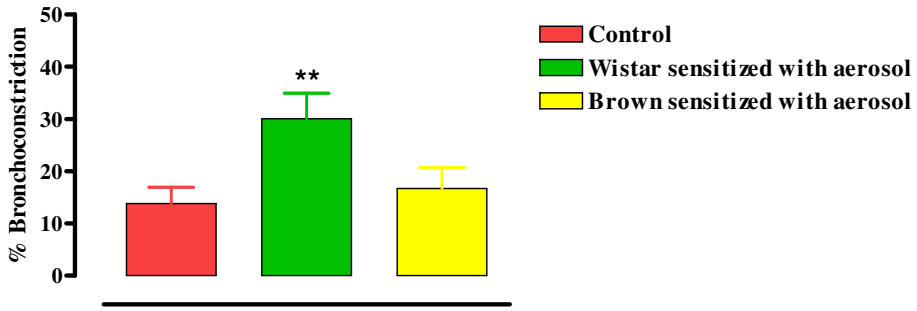


Figure 21: Bronchoconstrictor effect of adenosine (3 mg/kg iv.) in Wistar and Brown Norway rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA; **p < 0.01 vs. control. Dunnett's test (n = 10-17).

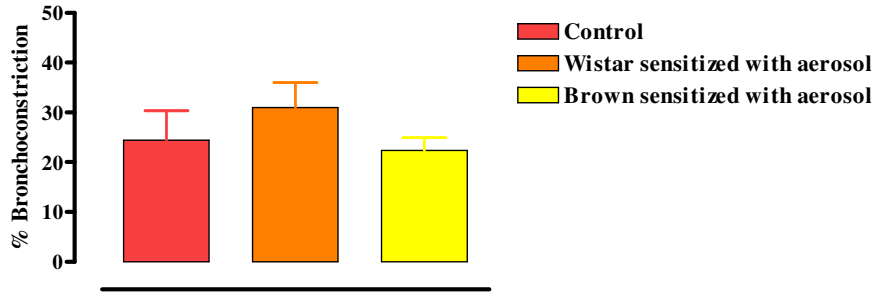


Figure 22: Bronchoconstrictor effect of carbachol (10 µg/kg iv.) in Wistar and Brown Norway rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA; p < 0.05 vs. control. Dunnett's test (n = 10-16).

2.2.2. Effect of allergen challenge on bronchial response to spasmogen agents

Following these preliminary results, all successive experiments were performed on Wistar rats. In control naïve rats ovalbumin (1 mg/kg iv.) caused a bronchoconstriction of 16.65 ± 3.54 % (n=17), that was significantly augmented

in rats sensitized and challenged with ovalbumin (36.65 ± 5.47 %, n=17; $p < 0.01$), while no difference was observed between control rats and rats sensitized and challenged with saline (10.00 ± 1.52 %, n=10; $p > 0.05$) (Figure 23). Similarly, in control naïve rats adenosine (3 mg/kg iv.) caused a bronchoconstriction of 13.82 ± 3.09 % (n=17), that was significantly augmented in rats sensitized and challenged with ovalbumin (30.06 ± 4.92 %, n=16; $p < 0.01$), while no difference was observed between control rats and rats sensitized and challenged with saline (6.40 ± 2.38 %, n=10; $p > 0.05$) (Figure 24). On the contrary, there was no difference in the bronchial response to carbachol (10 µg/kg iv.) among groups. (Figure 25).

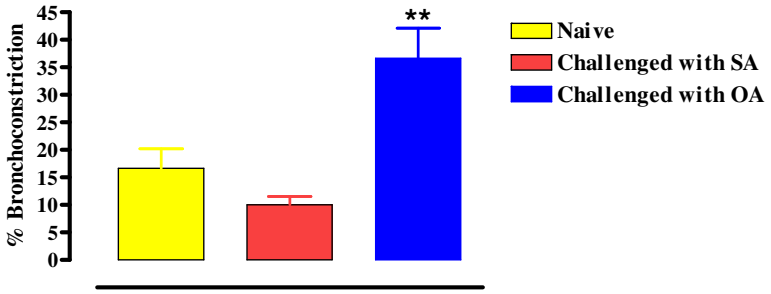


Figure 23: Bronchoconstrictor effect of ovalbumin (1 mg/kg iv.) in Wistar rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); **p < 0.01 vs. control. Dunnett's test (n = 10-17).

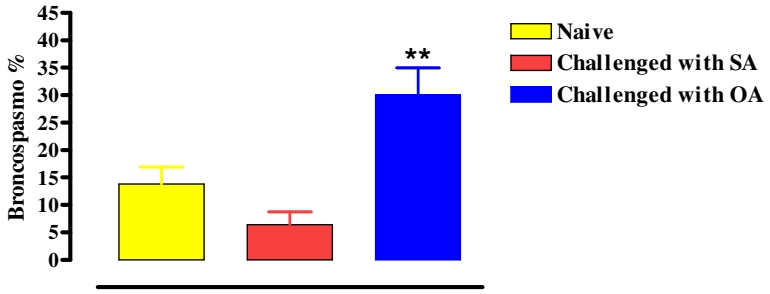


Figure 24: Bronchoconstrictor effect of adenosine (3 mg/kg iv.) in Wistar rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); **p < 0.01 vs. control. Dunnett's test (n = 10-17).

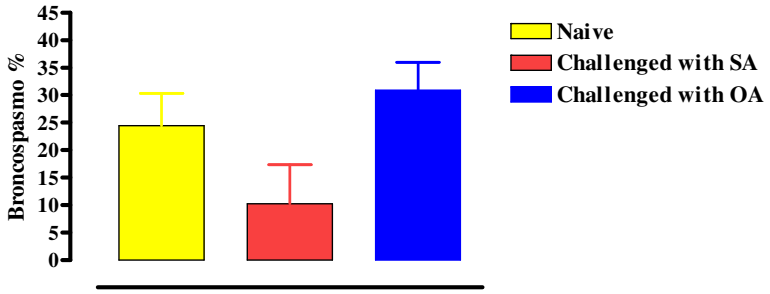


Figure 25: Bronchoconstrictor effect of carbachol (10 µg/kg iv.) in Wistar rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); p > 0.05 vs. control. Dunnett's test (n = 10-16).

2.2.3. Effects of A_1 receptor blockade on bronchial response to spasmogen agents

Pre-treatment with the A_1 receptor antagonist DPCPX significantly reduced the bronchial response to ovalbumin and adenosine in sensitized rats

challenged with ovalbumin, compared to control animals pre-treated with the vehicle (ovalbumin, 22.20 ± 6.08 % vs. 54.17 ± 6.38 %, $p < 0.01$; adenosine 14.60 ± 3.29 % vs. 45.75 ± 5.23 %, $p < 0.01$. Student's *t*-test, n=4-6) (Figure 26 and 27). No difference was observed in the bronchial response to carbachol (43.00 ± 10.24 % vs. 63.33 ± 6.89 %, $p > 0.05$. Student's *t*-test, n=5) (Figure 28).

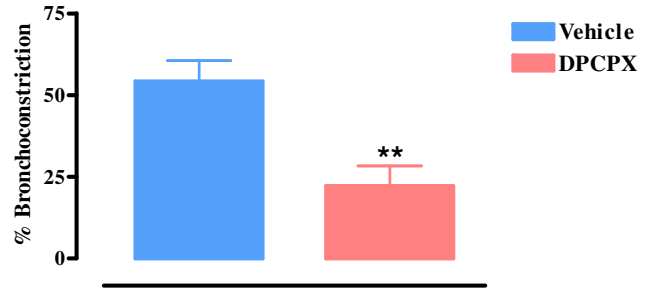


Figure 26: Effect of the adenosine A_1 receptor antagonist, DPCPX (100 µg/kg given iv. 5 min. prior to ovalbumin), on response to ovalbumin (1 mg/kg iv.) in sensitized rats challenged with ovalbumin; **p < 0.01. Student's *t*-test for unpaired data (n = 4-6).

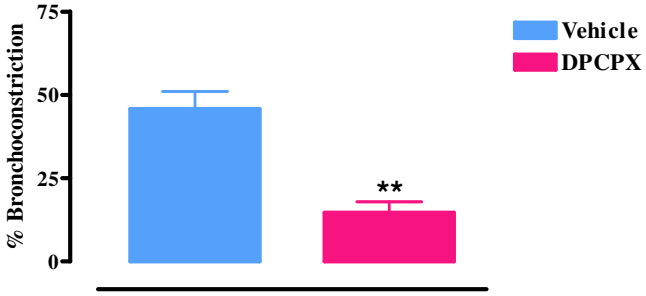


Figure 27: Effect of the adenosine A_1 receptor antagonist, DPCPX (100 µg/kg given iv. 5 min. prior to ovalbumin), on response to adenosine (3 mg/kg iv.) in sensitized rats challenged with ovalbumin; **p < 0.01. Student's *t*-test for unpaired data (n = 4-6).

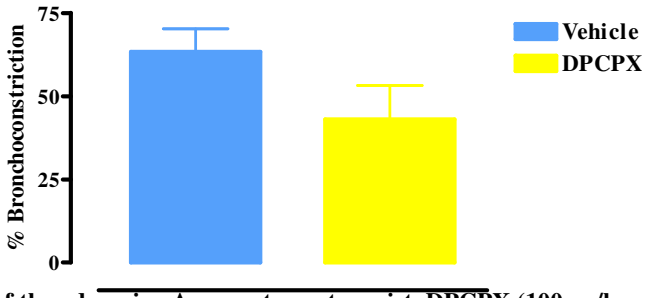


Figure 28: Effect of the adenosine A_1 receptor antagonist, DPCPX (100 µg/kg given iv. 5 min. prior to ovalbumin), on response to carbachol (10 µg/kg iv.) in sensitized rats challenged with ovalbumin; $p > 0.05$. Student's *t*-test for unpaired data (n = 4-6).

