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ADENOSINE RECEPTORS IN HEALTH AND DISEASE

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Abstract

Adenosine (Ado) is an endogenous nucleoside released from almost all cell types. It exerts neuroprotective and anti-inflammatory functions by acting through four receptor subtypes A_1, A_{2A}, A_{2B} and A_3 (ARs). These receptors differ in their affinity for Ado, in the type of G protein that they recruit and finally in the downstream signalling that are activated in target cells. The levels of Ado in the interstitial fluid are in the range of 20-200 nM, but many pathophysiological conditions such as asthma, neurodegenerative disorders, chronic inflammatory diseases and cancer are associated with changes in Ado levels. The primary aim of Ado is to reduce tissue injury and promote repair by different receptor-mediated mechanisms, including the increase of oxygen supply/demand ratio, anti-inflammatory effects and stimulation of angiogenesis. The investigation of ARs and their ligands is a rapidly growing field; there is extensive evidence for the involvement of ARs in the physiological regulation of several homeostatic processes and their implication in the ethiology of many diseases. The aim of this work was to analyse the expression of ARs and the signalling pathway, transcription factors and cytochines activated by them in different pathophysiological conditions linked to hypoxic and inflammatory conditions.

At first, the role of ARs was studied in healthy and aneuploid pregnancies to understand reasons of spontaneous abortion (SA). It has been suggested that the causes of SA in aneuploidy are no different to those in euploidy, with the increased frequency in the former perhaps being ascribable to a genetically-determined imbalance in the mediators of placental perfusion and uterine contraction. So aneuploidy can be used as a model of this event. The ado transduction cascade appears to be disturbed in Trisomy 21 (TR21) through reduced expression of A_{2B}AR and A_1AR. These anomalies may be implicated in complications such as fetal growth restriction, malformation and/or SA, well known features of aneuploid pregnancies. Therefore A_1AR and A_{2B}AR could be potential biomarkers able to provide an early indication of SA risk and their stimulation may turn out to improve fetoplacental perfusion by increasing nitric oxide (NO) and vascular endothelial growth factor (VEGF).

Next, the effects of ARs in glial cells under inflammatory conditions have been investigated. Glial cells, astrocytes and microglia, are important contributors to inflammatory immune responses and hypoxia-inducible factor 1 (HIF-1α) is the key transcription factor that is upregulated in response to hypoxia and inflammatory stimuli. Ado, through A_1AR and A_3AR activation, reduces lipopolysaccharide (LPS)-stimulated

HIF-1α mRNA expression and protein accumulation by inhibiting LPS-triggered p42/p44 mitogen-activated protein kinase (p44/42 MAPK) and serine/threonine protein kinase (Akt) phosphorylation in normoxic and hypoxic conditions. This leads to an inhibition of genes involved in inflammation like Inducible Nitric Oxide Synthase (iNOS) and A_{2B}ARs, that are stimulated by LPS and further increased by LPS in concert with hypoxia, whilst does not affect angiogenesis and metabolic related genes, with the exception of glucose transporter 1 (GLUT1) and hexokinase 2 (HK2) that are stimulated by LPS in normoxia and reduced by A_1AR and A_3AR activation. These findings add a new molecular pathway activated by Ado in astrocytes to give a reduction of genes involved in inflammation and hypoxic injury that may cohexist in stroke, ischemia and other central nervous system (CNS) disorders.

Adenosine Receptors

in Health and Disease

Adenosine

In 1929, Drury and Szent-Györgyi first reported the concept of Adenosine (Ado) acting as an extracellular signalling molecule (Drury and Szent-Gyorgyi, 1929). Ado is an endogenous nucleoside modulator released from almost all cell types (Gessi et al., 2011). Ado plays a central role as a structural element of nucleic acids and in the energy metabolism of all living organism. The physiological effects of Ado were first described in the cardiovascular system and gastrointestinal tract (Drury and Szent-Gyorgyi, 1929). It is composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond (Fig. 1). Ado accumulates in the extracellular space in response to metabolic stress and cell damage, and elevations in extracellular Ado are found in conditions of ischaemia, hypoxia, trauma asthma, neurodegenerative disorders, chronic inflammatory diseases and cancer (Linden, 2001; Fredholm et al., 2001; Gessi et al., 2011; 2013). The rapid release of Ado in response to tissue-disturbing stimuli has a dual role in modulating homeostasis. First, extracellular Ado represents a pre-eminent alarm molecule that reports tissue injury in an autocrine and paracrine manner to surrounding tissue. Second, extracellular Ado generates a range of tissue responses that can be generally viewed as organ protective thereby mediating homeostasis. Ado elicits its physiological responses by binding to and activating one or more of the four transmembrane Ado receptors (ARs), denoted A_1, A_{2A}, A_{2B} and A_3. A prominent body of evidence supports the notion that the ability of Ado, acting at its receptors, to control the immune and inflammatory systems plays a key role in the modulatory effects of Ado in both health and disease. There are many promising emerging therapeutic approaches that are focused on the modulation of Ado in the immune system (Hasko et al., 2008). Ado also has receptor-indipendent effects, because extracellular Ado can cross the cell membrane and have a role in less well-defined intracellular mechanisms, including the AMP-activated protein kinase (AMPK), adenosine kinase (AK) and S-adenosylhomocysteine hydrolase pathway (SAHase) (Antonioli et al., 2013).
Normally, it is present in body fluids in concentrations 20–200 nM, but in response to stress elevated levels of Ado, up to 300 μM are produced and released (Fredholm, 2010; Burnstock, 2008; Lopes et al., 2011).

Ado can be generated intracellularly by hydrolysis of AMP or SAHase. The former is mediated by intracellular soluble 5′ nucleotidase (5′NT) and the rate of Ado formation is largely controlled by the level of adenosine monophosphate (AMP), which provides a direct

link to energy metabolism (Newby et al., 1985). Interestingly, Ado can be converted back to AMP via the action of AK and together 5′ NT and AK generate a potential futile cycle that ensures that there will always be a not insubstantial amount of intracellular Ado. The other way in which intracellular Ado is formed depend on the action of SAHase. S-Adenosyl-L-homocysteine (SAH) levels are increased with increasing rates of S-adenosylmethionine-dependent transmethylation. Interestingly, the enzyme can also catalyze the reverse reaction, i.e., formation of SAH from Ado and homocysteine and this has been used to generate an estimate of intracellular Ado concentration of around 100 nMol/L under basal conditions (Deussen et al., 1988). Indeed, whenever there is an imbalance between the rate of Adenosine triphosphate (ATP) synthesis and ATP utilization AMP tends to rise and consequently Ado formation increases. This can occur under extreme physiological conditions such as heavy exercise, increases in nerve activity, or reduced ambient oxygen, e.g., at elevated locations. It will also occur pathologically, e.g., in ischemia, local trauma, or in the interior of a solid tumor (Fredholm, 2007; Sitkovsky, 2009).

Cell membrane–embedded nucleoside trasportes, which include equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transportes (CNTs), shunt extracellular Ado into the intracellular space, thereby terminating ARs signaling. ENTs, which carry nucleosides along their concentration gradient across cell membranes, include four subtypes: ENT1, ENT2, ENT3 e ENT4. CNTs, which include CNT1, CNT2 and CNT3, mediate the intracellular influx of nucleoside against their concentration gradient by using the sodium ion gradient, which occurs across the cell membrane, as a source of energy (Antonioli et al., 2013). Two enzymes play a key role in catabolizing Ado: Ado deaminase (ADA) and AK and they are responsible for an extremely short half-life of Ado in circulation (Fredholm et al., 2001; 2011; Eltzschig, 2009). AK is critically important in maintaining the physiological levels of Ado low, and also in maintaining depots of adenine nucleotides (Boison, 2006; Fredholm, 2007). However, some cells also possess concentrating transporters that drive Ado intracellularly by an ion gradient (Young et al., 2008). Extracellular Ado can be generated from extracellular degradation of adenine nucleotides via CD39 and ATP diphosphohydrolase (Deaglio and Robson, 2011) and CD73-5′nucleotidase (Colgan et al., 2006). In addition, some cells express ecto-enzymes that convert 2′, 3′,or 3′5′-cAMP to AMP for further conversion to Ado (Verrier et al., 2011). Although not all cells express these enzymes (Langer et al., 2008), they are present in most cellular microenvironments and provide a means for rapid degradation of extracellular nucleotides. Indeed, it appears that most cells can under some circumstances release nucleotides via exocytosis, specialized transporters or connexin or

pannexin hemichannels. There is also sometimes exocytotic release from vesicles that in addition store hormones or neurotransmitters (Lazarowsky, 2012), and perhaps even more interestingly the release of ATP can occur without the release of hormone when hormone storage granules transiently contact the cell membrane in a so called kiss-and-run encounter (MacDonald et al.,2006; Fredholm, 2013).

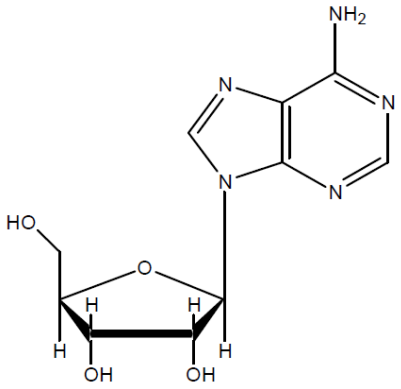

Figure 1: Chemical Structure of Adenosine

G protein–coupled receptors (GPCRs)

Ado is the endogenous ligand of four types of GPCRs, designated A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2011).

GPCRs comprise the largest protein superfamily in mammalian genomes. They share a common seven-transmembrane (7TM) topology and mediate cellular responses to a variety of extracellular signals ranging from photons and small molecules to peptides and proteins (Lagerstrom and Schioth, 2008). Diversity of the extracellular ligands is reflected in the structural diversity of more than 800 human GPCRs, which can be grouped into five major families and numerous subfamilies on the basis of their amino acid sequences. Subdivision on the basis of sequence homology allows the definition of rhodopsin (Class A), secretin (Class B), adhesion, glutamate (Class C) and Frizzled receptor families (Friedriksson et al., 2003). During the past few years, crystallography of GPCR has experienced exponential growth, resulting in the determination of the structures of 16 distinct receptors, 9 of them in 2012

alone (Katrick et al., 2013). Signal transduction by GPCRs is fundamental for most physiological processes - from vision, smell, and taste to neurological, cardiovascular, endocrine, and reproductive functions - thus making the GPCR superfamily a major target for therapeutic intervention (Overington et al., 2006). Current drug discovery efforts aim both to improve therapies for more than 50 established GPCR targets and to expand the list of targeted GPCRs (Lappano and Maggiolini, 2011; Katrick et al., 2013).

GPCRs share a common 7 TM α-helix architecture and couple to G-proteins (Lefkiowitz, 2004). The TM α-helices are connected by alternating three extracellular (EL) and cytoplasmic (CL) loops (EL1–EL3 and CL1–CL3), with the N-terminus (NT) extracellular and the C-terminus (CT) intracellular, arranged in an anticlockwise fashion as viewed from the extracellular surface. Binding of a stimulus, the so-called 'first messenger', to the extracellular or TM domains of a GPCR triggers conformational changes in the 7TM structure that are transmitted through the intracellular receptor domains to promote coupling between the receptor and its cognate heterotrimeric G-proteins. The receptor stimulates G-protein activation by catalysing the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the Gα subunit and dissociation of the GTP-bound Gα subunit from the Gβγ subunit heterodimer. Once dissociated, free Gα-GTP and Gβγ subunits regulate the activity of enzymatic effectors, such as adenylate cyclases (AC), phospholipase C (PLC) isoforms and ion channels, to generate small molecules, the "second messengers". The second messengers, in turn, control the activity of protein kinases that regulate key enzymes involved in intermediate metabolism. Hydrolysis of GTP to GDP within Gα and subsequent reassociation of Gα-GDP and Gβγ completes the G-protein cycle (Fig.2) (Oldham et al., 2008; Ding et al., 2013)

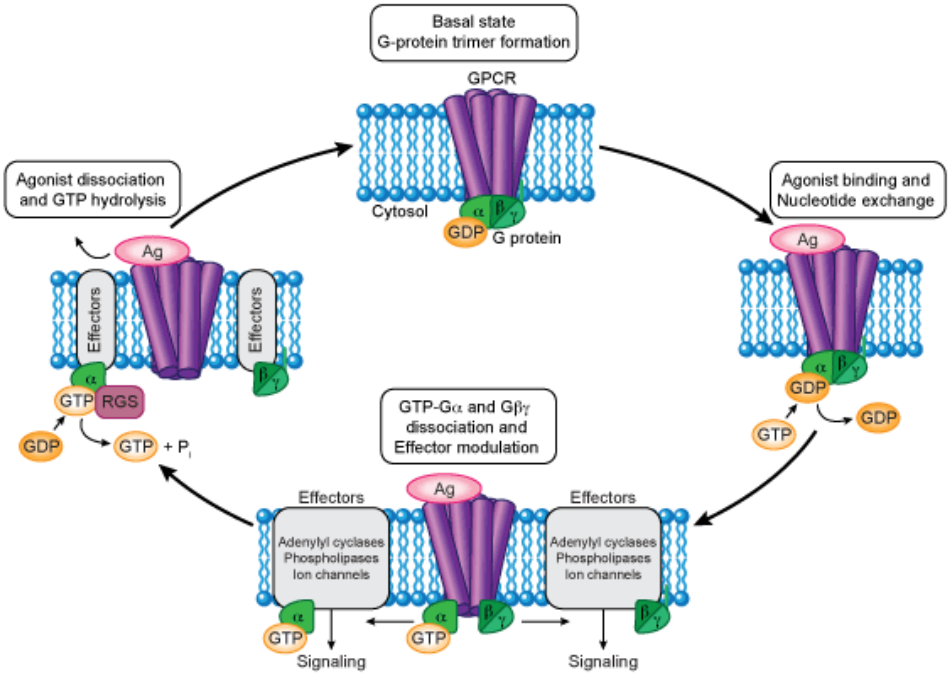

Figure 2: GPCR activation cycle.

