Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN

"Farmacologia e Oncologia Molecolare"

CICLO XXII

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ADENOSINE RECEPTORS MODULATION OF INFLAMMATORY CELLS: THE FOAM CELLS HISTORY

Settore Scientifico Disciplinare BIO/14

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Anni 2007/2009

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GENERAL INTRODUCTION

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. Adenosine is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond **(Figure 1)**.

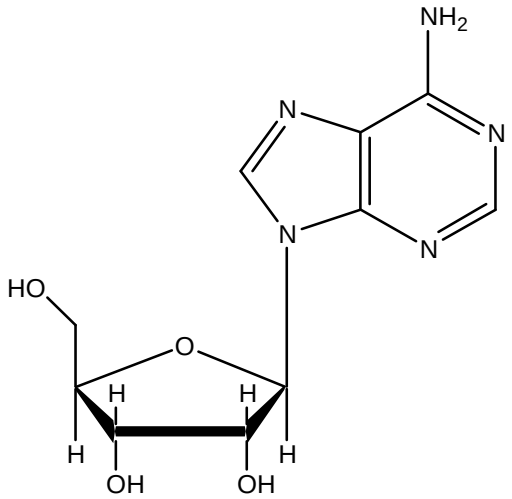

Figure 1: Adenosine structure

Adenosine is present in every cell of the body and its cytoplasmic concentration is tightly regulated. The levels of adenosine are determined primarily from the dephosphorylation of its immediate precursor, adenosine monophosphate (AMP). Precursors of AMP include cyclic AMP (cAMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP). ATP is co-released with other neurotransmitters from presynaptic vesicles and is also produced by mast cells, basophiles and endothelial cells and as result of cellular damage. ADP is derived from activates platelets, while cyclic AMP serves as a second messenger in most cells. In normoxic condition the estimated ratio of ATP: AMP is approximately 50:1; when the total ATP levels decrease, for example in ischemic tissues where the oxygen supply is strongly

reduced, there are a large production and so increase in AMP and adenosine (Linden, 1994). It is so clear that the important role of adenosine is to protect the cell when a decrease of oxygen supply could compromise the physiological cellular function and survival. Adenosine nucleotide is degraded by a series of ectonucleotidases. One such enzyme, 5'-nucleotidase, catalyses the conversion of AMP to adenosine during increased cellular metabolism. 5'-nucleotidase is found both extracellularly (attached to the plasma membrane by glycosyl-phosphatidylinositol anchors) and in the cytosol. Regulation of the activity and/or expression of this enzyme is critical for regulation of the levels of adenosine.

Adenosine is also produced from the hydrolysis of S-adenosylhomocysteine (SAH), by S-adenosylhomocysteine hydrolase. This mechanism is responsible of a significant portion of the adenosine present under resting conditions. S-adenosylhomocysteine also serves as an intracellular binding protein for adenosine, thereby protecting the nucleoside from degradation. Adenosine is rapidly cleared from the extracellular space through a bi-directional facilitates transporter that is sensible to the drug dipyridamole. So, the administration of dipyridamole increases interstitial adenosine levels, in accordance to the pharmacological actions of this drug, which include coronary vasodilatation, sedation and anticonvulsant action. Under normoxic conditions, adenosine is phosphorylated by adenosine kinase to AMP and subsequently to ATP to restore the nucleotide pool. However, under conditions of increased metabolic stress, the increased levels of adenosine easily saturate adenosine kinase and excess adenosine is metabolised to inosine and hypoxanthine by adenosine deaminase **(Figure 2)**.

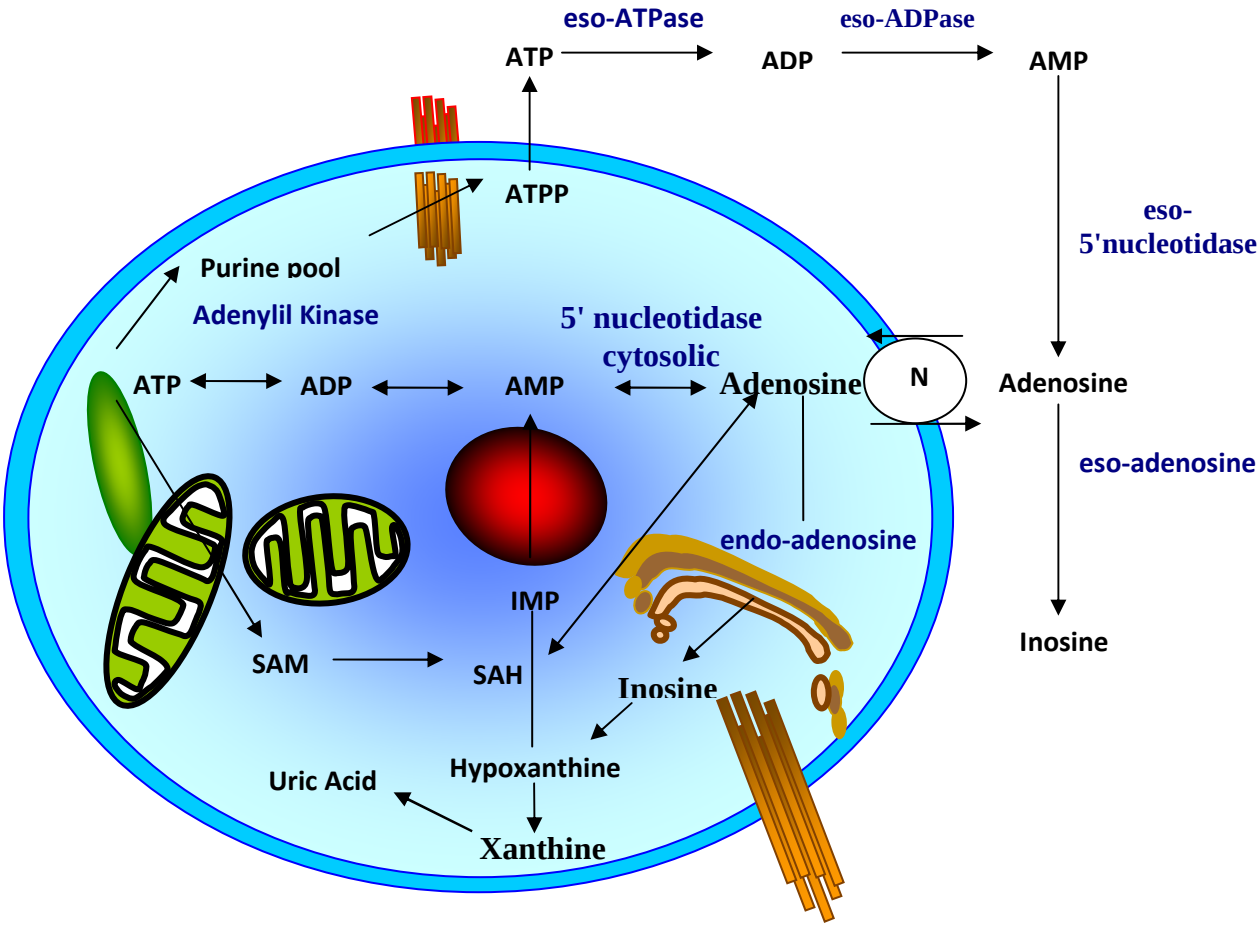

Figure 2: Metabolism of adenosine

Adenosine receptors

A large number of hormones, neurotransmitters and neuromodulators that regulate the interactions between cells, exert their effects by interacting with specific G protein-coupled receptors (GPCRs). In 1978, Burnstock proposed the existence of almost two types of receptors for purines, called P1 and P2. This distinction was based on the order of potency of various nucleotides and nucleosides: P1 identifies the family of receptors more sensitive to adenosine, whereas P2 receptors are preferentially activated by ATP and ADP (Burnstock, 1978). A further criterion of differentiation between P1 and P2 receptors is based on different sensitivity to antagonists with xanthine structure. In particular P1 receptors are inhibited competitively by drugs such as caffeine, xanthine, theophylline and theobromine, which are inactive on P2 receptors. This general division into P1 and P2 receptors has been the basis for the current classification and nomenclature of these receptors. Each of the two families includes several receptor subtypes identified by pharmacological profile, the mechanism for translating the signal and the molecular structure (Fredholm et al., 2001). P1 receptors are divided in 4 subtypes, localized at the cytoplasmic membrane (A_1, A_{2A}, A_{2B}, A_3) and have a variable distribution in different systems (central nervous system, cardiovascular, renal, respiratory, immune and gastrointestinal), where they modulate normal biological functions (Ralevic and Burnstock, 1998). Previously, adenosine receptors (ARs) have been classified according to their molecular structure, their pharmacological profile and their mechanism of signal transduction (Fredholm et al., 2001). Each subtype is coupled to a particular type of G protein, which may be stimulatory (Gs) or inhibitory (Gi). In some tissues, A_1 and A_3 receptors are able to modulate the activity of phospholipase C (PLC) and, in the case of A_1 receptors, ion channels for Ca^{2+} or K^+.Considering the overall protein structure, ARs display the topology typical of GPCRs. Sequence comparison between the different GPCRs revealed the existence of different receptor families sharing no sequence similarity even if specific fingerprints exist in all GPCR classes. However, all these receptors have in common a central

core domain consisting of seven transmembrane helices (TM1-7), with each TM composed of 20–27 amino acids, connected by three intracellular (IL1, IL2, and IL3) and three extracellular (EL1, EL2, and EL3) loops. Two cysteine residues (one in TM3 and one in EL2), which are conserved in most GPCRs, form a disulfide link which is possibly crucial for the packing and for the stabilization of a restricted number of conformations of these seven TMs. Aside from sequence variations, GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain, and their intracellular loops. Each of these domains provides very specific properties to these receptor proteins. Particularly, consensus sites for N-linked glycosylation exist on the extracellular regions of ARs, although the precise location of the sites for this post-translational modification varies amongst the AR subtypes. The carboxyl-terminal tails of the A_1AR, A_{2B}AR, and A_3AR, but not A_{2A}AR, possess a conserved cysteine residue that may putatively serve as a site for receptor palmitoylation and permit the formation of a fourth intracellular loop (Moro et al., 2005). The A_1AR, A_{2B}AR, and A_3AR are very similar in regard to the number of amino acids composing their primary structure, and in general, these AR subtypes are among the smaller members of the GPCR family. For example, the human homologs of the A_1AR, A_{2B}AR, and A_3AR consist of 326, 328, and 318 amino acid residues, respectively. Conversely, the human A_{2A}AR is composed of 409 amino acids. It should be noted that the size of ARs deduced from their primary amino acid structure frequently is not consistent with the mass estimated by polyacrylamide gel electrophoresis of the expressed proteins. The post-translational glycosylation of ARs, which may vary in a cell type-dependent fashion, likely accounts for these discrepancies. The human A_1AR and human A_3AR display 49% overall sequence identity at the amino acid level, while the human A_{2A}AR and human A_{2B}AR are 45% identical (Fredholm et al, 2001).

A_1 adenosine receptors

A_1 receptors were purified in several species including man, rat, bovine, rabbit. The human A_1 receptor gene is localised on 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). This receptor subtype has one or two glycosylation sites on the second extracellular domain, a potential site for acetylation of fatty acids on the carboxyl tail, several phosphorylation sites and a cluster of serine and threonine residues on the C-terminal portion (Linden, 1991). Molecular biology techniques demonstrated that the A_1 receptors throught the interaction with Gi or Go proteins mediate the inhibition of adenylate cyclase, but can also inhibit G protein-coupled activation of voltage dependent Ca^{2+} channels and induced PLC activation (Stiles, 1992) **(Figure 3)**.

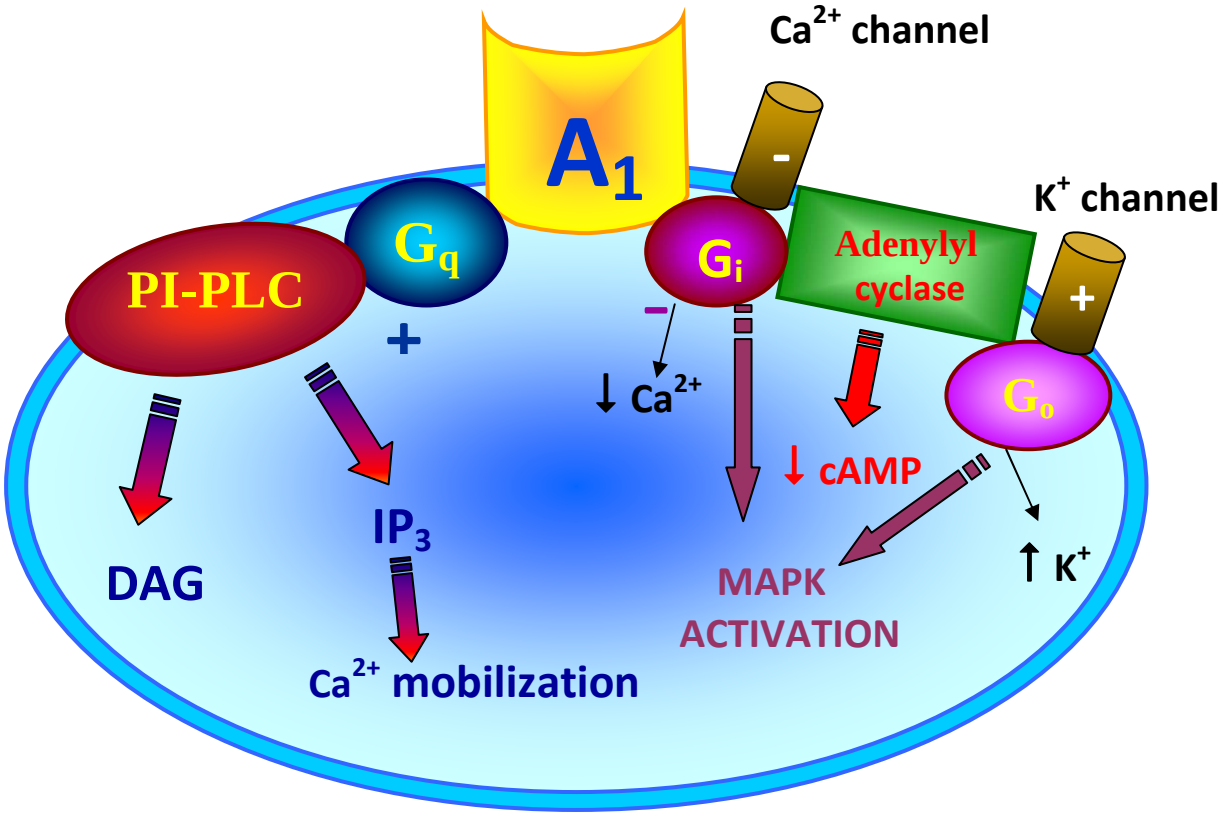

Figure 3: Adenosine A_1 receptor and its signal transduction pathway

A_1 adenosine receptor also determines the activation of the family of extracellular signal-regulated kinase 1/2 (ERK 1/2) (Fredholm et al., 2001a). Adenosine A_1 receptors are widely expressed in both central nervous system (CNS) and peripheral tissues (Schindler et al., 2005). In the CNS, they are present in both pre- and post-synaptic regions. The highest density was found in the cortex, cerebellum and hippocampus. In the heart, adenosine A_1 receptors predominate in the myocardium and in the sinus and atrioventicular nodes and are involved in responses that reduce oxygen demand by directly decreasing heart rate, force of contraction and conduction of action potential (through opening of K^+-channels) as well as by indirect antiadrenergic effects (through inhibition of adenylyl cyclase). High levels of adenosine A_1 receptors are observed in adipose tissue where its mediate a lipolytic action (Linden et al., 1991). Adenosine interacting with this receptor subtype posses the ability to reduce the free fatty acids in the blood; this advantageous aspect of adenosine could be useful for the treatment of type 2 diabetes. Adenosine A_1 receptors are expressed in quite high density on the specialized cells of the thyroid, spinal cord, eye, adrenal gland, kidney, lung, pancreas and liver **(Table 1)**.

Table 1: Effects mediated by A_1 adenosine receptors

DISTRIBUTION	EFFECTS	REFERENCES
SNC	Depression of excitotoxic neuronal damage Inhibition neurotransmitter release Neuroprotective effects	Wardas, 2002 Latini et al., 2001 Liu et al., 2005
HEART	Anti-ischemic effects Beneficial effects on cardiac hypertrophy and heart function	Roscoe et al., 2000 Liao et al., 2003
KIDNEY	Inhibition of glomerular filtration rate Inhibition of renin secretion Increase of sodium reabsorbition	Ren et al., 2001 Schweda et al., 2005 Gottlieb et al., 2002
PANCREAS	Increase of glucose uptake	Xu et al., 1998
AIRWAYS	Bronchoconstriction	Polosa, 2002
ADIPOSE TISSUE	Control of lipolysis	Fruhbeck et al., 2001
NEUTROPHILS	Increase of chemotaxis, margination and endothelium adhesion Increase of phagocytosis and ROS release	Gessi et al., 2000; Polosa, 2002 Sullivan et al., 2001

A_2 adenosine receptors

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. A_2 receptors are coupled to the intracellular transduction pathway through G_s proteins and stimulates the activation of adenylate cyclase resulting in the elevation of intracellular cAMP (Olah and Stiles, 1995; Moreau and Huber, 1999) **(Figure 4)**. Activation of A_2 receptors also increases mitogen-activated protein kinase (MAPK) activity. Adenosine agonists through activation of ERK1/2 using the cAMP-ras-MEK1 pathway, exert mitogenic effects on human endothelial cells via the adenosine A_{2A} subtype. However, the signaling pathways used by A_{2A} receptor seem to vary with the cellular background and the signaling machinery of each cell. The adenosine A_{2B} receptor subtype has been shown to activate not only ERK1/2 but also stress-activated protein kinases (SAPK), such as p38 and jun-N-terminal kinase (JNK) (Fredholm et al., 2001a). In most cell types the A_{2A} subtype inhibits intracellular calcium levels whereas the A_{2B}, via G_q proteins are linked with the stimulation of phospholipase C and induces calcium increase (Feostikov and Biaggioni, 1997).

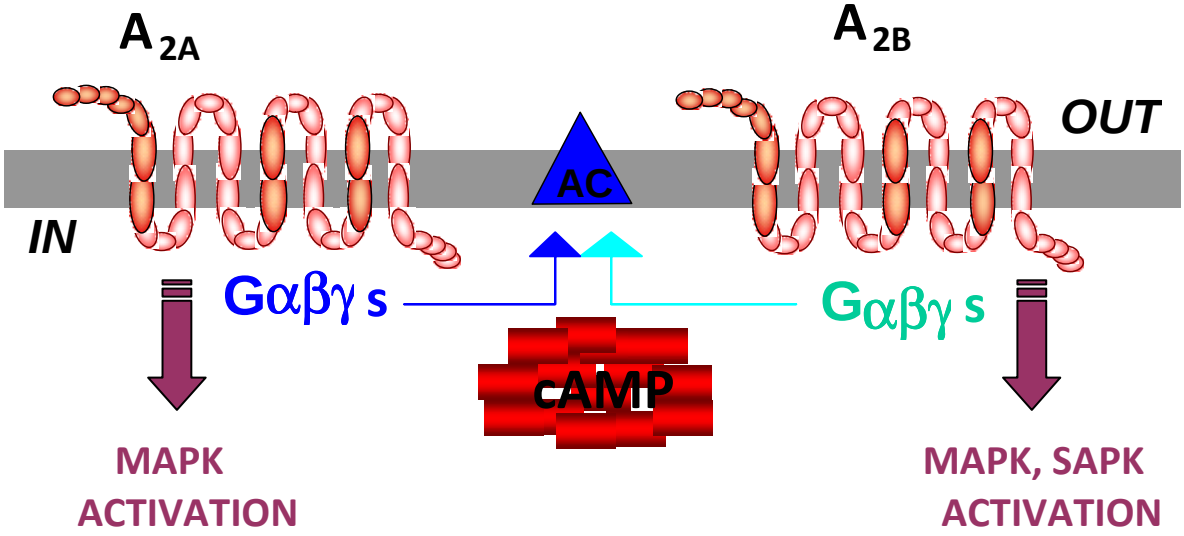

Figure 4: Adenosine A_2 receptor and their signal transduction pathways

A_{2A} adenosine receptors

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 (Gessi et al., 2000). It is well recognized that adenosine can exert powerful effects on the immune system; this molecule has been identified as an important endogenous immunosuppressing regulator as it has been demonstrated that the lack of the enzyme adenosine deaminase (ADA) is responsible for severe immunodeficient disease (Hirschhorn R., 1995; Apasov et al., 1995). A number of effects ascribed to adenosine on lymphocyte function, such as inhibition of interleukin-2 (IL-2) production, cell proliferation and major histocompatibility complex-restricted cytotoxicity, appear to be mediated through G_s-coupled A_{2A} receptors (Polmar et al., 1990). Moreover A_{2A} adenosine receptors are expresses in human neutrophils were they inhibits the respiratory burst, assayed as superoxide anion production (O_2^-) from cells stimulated by the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (FMLP) (Dianzani et al., 1994). Despite the beneficial aspects of quenching inflammation the described A_{2A} adenosine receptors-mediated down-regulation of the immune response is potentially dangerous on its own. Indeed, the premature inhibition of immune cell function may allow pathogens to survive and as a result the overall damage to the organism will be greater. On the other hand, the lack of A_{2A} adenosine receptors signaling may result in excessive damage with important biological consequences. Thus, a balance between the need to destroy invading pathogens and the desire to protect tissue from excessive damage may ultimately be dependent on the level of the activation of A_{2A} receptors. Manipulation of inflammatory processes may include not only efforts to inhibit inflammation, but also the development of approaches to enhance local inflammatory processes **(Table 2)**.

Table 2: Effects mediated by A_{2A} adenosine receptors

DISTRIBUTION	EFFECTS	REFERENCES
SNC	Protection against dopaminergic cell loss in Parkinson's desease Increase acetylcholine, glutamate, aspartate, dopamine, and norepinephrine release	Simon et al., 2008 Riberio et al., 2000
HEART	Increase coronary flow Protection against ischemia-reperfusion injury	Pacher et al., 2007 Peart et al., 2007
BLOOD VESSELS	Coronary vasodilatation Inhibition of IL-6,IL-8, IL-12 production	Glover et al., 2001 Haskò et al., 2000
PLATELETS	Inhibition of platelet aggregation	Gessi et al., 2000
IMMUNE CELLS	Decrease of ROS release and cytotoxic activity Inhibition of neutrophils activation and O_2^- production stimulated by fMLP Regulation of several cytokines production Inhibition of IL-2 production in lymphocytes	Pierce et al., 2001 Gessi et al., 2000 McColl et al., 2006 Erdmann et al., 2005
MONOCITES/ MACROPHAGES	Inhibition of TNF-α, IL-6, IL-8 and IL-10 production	Haskò et al., 2000 Ryzhov et al., 2008
LUNGS	Ant-inflammatory effects	Fozard et al., 2002
LIVER	Reduction of hepatic ischemia reperfusion injury	Lappas et al., 2006

A_{2B} adenosine receptors

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 A_{2B} receptors, on the contrary with the other adenosine receptor subtypes, are characterized by a low density and it needs high adenosine levels to their activation. (Fredholm et al., 2001b). 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).

A_{2B} adenosine receptor has been identified in several areas including in the brain, human bronchial epithelium, endothelial cells, muscle cells, neurons, glial cells, fibroblasts and mast cells (Gessi et al., 2006). A_{2B} receptors, predominantly expressed in human microvascular cells, modulate expression of angiogenic factors (Feoktistov et al., 2002). The A_{2B} adenosine receptor subtype appears to mediate the actions of adenosine to increase growth factor production (VEGF) and cell proliferation of human retinal endothelial cells (HREC). Adenosine activates the A_{2B} adenosine receptor in HRECs, which may lead to neovascularization by a mechanism involving increased angiogenic growth factor expression (Grant et al., 1999). On this light, A_{2B} adenosine receptor inhibition may offer a way to inhibit retinal angiogenesis and provide a novel therapeutic approach to treatment of diseases associated with aberrant neovascularization, such as diabetic retinopathy and retinopathy of prematurity (Grant et al., 2001). Adenosine causes inhibition of cardiac fibroblasts growth and of vascular smooth muscle cells by activating A_{2B} adenosine receptors (Dubey et al., 1999, 2000, 2001). Exogenous and endogenous adenosine inhibits both collagen production and cellular hypertrophy induced by fetal calf serum, most likely via A_{2B} receptors (Dubey et al., 1998). Thus, A_{2B} adenosine receptors may play a critical role in regulating cardiac remodeling associated with cardiac fibroblasts proliferation. Pharmacological or molecular biological activation of A_{2B} adenosine receptors may prevent cardiac remodeling associated with

hypertension, myocardial infarction, and myocardial reperfusion injury after ischemia. It is interesting to note that, in contrast to smooth muscle cells, A_{2B} receptors induce growth of endothelial cells (Sexl et al., 1995; Grant et al., 1999). Even if A_{2B} receptor stimulation improves the cell proliferation of peripheral microvessels and exerts opposite effect on cardiac tissue and capillaries, we can hypothesize that, as consequence of this ability to promote the endothelial cell proliferation, A_{2B} may contribute to tumor growth and spreading by inducing neovascularization in the area surrounding the tumor masses. Moreover A_{2B}ARs have been implicated in the regulation of mast cell secretion and, gene expression, intestinal function, neurosecretion, vascular tone and in particular asthma (Varani et al., 2005) **(Table 3)**.

Table 3: Effects mediated by A_{2B} adenosine receptors

DISTRIBUTION	EFFECTS	REFERENCES
SNC	IL-6 release from astrocytes and protection during CNS injury	Haskò et al., 2005
HEART	Inhibition of cardiac fibroblasts growth	Dubey at al., 2000, 2001
BLOOD VESSELS	Vasodilatation Inhibition of ICAM-1 and E-selectin expression Prevention endothelial cell-mediated inflammatory events Promotion of angiogenesis	Yang et al., 2006 Grant et al., 2001 Feoktistov et al., 2002
INTESTINAL EPITHELIAL CELLS	Regulation Cl^- secretion Release of IL-6	Kolachala et al., 2008 Sitaraman et al., 2001
MAST CELLS	Increase of degranulation, histamine release and IL-8 secretion	Polosa et al., 2006
AIRWAYS	Increase of IL-6, IL-19, TNF-α production	Zhong et al., 2004, 2006
MONOCITES/ MACROPHAGES/ NEUTROPHILS/ LYMPHOCYTES	Down-regulation of IFN-γ-induced MHC class II expression and inducible nitric oxide synthase production (iNOS) Inhibition of monocyte colony stimulating factor (M-CSF)-induced macrophage proliferation	Xaus et al., 1999 Gessi et al., 2005

A_3 adenosine receptors

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). The various effects of A_3 receptor agonists, in vitro and in vivo, seem to be dual and opposite, depending on the level of receptor activation (Jacobson et al., 1998). The A_3AR mRNA is being expressed in testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon and eye of rat, sheep and humans. However, marked differences exist in expression levels within and among species. In particular rat testis and mast cells express high concentrations of A_3AR mRNA, while low levels have been detected in most other rat tissues (Gessi et al., 2008). Lung and liver have been found as the organs expressing high levels of A_3AR mRNA in human, while low levels have been found in aorta and brain. Lung, spleen, pars tuberalis and pineal gland expressed the highest levels of A_3AR mRNA in sheep. The presence of A_3AR protein has been evaluated through radioligand binding, immunoassay or functional assay in a variety of primary cells, tissues and cell lines (Gessi et al., 2008). In the mouse brain a widespread, relatively low level of A_3AR binding sites was found (Jacobson et al., 1993). Similar data were obtained in the rat and in gerbil and rabbit brain. Electrophysiological and biochemical evidence suggested the presence of A_3ARs in the rat hippocampus and cortex, and functional studies also indicated its presence in the brain. In cardiomyocytes, there was no direct evidence of the presence of A_3ARs but several studies reported that it was responsible for cardioprotection in a variety of species and models, including isolated cardiomyocytes and isolated myocardial muscle preparations (Peart and Headrick, 2007). In lung parenchyma and in human lung type 2 alveolar-like cells (A549), the A_3AR was detected through radioligand binding and immunohistochemical assays (Varani et al., 2006). The classical pathways associated with A_3AR activation are the inhibition of adenylyl cyclase activity, through the coupling

with G_i proteins, and the stimulation of PLC, inositol triphosphate (IP3) and intracellular calcium, via Gq proteins (Fredholm et al., 2001) **(Figure 5)**. However, more recently additional intracellular pathways have been described as relevant for A_3AR signaling. For example, in the heart, A_3AR mediates cardioprotective effects through ATP-sensitive potassium (KATP) channel activation. Moreover, it is coupled to activation of RhoA and a subsequent stimulation of phospholipase D (PLD), which in turn mediates protection of cardiac myocytes from ischemia (Mozzicato et al., 2004). In addition, in different recombinant and native cell lines, A_3AR is involved, like the other adenosine subtypes, in the modulation of MAPK activity (Schulte and Fredholm, 2003). A_3AR signaling in Chinese Hamster Ovary cells transfected with human A_3AR (CHO-hA3) leads to stimulation of extracellular signal-regulated kinases (ERK1/2). In particular, A_3AR signaling to ERK1/2 depends on $\beta\gamma$ release from pertussis toxin (PTX)-sensitive G proteins, phosphoinositide 3-kinase (PI3K), Ras and mitogen-activated protein kinase kinase (Schulte and Fredholm, 2003). Recently, it has been shown that A_3AR activation leads to an increase in c-Jun N-terminal kinase (JNK) phosphorylation in U87MG glioblastoma cells (Gessi et al., 2010). It has been reported that A_3AR activation is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma cells. This implies the deregulation of the Wnt signaling pathway, generally active during embryogenesis and tumorigenesis to increase cell cycle progression and cell proliferation (Fishman et al., 2002). Involvement of the PI3K/PKB pathway has been linked with preconditioning effects induced by A_3AR activation in cardiomyocytes from newborn rats (Germack and Dickenson, 2005). Subsequently it has been demonstrated that CREB phosphorylation occurs through both Akt-dependent and -independent signaling. Activation of PI3K-Akt-pBAD by A_3AR has been observed recently in glioblastoma cells leading to cell survival in hypoxic conditions (Merighi et al., 2007). Further studies indicate that A_3AR activation by interfering with PKB/Akt pathways can decrease interleukin-12 (IL-12) production in human monocytes (la Sala et al., 2005). Collectively, these findings demonstrate that several intracellular mechanisms are involved following A_3AR stimulation, the understanding of which may be essential

and crucial for explaining the different aspect of its activation **(Table 4)**.