2.2.4. Effect of ovalbumin and adenosine on bronchial tissue *in vitro*

Adenosine (300 µM) administered before and after ovalbumin caused contraction of bronchial tissues obtained from sensitized rats challenged with ovalbumin or saline and from naïve rats, without any significant difference among animal groups. Tissues from sensitized rats challenged with saline responded only weakly to ovalbumin (10 µg) ($p > 0.05$ vs zero; one sample Student's *t*-test), while bronchi taken from sensitized animals challenged with allergen manifested a greater constrictor response to ovalbumin ($p<0.05$ vs zero; one sample Student's *t*-test); no response to ovalbumin was observed in bronchi from naïve rats (Figure 29).

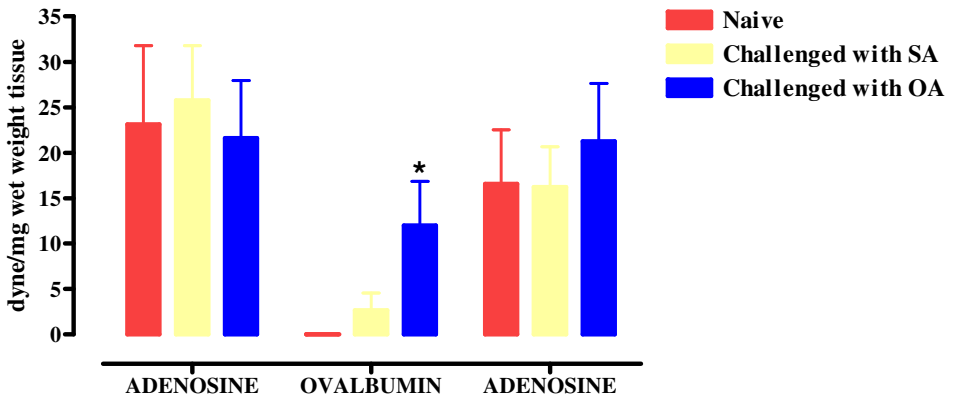


Figure 29: Effect of ovalbumin (10 µg) and adenosine (300 µM given before and after ovalbumin) on bronchial tissues from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); *p < 0.05 vs. zero. One sample Student's *t*-test (n = 10-14).

2.2.5. Salbutamol-induced bronchorelaxation *in vitro*

Salbutamol caused a concentration-dependent relaxation of tissues from naïve rats and rats sensitized to ovalbumin with and without allergen challenge, but the sigmoidal concentration-response curve to salbutamol on bronchi from sensitized rats challenged with ovalbumin was lower than those obtained on bronchi from sensitized rats challenged with saline and from naïve rats, reflecting a reduction of the maximum relaxant effect of salbutamol following allergen challenge (Figure 30).

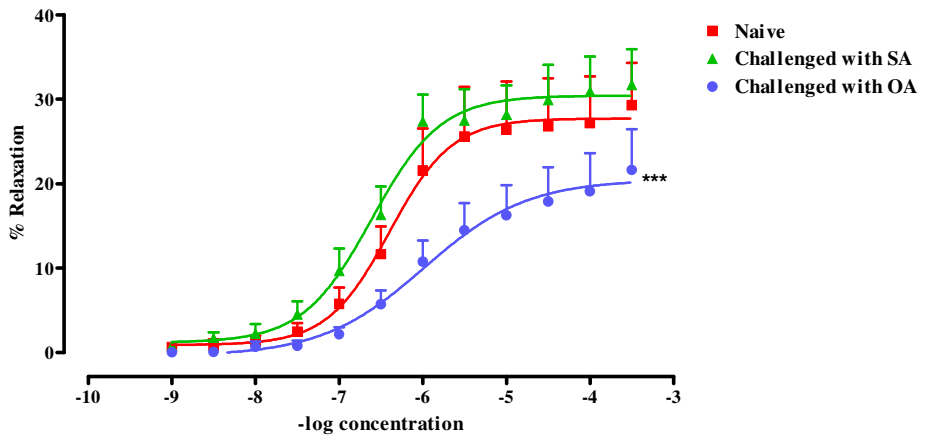


Figure 30: Effect of salbutamol (0.001-0.4 µM) on bronchial tissues pre-contracted with carbachol (1 µM) from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); summary $p < 0.001$. Two way ANOVA (n = 10-14).

2.2.6. Western Blot of adenosine receptors and β_2-adrenergic receptor

Pulmonary tissue of sensitized rats challenged with ovalbumin presented an increased proteic expression of adenosine A_1 receptor, compared to the proteic expression of pulmonary tissue of sensitized rats challenged with saline and naïve rats (Figure 31). A_{2A} receptor proteic expression was increased on pulmonary tissue from sensitized rats challenged with saline but not with allergen (Figure 32); on the contrary, no difference was observed in the proteic expression of adenosine A_{2B} and A_3 receptors, and of β_2-adrenergic receptor (Figure 33, 34,35).

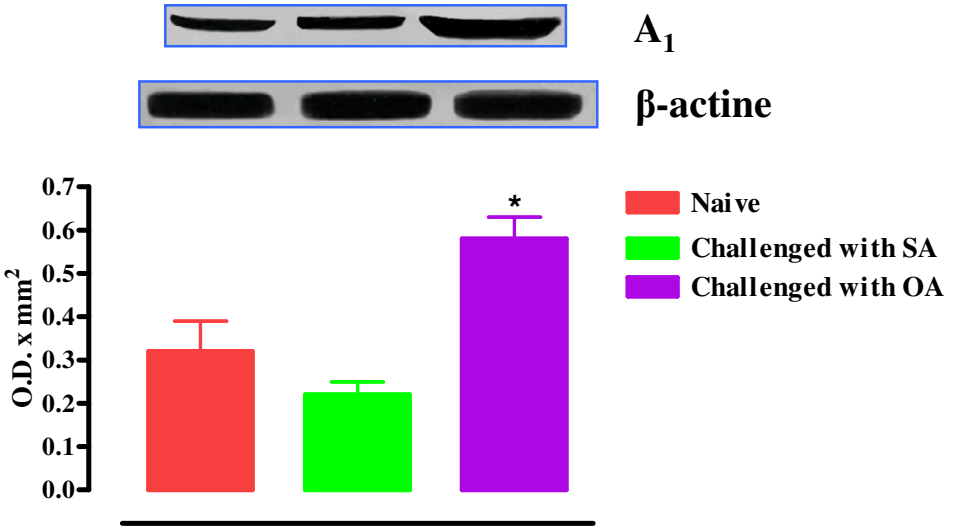


Figure 31: Detection of the adenosine A_1 receptor and optical density analysis in lungs from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); *p < 0.05 vs. naive. Dunnett's test (n = 3-4).

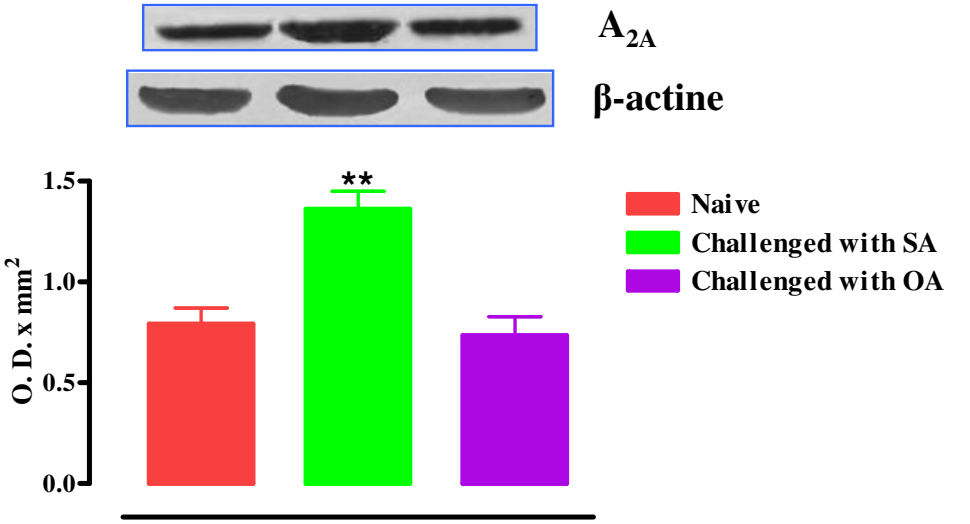


Figure 32: Detection of the adenosine A_{2A} receptor and optical density analysis in lungs from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); **p < 0.01 vs. naive. Dunnett's test (n = 3-4).

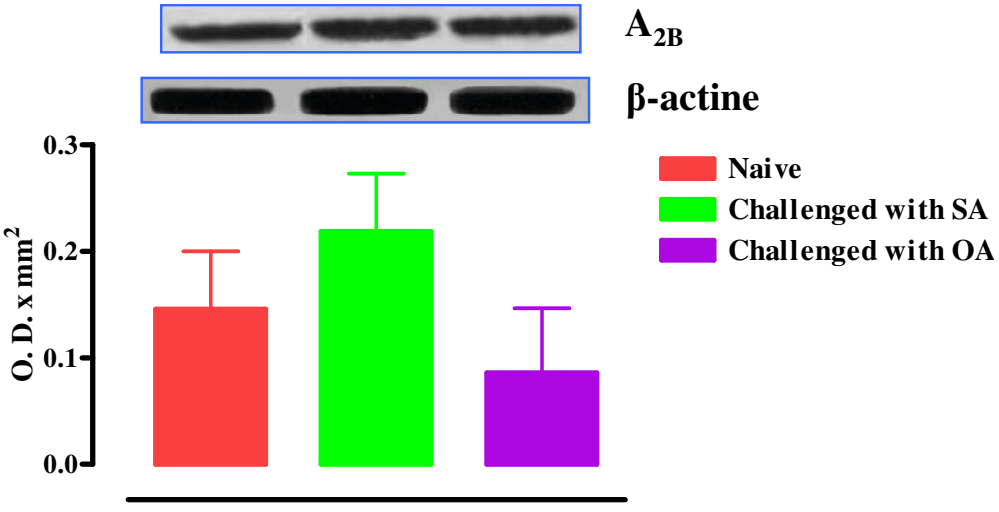


Figure 33: Detection of the adenosine A_{2B} receptor and optical density analysis in lungs from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); p > 0.05 vs. naive. Dunnett's test (n = 3-4).