ARs, like other class A GPCRs, have long been thought to exclusively occur in a monomeric state. Monomeric receptors are sufficient to induce signaling (Whorton et al., 2008). At least some studies suggest signaling via dimers occurs only at higher receptor densities (White et al., 2007). More recently, however, evidence is accumulating that ARs can form dimeric or, more generally speaking, multimeric or oligomeric structures. Through self-association, homo-oligomers ("homomers") can be formed. Hetero-oligomerization leading to "heteromers" may be the consequence of the association between ARs and preferred partners, mostly other GPCRs, including other AR subtypes (Fredholm et al., 2011).

Adenosine receptors (ARs)

Extracellular purines (Ado, ATP, and ADP) and pyrimidines (uridine diphosphate (UDP) and uridine triphosphate (UTP) comprise a family of molecules that exert a variety of important physiological functions via the activation of cell-surface receptors termed purine receptors. Although the physiologic effects of Ado and ATP have been recognized for over 80 years, purinergic receptors were first described in 1976 and two subfamilies were identified: P1 or

ARs (selective for Ado), and P2 or nucleotide receptors (selective for ATP, ADP and UTP, which act as extracellular signaling molecules) (Mediero and Cronstein, 2013).

Four members of the Ado/P1 receptor family have now been cloned and characterized from a variety of species: A_1, A_{2A}, A_{2B}, and A_3, and selective agonists and antagonists have been identified. All P1 receptors couple to G-proteins, and modulate AC activity in an inhibitory (A_1, A_3) or stimulatory (A_{2A}, A_{2B}) fashion, resulting in cyclic adenosine monophosphate (cAMP) changes (Fig.3). P2 receptors are divided into two families: P2X and P2Y, based on molecular structure, transduction mechanisms, and pharmacological properties (Burnstock et al., 2012).

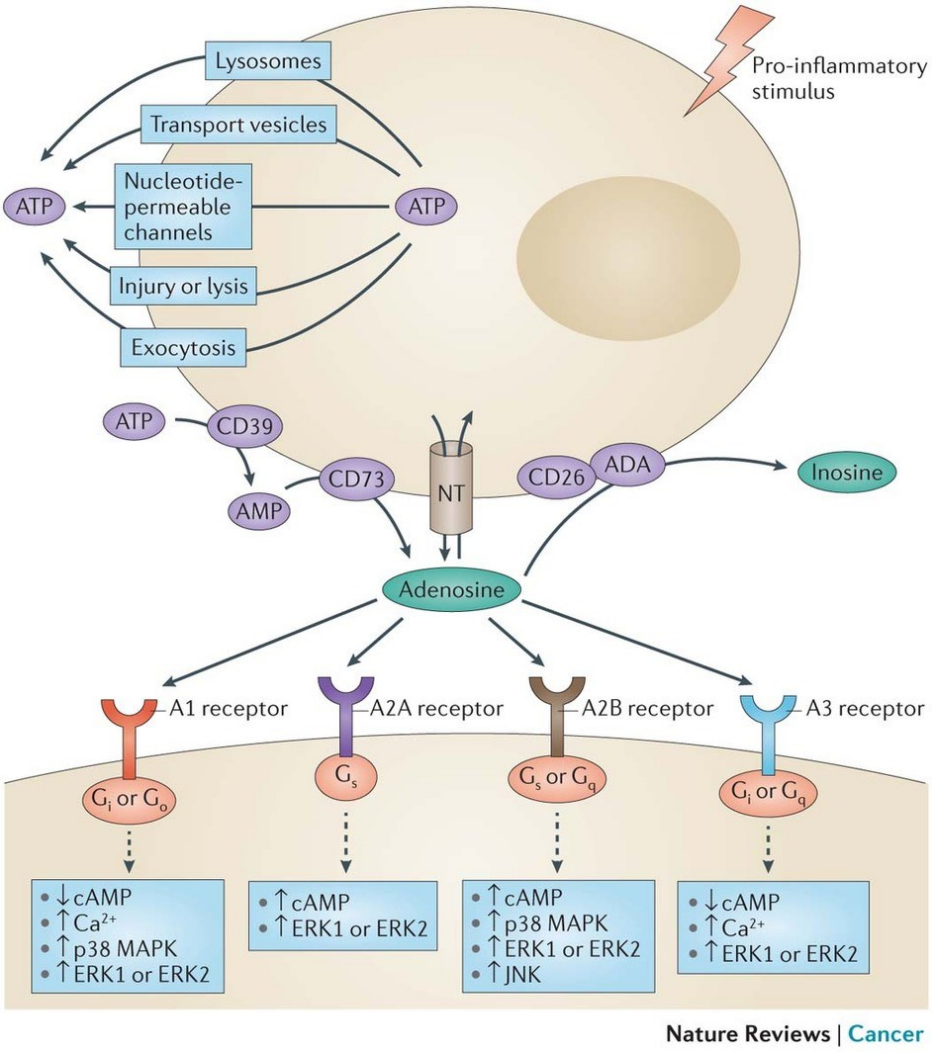

Figure 3: Ado synthesis and receptor activation in the cell.
(Antonioli et al., Nature Reviews Cancer 13, 842–857;2013)

Each AR subtype has a different pattern of tissue expression and ligand binding properties. In cell-based systems, A_1ARs have the highest affinity for Ado (K_i=10 nmol/L). The K_i values for Ado for A_{2A}AR A_{2B}AR, and A_3AR are 200, 2000, and 10000 nmol/L, respectively, for the human receptors. A_3ARs are also activated by the Ado metabolite inosine (K_i 2300 nmol/L) (Rivkees and Wendler, 2012).

Many biological functions have been attributed to Ado signaling. For example, the heart rate-slowing effects of intravenous Ado that is used for patient treatment of supraventricular tachycardia are mediated through the A_1AR (Delacretaz, 2006). The A_{2A}AR is expressed on inflammatory cells: pharmacologic studies provided critical evidence that the activation of A_{2A}AR on neutrophils attenuates inflammatory responses (Gessi et al., 2000; Ohta and Sitkovsky, 2001). The A_{2B}AR is involved in hypoxia-adaptive responses (Kong et al., 2006; Eltzschig et al., 2003; 2004), for example, during myocardial ischemia (Eckle et al., 2012), Acute Kidney Injury (AKI) (Grenz et al., 2012a, Bauerle et al., 2011) or intestinal inflammation (Grenz et al., 2012b, Frick et al., 2009) - such as that occurring during inflammatory bowel disease. A_3AR has been suggested as a tumoral marker (Gessi et al., 2004) and is involved in the inhibition of cancer growth (Fishman et al., 2004)

A_1 Adenosine Receptor

The A_1AR has been cloned from several animal species, including humans, and is known to bear a close structural similarity across the species (Ravelic and Burnstock, 1998). As for signal transduction, the A_1AR is coupled to members of the G_i/G_o family of G proteins, whereby it induces inhibition of AC activity (Van Calker et al., 1979). In addition, it is thought to activate PLCβ, which is known to increase inositol 1,4,5-triphosphate (IP_3) and intracellular Ca^{2+}. A_1AR is coupled to pertussis-toxin-sensitive potassium channels as well as K_{ATP} channels, particularly in cardiac tissue and neurons. Moreover, it may inhibit Q, P and N-type Ca^{2+} channels and modulate extracellular signal-regulated protein kinases (ERKs) (Fredholm et al., 2001). Furthermore, a role for the β-arrestin1/ERK mitogen-activated protein kinase (MAPK) pathway in regulating A_1AR desensitization and recovery has recently been reported (Jajoo et al., 2010).

A_1AR and central nervous system (CNS)

The A_1AR is widely distributed throughout the CNS, featuring particularly high levels in the brain cortex, cerebellum and hippocampus, as well as the dorsal horn of the spinal cord. It is present in both pre- and post-synaptic terminals, and modulates the activity of the nervous system at a cellular level. At the presynaptic level it mediates inhibition of neurotransmitter release, while at the postsynaptic level it induces neuron hyperpolarization. Thus, activation of A_1AR via Ado is responsible for sedative, anticonvulsant, anxiolytic and locomotor-depressant effects. Moreover, endogenous Ado levels are sufficient to tonically activate inhibitory A_1AR, and caffeine, perhaps the most commonly used drug in the world, mediates its excitatory effects by antagonising this inhibition. Ado also has a fundamental role to play in analgesia (Eltzschig et al., 2009); indeed, both spinal and systemic administration of Ado or its analogs produces anti-nociception by A_1AR activation in a variety of animal models (Boison, 2007; Gong et al., 2010; Nascimento et al., 2010; Sowa et al., 2010). These antinociceptive effects may be mediated by the inhibition of intrinsic neurons by an increase in K^+ conductance and presynaptic inhibition of sensory nerve terminals, which would theoretically hinder the release of substance P and glutamate. Likewise, attenuation by N-methyl-D-aspartate (NMDA)-induced production of NO may also be involved. Furthermore, Ado has been shown to mediate opioid analgesia (Gan and Habib, 2007). In addition, it has recently been reported that allopurinol, a potent inhibitor of the enzyme xanthine oxidase used primarily in the treatment of hyperuricemia and gout, induces anti-nociception related to Ado accumulation, an effect that is completely prevented by A_1AR blockade (Schmidt et al., 2009). Compounds that are able to enhance the activity of the A_1AR mediated by the endogenous ligand within specific tissues may have potential therapeutic advantages over non-endogenous agonists, due to allosteric modulation of GPCRs. As allosteric enhancers act only on the agonist A_1AR-G protein ternary complex, limiting their action to sites and times of Ado accumulation, the use of these drugs to increase the responsiveness of the A_1AR to endogenous Ado at sites of its production is an appealing alternative to activation by exogenous agonists (Romagnoli et al., 2010), especially as the former approach minimizes side effects such as dyspnea, chest pain, atrioventricular blockage or bronchospasm.

A_1AR and the respiratory system

A_1ARs are responsible for many effects induced by Ado, not only in the CNS but also in peripheral tissues (Russo et al., 2006; Baraldi et al., 2008). In particular, this signaling nucleoside has been implicated in the regulation of asthma and chronic obstructive pulmonary

disease (COPD) (Russo et al., 2006); Ado levels are elevated in the asthmatic lungs to an extent that can be directly correlated with the degree of inflammatory insult (Brown et al., 2008a). Unsurprisingly, therefore, A_1AR expression is also increased in the epithelium and airway smooth muscle of human asthmatics (Brown et al., 2008b). Early evidence of A_1AR is involvement in asthma was provided by studies on allergic rabbit models, where the Ado-induced acute bronchoconstrictor response was attenuated by pretreatment with A_1AR antagonists. Accordingly in human airway tissue and bronchial smooth muscle cells, activation of A_1AR has been shown to produce effects that cause airway hyper-responsiveness. In particular, activation of A_1AR in human airway epithelial cells causes an increase in the expression of the Mucin 2 (MUC 2) gene, which is responsible for mucus hypersecretion. Moreover, activation of A_1AR is known to produce pro-inflammatory effects on various types of human cells (Ponnoth et al., 2010). As a whole, these effects of A_1AR in humans suggest A_1AR as an important therapeutic target in human asthma (Ethier and Madison, 2006; Baraldi et al., 2008; Wilson, 2008). Indeed, the non-selective AR antagonists theophylline and doxofylline have been launched as bronchodilators for the treatment of various respiratory disorders (Press et al., 2007). Paradoxically, however, findings in ADA-deficient mice suggest the occurrence of anti-inflammatory actions of Ado in the lung, mediated through chronic A_1AR activation of macrophages (Sun et al., 2005). Likewise, it has been recently reported that A_1AR inhibits transendothelial and transepithelial polymorphonuclear cell migration in a murine model of lipopolysaccharide (LPS)-induced lung injury, presumably by reducing the release of chemotactic cytokines into the alveolar space. In addition, A_1AR is involved in decreasing microvascular permeability and leukocyte transmigration in endothelial cells (Ngamsri et al., 2010), suggesting also a protective and anti-inflammatory role for A_1AR (Gazoni et al., 2010).