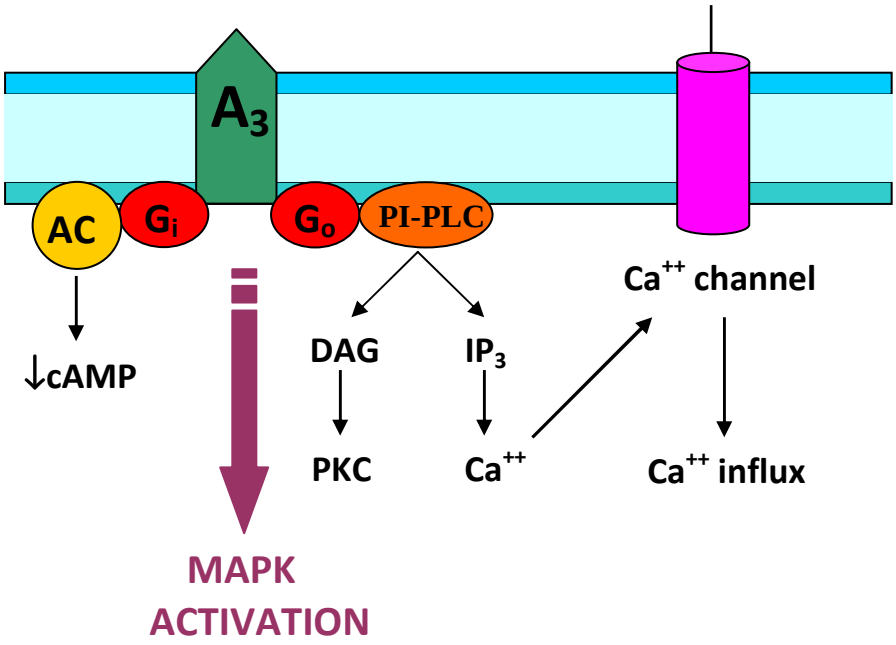

Figure 5: Schematic representation of intracellular pathways coupled to adenosine A_3 receptors

Table 4: Effects mediated by A_3 adenosine receptors

DISTRIBUTION	EFFECTS	REFERENCES
SNC	Reduction in the degree of spontaneous apoptotic cell death Neuro-protective effect in epilepsy, trauma and brain ischemia	Abbracchio et al., 1998 Abbracchio et al., 1999
HEART	Protection of myocytes from ischemia	Mozzicato et al., 2004
MAST CELLS	Antigen-mediated mast cell degranulation.	Fredholm et al., 2001
IMMUNE CELLS	Eosinophils: inhibition of degranulation Neutrophils: inhibition of oxidative burst and degranulation Monocytes: inhibition of superoxide anion generation; suppression of TNF-α and IL-12 release Mast cells: release of allergic mediators	Fossetta et al., 2003 Gessi et al., 2002 Broussas et al., 1999 La Sala et al., 2005 Gessi et al., 2004a
LUNG	Inhibition of degranulation and O_2^- release in eosinophils	Ezeamuzie et al., 1999
TUMOR CELLS	Inhibition of tumour growth Inhibition of A375 human cells proliferation Stimulation of glioblastoma cell survival in hypoxic conditions	Gessi et al., 2004b Merighi et al., 2005 Merighi et al., 2007

Therapeutic potential

Adenosine and cardiovascular system

A_1AR is the most extensively studied and well characterized of the adenosine receptor subtypes in relation to cardiac protection. The A_1AR is best understood insofar for its effects on injury and in terms of receptor-coupled kinase/protein signaling. Originally, adenosine (via A_1AR activation) was thought to induce myocardial protection through preservation of ATP (and improved nucleotide repletion on reperfusion), stimulation of glycolysis, and normalization of the hearts so-called “oxygen supply/demand ratio” (Ely and Berne, 1992). Subsequent investigations have identified essential protein kinase signaling cascades together with putative end-effectors (including the mitochondrial KATP channel), in the protective and preconditioning actions of A_1ARs.

Adenosine enhances tolerance to ischemia via metabolic substrate effects (Headrick et al., 2003). Adenosinergic cardioprotection in ischemic-reperfused hearts involves reductions in oncotic and apoptotic death, and improved functional outcomes (Willems et al., 2005). Recent work supports differential effects of acute adenosine vs. transient adenosinergic preconditioning, consistent with multiple pathways of protection (Peart and Headrick, 2003). In terms of cellular targets, adenosine appears to directly protect cardiomyocytes or myocardial tissue (likely via A_1 and A_3ARs), and additionally protects via limiting inflammation and injurious interactions between inflammatory cells and vascular and myocardial tissue. The different cardioprotective effects of AR agonism have been verified in animal and human tissue (Willems et al., 2005). However, few studies have addressed the possibility that altered AR-mediated protection might underlie specific cardiovascular disorders, though there is evidence to support this. Hypertrophic hearts, for example, display abnormal adenosinergic signaling, and dysregulated adenosine formation. Interestingly, ARs impact on many processes implicated in cardiovascular "aging", regulating Ca^{2+} influx and oxidant injury, substrate metabolism, angiogenesis, myocardial fibrosis, and apoptotic

processes (Willems et al., 2005). Given evidence of a role for ARs in intrinsic cardioprotection, mediation of preconditioning, and modifying the above-mentioned processes, alterations in AR signalling could contribute both to ischemic intolerance and emergence of other features of aged myocardium. All ARs are considered to be expressed within cardiovascular cells. Studies in different species verify endogenous adenosine contributes to intrinsic ischemic tolerance, and support cardioprotective roles for A_1ARs in vitro and in vivo, and for A_{2A}ARs in vivo (Willems et al., 2005). Anti-ischemic effects of A_1ARs appear direct (at cardiomyocytes), since similar protection is observed in isolated hearts, cardiomyocytes, and in vivo (Roscoe et al., 2000). Protective A_{2A}AR effects involve modulation of vascular function, platelet adhesion and neutrophil activation. There is currently no direct evidence for acute A_{2B}AR mediated cardioprotection, partially due to lack of selective A_{2B}AR agonists/antagonists. In contrast to A_1 and A_{2A}ARs, there is little evidence that intrinsically activated A_3ARs mediate protection. A_3AR antagonists have no effect on ischemic outcomes in myocytes or hearts (Maddock et al., 2002).

Adenosine and inflammation

The adenosine receptor system has evolved as both a rapid sensor of tissue injury and the major 'first-aid' machinery of tissues and organs (Haskò et al., 2008). Adenosine receptor activation thus preserves tissue function and prevents further tissue injury following an acute injurious insult, such as reperfusion injury, actions in which the immune system has a paramount role. This primordial protective function of the adenosine receptor system following acute insults can, however, be overshadowed by its reduced ability to protect against chronic insults. In addition, in certain chronic disease states, such as asthma, the adenosine receptor system can even exacerbate tissue dysfunction (Haskò et al., 2008).

The effect of adenosine on cytokine production by macrophages has attracted considerable attention, because macrophage-derived cytokines are crucial initiators and orchestrators of immune responses. As tumour necrosis factor α (TNF-α) was one of the first cytokines to be discovered, a substantial

body of information has accumulated regarding the ability of adenosine receptor activation to limit TNF-α production following macrophage activation. Recent studies using adenosine-receptor knockout mice have painted a detailed but still no complete picture of the receptors involved. Several studies agree that the A_{2A} receptor is the primary and dominant adenosine receptor subtype that mediates inhibition of TNF-α (Haskò et al., 2000; Kreckler et al., 2006; Ryzhov et al., 2008). A role for other receptors was postulated based on the observation that adenosine, and the agonists NECA and IB-MECA (CF-101) can each inhibit, albeit to a lesser extent, TNF-α production even in A_{2A}-receptor knockout mice (Haskò et al., 2000; Kreckler et al., 2006). A study using a combined approach of using A_{2A} receptor knockout mice and the A_{2B} receptor antagonist MRS 1754 supports a role for A_{2B} receptors as the other inhibitory receptor (Kreckler et al., 2006). However, it appears that A_{2B} receptors become operational only when their effect is not masked by A_{2A} receptors, because both MRS 1754 and genetic deletion of A_{2B} receptors in the presence of functional A_{2A} receptors fails to affect the suppression of TNF-α production (Kreckler et al., 2006; Ryzhov et al., 2008).

Adenosine is also a potent modulator of neutrophil function and it has long been appreciated that adenosine, by activating its receptors, regulates stimulated production of reactive oxygen species by these cells and phagocytosis (Cronstein et al., 1983, 1985; Varani et al., 1998). Individual neutrophils do not produce large quantities of cytokines; however, because of the large numbers of accumulated neutrophils the cumulative contribution to pro-inflammatory cytokine levels at a given site is large. Adenosine, acting at A_{2A} receptors, regulates the production of a range of cytokines including TNF-α, macrophage inflammatory protein (MIP)-1α (also known as CCL3), MIP-1β (CCL4), MIP-2α (CXCL2) and MIP-3α (CCL20) (McColl et al., 2006). Neutrophils are recruited to inflammatory sites by the post-capillary venular endothelium, which alters the expression of adhesive molecules on its surface to capture neutrophils from the circulation. Adenosine, via A_{2A} receptors, inhibits the adhesion of neutrophils to the endothelium by decreasing the expression and stickiness of the adhesion molecules expressed on neutrophils (Cronstein et al., 1992; Sullivan et al.,

2004; Zhao et al., 1996). By contrast, A_1 receptors promote neutrophil adhesion to different adhesive molecules on the endothelium and on other surfaces (Cronstein et al., 1992). Once in the tissue neutrophils migrate along gradients of chemoattractants. In addition, neutrophils cluster their A_3 receptors at the leading edge of the cell and release ATP, which is converted at the cell surface to adenosine, which then acts in an autocrine manner to stimulate migration (Chen et al., 2006). At inflamed sites neutrophils undergo apoptosis and adenosine, acting at A_{2A} receptors, prevents neutrophils from undergoing apoptosis (Mayne et al., 2001; Walker et al., 1997; Yasui et al., 2000). Thus, virtually every function carried out by neutrophils is regulated by adenosine and its receptors.

In addition to regulating lymphocyte function indirectly by stimulating adenosine receptors on innate immune cells such as dendritic cells, adenosine can also directly affect lymphocyte responses by binding and activating adenosine receptors on lymphocytes. A number of recent studies using adenosine-receptor-knockout mice have evaluated the effect of adenosine receptor activation on various lymphocyte functions. The consensus emerging from these studies, as well as pharmacological studies, is that A_{2A} receptors are the dominant adenosine receptors in dictating lymphocyte responses. Similar to CD4+ cells, adenosine inhibits IL2 production by both polarized type 1 cytotoxic T (TC1) and TC2 CD8+ cells, the effect of which was proposed to proceed through A_{2A} receptors based on pharmacological evidence (Erdmann et al., 2005). However, the production of neither TC1 (IFN-γ) nor TC2 (IL4 and IL5) cytokines was influenced by A_{2A} receptor activation. In addition, pharmacological A_{2A} receptor activation failed to reduce TC1 or TC2 cell cytolytic function (Erdmann et al., 2005), which suggests that another subtype, possibly the A_3 receptor, may mediate the anti-cytotoxic effect of adenosine noted in prior studies (Koshiba et al., 1997; Hoskin et al., 2002). Indeed the expression of A_3 receptors in CD4+ and CD8+ cells is up-regulated after T cells activation with phytohemagglutinin (PHA) (Gessi et al., 2004a). In contrast to these results with CD8+ cytotoxic cells, recent data with A_1-, A_{2A}- and A_3-receptor-knockout mice support a primary role for A_{2A} receptors in preventing the cytolytic activity of IL2-activated natural killer (NK) cells (Raskovalova et al., 2005). In conclusion lymphocyte function is potently regulated by

A_{2A} receptors, suggesting that the anti-inflammatory effects of A_{2A} receptor agonists in animal models of autoimmunity and ischaemia are mediated, in part, by targeting lymphocytes.

Adenosine and airways

A role for adenosine in pulmonary disease was first suggested when it was found that adenosine and related synthetic analogues were potent enhancers of IgE-dependent mediator release from isolated rodent mast cells (Holgate et al., 1980). A few years later, adenosine administered by inhalation was shown to be a powerful bronchoconstrictor of asthmatic but, importantly, not of normal airways (Cushley et al., 1983). Further work showed that both allergic and non-allergic asthmatics responded in a similar way and that the effect was also seen with adenosine 5′-monophosphate (AMP), ADP and ATP (Basoglu et al., 2005). Elevated levels of adenosine are present in chronically inflamed airways; they have been observed both in the bronchoalveolar lavage fluid and the exhaled breath condensate of patients with asthma (Caruso et al., 2006). Adenosine levels are also increased after allergen exposure and during exercises in atopic individuals. The observed increase in tissue levels of adenosine suggests that adenosine signaling could regulate important features of chronic inflammatory disorders of the airways, including asthma and chronic obstructive pulmonary disease (COPD). Consistent with the hypothesis of adenosine playing an important role in the pathogenesis of chronic inflammatory disorders of the airways, mice deficient in adenosine deaminase (ADA) develop severe pulmonary inflammation and airway remodeling in association with elevated adenosine concentrations in the lung (Blackburn et al., 2000). The pulmonary phenotype in ADA-deficient mice consists of airway accumulation of eosinophils and activated macrophages, mast cell degranulation, mucus metaplasia in the bronchial airways, and emphysema-like devastation of the lung parenchyma. Although these histological traits do not completely resemble those of human asthma, the ADA-deficient mouse model is a useful tool to study the pathogenic role of adenosine in chronic airway inflammation. The central role of adenosine in chronic lung inflammation is also supported by

studies carried out in mice that have increased levels of interleukin IL-13 in the lung. These mice develop inflammation, fibrosis and alveolar destruction in association with elevated adenosine concentrations in the lung (Caruso et al., 2006). Treatment with ADA to prevent the increase in adenosine concentrations resulted in a marked decrease in the severity of the pulmonary phenotype, suggesting that adenosine mediates IL-13-induced inflammation and tissue remodeling. Blockade of adenosine re-uptake by administration of dipyridamole has been used in humans to test the hypothesis that the accumulation of extracellular adenosine functionally modulates important features of the asthmatic response. In addition, it has been shown that a rapid increase in sputum eosinophilia occurs when asthmatics are exposed to adenosine by means of a provocation test with AMP (van der Berge et al., 2003). Taken together, these observations indicate that adenosine is likely to play an important role in asthma and COPD through interaction with specific cell-surface receptors. Expression of the four identified adenosine receptors has been shown in a large number of proinflammatory and structural cells and recently in the peripheral lung parenchyma of patients with COPD. The affinity of A_1, A_{2A}, and A_3ARs, studied by means of saturation binding assays, was substantially decreased in patients with COPD, whereas their level of expression appears to be increased. Conversely, the affinity of A_{2B}ARs was not altered, but the density was significantly decreased in patients with COPD (Varani et al., 2006). This suggests that adenosine signalling play an important but rather complex role in COPD. Hence, adenosine responses are not only dictated by the bioavailability of the nucleoside but also by the pattern of adenosine receptor expression, which is known to be finely modulated by physiological and/or pathological tissue environments. Stimulation of A_1ARs promotes activation of human neutrophils and enhances neutrophil adhesion to the endothelium in vitro, suggesting a pro-inflammatory role for this receptor (Cronstein et al., 1985, 1990, 1992). However, in ADA/A_1ARs double knockout mice, the lack of A_1ARs results in enhanced pulmonary inflammation, mucus metaplasia, alveolar destruction and earlier death from respiratory distress, indicating a protective function. Activation of A_{2A}ARs on activated immune cells by adenosine appears to largely suppress the inflammatory

response. In human neutrophils, stimulation of A_{2A}ARs reduces neutrophil adherence to the endothelium, inhibits formyl-Met-Leu-Phe (fMLP)-induced oxidative burst and inhibits superoxide anion generation (Fredholm et al., 1996). In monocytes and macrophages, activation of A_{2A}ARs inhibits lipopolysaccharide-induced tumour necrosis factor-α expression. Therefore, A_{2A}AR agonists might have anti-inflammatory effects in diseases such as COPD, where neutrophil- and monocyte-mediated tissue injury is implicated (Caruso et al., 2006). Initial evidence for the role of A_{2B}ARs in asthma and COPD came from pharmacological studies of enprofylline, a methylxanthine structurally related to theophylline (Feoktistov and Biaggioni, 1995). It was proposed that the A_{2B}AR might be the therapeutic target in the long-term clinical benefit achieved with relatively low doses of theophylline and enprofylline. Recently, A_{2B}ARs have been shown to mediate several pro-inflammatory effects of adenosine in the large majority of inflammatory and structural cells of the lung. For example, functional human A_{2B}ARs have been identified in mast cells, bronchial smooth muscle cells and lung fibroblasts. In these cells, adenosine, via activation of A_{2B}ARs, increases the release of various inflammatory cytokines, which induce IgE synthesis from human B lymphocytes and promote differentiation of lung fibroblasts into myofibroblasts. Such findings provide support for the view that activation of A_{2B}ARs could enhance the inflammatory response associated with asthma and that selective blockade of these receptors would be potentially beneficial in the treatment of asthma and other pulmonary inflammatory diseases. The functional significance of the A_3AR in the pathogenesis of chronic inflammatory airway diseases remains controversial largely owing to major species differences (Caruso et al., 2006).

Adenosine and cancer

One of the difficulties in treating most of the common cancers (colon, lung, breast, prostate, etc.) is that they form solid tumors. The individual cancer cells, being different from normal cells, form a tissue mass that behaves in a radically different way from normal tissues in the body. This is

because the major cell population (the cancer cells) has grown in a way that is out of step with all of the other cells that would normally form a supportive network. In particular, the growth of the cancer is not coordinated with the development of a proper blood supply. The vascular network of a tumor is usually inadequate, the blood vessels are often too few in number, the network is improperly branched, and their calibre is not well controlled. This means that the blood supply is inadequate. Consequently, most solid tumors do not receive sufficient oxygen and the cells are hypoxic. Specifically, hypoxia is conducive to adenine nucleotide breakdown, which is responsible for the adenosine release (Vaupel et al., 2001). As a consequence, adenosine accumulates to high levels in hypoxic tissues. In particular, it is recognized that significant levels of adenosine are found in the extracellular fluid of solid tumors, suggesting a role of adenosine in tumor growth (Merighi et al., 2003). Adenosine, released from hypoxic tissue, is thought to be an angiogenic factor that links altered cellular metabolism, caused by oxygen deprivation, to compensatory angiogenesis. Angiogenesis (or neovascularization) begins with the migration of endothelial cells, originating from capillaries, into the tissue being vascularized. Adenosine has been reported to stimulate or inhibit the release of angiogenic factors depending on the cell type examined (Burnstock, 2006). On one hand, adenosine is known to cause the synthesis of vascular endothelial growth factor (VEGF) and increase the proliferation of endothelial cells obtained from the aorta, coronary vessels, and retina (human retinal endothelial cells, HREC). In particular, adenosine has been shown to induce the DNA synthesis in cultures of human umbilical vein endothelial cells (HUVEC) (Burnstock, 2006).

In the human leukemia HL60, human melanoma A375, and human astrocytoma cells, adenosine at millimolar concentrations caused apoptosis. It seems likely that apoptosis is mediated by the intracellular actions of adenosine rather than through surface receptors (Merighi et al., 2002). It has been argued that the effect of high adenosine concentration might be subsequent to uptake of adenosine by the cell and intracellular accumulation of AMP, leading to caspase activation (Merighi et al., 2003).

In many cases, tumor-induced immune suppression is mediated by soluble inhibition factors or cytokines elaborated by the tumor cells. Extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine, suggesting that this autacoid constitutes an important local immunosuppressant within the microenvironment of solid tumors.

Antigen-presenting cells such as dendritic cells and macrophages are specialized to activate naïve T-lymphocytes and initiate primary immune responses. Adenosine inhibits interleukin-12 (IL-12) and tumor necrosis factor-α production in dendritic cells and in macrophages impairing T-cell priming and suppressing the anticancer immune response. Furthermore, adenosine impairs the induction and expansion of cytotoxic T-lymphocytes and the antitumor activity of natural killer (Hoskin et al., 2002).

Differential effects of adenosine on normal and cancer cells have been previously reported, showing that the proliferation of lymphocytes derived from patients with chronic lymphocytic leukemia was inhibited by adenosine, whereas the proliferation of lymphocytes from healthy people was inhibited to a lesser extent. In vivo studies have shown that adenosine exerts a profound inhibitory effect on the induction of mouse cytotoxic T-cells, without substantially affecting T-cell viability (Hoskin et al., 2002). Adenosine sustains a complex role in the immune system activity, because when given to mice pretreated with cyclophosphamide it demonstrated a myeloprotective effect by restoring the number of white blood cells and the percentage of neutrophils as compared with normal values. Furthermore, it has been demonstrated that the elevation of the extracellular adenosine concentrations induced a radioprotective effect in mice by the stimulation of hematopoiesis in the bone marrow and the spleen. In support of this myelostimulatory role, it has been demonstrated that adenosine enhances cycling of the hematopoietic progenitor cells (Pospísil et al., 2001).

The ability of adenosine to specifically inhibit tumor cell growth in vitro and in vivo suggests that the activation and/or blockade of the pathways downstream of adenosine receptors may contribute to tumor development. Furthermore, the extracellular adenosine concentration may be a crucial

factor in determining the cell progression pathway, either in the apoptotic or in the cytostatic state (Merighi et al., 2003).

Adenosine accumulates at high levels in hypoxic regions of solid tumours, and several lines of evidence indicate that the A_3 receptor is overexpressed in several solid tumors suggesting that it may be a tumor marker (Gessi et al., 2004b). Furthermore it has been reported that it plays a pivotal role in the adenosine-mediated inhibition of tumour cell proliferation (Merighi et al., 2005). Therefore clinical trials have demonstrated the possibility of using A_3 agonists for treatment of cancer (Fishman et al., 2002).

Adenosine and central nervous system

Adenosine levels in the brain extracellular space increase dramatically during metabolically stressful conditions, such as ischemia, seizures, or trauma. Adenosine, acting via its receptors, modulates excitability in the central nervous system (CNS) and has a role in mechanisms of seizure susceptibility, sleep induction, basal ganglia function, pain perception, cerebral blood flow, and respiration (Benarroch, 2008).

Adenosine functions as a natural sleep-promoting agent accumulating during periods of sustained wakefulness and decreasing during sleep. It was suggested that adenosine participates in resetting of the circadian clock by manipulations of behavioural state. Indeed, A_1ARs of the suprachiasmatic nucleus regulate the response of the circadian clock to light (Elliott et al., 2001). The sleep inducing properties of adenosine is in line with its A_1AR-mediated inhibitory action and may involve multiple neuronal populations in the central nervous system; however, the actions upon the basal forebrain nuclei involved in sleep and arousal appear to be particularly important (Ribeiro et al., 2002).

In healthy humans, caffeine inhibits psychomotor vigilance deficits from sleep inertia, a ubiquitous phenomenon of cognitive performance impairment (van Dongen et al., 2001). It thus emerges that there exists a potential role of adenosine-related compounds and of A_1AR agonists as sleep

promoters and adenosine receptor antagonists as arousal stimulators. Adenosine A_1AR agonists have anxiolytic activity in rodent models of anxiety, whereas caffeine and the adenosine A_1AR selective antagonist, cyclopentyltheophylline, have anxiogenic properties. In accordance with the notion that synaptic plasticity is the basis for learning and memory in different brain areas, adenosine correspondingly modulates behaviour in various learning and memory paradigms, and adenosine A_1AR antagonists have been proposed for the treatment of memory disorders (Ribeiro et al., 2002). Cognitive effects of caffeine are mostly due to its ability to antagonise A_1ARs in the hippocampus and cortex, the brain areas mostly involved in cognition, positive actions of caffeine on information processing and performance might also be attributed to improvement of behavioural routines, arousal enhancement and sensorimotor gating (Fredholm et al., 1999).

One of the first pathophysiological roles proposed for adenosine was as an endogenous anti-convulsant (Dragunow et al., 1986). Limitations of the use of adenosine receptor agonists as anti-convulsant drugs are due to their pronounced peripheral side effects as well as central side effects, like sedation.

Phase I clinical safety studies in healthy volunteers showed that intrathecal adenosine administration attenuated several types of experimental pain without causing significant side effects. Allosteric modulation of adenosine receptors, namely of A_1ARs, has been attempted with success with the objective of developing drugs that by sinergising with endogenous adenosine action could have minimal side effects in the absence of adenosine. A further advantage of allosteric modulators is that they usually possess some degree of tissue selectivity. Allosteric modulation of adenosine A_1ARs reduces allodynia, and, more interesting, the allosteric modulator T62 was effective not only after intrathecal injection but also after systemic administration, which reinforces the interest of adenosine-related compounds as putative drugs for the treatment of chronic pain associated with hyperalgesia and allodynia (Pan et al., 2001). A_1AR agonists were conclusively shown to attenuate ischemic or excitotoxic neuronal damage both in vitro (cell cultures, brain slices) and in vivo in different models of ischemia/hypoxia (Wardas, 2002).

Studies in knockout mice or by using pharmacologic blockade indicate that A_{2A}ARs in the striatum modulate locomotor activity (Benarroch 2008). Activation of A_{2A}ARs results in increased GABA release in the globus pallidus, which would lead to disinhibition of the subthalamic nucleus (Ribeiro et al., 2002). The A_{2A}ARs are also coexpressed with A_1ARs in glutamatergic corticostriate terminals and antagonize the presynaptic inhibitory effect of the A_1ARs on glutamate release in the striatum. Epidemiologic and laboratory data suggest that caffeine may reduce the risk of Parkinson's disease by preventing degeneration of nigrostriatal dopaminergic neurons. Furthermore, caffeine and selective A_{2A}AR antagonists protect against dopaminergic cell loss in several toxin models of Parkinson's disease (Simon et al., 2008).

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AIM OF THE THESIS

Adenosine is a potent extracellular messenger that is produced in high concentrations under metabolically unfavourable conditions, like hypoxia or ischaemia. Tissue hypoxia, consequent to a compromised cellular energy status, is followed by the enhanced breakdown of ATP leading to the release of adenosine. Through the interaction with A_1 and A_2 membrane receptors, adenosine is devoted to the restoration of tissue homeostasis, acting as a retaliatory metabolite. Several aspects of the immune response have to be taken into consideration and even though in general it is very important to dampen inflammation, in some circumstances, such as the case of cancer, it is also necessary to increase the activity of immune cells against pathogens. Therefore, adenosine receptors that are defined as "sensors" of metabolic changes in the local tissue environment may be very important targets for modulation of immune responses and drugs devoted to regulating the adenosinergic system are promising in different clinical situations.

The first studies aimed at elucidating the mechanisms by which the absence of ADA leads to immunodeficiency first suggested the presence of adenosine receptors on lymphocytes to suppress or dampen the immune response. Therefore, to understand the pathophysiological implications of adenosine-triggered effects in T cells we review the main actions attributed to adenosine by receptor subtype activation. Adenosine through the interaction with A_2 and A_3 receptors plays a crucial role in inflammation and in the regulation of immune cells. The most important therapeutic implication is that A_{2A} receptors, proposed as "natural" brakes of inflammation, appear to represent a promising pharmacological target to treat a wide variety of diseases characterized by a strong immunoinflammatory component (chapter 1).

Then we moved to discuss the involvement in inflammation of A_3 adenosine receptor, that is evident from the large amount of experimental work carried out in peripheral blood cells of the immune system and in a variety of inflammatory conditions. Following a detailed analysis of the literature the A_3 adenosine receptor subtype appears to play a complex role as both pro and anti-inflammatory effects have been demonstrated depending not only on the cell types investigated but also on the

model of inflammation used and the species considered. This chapter will discuss developments in our understanding of the role of adenosine A_3 receptor activation in the function of the different types of cells of the immune system including neutrophils, eosinophils, lymphocytes, monocytes, macrophages and dendritic cells (chapter 2).

Finally we have characterized the role of adenosine in atherosclerosis, a chronic inflammatory desease, in large part due to the accumulation of macrophage white blood cells and promoted by low-density lipoproteins (LDL). Adenosine released from ischemic and hypoxic tissues interacts with four extracellular G protein-coupled receptors, A_1, A_{2A}, A_{2B}, and A_3. All the four adenosine subtypes have been recently associated to the modulation of angiogenesis. Therefore due to the link between ado, inflammation and angiogenesis and the increasing evidence that these factors play a role in atherogenesis we thought to investigate HIF-1α, VEGF, IL-8 and FC formation by ado receptors in human macrophages and in an *"in vitro"* model of human FC (chapter 3).

CHAPTER 1:

Adenosine and lymphocyte regulation

Introduction

Adenosine is an endogenous purine nucleoside that is constitutively present at low levels outside the cells but might dramatically increase its concentrations following metabolic stress conditions like those induced by hypoxia or ischaemia. After its release adenosine induces its biological effects through the interaction with four cell surface receptors classified by molecular, biochemical and pharmacological data into four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2001). Each of these receptors, with the exception of the A_1 subtype, are expressed on human and mouse T lymphocytes and it appears that their activation represents a potent endogenous immunosuppressive pathway that regulates the excessive immune response against potent external insults. The interest in the immunomodulatory effects of adenosine arose after the discovery that hereditary deficiency of the enzyme adenosine deaminase (ADA) was associated with severe combined immune deficiency disease (SCID) (Hershfield, 2005). ADA converts adenosine to inosine and deoxyadenosine to deoxyinosine and its activity is greater in T cells in comparison with B cells or erythrocytes. ADA deficiency is one of the most severe immunodeficiencies due to the sensitivity of T cells to the accumulation of the ADA substrates adenosine and 2′-deoxyadenosine. SCID is a disease characterized by severe lymphocytopenia, affecting both B and cells, and a marked susceptibility to infection. The accumulation of adenosine and the activation of adenosine receptors in T cells may lead to the depletion of lymphocytes and impairment of their function (Buckley 2004; Hershfield, 2004). Moreover, ADA deficiency in mice causes impairment of intrathymic T cell development and enhanced thymocyte apoptosis supporting the hypothesis that adenosine is responsible for depletion of T cells occurring in ADA SCID (Apasov et al., 2001). Indeed, the studies aimed at elucidating the mechanisms by which the absence of ADA leads to immunodeficiency first suggested the presence of adenosine receptors on lymphocytes to suppress or dampen the immune response (Wolberg et al., 1975). Therefore, to understand the pathophysiological implications of adenosine-triggered effects in T cells we intend to review the main actions attributed to adenosine by receptor subtype activation.

Adenosine metabolism and adenosine receptor activation under physiological and pathological conditions

Adenosine has several physiological effects through the interaction with four known adenosine receptors, A_1 and A_3 that through the interaction with Gi/0 inhibit adenylyl cyclase activity, and A_{2A} and A_{2B} that increase cAMP production (a strong immunosuppressive agent) via Gs. In addition, they can modulate the activity of phospholipase C, D, A_2, cGMP, K^+ and Ca^{2+} channels and mitogenactivated protein kinases (MAPKs) regulating a variety of cellular effects (Fredholm et al., 2001). It has been suggested that adenosine receptors act as "sensors" and that extracellular adenosine acts as a "reporter" of metabolic changes in the local tissue environment (Sitkovsky et al., 2005) Adenosine concentrations are normally regulated through its catabolism by ADA and through phosphorylation by adenosine kinase. In ADA deficiency the capacity of adenosine kinase is saturated and adenosine levels increase from 100–300 nM to 10 μM (Hershfield 2005), which could excessively stepwise activate all adenosine receptors. Moreover, an increase of intracellular adenosine inhibits the hydrolysis of S adenosylhomocysteine that is a potent inhibitor of transmethylation reactions. The lack of ADA leads also to the accumulation of deoxyadenosine, a product of DNA degradation, that in contrast to adenosine does not play a regulatory role in physiological conditions but determines an increase in deoxyATP that in turn inhibits DNA synthesis and activates apoptosis suggesting the possibility that deoxyadenosine itself may also have pathological effects (Hershfield 2005). Inflammatory tissue conditions are often associated with a low oxygen tension, or hypoxia, that is the cause of an associated production of adenosine in this environment. Therefore, the prevalent activity of 5'nucleotidase over activity of adenosine kinase (Decking 1997; Ledoux 2003) occurring in hypoxic conditions is responsible for a significant adenosine accumulation in sites of tissue injury (Lukashev et al., 2004). 5′Nucleotidase represents the major enzyme responsible for the extracellular production of adenosine from AMP and has a critical role in the functional activation of T cells. Deficiency of 5′nucleotidase activity has been associated with a number of immunodeficiency diseases, such as lymphoproliferative

disorders and systemic lupus erythematosus. Abnormal levels of these enzymes may be associated with an autoimmune pathology (Vivekanandhan et al., 2005). Therefore, under normal conditions adenosine, which is continuously produced intracellularly and extracellularly and maintained at low intracellular levels (about 100 nM) through the metabolic activity exerted by adenosine kinase and adenosine deaminase, interacts with the high-affinity A_1 and A_{2A} receptor subtypes. In ADA deficiency or in hypoxic, ischaemic or inflamed conditions, the intracellular production of adenosine is increased at very high micromolar concentrations and transported across cell membranes by specific agents finally leading to the stepwise activation of all adenosine receptors, including the lowaffinity A_{2B} and A_3 subtypes. Adenosine has been classified as a "retaliatory metabolite" as a consequence of its ability to mediate an autoregulatory loop, the function of which is to protect organs from injury following the initiating stressful stimuli (Newby 1984; Hasko et al., 2004). There are different mechanisms by which adenosine receptor activation protects organs. First, it decreases the energy demand of the tissue, second it can augment oxygen and nutrient supply through vasodilation, and finally, it regulates the exuberant immune response to harmful external agents. As basal adenosine levels have only a marginal influence on the immune response, ischaemic and inflammatory conditions represent scenarios in which adenosine levels become high enough to have significant immunomodulatory and specifically immunosuppressive effects (Gessi et al., 1999).