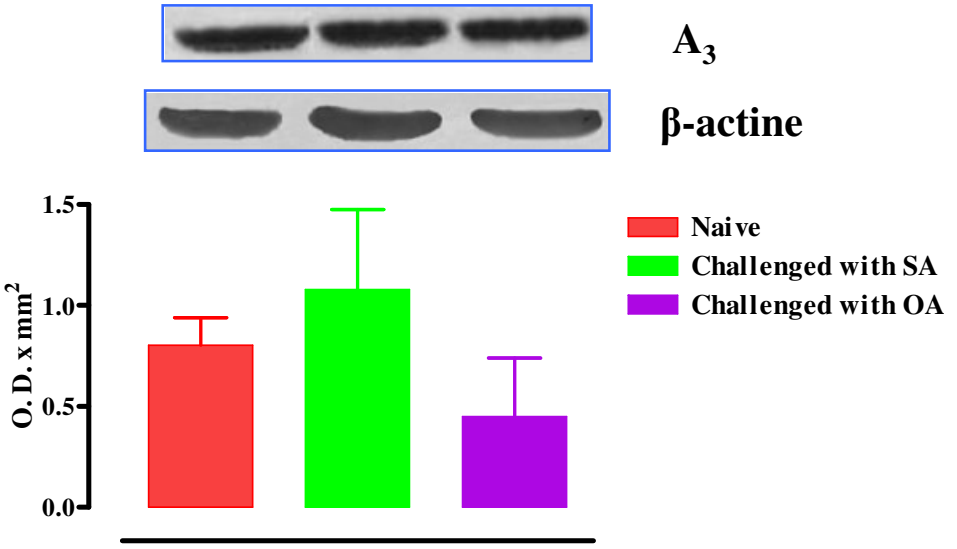


Figure 34: Detection of the adenosine A_3 receptor and optical density analysis in lungs from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); $p > 0.05$ vs. naive. Dunnett's test (n = 3-4).

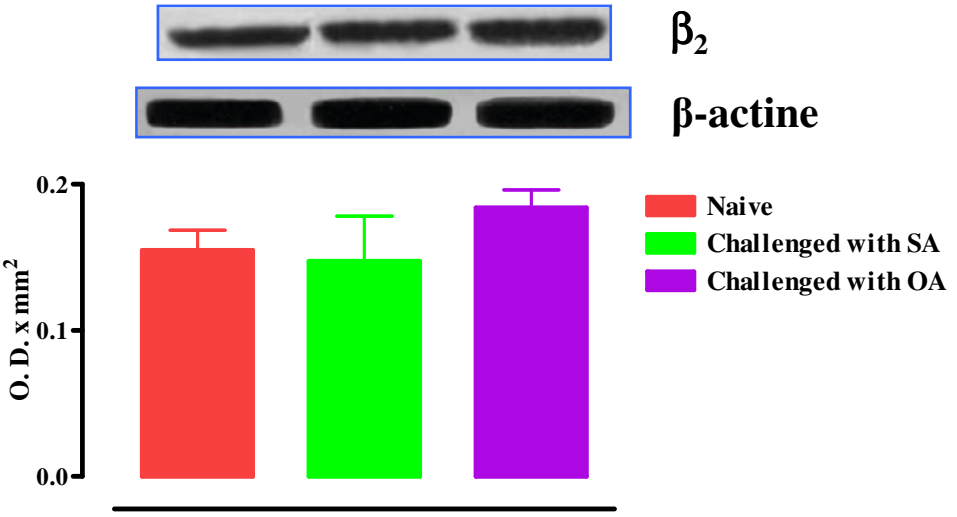


Figure 35: Detection of the β_2-adrenergic receptor and optical density analysis in lungs from rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA or saline (SA); $p > 0.05$ vs. naive. Dunnett's test (n = 3-4).

2.2.7. Histological analysis

In naïve rats lung sections were free of inflammation and edema (Figure 36 A), and sensitized rats challenged with saline showed a similar profile (data not shown). In sensitized rats challenged with ovalbumin, peribronchial and perivascular edema and inflammatory cell infiltration were observed (Figure 36 B and C).

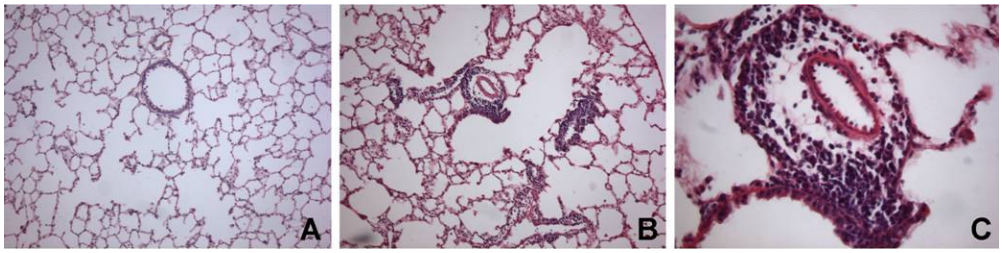


Figure 36: Histological analysis of haematoxylin and eosin (H&E)-stained lung sections from naïve rats (A) and rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA (B and C); original magnification: A and B x 100, C x 400.

2.2.8. Immunoistochemical analysis

Bronchial tissues from sensitized rats challenged with ovalbumin showed a strong positive immunoreactivity for adenosine A_1 receptor compared to tissues obtained from sensitized rats challenged with saline and from naive animals; the enzyme was preferentially localized in the bronchial smooth muscle (Figure 37). On the contrary, bronchial tissue from sensitized rats challenged with saline and naïve rats showed a strong positive immunoreactivity in the smooth muscles for β_2-adrenergic receptor, compared to tissues from sensitized rats challenged with ovalbumin (Figure 38). No positive immunoreactivity for adenosine A_{2A}, A_{2B} A_3 receptors was observed in bronchi from these groups of animals (Figure 39, 40, 41).

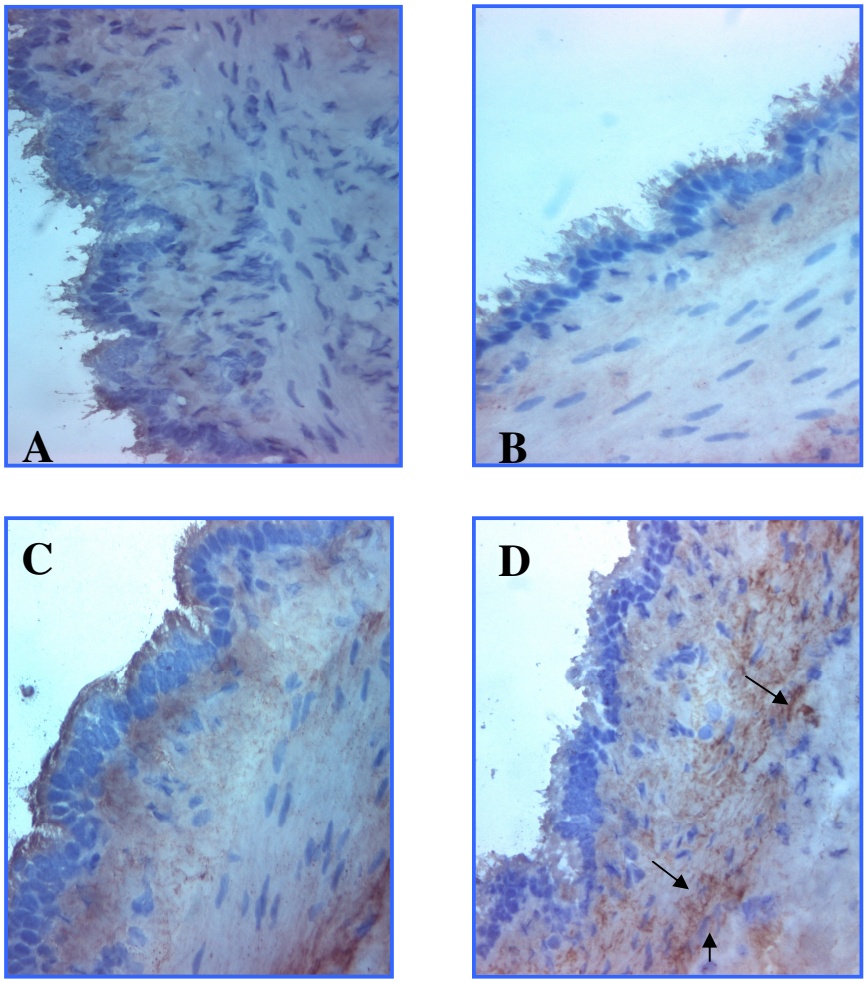


Figure 37: Immunohistochemical localization of adenosine the A_1 receptor in bronchi from naïve rats (B), sensitized rats challenged with saline (C) and sensitized rats challenged with ovalbumin (D). A is negative control; original magnification: x 40.