A_1AR and the cardiovascular system

At a cardiovascular level, A_1ARs mediate negative chronotropic, dromotropic and inotropic effects. A_1AR subtypes located on sinoatrial and atrioventricular nodes can cause bradycardia and heart block, respectively, while their negative inotropic effects include a decrease in atrial contractility and action potential duration. Recently, it has been shown that the selective deletion of the A_1AR abolishes the heart-rate slowing effects of intravascular Ado in vivo (Koeppen et al., 2009). Stimulation of A_1AR in the heart, on the other hand, exerts a cardioprotective effects by inhibiting norepinephrine release from sympathetic nerve endings (Schutte et al., 2006). Ado also protects tissues through ischemic preconditioning (IPC), a brief period of ischemia and reperfusion that can protect the myocardium against infarction

from a subsequent prolonged ischemic insult. This response, which has been most widely investigated in the heart, but also occurs in other tissues (Schneyvays et al., 2005; Grenz et al., 2007), is brought about by the activation of A_1AR, protein kinase C (PKC) and mitochondrial K_{ATP} channels (Kiesman et al., 2009; Solenkova et al., 2006). A_1AR agonists, for example, tecadenoson (N6-[3(R)- tetrahydrofuranyl]adenosine), are in development for arrhythmias and atrial fibrillation; clinical studies with intravenous tecadenoson suggest that it may slow the speed of atrio ventricular nodal conduction by selectively stimulating the A_1AR, and may prevent blood pressure dropping by failing to stimulate the A_{2A}AR (Yldiz et al., 2007). In the kidney, on the other hand, A_1AR mediates vasoconstriction, decrease in glomerular filtration rate and the inhibition of both renin secretion and neurotransmitter release. Thus, A_1AR antagonists represent a novel class of agents for potential use in the treatment of hypertension and edema (Vallon et al., 2006). In fact, A_1AR antagonists are more effective diuretics and natriuretics than thiazides, with the added advantage of reducing the potassium wastage and reductions of renal blood flow and glomerular filtration rate seen with the latter drugs (Zhou and Kost, 2006). Furthermore, evidence from genetically altered mice indicates that transcellular NaCl transport induces the generation of Ado, which, in conjunction with angiotensin II, elicits afferent arteriolar constriction through A_1AR activation (Sun et al., 2001; Schnermann and Briggs, 2008). Moreover, clinical trials, albeit in a limited number of subjects, have demonstrated that A_1AR antagonists produce natriuretic and hypotensive effects in hypertensive patients and attenuated the furosemide-induced decline of renal hemodynamic function in heart failure patients. Hence, selective A_1AR antagonists targeting renal microcirculation are currently under development for the treatment of both chronic and acute heart failure. One of these novel pharmacological agents, rolofylline (1,3-dipropyl-8-(2-nor-1-adamantyl) xanthine, KW-3902), facilitates diuresis and preserves renal function through A_1AR antagonism in patients with acute decompensated heart failure and renal dysfunction; pilot data also suggest beneficial effects on symptoms and short-term outcomes (Slawsky and Givertz, 2009). Nevertheless, despite several studies showing improvement of renal function and/or increased diuresis with A_1AR antagonists, particularly in chronic heart failure, these findings were not confirmed in the large Phase III trials PROTECT 1 and 2 (Placebo-controlled Randomized study of the selective A_1 antagonist Rolofylline for patients hospitalized with acute heart failure and volume Overload to assess Treatment Effect on Congestion and renal funcTion) in acute heart failure patients. In fact, the pooled/meta-analysis of two studies demonstrated that treatment with rolofylline was associated with poor outcomes due to worsening renal function in patients with acute decompensated heart failure (Watherley et al., 2010; Voors et al., 2011). Nonetheless, lessons

can be learned from these and other studies, and there is still hope for a clinical role for A_1AR antagonists (Hocher et al., 2010; 2011).

A_1AR and inflammation

Indeed, several studies have demonstrated that A_1AR activation has a protective function in vivo, inhibiting necrosis, inflammation and apoptosis. Moreover, A_1AR has been implicated as potent anti-inflammatory mediator in various kidney, heart, liver, lung and brain injury models (Gazoni et al., 2010; Pang et al., 2011). In particular A_1AR activation appears to protect against hepatic injury by upregulation and phosphorylation of heat shock protein 27, a member of a family of chaperone proteins that serves to defend against cell damage (Chen et al., 2009). A critical role for Ado in bone homeostasis via interaction with A_1AR has also been recently reported. In particular, due to the stimulatory effect played by A_1AR on osteoclast function and formation, antagonists of this receptor may be able to prevent the bone loss associated with inflammatory diseases and menopause (Kara et al., 2010a; 2010b).

Activation of A_1AR inhibits lipolysis and lowers plasma concentrations of free fatty acids by inhibiting AC and downstream cAMP formation. Unfortunately, however, the majority of full A_1AR agonists are plagued by significant cardiovascular effects. Hence, selective partial A_1AR agonists have been developed (Dhalla et al., 2007a; 2007b). One such compound, CVT-3619 (2-{6-[((1R,2R)-2-hydroxycyclopentyl) amino] purin-9-yl(4S,5S,2R,3R)-5-[(2-fluorophenylthio)- methyl]oxolane- 3,4-diol), is a partial A_1AR agonist that has anti-lipolytic effects at concentrations that do not provoke cardiovascular symptoms (Fatholahi et al., 2006; Shearer et al., 2009). A further advantage of these partial agonists is that they are accompanied by a minimal risk of ARs desensitization in response to chronic drug exposure (Vallon et al., 2006; Shearer et al., 2009). The A_1AR receptor ligand candidates for novel therapeutic treatments currently undergoing clinical studies are listed in Table 1.

Table 1: Progress of A_1AR ligands as novel therapeutic treatments (Gessi et al., Expert Opin. Investig. Drugs:20;1591-1609; 2011)

Pathology	Drug name	Type of ligand	Development status
Arrhythmia, atrial fibrillation	Capadenoson	Agonist	Phase II trial
	Selodenoson	Agonist	Phase II trial
	Tecadenoson	Agonist	Phase III trial
Heart failure, congestive	Derenofylline	Antagonist	Phase II trial
Chronic heart failure	Tonapofylline	Antagonist	Phase III trial
Asthma	Doxofylline	Antagonist	Launched: 1987
	Theophylline	Antagonist	Launched: 1939
COPD	Theophylline	Antagonist	Phase II trial
Renal failure	Rolofylline	Antagonist	Phase III trial
	Derenofylline	Antagonist	Phase II trial
Diabetes	CVT-3619	Partial agonist	Phase I trial
Glaucoma	PJ-875	Agonist	Phase II trial
Lipoprotein disorders	CVT-3619	Partial agonist	Phase I trial
Neuropathic pain	T-62	Allosteric enhancer	Phase II trial

A_{2A} Adenosine Receptor

Of the four ARs, A_{2A}ARs have taken center stage as the primary anti-inflammatory effectors of extracellular Ado (Hasko and Pacher, 2008). The gene for the A_{2A}AR has been cloned from several species including dog, rat, human, guineapig and mouse, and has demonstrated a high degree of homology among human, mouse and rat (Baraldi et al., 2008). The A_{2A}AR stimulates AC activity through its coupling with G_s proteins; this leads to the activation of cAMP-dependent protein kinase A (PKA), which in turn phosphorylates and activates various receptors, ion channels, phosphodiesterases and phosphoproteins such as cAMP response element-binding protein (CREB) and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32). PKC triggering by A_{2A}AR activation has also been reported. In the brain striatum, the A_{2A} subtype stimulates G_{olf}, another member of the G_s subfamily of G proteins. In addition, A_{2A}AR can interact with different types of Ca^{2+} channels to either increase intracellular Ca^{2+} or decrease Ca^{2+} influx and, like the other Ado subtypes, it is involved in the modulation of ERK activity. Due to a long carboxy-terminal domain, the A_{2A}AR possesses a greater molecular mass (45 kDa) in comparison with the other subtypes (36-37 kDa). The A_{2A}AR C-terminus has been defined as a crowded place where various accessory proteins, such as D2-dopamine receptors, α-actinin, ADP-ribosylation factor nucleotide site opener, ubiquitin-specific protease 4 and translin-associated protein X, may interact. In fact, it is thought that the lack or the presence of such varied partners may explain the conflicting effects resulting from A_{2A}AR activation, for example, neuroprotection versus neurotoxicity (Sun et al., 2006). Recently, it has been demonstrated that agonist-bound

A_{2A}AR structures possess common GPCR activation features (Lebon et al., 2011). GPCRs have similar structures, consisting of 7TM helices containing well-conserved sequence motifs, indicating that they are activated by a common mechanism. Recently described structures of β-adrenoceptors highlight residues in TM region 5 (H5) that initially bind specifically to agonists rather than to antagonists, suggesting that these residues play an important role in agonist-induced activation of receptors. In this context, important information concerning the different interactions occurring between agonists and antagonists with the A_{2A}AR has recently come to light. In particular, agonists contain a ribose group that extends deep into the A_{2A}AR ligand binding pocket, where it forms polar interactions with conserved residues in H7 and non-polar interactions with residues in H3. In contrast, an inverse agonist fails to interact with any of these residues; indeed, comparison with agonist bound structures indicates that these compounds sterically prevent conformational change in H5, thereby acting as inverse agonists. Furthermore, comparison of agonist-bound structures of A_{2A}AR with agonist-bound structures of β-adrenoceptors has inferred that the contraction of the ligand-binding pocket caused by inward motion of helices 3, 5 and 7 may be a feature common to the activation of all GPCRs. It is evident that this detailed new structural information regarding these receptors has the potential to hugely influence rational drug design.

A_{2A}AR and the CNS

A_{2A}AR is found ubiquitously throughout the body, but its expression is particularly common in the immune system and the striato-pallidal system of the brain (Fredholm et al., 2001). A_{2A}AR localization in basal ganglia is restricted to the GABA-contained neurons of the indirect pathway projecting from the caudate putamen to the globus pallidus, which also selectively expresses the D_2 dopamine receptor and the peptide encephalin (Jenner et al., 2009). This explains why several studies have investigated the possible involvement of A_{2A}AR in the pathogenesis of neuronal disorders, including Huntington's chorea and Parkinson's disease (Varani et al., 2007; 2010; Simola et al., 2008; LeWitt et al., 2008; Ramlackhansing et al., 2011). In fact, changes in A_{2A}AR expression and signaling have been reported in various experimental models of Huntington's disease, and an aberrant amplification of A_{2A}-stimulated AC response has been demonstrated in striatal-derived cells engineered to express mutant Huntingtin protein. Moreover, a subsequent study also demonstrated an abnormal increase of A_{2A}AR density in the peripheral blood cells of Huntington's patients, as compared with age-matched healthy subjects (Varani et al., 2007). This suggests that the aberrant A_{2A}AR phenotype may represent a novel biomarker of Huntington's disease, which would be extremely useful in monitoring disease progression and

assessing the efficacy of novel neuroprotective approaches, not to mention providing grounds for further scientific investigation. Comparison of striatal A_{2A}AR binding and AC activity in one of the best-characterized animal models of Huntington's disease, R6/2 mice, in this case of different developmental ages, showed a transient increase in A_{2A}AR density and A_{2A}AR-dependent cAMP production at early presymptomatic ages with respect to age-matched wild-type animals (Varani et al., 2007). A_{2A}AR in the CNS is also been implicated in the modulation of motor functions. Hence, A_{2A}AR antagonists are a useful alternative to dopaminergic drugs in the treatment of Parkinson's disease (Simola et al., 2008). Accordingly, the A_{2A}AR antagonist istradefylline (8-[2(E)-(3,4-Dimethoxyphenyl)vinyl]-1,3-diethyl- 7-methylxanthine; KW-6002) has now been pre-registered as a Parkinson's disease treatment in North America by Kyowa Hakko Kirin (LeWitt et al., 2008). Indeed, it has been extensively demonstrated that A_{2A}AR antagonists have the potential to reverse motor deficits and enhance dopaminergic treatments in animal models of Parkinson's disease. Furthermore, istradefylline, for example, in combination therapy with levodopa or dopamine agonists, has been shown to improve the symptoms of the disease in a Parkinsonian monkey model without either increasing the incidence or severity of dopaminergic related side effects or triggering or aggravating dyskinesia. In particular, an upregulation of A_{2A}AR has been reported in Parkinson's patients with levodopa-induced dyskinesias (LIDs), suggesting that A_{2A}AR antagonists could be used, in combination with a reduction in the dosage of levodopa, to manage LIDs (Ramlackhansingh et al., 2011). The presence of an A_{2A}AR alteration in the postmortem putamen of Parkinson's disease patients as compared to healthy controls has also been demonstrated, confirming that A_{2A}AR plays a key role in this neurological pathology. Furthermore, a selective increase of A_{2A}AR density in the peripheral circulating cells of patients affected by Parkinson's disease has been observed. As a whole, these data show that A_{2A}AR alteration is a property common to both peripheral circulating cells and the putamen fraction in Parkinson's disease, confirming that lymphocytes or neutrophils could represent a mirror of the CNS (Varani et al., 2010). In addition, A_{2A}AR antagonists have been shown to attenuate neurotoxicity induced by kainite and quinolinate (Baraldi et al., 2008).