Adenosine inhibits lymphocyte activation through A_2 receptor activation

A body of literature concerning in vitro and in vivo studies clearly shows the beneficial role of adenosine as an immune modulator. Activation of T lymphocytes starts from the recognition of antigen by T cell antigen receptor and CD4 or CD8 coreceptors that initiates a cascade of signalling events resulting in cytokine secretion, cellular cytotoxicity and T cell proliferation. The immunosuppressive effects of adenosine on cytotoxic T lymphocyte (CTL) actions may be explained by activation of A_{2A} receptors followed by sustained increases in cAMP that, in turn,

antagonize T cell receptor (TCR)-triggered signalling. In particular, adenosine modulates TCR-mediated interleukin (IL)-2 production, expression of CD25 and CD69, granule exocytosis, Fas ligand up-regulation and cell proliferation through the interaction with A_{2A} receptors (Huang et al., 1997; Koshiba et al., 1997).

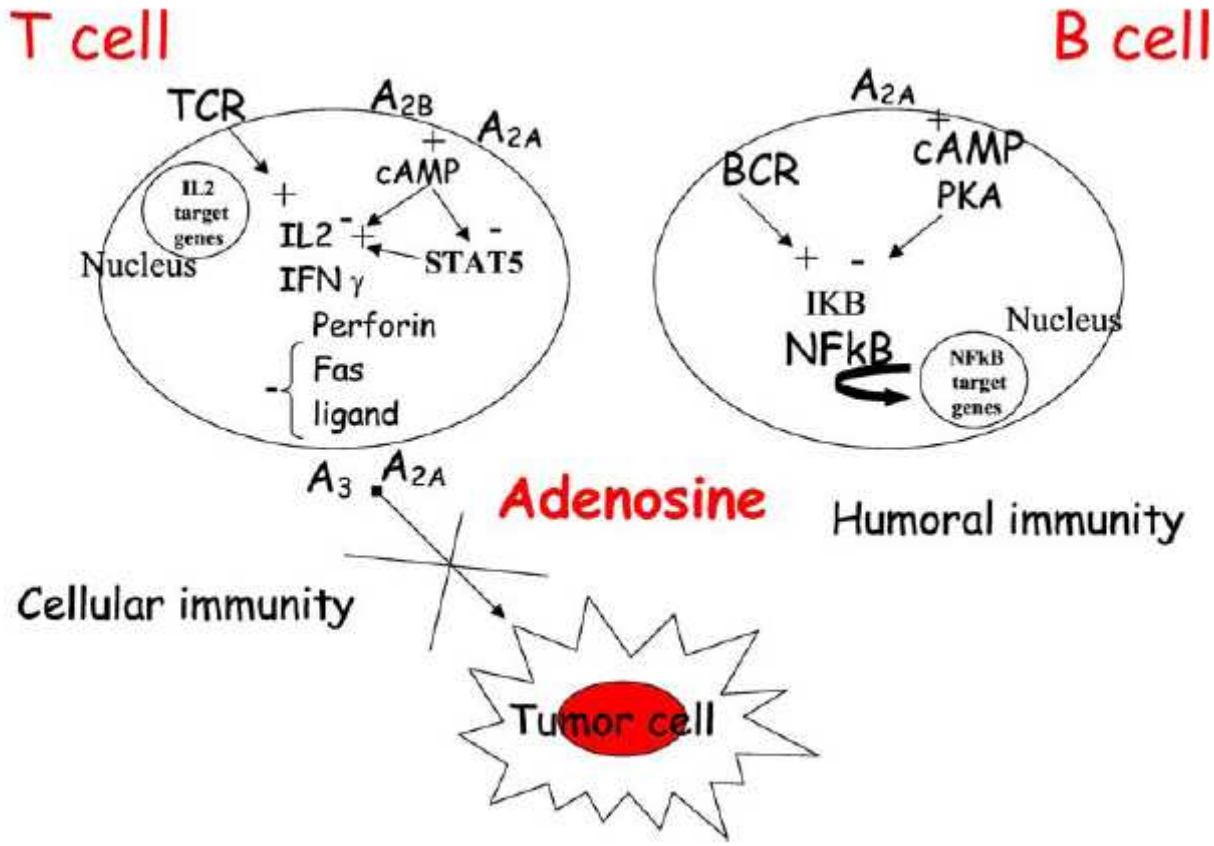

Figure 1: Role of adenosine receptors in the regulation of immune responses

The predominant expression of A_{2A} receptors, which has been established in functional assays using selective agonists and antagonists of A_{2A} receptors, was confirmed by Northern blot studies (Huang et al., 1997; Koshiba et al., 1997). The role of A_{2A} adenosine receptors in the regulation of the immune response has been investigated by determining the expression levels of this receptor in different subsets of functional lymphocytes (Koshiba et al., 1999). The levels of expression of A_{2A} receptors have been found to be much higher among T than B cells. T cell subsets are distinguished

by the expression of TCR coreceptor molecules CD8+ and CD4+ involved in recognition of class I and class II major histocompatibility complex, respectively. More CD4+ than CD8+ T cells express A_{2A} adenosine receptors. CD8+ T cells are mostly cytotoxic effector cells, whereas CD4+ cells have been implicated in T-helper cell activities. Studies of T-helper cell subsets (TH1 and TH2) reveal that lymphokine-producing cells are much more likely to express A_{2A} receptors than are cells that do not produce lymphokines. A possible explanation was that inhibitory A_{2A} receptors are induced selectively in cells that produce cytokines, as a means of limiting cytokine release (Koshiba et al., 1999). In agreement with these results, it has been reported that activation of CD4+ lymphocytes induced A_{2A} receptor mRNA expression and resulted in a dose-dependent inhibition of TCR-mediated interferon gamma (IFN-γ) production, which is the most important effector molecule synthesized by Th1 cells (Lappas et al., 2005). Recently, the effect of adenosine on IL-2 receptor-associated signalling pathways was explored in T lymphocytes by Zhang and co-workers (Zhang et al., 2004). IL-2 is an immunomodulatory molecule crucial for the proliferation of activated T cells, and its receptor consists of three subunits indicated as alpha, beta and gamma. Signalling through this receptor for the transduction of a proliferative signal includes Jak1, Jak3 and STAT5. Jak1 and Jak3 activation leads to phosphorylation of tyrosine residues on IL-2 receptors. Primed by these events the IL-2 complex generates two major proliferative signals. One of these signals is mediated by the adaptor protein Shc and activates p42/p44 MAPK and phosphatidylinositol 3-kinase (PI3K) signalling pathways. The second proliferative signal transduced by the IL-2 receptor complex involves the transcription factor STAT5 that after phosphorylation translocates to the nucleus to control gene expression. Adenosine, by activating A_{2A} and A_{2B} receptors, inhibits IL-2-dependent proliferation through inhibition of STAT5 phosphorylation. Because T cells play a major role in antitumour immunity and considering that adenosine is highly increased on the microenvironment of solid carcinomas, the adenosine-induced defect in IL-2-stimulated proliferative signalling would be expected to result in a defective T lymphocyte response in cancer patients and generally in impaired antitumour immunity **(Figure 1)**. Impairment of proliferation has been recently

investigated also in anergic B lymphocytes (Minguet et al., 2005). In B cells adenosine inhibits activation of NF-kappa B in response to B cell antigen receptor (BCR) triggering (Fig. 1). Stimuli known to enhance 3′,5′-cyclic adenosine monophosphate (cAMP) are capable of selectively suppressing the activation both of NF-kB downstream of the BCR and Toll-like receptor 4 in splenic B lymphocytes. This suppression is reached by blocking phosphorylation and subsequent degradation of the inhibitor of NF-kappa B. This suggests that adenosinemediated signals represent an important step in the molecular decision process controlling inflammation versus anergic immune responses. In this way adenosine might affect B cell dysfunction in ADA deficiency and may explain defective B cell proliferation and activation found in ADA-deficient mice (Aldrich et al., 2003). Convincing evidence of a link between adenosine receptors and NF-kB activation comes also from in vivo studies performed in A_{2A} adenosine receptor knockout (KO) mice. Lipopolysaccharide (LPS), given to these animals, induced a greater inflammatory response and a higher binding of NF-kB to nuclear DNA and consequently a higher cytokine gene transcription in macrophages (Lukashev et al., 2004).

In vivo evidence of the immunosuppressive effects played by adenosine through A_{2A} receptors

Although it has been demonstrated that adenosine in vitro acted as an immunosuppressing agent it was not clear, until genetically modified mice were available, whether its effects were present also under pathophysiological conditions in vivo. There are many G protein-coupled receptors that increase cAMP levels showing immunosuppressive pharmacological effects and that have been considered as potential anti-inflammatory agents, e.g. catecholamines, prostaglandins, dopamine and histamine. However, although pharmacological evidence underlines their importance as potential immunosuppressive molecules, there is no real proof whether their recruitment does occur during physiological control of inflammation in vivo. Such evidence has been obtained for adenosine A_{2A} receptors. In wildtype mice, activation of A_{2A} receptors blocked tissue damage

induced by inflammation demonstrating its role in counteracting inflammation (Cronstein 1994; Hasko et al., 1996). More recently, the availability of A_{2A} receptor-deficient mice in models of acute liver inflammation and sepsis provided the first conclusive in vivo evidence of A_{2A} receptors playing a critical role in the suppression of acute inflammation. Evaluation of T cell-, macrophage- and cytokine-dependent tissue injury in A_{2A} adenosine receptor-deficient mice revealed a dramatic increase of local tissue damage and an increase of proinflammatory cytokines such as tumour necrosis factor α (TNF-α), IFN-γ and IL-12 in these mice, but not in wild-type animals (Ohta et al., 2001; Sitkovsky 2003). Moreover, low doses of inflammatory stimuli, which were not damaging in wild-type animals, were detrimental in terms of liver status and cytokine levels in knockout mice. These data furnished the first indisputable demonstration of an in vivo role played by A_{2A} adenosine receptor in the regulation of inflammation and underlined a non-redundant role in the down-regulation of inflammation in vivo. Importantly, A_{2A} receptors seem to be essential as demonstrated by the failure of all other anti-inflammatory mechanisms, e.g. β-adrenergic and prostaglandin receptors, to overcome the lack of A_{2A} receptors in preventing severe tissue injury. Experiments performed in thymocytes and T cells from heterozygous A_{2A} receptor mice showed that there was a correlation between reduction of A_{2A} receptors and reduction of cAMP response of the cells after activation with adenosine and suggesting the lack of A_{2A} spare receptors in T cells and a lack of compensation by the A_{2B} receptors (Armstrong et al., 2001). Also the level of apoptosis of thymocytes from these animals was less pronounced following A_2A activation in comparison to that obtained in wild-type mice. The expression of other adenosine subtypes like A_1, A_{2B} or A_3 did not compensate for the lack of A_{2A} in lymphoid organ of deficient mice as suggested by real-time reverse transcription polymerase chain reaction (RT-PCR) experiments indicating again that in acute models of inflammation, the A_{2A} receptors play a crucial and non-redundant role in the protection of tissue from damage derived by excessive inflammation (Lukashev et al., 2003). It has been also suggested that in non-exacerbated inflammatory conditions different anti-inflammatory stimuli, such as other adenosine receptor subtypes and other anti-inflammatory mediators, may act

in concert to reduce the extent of damage. Therefore, based on the evidences reported above, the treatment of a series of diseases such as sepsis, wound healing and rheumatoid arthritis, might improve following A_{2A} receptor stimulation. Indeed, methotrexate (MTX), which is used for treatment of rheumatoid arthritis, may mediate its effects through the release of endogenous adenosine (Khan et al., 2002). Even though its mechanism of action remains unclear, it has been shown that MTX caused a dose-dependent suppression of T cell activation and adhesion molecule expression, and this was not due to lymphocyte apoptosis. The suppression of intercellular adhesion molecule (ICAM)-1 was adenosine and folate dependent, suggesting that the suppression of T cell activation and T cell adhesion molecule expression, rather than apoptosis, mediated in part by adenosine or polyglutamated MTX or both, are important mechanisms in the anti-inflammatory action of MTX (Johnston et al., 2005). Besides methotrexate, there is evidence that other therapeutic agents, such as sulfasalazine and FK-506, could exert their anti-inflammatory effects by promoting adenosine release (Gadangi et al., 1996; Hwang et al., 2001).

Adenosine inhibits killer T cell activation via A_3 and A_{2A} stimulation: implications in cancer

The ability of immune cells to recognize and eliminate tumour cells is fundamental for successful host defence against cancer. It has been suggested that adenosine, the concentration of which increases within hypoxic regions of solid tumours, may interfere with the recognition of tumour cells by cytolytic effector cells of the immune system (Blay et al., 1997; Merighi et al., 2003). Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumour-induced immunosuppression. In 1994 Hoskin and co-workers demonstrated that colon adenocarcinoma cells inhibited anti-CD3-activated killer cell induction through the production of a tumour-associated soluble factor that was distinct from transforming growth factor beta or

prostaglandins (Hoskin et al., 1994a,b). In the same period these authors indicated adenosine as a possible inhibitor of killer T cell activation in the microenvironment of solid tumours (Hoskin et al., 1994b) and showed that 2-chloroadenosine (2CA), a stable analogue of adenosine, reduced MHC-unrestricted killing of P815 tumour target cells by anti-CD3-activated killer (AK) lymphocytes. 2CA exerted this effect by interfering with the recognition/adhesion phase of cytolysis. Treatment with dipyridamole to block cellular uptake of 2CA increased the inhibition of cytolysis, suggesting the involvement of a cell surface receptor. However, neither DPCPX nor DMPX, the A_1 and A_2 receptor antagonists, respectively, were able to reduce the inhibitory effect of 2CA on AK lymphocyte function. Similarly, the nonselective A_1 and A_2 receptor blockers, theophylline and 8-phenyltheophylline, had no effect on 2CA-mediated inhibition of AK cell activity. These data clearly demonstrated that 2CA inhibited the cytolytic activity of AK lymphocytes by interacting with a novel non-A_1/A_2 cell surface receptor (Hoskin et al., 1994c). Then, MacKenzie and co-workers (MacKenzie et al., 1994) evaluated the adhesion of murine spleen-derived anti- CD3-activated killer (AK) lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells. Adenosine, in the presence of the adenosine deaminase inhibitor coformycin, reduced adhesion by up to 60%. The inhibitory effect of adenosine was exerted on AK cells and not on the MCA-38 targets and was mediated by cell surface receptors as adenosine-induced inhibition of adhesion was not abrogated following treatment with dipyridamole, a blocker of adenosine uptake. The agonist potency profile indicated that the A_3 receptor subtype might be responsible for the inhibition of adhesion. The authors suggested that this mechanism of immunosuppression, secondary to tissue hypoxia, may be important in the resistance of colorectal and other solid cancers to immunotherapy. In addition, the same authors demonstrated that adenosine exerts a strong inhibitory effect on the induction of mouse cytotoxic T cells (Hoskin et a., 2002). Diminished tumouricidal activity correlated with reduced expression of mRNAs coding for granzyme B, perforin, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) **(Figure 1)**. IL-2 and IFN-γ synthesis by AK-T cells was also inhibited by adenosine. Also in this case the inhibitory effect of adenosine on AK-T cell

proliferation was blocked by an A_3 receptor antagonist, suggesting that adenosine acts through A_3 receptors to prevent AK-T cell induction. Tumour-associated adenosine may act through the same mechanism to impair the development of tumour-reactive T cells in cancer patients. Therefore, the suppression of T killer cell function suggests that adenosine may act as a local immunosuppressant within the microenvironment of solid tumours. The same authors reported that adenosine partially inhibits the interaction of T lymphocytes with tumour cells by blocking the function of integrin α4β7, which is the major cell adhesion molecule involved in the adhesion of T cells to syngeneic MCA-38 adenocarcinoma cells (MacKenzie et al., 2002). Importantly, involvement of α4β7 has also been postulated in leukocyte localization in inflammatory disorders such as asthma, intestinal inflammation and rheumatic disorders and may be regulated by adenosine (Walsh et al., 1996; Michetti et al., 2000; Elewaut et al., 1998). As adenosine is known also for its anti-inflammatory effects in addition to its immunosuppressive actions, it is possible to hypothesize that adenosine-mediated inhibition of adhesion through α4β7 found in T cells may be important in a variety of other inflammatory diseases. Tumour microenvironment can suppress the function of tumour-infiltrating T cells. Therefore, the effect of adenosine has been investigated on the expression of costimulatory molecules by T cells in resting and activated conditions. One of the most important costimulatory molecules present on the T cells' surface are CD2 and CD28 acting in concert to achieve optimal costimulation of T lymphocytes during interaction with antigen-presenting cells. It has been demonstrated that adenosine interfered with activation-induced expression of the costimulatory molecules CD2 and CD28 and that their up-regulation was IL-2 dependent. This effect could not be attributed to cyclic AMP (cAMP) accumulation resulting from the stimulation of adenylyl cyclase-coupled adenosine receptors, even though cAMP at concentrations much higher than those generated following adenosine stimulation was inhibitory for both CD2 and CD28 expression. Therefore, it has been proposed that adenosine interferes with IL-2-dependent T cell expression of costimulatory molecules via a mechanism that does not involve the accumulation of intracellular cAMP and through a cell surface receptor that is coupled to signalling pathways

different from those involving adenylyl cyclase activity, possibly the A_3 subtype (Butler et al., 2003). However, the relevance of adenosine-mediated pathways in tumour-infiltrating T cells, considering the expression on T cells of adenosine deaminase in association with CD26 (dipeptidyl aminopeptidase), remains to be elucidated (Dong et al., 1996). CD26 is a multifunctional type II cell surface glycoprotein widely expressed on T, B and natural killer (NK) cells. One of its functions is that of an adenosine deaminase binding site. Binding of ADA to CD26 is capable of reducing the local concentration of adenosine and has an enzymatic role in protecting T cells from an adenosine-mediated inhibition of proliferation [Dong et al., 1996; Gorrell et al., 2001). Indeed, it has been reported that adenosine deaminase activity is significantly lower in the peripheral lymphocytes of cancer patients, suggesting increased susceptibility of T cells to adenosine-mediated inhibition within the tumour microenvironment (Dasmahapatra et al., 1986). Moreover, cytotoxic T cells show very low levels of adenosine deaminase activity, leaving us to hypothesize that cytotoxic cells would be quite sensitive to the immune inhibitory effect of adenosine accumulated within tumours. In this regard, it is relevant to underline that the presence of the A_3 adenosine receptor has been demonstrated in Jurkat cells, a human leukaemic cell line, where its level is very high, and in human lymphocytes in which an up-regulation occurs after T cell activation (Gessi et al., 2001, 2004). In addition to the A_3 receptors, antigen activation has been shown to alter the expression of other adenosine subtypes. For example it has been reported that A_{2B} receptors are also up-regulated by phytohemagglutinin (PHA) and/or anti-CD3 in both CD4+ and CD8+ cells (Mirabet et al., 1999), and as for A_{2A} subtypes there are contrasting reports suggesting both that the activation process increases their expression predominantly in CD8+ and/or CD4+ T cells (Koshiba et al., 1999; Lappas et al., 2005). These data suggest that adenosine may stepwise recruit different adenosine receptors following antigen activation. In human lymphocytes, e.g. A_{2A} and A_{2B} receptors are coexpressed, leading to synergy in cAMP production (Gessi et al., 2005); in addition, adenosine may mediate different signals if each of these receptors is linked to different effector molecules, as is the case of the A_3 subtype. In contrast to the immunosuppressive role of adenosine

in the environment of solid tumours, recent findings from in vivo studies of Fishmans' group report a stimulatory effect of A_3 agonists in the synthesis of IL-12 that is dependent from inhibition of cAMP levels and protein kinase A expression. This cytokine is a potent stimulatory agent of NK cells and is a cytotoxic factor that exerts a potent antitumour effect in vivo. It induces IFN-+ production by activated T and NK cells and augments cytotoxic activity of these cells via perforin, Fas and TRAIL-dependent mechanisms. Therefore, it enhances NK cell activity and probably NK cell-mediated destruction of tumour cells (Harish et al., 2003). This antitumour effect played in immune cells is in line with other findings of the same group demonstrating a direct inhibitory action of A_3 receptor activation on tumour cell growth (Fishman et al., 2001). However, in general, it has to be remarked that contrasting results obtained from pharmacological studies may be explained by the limitations due to the poor selectivity of agonists and antagonists for adenosine receptors. Recent data obtained from studies using adenosine receptor KO mice examined the capability of adenosine and its analogues to inhibit the ability of lymphokine-activated killer cells (LAK) to kill tumour cells. This work demonstrated that adenosine and adenosine A_{2A} ligands suppress the cytotoxicity of LAK cells in parallel with their ability to increase cAMP levels. These effects were produced by interfering with both perforin-mediated and Fas ligand-mediated killing pathways. Studies with LAK cells generated from A_1 and A_3 adenosine receptor KO mice indicated the lack of any involvement of these adenosine subtypes in the inhibitory effect exerted by adenosine, whereas LAK cells obtained from A_2 adenosine receptor KO mice were resistant to the inhibitory effect of this nucleoside. Only very high concentrations of the nonselective agonists NECA or CADO produced mild inhibition of LAK cytotoxicity that were possibly induced through A_{2B} activation, suggesting a predominant role of A_{2A} subtype in inhibition of LAK cell toxicity (Raskovalova et al., 2005). Therefore, adenosine has been indicated as an important intra-tumour factor that inhibits the effector function of NK and T cells and protects tumours from immune destruction. Being that these effects are mediated by A_{2A} receptors, the authors suggest the introduction of A_{2A} antagonists to increase the efficacy of immunotherapy.

Conclusion and perspectives

The evidences summarized in this review indicate that adenosine through the interaction with A_2 and A_3 receptors plays a crucial role in the regulation of immune cells. The most important therapeutic implication arising from the data summarized above is that A_{2A} receptors, proposed as "natural" brakes of inflammation, appear to represent a promising pharmacological target to treat a wide variety of diseases characterized by a strong immunoinflammatory component. On the other hand, it may be advantageous in some circumstances to enhance certain aspects of inflammation in order to eliminate the causative agent, as in the case of cancer. In fact, it has to be remarked that tumour defence mechanisms are akin to inflammatory processes. Solid tumours, due to their abnormal vasculature, are often hypoxic and show increased levels of adenosine that may be an important mediator of tumour-associated immunosuppression. It is likely that killer T cells that may be recruited against cancer cells fail to act in an effective manner due to the high level of adenosine in the tumour microenvironment. Because several of these immunosuppressive functions have been attributed to the stimulation of A_3 and A_{2A} receptors, expressed on the surface of T cells, adenosine antagonists of these subtypes may be potentially useful in the immunotherapy of cancer.

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CHAPTER 2:

A_3 adenosine receptor regulation of cells of the immune system and modulation of inflammation

Introduction

The interest in the elucidation of A_3 adenosine receptor involvement in inflammation is evident from the large amount of experimental work carried out in peripheral blood cells of the immune system and in a variety of inflammatory conditions. Following a detailed analysis of the literature the A_3 adenosine receptor subtype appears to play a complex role as both pro and anti-inflammatory effects have been demonstrated depending not only on the cell types investigated but also on the model of inflammation used and the species considered (Gessi et al., 2008). This chapter will discuss developments in our understanding of the role of adenosine A_3 receptor activation in the function of the different types of cells of the immune system including neutrophils, eosinophils, lymphocytes, monocytes, macrophages and dendritic cells.

A_3 adenosine receptor effects on neutrophil function

Neutrophils represent a larger percentage of circulating leukocytes than any other cell type. They are the first white blood cells to arrive at an injured or infected site. Neutrophils arise in the bone marrow and then must traverse the vasculature to arrive at the sites of injury. They leave the circulation at the level of the postcapillary venules following specific interaction with endothelium. Once in the extravascular space, neutrophils follow a gradient set up by chemoattractants, such as activated complement components, cytokines, lipids or bacterial products by means of specific cell surface receptors. Although the primary role of the neutrophil is to rid the body of injurious organisms and clean up the debris after tissue injury, the extracellular release of any of the contents of the phagolysosome or the generation of toxic oxygen metabolites into the extracellular space can lead to destruction of normal, uninjured cells surrounding the infected site. It is the destruction of the surrounding tissue by overactive neutrophils that adds so greatly to tissue destruction in the setting of reperfusion injury. Adenosine, acting through its cell surface receptors, is a potent regulator of neutrophil function.

The first report implicating a role for A_3 receptors in human neutrophils came in 1997 following investigations into the effect of adenosine and its more selective analogues on neutrophil degranulation in human whole blood (Bouma et al., 1997). Adenosine inhibited concentration-dependently the LPS- and TNF-alpha-induced release of the azurophilic granule proteins with an IC_{50} in the µmolar range. The inhibitory effects of adenosine were partially blocked by the A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine, the A_1/A_2 antagonist 8(p-sulfophenyl)theophyline, and the A_1/A_3 antagonist xanthine amine congener, but not by the A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine. The highly selective A_3 agonist N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide and the nonselective agonist 2-chloroadenosine reduced degranulation more potently than the A_1 agonist N6-cyclopentyladenosine. The inhibitory effects of N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide and 2-chloroadenosine were strongly reversed by xanthine amine congener, but were not affected by 8(p-sulfophenyl)theophyline. These data suggest that adenosine acted via A_2 as well as A_3 receptors to inhibit neutrophil degranulation. However, activation of A_3 receptors in canine neutrophils did not attenuate superoxide anion production but reduced platelet-activating factor-stimulated neutrophil adherence to coronary endothelium suggesting that it might be a novel target for treatment of myocardial ischemia and reperfusion (Jordan et al., 1999).

Subsequent binding and functional studies showed that human neutrophils expressed A_3 receptors which were coupled to the inhibition of adenylyl cyclase and calcium signalling (Gessi et al., 2002). However in the case of calcium the high micromolar doses of the A_3 agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide (Cl-IB-MECA) and the A_3 antagonist 5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [1,5-c] pyrimidine (MRE 3008F20) needed to stimulate or block Ca^{2+} mobilization respectively, were not completely consistent with the involvement of an A_3 receptor. Similar effects of Cl-IB-MECA in mobilizing Ca^{2+} have been found in several cell systems a finding that is difficult to reconcile with the high affinity of this selective A_3 agonist in binding and cAMP inhibition assays (Kohno et al.,

1996a, b; Jacobson, 1998; Reeves et al., 2000; Reshkin et al., 2000; Shneyvays et al., 2000; Gessi et al., 2001; Suh et al., 2001; Merighi et al., 2001). The reason why high, nonselective doses of Cl-IB-MECA are needed to stimulate Ca^{2+} mobilization remains unknown. A contribution of other mechanisms other than A_3 receptor stimulation cannot be excluded. Importantly, for the first time it was suggested that both A_3 and A_{2A} receptors contribute to the inhibition of oxidative burst, an indication of anti-inflammatory activity (Gessi et al., 2002). Using this readout, alterations of A_3 adenosine receptors in human neutrophils exposed to low frequency, low energy pulsing electromagnetic fields (PEMFs) has been reported. There is considerable interest in the use of PEMFs in clinical practice since the date correlate well with inflammatory conditions. Saturation experiments after treatment with PEMFs revealed that the A_3 receptor density in human neutrophils was increased. Consistent with this in functional assays Cl-IB-MECA and N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA) were able to inhibit cyclic AMP accumulation and their potencies were increased after exposure to PEMFs. These results indicated that in human neutrophils treated with PEMFs there are significant alterations in the A_3 adenosine receptor density and functionality (Varani et al., 2003). The upregulation cannot be ascribed to the synthesis of new receptors since the duration of PEMF treatment was too short. The upregulation of A_3 adenosine receptors is most likely due to a translocation of this receptor subtype to the membrane surface. It is of interest that PEMFs treatment also modified the binding parameters of the A_{2A} adenosine receptors but not those of α_2, β_2 adrenergic and μ, k opioid receptors suggesting a relationship between adenosine receptor-mediated anti-inflammatory effects and PEMF exposure (Varani et al., 2002).

An up-regulation of the A_3 adenosine receptor has also been observed in neutrophils obtained from patients with colorectal cancer in comparison with healthy subjects. This overexpression was found to reflect at peripheral level the same up-regulation found in the tumoral tissue from the colon in comparison to healthy mucosa, suggesting that peripheral A_3 adenosine receptors in neutrophils might represent potential marker for revealing colorectal cancer (Gessi et al., 2004a). It was also

found that in a small cohort of subjects A_3 receptor expression of circulating blood cells normalizes after surgical treatment, consistent with the negative results of follow-up evaluation with carcinoembryonic antigen (CEA), computed tomography scan, and colonoscopy. Hence, the improved health of patients after surgical resection seems to be associated with restoration of a normal adenosinergic system, at least in terms of A_3 receptor expression. These findings might be used for clinical applications. In particular, examination of neutrophil A_3 expression (for example, in addition to CEA determination) could play a role in the screening of high-risk individuals or in the follow-up of patients after surgical resection.

Recently Chen et al. reported that migrating human neutrophils secrete ATP at the leading edge, which signals via P2Y2 receptors to amplify chemoattractant signals (Chen et al., 2006a). Neutrophils rapidly hydrolyze released ATP to adenosine which then acts via A_3 receptors, which are recruited to the leading edge, to promote cell migration. In resting cells, A_3 receptors appear to be located primarily in intracellular compartments associated with granules (Chen et al., 2006a). Upon cell stimulation with chemoattractant, A_3 receptors are rapidly mobilized at the leading edge to promote chemotaxis. Thus, ATP release and autocrine feedback through P2Y2 and A_3 receptors provides signal amplification and controls gradient sensing and migration of neutrophils. Interestingly, chemotaxis of neutrophils obtained from A_3 receptor knockout (KO) animals is inhibited. In contrast, A_{2A} receptors are uniformly distributed across the cell surface and cell polarization does not seem to change this distribution pattern (Chen et al., 2006a). This suggests that the inhibitory A_{2A} receptors may function to globally suppress pseudopod formation across the entire cell surface of neutrophils, except at the leading edge, where A_3 adenosine receptor counteract the suppressive action of A_{2A} receptors (Chen et al., 2006a; Linden, 2006; Junger, 2008). However, these findings were questioned recently by Hoeven et al. (2008) who demonstrated that A_3 adenosine receptor activation is responsible for inhibition of superoxide production and chemotaxis of mouse bone marrow neutrophils, suggesting that the A_3 receptor may contribute to the anti-inflammatory actions of adenosine. Although there are many differences between this study and the

earlier work, including the species difference (mouse vs human), the pharmacological agents used to stimulate the A_3 adenosine receptor (CP-532,903 versus IB-MECA), the methods used to isolate/culture murine neutrophils, the stimulation protocols (including the time and duration of pretreatment of cells with agonists), and the state of cell priming, a definite explanation for the differences in results obtained in these studies remains unclear.