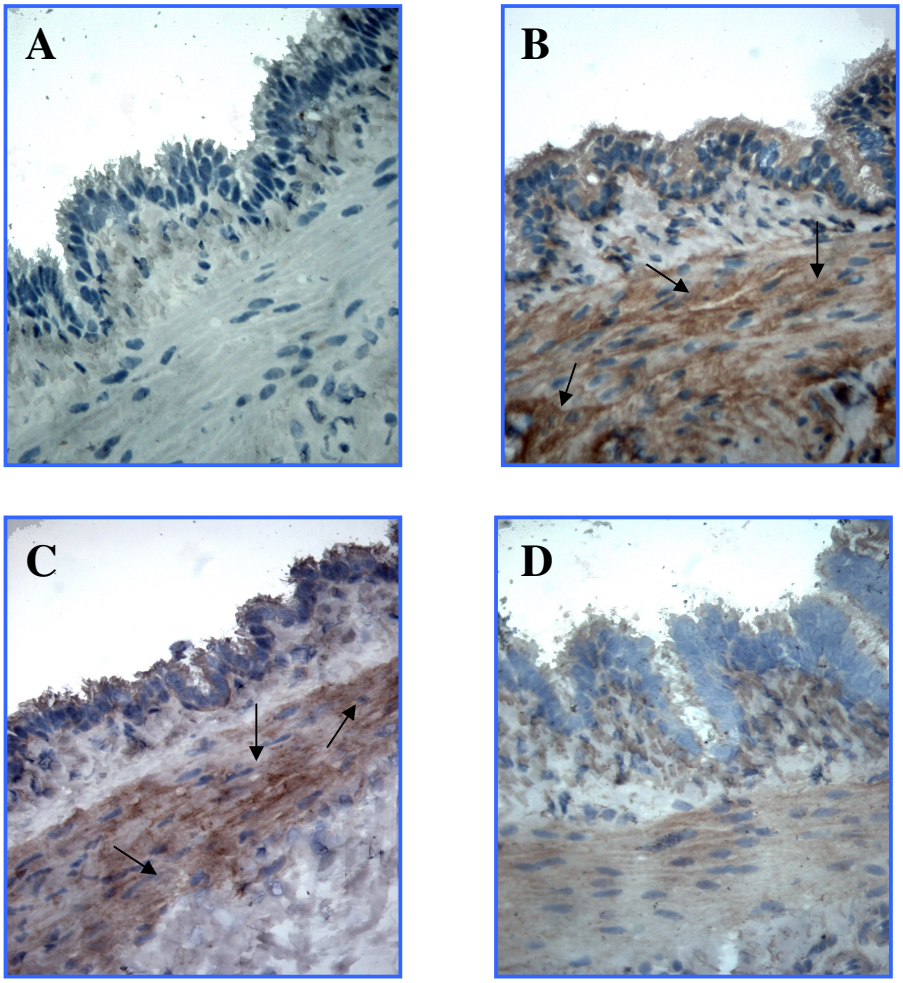


Figure 38: Immunohistochemical localization of adenosine the β_2-adrenergic receptor in bronchi from naïve rats (B), sensitized rats challenged with saline (C) and sensitized rats challenged with ovalbumin (D). A is negative control; original magnification: x 40.

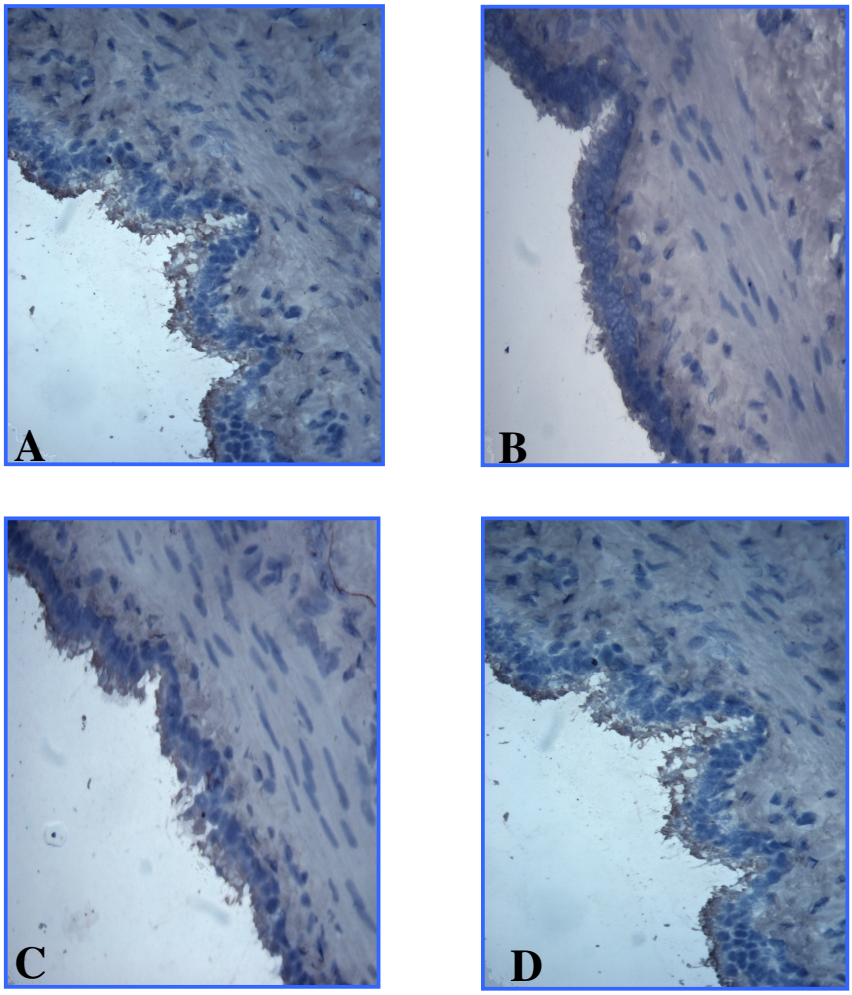


Figure 39: Immunohistochemical localization of adenosine the A_{2A} receptor in bronchi from naïve rats (B), sensitized rats challenged with saline (C) and sensitized rats challenged with ovalbumin (D). A is negative control; original magnification: x 40.

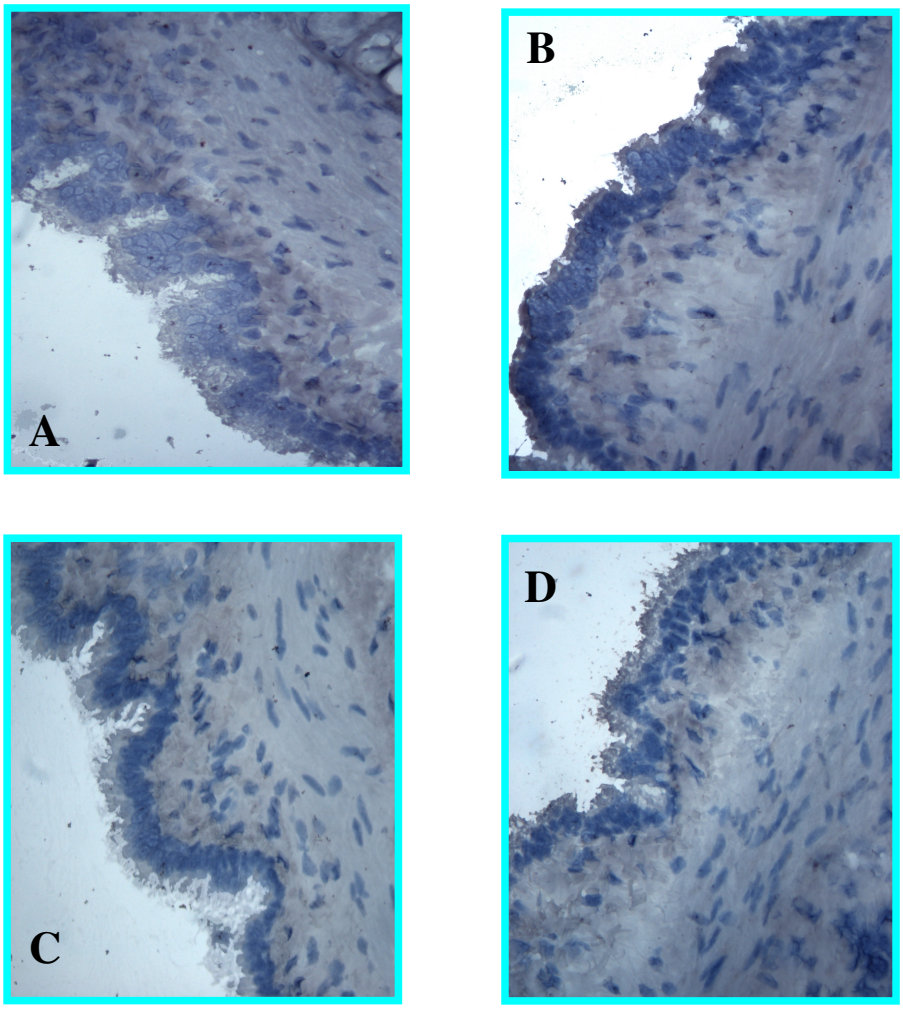


Figure 40: Immunohistochemical localization of adenosine the A_{2B} receptor in bronchi from naïve rats (B), sensitized rats challenged with saline (C) and sensitized rats challenged with ovalbumin (D). A is negative control; original magnification: x 40.