A_{2A}AR and the cardiovascular system

Ado has also been shown to confer important protective effects on the cardiovascular system. Regadenoson (2-[4-(Nmethylcarbamoyl)- 1H-pyrazol-4-yl]Ado), a short-acting, selective A_{2A}AR agonist, has already been approved as an adjunctive pharmacological stress agent for myocardial perfusion imaging studies, and was accordingly launched in the US in 2008 by Astellas Pharma. More recently, the safety and good tolerance of this drug, as well as the lack

of significant adverse cardiovascular events, has been demonstrated in post-heart transplant patients (Cavalcante et al., 2011). In addition, the results of a Phase III study, began in November 2009, to compare the safety and efficacy of Ado versus Apadenoson, another A_{2A}AR agonist, in single-photon emission CT myocardial perfusion imaging in patients with coronary artery disease is eagerly awaited in September 2011 (ClinicalTrials.gov Identifier: NCT00990327) (Kern et al., 2006; Bayes, 2007).

A_{2A}AR, inflammation and the immune system

Activation of the A_{2A}AR subtype on a wide range of cells, namely platelets, coronary smooth muscle cells, endothelial cells, monocytes/macrophages and foam cells, has been shown to result in vasodilation, neo-angiogenesis, inhibition of proinflammatory cytokine production and the reduction of plaque formation (Belardinelli et al., 1998; Varani et al., 2000; Gessi et al., 2000; Blackburn et al., 2009; Bingham et al., 2010). Substantial lines of evidence have suggested that the majority of anti-inflammatory effects of endogenous Ado are mediated by A_{2A}AR (Blackburn et al., 2009; Ohta and Sitkovsky, 2009). In particular, the dominant mechanism involved is likely to be the suppression of cytokine and chemokine expression by immune cells through A_{2A}AR activation. In particular, Ado regulates the production of tumor necrosis factor (TNF-α) and macrophage inflammatory proteins (MIP)-1 α, MIP-1β, MIP-2 α and MIP-3 α, acting via neutrophil A_{2A}AR (McColl et al., 2006). As previously mentioned, there is a particularly strong presence of A_{2A}AR in the immune system. Studies on A_{2A}knockout (KO) models have shown that A_{2A}AR activation inhibits interleukin (IL-2) secretion by naive $CD4^+$ T cells, thereby reducing their proliferation, which confirms the immunosuppressive effects of A_{2A}AR stimulation (Naganuma et al., 2006; Sevigny et al., 2007). Indeed, one of the mechanisms by which immunosuppression is induced is T-regulatory cell triggering of CD39 expression in order to generate Ado (Deaglio et al., 2007; Borsellino et al., 2007). Although A_{2A}ARs are generally viewed as negative regulators of immune cells, including activated T cells, it has recently been reported that A_{2A}AR activation by Ado protects $CD4^+$ T lymphocytes against activation-induced cell death. Because activation-induced cell death can be viewed as a process that terminates an immune response, the fact that it is prevented by A_{2A}AR indicates that A_{2A}AR activation can actually prolong immune processes, suggesting that the role of these receptors in regulating immune responses is more complex than previously thought. It is evident, then, that further studies aimed at determining the precise role of the antiapoptotic effect of A_{2A}AR activation in the regulation of T-cell-mediated immune responses are required (Himer et al., 2010). It has also been demonstrated that A_{2A}ARs play an important role in the promotion of wound healing and

angiogenesis (Ahmad et al., 2009; Ernens et al., 2010). Moreover, A_{2A}AR and A_3AR are responsible for the anti-inflammatory actions of methotrexate (MTX) in the treatment of inflammatory arthritis (Montesinos et al., 2006; Chan and Cronstein, 2010). In rheumatoid arthritis patients, Ado has been reported to suppress the elevated levels of pro-inflammatory cytokines, including TNF-α and IL-1β. In a recent study, an upregulation of A_{2A}AR and A_3AR receptors was found in lymphocytes and neutrophils obtained from early rheumatoid arthritis patients and MTX-treated patients. This alteration was associated with high levels of TNF-α and Nuclear Factor-KappaB (NF-kB) activation. Interestingly, the treatment with anti-TNF-α drugs normalized A_{2A}AR and A_3AR expression and functionality (Varani et al., 2009). These data consolidate the involvement of A_{2A}AR and A_3AR in rheumatoid arthritis and support the importance of these receptors in human diseases characterized by a marked inflammatory component. Activation of the A_{2A}AR during reperfusion of various tissues has been found to markedly reduce ischemia-reperfusion injury. In particular, in a model of ischemia-reperfusion injury in the lung, that is, A_{2A}AR stimulation with the selective agonist trans-4-[3-[6-amino-9-[(2R,3R,4S,5S)-5-(N-ethylcarbamoyl)- 3,4-dihydroxytetrahydrofuran-2-yl]-9H-purin- 2-yl]-2-propynyl]cyclohexanecarboxylic acid methyl ester (apadenoson) is associated with decreased inflammation and greatly protects mouse lung from injury, when administered at the time of reperfusion (Gazoni et al., 2010). It has been widely reported that hypoxia-induced accumulation of Ado may represent one of the most fundamental and immediate tissue-protection mechanisms, with A_{2A}AR triggering signals in activated immune cells. In these regulatory mechanisms, oxygen deprivation and extracellular Ado accumulation serve as “reporters”, while A_{2A}AR serve as “sensors” of excessive tissue damage (Sitkovsky et al., 2004). The hypoxia-adenosinergic tissue-protecting mechanism is provoked by inflammatory damage to blood vessels, interruption in oxygen supply, low oxygen tension (i.e., hypoxia) and by the hypoxia-driven accumulation of extracellular Ado acting via immunosuppressive, cAMP-elevating A_{2A}ARs (Sitkovsky, 2009).

A_{2A}AR and the digestive system

Another area where A_{2A}AR signaling has received attention as a potential therapeutic target is the gastrointestinal tract; studies have highlighted the protective effects of A_{2A}AR activation in various animal models of colitis, and these protective effects can be ascribed to two major mechanisms: decreased inflammatory-cell infiltration and increased activity of regulatory T cells (Nagamuna et al., 2006; Hasko and Pacher, 2008). A_{2A}AR stimulation was found, in rats, to attenuate gastric mucosal inflammation induced by indomethacin. This effect was

obtained by blocking secondary injury caused by stomach inflammation, through a reduction of myeloperoxidase and pro-inflammatory cytokines (Koizumi et al., 2009).

A_{2A}AR and the respiratory system

Increased Ado levels have been found in the lungs of individuals with asthma or COPD, and ARs are known to be expressed on most, if not all, inflammatory and stromal cell types involved in the pathogenesis of these diseases (Polosa and Blackburn, 2009). In addition, pharmacological treatment of allergic rats with an A_{2A}AR agonist has been shown to result in diminished pulmonary inflammation. Moreover, a recent study in an ADA deficient model has demonstrated that genetic removal of A_{2A}AR leads to enhanced pulmonary inflammation, mucus production and alveolar airway destruction (Mohsenin et al., 2007). Furthermore, A_{2A}AR induced on Invariant Natural killer T (iNKT) and NK cells can reduce pulmonary inflammation in mice with sickle-cell anemia, improving baseline pulmonary function and preventing hypoxia-reoxygenation-induced exacerbation of pulmonary injury (Wallace and Linden, 2010). These findings further confirm the involvement of A_{2A}AR in the anti-inflammatory networks of the lung. In addition, a study performed in peripheral lung parenchyma has demonstrated that ARs affinity and/or density are altered in patients with COPD, as compared to smokers with normal lung function. Moreover, a significant correlation was found between the density and affinity of ARs and the forced expiratory volume in 1s:forced vital capacity ratio, a widely used index of airflow obstruction. In particular, A_{2A}ARs, as well as A_3ARs, were found to be upregulated in COPD patients (Varani et al., 2006). This alteration may represent a compensatory response mechanism and could contribute to the anti-inflammatory effects mediated by stimulation of these receptors. Given the central role of inflammation in asthma and COPD, substantial preclinical research activity with the aim of understanding the function of A_{2A}AR in models of airway inflammation is underway. A list of A_{2A}AR ligands currently undergoing clinical trials as novel therapeutic treatments is reported in Table 2.

Table 2: Progress of A_{2A}AR ligands as novel therapeutic treatments (Gessi et al., Expert Opin. Investig. Drugs:20;1591-1609; 2011).

Pathology	Drug name	Type of ligand	Development status
Huntington's disease	[^{123}I]MNI-420	Antagonist	Phase I trial
Parkinson's disease	Istradefylline	Antagonist	Pre-registered
	Preladenant	Antagonist	Phase III trial
	ST-1535	Antagonist	Phase I trial
	SYN-115	Antagonist	Phase II trial
	[^{123}I]MNI-420	Antagonist	Phase I trial
Coronary artery disease diagnosis	Regadenoson	Agonist	Launched: 2008
	Apadenoson	Agonist	Phase III trial
	Binodenoson	Agonist	Pre-registered
Hypertension	YT-146	Agonist	Phase II trial
Sickle-cell disease	Regadenoson	Agonist	Phase I trial
COPD	Apadenoson	Agonist	Phase I trial
Asthma	Apadenoson	Agonist	Phase I trial
Ulcers diabetic	Sonedenoson	Agonist	Phase II trial

A_{2B} Adenosine Receptor

A_{2B}ARs have been cloned from the rat hypothalamus, human hippocampus and mouse mast cells. The tissue distribution of A_{2B}AR was initially reported in peripheral organs such as the bowel, bladder, lung and vas deferens. As for the brain, mRNA and protein have been detected in hippocampal neurons and glial cells, but not in microglial cells (Colgan et al., 2006). Following initial studies indicating selective induction of A_{2B}AR by hypoxia, analysis of the cloned human A_{2B}AR promoter identified within it a functional hypoxia-responsive region, including a functional binding site for hypoxia inducible factor 1 (HIF-1) (Kong et al., 2006, Yang et al., 2010).The same study demonstrated transcriptional coordination of A_{2B}AR by HIF-1α, and amplified Ado signaling during hypoxia, suggesting an important link between hypoxia and metabolic conditions related to inflammation and angiogenesis (Cohen et al., 2010). A_{2B}AR have long been known to couple to AC activation through G_s proteins. However, an association between A_{2B}AR and other intracellular signaling pathways, including Ca^{2+} mobilization through G_q proteins and MAPK activation, has been demonstrated (Colgan et al., 2006). A_{2B}AR-induced stimulation of PLC results in mobilization of intracellular calcium and promotion of IL-8 production in human mast cells (HMC)-1 cells (Feoktikov and Biaggioni, 1995). Stimulation of A_{2B}AR mediates the release of IL-6 from astrocytes. Due to the neuroprotective effect of IL-6 against hypoxia and glutamate neurotoxicity, activation of A_{2B}AR subtype provides a damage-control mechanism

during CNS injury (Hasko et al., 2005). Functional studies have identified A_{2B}AR in airway smooth muscle, fibroblasts, glial cells, gastrointestinal tract, vasculature and platelets.

A_{2B}AR and the cardiovascular system

Vascular A_{2B}AR may be associated with vasodilatation in both smooth muscle and the endothelium; this subtype plays a particularly important role in the modulation of vasodilatation in certain vessels such as the mesenteric, pulmonary and coronary arteries, but not in others where the A_{2A}AR effect predominates (Feng and Navar, 2010). In juxtamedullary afferent arterioles, both A_{2A}AR and A_{2B}AR are functionally expressed, and via the latter, the powerful vasodilatory action of Ado is exerted, counteracting A_1AR-mediated vasoconstriction (Wakeno et al., 2006). Furthermore, in mIMCD-K2 cells, a murine model system for the renal inner medullary collecting duct, Ado stimulates Cl^- secretion through the cystic fibrosis transmembrane conductance regulator by activating apical A_{2B}AR and signaling through cAMP/PKA. This suggests that the A_{2B}AR pathway may provide one mechanism for enhancing urine NaCl excretion in the setting of high dietary NaCl intake (Rajagopal et al., 2010; Philipp et al., 2006). Activation of A_{2B}AR may also prevent cardiac remodeling after myocardial infarction (Kuno et al., 2007). Protection from infarction has been also attributed to A_{2B}AR in ischemic post-conditioning, through a pathway involving PKCε and Phosphoinositide 3-kinase (PI3K) (Kuno et al., 2008; Methner et al., 2010; Koda et al., 2010; Yang et al., 2010a). Furthermore, A_{2B}AR/A_3AR are known to mediate the cardioprotective effects induced by ischemic pre-conditioning through PKCε, aldehyde dehydrogenase type-2 (ALDH2) activation and renin inhibition (Koda et al., 2010). Finally, a new role for the A_{2B}AR has been discovered in the regulation of platelet function. In particular, upregulated A_{2B}AR have been found to modulate ADP receptor expression and inhibit agonist-induced aggregation in platelets under stress in vivo (Yang et al., 2010b).