Consistent with a pro-inflammatory role of A_3 adenosine receptors in human neutrophils it has been demonstrated that A_3 receptors together with P2Y subtypes mediate neutrophil elastase release induced by hypertonic saline (Chen et al., 2006b). Hypertonic saline holds promise as a novel resuscitation fluid for the treatment of trauma patients because it inhibits polymorphonuclear neutrophil activation and thereby prevents host tissue damage and associated post-trauma complications. However, under certain conditions of cell activation, hypertonic saline can increase neutrophil degranulation, which could exacerbate tissue damage in trauma victim (Chen et al., 2006b). The cellular mechanism by which hypertonic saline increases degranulation involves elastase release and ERK and p38 MAPK activation when hypertonic saline is added after submaximal activation of neutrophils with formyl peptide (fMLP) or phorbol ester (PMA). Agonists of P2 nucleotide and A_3 adenosine receptors mimicked these enhancing effects of hypertonic saline, whereas antagonists of A_3 receptors or removal of extracellular ATP with apyrase diminished the response to hypertonic saline suggesting that hypertonic saline upregulates degranulation via ATP release and positive feedback through P2 and A_3 receptors. It has been hypothesized that these feedback mechanisms can serve as potential pharmacological targets to fine-tune the clinical effectiveness of hypertonic saline resuscitation (Chen et al., 2006b). In this context, it has been shown that A_3 receptor activation may diminish the efficacy of hypertonic saline in a mouse model of acute lung injury after sepsis (Inoue et al., 2008a). Acute lung injury in wild-type mice treated with hypertonic saline 60 min after sepsis induction, through cecal ligation and puncture (CLP), was significantly greater than in wild-type mice pretreated for 5 and 15 min with hypertonic saline. Parallel experiments aimed at evaluating the expression of A_3 receptors in human neutrophils treated

with hypertonic saline either 10 min before or after stimulation with formyl methionylleucyl-phenylalanine (fMLP) reveal that in the first condition A_3 receptor expression was reduced whilst in the second one it was markedly increased. These findings show that the opposing effects of hypertonic saline *in vivo* correlate with differences in the cell surface expression of A_3 receptors, suggesting that the enhancing effects of hypertonic saline are a result of increased A_3 receptor expression of stimulated neutrophils. The aggravating effect of delayed hypertonic saline treatment was absent in A_3 receptor knockout (KO) mice. Similarly, mortality in wild-type mice with delayed hypertonic saline treatment was significantly higher than in animals treated with hypertonic saline before CLP. Mortality in A_3 receptor KO mice remained at only 50% regardless of timing of hypertonic saline administration. These findings suggest that A_3 antagonists could improve the efficacy of hypertonic saline resuscitation by reducing side effects in patients whose polymorphonuclear neutrophils are activated before hypertonic saline treatment. The role of A_3 and P2Y2 receptors in neutrophil sequestration in the lungs in a mouse model of sepsis has also been demonstrated (Inoue et al., 2008b). Sepsis was induced by CLP using wild type mice, homozygous A_3 receptor KO mice, and P2Y2 receptor KO mice. The data suggest that A_3 and P2Y2 receptors are involved in the influx of neutrophils into the lungs after sepsis. Neutrophil sequestration in the lungs reached a maximum 2 h after CLP and remained significantly higher in wild type mice compared with A_3 KO and P2Y2 KO mice. Survival after 24 h was significantly lower in WT mice than in A_3 KO or P2Y2 KO mice. Thus, pharmaceutical approaches that target these receptors might be useful to control acute lung tissue injury in sepsis.

It has been recognized that the inflammatory response to infection depends on the coordinated interaction of the adenine nucleotides, ATP, ADP and adenosine released by damaged tissue (Linden, 2006). Therefore the contribution of A_3 receptors expressed in neutrophils, must be in concert with the other purinergic receptors to allow neutrophil adhesion, extravasation and chemotaxis. Neutrophils express predominantly A_{2A} and A_3 receptors which have opposite effects on these cells. In this chapter we have described how neutrophils following gradients of ATP and

adenosine initiate and increase the speed of chemotaxis via P2Y and A_3 receptors, respectively and that the A_{2A} may amplify gradient signals by inhibiting chemotaxis at membrane region distant from the leading edge where the A_3 receptor predominates and increases chemotaxis. This seems possible given that the affinity of adenosine for the A_{2A} receptor is several orders of magnitude higher than its affinity for the A_3 receptors. Thus, differences in external adenosine concentrations in the environment surrounding migrating neutrophils may contribute to the regulation of chemotaxis (Chen et al., 2006a). A coordinated activity of A_{2A} and A_3 receptors has also been found with respect to degranulation and superoxide anion production in human neutrophils where both receptors cooperate to fine-tune the inflammatory response (Bouma et al., 1997; Gessi et al., 2002). However, it is important that the inhibitory effect exerted by A_{2A} and A_{2B} receptors on chemotaxis and adhesion to endothelial cells, respectively can overcome the stimulatory effect exerted by A_3 when excessive influx of neutrophils damages host tissues (Zhang et al., 2006). After activation of A_3 receptors opposite effects on inflammation have been reported depending essentially on the response considered, the experimental conditions and the species used. It is relevant to underline that by comparing the studies performed in human neutrophils both anti and proinflammatory effects have been demonstrated **(Figure 1)**. Therefore caution should be used before proposing A_3 agonists as anti or proinflammatory agents until a more definite role of this receptor has been defined.

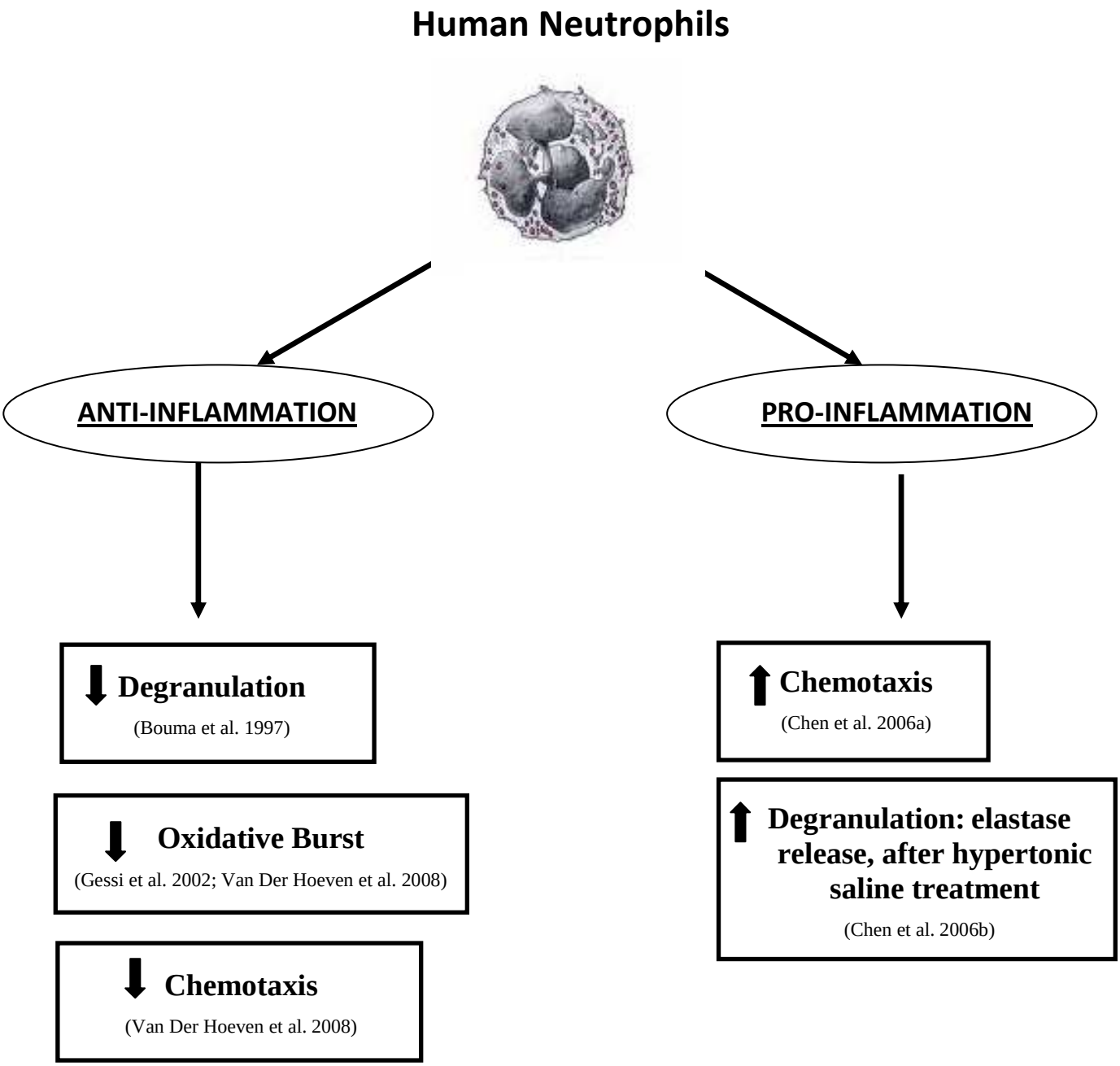

Figure 1: Effects of A_3 adenosine receptors in neutrophils

A_3 adenosine receptor effects on eosinophil function

Eosinophils are one of the immune system components responsible for combating infection. Along with mast cells, they also control mechanisms associated with allergy and asthma. Eosinophils develop and mature in the bone marrow. They differentiate from myeloid precursor cells in response to the cytokines interleukin 3 (IL-3), interleukin 5 (IL-5), and granulocyte macrophage colony-stimulating factor (GM-CSF). Eosinophils produce and store many secondary granule proteins prior to their exit from the bone marrow. After maturation, eosinophils circulate in blood and migrate to inflammatory sites in tissues, in response to chemokines such as CCL11 (eotaxin-1), CCL24 (eotaxin-2), CCL5 (RANTES), and leukotriene B4 (LTB4). At these infectious sites, eosinophils are activated by Type 2 cytokines released from a specific subset of helper T cells (T_h2); thus IL-5, GM-CSF, and IL-3 are important for eosinophil activation as well as maturation. Following activation, eosinophils release the contents of small granules within the cellular cytoplasm, which contain many chemical mediators, such as histamine and proteins such as eosinophil peroxidase, RNase, DNases, lipase, plasminogen, and major basic protein that are toxic to both parasite and host tissues (Gleich and Adolphson, 1986).

A_3 receptors are present on human eosinophils and couple to signalling pathways that lead to cell activation (Kohno et al., 1996a; Reeves et al., 2000). Despite this it has not proven easy to demonstrate the functional consequences of activation of these sites (Reeves et al., 2000). Nevertheless, the chronic inflammation in asthma is characterised by extensive infiltration of the airways by activated eosinophils (Holgate, 1999; Pearlman, 1999) and it remains possible that the elevated adenosine concentrations associated with asthma would contribute to eosinophil activation through stimulation of A_3 receptors. In addition, it has been speculated that activation of A_3 receptors may protect eosinophils from apoptosis (Gao et al., 2001). Thus, blockade of A_3 receptors may reduce the numbers of eosinophils and their activation thereby reducing the pro-inflammatory burden in the lung. Consistent with this, following 6 weeks treatment of mild asthmatics with

theophylline there was a significant reduction in the number of activated eosinophils beneath the epithelial basement membrane (Sullivan et al., 1994). Significantly, the average blood levels in this study (37 µM) were within the range of the affinity of theophylline for the human A_3 receptor. Moreover, it has been reported that activation of A_3 receptors mediates inhibition of eosinophil chemotaxis (Knight et al., 1997). The authors argue that since adenosine levels are highest at the site of inflammation, A_3 receptor activation would be pro-inflammatory by inhibiting eosinophil migration away from the sites of inflammation. Clearly, however, inhibition of chemotaxis could be pro- or anti-inflammatory. In line with a pro-inflammatory role, a high expression of A_3 receptor transcripts has been found in eosinophilic infiltrates of the lungs of patients with asthma and chronic obstructive pulmonary disease (COPD) (Walker et al., 1997). Interestingly, similar findings were seen in the lungs of adenosine deaminase deficient ($ADA^{-/-}$) mice that showed adenosine-mediated lung disease. Treatment of $ADA^{-/-}$ mice with MRS 1523, a selective A_3 receptor antagonist, prevented airway eosinophilia and mucus production. Similar results were obtained in the lungs of ADA/ A_3 receptor double KO mice, suggesting that A_3 receptor signalling plays an important role in regulating chronic lung disease and that A_3 receptor antagonism may be useful for reducing eosinophilia (Young et al., 2004). However these results contrast with those from experiments performed in human eosinophils *ex vivo*, where chemotaxis, degranulation and superoxide anion production were reduced by A_3 receptor activation (Knight et al., 1997; Walker et al., 1997; Ezeamuzie and Philips, 1999). This discrepancy was later attributed to the *ex vivo* nature of the chemotaxis experiments and implied that diminished airway eosinophilia seen in the lungs of $ADA^{-/-}$ mice following disruption of A_3 receptor is not a direct effect on the eosinophils but be due to the modulation of key regulatory molecules from other cells that express A_3 receptors and that affect eosinophil migration (Young et al., 2004). For example A_3 receptors are expressed on murine mast cells, airway macrophages and epithelial cells, all of which might affect eosinophil migration. However levels of key regulatory cytokines such as IL-5 and IL-13, or chemokines including eotaxin I, thymus- and activation-regulated chemokine (TARC) and monocyte chemotactic protein-3

(MCP3) were not affected by A_3 receptor deletion in $ADA^{-/-}$ mice, pointing perhaps to the involvement of A_3 receptor in the regulation of other key modulators of eosinophil migration such as cell adhesion molecules, extracellular matrix elements and proteases (Young et al., 2004). In contrast to a pro-inflammatory role of the A_3 subtype implied by the work of Young and colleagues cited above, the involvement of the A_3 adenosine receptor in a bleomycin model of pulmonary inflammation and fibrosis seems to indicate an anti-inflammatory effect (Morschl et al., 2008). Analysis of A_3 adenosine receptor KO mice revealed enhanced pulmonary inflammation including an increase in eosinophils and a selective up-regulation of eosinophil related chemokines and cytokines in the lungs of A_3 adenosine receptor KO mice exposed to bleomycin. This increase in eosinophil numbers was accompanied by a decrease in the eosinophil peroxidase activity in lavage fluid from A_3 adenosine receptor KO mice exposed to bleomycin, an observation suggesting the A_3 adenosine receptor is necessary for eosinophil degranulation in this model. Together these results suggest that the A_3 adenosine receptor mediates anti-inflammatory functions in the bleomycin model, and is also involved in regulating the production of mediators that can impact fibrosis (Morschl et al., 2008). The effects obtained in human eosinophils after A_3 receptor activation including inhibition of chemotaxis, degranulation, oxidative burst and the effects obtained from *in vivo* models of lung disease such as eosinophilia and mucus production are summarized in **Figure 2**.

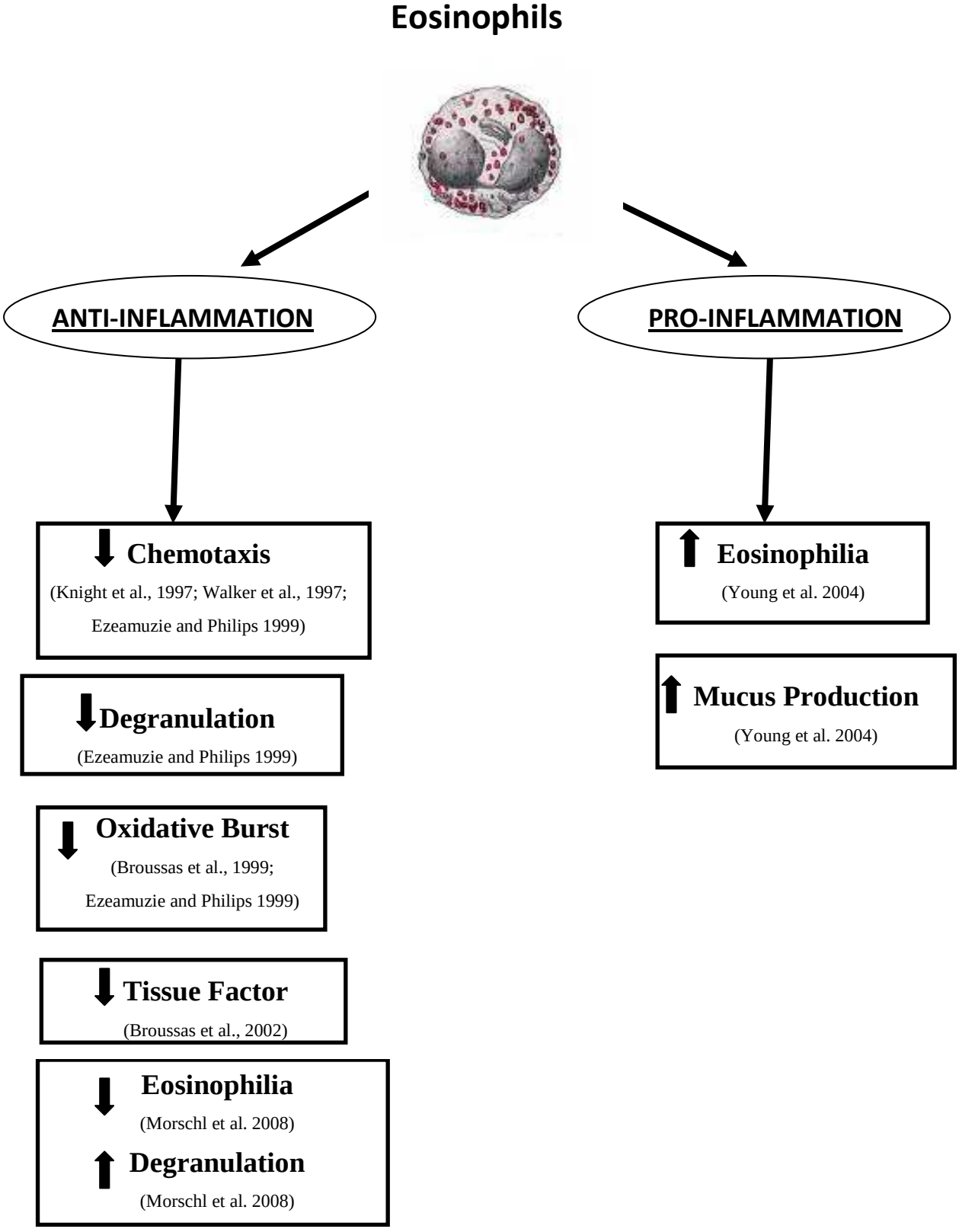

Figure 2: Effects of A_3 adenosine receptors in eosinophils

A_3 adenosine receptor effects on lymphocyte function

The ability of immune cells to fight tumor cells is fundamental for successful host defence against cancer. Adenosine, whose concentration increases within hypoxic regions of solid tumors, may interfere with the recognition of tumor cells by cytolytic effector cells of the immune system (Blay et al., 1997; Merighi et al., 2003). Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumor-induced immunosuppression. It has been demonstrated that colon adenocarcinoma cells inhibited anti-CD3-activated killer cell induction through the production of a tumor-associated soluble factor that was distinct from transforming growth factor beta or prostaglandins (Hoskin et al., 1994a). As a result, adenosine was suggested as a possible inhibitor of killer T-cell activation in the microenvironment of solid tumours (Hoskin et al., 1994b; Hoskin et al., 1994c). Indeed, evaluating the adhesion of murine spleen-derived anti-CD3-activated killer (AK) lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells it was found that adenosine reduced adhesion by up to 60% (MacKenzie et al., 1994). The inhibitory effect of adenosine was exerted on AK cells and not on the MCA-38 targets and the agonist potency profile indicated that the A_3 receptor subtype might be responsible for the inhibition of adhesion. The authors suggested that this mechanism of immunosuppression, secondary to tissue hypoxia, may be important in the resistance of colorectal and other solid cancers to immunotherapy. In addition the same authors demonstrated that adenosine plays a strong inhibitory effect on the induction of mouse cytotoxic T cells (Hoskin et al., 2002). Diminished tumoricidal activity correlated with reduced expression of mRNAs coding for granzyme B, perforin, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL). Interleukin-2 (IL-2) and interferon-γ (IFN-γ) synthesis by AK-T cells was also inhibited by adenosine. The inhibitory effect of adenosine on AK-T cell proliferation

was also blocked by an A_3 receptor antagonist suggesting that adenosine acts through A_3 receptors to prevent AK-T cell induction. Tumor-associated adenosine may act through the same mechanism to impair the development of tumor-reactive T cells in cancer patients. Therefore the suppression of T-killer cell function suggests that adenosine may act as a local immunosuppressant within the microenvironment of solid tumors. Subsequently it was reported that adenosine partially inhibits the interaction of T lymphocytes with tumor cells by blocking the function of integrin $\alpha 4\beta 7$ which is the major cell adhesion molecule involved in the adhesion of T cells to syngeneic MCA-38 adenocarcinoma cells (MacKenzie et al., 2002). The effect of adenosine has been investigated on the expression of costimulatory molecules by T cells in resting and activated conditions. The most important costimulatory molecules present on the T cells surface are CD2 and CD28 acting in concert to achieve optimal costimulation of T lymphocytes during interaction with antigen presenting cells. It has also been demonstrated that adenosine interferes with activation-induced expression of the co-stimulatory molecules CD2 and CD28 by an IL-2 dependent mechanism but not involving the accumulation of intracellular cAMP and possibly by activating the A_3 subtype (Butler et al., 2003). Subsequently the inhibitory effect mediated by adenosine on the ability of LAK cells to kill tumor cells was attributed essentially to the cAMP-elevating A_{2A} receptor whilst no evidence of the involvement of cAMP inhibitory A_1 or A_3 subtypes in the regulation of the cytotoxic activity of LAK cells was found (Raskovalova et al., 2005). Indeed, it has been suggested that hypoxic cancerous tissues may be protected by the same hypoxia→adenosine→A_{2A} receptor pathway that was recently shown to be critical and nonredundant in preventing excessive damage of normal tissues by overactive immune cells *in vivo* (Ohta et al., 2001).

In contrast to the immunosuppressive role of adenosine in the environment of solid tumors, it has been reported that A_3 receptor activation stimulates the proliferation of murine bone marrow cells *in vitro*. This effect was induced through the G-CSF production by human peripheral blood mononuclear cells (PBMC) mediated by adenosine. The finding was confirmed *in vivo*

experiments, which revealed an increase in leukocyte and neutrophil numbers when adenosine was administered before chemotherapy (Fishman et al., 2000). The molecular mechanisms underlying G-CSF production included the upregulation of the PI3K, PKB/Akt and NF-kB pathways (Bar-Yehuda et al., 2002). In addition, it has been observed that Cl-IB-MECA increases the activity of NK cells in naïve and tumor bearing mice through the induction of IL-12; this effect was dependent on inhibition of cAMP levels and PKA expression. IL-12 is a potent stimulant of NK cells and is a cytotoxic factor that exerts a potent anti-tumor effect *in vivo*. It induces IFN-γ production by activated T and NK cells and augments cytotoxic activity of these cells via perforin, Fas and Trail-dependent mechanisms. Therefore, A_3 receptor activation enhances NK cell activity and probably NK cell-mediated destruction of tumor cells (Harish et al., 2003). The expression of A_3 receptor was also investigated in resting and activated lymphocytes (Gessi et al., 2004b). Activated human lymphocytes undergo a rapid induction of both transcript and protein of A_3 receptors. The kinetics of this up-regulation revealed that even at earlier time points, the increase was present only in $CD4^+$ cells, whereas it was not changed in $CD8^+$ cells. Therefore, it is possible that in humans, as in mice (Hoskin et al., 2002), A_3 receptors play an immunosuppressive role in $CD8^+$ T cells, but their up-regulation in $CD4^+$ cells strongly suggests that they might also be implicated in T helper cell activities. One method of increasing the number of A_3 receptors on the cell membrane is to increase the accumulation of mRNA encoding the A_3 subtypes. As evaluated by means of real-time RT-PCR experiments, activation of T cells with PHA rapidly increased the level of A_3 message in the $CD4^+$ subset, but not in the $CD8^+$ cells. This increase in A_3 receptor mRNA, which could occur as a result of an increase in transcription and/or an increase in mRNA stability, is likely to be responsible for the increased synthesis of receptor proteins as detected by means of binding and Western blot studies. The rapid up-regulation of A_3 receptors functionally coupled to adenylyl cyclase in activated T cells may indicate another potential example of biological significance for adenosine-mediated responses in T cells.

An overexpression of A_3 receptors has also been detected in lymphocytes of patients with colorectal cancer. Interestingly, the existence of A_3 receptors was previously demonstrated on Jurkat cells, a human leukemic cell line, where they were associated with inhibition of adenylyl cyclase activity and calcium modulation (Gessi et al., 2001). Blood lymphocytes obtained from 30 colorectal cancer patients showed a >3-fold overexpression of A_3 receptors compared with blood cells from healthy donors, in line with the data found in tissues. No association was found with stage of the disease, tumor site, patient age, or gender. Even though the mechanism of this up-regulation are not known it is interesting that binding data from tissues, as in circulating blood cells, discriminate between small-sized adenomas and cancer, suggesting that A_3 receptor may be a requirement for colorectal tumor progression. These receptors may represent, like those in neutrophils, tumoral markers due to their higher expression in comparison to that observed in healthy subjects. This suggests that peripheral blood cells mirror at the peripheral level the higher levels of the A_3 receptor found in colorectal cancer. However the selectivity of the A_3 receptor as a tumoral marker may be of only limited value because a similar phenomenon has been confirmed in patients with rheumatoid arthritis. Thus the A_3 receptor was overexpressed in PBMC of patients with rheumatoid arthritis compared to healthy subjects and was directly correlated to an increase in NF-kB in the same cells (Madi et al., 2007). Similar data were found in phytohemagglutinin and lipopolysaccharide-stimulated PBMC from healthy subjects suggesting that receptor upregulation is induced by inflammatory cytokines controlling the expression of the A_3 adenosine receptor transcription factor NF-kB (Madi et al., 2007). It seems that the A_3 adenosine subtype found in PBMC obtained from peripheral blood may not represent a specific tumoral marker but more generally a marker for inflammation.

In conclusion, it is well established that extracellular adenosine has the potential to be an important inhibitor of tumor cell destruction by NK and LAK cells within the microenvironment of solid tumors by signaling primarily through A_{2A} and A_3 adenosine receptors on the surface of T cells (Hoskin et al., 2008). However after the demonstration that

genetic deletion of immunosuppressive A_{2A} and A_{2B} receptors or their pharmacological inactivation can prevent the inhibition of anti-tumor T cells by the hypoxic tumor and facilitate full tumor rejection, several reviews focused on the relevance of A_{2A} and in minor part of A_{2B} adenosine subtypes to improve the effectiveness of immune-based cancer therapies (Ohta et al., 2006; Lukashev et al., 2007; Sitkovsky et al., 2008a, b). In contrast to the well described mechanisms by which A_{2A} adenosine receptor signaling blocks T cell activation and effector function, little is known about the mechanism of A_3 adenosine receptor-mediated T cell inhibition. Moreover, while the importance of A_{2A} adenosine receptor signaling in adenosine-mediated suppression of T cell responses has been confirmed using A_{2A} adenosine receptor-deficient mice (Lukashev et al., 2003), similar confirmatory studies have not yet been performed with A_3 adenosine receptor deficient mice. Additional studies need to be performed in human lymphocytes as almost all the functional effects attributed to A_3 receptor activation are derived from studies carried out in mice species **(Figure 3)**. The identification of adenosine receptor subtypes and/or signal transduction pathways through which adenosine exerts its inhibitory effects on cell-mediated anti-tumor immune responses may allow for the development of novel “anti-adenosinergic” approaches that increase the effectiveness of therapeutic cancer vaccines and other immune-based cancer therapies.

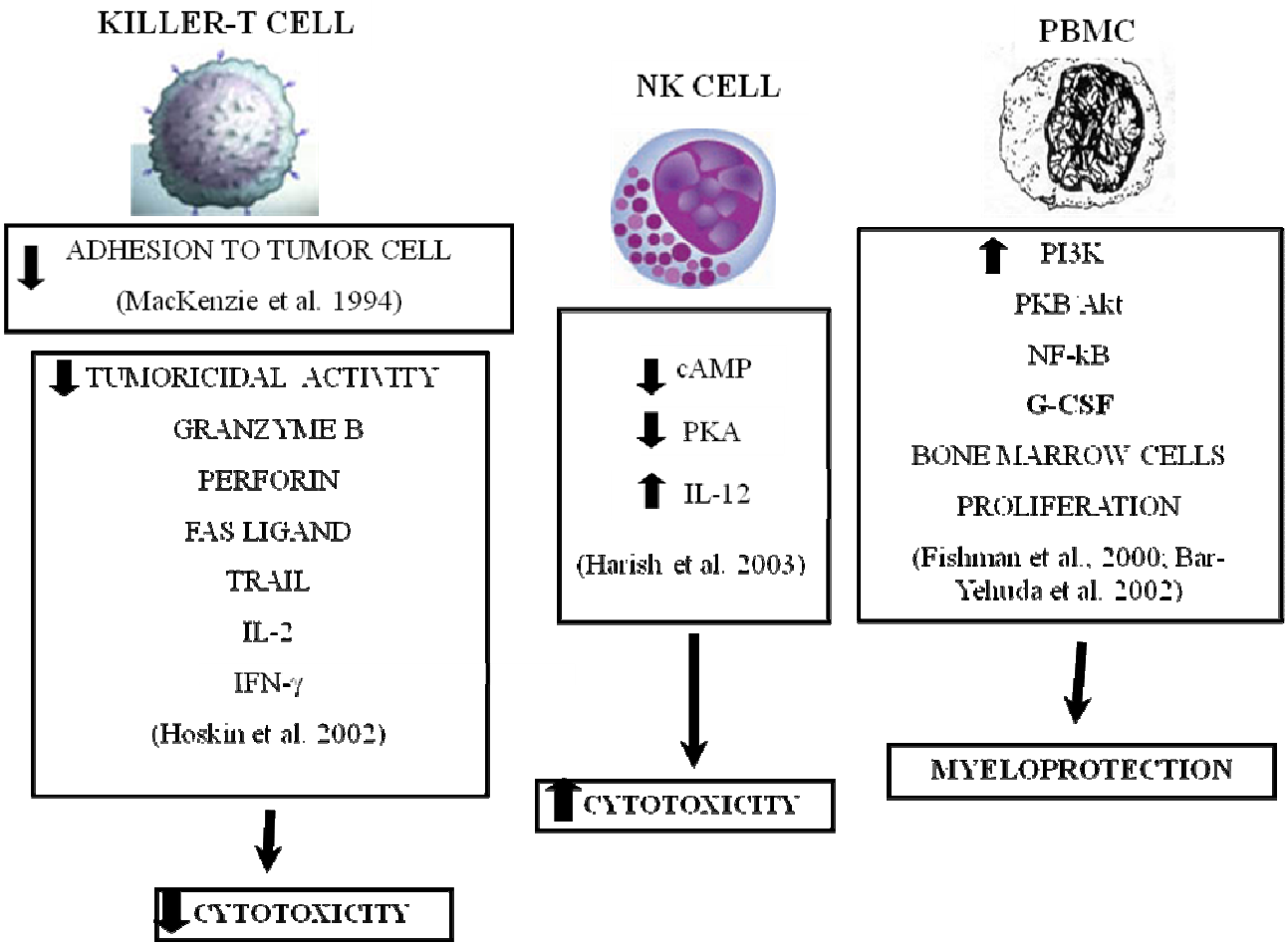

Figure 3: Effects of A_3 adenosine receptors in lymphocytes

A_3 adenosine receptor effects on monocyte-macrophage function

In vivo and *in vitro* studies in animal systems led to the concept of the mononuclear-phagocyte system as a cell system involved in host defenses, phagocytosis, and antigen presentation and processing (Douglas, 1999). Following Metchnikoff's development of phagocyte theory, Wright described opsonins as factors in serum that facilitated phagocytosis. Aschoff defined the reticuloendothelial system as a cellular system in which tissue macrophages and monocytes share important functional characteristics, namely, phagocytic ability and adhesiveness to glass. Subsequently, the histologic development of silver stains by Del Rio-Hortega defined a type of macrophage-related cell in the brain, the microglia. In the mid-1960s, the late Zanvil Cohn and his collaborators carried out seminal studies of mononuclear phagocytes leading to concepts of macrophage differentiation, activation, secretion, and the relationship of macrophages to antigen presentation and processing (for further details see Douglas, 1999).