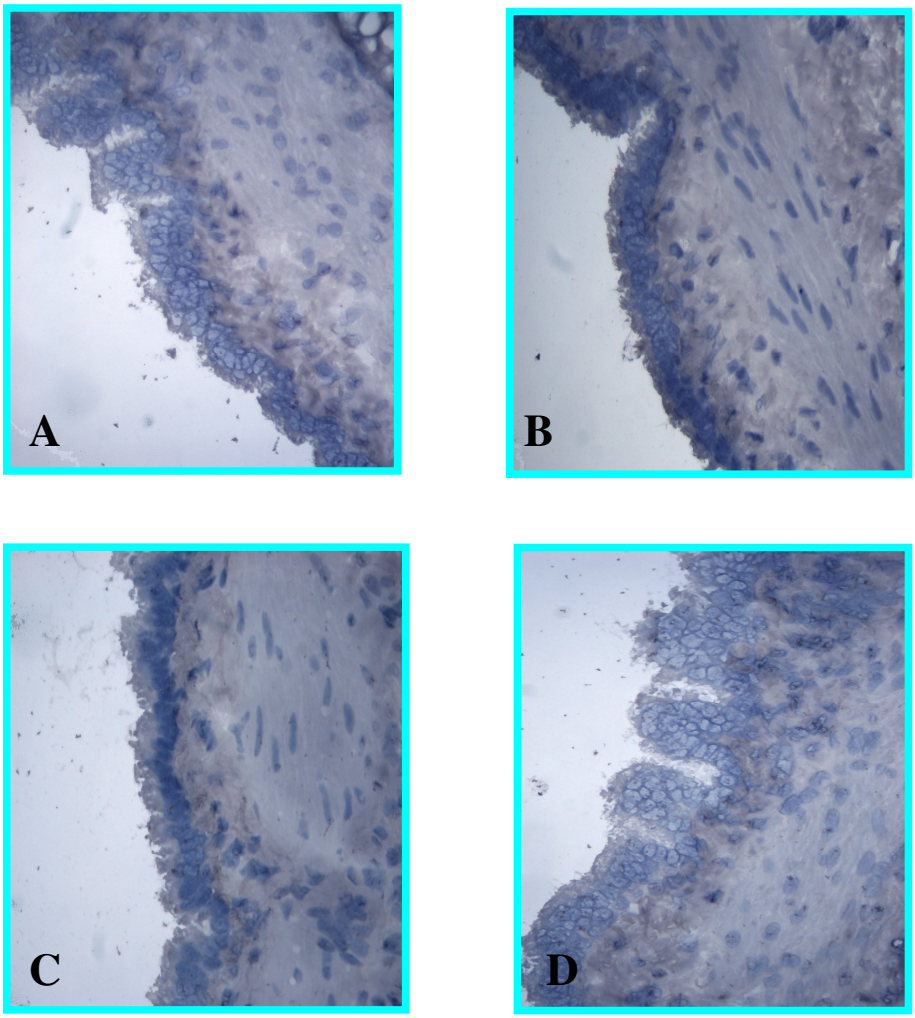


Figure 41: Immunohistochemical localization of adenosine the A_3 receptor in bronchi from naïve rats (B), sensitized rats challenged with saline (C) and sensitized rats challenged with ovalbumin (D). A is negative control; original magnification: x 40.

3. ADENOSINE AND MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

3.1. Materials and methods

3.1.1. Materials

Ketamine and xylazine were purchased by Sigma (Italy). Polyclonal goat antibodies to A_1 and A_3 receptors were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal mouse antibody to hypoxia inducible factor -1 α (HIF-1α) was purchased from Chemicon International Inc. (Temecula, CA). anti-goat and anti-mouse IgG biotinylated were purchased from Dako (Denmark). All salts and formalin were purchased from Carlo Erba (Italy).

3.1.2. Surgical preparation

Male Wistar rats (250-300g; Harlan, Italy) were anesthetized with ketamine 100 mg/kg ip. and xylazine 5 mg/kg ip.; rats were then placed in a supine position with their paws taped to the operating table. The trachea was cannulated and artificial respiration was maintained by a respirator with a frequency of 100 strokes/min and a tidal volume of 1 ml/100 g. A midline skin incision was made with reflection of the skin from the midsternal line; the chest was opened by a lateral cut along the left side of the sternum, cutting through the ribs to approximately the midsternum The animals were slightly rotated to the right to expose the left ventricle, and the auricle of the left atrium was slightly

retracted, exposing the entire left main coronary artery system; ligation was performed with a 6-0 silk suture (Ethicon®) passed with a tapered needle underneath the left anterior descending coronary artery (LAD), and a cotton tubing was placed on top of the vessel and a knot was tied on top of the cotton tubing to occlude the coronary artery. After occlusion for a period of 30 minutes, reperfusion was induced by cutting the knot on top of the cotton tubing; the chest cavity was closed and rats were allowed to recover. Twenty four hours later, rats were stunned by exposition to CO_2, the chest cavity was re-opened and the heart was rapidly removed for in vitro study.

All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associate guidelines in the European Community Council Directive of 24 November 1986 (86/609/ECC).

3.1.3. Experimental protocol

Rats were randomly assigned to one of the three groups: sham-operated animals underwent identical surgical procedure but without ligation of the LAD; rats underwent ischemia (30 minutes) and immediate removal of the heart; rats underwent ischemia (30 minutes) and subsequent reperfusion (24 hours).

3.1.4. Western Blot analysis

From the three groups of animals, heart ischemic areas were removed, frozen in liquid nitrogen and stored; tissue samples were then defrosted, weighed and homogenized with a Polytron homogenizer (3 cycles of 30 seconds at the maximum speed) in a lysis buffer containing: NaCl, 150 mM; sodium

ortovanadate, 100 μM; β-glycerophosphate, 50 mM; EDTA, 10 mM; DTT, 1 mM; PMSF, 1 mM; leupeptin, 10 μg/ml and aprotinin, 10 μg/ml. Centrifugations and protein concentration measurement were performed as described above; protein samples (100 μg) were briefly boiled and subjects to electrophoresis on an SDS 10% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane using standard procedure (described above). The membranes were placed in 5 % (w/v) non-fat milk and 0.1 % (w/v) BSA in PBS-Tween 20 0.1 % for 2 hours at room temperature and then incubated with a polyclonal goat antibody to A_1 receptor (1 : 500), to A_3 receptor (1 : 250) or with a polyclonal mouse antibody to HIF-1α receptor (1 : 1000), all diluted in in PBS-Tween 20 0.1 % containing 5 % non-fat milk and 0.1 % BSA, overnight at 4 °C. Detection blots were then washed with PBS-Tween 20 0.1% at 5 min intervals for 30 minutes and incubated with a secondary anti-goat (1 : 5000) or anti-mouse (1 : 20000) antibody, both conjugated with horseradish peroxidase (HRP) for 2 hours at 4 °C. After 5 washes, the immunoreactive bands were visualised using an enhanced chemiluminescence (ECL) system, as described by the manufacturer.

3.1.5. Statistical analysis

All data are expressed as mean ± S.E.M. and analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test. A value of $p < 0.05$ was considered significant.

3.2. Results

3.2.1. Western Blot of HIF-1α and adenosine A_1 and A_3 receptors

HIF-1α protein expression was weakly augmented ($p < 0.05$) in hearts underwent ischemia of 30 minutes, compared to tissues from sham rats, while expression of this protein was strongly increased ($p < 0.01$) in hearts underwent ischemia of 30 minutes and subsequent reperfusion of 24 hours (Figure 42). A_1 receptor protein expression was augmented in hearts underwent ischemia of 30 minutes, compared to tissues from sham rats, while expression of this protein was not modified in hearts underwent ischemia of 30 minutes and subsequent reperfusion of 24 hours (Figure 43); A_3 receptor protein expression was reduced in hearts underwent ischemia alone and in hearts underwent ischemia *plus* reperfusion, compared to tissues from sham-operated rats (Figure 44).

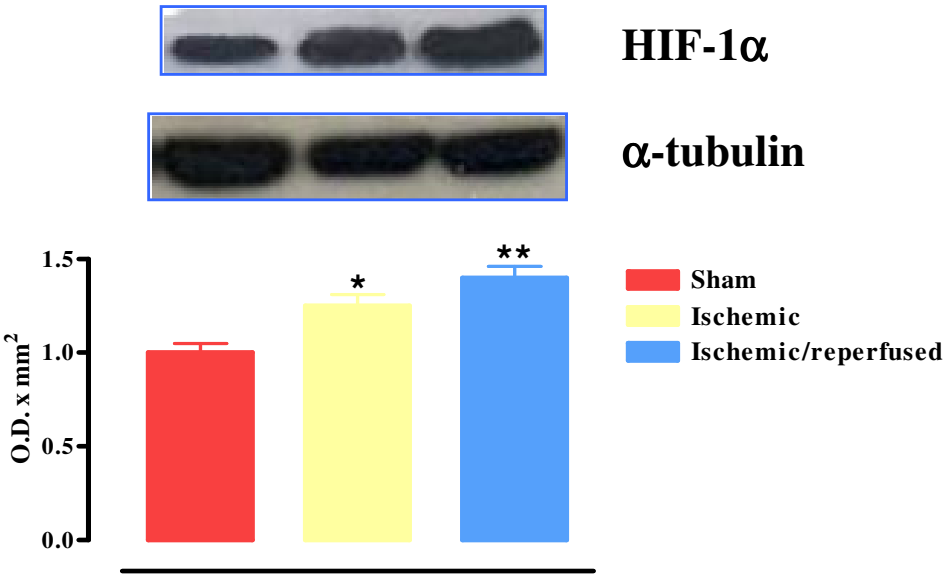


Figure 42: Detection of HIF-1α and optical density analysis in ischemic and ischemic/reperfused hearts; *p < 0.05, **p< 0.01 vs. control. Dunnett's test (n = 3).

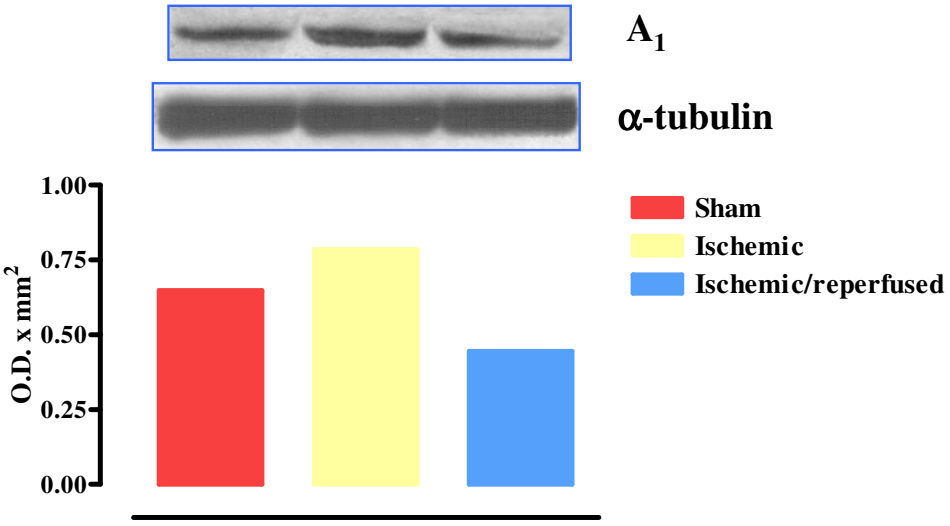


Figure 43: Detection of adenosine A_1 receptor and optical density analysis in ischemic and ischemic/reperfused hearts.