A_{2B}AR and the digestive system

According to mRNA analysis, which has revealed large amounts of A_{2B}AR in the cecum and large intestine, A_{2B}AR trigger an increase in cAMP levels in intestinal epithelial cells, which in turn provokes Cl^- secretion. This pathway results in the movement of isotonic fluid into the lumen, a process that naturally serves to hydrate the mucosal surface but, in extreme cases, produces secretory diarrhea (Strohmeier et al., 1995). Moreover, it has recently been reported that Ado increases HCO_3^- secretion in intact epithelium in vivo through the activation of A_{2B}ARs expressed in the brush border membrane of duodenal villi (Ham et al., 2010). A_{2B}AR stimulation of intestinal epithelial cells, by increasing intracellular cAMP levels, also leads to

IL-6 transcription via activation of the Activating transcription factors (ATF) and CREB and CCAAT/enhancer-binding protein beta (C/EBPb) (NF-IL-6) transcription factor systems. The physiological relevance of this response lies in the fact that it provides an amplification mechanism for intestinal inflammation, as neutrophils transmigrating through the epithelial cell layer release Ado, which in turn induces the production of the neutrophil-activating IL-6. This amplification loop is further enhanced by the rapid increase in the surface expression of A_{2B}AR that occurs after stimulation of these cells with Ado, a phenomenon made possible by the prompt recruitment of preformed A_{2B}AR from intracellular stores (Sitaraman et al., 2001). Furthermore, epithelial A_{2B} mRNA and protein have been found to be upregulated via TNF-α in colitis, through a posttranscriptional mechanism involving microRNA (Kolachala et al., 2010). Accordingly, A_{2B}AR gene deletion has been found to attenuate murine colitis in mice (Kolachala et al., 2008). Conversely, however, recent studies combining pharmacological and genetic approaches have demonstrated that Ado signaling via the A_{2B}AR dampens mucosal inflammation and tissue injury during experimental colitis or intestinal ischemia (Frick et al., 2009). It has also been reported that A_{2B}AR play a central regulatory role in IL-10 modulation during the acute inflammatory phase of dextran sodium sulfate colitis, thereby implicating the A_{2B}AR expressed on intestinal epithelial cells as an endogenously protective protein (Eltzschig et al., 2009). The reason for these conflicting results is not clearly understood, although possible explanations may include inter-study disparity in colitis protocol, A_{2B}AR-deleted murine strains or animal housing conditions, the last leading to, for example, differences in the bacterial flora of the mice. Hence, additional comparison of the individual mouse strains tested is necessary if some of these apparent discrepancies are to be rectified.

A_{2B}AR and the respiratory system

Recently, A_{2B}ARs have been implicated in the mediation of several pro-inflammatory effects of Ado in inflammatory cells of the lung. A_{2B}ARs have been reported to mediate degranulation and activation of canine mastocytoma and HMCs, thereby potentially playing a role in allergic and inflammatory disorders (Polosa and Blackburn, 2009). Ado constricts the airways of asthmatic patients through the release of histamine and leukotrienes from sensitized mast cells (Hasko et al., 2009); although the receptor involved seems to be the A_3R in rats, it is the A_{2B}AR that is implicated in humans. Accordingly, A_{2B}AR antagonists potently inhibit the activation and degranulation of HMCs induced by Ado (Sun et al., 2006). In addition to mast cells, functional A_{2B}ARs have been found in bronchial smooth muscle cells and lung fibroblasts. In these cells, Ado, through stimulation of the A_{2B} subtype, increases the release of various inflammatory cytokines, lending weight to evidence that A_{2B}ARs play a key

role in the inflammatory response associated with asthma. Furthermore, it has been reported that, through A_{2B}AR activation, Ado-differentiated dendritic cells have impaired allostimulatory activity and express high levels of angiogenic, pro-inflammatory, immune suppressor and tolerogenic factors, including vascular endothelial growth factor (VEGF), IL-8, IL-6, IL-10, cyclooxygenase (COX-2), Transforming growth factor beta (TGF-β) and indoleamine 2,3-dioxygenase (Ben Addi et al., 2008; Novitskiy et al., 2008). Moreover, using ADA KO animals, it has been shown that Dendritic Cells (DCs) with a pro-angiogenic phenotype are highly abundant in vivo under conditions associated with elevated levels of extracellular Ado. The first evidence for the involvement of A_{2B}AR in asthma was provided by studies concerning the selectivity of enprofylline, a methylxanthine structurally related to theophylline (Feoktisov et al., 1998), and further support came from research demonstrating the presence of A_{2B}AR on various type of cells involved in cytokine release in asthmatic disease, such as smooth muscle cells, lung fibroblasts, endothelial cells, bronchial epithelium and mast cells. Expression of A_{2B}AR has also been found in the mast cells and macrophages of patients affected by COPD (Varani et al., 2006). In another study, activation of A_{2B}AR in the HMC-1 mast cell line provoked an increase in IL-8 release in vitro (Feoktistov et al., 1995).

A_{2B}AR and inflammation

Recently, it has been reported that ADA-deficient mice treated with the selective A_{2B}AR antagonist 3-ethyl-1-propyl- 8-[1-[3-(trifluoromethyl)benzyl]-1H-pyrazol-4-yl]xanthine (CVT-6883) showed reduced elevations in pro-inflammatory cytokines and chemokines as well as mediators of fibrosis and airway destruction (Yang et al., 2006). Interestingly, other authors have investigated the role of A_{2B}AR in inflammation in vivo (Schingnitz et al., 2010). In particular, a study carried out on A_{2B}AR KO mice, in which exon 1 of the A_{2B}AR was replaced by a reporter gene, consented examination of endogenous A_{2B}AR expression in various tissues and cell types. The results of this study show that there is abundant reporter expression in the vasculature and in macrophages. This new animal model emphasizes a role for the A_{2B}AR not only in attenuating inflammation through the regulation of pro-inflammatory cytokine production, but also in inhibiting leukocyte adhesion to the vasculature. Contrasting with the function of A_{2B}AR in vasodilation, the A_{2B}AR KO mice have normal blood pressure (Yang et al., 2006). This apparent contradiction between pro- and anti-inflammatory effects exerted by A_{2B}ARs may be related to differences between acute and chronic inflammation, that is, an A_{2B}AR agonist may protect against acute endotoxin-mediated lung toxicity while chronic accumulation of Ado could induce lung lesions. Studies

with a specific $A_{2B}AR$ agonist, 2-[6-amino-3,5- dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl] acetamide (BAY 60-6583), have demonstrated attenuation of lung inflammation and pulmonary edema in wild type but not in $A_{2B}AR$ KO mice, thereby suggesting the $A_{2B}AR$ as a potential therapeutic target in the treatment of endotoxin-induced forms of acute lung injury (Schingnitz et al., 2010). Furthermore, the dependence of epithelial ciliary motility and pulmonary clearance on $A_{2B}AR$ activation has recently been reported (Allen-Gipson et al., 2011).

$A_{2B}AR$ and cancer

$A_{2B}ARs$ play a role in cancer development by modulating both anti- and pro-tumoral effects. In particular, A_{2B} receptor stimulation inhibits ERK1/2 phosphorylation in breast cancer cells, whilst it increases angiogenesis, proliferation, IL-8, VEGF and basic fibroblast growth factor in endothelial, foam and tumor cells (Gessi et al., 2010a). Recently, it has been reported that hypoxia-induced apoptosis of T cells is mediated by $A_{2A}AR$ and $A_{2B}AR$, and that blocking the $A_{2A}AR$ signaling pathways can increase the anti-apoptotic function of T cells; this appears to suggest a new strategy for improving anti-tumor defences (Sun et al., 2010). A list of $A_{2B}AR$ ligands currently undergoing clinical trials as novel therapeutic treatments is reported in Table 3.

Table 3: Progress of $A_{2B}AR$ ligands as novel therapeutic treatments (Gessi et al., Expert Opin. Investig. Drugs:20;1591-1609; 2011).

Pathology	Drug name	Type of ligand	Development status
Asthma	CVT-6883	Antagonist	Phase I trial
	GS-6201	Antagonist	Phase I trial
Diarrhea	CVT-6883	Antagonist	Phase I trial
	CVT-6883	Antagonist	Phase I trial

A_3 Adenosine Receptor

The A_3AR is the only Ado subtype to be cloned before its pharmacological identification. It was originally isolated as an orphan receptor from rat testes that possessed 40% sequence homology with canine A_1 and A_{2A} subtypes. Homologues of the rat striatal A_3AR have been cloned from sheep and humans showing, however, a large interspecies difference in A_3 structure, that is, the rat A_3AR possesses only 74% sequence homology with sheep and humans (Baraldi et al., 2008; Baraldi and Borea, 2000). A_3AR activation inhibits AC activity

by coupling with G_i proteins. In the rat mast cell line RBL-2H3 and rat brain, A_3AR stimulation activates PLC through G_q proteins. Moreover, in some cells A_3AR may also activate the MAPK signaling pathway, which is critical to the regulation of cell proliferation and differentiation (Raman et al., 2007). The A_3AR is widely distributed and its mRNA is expressed in testis, lung, kidneys, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, proximal colon, eye of rat, sheep and humans (Jacobson, 1998; Gessi et al., 2008).

A_3AR and the CNS

Interestingly, a dual role of A_3AR has been reported in the brain. In particular, it seems that chronic pre-ischemic administration of the agonist 1-deoxy-1-[6-[[(3-iodophenyl)methyl] amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide (CF-101, IB-MECA) induces significant neuronal protection and reduction of the subsequent mortality, in contrast with the pronounced worsening of neuronal damage and post-ischemic mortality which accompanies acute administration of the drug. Furthermore, A_3ARs also seem to play a role in a number of CNS functions, as revealed by mice featuring functional deletions of the A_3AR, including nociception, locomotion, behavioral depression and neuroprotection. Consistent with previous reports of the neuroprotective actions of A_3AR agonists, A_3AR KO mice show an increase in neurodegeneration in response to repeated episodes of hypoxia, thereby suggesting that A_3AR agonists may be useful in the treatment of ischemic and degenerative conditions of the CNS (Fedorova et al., 2003).

A_3AR and the cardiovascular system

To date, several studies have provided evidence to support the theory that activation of A_3AR is crucial for cardioprotection during and following ischemia-reperfusion, and it is likely that a considerable proportion of the Ado-mediated cardioprotective effects, once largely attributed to the A_1AR, may now be in partly ascribable to A_3AR activation (Ge et al., 2006). In fact, the cardioprotective effects of low levels of A_3AR have been detected in transgenic mice, which showed no adverse effects, although higher levels of A_3AR expression did lead to the development of dilated cardiomyopathy (Black et al., 2002). Similar data were observed in the case of A_1AR overexpression (Funakoshi et al., 2006). The molecular mechanism of A_3AR cardioprotection has been attributed to regulation of K_{ATP} channels. Moreover, as previously mentioned, a signaling cascade initiated by A_{2B}/A_3 subtypes that triggers PKC-mediated ALDH2 activation in cardiac mast cells contributes to IPC-induced cardioprotection by preventing mast cell renin release and the dysfunctional consequences of local renin angiotensin system (RAS) activation. Thus, unlike classic IPC, in which cardiac

myocytes are the main target, cardiac mast cells are the critical site for the development of the cardioprotective anti-RAS effects of IPC (Koda et al., 2010). A role of NO in A_3AR -mediated cardioprotection has been also reported. In particular, the involvement of inducible NO synthase (iNOS) as a downstream effector of the PI3K signaling cascade after activation of A_3AR at reperfusion has been demonstrated (Karjian et al., 2006; 2008; Hussain et al., 2009). Furthermore, A_3AR stimulation restores vascular reactivity after hemorrhagic shock through a ryanodine receptor-mediated and calcium-activated potassium channel-dependent pathway (Zhou et al., 2010). Recently, it has been shown that Ado in hypoxic foam cells stimulates HIF-1α accumulation by activating all ARs. HIF-1α modulation appears to involve ERK1/2, p38 MAPK and Akt phosphorylation in the case of A_1AR, A_{2A}AR and A_{2B}AR, while only ERK 1/2 activation is implicated in the case of A_3AR. Furthermore, Ado, through the activation of A_3AR and A_{2B}AR, stimulates VEGF secretion in a HIF-1α-dependent way. Finally, Ado stimulates foam cell formation, and this effect is strongly reduced by A_3AR and A_{2B}AR blockers and by HIF-1α silencing. This study provides the first evidence that A_3AR, A_{2B}AR mixed A_3/A_{2B} antagonists may be useful in blocking important steps in Ado-induced atherosclerotic plaque development (Gessi et al., 2010a).