Adenosine has been investigated as an endogenous regulator of monocyte-macrophage functions. The effects produced by A_3AR activation of macrophages seem to indicate an anti-inflammatory effect of this adenosine subtype. For example, the A_3ARs suppress TNF-α release induced by the endotoxin CD14 receptor signal transduction pathway from human monocytes and murine J774.1 macrophages (Le Vraux et al., 1993; McWhinney et al., 1996). Moreover in a macrophage model the A_3AR was the prominent subtype implicated in the inhibition of LPS-induced TNF-α production (Sajjadi et al. 1996). This effect was associated with changes in stimulation of the activator protein-1 (AP-1) transcription factor, whereas it was independent of MAPKs, NF-kB, PKA, PKC and PLC. The inhibitory effect induced by the A_3AR on TNF-α production was also assessed in A_3KO mice where the A_3 agonist was unable to reduce TNF-α levels in contrast to its effect in wild type animals (Salvatore et al., 2000). In contrast, in BV2 microglial cells the A_3-mediated inhibition of LPS-induced TNF-α expression was associated with the inhibition of LPS-induced activation of the PI3-kinase/Akt and NF-kB pathways (Lee et al., 2006). Recently it has been reported that in mouse

RAW 264.7 cells the A_3 subtype inhibits LPS-stimulated TNF-α release by reducing calcium-dependent activation of NF-kB and ERK 1/2 (Martin et al., 2006). In contrast, in peritoneal macrophages, isolated from A_3 KO mice, the ability of IB-MECA to inhibit TNF-α release was not altered in comparison to wild type mice (Kreckler et al., 2006). In this study, the inhibitory effect was exerted through the activation of A_{2A} and A_{2B} agonists as has been recently demonstrated in human monocytes (Zhang et al., 2005; Haskó et al., 2007). The discrepancy observed among these papers cannot be the consequence of species differences, since in both cases mouse cells were used. Other factors, including the source of the cells and/or the inflammatory stimulus used, may be responsible. However in spite of these contrasting results, one of the most likely therapeutic applications of the regulatory role of A_3 activation on TNF-α release is in the treatment of arthritis. More recent studies show that A_3AR agonists exert significant effects in different autoimmune arthritis models by suppression of TNF-α production (Baharav et al., 2005). The molecular mechanisms involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis include receptor downregulation and de-regulation of the PI3K-NF-kB signalling pathway (Fishman et al., 2006; Madi et al., 2007). Thus, A_3AR activation by IB-MECA inhibited macrophage inflammatory protein (MIP)-1α, a C-C chemokine with potent inflammatory effects, in a model of collagen-induced arthritis, providing the first proof of concept of the adenosine agonists utility in the treatment of arthritis (Szabò et al., 1998). Other anti-inflammatory effects involving A_3 receptors activation include inhibition of fMLP-triggered respiratory burst and tissue factor expression by human monocytes (Broussas et al., 1999, 2002). Recently, it has been reported that, adenosine may be involved in ventricular remodeling by stimulating Matrix metalloproteinase-9 (MMP-9) production in human macrophages following A_3 receptor activation (Velot et al., 2008). MMP-9 plays an important role in ventricular remodelling after acute myocardial infarction (MI). Adenosine enhanced MMP-9 production when macrophages were activated by hypoxia or Toll-like receptor-4 ligands such as lipopolysaccharide, hyaluronan, and heparan sulfate. The effect of adenosine was replicated by the A_3 agonist IB-MECA and inhibited by silencing the A_3AR through the use of RNA

interference. Interestingly, it was found that MMP-9 expression was higher in blood cells from patients with acute MI compared with healthy volunteers with important implications for therapeutic strategies targeting adenosine receptors in the setting of MI (Velot et al., 2008).

In conclusion as for the role of A_3 receptors in the inhibition of TNF-α production in macrophages discrepant results have been obtained and not only due to the different species considered. For example some studies attributed reduction of TNF-α to A_3 receptors either in human and mouse species (Sajjadi et al., 1996; McWhinney et al., 1996), whilst other found this effect to be mediated essentially by A_{2A} and in minor part by A_{2B} without the involvement of the A_3 receptors again in both human and mouse species (Zhong et al., 2005; Kreckler et al., 2006). Therefore it is difficult in this case to verify the relevance of the A_3 receptor-induced cellular response when other adenosine subtypes like A_{2A} and A_{2B} are also activated. As for the effects exerted by the A_3 subtype in human monocytes and macrophages it is possible to find support for an anti-inflammatory role for this receptor as attested by reduction of tissue factor, oxidative burst and perhaps TNF-α release. Also the recent discovery of an increase in MMP9 supports a role for A_3 agonists in the therapy of myocardial infarction (Velot et al., 2008) **(Figure 4)**.

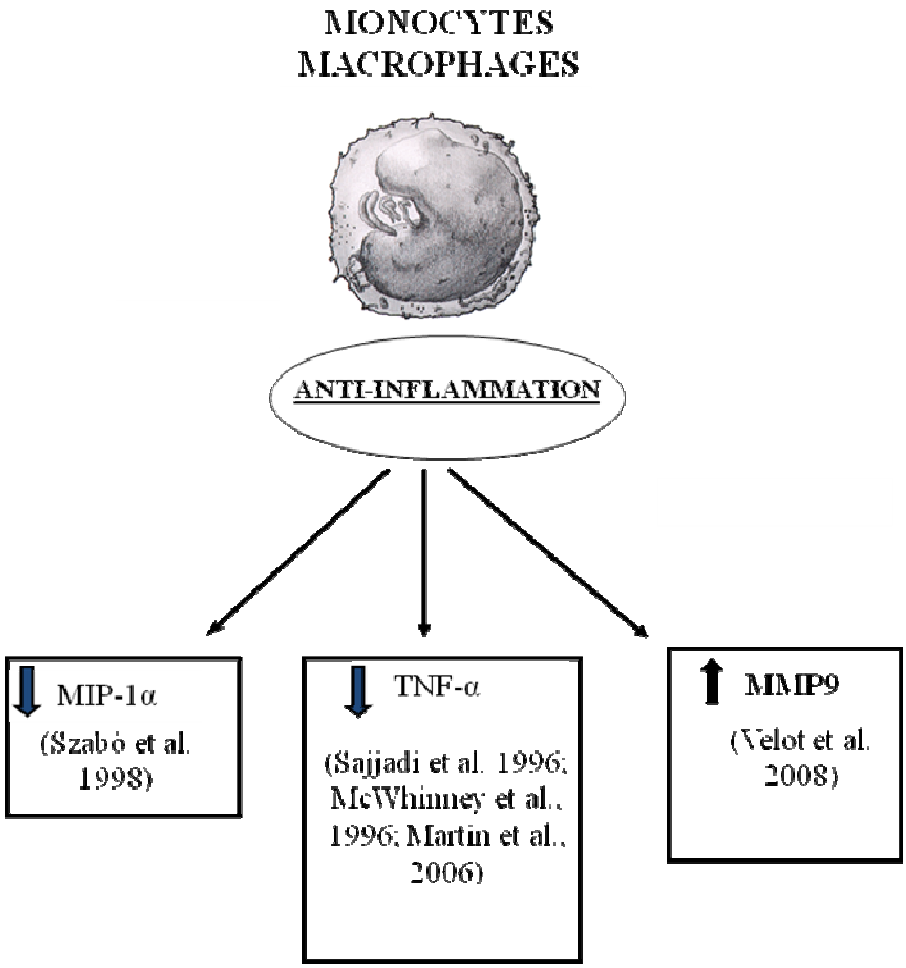

Figure 4: Effects of A_3 adenosine receptors in monocytes-macrophages

A_3 adenosine receptor effects on dendritic cell function

Dendritic cells are antigen-presenting cells specialized to activate naive T lymphocytes and initiate primary immune responses (Steinman, 1991; Hart, 1997; Banchereau,et al., 1998). Dendritic cells originate from hemopoietic stem cells and migrate into peripheral tissues. Dendritic cells reside in an immature form in unperturbed tissue, where they are capable of taking up antigens but weak at stimulating T cells. Under the influence of a variety of so-called danger signals including pathogens; dying cells; soluble CD40 ligand; cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), or bacterial products such as LPS dendritic cells undergo a process of differentiation known as maturation. Thereafter, they migrate to the T-cell areas of secondary lymphoid organs. This maturation process is associated with reduced phagocytic and endocytic activity, increased membrane expression of major histocompatibility complex and co-stimulatory molecules, production of cytokines such as interleukin 12 (IL-12), and acquisition of potent T-cell-stimulating functions. Depending on the conditions, dendritic cells can stimulate growth of a variety of T-cell subsets. In the presence of IL-12, they support the growth of Th1 cells, whereas with IL-4 dendritic cells induce Th2-cell differentiation. In recent years it has become clear that A_3 adenosine receptors play a role in regulation of various activities of dendritic cells.

The expression and function of adenosine receptors in human dendritic cells has been investigated by using reverse transcriptase-polymerase chain reaction and functional experiments (Panther et al., 2001). mRNA expression of the A_3 receptor has been detected in immature dendritic cells together with A_1 and A_{2A} receptors. Adenosine, IB-MECA and also the A_1 agonist CHA, induced Ca^{2+} transients as well as actin polymerization and chemotaxis but only in immature dendritic cells. These findings suggest that adenosine may control proinflammatory activities of dendritic cells and regulate their accumulation at target sites. Maturation of dendritic cells is accompanied by a loss of the adenosine responses such as Ca^{2+} transients, actin polymerization, and migration. Unequivocal evidence of cell surface expression of the A_3 receptor in immature dendritic cells was obtained from

[^{125}I]ABMECA binding experiments. Saturation isotherms indicated a Bmax of approximately 300 fmol/mg membrane protein, and competition for the radioligand of a variety of adenosine receptor ligands categorically identified the binding site as the A_3 receptor (Fossetta et al., 2003). Moreover through fluorometric imaging plate reader (FLIPR)-based analysis of calcium mobilization it was shown that the A_3 adenosine receptor is coupled to calcium mobilization in a pertussis toxin-dependent way. Interestingly these authors demonstrated that adenosine is much more potent at the A_3 receptor than had been appreciated, being active in the low nanomolar range. Generally, adenosine has been regarded as a low potency agonist of the A_3 receptor, with apparent affinities ranging from 300 nM to 1 µM (Fredholm et al., 2001). The presence of functional A_3 receptors has been observed in XS-106, a mouse dendritic cell line, where they were coupled negatively to adenylyl cyclase and to stimulation of p42/p44 mitogen-activated protein kinase phosphorylation. Adenosine A_3 receptor activation also inhibits lipopolysaccharide-induced TNF-α release from XS-106 cells as already reported in macrophages (McWhinney et al., 1996; Dickenson et al., 2003). At present, the signal transduction pathway involved in adenosine A_3 receptor-mediated inhibition of TNF-α release from XS-106 cells (and see above macrophages) is unclear. Inhibition of TNF-α release is usually associated with Gs-protein-coupled receptor-mediated cyclic AMP production. Interestingly, adenosine A_3 receptors have been shown to induce an increase in intracellular calcium and potentiate Ca^{2+} currents via protein kinase A activation in A6 renal cells (Reshkin et al., 2000) and hippocampal CA_3 pyramidal neuronal cells (Fleming and Mogul, 1997). In addition, activation of the adenosine A_3 receptor stimulates cyclic AMP production in human eosinophils (Ezeamuzie and Philips, 2003). However, in XS-106 cells, Cl-IB-MECA did not stimulate cyclic AMP accumulation indicating that the adenosine A_3 receptor is not directly coupled to Gs-protein/cyclic AMP accumulation in XS-106 cells. Finally, the transcript for the A_3 adenosine receptor was elevated more than 100-fold in immature dendritic cells compared with monocyte precursors. A_3 receptor transcript was substantially diminished by LPS-induced maturation of immature dendritic cells. The strict dependence of A_3 receptor expression on the immature cells suggests that the A_3

receptor could also be involved in the maintenance of the immature phenotype, and its abrupt disappearance may be crucial for transition to a fully activated dendritic cell (Fossetta et al., 2003).

The relevance of the A_3 receptor over the other adenosine subtypes in immature human dendritic cells is attested to by different studies demonstrating a role for this receptor in the increase of intracellular calcium, actin polymerization and chemotaxis (Panther et al., 2001; Fossetta et al., 2003) **(Figure 5)**. However a loss of the A_3 and an increase of the A_{2A} receptor has been reported during maturation of dendritic cells. This switch has been interpreted as a protective effect of adenosine in the context of tissue injury as A_{2A} activation plays an inhibitory role on dendritic cells migration. In this way adenosine could counterbalance inflammatory stimuli by delaying the arrival of mature dendritic cells to lymph nodes, thereby impairing the initiation of immune responses and reducing the potentially detrimental effects of chronic cell activation responsible for tissue damage and disease.

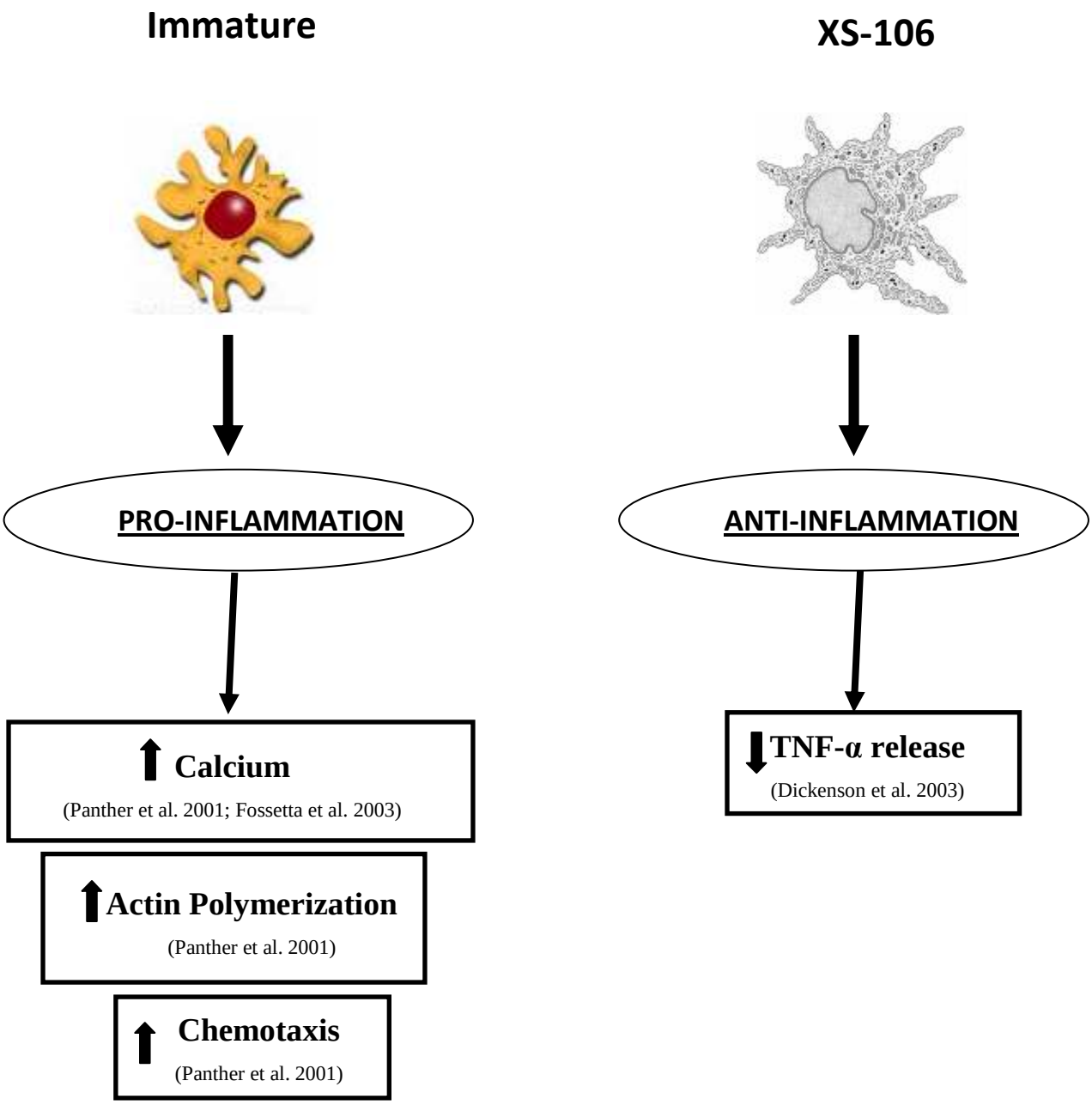

Figure 5: Effects of A_3 adenosine receptors in dendritic cells

Conclusions

The data summarized in this chapter show that A_3 receptors are present in immune cells and are indeed involved in the physiopathologic regulation of inflammatory and immune processes. However results from *in vitro* and *in vivo* studies in experimental animals suggest activation of the A_3 subtype can be both pro or anti-inflammatory depending on:

- the cell type examined e.g. neutrophil, eosinophil, macrophage, T cell, dendritic cell;
- the cellular model used e.g. in vitro or ex vivo; transgenic animals;
- the response investigated e.g. degranulation, oxidative burst, migration, maturation, cytokine production;
- the species considered e.g. human or animal;
- the presence and functional roles of other adenosine receptor subtypes.

Even though it seems that in each cell type examined contrasting effects have been reported, the results reviewed here offer the background for possible new therapeutic strategies for a number of inflammatory conditions such as sepsis, asthma and autoimmune disorders including rheumatoid arthritis, Crohn's disease and psoriasis. Indeed at the moment there are A_3AR agonists in clinical development for rheumatoid arthritis. Unfortunately there are no A_3AR antagonists in clinical development but a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, and stroke, waiting to enter the clinical arena (Baraldi et al. 2008). Future studies aimed at elucidating new effects of the A_3 subtype in the modulation of important inflammatory responses in the different peripheral blood cells are likely to reveal exciting new potential therapeutic applications of A_3 agonists and/or antagonists.

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CHAPTER 3:

Adenosine modulates HIF-1α, VEGF, IL-8 and foam cells formation in a human model of hypoxic foam cells

GENERAL INTRODUCTION

Main features of atherosclerotic lesions

Atherosclerotic lesions are asymmetric focal thickenings of the innermost layer of the artery, the intima **(Figure 1)**. They consist of cells, connective-tissue elements, lipids, and debris (Stary et al., 1995). Blood-borne inflammatory and immune cells constitute an important part of an atheroma, the remainder being vascular endothelial and smooth-muscle cells. The atheroma is preceded by a fatty streak, an accumulation of lipid-laden cells beneath the endothelium (Stary et al., 1994). Most of these cells in the fatty streak are macrophages, together with some T cells. Fatty streaks are prevalent in young people, never cause symptoms, and may progress to atheromata or eventually disappear. In the center of an atheroma, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of smooth-muscle cells and a collagen-rich matrix. T cells, macrophages, and mast cells infiltrate the lesion and are particularly abundant in the shoulder region where the atheroma grows (Stary et al., 1995; Jonasson et al., 1986; Kovanen et al., 1995). Many of the immune cells exhibit signs of activation and produce inflammatory cytokines (Kovanen et al., 1995; Hansson et al., 1989; van der Wal et al., 1994; Frostegard et al., 1999). Myocardial infarction occurs when the atheromatous process prevents blood flow through the coronary artery. It was previously thought that progressive luminal narrowing from continued growth of smooth-muscle cells in the plaque was the main cause of infarction. Angiographic studies have, however, identified culprit lesions that do not cause marked stenosis, (Hackett et al., 1995) and it is now evident that the activation of plaque rather than stenosis precipitates ischemia and infarction. Coronary spasm may be involved to some extent, but most cases of infarction are due to the formation of an occluding thrombus on the surface of the plaque (Davies et al., 1995). Plaque rupture is the major cause of coronary thrombosis because it exposes prothrombotic material from the core of the plaque — phospholipids, tissue factor, and platelet-adhesive matrix molecules — to the blood **(Figure 1)**. Ruptures preferentially occur where the fibrous cap is thin and partly destroyed. At these sites, activated immune cells are abundant (van der Wal et al., 1994). They produce numerous inflammatory molecules and proteolytic enzymes that can weaken the cap and

activate cells in the core, transforming the stable plaque into a vulnerable, unstable structure that can rupture, induce a thrombus, and elicit an acute coronary syndrome.

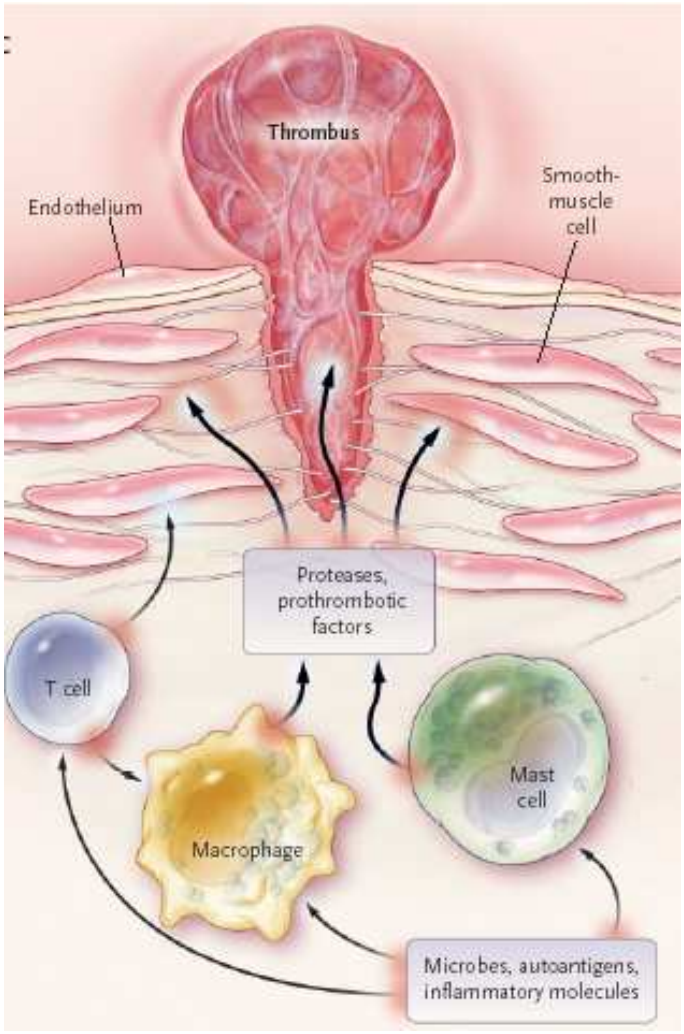


Figure 1: Consequences of the activation of immune cells in a coronary plaque

Role of endothelial activation, adhesion molecules and chemokines

Studies in animals and humans have shown that hypercholesterolemia causes focal activation of endothelium in large and medium-sized arteries. The infiltration and retention of LDL in the arterial intima initiate an inflammatory response in the artery wall (Skalen et al., 2002) **(Figure 2)**. LDL is a globular molecule with an highly-hydrophobic core consisting of polyunsaturated fatty acid and esterified cholesterol molecules. This core is surrounded by a shell of phospholipids, unesterified cholesterol and a single copy of apolipoprotein B-100 molecule. Cholesterol can move in the bloodstream only by being transported by lipoproteins. Modification of LDL, through oxidation or enzymatic attack in the intima, leads to the release of phospholipids that can activate endothelial cells, (Leitinger, 2003) preferentially at sites of hemodynamic strain (Nakashima et al., 1998). Patterns of hemodynamic flow typical for atherosclerosis-prone segments (low average shear but high oscillatory shear stress) cause increased expression of adhesion molecules and inflammatory genes by endothelial cells (Dai et al., 2004). Therefore, hemodynamic strain and the accumulation of lipids may initiate an inflammatory process in the artery. The platelet is the first blood cell to arrive at the scene of endothelial activation (Massberg et al., 2003). Its glycoproteins Ib and IIb/IIIa engage surface molecules on the endothelial cell, which may contribute to endothelial activation. Inhibition of platelet adhesion reduces leukocyte infiltration and atherosclerosis in hypercholesterolemic mice (Massber et al., 2003). Activated endothelial cells express several types of leukocyte adhesion molecules, which cause blood cells rolling along the vascular surface to adhere at the site of activation (Eriksson et al., 2002). Since vascular-cell adhesion molecule 1 (VCAM-1) is typically up-regulated in response to hypercholesterolemia, cells carrying counterreceptors for VCAM-1 (monocytes and lymphocytes) preferentially adhere to these sites (Cybulsky et al., 1991). Once the blood cells have attached, chemokines produced in the underlying intima stimulate them to migrate through the interendothelial junctions and into the subendothelial space. Genetic abrogation or pharmacologic blockade of certain chemokines and adhesion

molecules for mononuclear cells inhibits atherosclerosis in mice (Boring et al., 1998; Gu et al., 1998; Lesnik et al., 2003; Lutters et al., 2004).

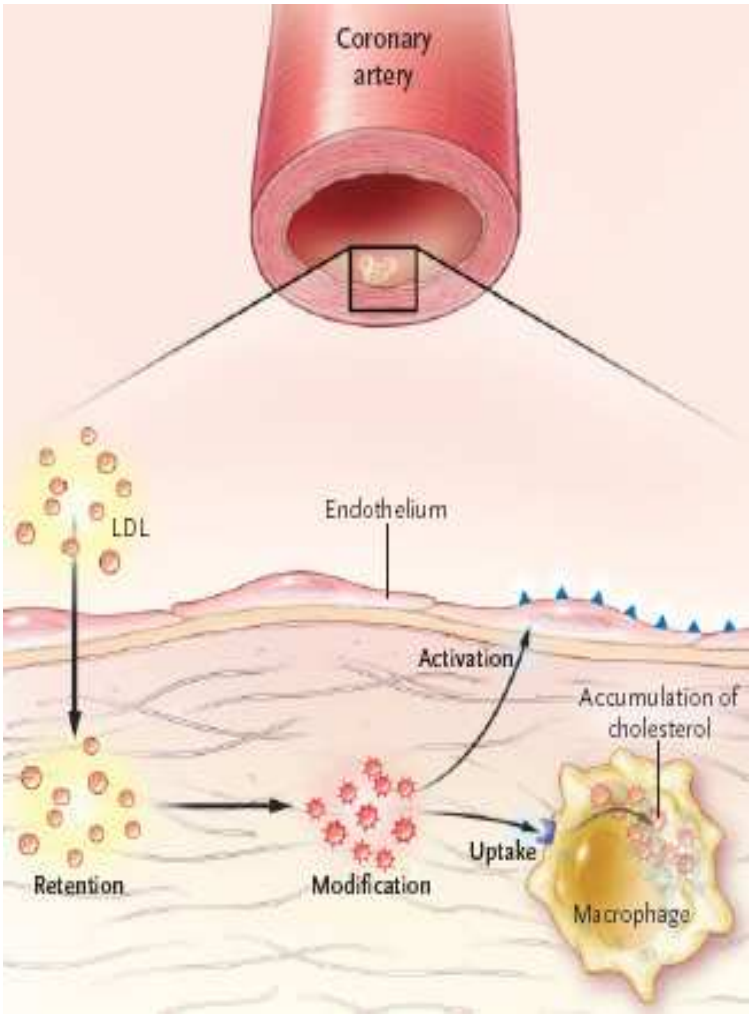


Figure 2: Effect of LDL infiltration on inflammation in the artery

Macrophages in the Developing Plaque

A cytokine or growth factor produced in the inflamed intima, macrophage colony-stimulating factor (MCS-F), induces monocytes entering the plaque to differentiate into macrophages **(Figure 3)**. This step is critical for the development of atherosclerosis (Smith et al., 1995) and is associated with up-regulation of pattern-recognition receptors for innate immunity, including scavenger receptors and toll-like receptors (Peiser et al., 2002; Janeway et al., 2002). Scavenger receptors internalize a broad range of molecules and particles bearing molecules with pathogen-like molecular patterns (Peiser et al., 2002). Bacterial endotoxins, apoptotic cell fragments, and oxidized LDL particles are all taken up and destroyed through this pathway. If cholesterol derived from the uptake of oxidized LDL particles cannot be mobilized from the cell to a sufficient extent, it accumulates as cytosolic droplets. Ultimately, the cell is transformed into a foam cell, the prototypical cell in atherosclerosis. Toll-like receptors also bind molecules with pathogen-like molecular patterns, but in contrast to scavenger receptors, they can initiate a signal cascade that leads to cell activation (Janeway et al., 2002). The activated macrophage produces inflammatory cytokines, proteases, and cytotoxic oxygen and nitrogen radical molecules.