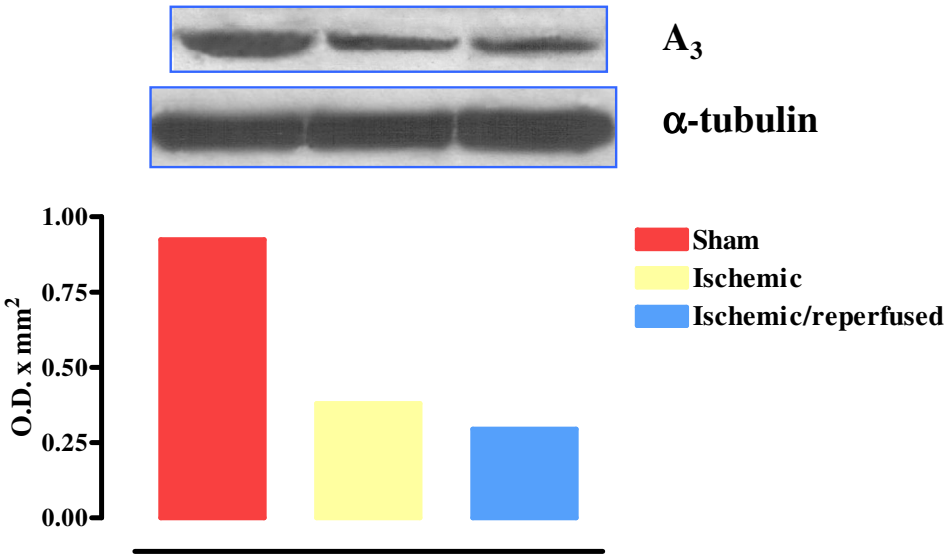


Figure 44: Detection of adenosine A_3 receptor and optical density analysis in ischemic and ischemic/reperfused hearts.

4. DISCUSSION

4.1. Adenosine A_{2A} receptor and acute inflammation

The adenosine A_{2A} receptor is expressed on several inflammatory cells and its activation results in the inhibition of these cell function (Linden, 2006); thus, recently much attention has been focused on the possible antinflammatory effect of adenosine A_{2A} receptor agonists (Lappas et al., 2005).

In order to investigate on the role of adenosine A_{2A} receptor in acute inflammation, we evaluated the effect the synthetic A_{2A} receptor selective agonist, CGS 21680, on carrageenan-induced rat paw oedema, a classical model of acute inflammation widely used to test the potential antinflammatory of new molecules. Our results show that CGS 21680, administered intraperitoneally immediately before oedema induction, reduces, in a dose-dependent manner, local acute inflammation induced by carrageenan injection in the rat paw; at the highest dose (2 mg/kg) used, CGS 21680 completely blocks oedema development. To assess the specificity of the response to CGS 21680, we also treated rats with the synthetic A_{2A} receptor selective antagonist, ZM 241385, in combination with CGS 21680. We observed that the inhibitory effect of CGS 21680 on carrageenan oedema was reverted by the concomitant administration of ZM 241385, confirming that CGS 21680 effect is mediated through systemic A_{2A} receptor activation; on the contrary, ZM 241385 alone did not modify the extent of inflammation.

Moreover, as marker of neutrophil accumulation (Mullane et al., 1985), we measured myeloperoxidase activity in the inflamed paws; we found that the

intraperitoneal treatment with CGS 21680 inhibited, in a dose-dependent manner, the increase in MPO activity in the inflamed tissue; the effect of the highest dose (2 mg/kg) of CGS 21680 was highly significant. This finding suggests that A_{2A} receptor activation mediated by CGS 21680 might inhibit acute local inflammation, at least in part, by inhibiting neutrophil sequestration and function; this result is in accordance with evidence that neutrophils express adenosine A_{2A} receptors on their membrane surface and with the previous findings showing that the antinflammatory effects of A_{2A} agonists are due to inhibition of neutrophil sequestration (Ross et al., 1999; Sullivan et al., 2004; Odashima et al., 2005; Linden et al., 2006).

The expression and the function of adenosine receptors are regulated by endogenous factors involved in the inflammatory response, such as growth factors (Arsalan et al., 1997; Navarro et al., 1999), glucocorticoids (Svenningsson and Fredholm, 1997; Ren and Stiles, 1999) and cytokines (Khoa et al., 2001; Trincavelli et al., 2002). In particular, inflammatory cytokines up-regulate the function and expression of A_{2A} receptors in human monocytic THP-1 cells (Khoa et al., 2001) and in rat PC12 cells (Trincavelli et al., 2002), while nerve growth factor down-regulates A_{2A} receptor expression in PC12 cells (Malek et al., 1999). In our work, Western Blot analysis of inflamed paws shows that there is a time-dependent increase in A_{2A} receptor proteic expression in the inflamed tissue, with an early increase starting 1 hour following the oedema induction, peaking between the third and the fourth hour and declining at 5 hours; this result suggests that A_{2A} adenosine receptor upregulation in the inflamed tissue might represent receptor

functionality and might be involved in the regulation of downstream regulatory proteins.

Following systemic treatment with CGS 21680, concomitant to edema and MPO activity inhibition in the paw, we observed that A_{2A} proteic expression was down-regulated compared to the receptor expression measured in inflamed paws from non-treated animals. These findings strongly suggest that A_{2A} adenosine receptor expression *in vivo*, likely regulated by endogenous factors involved in inflammation, might be part of a homeostatic mechanism able to limit tissue damage following inflammation. In this respect, it has been demonstrated that in mice lacking A_{2A} receptor there is an exacerbation of inflammation compared to wild type mice (Ohta and Sitkovsky, 2001).

We have also evaluated the effect of CGS 21680 when administered locally, to better investigate on the antinflammatory effect of this drug; interestingly, CGS 21680 is active either when given locally and when given systemically, indicating that this drug might negatively modulate an inflammatory reaction through the A_{2A} receptor activation at the site of inflammation, even if other mechanisms forward from the lesion are probably involved, for example the systemic release of mediator(s).

Taken together, our results confirm the importance of adenosine A_{2A} receptor in the modulation of inflammation and suggest that agonists at this receptor may act as antinflammatory drugs with possible high clinical potential.

4.2. Adenosine receptors and airway hyperreactivity

The heightened sensitivity of asthmatics to a range of stimuli (immunological, physical and pharmacological) that ordinarily are without effect in normal subjects is a defining characteristic of asthma (Woolcock 1997); this phenomenon is termed bronchial, or airway, hyperreactivity, and results in facilitation of bronchospasm that contributes to the airway obstruction characteristic of asthma (Cockcroft , 1997). The phenomenon is particularly well exemplified by the broncocostrictor response to adenosine which is prominent in asthmatic patients and generally not present in control subjects (Cushley et al., 1983); indeed, bronchoconstriction to adenosine was demonstrated in a number of animal models, but with contrasting results.

Several rat strains have been used in experimental animal models to mimic airway hyperresponsiveness seen in asthmatics; in this study we performed preliminary experiments in two rat strains, Brown Norway and Wistar, in order to compare bronchial reactivity following sensitization. Although several authors develop the allergic asthma model in the Brown Norway rat strain, since this strain is known to have a highly susceptive immune system (Hannon et al., 2001; Tigani et al., 2003), in our experimental study we were able to induce airway hyperreactivity in Wistar rats but not in Brown Norway rats. Indeed, our results *in vivo* show that only Wistar rats actively sensitized and aerosolized with ovalbumin (according to our experimental protocol of sensitization and allergen challenge) show an increased bronchial response to both ovalbumin (allergen) and adenosine compared to control naïve rats; this bronchial hyperreactivity cannot be elicited by sensitization and further allergen challenge in Brown Norway rats (as

summarized in Figure 45). Our results are in agreement with those obtained previously by other authors showing that Wistar appears to be the best rat strain to induce airway hyperreactivity (Chiba and Misawa, 1993). Thus, we performed all the following experiments on Wistar rats.

Our findings demonstrate that sensitization and subsequent challenge with ovalbumin elicits, only in Wistar rats, bronchial hyperreactivity that is proven by an increased bronchoconstriction in response to both adenosine and allergen. On the contrary, we didn't find any significant difference in the response to carbachol (10 µg/kg) among animal groups; we used carbachol administration as a control of bronchial reactivity at the end of each single *in vivo* experiment. In addition, histological analysis showed evidence of peribronchial and perivascular inflammation with cell infiltration in the lung only from rats sensitized and aerosolized; this finding shows that ovalbumin challenge is essential also to induce features of pulmonary inflammation.

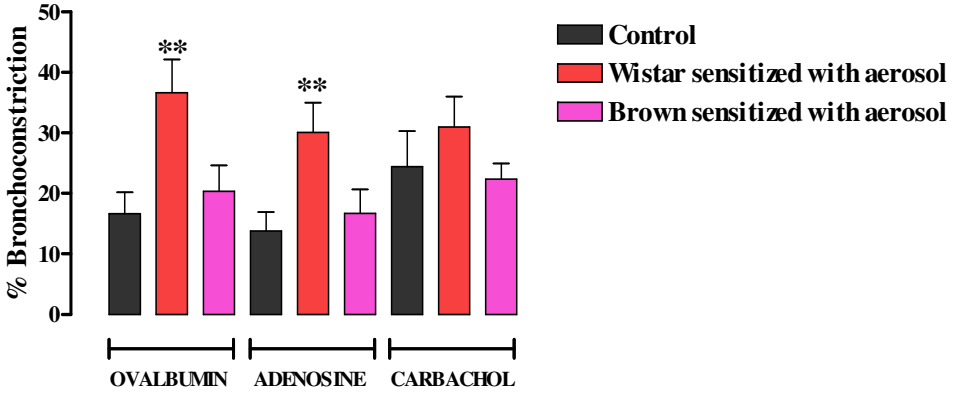


Figure 45: Bronchoconstrictor effect of ovalbumin (1 mg/kg iv.), adenosine (3 mg/kg iv.) and carbachol (10 µg/kg iv.) in Wistar and Brown Norway rats actively sensitised to ovalbumin (OA) and challenged 24 hours previously with aerosol of OA; **p < 0.01 vs. control. Dunnett's test (n = 10-17).