A_3AR and the respiratory system

In addition to reducing injury in myocardial and vascular tissues, other beneficial anti-inflammatory actions have been attributed to the A_3 subtype, with particular relevance to the respiratory system. For example, A_3ARs are expressed in human neutrophils where, together with A_{2A}AR, they are involved in the reduction of superoxide anion generation; they have also been implicated in the suppression of TNF-α release induced by endotoxin from human monocytes (Gessi et al., 2002). In neutrophils, however, A_3ARs also play a role in chemotaxis, in conjunction with P2Y receptors (Chen et al., 2006, Linden, 2006). Moreover, A_3AR activation seems to inhibit degranulation and superoxide anion production in human eosinophils. Indeed, transcript levels for the A_3 subtype are elevated in the lungs of asthma and COPD patients, where expression is localized to eosinophilic infiltrates. Similar evidence has also been observed in the lungs of ADA KO mice exhibiting Ado-mediated lung disease. Treatment of ADA KO mice with 3-propyl-6-ethyl- 5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS 1523), a selective A_3AR antagonist, prevented airway eosinophilia and mucus production (Young et al., 2004). Nevertheless, these findings contrast sharply with the results of experiments performed in human eosinophils ex vivo, where chemotaxis was reduced by A_3AR activation, suggesting that significant differences exist between the impact of A_3AR signaling on eosinophil migration ex vivo and in the whole

animal (Ezeamuzie and Philips, 1999). More recently, the involvement of the A_3AR in a bleomycin model of pulmonary inflammation and fibrosis has been explored. Results demonstrated that A_3AR KO mice exhibit enhanced pulmonary inflammation that involves an increase in eosinophils. Accordingly, a selective upregulation of eosinophil-related chemokines and cytokines was seen in the lungs of A_3AR KO mice exposed to bleomycin, thereby suggesting that the A_3AR performs anti-inflammatory functions in the bleomycin model (Morschl et al., 2008). Nonetheless, the role of the A_3AR in the human lung, and indeed in asthma, still remains to be clarified. In general, receptor knockouts have provided significant new insights into Ado's control of complex physiological (e.g., cognition) and pathological (e.g., neuroinflammation) phenomena, suggesting that further studies in these animal models would help in obtaining a clearer picture of the role of A_3AR in inflammatory lung disease. What is clear, however, is that the expression of the A_3AR in asthmatic airways is predominantly located in eosinophils (Brown et al., 2008a; Gessi et al., 2008; Wilson et al., 2008).

A_3AR and cancer

A_3AR ligands appear to have found very interesting applications in cancer therapies, and the possibility that A_3AR plays a role in the development of cancer has aroused considerable interest in recent years (Merighi et al., 2003; Gessi et al., 2011). The A_3 subtype has been implicated in regulation of the cell cycle, and both pro- and antiapoptotic effects have been reported, depending on the level of receptor activation (Jacobson, 1998; Merighi et al., 2005a; Kim et al., 2010; Gessi et al., 2007; Taliani et al., 2010; Varani et al., 2011). Furthermore, the involvement of A_3AR activation in inhibition of tumor growth has been demonstrated both in vitro and in vivo, leading to clinical trials being developed to test the efficacy of A_3AR agonists in cancer treatment. The molecular mechanisms involved in the anticancer effects induced by A_3AR agonists include regulation of the WNT pathway (Fishman et al., 2004), and it has been reported that Ado upregulates HIF-1α protein expression and VEGF protein accumulation by activating the A_3 subtype in tumoral cells (Merighi et al., 2005b, 2006, 2007a, 2007b). In contrast, A_3AR blockade by etoposide and doxorubicin potentiates inhibition of VEGF secretion and affects HIF-1 expression in human melanoma cancer cells. This finding appears to infer the possibility of using AR antagonists to improve the ability of chemotherapeutic drugs to block angiogenesis (Merighi et al., 2009). The reason why both agonists and antagonists appear to be useful anticancer drugs in in vitro studies is not clear. It may be that the different ligands provoke the same response as a consequence of a desensitization process undergone by the receptor after agonist stimulation, or different

experimental conditions linked to oxygen concentrations may be implicated. Hence, A_3AR antagonists should be tested on animal cancer models in order to shed light on this important issue. As regards studies on human tissues, overexpression of the A_3 subtype has been demonstrated, as compared to normal mucosa, in colon cancer samples obtained from patients undergoing surgery. This overexpression was also reflected in peripheral blood cells, thereby rendering this Ado subtype a possible marker for cancer detection (Gessi et al., 2004a, 2004b). Moreover, in a further study, it has been shown that A_3AR mRNA expression is upregulated in hepatocellular carcinoma (HCC) tissues in comparison to adjacent normal tissues (Bar-Yehuda et al., 2008). Remarkably, upregulation of A_3AR was also noted in Peripheral blood mononuclear cells (PBMCs) derived from these HCC patients with respect to levels in healthy subjects. These results provide further evidence that A_3AR in PBMCs reflects receptor status in remote tumor tissue and may, therefore, represent a useful biomarker (Gessi et al., 2004b). The A_3AR also reduces the ability of prostate cancer cells to migrate in vitro and metastasize in vivo. In particular, it has been reported that activation of the A_3AR in these cells reduces PKA-mediated stimulation of ERK1/2, leading to lower levels of NADPH oxidase activity and cancer cell invasiveness (Jajoo et al., 2009). In a different study, the biological functions of Ado in Matrix Metallopeptidase 9 (MMP-9) regulation in U87MG human glioblastoma cells were investigated, revealing an increase of glioblastoma cell invasion (Gessi et al., 2010b). In this case, A_3AR stimulation induced an increase of MMP-9 levels in U87MG cells by phosphorylation of ERK1/2, c-Jun N-terminal kinases (c-JNK)/stress-activated protein kinase, protein kinase B (PKB)/Akt and activator protein 1 (AP-1). A_3AR is thus far the only AR to have been involved in “in vivo” cancer studies (Press et al., 2007), and the principal findings are summarized below. Studies performed to date include syngeneic, xenograft, orthotopic and metastatic experimental animal models utilizing IB-MECA and 2-chloro-N6-(3-iodobenzyl)Ado-5¢-Nmethylcarboxamide (CF-102, Cl-IB-MECA) as the therapeutic agents in melanoma, colon, prostate and HCCs. In all experimental animal models, A_3AR agonists were administered orally thanks to their stability and bioavailability profile. Oral administration of 10-100 μgkg^{-1} IB-MECA and Cl-IB-MECA once or twice daily inhibited the growth of primary B16-F10 murine melanoma tumors in syngeneic models. Moreover, in an artificial metastatic model, IB-MECA inhibited the development of B16-F10 murine melanoma lung metastases. The specificity of the response was demonstrated by the administration of an A_3AR antagonist, which reversed the effect of the agonist. Furthermore, in combination with the chemotherapeutic agent cyclophosphamide, both IB-MECA and Cl-IB-MECA induced an additive antitumoral effect on the development of B16-F10 melanoma lung metastatic foci.

Oral administration of 10-100 μgkg^{-1} IB-MECA once or twice daily also inhibited the growth of primary CT-26 colon tumors. Furthermore, in xenograft models, IB-MECA inhibited the development of HCT-116 human colon carcinoma and PC3 human prostate carcinoma in nude mice. In these studies, the combined treatment of IB-MECA and 5-fluorouracil resulted in an enhanced antitumoral effect. Additionally, IB-MECA has also been shown to increase the cytotoxic index of taxol in PC3 prostate carcinoma-bearing mice and appears to be an efficacious inhibitor of liver metastases of CT-26 colon carcinoma cells inoculated in the spleen. Finally, Cl-IB-MECA treatment apparently inhibits hepatocellular tumor growth in a dose-dependent fashion (Bar-Yehuda et al., 2008; Fishman et al., 2009).

A_3AR and inflammation

In addition, a role for A_3AR in inflammation has been reported in literature. In arthritis, A_3AR activation shows beneficial effects by suppression of TNF-α production (Fishman et al., 2009). A_3ARs suppress TNF-α release induced by endotoxin CD14 receptor signal transduction pathway from human monocytes and murine J774.1 macrophages. 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 AP-1 transcription factor, whereas it was independent on MAPK, NF-kB, PKA, PKC and PLC. This was not confirmed in BV2 microglial cells where A_3-mediated inhibition of LPS-induced TNF-α expression was associated with the inhibition of LPS-induced activation of PI3K/Akt and NF-kB pathway (Lee et al., 2006a). The inhibitory effect induced by A_3AR on TNF-α production was also assessed in A_3KO mice, where the A_3AR agonist was unable to reduce TNF-α levels in contrast with its effect in wild-type animals (Salvatore et al., 2000). 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_3KO 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}AR and A_{2B}AR agonists as recently demonstrated also in human monocytes (Hasko et al., 2007). The discrepancy observed among these papers might not depend on species differences, being in both cases mouse cells, but by other factors including the source of the cells and/or the inflammatory stimulus used. However, in spite of these contrasting results, one of the best potential therapeutic applications of the regulatory role of A_3AR activation on TNF-α release has been found in the treatment of arthritis. A_3AR agonists exert significant antirheumatic effects in different autoimmune arthritis models by

suppression of TNF-α production. The molecular mechanism involved in the inhibitory effect of IB-MECA on adjuvant-induced arthritis included receptor downregulation and deregulation of the PI3K-NF-kB signaling pathway (Fishman et al., 2006; Madi et al., 2007). Previous studies also demonstrated that A_3AR activation inhibited MIP-1α, which is a C-C chemokine with potent inflammatory effects, in a model of collagen induced arthritis, providing the first proof of concept of the utility of Ado agonists in the treatment of arthritis (Gessi et al., 2008). In agreement with an anti-inflammatory role for the A_3AR, it has been recently demonstrated that A_3AR activation decreases mortality and renal and hepatic injury in murine septic peritonitis (Lee et al., 2006b). Higher levels of endogenous TNF-α were observed in A_3KO mice after sepsis induction, in comparison to wild-type animals and IB-MECA significantly reduced mortality in mice lacking the A_1AR or A_{2A}AR but not the A_3AR, demonstrating specificity of the A_3AR agonist in activating A_3 subtype and mediating protection against sepsis induced mortality (Lee et al., 2006b). Recently, in a mouse model of cecal ligation and puncture-induced sepsis, A_3AR blockade reduces acute lung injury and polymorphonuclear leukocytes accumulation in lung tissue (Inoue et al., 2010). A similar mortality reduction associated with a decrease of IL-12 and Interferon (IFN-γ) production induced by A_3AR activation was observed in endotoxemic mice. In addition, a reduced inflammation and increased survival following A_3AR activation in two murine models of colitis has been reported. Furthermore, a protective role for A_3AR in lung injury following in vivo reperfusion has been observed (Matot et al., 2006). This effect has been attributed to the stimulation of A_1AR, A_{2A}AR and A_3AR leading to increased lung compliance and oxygenation, decreased pulmonary artery pressure, decreased neutrophil infiltration, decreased edema and reduced TNF-α production (Gazoni et al., 2010). ARs have been implicated in many ocular and systemic ischemic diseases (e.g., retinal ischemia). The A_3 KO mouse showed lower intracellular pressure suggesting a role for A_3AR antagonists in the therapy of glaucoma (Yang et al., 2005). Accordingly, nucleoside-derived antagonists to A_3ARs lower mouse intraocular pressure and act across species (Wang et al., 2010). Furthermore, retinal ganglion cells express A_3AR. Agonists for the A_3AR prevented the Ca^{2+} rise and cell death, which accompanied activation of the P2X7 and NMDA receptors suggesting a neuroprotective potential of A_3AR agonists for glaucoma treatment. These findings have been confirmed in in vivo experiments (Zhang et al., 2006a; 2006b, 2010; Hu et al., 2010). Importantly, studies from Phase II clinical trial reveal that CF-101, given orally, was well tolerated and induced a statistically significant improvement in patients with moderate to severe dry eye syndrome. These data and the anti-inflammatory characteristic of CF-101 support further studies of the drug as a potential treatment for the signs and symptoms

of dry eye syndrome (Avni et al., 2010). A list of A_3AR ligand candidates for novel therapeutic treatments currently in clinical studies is reported in Table 4.

Table 4: Progress of A_3AR ligands as novel therapeutic treatments (Gessi et al., Expert Opin. Investig. Drugs:20;1591-1609; 2011).

Pathology	Drug name	Type of ligand	Development status
Osteoarthritis	CF-101	Agonist	Phase II trial
Rheumatoid arthritis	CF-101	Agonist	Phase I trial
Hepatocellular carcinoma	CF-102	Agonist	Phase I–II trials
Dry eye syndrome	CF-101	Agonist	Phase II trial
Glaucoma	CF-101	Agonist	Phase II trial
Uveitis	CF-101	Agonist	Phase I trial
Liver disorders	CF-102	Agonist	Phase I trial
Hepatitis C	CF-102	Agonist	Phase I–II trials
Psoriasis	CF-101	Agonist	Phase II–III trial

Conclusion

In conclusion, a considerable amount of data from in vitro and in vivo experiments suggest that AR ligands regulate the course of numerous diseases and conditions (Tables 1,2,3,4), including pain, cancer, inflammation, neurological, cardiovascular and respiratory diseases, making ARs potential targets for therapeutic intervention in several pathological conditions.