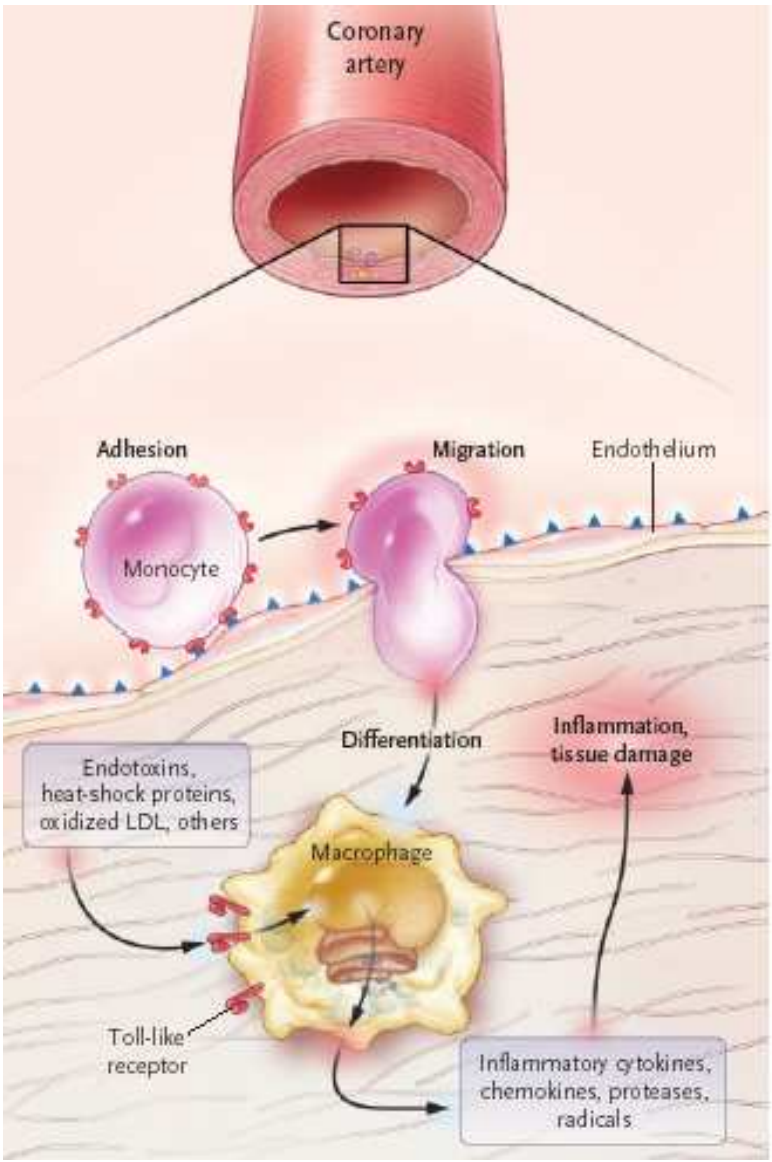


Figure 3: Role of macrophage inflammation of the artery

T-Cell activation and vascular inflammation

Immune cells, including T cells, antigen-presenting dendritic cells, monocytes, macrophages, and mast cells, patrol various tissues, including atherosclerotic arteries, in search of antigen (Hansson, 2001). A T-cell infiltrate is always present in atherosclerotic lesions **(Figure 4)**. Such infiltrates are predominantly CD4+T cells, which recognize protein antigens presented to them as fragments bound to major-histocompatibility- complex (MHC) class II molecules. CD4+ T cells reactive to the disease-related antigens oxidized LDL, heat-shock protein (Caligiuri et al., 2001), and chlamydia proteins have been cloned from human lesions (Xu, 2002; de Boer et al., 2000). A minor T-cell subpopulation, natural killer T cells, is prevalent in early lesions. CD8+ T cells restricted by MHC class I antigens are also present in atherosclerotic lesions (Hansson, 2001). These cells typically recognize viral antigens, which may be present in the lesions. When the antigen receptor of the T cell is ligated by antigen, an activation cascade results in the expression of a set of cytokines, cell-surface molecules, and enzymes. The atherosclerotic lesion contains cytokines that promote a Th1 response (rather than a Th2 response) (Frostegard et al., 1999). Activated T cells therefore differentiate into Th1 effector cells and begin producing the macrophage-activating cytokine interferon-γ **(Figure 4)**. Interferon-γ improves the efficiency of antigen presentation and augments synthesis of the inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Szabo et al., 2003). Acting synergistically, these cytokines instigate the production of many inflammatory and cytotoxic molecules in macrophages and vascular cells (Hansson, 2001). All these actions tend to promote atherosclerosis. Cytokines of the Th2 pathway can promote antiatherosclerotic immune reactions (Binder et al., 2004). However, they may also contribute to the formation of aneurysms by inducing elastolytic enzymes (Shimizu et al., 2004). Therefore, switching the immune response of atherosclerosis from Th1 to Th2 may not necessarily lead to reduced vascular disease. T-cell cytokines cause the production of large amounts of molecules downstream in the cytokine cascade. As a result, elevated levels of interleukin- 6 (IL-6) and C-reactive protein may be detected in the peripheral circulation. In this way, the activation of a limited

number of immune cells can initiate a potent inflammatory cascade, both in the forming lesion and systemically.

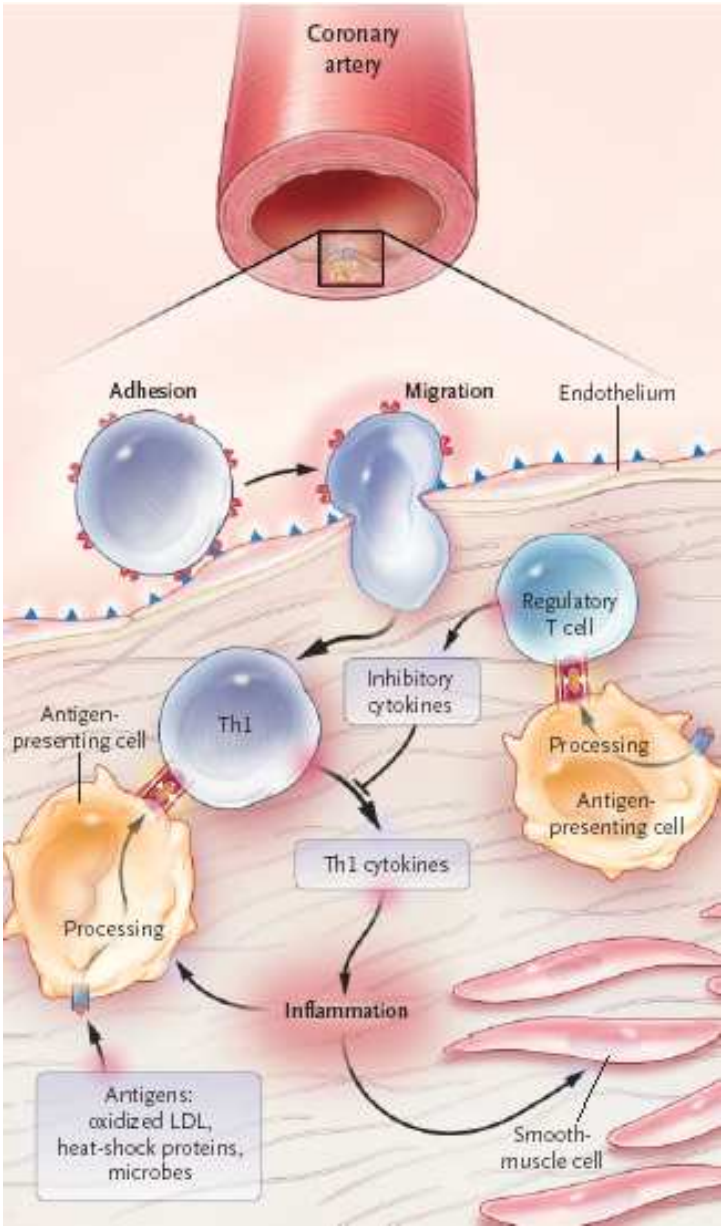


Figure 4: Effects of T-Cell activation on plaque inflammation

Mechanisms of plaque rupture

What causes a silent atherosclerotic lesion to rupture?

Activated macrophages, T cells, and mast cells at sites of plaque rupture (Kovanen et al., 1995; van der Wal et al., 1994; Moreno et al., 1994) produce several types of molecules — inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules — that can destabilize lesions. They inhibit the formation of stable fibrous caps, attack collagen in the cap, and initiate thrombus formation (Hansson et al., 1989; Saren et al., 1996; Mach et al., 1997). All these reactions can conceivably induce the activation and rupture of plaque, thrombosis, and ischemia. Two types of proteases have been implicated as key players in plaque activation: matrix metalloproteinases (MMPs) and cysteine proteases (Jons et al., 2003; Liu et al., 2004). Several members of these families of enzymes occur in the plaque and may degrade its matrix. MMP activity is controlled at several levels: inflammatory cytokines induce the expression of MMP genes, plasmin activates proforms of these enzymes, and inhibitor proteins (tissue inhibitor of metalloproteinase) suppress their action. Similarly, cysteine proteases are induced by certain cytokines and checked by inhibitors termed “cystatins” (Liu et al., 2004). Several of these molecules play decisive roles in the formation of aneurysms, as shown by experiments in gene-targeted mice. However, mechanistic studies in models of atherosclerosis have yielded complex results, with certain MMPs reducing rather than increasing the size of the lesions. At the same time, these enzymes clearly affect the composition of plaque. Therefore, they may represent future therapeutic targets. Study of plaque rupture in animal models should be helpful in determining the role of these proteases in the activation of plaque and myocardial infarction.

Risk Factors

Reducing risk factors is the primary clinical approach for the prevention of cardiovascular disease (Kavey et al., 2006). Risk factors of cardiovascular disease are divided in modifiable and non modifiable. Modifiable risk factors for atherosclerosis include tobacco use, high cholesterol levels, high blood pressure, diabetes, obesity, physical inactivity, and diet. One of the most important modifiable risk factors is smoking. A smoker‘s risk of developing coronary artery disease is directly related to the amount of tobacco smoked daily. The risk of a heart attack is increased threefold in men and sixfold in women who smoked (Stemme et al., 1995) or more cigarettes per day compared with nonsmokers. Tobacco use decreases the level of high-density lipoprotein (HDL) cholesterol and increases the level of low-density lipoprotein (LDL) cholesterol. An high level of LDL cholesterol is another important modifiable risk factor, so diet become very important. Cholesterol levels also increase with age and are normally higher in men than in women, although levels increase in women after menopause. Diabetes is yet another risk factor for atherosclerosis of growing importance. The hyperglycemia associated with diabetes can lead to modification of macromolecules, for example, by forming advance glycation end products (AGE)(Schmidt et al., 1999) that can augment the production of proinflammatory cytokines and other inflammatory pathways in vascular endothelial cells. Beyond the hyperglycemia, the diabetic state promotes oxidative stress mediated by reactive oxygen species and carbonyl groups (Baynes et al., 1999). Obesity not only predisposes to insulin resistance and diabetes, but also contributes to atherogenic dyslipidemia. High levels of free fatty acids originating from visceral fat reach the liver through the portal circulation and stimulate synthesis of the triglyceride-rich lipoprotein VLDL by hepatocytes. Adipose tissue can also synthesize cytokines such as TNF-α and IL-6 (Yudkin et al., 1999). In this way obesity itself promotes inflammation and potentiates atherogenesis independently of effects on insulin resistance or lipoproteins. Risk factors that cannot be modified include having a family history of early atherosclerosis, advancing age, and male sex. Men have a higher risk than women,

although women who have coronary artery disease are more likely to die than men who have the disease (Rosamond et al., 2007).

INTRODUCTION

Macrophage foam cell formation is an important process in atherosclerotic plaque development (Pennings et al., 2006). Atherosclerosis is initiated by dysfunction of endothelial cells at lesion-prone sites in the walls of arteries, which results in monocyte infiltration into the arterial intima. These cells differentiated into macrophages, which then internalize large amounts of oxidized low-density lipoprotein forming cholesterol-laden macrophages called “foam cells” (FC), which in turn give rise to fatty streaks in the arterial wall (Lusis, 2000). As the atherosclerotic lesion develops, the arterial wall tickness increases and oxygen diffusion into the intima is markedly reduced. These hypoxic regions contain large number of FC revealing that these cells experience hypoxia during the development of atherosclerotic lesions (Bjornheden et al., 1999; Murdoch et al., 2005). Hypoxia-inducible factor-1 (HIF-1), the most important factor involved in the cellular response to hypoxia, is an heterodimeric transcription factor composed of an inducibly-expressed HIF-1α subunit and a constitutively-expressed HIF-1β subunit (Semenza, 2001). It is well established that HIF plays a major role in vascular endothelial growth factor (VEGF) expression and angiogenesis, mediating important alterations associated with atherogenesis and angiogenic activity of macrophages (Paul et al., 2004; Sluimer et al., 2008). Moreover, under atherogenic conditions, the high expression of HIF-1 in macrophages promotes FC formation and atherosclerosis (Jiang et al., 2007).

Recently, it has been shown that another angiogenic chemokine, Interleukin-8 (IL-8) is up-regulated by FC located in hypoxic areas in rabbit and human atherosclerotic plaques (Murdoch et al., 2005). Hypoxia-induced secretion of IL-8 from FC may lead to the recruitment of smooth muscle, vascular endothelial and T cells into the atherosclerotic plaques and thus to plaque progression (Wang et al., 1996). It has been also demonstrated that in vascular endothelium, under hypoxia, IL-8 expression is increased by HIF (Conway EM., 2003/2004; Kim et al., 2006). However, the relationships between HIF and IL-8 has been questioned by other authors (Loboda et al., 2006).

Adenosine (Ado) is a proangiogenic purine nucleoside released from ischemic and hypoxic tissues. Under these conditions, it is released into the extracellular space and signals through the stimulation of four extracellular G protein-coupled receptors named A_1, A_{2A}, A_{2B}, and A_3 (ARs) (Jacobson et al., 2006). All the four adenosine subtypes have been recently associated to the modulation of angiogenesis. Therefore due to the link between ado, inflammation and angiogenesis and the increasing evidence that these factors play a role in atherogenesis we thought to investigate HIF-1α, VEGF, IL-8 and FC formation by ado receptors in human macrophages and in an *"in vitro"* model of human FC (Yu et al., 2003).

EXPERIMENTAL PROCEDURES

Materials

5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2(2furyl)-pyrazolo-[4,3e]-1,2,4-triazolo [1,5-c] pyrimidine ([3H]MRE 3008F20, specific activity 67 Ci mmol-1), N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy] acetamide] ([3H]MRE 2029F20, specific activity 123 Ci mmol-1) were obtained from Amersham International (Buckinghamshire, UK), (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,32][1,3,6]triazinyl-amino] ethyl)-phenol) ([3H]ZM 241385, specific activity 20 Ci mmol-1) was purchased from Tocris (Boston, Mass; USA) and 1,3-dipropyl-8-cyclopentyl-xanthine ([3H]DPCPX, specific activity 120 Ci mmol-1) was derived from NEN Research Products (Boston, Mass; USA). 7-(2-phenylethyl)-2- (2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine (SCH 58261), 1-Deoxy-1-[6-{4- [(phenylcarbamoyl)-methoxy]phenylamino}-9H-purin-9-yl]-N-ethyl-b-D-ribofuranuronamide (Compound 24) were synthesized by Prof. P.G. Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Italy). Phorbol myristate acetate (PMA), erythro-9-(2-hydroxy-3- nonyl)adenine (EHNA), cyclohexyl-adenosine (CHA), 2-[p-(carboxyethyl)-phenethylamino]- NECA (CGS 21680), *N6* -(3iodobenzyl)-2-chloroadenosine-5'N-methyluronamide (Cl-IB-MECA), rapamycin were purchased by Sigma Aldrich (Milano, Italy). The antibodies for A1, A2A, A2B adenosine receptor subtypes were purchased from Alpha Diagnostic (S. Antonio, Texas, USA) and the antibody for A3 was from AVIVA System Biology (Milano, Italy). Human anti-HIF-1a and HIF-1b antibodies were obtained from BD Transduction Laboratories (Milano, Italy). The Anti- ACTIVE_ mitogen-activated protein kinase anti-ERK1/2 (pAb) was from Promega (Milano, Italy). Phospho-Akt (Ser473) and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Milano, Italy). Ficoll-Hypaque was obtained by Amersham Pharmacia Biotech AB. The assays-on-demandTM. Gene expression Products NM 000674, NM 000675, NM 000676 and NM 000677 for A1, A2A, A2B and A3 adenosine subtypes

and the assays-on-demandTM Gene expression Products Hs00936368_m1, Hs00173626_m1, Hs00174103_m1, Hs00765620_m1, Hs99999906_m1, for HIF-1_, VEGF, IL-8, Aldolase A and Phosphoglycerate kinase (PGK) were purchased from Applera (Milano, Italy). A1, A2A, A2B, A3AR, HIF-1_ small interfering RNAs **(**siRNAs) and HIF-2_ antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear Extract Kit and HIF-1_ binding activities were from Active Motif (Belgium). All other reagents were of analytical grade and obtained from commercial sources.

Methods

Cell culture

The human myelomonocytic cell line U937 was obtained from ATCC and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), 100 U/ml penicillin, 100 µg/ml streptomycin, at 37C° in 5% CO_2/95% air.

Preparation of human macrophages (HM) from peripheral blood

Peripheral blood mononuclear cells were isolated from buffy coats by the Ficoll-Hypaque gradient (Amersham Pharmacia Biotech AB) and centrifuged at 2200 rpm for 15 min. (Gessi et al., 2004). The human peripheral blood mononuclear cells were isolated and removed from the Ficoll-Hypaque gradients. Subsequently, they were washed in 0.02 M phosphate-buffered saline at pH 7.2 containing 5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. Finally, they were decanted into a culture flask in RPMI 1640 medium containing 2 mM glutamine, 5% human AB serum (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin and placed in a humidified incubator (5% CO_2). Monocytes were selected by adhesion and differentiated into HM over 5-7 days.

Hypoxic treatment

Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2 (MiniGalaxy, RSBiotech, Irvine, Scotland).

FC formation

U937 cells were induced to differentiate into HM by treatment with 40 nM phorbol myristate acetate (PMA) for 72 hours. Before use oxLDL (Intracel, Frederick, MD) were dialyzed against 1 liter of 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 12 h at 4°C, then against RPMI 1640 medium (two changes, 1 liter/each change) for 24 h at 4°C. All dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular wheight cut-off). After dialysis, lipoproteins were sterilized by passing them through a 0.45 µm (pore-size) filter and added (50 or 100 µg/ml) to PMA-treated U937 cells for 48 h in serum-free RPMI (Kruth et al., 2002). Then all treatments to the cells with ado were carried out in the presence of the adenosine deaminase (ADA) inhibitor, erythro-9-(2- hydroxy-3-nonyl)adenine (EHNA) 5 µM and those with ado agonists were performed in the presence of ADA.

Real-time RT-PCR

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assay was performed as previously described (Higuchi et al., 1993). The assays-on-demandTM Gene expression Products NM 000674, NM 000675, NM 000676 and NM 000677 for A_1, A_{2A}, A_{2B} and A_3 ARs were used, respectively. Quantification of ARs messages was made by interpolation from standard curve of Ct values generated from the plasmid dilution series (Gessi et al., 2007). Analogue results were obtained when the expression level of ARs was normalized to that of β-actin. For the real-time RT-PCR of HIF-1α, VEGF, IL-8, Aldolase A and PGK the assays-on-demand^{TM} Gene expression Products Hs00936368_m1, Hs00173626_m1, Hs00174103_m1, Hs00765620_m1, Hs99999906_m1 were used, respectively. For the real-time

RT-PCR of the reference gene the endogenous control human β-actin kit was used, and the probe was fluorescent labeled with VIC^{TM} (Applera).

Binding experiments

Binding assays were carried out as reported previously (Gessi et al., 2007). In saturation experiments, membranes (70 µg of protein/assay) were incubated with 50 mM Tris HCl buffer (10 mM $MgCl_2$ for A_{2A}; 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine for A_{2B} and 10 mM $MgCl_2$, 1 mM EDTA for A_3) pH 7.4 and increasing concentrations of 1,3-dipropyl-8-cyclopentylxanthine ([3H]DPCPX) (0.4-40 nM); (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,32]-[1,3,6]- triazinyl-amino] ethyl)-phenol) ([3H]ZM 241385) (0.3-30 nM); N-benzo[1,3]dioxol-5-yl-2-[5-(1,3- dipropyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide] ([3H]MRE 2029F20) (0.4-40 nM); 5-*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2furyl)- pyrazolo-[4,3e]-1,2,4-triazolo [1,5-c] pyrimidine ([3H]MRE 3008F20) (0.4-40 nM) to label A_1, A_{2A}, A_{2B} and A_3 ARs, respectively. The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

Western Blot Analysis

Cells were harvested and washed with ice-cold PBS containing 1 mM sodium orthovanadate. Cells were then lyzed in Triton lysis buffer. Equivalent amounts of protein (40 µg) were subjected to electrophoresis on 10% sodium dodecyl sulfate–acrylamide gel. The gel was then electroblotted on to a nitrocellulose membrane. Then the membranes were probed with specific antibodies (Merighi et al., 2005). ARs were evaluated by using specific antibodies towards human A_1, A_{2A}, A_{2B} (Alpha Diagnostic) and A_3 ARs (Aviva) (1:1000 dilution). In experiments aimed to detect HIF, western blot analyses were performed using antibody against HIF-1α (1:250 dilution) (BD Biosciences), HIF-2α (1:1000) (Novus Biologicals) and HIF-1β (1:1000 dilution) (BD Biosciences) in 5% non-fat dry milk in PBS/0.1% Tween-20 overnight at 4°C. For detection of phosphorylated proteins

antibodies specific for phosphorylated (Thr183/Tyr185) p44/p42 MAPK (1:5000 dilution), phosphorylated (Thr180/Tyr182) p38 MAPK (1:1000 dilution) and phosphorylated Akt (Ser473) (1:1000 dilution) were used. The protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Tubulin (1:250) was used to ensure equal protein loading. Immunoreactivity was assessed and quantified by using a VersaDoc Imaging System (Bio-Rad).

HIF-1 DNA binding activity

Nuclear extracts from U937, FC and HM were prepared by using the Nuclear Extract Kit (Active Motif) and HIF-1α binding activities in the nuclear extracts were detected by using an ELISA-based HIF binding kit (TransAM™ HIF-1, Active Motif) according to the manufacturer's recommendations.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of VEGF and IL-8 protein secreted by the cells in the medium were determined by VEGF and IL-8 ELISA kits (R&D Systems) according to the manufacturer's instructions. The data were presented as mean ± SD from four independent experiments.

Knockdown of ARs and HIF-1α by small interfering RNA (siRNA)

FC were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of siRNA was performed at a concentration of 100 nM using Lipofection 2000 in Opti-MEM (Invitrogen). A non-specific random control ribonucleotide sense strand (5'-ACU CUA UCU GCA CGC UGA CdTdT-3') and antisense strand (5'-dTdT UGA GAU AGA CGU GCG ACU G-3') were used under identical conditions as already reported (Merighi et al., 2005). A_1, A_{2A}, A_{2B}, A_3AR and HIF-1α siRNAs were from Santa Cruz Biotechnology (Santa Cruz, CA).

Oil red O-stain analysis

Treatment of PMA-differentiated U937 cells with ado and ado ligands was performed before addition of oxLDL. After exposition to oxLDL under hypoxia for 24 h cells were fixed in saline-buffered 4% paraformaldehyde solution for 15 min and then air dried. Oil red O (in 60% isopropanol) staining was done for 15 min essentially as described before (Kalayoglu et al., 1998). Cells were viewed under a bright-field microscope in 100 X fields using a Nikon's Eclipse E800 microscope. FC were defined as macrophages in which cytoplasm was filled with Oil Red O-stainable lipid droplets.

MTS Assay

The MTS assay was performed to determine foam cells viability according to the manufacturer's protocol from the CellTiter 96 AQueous One Solution (Promega) cell proliferation assay. Cells (10^5) were plated in 24-multiwell plates; 500 µl of complete medium was added to each with ado. The cells were then incubated for 24 h. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 570 nm. Experiment was repeated three times.

Statistical analysis

All values in the figures and text are expressed as mean ± standard error (S.E.) of N observation (with N ³ 3). Data sets were examined by analysis of variance (ANOVA) and Dunnett's test (when required). A P-value less than 0.05 was considered statistically significant.

RESULTS

Expression of ARs mRNA in U937, HM and FC

mRNA expression of ARs was evaluated in U937, HM and FC in normoxia and hypoxia. Hypoxia induced a significant increase of A_{2B}ARs in all the three cellular models investigated, whilst did not change the level of the other ARs **(Figure 5 A-D)**.

Expression of ARs protein in U937 cells, HM and FC

The protein evaluation of all ARs was examined, through immunoblots, in U937, HM and FC in normoxia and hypoxia. We observed the presence of all ARs in the cells investigated according to mRNA data, as reported in **Figure 5 E-H**. These results were also confirmed by [^{3}H]DPCPX, [^{3}H]ZM 241385, [^{3}H]MRE 2029F20 and [^{3}H]MRE 3008F20 radioligands, used in receptor binding studies to evaluate affinity and density values of $A_{1,}$ $A_{2A,}$ A_{2B} and A_3 ARs, respectively **(Table 1)**.

Ado induces HIF-1α protein accumulation

To evaluate the effect of ado on HIF-1α FC, HM and U937 cells **(Figure 6)** were incubated with ado 100 μmol/L in normoxia and hypoxia. In hypoxia ado stimulated HIF-1α accumulation, time-dependently, in all cells investigated. As for normoxia, ado effect slightly appears after 24 hours in FC, whilst HIF-1α protein was undetectable in HM and in U937 cells. In FC the effect was similar with 50 or 100 μg/mL of oxLDL (data not shown), therefore the concentration of 50 μg/mL was used in all experiments. No changes in cell viability were observed after treatment of cells with ado 100 μmol/L for 24 h of hypoxia (data not shown). Furthermore, treatment of ado stimulated, in a time-dependent way, HIF-1α DNA binding activity in hypoxia and also induced a minor but statistically significant effect in normoxia in FC, HM and U937 cells **(Figure 7)**. Ado did not affected HIF-1α mRNA levels in normoxia and after 2 hours hypoxia whilst it induced a slight

increase of 1.6±0.1, 1.9±0.1 and 1.5±0.1 fold after 4, 8, 24 hours of hypoxia, respectively ($P<0.05$ *vs* control); following addition of actinomycin D (actD), ado did not increase HIF-1α mRNA excluding a role in mRNA stability **(Figure 8A)**. The lack of mRNA modulation after 2 h, time at which ado start to affect protein increase, suggests that ado does not affect transcription. Furthermore, we evaluated the ado-induced regulation of HIF-2α in hypoxia. **Figure 8B** shows that ado slightly increased HIF-2α and this effect was blocked by actD suggesting that at variance with HIF-1α, HIF-2α was transcriptionally regulated by ado.

Then we investigated the ado modulation of HIF-1α protein stability in hypoxia and normoxia. Ado in normoxia, at variance with hypoxia, increases HIF-1α stability; furthermore rapamycin, inhibitor of mTOR pathway, reduced ado effect suggesting also an increase in translation **(Figure 9A-C)**. However as the ado effect on HIF-1 was most evident in hypoxia all the other experiments were carried out in this condition for 4 hours.

Involvement of ARs in ado-induced HIF-1α expression

To evaluate which AR was involved in the ado-induced HIF-1α expression we treated FC with antagonists of ARs before addition of ado in hypoxia **(Figure 10A)**. Ado effect was partially antagonized by 100 nmol/L DPCPX, SCH 58261, MRE 2029F20 and MRE 3008F20 suggesting the involvement of A_1, A_{2A}, A_{2B} and A_3 ARs, respectively. Therefore we evaluated the effect of high affinity agonists, CHA, CGS 21680, Compound 24 (Baraldi et al., 2007) and Cl-IB-MECA on HIF-1α accumulation. Probes selectivity is provided in **table 2**. All the agonists were able to induce HIF-1α in FC **(Figure 10B)**. Analogous results were obtained in U937 cells and in HM (data not shown). Therefore in the second part of the work we focused our attention on FC.

To further ascertain the involvement of the different ARs in the ado-induced HIF-1α accumulation we knocked-down ARs. After 48 and 72 hours posttransfection with siRNA targeting each AR,

mRNA and protein levels were significantly reduced; the specificity of a given siRNA to the other AR subtypes is also shown in **figure 11**. Treatment of cells with siRNAs for A_1, A_{2A}, A_{2B} and A_3 subtypes reduced the effect of ado on HIF-1α modulation supporting again a role for all ado subtypes in this effect; silencing of all ARs together abrogated the ado-mediated increase of HIF-1α protein **(Figure 10C)**.

Involvement of MAPK and Akt pathways in ARs-induced modulation of HIF-1α

To investigate the role of MAPK and Akt kinases in ARs-induced HIF-1α accumulation, we performed experiments with U0126, SB202190 and SH-5, inhibitors of MEK1/2, p38 MAPK, and Akt respectively, in FC. All the blockers were able to abrogate the effect induced by A_1, A_{2A} and A_{2B} agonists, whilst the A_3-mediated HIF-1α accumulation was antagonized only by U0126 **(Figure 12A)**. Addition of CHA, CGS 21680 and Compound 24 induced a concentration-dependent increase of pERK1/2, pp38 and pAkt, whilst Cl-IB-MECA was involved only in ERK1/2 phosphorylation **(Figure 12B)**.

ARs induce VEGF increase in Hypoxia

We tested VEGF production by FC after ado treatment for 24 h of hypoxia. Ado 100 μmol/L increased VEGF levels of 165±10% and the effect was strongly reduced by MRE 2029F20 and MRE 3008F20 100 nmol/L suggesting the involvement of A_{2B} and A_3ARs and inhibited to a lesser extent by the A_{2A} antagonist **(Figure 13A)**. DPCPX 100 nmol/L produces a moderate blunting of ado-induced VEGF release, but at this concentration it can have antagonistic actions against A_{2B} receptors see supplemental Table 2. Indeed a lower dose of DPCPX 10 nmol/L did not reduce ado effect (161±10%). U0126 and SB202190 followed by SH-5 were able to block the ado increase on VEGF levels. Treatment of the cells with siRNA of HIF-1α abrogated the VEGF increase induced

by ado suggesting that the nucleoside was acting through HIF-1α modulation **(Figure 13B)**. The increase induced by ado 100 µmol/L on VEGF was also observed at mRNA level (2.4±0.2 fold of increase, P<0.05 *vs* control). Other HIF-1α-responsive genes, aldolase and PGK were increased at mRNA level after ado treatment for 24 hours of 4.5±0.2 and 1.8±0.2 fold, respectively and the effect was abrogated in the presence of HIF-1α siRNA, 1.1±0.1 and 1.0±0.1 fold, respectively (P<0.05 *vs* control).

A_{2B}AR induces IL-8 increase in Hypoxia

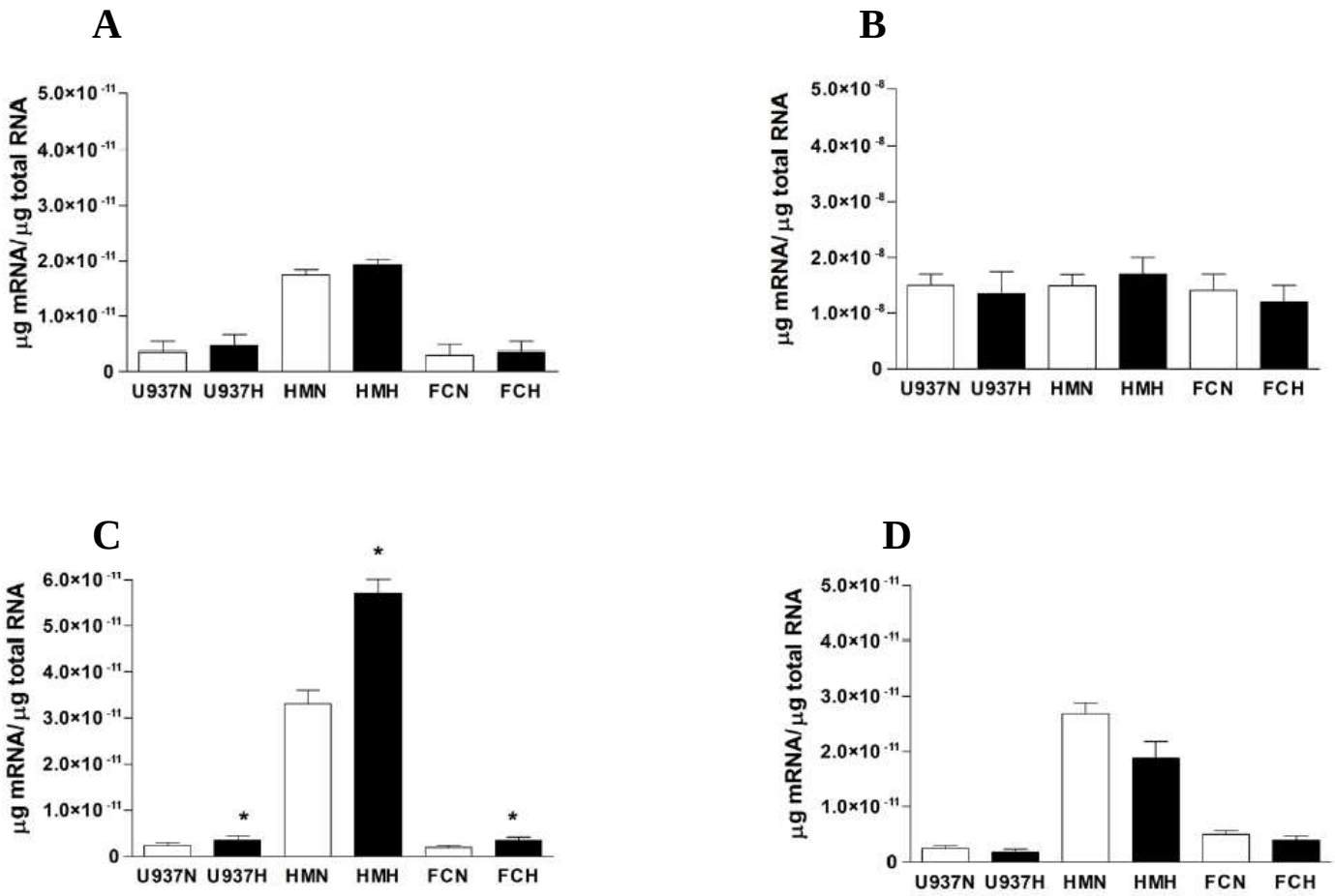
We tested IL-8 production by FC after ado treatment for 24 h in hypoxia. Ado 100 µmol/L increased IL-8 levels of 158±10 % and the effect was blocked by MRE 2029F20 or A_{2B} silencing, but not by DPCPX, SCH 58261 and MRE 3008F20 **(Figure 13C-D)**. A dose-response curve of Compound 24 revealed an EC_{50} value of 58±6 nmol/L for stimulation of IL-8 secretion. The effect of Compound 24 1 µmol/L (142±8% of IL-8 secretion) was completely blocked by MRE 2029F20 (102±6% of IL-8 secretion). All these data suggest the involvement of A_{2B} subtype in this response. U0126, SB202190 and SH5 were able to revert the ado increase on IL-8 levels suggesting a role for ERK 1/2, p38 and Akt pathways **(Figure 13D)**. Finally, treatment of cells with siRNA of HIF-1α for 72 hours before stimulation with ado shows that IL-8 modulation was not affected by HIF-1α silencing **(Figure 13D)**. IL-8 was not altered by ado at mRNA level (1.14±0.1 fold of increase versus control).