There is evidence that exposition to allergen subsequent to sensitization is crucial to induce bronchial hyperreactivity and that bronchoconstriction in response to adenosine is a marker of airway hyperreactivity, both in experimental animals and in humans (Hannon et al., 2001); however, the mechanism at the basis of adenosine-induced bronchoconstriction is still unknown. We evaluated the bronchial response to spasmogen agents in rats sensitized with and without ovalbumin challenge to assess a possible difference among animal groups; we observed that sensitization *per se* does not increase the sensitivity of the airways to ovalbumin and adenosine while allergen exposure appears to be crucial. This finding is in accordance with clinical observations about asthma; in fact, asthmatics undergo regular exposure to allergen, a situation which would resemble more that of sensitised aerosolised animals than that of animals sensitised but not exposed to allergen.

The mechanism at the basis of bronchial hyperreactivity and adenosine-induced bronchoconstriction in humans and sensitized animals has not yet been elucidated, nor it has been established which adenosine receptor is involved, but there is evidence that the receptor responsible varies among animal species. Indeed, it has been suggested that adenosine-induced bronchoconstriction is mediated by A_1 and A_{2B} receptors in rat and mouse; A_3 receptor in rat, guinea pigs and mouse; A_{2B} receptor in humans (Spicuzza et al., 2006); however, more investigations are needed to clarify this scenario. It has been supposed that adenosine and A_1 receptor are central players in the development of airway hypersensitivity and the inflammatory response to allergen (Richardson, 1997). It was reported that two interesting changes that occur in the human lung with the

onset of asthma are an increase in the mucosal concentration of adenosine and the "appearance" of the A_1 receptor role (Richardson, 1997). It was also reported that it is very difficult to detect this receptor in normal lung tissue (Dixon, 1996), but in asthmatic lungs its presence is associated with the ability of adenosine to induce bronchoconstriction (not seen in normal lungs).

By Western Blot analysis, we observed that proteic expression of adenosine A_1 receptor on the lung of rats sensitised and aerosolised with ovalbumin was increased compared to the receptor expression on lung of sensitized rats but not challenged with allergen; immunohistochemical analysis shows that the receptor is localized on bronchial smooth muscles. This finding is in agreement with an other work that showed an increased expression of A_1 receptor in small airways from allergic rabbits compared with that in small airways from normal rabbits (Ali et al., 1994); moreover, asthmatic patients are believed to have increased expression of A_1 receptors in the lung (Tilley and Boucher, 2005). Here, we demonstrate that in sensitized rats challenged with allergen the increased airway hyperrectivity is associated to an increased A_1 receptor expression localized on bronchial smooth muscles. To evaluate whether bronchial A_1 receptor was responsible for airway hyperreactivity observed in sensitized allergen challenged rats, we performed also experiments in groups of rats pre-treated with the synthetic A_1 receptor selective antagonist, DPCPX.

Interestingly, our data show that DPCPX reduces not only adenosine-induced bronchoconstriction in sensitized rats aerosolized with allergen, but also allergen-induced bronchoconstriction, in the same group of rats. This result strongly suggests that adenosine A_1 receptor contributes to airway hyperreactivity

caused by exposure to allergen of sensitized rats. Our findings are in agreement with emerging experimental data providing evidence for A_1 receptor as an important target in bronchial asthma (Obiefuna et al., 2005). Moreover, positive results in human asthmatics have been obtained from clinical trials with an antisense oligonucleotide to adenosine A_1 receptor (Ball et al., 2003). Furthermore, recently it has been shown an increased expression of A_1 receptor on bronchial epithelium and airway smooth muscle of asthmatic subjects (Brown et al., 2007).

It is now also important to understand how and where adenosine A_1 receptor exerts its effects in the human lung; there is already evidence that adenosine can activate mast cells, and this has been considered the main mechanism of bronchospasm in asthmatics (Holgate et al., 1987). But in normal lungs, A_3 receptors mediate the adenosine-dependent activation of mast cells, while it is not clear if A_1 receptors mediate mast cell activation in asthmatic lungs; moreover, it has been shown that A_1 receptor elicits bronchospasm even in absence of mast cell activation (Meade et al., 1996). In sensitized guinea pigs, it has been recently suggested that adenosine-induced bronchoconstriction is dependent on a neuronal mechanism through A_1 receptor activation (Keir et al., 2006). Thus, to date it is known that adenosine A_1 receptor mediated bronchoconstriction may involve effects on neural pathways, smooth muscle and inflammatory cells. Taken together, our *in vivo* results confirm a provocative role for the adenosine A_1 receptor in airways, while its block could be beneficial in asthma therapy.

Western Blot analysis also showed increased expression of the A_{2A} receptor in lungs from sensitised rats, returning to control levels after allergen challenge. However, this increased A_{2A} proteic expression was not paralleled by receptor immunoreactivity on bronchial tissue. Thus, we guess that the increased A_{2A} proteic expression in lung homogenates from sensitized non-allergen challenged rats could reflect receptor expression on cells present in the pulmonary tissue. It was recently shown (Morello et al., 2006) that inflammatory stimuli, such as IL-1β and TNF-a, increase the expression of the A_{2A} gene and protein levels in lung epithelial cells *in vitro*. Our experiments, described above, on carrageenan-induced rat paw edema demonstrate that acute inflammation is associated to an increased A_{2A} receptor expression that likely plays a protective role able to limit tissue damage. One could speculate that in sensitized rats not exposed to allergen the increased A_{2A} receptor pulmonary expression is involved in the protection from airway hyperreactivity. Such an effect of A_{2A} would be loss following allergen challenge and A_1 receptor up-regulation. However, this point needs to be further investigated.

It has also been shown an increased A_{2B} receptor expression in peripheral blood mononuclear cells from asthmatic patients (Zhou et al., 2001) and, more recently, in airways and lungs of smokers with COPD (Varani et al., 2006). In addition, up-regulation of adenosine A_3 receptor is thought to occur in sensitized guinea pigs and humans with inflammatory airway disorders (Mundell et al., 2001; Walker et al., 1997). However we observed no difference in the proteic expression of the adenosine A_{2B} and A_3 receptors on lungs from the three groups

of animals studied, and no immunoreactivity was observed of these receptors in bronchi from the three animal groups.

We also performed *in vitro* experiments, on isolated bronchi from rats sensisitized and challenged with aerosolized ovalbumin *ex vivo*. The significant augmentation of the bronchoconstrictor response to allergen was reproduced in isolated bronchi obtained from *ex vivo* sensitized rats challenged with ovalbumin; conversely, there was no difference in response to adenosine on isolated bronchi obtained from the three different animal groups. Thus, the bronchial hyperreactivity conferred by allergen challenge, and evaluated as an increased response to adenosine, is lost on isolated bronchi. This finding would suggest that *in vivo* the increased bronchoconstriction in response to adenosine is likely dependent upon an indirect mechanism probably activated following A_1 stimulation. We do not know what mechanism and which mediators are involved, neither whether this indirect mechanism is wholly or only partly responsible for the response to adenosine. However, evidence that *in vivo* treatment with DPCPX, the selective A_1 antagonist, only inhibits but not abolishes the increased response to adenosine supports the hypothesis of an adjunctive mechanism apart from A_1 involvement.

There is evidence that asthmatic subjects present a reduced bronchial response to β_2-agonsits, whose cause is still a matter of debate. The "β-adrenergic theory", as proposed by Szentivanyi in 1968, tried to explain with β-adrenergic dysfunction the cause of bronchial hyperreactivity and of all atopic diseases. It was found that asymptomatic asthmatic patients presented a number of β-receptors similar to that of a normal control group, while symptomatic asthmatic

patients presented a significantly decreased receptor number when compared to both groups, suggesting that this difference could be cause of the disease (Gamboa et al., 1991); however, in another study, it was found a significant decrease of β-receptor numbers after bronchial allergic provocation test in asymptomatic asthmatic patients, thus suggesting that the reduced β-adrenoceptor number would likely be the consequence and not the cause of atopic disease (Gamboa et al., 1990). Furthermore, the reduced response of asthmatics to β_2-agonists has also been attributed to receptor desensitization due to a prolonged exposure to the agonists, or to genetic polymorphisms leading to variants of β_2-recptors with different functional properties (Broadley, 2006).

We assessed the response to salbutamol of isolated bronchi from *ex vivo* sensitized rats, to evaluate possible differences among animal groups under investigation. Our data show that salbutamol-induced relaxation on isolated bronchi from rats sensitised and aerosolised with ovalbumin was reduced in comparison to the response obtained on bronchi isolated from naïve and from sensitized rats not exposed to allergen. In parallel, immunohistochemical analysis showed a reduced immunoreactivity for β_2-adrenoceptor on bronchial smooth muscle from group of rats exposed to allergen, compared to the other groups, that could be likely caused by receptor desensitization with a mechanism of internalization, sequestration or receptor breakdown, leading to the loss of receptors from cell surface (Johnson et al., 1998). Our results suggest that the reduced β_2-adrenoceptor number on airway smooth muscle would account for the reduced response to salbutamol and would be consequent to allergen exposure. Thus, our findings further support the hypothesis of early clinical studies

suggesting that decrease of β_2-receptors could be the consequence of atopic diseases and secondary to the immunological events that follow antigen-antibody reactions and cell membrane changes (Gamboa et al., 1990; Gamboa et al., 1991; Oehling et al., 1991). There is evidence that bronchial hyperreactivity following allergen exposure could be linked to the reduced β_2-receptor number or sensitivity (Nielson et al., 1992); but the mechanism at the basis of β-adrenergic desensitization has not yet been clarified; however, several inflammatory mediators likely present in asthmatic airways have been shown to be involved (Shore and Moore, 2002; Hirst et al., 2003; Rovati et al., 2006).