The investigation of ARs and their ligands is a rapidly growing field; there is now extensive evidence for the involvement of ARs in the physiological regulation of several homeostatic processes and their implication in the etiology of many diseases. It is understandable, therefore, that these compounds are having an increasing impact on the drug discovery and development process. Indeed, over the past 30 years, a considerable body of research has resulted in the identification of clinical candidates for AR agonism, partial agonism and antagonism (Figure 4).

Furthermore, it is exciting to note that several molecules from the purine world have completed the long road of drug development. In particular, regadenoson is already commercially available, while binodenoson (2-[2 (cyclohexylmethyl)hydrazino]Adenosine) and istradefylline have now been pre-registered. Furthermore, other molecules such as tecadenoson, rolofylline, tonapofylline (3-[4-(1,3-dipropylxanthin-8-yl)bicyclo[2.2.2] oct-1-yl]propionic acid) and CF-101 are at an advanced stage of clinical trials and, with luck, could soon become widely available. Despite this encouraging progress, however, many issues surrounding ARs still remain to be clarified. In fact, the opportunities for further research are almost boundless as all four ARs have been detected in association with a host of mammalian tissues and appear to control the pathogenesis of inflammatory and degenerative diseases in

many organ systems. This offers ripe opportunities for the development of AR agonists/antagonists for multiple indications, but also presents the challenge of making sure these compounds are devoid of side effects due to the action of AR subtypes on other organ systems. Fortunately, however, recent developments in the field of ARs agonists and antagonists have helped scientists to design and develop more specific and safer lead and back-up candidates for clinical development. Whereas the majority of these novel molecules are being considered as therapeutic agents for a range of pathological conditions, including Parkinson's disease, neurological disorders, stroke, heart and renal failure, asthma and cancer, others are aimed at basic scientific applications, such as receptor probes and radioligands (Varani et al., 2000a; Gessi et al., 2005a). Indeed, based on the significant scientific and clinical advances summarized in this thesis, purine scientists do seem to be getting closer to their goal: the incorporation of Ado ligands into drugs with the ability to improve human health and even save lives.

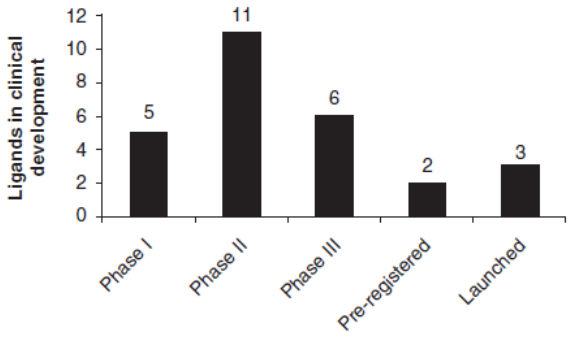

Figure 4: Bar graph showing the progress of ARs ligands in clinical development (Gessi et al., Expert Opin. Investig. Drugs:20;1591-1609; 2011).

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Downregulation of A_1 and A_{2B} ARs

in human trisomy 21 mesenchymal cells from first-trimester chorionic villi

Introduction

15% of human pregnancies are known to end in spontaneous abortion (SA) before 12 weeks of gestation, and immunity, angiogenesis and apoptosis-related genes have all been implicated. In aneuploidy, however, the reported percentage of SA is much higher (Vesce et al., 2002). One possible reason could be that the chromosomal abnormality itself leads to miscarriage, but if this is the case, the pathogenic mechanism is still unknown. It has also been suggested that the causes of SA in aneuploidy are no different to those in euploidy, with the increased frequency in the former perhaps being ascribable to a genetically-determined imbalance in the mediators of placental perfusion and uterine contraction (Vesce et al., 2002). In this scenario, mediators such as VEGF and NO may be involved; indeed, a critical role has been reported for both in placental angiogenesis (Levine et al., 2004; Carmeliet et al., 1996). During gestation, angiogenesis occurs extensively in the placenta and villi to supply the fetus with oxygen and nutrition. This vascular development during embryonic and fetal growth in utero is triggered by hypoxia, a condition that is also known to increase the levels of Ado (Simon and Keith, 2008). This important hormone is locally released from metabolically active cells, or generated extracellularly by the degradation of ATP. Acting through its AR subtypes A_1, A_{2A}, A_{2B} and A_3, this nucleoside has been shown to regulate a wide variety of physiological processes, including angiogenesis in hypoxic tissues (Fredholm et al., 2011). In particular, Ado plays an important role in the regulation of VEGF from placental villi in hypoxic conditions, and it also increases NO be a major factor in maintaining normal fetoplacental function (Escudero and Sobrevia, 2008; George et al., 2010).

Angiogenesis

During normal pregnancy, dramatically increased feto- and utero-placental blood flows are highly correlated with fetal growth and survival as well as neonatal birth weights and survivability (Rosenfeld et al., 1974; Reynolds and Redmer, 1995; Reynolds et al., 2005). These increased blood flows are primarily caused by angiogenesis, vasodilatation, and vascular remodeling (Barcroft and Barron, 1946; Magness and Zheng, 1996; Osol and Mandala, 2009). Two potent angiogenic factors, fibroblast growth factor 2 (FGF2) and

VEGFA, are key factors regulating placental vascular growth, vasodilatation, and vascular remodeling (Klagsbrun and D'Amore, 1991; Ferrara et al., 2003; Osol and Mandala, 2009). Local endothelial production of vasodilators such as NO is increased during pregnancy in temporal association with increases in the production of placental angiogenic factors and may play an active role in the integral regulation of placental angiogenesis and vasodilatation (Myatt, 1992; Magness and Zheng, 1996; Sladek et al., 1997; Magness, 1998; Szukiewicz et al., 2005; Brownbill et al., 2007; Sprague et al., 2010). VEGFA is also a member of a family of structurally homologous growth factors with a potent angiogenic activity for vascular endothelial cells (Ferrara et al., 2003). Biological actions of VEGFA are initiated upon binding to its high affinity receptors including VEGFR1 (Flt1) and VEGFR2 (Gille et al., 2000; Ferrara et al., 2003). VEGFR2 is the major signal transducer of VEGFA, responsible for mediating VEGFA-stimulated major steps of angiogenesis (endothelial cell proliferation and migration) and vasodilatation, whereas VEGFR1 may inhibit VEGFR2-mediated endothelial functions (Gille et al., 2000; Ferrara et al., 2003). However, knocking down either of these receptors in the mouse impairs vascular growth and development during the early embryonic stage, ultimately leading to embryonic death, indicating that both VEGFR1 and VEGFR2 are important for vascular formation and growth during early embryonic stage (Fong et al., 1995; Shalaby et al., 1995). Over the past two decades, it has become clear that apart from being a potent vasodilator (Dulak and Jozkowicz, 2003), NO is also a key mediator of angiogenesis (Bussolati et al., 2001; Hida et al., 2004). Expression of endothelial NO synthase (eNOS) and iNOs has been identified in the placenta of human, rhesus monkey, rat, and sheep (Conrad et al., 1993; Zarlingo et al., 1997). In association with robust fetoplacental angiogenesis (Reynolds and Redmer, 1995; Magness and Zheng, 1996), the NO level was increased in maternal circulation as pregnancy progresses in sheep (Vonnahme et al., 2005), and in late human pregnancy (Williams et al., 1997; Wang and Zheng, 2012).

Adenosine and Angiogenesis in pregnancy

The role of Ado in the placental vessels remains poorly investigated. Reid (Reid et al., 1990) reported that Ado has a biphasic response in the ovine fetal placental vasculature, an observation that may indicate the expression of a mixed ARs population along the placental vasculature or signaling mechanisms at variance. On the other hand, ATP contracts superficial

chorionic vessels from the human placenta (Huidobro and Valdecantos, 2000; Valdecantos et al., 2003). Considering that ATP is rapidly inactivated by releasable ectonucleotidases (Westfall et al., 2002), the possibility exists that even though the placenta lacks sympathetic perivascular nerves, the ATP released to the fetal-placental circulation will be rapidly degraded to Ado and modulate the ATP vasomotor response. The Ado-induced vasoconstriction of human chorionic vessels is mediated by the activation of A_{2B}AR, an effect that appears to be related to the synthesis of an arachidonate metabolite that ultimately activates a thromboxane receptor in the vascular smooth muscles (Donoso et al., 2005). Ado stimulates endothelial cell proliferation and migration via activation of A_{2A} and/or A_{2B} ARs involving increased expression of angiogenic factors, including VEGF (Feoktistov et al., 2004; Adair, 2005; Ryzhov et al., 2007; Fang and Olah, 2007). VEGF is the unique mitogen that acts specifically in endothelial cells by activating VEGFR (Olsson et al., 2006; Kerbel, 2008). Activation of VEGFR triggers intracellular signalling pathways that involve increased NO synthesis (Roy et al., 2008; Casanello et al., 2007), a gas that seems to be determinant in endothelial cell proliferation in the fetal-placental unit (Escudero and Sobrevia, 2008). Ado could contribute up to 50-70% of the angiogenic response in some condition such as hypoxia (Adair, 2005) via a direct mitogenic effect on endothelium (Auchampach, 2007) or by regulating the production of pro-angiogenic substances, such as VEGF and IL-8 (Feoktistov et al., 2002; 2004; Clark et al., 2007) or anti-angiogenic factors such as thrombospondin 1 (Desai et al., 2005) from endothelial and immune cells (Auchampach, 2007). Recently, it has been reported that 5'-N-ethylcarboxamidoadenosine (NECA) increased intracellular cAMP level due to activation of G_s proteins leading to ERK1/2 activation (i.e. phosphorylation) in human umbilical vein endothelial cells (HUVEC). NECA effect was associated with A_{2B}AR activation rather than other receptors since the selective A_{2B}AR antagonist MRS-1754 (N-(4-cyano-phenyl)-2-[4- (2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8- yl)-phenoxy]acetamide) blocked NECA effect on ERK1/2 (Fang and Olah, 2007). Interestingly, a reduced A_{2B}AR expression induced by the use of a ribozyme selectively designed, blocked NECA effect on human retinal endothelium migration and mouse endothelial proliferation (Afzal et al., 2003). Other studies show that NECA via A_{2B}AR activation increases the VEGF gene promoter activity, as well as VEGF mRNA level and protein abundance in the cultured medium in human microvascular endothelial cell line 1 (HMEC-1) (Feoktistov et al., 2002). It has also been shown that expression and protein abundance of VEGF is increased in HUVEC cultured in its physiological oxygen content (i.e. ~5% O_2) exposed to NECA (Feoktistov et al., 2004). More recently, using primary cultures of human monocytes it has been shown that A_1AR activation by the selective agonist CPA (N6-cyclo-pentylAdenosine) increased the

VEGF level, an effect blocked with the selective antagonists WRC-0571 (N6-[endo-2'-(endo-5'- hydroxy)norbornyl]-8-(N-methylisopro-pylamino)-9-methyladenine) and CPX (8-cyclopentyl-1,3-dipropylxanthine) (Clark et al., 2007). In addition, interaction between Ado and VEGF had been also suggested in in vivo models where ado infusion (0.14 mg/kg/min, 6 h) increased (3-fold) the VEGF plasma level in humans (Adair et al., 2005).Thus, Ado could activate ARs, probably A_{2A} and/or A_{2B} subtypes, increasing cAMP intracellular levels and ERK1/2 phosphorylation to induce VEGF expression and proliferation of human microvascular endothelium (Escudero et al., 2009).

Aim of the thesis

Although ARs expression has been characterized in human placenta from normal pregnancies, no data are as yet available concerning expression and signaling cascades triggered by ARs in aneuploidies (Fox and Kurpis, 1983; Schocken and Schneider, 1986). Nevertheless, as previously mentioned, aneuploidies very often end in SA, making them a good experimental model for potentially shedding light on the mechanism regulating this event. The aim of this study was therefore to investigate the expression of ARs in first-trimester chorionic villi (CV) and isolated mesenchymal cells (MC) from both euploid (E) and Trisomy 21 (TR21) pregnancies, one of the most frequent autosomal aneuploidy; viable cells, namely those obtained via routine chorionic villus sampling, rather than SA tissue, were chosen, as any alteration of ARs in the latter could be a consequence rather than the cause of miscarriage. The rationale behind the study was that elucidating the role of Ado in the modulation of important proangiogenic molecules like VEGF and NO in aneuploid pregnancies may also shed light on the proteins and pathways involved in SA in E pregnancy.