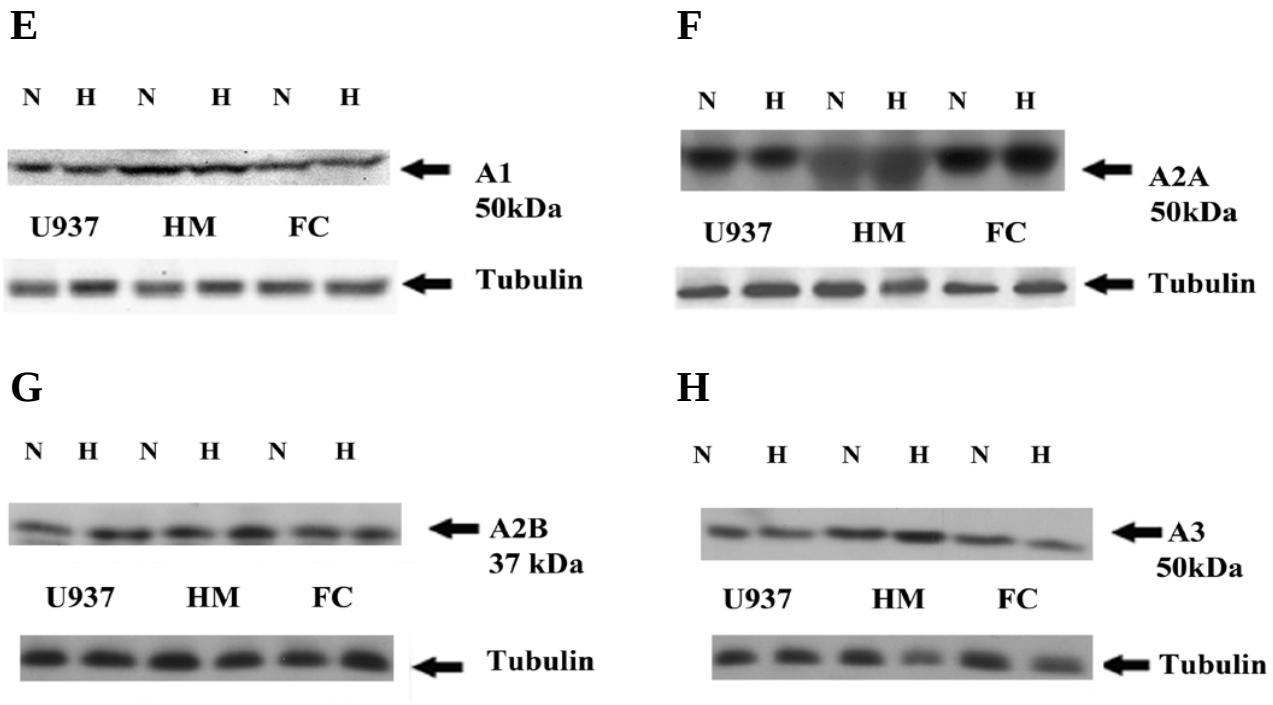
Oil red O staining in FC

U937 cells without oxLDL did not contain high levels of neutral lipids and were not stained with Oil red O, a dye specific for neutral lipids **(Figure 14)**. After treatment of U937 cells with 50 µg/mL ox-LDL for 24 h, we observed FC formation characterized by large cytoplasmic lipid droplets. This effect was increased after incubation with ado 100 µmol/L, not significantly affected by DPCPX and

SCH 58261 and strongly blocked by MRE 2029F20 and MRE 3008F20 antagonists and HIF-1α silencing, suggesting the involvement of HIF-1α and A_{2B} and A_3 ARs in the ado-induced FC formation. Also the high affinity A_{2B} and A_3 agonists were able to increase FC formation **(Figure 14)**.

Figure 5 - mRNA and protein expression of ARs in U937 cells, HM and FC in normoxia (N) and hypoxia (H): Bargraph showing µg mRNA/µg total RNA of human A_1, A_{2A}, A_{2B} and A_3 ARs (A-D, respectively). ARs detection by western blot analysis (E-H). Cellular extracts were prepared and subjected to immunoblot assay using anti-A_1, A_{2A}, A_{2B} and A_3 antibodies. Tubulin shows equal loading protein. Values are the means and vertical lines S.E. of the mean of four separate experiments performed in triplicate. *P<0.05 compared with normoxia.




E
N H N H N H
A1
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Tubulin
F
N H N H N H
A2A
50kDa
U937 HM FC
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G
N H N H N H
A2B
37 kDa
U937 HM FC
Tubulin
H
N H N H N H
A3
50kDa
U937 HM FC
Tubulin

Table 1- Affinity (KD, nM) and density (Bmax, fmol/mg of protein) values of A_1, A_{2A}, A_{2B} and A_3. ARs evaluated through [3H]DPCPX, [3H]ZM 241385, [3H]MRE 2029F20 and [3H]MRE 3008F20 radioligands, respectively, in PMA-treated U937, HM and FC cells in normoxia (N) and hypoxia (H).

	A_1		A_{2A}		A_{2B}		A_3	
	K_D (nM)	Bmax (fmol/mg prot)	K_D (nM)	Bmax (fmol/mg prot)	K_D (nM)	Bmax (fmol/mg prot)	K_D (nM)	Bmax (fmol/mg prot)
U937 N	4.0±0.3	52±6	2.8±0.3	62±9	4.3±0.4	33±3	1.5±0.1	235±26
U937 H	4.4±0.4	80±10	2.5±0.2	57±8	4.1±0.5	73±6	2.0±0.1	267±28
HM N	2.8±0.3	85±9	2.2±0.3	109±12	4.9±0.3	173±15	4.5±0.5	254±24
HM H	2.8±0.4	83±10	2.3±0.3	90±10	4.8±0.6	240±18	4.8±0.6	360±33
FC N	3.3±0.5	78±10	2.1±0.1	84±9	2.0±0.2	90±8	1.7±0.1	250±30
FC H	3.7±0.6	102±12	2.2±0.1	75±7	1.98±0.2	140±12	2.3±0.1	275±32

Figure 6 - Time course of HIF-1α modulation induced by ado. Effect of 100 µmol/L Ado on HIF-1α protein expression (panel A-C) in FC, HM and U937 cells, respectively, in normoxia (N) and hypoxia (H). HIF-1β shows equal loading protein. Densitometric quantification of HIF-1α western blots is the mean ± S.E. values (N=3); *P<0.05 compared with 24 h normoxia in FC or with 2 h hypoxia in HM and U937 cells in the absence of Ado; #P<0.05 compared with cells in the absence of Ado at each time.

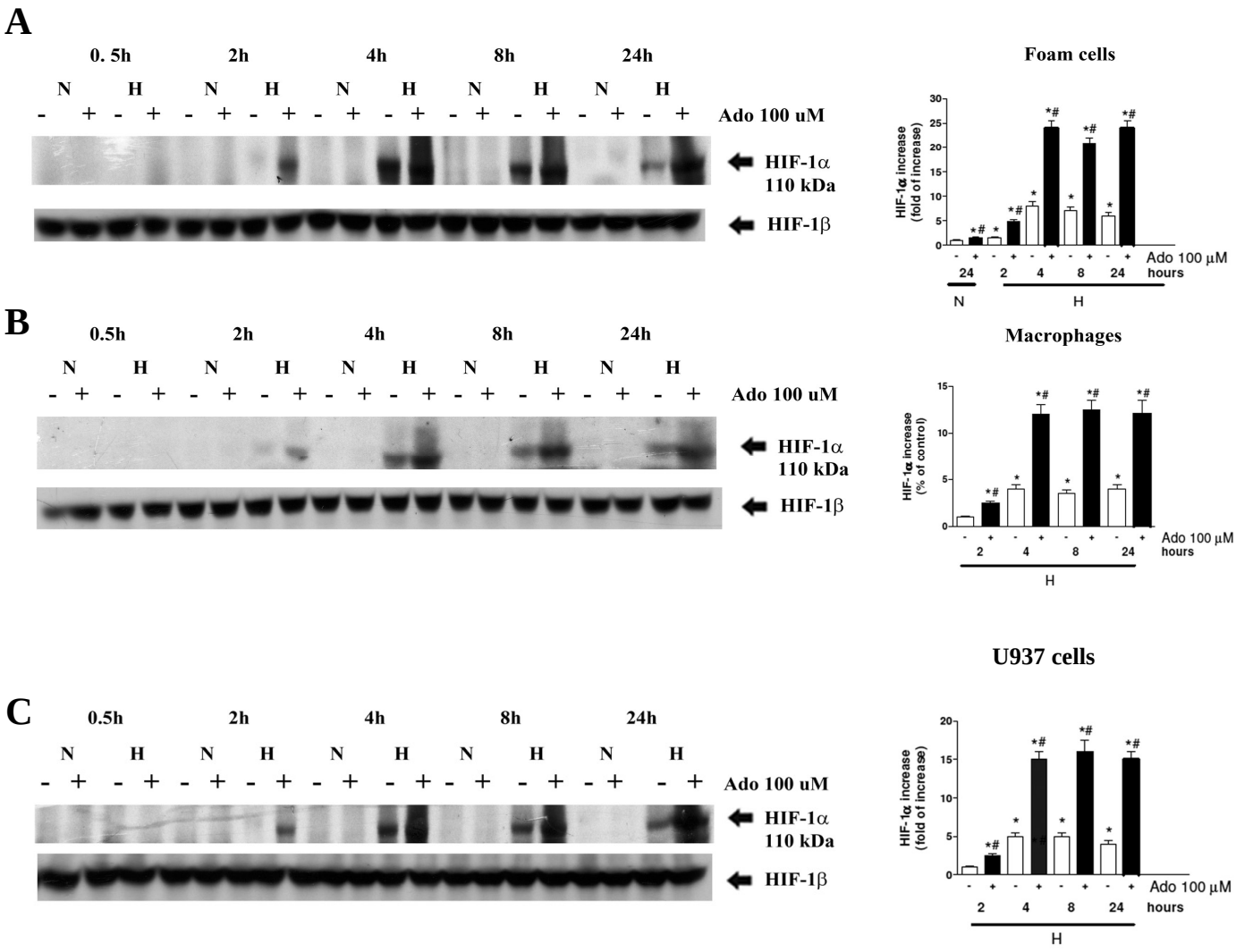


Figure 7 - HIF-1α DNA binding activity (panel A-C) in FC, HM and U937 cells, respectively, in normoxia (N) and hypoxia (H). DNA binding activity data are means ± S.E (N=3); *P<0.05 compared with 24 h normoxia in FC and U937 cells or with 4 h normoxia in HM in the absence of Ado; #P<0.05 compared with cells in the absence of Ado at each time.

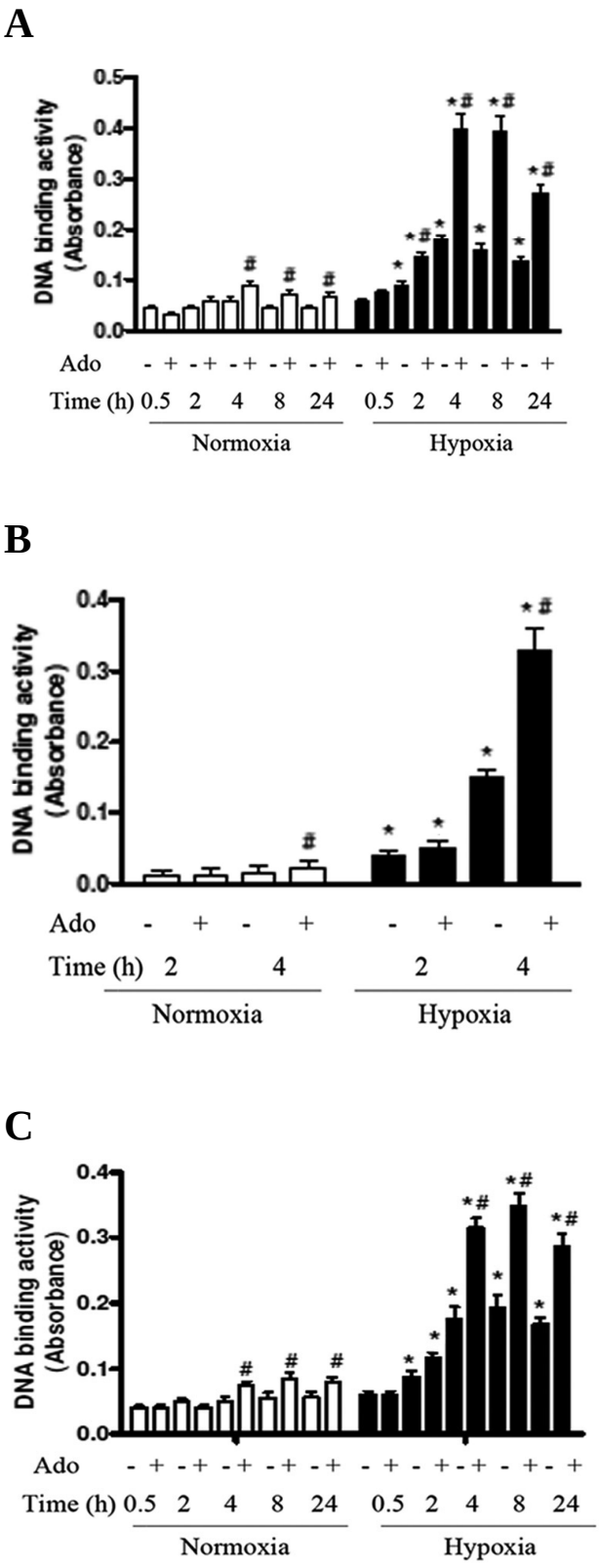


Figure 8 - Effect of actinomycin D (ActD) on HIF-1α and HIF-2α in foam cells. Real-time RTPCR analysis of HIF-1α mRNA level. Total RNA was extracted from cells treated in the absence or in the presence of Ado for 2, 4, 8, 24 h hypoxia before and after addition of ActD (10 µg/ml). Data were expressed as fold of increase vs control arbitrarily fixed as 1 (cells in the absence of Ado). *P<0.05 compared with control (A). Western blot analysis of HIF-1α and HIF-2α protein level after treatment with 100 µmol/L Ado in the absence and in the presence of ActD. Densitometric quantification of western blots is the mean ± S.E. values (N=3); *P<0.05 compared with control (cells in the absence of Ado) (B).

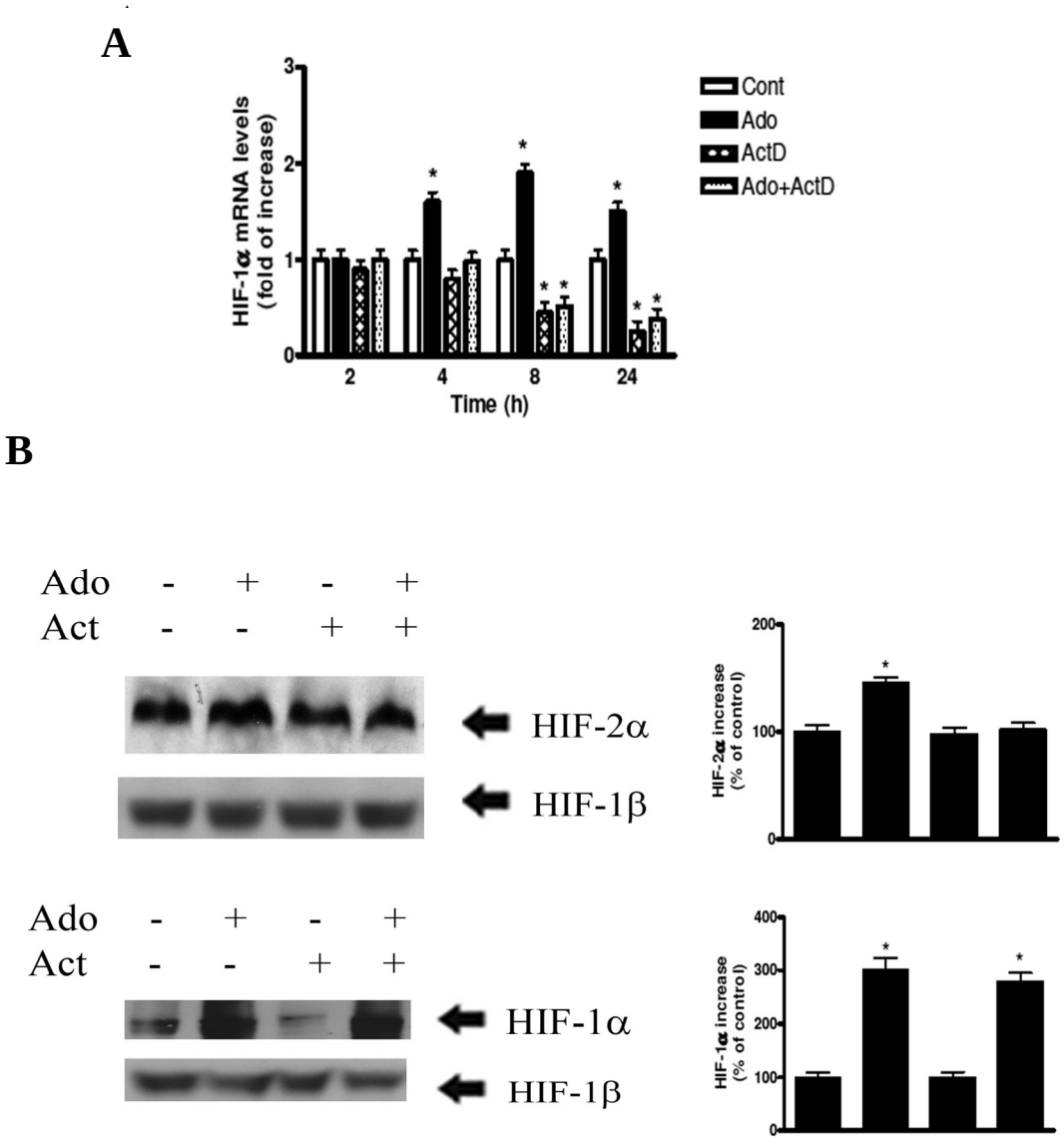
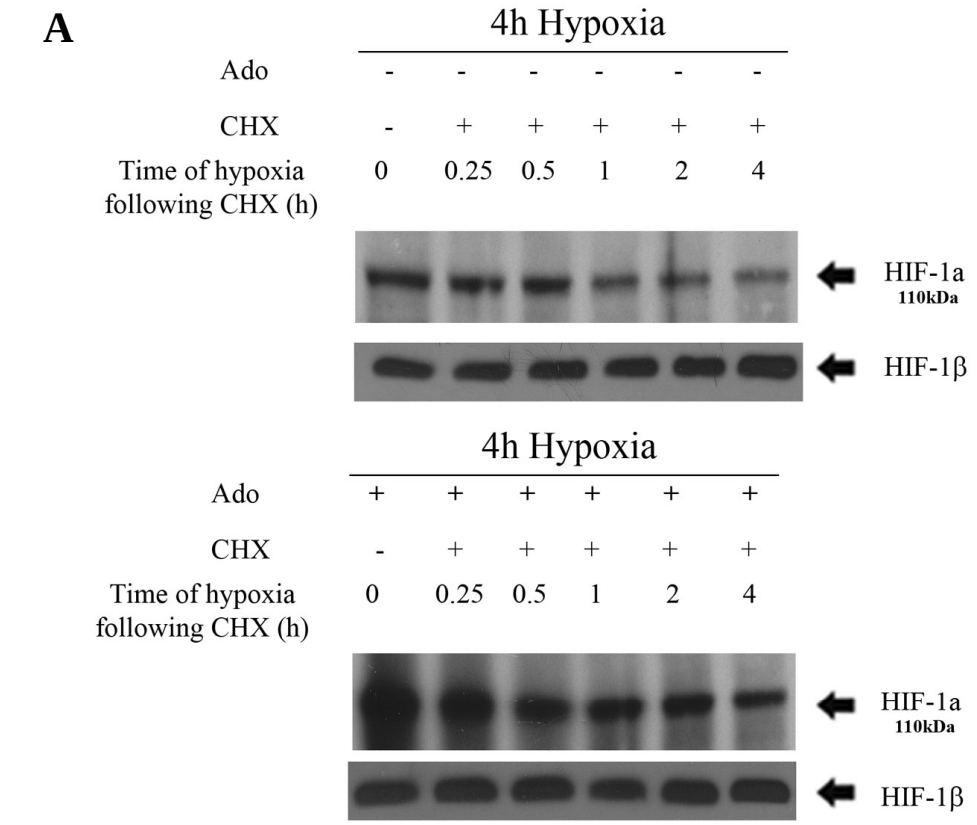
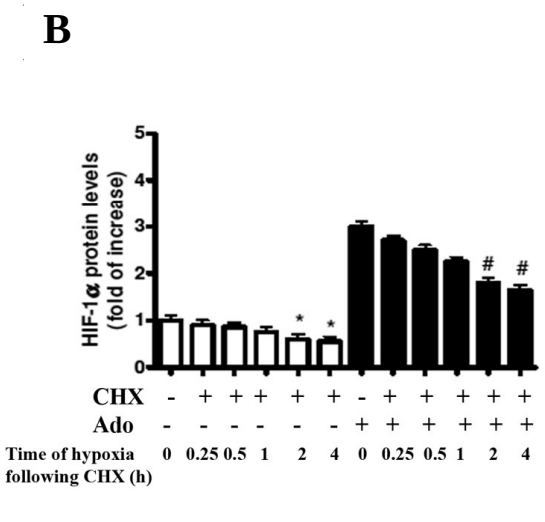


Figure 9 - Effect of cycloheximide (CHX) on induction of HIF-1α in foam cells. Western blot analysis of HIF-1α protein level. Cells were treated in hypoxia without or with 100 µmol/L ado for 4 hours, 1 µmol/L CHX was added to inhibit new HIF-1α protein synthesis, and incubation was continued for 0.25, 0.5, 1, 2 and 4 h (A). Densitometric quantification of HIF-1α western blots is the mean ± S.E. values (N=3); *P<0.05 compared with control (cells in the absence of Ado and CHX treated in hypoxia for 4 h), # P<0.05 compared with control (cells in the absence of CHX treated in hypoxia plus Ado for 4 h) (B). Effect of CHX and 100 nmol/L rapamycin on HIF-1α DNA binding activity induced by ado in normoxia (C). Cells were treated in normoxia without or with 100 µmol/L ado for 8 hours, 1 µmol/L CHX was added to inhibit new HIF-1α protein synthesis, and incubation was continued for 0.25, 0.5 and 1 h. Cells were treated in normoxia without or with 100 µmol/L ado for 8 hours in the presence of rapamycin. Nuclear extracts were isolated and subjected to a colorimetric assay. Means ± S.E (N=3); *P<0.05 versus control (cells in the absence of CHX treated in normoxia plus Ado for 8 h).





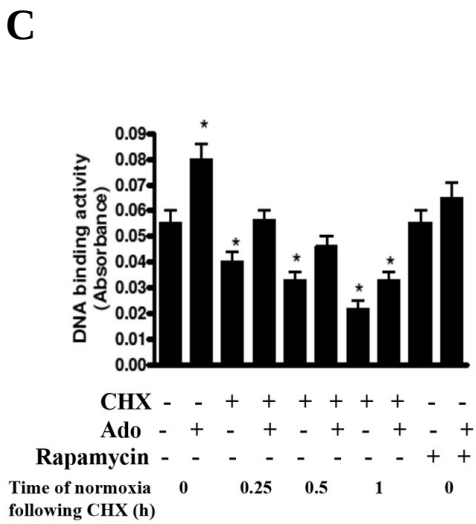


Figure 10 – Effect of AR ligands and ARs silencing on HIF-1α protein increase. Effect of Ado on HIF-1α protein accumulation and antagonism by 100 nmol/L MRE 3008F20 (selective A_3 antagonist), SCH 58261 (selective A_{2A} antagonist), DPCPX (A_1 antagonist) and MRE 2029F20 (selective A_{2B} antagonist) (A). HIF-1α accumulation in the absence (line 1, control) and in the presence of AR agonists (nmol/L) 10, 100 CHA (A_1 agonist, lines 2, 3); 500, 1000 CGS 21680 (A_{2A} agonist, lines 4, 5); 10, 100 Compound 24 (A_{2B} agonist, lines 6,7); 10, 100 Cl-IB-MECA (A_3 agonist, lines 8, 9) (B). Ado effect on HIF-1α in the absence (line 2) and in the presence of siRNA of A_1, A_{2A}, A_{2B}, A_3 ARs (lines 3, 4, 5, 6, respectively). Ado effect in the presence of siRNA of A_1, A_{2A}, A_{2B}, A_3 ARs together (siAdoRs) (line 7); cells transfected with control (C) ribonucleotides for 72 h (line 1) (C). Densitometric quantification of western blots is the mean ± S.E. values (N=3); *P<0.05 compared with the control; **P<0.05 compared with Ado.

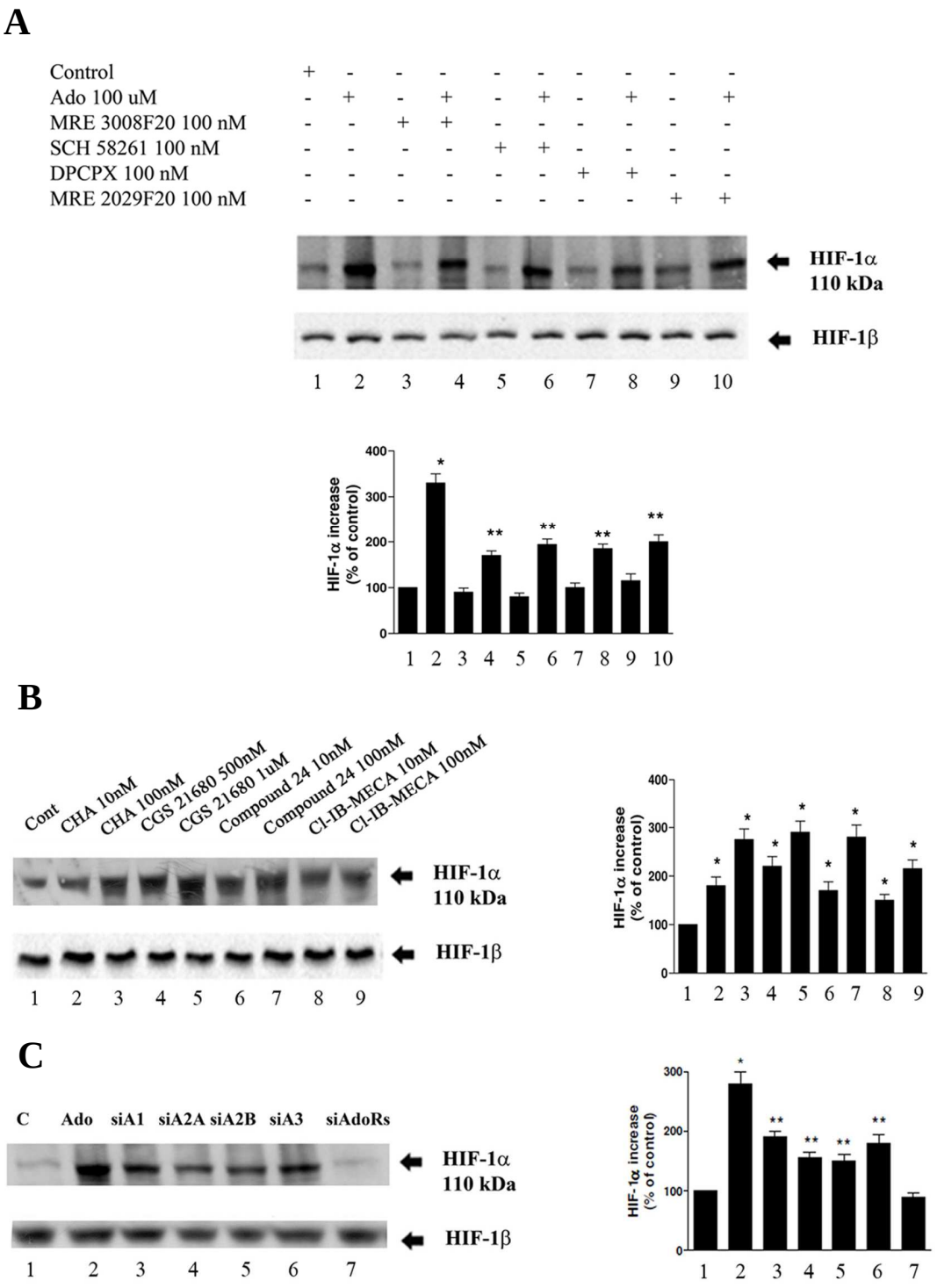
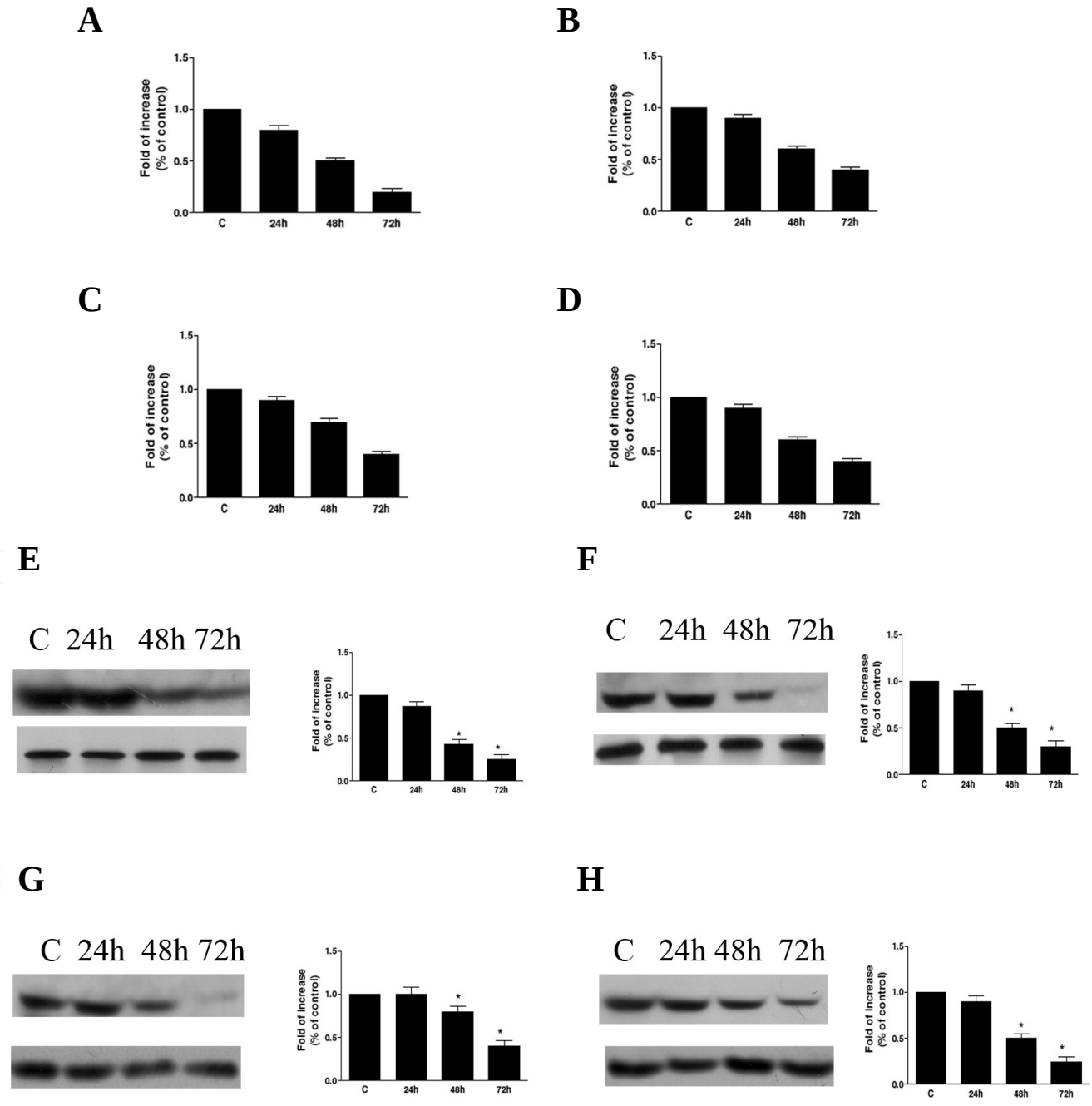


Table 2-Affinity (Ki, nM) of selected adenosine receptor agonists and antagonists to A_1, A_{2A}, A_{2B} and A_3 adenosine receptors.