In conclusion, our study suggests that allergen exposure of sensitized rats causes airway hyperresponsivenes to ovalbumin that might be related to a reduced number of β_2-adrenoceptor, likely dependent upon receptor desensitization evaluated as a reduced response to salbutamol. Conversely, hyperresponsiveness to adenosine, also caused by allergen exposure, cannot be explained by β_2-adrenoceptor desensitiziation since, under our experimental conditions, it is lost on isolated tissues; thus, in agreement with other authors (Broadley, 2006; Keir et al., 2006), we hypotesise that it is mostly due to an indirect mechanism probably involving sensory nerves and/or inflammatory cell activation.

4.3. Adenosine receptors and myocardial ischemia/reperfusion injury

The microenviroment of injured and inflamed tissues is characterized by low levels of oxygen and inflammation/injury-related hypoxia can be expected to

activate hypoxia-inducible factor 1 (HIF-1), an ubiquitous transcription factor, which functions as a master regulator of oxygen homeostasis (Semenza, 2001; Berra et al., 2006; Fandrey et al., 2006). Active HIF-1 is a heterodimer consisting of an inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit and regulating the expression, under pathological conditions, of numerous genes which encode proteins that are prosurvival in nature and can result in cytoprotection (Lefer, 2006). In fact previous studies demonstrated that stable expression or activation of HIF-1α can protect isolated cardiac myocytes and isolated hearts against ischemia/reperfusion injury (Date et al., 2005; Ockaili et al., 2005). In addition, it was found that HIF-1α could be considered a sensitive auxiliary diagnostic marker of hypoxia; in fact, its expression, as yet discussed, is very low in non-hypoxic tissues, including cardiac muscle, while its gene expression is up-regulated within several minutes after ischemia, including myocardial ischemia and remains high if the inducement continues (Li et al., 2007). We measured HIF-1α levels in rat cardiac tissue samples, expecting a modulation of this protein among animal groups; we found an augmented protein expression of HIF-1α in ischemic hearts ($p < 0.05$), and a strongly augmented expression of this factor in ischemic/reperfused hearts ($p < 0.01$), compared to non-ischemic hearts. This finding is in accordance also to an other recent work (Cai et al., 2007) which show that HIF-1 α is required for myocardial protection against ischemia/reperfusion injury.

It is now well established that adenosine functions as a potent intrinsic cardioprotective molecule; in particular, the binding of adenosine to A_1 receptor is the initial step in triggering this protective mechanism against myocardial

infarction (Yang et al., 2002). It was demonstrated that the effects of adenosine are limited by receptor number rather than by agonist concentration in ischemic murine myocardium (Matherne et al., 1997); in addition, it was shown, using transgenic techniques, that cardiac adenosine A_1 receptor over-expression activates endogenous protective mechanisms that provide the heart with increased resistance to ischemia/reperfusion injury (Yang et al., 2002). Furthermore, it has been shown that adenosine may regulate HIF-1 expression in cells under hypoxic and normoxic conditions (De Ponti et al., 2007; Kong et al., 2006). We measured proteic expression of the adenosine A_1 receptor to evaluate a possible change of its levels after myocardial ischemia and reperfusion; we found an augmented expression of A_1 receptor in ischemic hearts, returning to basal levels following reperfusion; this finding suggests that ischemia injury itself causes an over-expression of this receptor, that might operate as immediate cardioprotective mechanism after myocardial damage.

On the other hand, the functional importance of the adenosine A_3 receptor in modulating injury during myocardial ischemia remains to be determined; it was supposed that this receptor is involved in adenosine cardioprotection (Kilpatrick et al., 2001), but it was also shown that mice lacking functional A_3 receptor are more resistant to the development of irreversible ischemic injury, suggesting an injurious role for this receptor during myocardial ischemia, probably potentiating the inflammatory response (Guo et al., 2001). We measured proteic expression of the adenosine A_3 receptor to evaluate a possible change of its levels too, after myocardial ischemia and reperfusion; our results showed a reduction of A_3 protein expression after ischemia and also after 24 h reperfusion, suggesting that

ischemia/reperfusion injury itself causes a down-regulation of this receptor, with a restriction of its possible harmful action.

Taken together, our preliminary results suggest that adenosine A_1 and A_3 receptors might have opposite effects in modulating injury during myocardial ischemia; thus, as yet supposed (Yang et al., 2002), strategies designed to moderately modify the density of adenosine receptors in the myocardium might have clinical potential.

5. CONCLUSIONS

In this study, we have investigated on the role of adenosine and adenosine receptors in three different rat models involving the inflammatory-immune system: paw oedema (acute inflammation), airway hyperresponsiveness (immunological inflammation) and myocardial ischemia/reperfusion injury. Indeed, bronchial asthma and ischemia-reperfusion injury after myocardial infarction are disorders characterized by an underlying inflammatory process. Our results confirm a pivotal role of adenosine in inflammation and are further evidence for adenosine receptors as therapeutic targets of inflammatory disorders.

To date there is growing evidence that adenosine receptors could be promising therapeutic targets in a wide range of pathologies, including cardiovascular diseases (cardiac arrhythmia and ischemia), nervous system disorders (dementia, Parkinson's disease and other neurodegenerative diseases; hyperactivity, anxiety, schizophrenia and sleep disorders) renal system diseases, pulmonary disorders (bronchial asthma and chronic obstructive pulmonary disease), immune and inflammatory disorders, endocrine disorders (diabetes) and cancer.

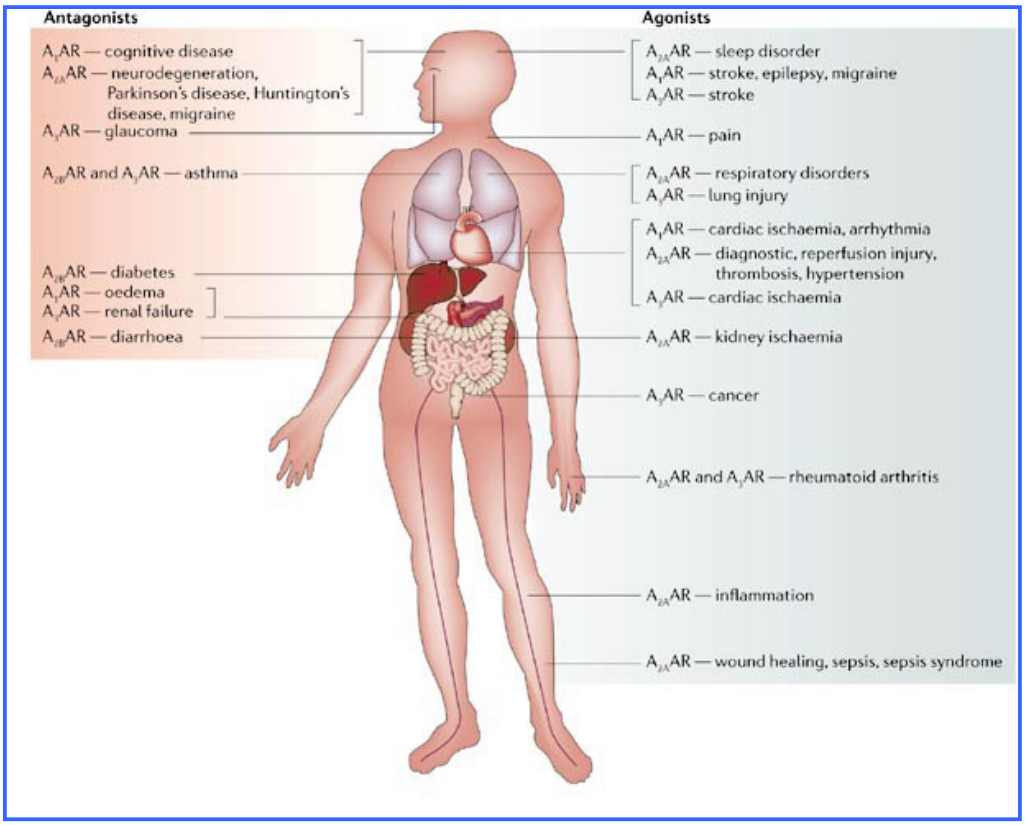


Figure 46: Novel disease targets for selective adenosine receptor ligands.

After more than three decades of pharmaceutical research, several therapeutic targets related to the "adenosinergic system" have been specified, while a considerable number of selective agonists and antagonists of adenosine receptors have been discovered, and some have been clinically evaluated; however, none among adenosine receptor modulators has yet received regulatory approval.

Indeed, it is surprising to note that only the same adenosine is to date in clinical use. In fact, Adenoscan® (adenosine 30 mg/10 ml, for iv. use) is indicated as an adjunct to thallium201 myocardial perfusion scintigraphy for coronary vasodilation in patients unable to exercise adequately; Krenosin® (or

Adenocard®; 6 mg/2 ml, for iv. use) is indicated in cardiac arrhythmia (conversion to sinus rhythm of paroxysmal supraventricular tachycardia (PSVT), including that associated with accessory bypass tracts (Wolff-Parkinson-White Syndrome). On the other hand, adenosine receptors are major targets of caffeine and other xanthines (theophylline and theobromine), the most "commonly consumed drugs" in the world.

One reason for this enigma is the ubiquity of adenosine receptors and the possibility of side effects; in addition, species differences in the affinity of putatively selective ligands complicated pre-clinical testing in animal models. Local administration, with selective activation and blockade of these receptors, could be a good strategy to overcome these obstacles; an other recent strategy is that of "neoceptors": reengineered adenosine receptors that can recognize uniquely modified nucleosides that are inactive at the native adenosine receptors. This neoceptor strategy is intended for eventual use in organ-targeted gene therapy and has yet led to development of neoceptors for adenosine A_{2A} and A_3 receptors (Jacobson and Gao, 2006).

In conclusion, recent advances in the understanding of the roles of the various adenosine receptor subtypes and in the development of selective and potent ligands, together with new findings in pathogenetic mechanisms of various diseases can make possible therapeutic applications of drugs interfering in the "adenosine system".

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