	A_1	A_{2A}	A_{2B}	A_3	Ref.
Agonists					
CHA	3.5	812	>1,000	83	7
CGS 21680	289	27	>10,000	67	8
DPA23	8.5	>1,000	7.3	38.4	9
Cl-IB-MECA	220	5,360	>100,000	1.4	8
Antagonists					
DPCPX	3.9	129	51	1,100	10
SCH 58261	549	1.1	>10,000	>10,000	10
MRE 2029F20	200	>1,000	5.5	>1,000	10
MRE 3008F20	1,200	141	2,100	0.82	10

Figure 11 - ARs silencing by siRNA transfection in FC. Relative ARs mRNA quantification,related to β-actin mRNA, by real-time RT-PCR; FC were transfected with siRNA of A_1, A_{2A}, A_{2B} and A_3 ARs (A-D, respectively) and cultured for 24, 48 and 72 h. Plots are mean ± S.E. values (N = 3); *P < 0.05 compared with the control (time = 0). Western blot analysis using anti A_1, A_{2A}, A_{2B} and A_3 receptor polyclonal antibodies (E-H, respectively), of protein extracts from FC treated with siRNA of each AR subtype and cultured for 24, 48 and 72 h. Tubulin shows equal loading protein. Specificity of adenosine receptors siRNAs (I). Western blot analysis using anti A_1, A_{2A}, A_{2B} and A_3 receptor polyclonal antibodies of protein extracts from FC transfected with control ribonucleotides (ctr.) or with siRNA of each AR subtype and cultured for 72 h. Tubulin shows equal loading protein.



I

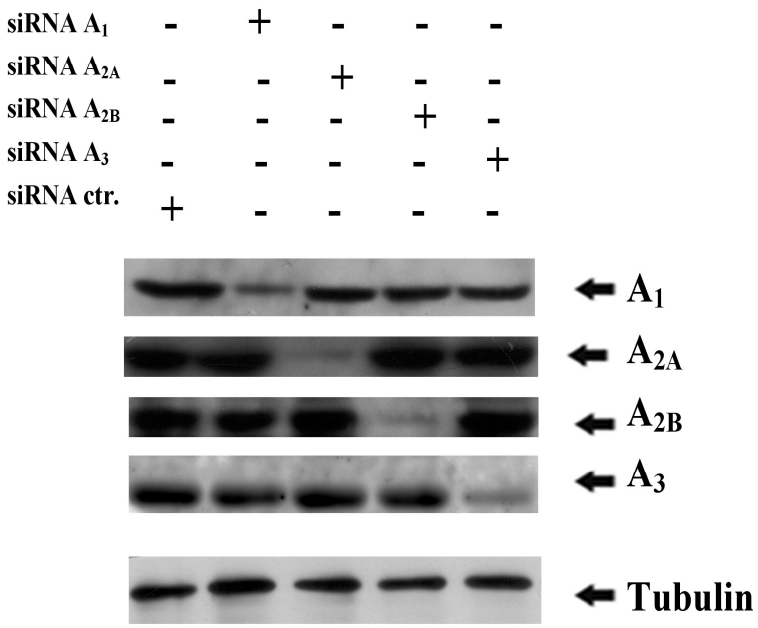
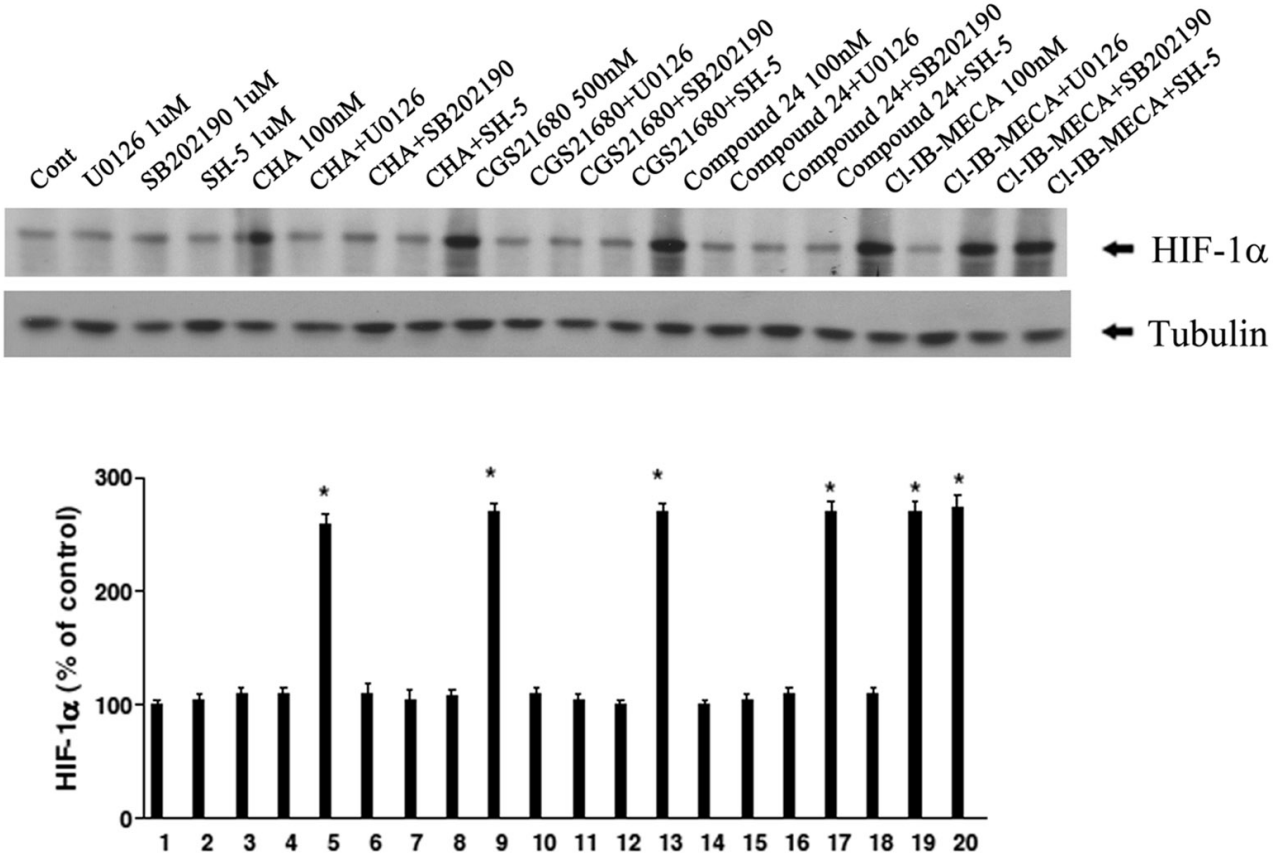

siRNA A1 - + - - -
siRNA A2A - - + - -
siRNA A2B - - - + -
siRNA A3 - - - - +
siRNA ctr. + - - - -
A1
A2A
A2B
A3
Tubulin

Figure 12 - Role of intracellular kinases in HIF-1α modulation induced by AR activation. FC were treated with nmol/L 100 CHA , 500 CGS 21680, 100 Compound 24, 100 Cl-IB-MECA in the absence (lines 5, 9, 13, 17, respectively) and in the presence of (1 µmol/L) U0126 (lines 6, 10, 14, 18, respectively), SB202190 (lines 7, 11, 15, 19, respectively), SH-5 (lines 8, 12, 16, 20). Line 1(control), line 2 (U0126), line 3 (SB202190), line 4 (SH-5) (A). Effect of ado agonists (nmol/L) 10, 100 CHA (lines 2, 3); 500, 1000 CGS 21680 (lines 4, 5); 10, 100 Compound 24 (lines 6, 7); 10, 100 Cl-IB-MECA (lines 8, 9) on ERK1/2, p38 and Akt phosphorylation (B). Densitometric quantification of western blots is the mean ± S.E. values (N=4); *P<0.05 compared with the control.

A



B

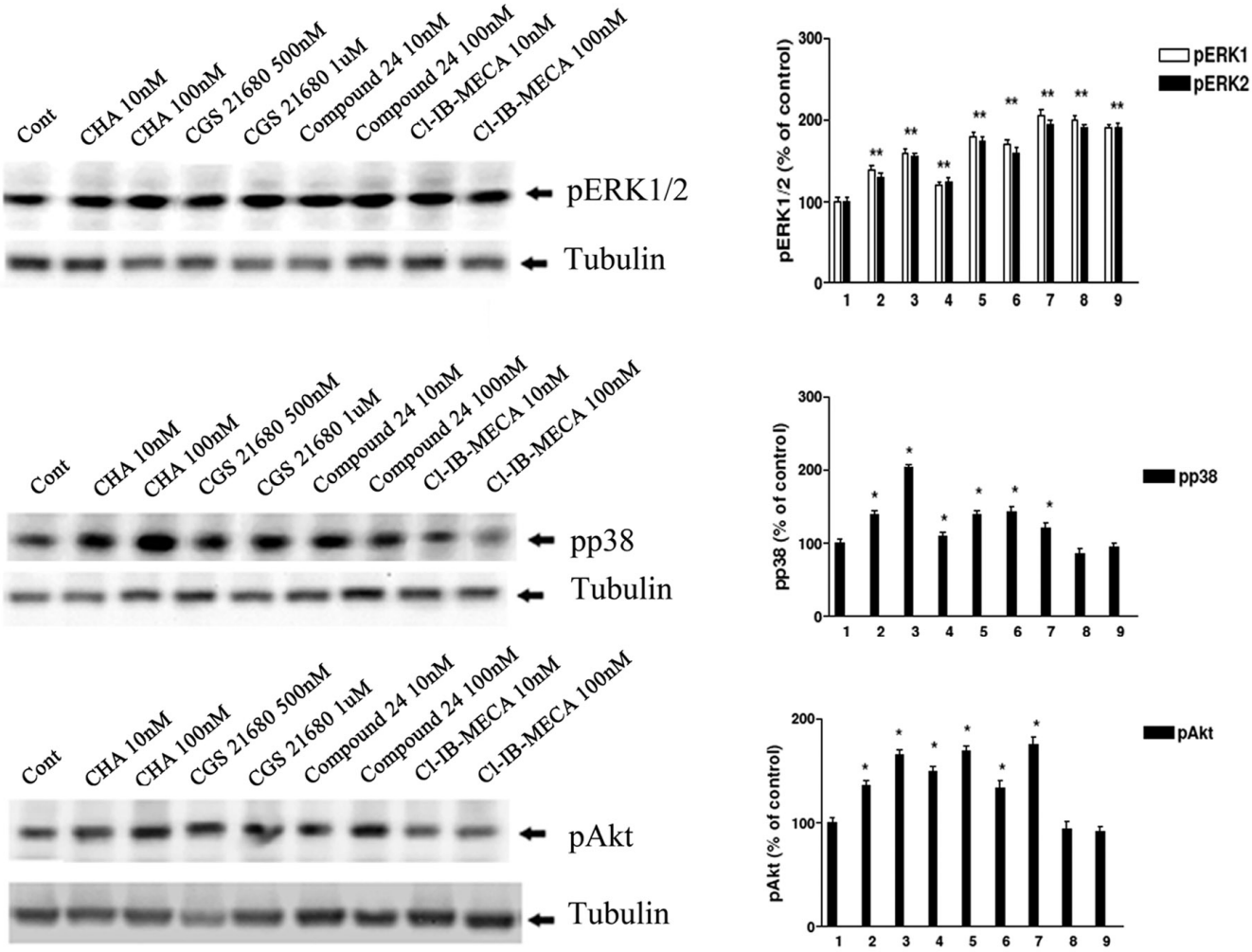

Cont
CHA 10nM
CHA 100nM
CGS 21680 500nM
CGS 21680 1uM
Compound 24 10nM
Compound 24 100nM
Cl-IB-MECA 10nM
Cl-IB-MECA 100nM
pERK1/2
Tubulin
pERK1/2 (% of control)
pERK1
pERK2
pp38
Tubulin
pp38 (% of control)
pp38
pAkt
Tubulin
pAkt (% of control)
pAkt

Figure 13 - Effect of ado on VEGF and IL-8 secretion. VEGF and IL-8 levels in FC treated with 100 µmol/L Ado in the absence and in the presence of 100 nmol/L DPCPX, SCH 58261, MRE 3008F20 and MRE 2029F20 (A, C). Role of 1 µmol/L ERK1/2, p38, Akt inhibitors and siRNA of HIF-1α in VEGF secretion induced by Ado (B); siRNA of HIF-1α was compared with cells transfected with control ribonucleotides for 72 h (-siRNA). Role of 1 µmol/L ERK1/2, p38, Akt and siRNA of HIF-1α and A_{2B} receptors in IL-8 secretion induced by Ado (D). Bargraphs are the means and vertical lines S.E. of the mean of four separate experiments performed in triplicate. *P<0.05 compared with control; #P<0.05 compared with ado.

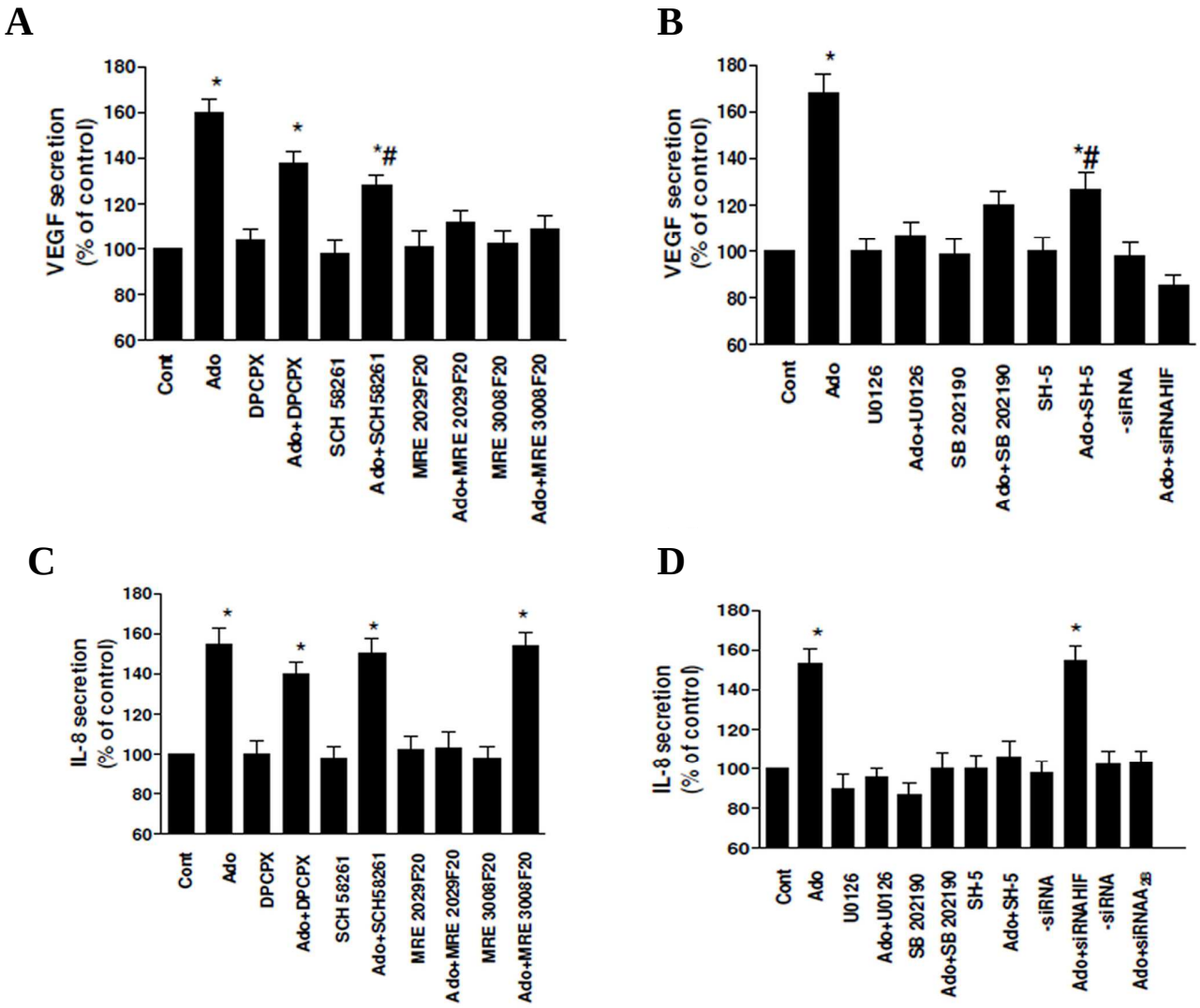
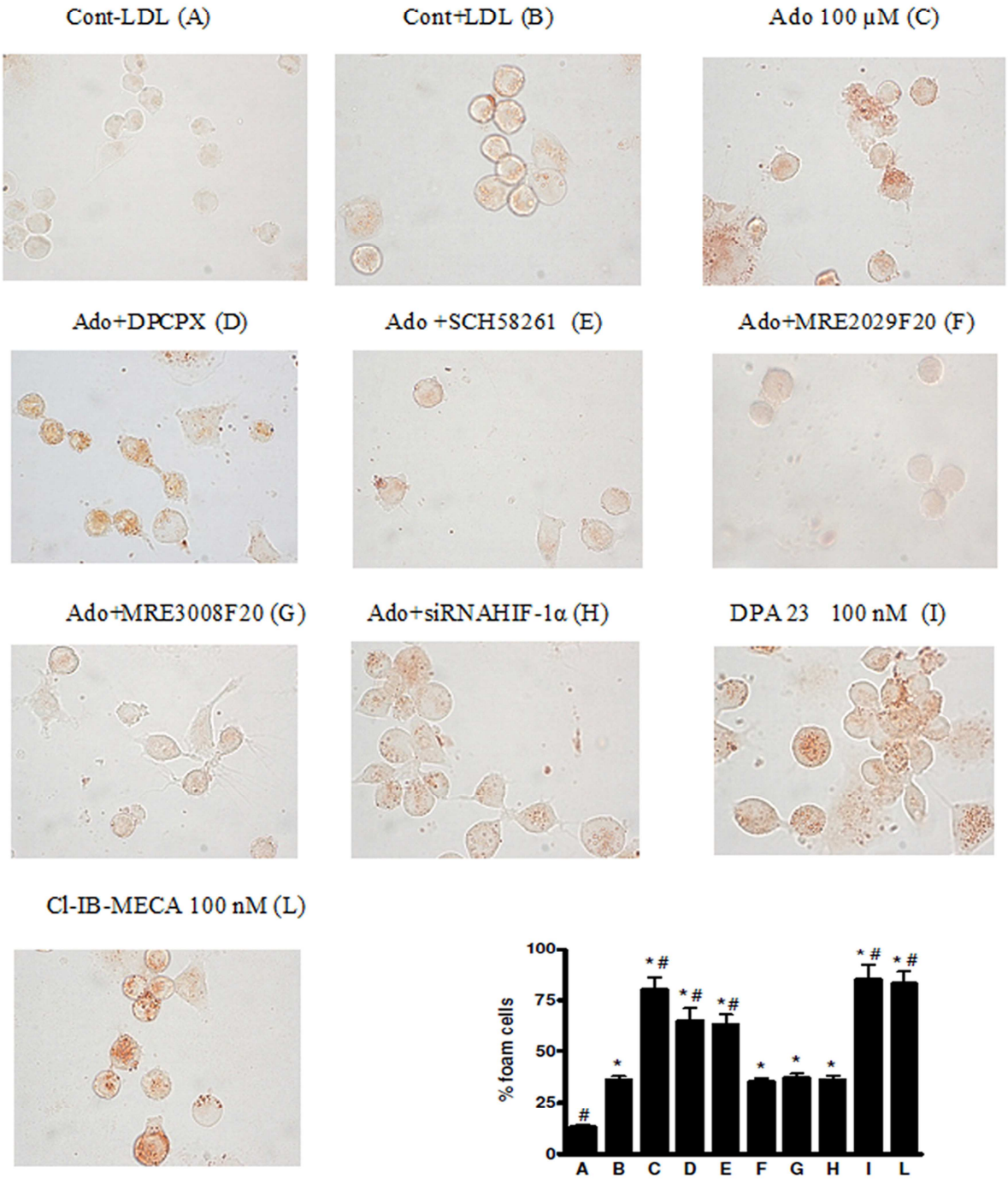


Figure 14 – Induction of FC formation by the U937 cells. Cells were stained for lipids with Oil red O in parallel cultures by incubation in the absence (A) or in the presence (B) of ox-LDL (50 µg/ml) followed by paraformaldehyde fixation. Effect of 100 µmol/L Ado on FC formation (C) and effect of 100 nmol/L ARs blockers (D-G) and HIF-1 silencing (H). Effect of A_{2B} and A_3 agonists on FC formation (I-L). Cells were viewed under a bright-field microscope in 100 X fields using a Nikon's Eclipse E800 microscope. Bargraph data expressed as the percentage of foam cells/total number of cells plated, are the mean ± S.E. values (N=3); *P<0.05 vs cells in the absence of oxLDL (A); #P<0.05 vs cells in the presence of oxLDL (B). Figure shows one representative experiment.



DISCUSSION

Hypoxia, HIF-1 and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis (Sluimer et al., 2008; Herrmann et al., 2006; Vink et al., 2007). Furthermore hypoxia stabilizes HIFs and leads to the accumulation of ado (Semenza 2001; Paul et al., 2004; Sluimer et al 2008; Jiang et al., 2007; Wang et al., 1996; Conway 2003/2004; Kim et al., 2006; Loboda et al., 2006; Jacobson et al., 2006; Yu et al., 2003; Gessi et al., 2004,2007; Kruth et al., 2002; Higuchi et al., 1993; Merighi et al., 2005; Kalayoglu et al., 1998; Baraldi et al., 2007; Herrmann et al., 2006; Vink et al., 2007; Blay et al., 1997). This study reports, for the first time, that ado increases HIF-1α protein levels in U937, HM and FC in hypoxia as already observed in cancer cells (Merighi et al., 2005, 2006, 2007). A_{2B} and A_3 subtypes play a major role in the VEGF increase and FC formation and only the A_{2B} is responsible for IL-8 stimulation induced by adenosine. The normoxic modulation of HIF-1α by ado was only barely appreciated by means of western blotting experiments. However, by evaluating the HIF-1 DNA binding activity through an ELISA assay, ado was able to induce a significant increase of this response in hypoxia and a lower but significant effect in normoxia, according to the elegant study by De Ponti et al. (De Ponti et al., 2007). This result also suggests that, in the case of low signals, ELISA approach on nuclear extract is more sensitive than western blot on whole cell extracts. The possibility that the nucleoside could increase HIF-1α gene expression in normoxia was rejected due to the lack of mRNA modulation induced by ado, whilst addition of the protein translation inhibitor CHX revealed an effect on protein stability that was not detectable under hypoxic conditions. Furthermore, addition of rapamycin, reduced the nucleoside effect on HIF-1 DNA binding activity in normoxia, suggesting that adenosine may play a role also in protein translation (De Ponti et al., 2007). In hypoxia, our results with inhibitors of transcription and translation suggest that ado stimulates HIF-1α protein levels essentially by increasing translation, as transcription and stability did not appear to be altered by the nucleoside, according to what reported in cancer cells (Merighi et al., 2005, 2006, 2007). The role of ARs in the nucleoside regulation of HIF-1α was investigated by using ARs blockers. DPCPX, SCH 58261, MRE 2029F20 and MRE

3008F20, used at 100 nmol/L, a dose that may be considered selective for A_1, A_{2A}, A_{2B} and A_3 ARs, (Baraldi et al., 2008) respectively, were able to reduce HIF-1α protein accumulation induced by ado. The involvement of all ARs was also confirmed by the increase of HIF-1α protein levels induced by high affinity AR agonists like CHA, CGS 21680, Compound 24 and Cl-IB-MECA for A_1, A_{2A}, A_{2B} and A_3 ARs, respectively. Furthermore, we found that silencing of A_1 or A_{2A} or A_{2B} or A_3 ARs was able to reduce HIF-1α modulation induced by ado and that the simultaneous knocking down of all four ARs abrogated the ado effect. Addition of oxLDL did not modify the responses of FC versus macrophages and U937 cells but we concentrated on FC because the effects of ado modulation of HIF-1α in this cellular type, crucial in atherosclerosis, have not been addressed before. However ox-LDL are recognized by different receptors than minimally-oxidized LDL (mm-LDL) and it is likely that alternative LDL ligands such as mm-LDL might have different effects, with greater relevance to atherosclerosis (Boullier et al., 2006). Different receptor subtypes have been reported to play a role in the ado-induced HIF-1α accumulation depending on the cellular model investigated (Merighi et al., 2005, 2006, 2007; De Ponti et al., 2007; Ramanathan et al., 2007; Alchera et al., 2008; Wendler et al., 2007). The results of this study suggest that HIF-1α accumulation may be triggered by all ARs in FC analogously to their effect in activating other intracellular signalling factors like ERK1/2 (Schulte et al., 2000). For example the ado-induced activation of myocardial ERK1/2 by statins has been found to involve A_1, A_{2A} and A_{2B} ARs in mice (Merla et al., 2007). It is well known that HIF-1 expression and activity, in addition to O_2 concentration, are also regulated by important signal transduction pathways including those involving ERK/MAPK and Akt (Semenza 2002). As these pathways are also modulated by ado, our aim was to investigate the intracellular signalling triggered by this nucleoside in HIF-1α modulation. Our results show the involvement of ERK1/2, p38 MAPK and Akt phosphorylation, whilst the Cl-IB-MECA effect was abrogated only by U0126. Indeed A_1, A_{2A} and A_{2B} receptors activate ERK1/2, p38 MAPK and Akt, whilst the A_3 subtype was involved in the modulation of ERK1/2. Several studies demonstrated a link between ado and HIF-1 at first in human cancer cell lines and then also in murine macrophages and in liver cells (Merighi et al., 2005,

2006, 2007; Ramanathan et al., 2007; Alchera et al., 2008). In most of these cases its accumulation was related to an increase of VEGF, which regulates important functions associated with angiogenesis. According to these results we found that ado increased VEGF levels through A_3 and A_{2B} receptors and to a lesser extent by the A_{2A} subtype and was dependent by HIF-1α, pERK1/2, pp38 MAPK and pAkt. Recently, it has been reported that HIF-1 is also linked to IL-8 expression in human endothelial cells, (Kim et al., 2006) whilst other authors point to different mechanisms of IL-8 regulation (Loboda et al., 2006) IL-8 is another crucial angiogenic factor found to be expressed by FC in human atheroma (Murdoch et al., 2005; Wang et al., 1996) and is also modulated by ado in different cellular models by activation of A_{2B}ARs (Feokistov et al., 2003). In this study we found that ado increased IL-8 secretion in hypoxic FC through activation of the only A_{2B} subtype. However, in agreement with other authors, its modulation was not dependent by HIF-1α accumulation suggesting that other transcription factors, possibly AP-1, may be involved (Loboda et al., 2006). Finally, as HIF-1 has been demonstrated to promote FC formation (Jiang et al., 2007), we evaluated the involvement of ado in FC development. Our results clearly demonstrate that ado increases FC formation and that this effect is strongly reduced by A_3 and A_{2B} antagonists and by silencing HIF-1α; this suggests that under hypoxic conditions, ado by increasing HIF-1α, through activation of A_3 and A_{2B} ARs, promotes FC formation. The marginal role of the A_{2A} antagonist in the modulation of this effect may be in line with recent relevant studies carried out by Reiss and Cronstein. These authors demonstrated that A_{2A} agonists in normoxic conditions inhibited foam cells formation in stimulated THP-1 macrophages by increasing expression of cholesterol 27-hydroxylase and adenosine 5'-triphosphate-binding cassette transporter A1, that are proteins involved in reverse cholesterol transport (Reiss et al., 2004). The same authors demonstrated that A_{2A} receptors were responsible for the atheroprotective effects induced by methotrexate (Reiss et al., 2008). Therefore it seems that adenosine by regulating FC may play both anti or pro-atherogenic effects depending on the receptors activated and the oxygen conditions present.

Altogether, these data suggest that in hypoxic conditions ado, through A_3 and A_{2B} ARs activation, induces HIF-1α protein accumulation thus leading to an increase of VEGF secretion and of FC formation; in addition the A_{2B} subtype is responsible for IL-8 accumulation. Therefore AR antagonists and in particular A_3 and A_{2B} or mixed A_3/A_{2B} blockers may be useful in order to block important steps in the atherosclerotic plaque development mediated by ado.

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RINGRAZIAMENTI

Giunta al termine di questo percorso desidero ringraziare ed esprimere la mia riconoscenza nei confronti di tutte le persone che, in modi diversi, mi sono state vicine e hanno incoraggiato e sostenuto le mie scelte. I miei più sentiti ringraziamenti vanno al Prof. P.A. Borea per avermi dato la possibilità di frequentare il dottorato nel suo laboratorio. Un ringraziamento speciale va alla Dott.ssa Gessi che con i suoi insegnamenti, in questi anni ha reso possibile la mia crescita professionale. Un grazie di cuore a tutti ragazzi del laboratorio che non sono mai stati semplici colleghi, ma veri e propri compagni di viaggio. Infine, il grazie più sentito è sicuramente per i miei genitori e Stefano che hanno rappresentato un costante punto di riferimento e mi hanno sempre sostenuto e incoraggiato con tanto amore.