Materials and Methods

Materials

[^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine ([^{3}H]DPCPX) (specific activity 120 Ci/mmol), was purchased by NEN Research Products(Boston, MA). [^{3}H] (4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo-[2,32][1,3,6]triazinyl-amino]ethyl)-phenol) ([^{3}H]ZM 241385) (specific activity 20 Ci/mmol), was furnished by Tocris (Boston, MA). [^{3}H]N-benzo[1,3]dioxol-5-yl-2-[5-(1,3-dipropyl-2,6-dioxo-2,3,6,7-tetrahyro-1 H-purin-8-yl)-1-methyl-1 H-pyrazol-3-yl-oxy]-acetamide] ([^{3}H]MRE 2029-F20) (specific activity 123 Ci/mmol) and [^{3}H]5-N-(4-methoxyphenylcarbamoyl)-amino-8-propyl-2-(2-furyl)-pyrazolo[4,3e] 1,2,4triazolo[1,5c]pyrimidine ([^{3}H]MRE 3008 F20) (specific activity 67 Ci/mmol), were synthesized at Amersham International (Buckinghamshire, UK). ARs small interfering RNA (siRNA) were from Santa Cruz DBA (Milano, Italy). RNAiFect Transfection Kit was purchased from Qiagen (Milano, Italy). Antibody against A_1 and A_3 ARs were obtained from Calbiochem Inalco (Milano, Italy). Antibody against A_{2A} was from Alpha Diagnostic, Vinci Biochem (Firenze, Italy). Antibody against A_{2B} was from Santa Cruz, Tebu-bio (Milano, Italy). VEGF ELISA kit was purchased from R&D Systems, Space Import–Export (Milano, Italy) and NO ELISA kit was furnished by Merck Chemicals (Nottingham, UK). CD45, CD34, CD14, CD73, CD90, CD13, CD31 antibodies were purchased from BD Biosciences (Milano, Italy), CD105 was from Space Import–export Milano (Milano, Italy). The Assays-on-demand™ Gene expression Products Hs00181231_m1, Hs00169123_m1, Hs00386497_m1, Hs00181232_m1, Hs01573922_m1, Hs00173626_m1 for A_1, A_{2A}, A_{2B}, A_3, CD73 and VEGF, respectively were purchased from Applied Biosystems (Monza, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milano, Italy).

Study subjects and sample collection

From 2008 to 2011 CV were collected from 71 pregnant women consecutively scheduled for sampling at 12 weeks of gestation. Pregnancies were categorized as follows: 41 E and 30 TR21. Informed consent was obtained in all cases, and the study was approved by the Ferrara S. Anna University Hospital Ethics Committee.

Membrane preparation from CV

Membrane preparation from 23 CV (13E and 10TR21) was carried out as described previously with minor modifications (Masnikosa et al., 2008). Briefly, tissue was homogenized in a polytron homogenizer with 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.5) containing 250 mM sucrose, 1 mM Ethylenediaminetetraacetic acid (EDTA) and 2 mM phenylmethyl-sulphonyl fluoride (PMFS). The homogenate was centrifuged at 600 ×g for 15 min. The pellet was discarded and the supernatant centrifuged at 18,000 ×g for 30 min. The supernatant obtained was further centrifuged at 100,000 ×g for 60 min. The crude membrane pellet was washed twice and finally resuspended in membrane buffer (50 mM HEPES, pH 7.5 containing 4 mM $MgCl_2$).

Binding studies

Saturation experiments of [^{3}H]DPCPX (0.1-30 nM), [^{3}H]ZM 241385 (0.1–30 nM), [^{3}H] MRE 2029 F20 (0.1–30 nM) and [^{3}H]MRE 3008 F20 (0.1–30 nM) to label A_1, A_{2A}, A_{2B} and A_3 ARs, respectively, were carried out inmembranes from CV as previously described (Gessi et al., 2010). 100 µl of membrane homogenate (80 µg of protein assay^{-1}) were incubated in duplicate, in a final volume of 250 µl in test tubes containing 50 mM Tris HCl buffer (10 mM $MgCl_2$ for A_{2A}, 10mM $MgCl_2$, 1mM EDTA, 0.1mM benzamidine for A_{2B} and 10 mM $MgCl_2$, 1 mM EDTA for A_3) pH 7.4, with 10–12 different concentrations of each selective radioligand. Non-specific binding was obtained by using 1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1 H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione (PSB 36), 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7 Hpyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 442416), 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB 603) and N-(2-Methoxyphenyl)-N'-[2-(3-pyrindinyl)-4-quinazolinyl]-urea (VUF 5574) 1 µM and at the K_D value for each radioligand was 31, 30, 32, 26%, respectively of total binding in E and 39, 30, 41, 34%, respectively in TR21 cells. Bound and free radioactivity were separated, after an incubation time of 2h at 4 °C, by filtering the assay mixture through Whatman GF/B glass-fiber filters using a cell harvester (Packard Instrument Company). The filter bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20.

Western blot analysis

AR expression was evaluated in CV samples (4E and 4TR21) and in mesenchymal cells (3E and 3TR21) by Western blot.Whole cell lysates, prepared as previously described were

resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane (Gessi et al., 2010). Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for A_1, A_{2A}, A_{2B} and A_3 ARs (1:1000 dilution) in 5% non-fat dry milk in PBS 0.1% Tween-20 overnight at 4–8°C. Membranes were washed and incubated for 1 hour at room temperature with peroxidase-conjugated species specific secondary antibodies. Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent. Tubulin (1:250) was used to mathematically normalize samples; then signals were expressed as % of control.

Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad). Mean densitometry data from independent experiments were normalized to the results in control cells. The data were presented as the mean±S.E.

MC isolation

MC cells were isolated from a total of 40 CVs (24E,16TR21) as previously described (Poloni et al., 2008). Only cells taken from back-up cultures were used after karyotype analysis. Cells were maintained in Chang medium D, supplemented with 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37° in 5% CO_2/ 95% air. All cell treatments with ARs ligands were performed in the presence of ADA.

Hypoxic treatment

Hypoxic exposure (24 h) was performed in a modular incubator chamber flushed with a gas mixture containing 1%O_2, 5%CO_2 and balance N_2 (MiniGalaxy, RSBiotech).

Immunophenotyping

MCs isolated from 3 separate CV samples were immunophenotyped. Analysis was performed using an EPICS-XL flowcytometer and EXPO32 software (Beckman Coulter). At least 10,000 events were collected per sample (Mirandola et al., 2011).

Real-time RT-PCR

Quantitative real-time RT-PCRwas performed as previously reported (Gessi et al., 2010). Total cytoplasmic RNA was extracted from MCs (13E, 8TR21) by the acid guanidinium thiocyanate phenol method. For the real-time RT-PCR of A_1, A_{2A}, A_{2B}, A_3 ARs, CD73 and VEGF the assays-on- demand™ Gene expression Products were used. As reference gene the

endogenous control human β-actin kit was used, and the probe was fluorescent-labeled with VIC™ (Applied Biosystems, Monza, Italy).

Immunofluorescence analysis

For HIF-1α detection cells were treated with NECA for 2 h, under hypoxia. MCs (5E, 5TR21) were washed two times with PBS, fixed in 10% paraformaldehyde for 10 min, permeabilized in a PBS solution containing 0.1% of Triton X-100 and incubated for 30 min with PBS plus 5% goat serum and 0.5% bovine serum albumin. The cells were then incubated O.N. at 4 °C in a humidified chamber with anti-HIF-1α Ab solutions (1:50) containing 0.5% of goat serum and 0.5% of bovine serum albumin in PBS. Excessive antibody was washed away with PBS and rabbit antibodies were detected with fluorescein isothiocyanate- labeled goat anti-rabbit IgG. Coverslips were stained with 4¢,6¢,-diamino- 2-phenyl-indole, mounted in DABCO glycerol-PBS and observed on Nikon fluorescent microscope (Eclipse 50i) as previously described (Merighi et al., 2012). Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Milan, Italy). Levels of hypoxic HIF-1 in euploid cells were the basis for calculation of the additional NECA-mediated increase and for HIF-1 staining in aneuploid cells. The mean intensity of each cell was obtained from the cells pixels that had a higher intensity than that of the mean background intensity. A mean of 150 cells was analyzed for each condition at 40X magnification, at fixed time exposure.

siRNA treatment of MCs

MCswere plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of ARs siRNA, was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit for 72 hours (Gessi et al., 2010). A non-specific 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.

Nitrite assay for mesenchymal cells

NOS activity in MCs (5E, 5TR21) was assessed indirectly by measuring nitrite (NO_2^-) accumulation in the cell culture media using a colorimetric kit (Calbiochem, Milan, Italy). At the end of the treatment period, the nitrite concentration in the conditioned media was determined according to a modified Griess method (Merighi et al., 2012). Briefly, the NADH dependent enzyme nitrate reductase was used to convert the nitrate to nitrite prior to quantification of the absorbance, measured at 540 nm by a spectrophotometric microplate

reader (Fluoroskan Ascent, Labsystems, Sweden). Sodium nitrite was used as the standard compound.

Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGF secreted by theMCs (5E, 5TR21) in the medium were determined by ELISA kits. In brief, subconfluent cells (40,000/ml) were seeded in 24-well plates andincubated in the presence of solvent or various concentrations of ado ligands for 24 hours. The medium was collected, centrifuged for 5 min at 900 g to remove floating cells and assayed for VEGF and NO content by ELISA according to the manufacturer's instructions. The data were presented as mean±SE from four independent experiments.

Statistical analysis

LIGAND, a weighted nonlinear least-squares curve-fitting program, was used for computer analysis of the data fromsaturation experiments (Gessi et al., 2010). Functional experiments were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve (GraphPAD Prism, San Diego, CA, USA). Data sets were analyzed using Student's t test or analysis of variance (ANOVA) and Dunnett's test (when required). A P-value of less than 0.05 was considered statistically significant. All values in the figures and text are expressed as mean±standard error (S.E.) of independent experiments and are indicated in the figure legends. Each experiment was performed by using the CV derived from one single donors, and was performed in duplicate (for binding and real-time PCR experiments) or in triplicate (for functional experiments). The experiments were repeated at least three times as indicated from n-values that represent the number of patients used.

Results

AR proteins in E and TR21 CV

Immunoblotting was used to investigate AR expression in CV biopsies from E and TR21 pregnancies. Expression of A_1, A_{2B} and A_3ARs was lower in TR21 CV with respect to E CV, while no difference in A_{2A}AR was observed between the two (Fig. 5A). The specificity of the A_{2B} antibody, recognizing a band of 50 kDa different from the predicted molecular wheight of 36 kDa, was assessed in both untransfected and transfected CHO cells as shown in supplemental Fig. 1. Saturation binding experiments in CV were carried out to evaluate affinity (K_D) and density (B_{max}) of ARs. [^{3}H]DPCPX saturation assays revealed A_1ARs with K_D of 2.4±0.3 and 1.9±0.2 nM, and B_{max} of 203±21 and 107±12 fmol/mg of protein in E and TR21 samples, respectively; [^{3}H]ZM 241385 saturation studies showed A_{2A}ARs with K_D of 2.5±0.3 and 2.8±0.3 nM, and B_{max} of 78±10 and 72±9 fmol/mg of protein in E and TR21 samples, respectively; [^{3}H]MRE2029F20 saturation experiments detected A_{2B}ARs with K_D of 2.8±0.3 and 3.2±0.3 nM, and B_{max} of 140±15, and 90±12 fmol/mg of protein in E and TR21 samples, respectively; [^{3}H] MRE3008F20 saturation assays revealed A_3ARs with K_D of 1.2±0.2 and 0.9±0.1 nM, and B_{max} of 168±20 and 95±10 fmol/mg of protein in E and TR21 samples, respectively (Fig. 5B). Scatchard plot analysis revealed the presence of an high affinity binding site for each radioligand as suggested by the linearity of the lines. Computer analysis of the data failed to show a significantly better fit to a two site than to a one site binding model, suggesting that under our experimental conditions, there was, primarily, a single class of high affinity binding sites.

Cell morphology

Primary cultures obtained from CV exhibited a population of embryonal MCs, as revealed by positive immunostaining towards CD13, CD73, CD90 and CD105, while hematopoietic and endothelial cell markers were undetectable (CD14, CD31, CD34, CD45). Flow cytometry demonstrated culture contamination by non-MC cells of less than 5% (Fig. 6).

AR mRNAs in CVMCs from E and A pregnancies

AR mRNAs were investigated in CVMCs from E and TR21 pregnancies. The order of expression detected in both was A_{2B}>A_1>A_{2A}AR. A_3AR was revealed after 37–38 PCR cycles, suggesting low levels of expression. A_1ARs were found to be downregulated in MCs of TR 21, (0.51±0.05) in comparison to euploid MCs; A_{2B}AR was reduced in TR 21

(0.52±0.05); as for A_{2A}ARs, they were expressed at similar levels to E in TR 21 (0.95±0.1) (Fig. 7A). CD73 expression was similar in E and A cells (0.90±0.1) (Fig. 7B).

AR proteins in E and A MCs

Western blot analysis was used to quantify ARs in MCs from CV of E and TR21 pregnancies. A_1 and A_{2B} ARs were found to be downregulated in TR21 MCs with respect to E MCs. A_{2A}ARs and CD73 were not significantly altered in TR21. A_3ARs were expressed at lowlevels in both E and A cells (Fig. 8).

ARs increase NO secretion in MCs

We evaluated NO production by MCs after treatment with the nonselective agonist NECA under hypoxic conditions (24 h). NECA raised NO levels by 459±50% and 466±48% in E and TR21 cells, respectively. Basal NO levels were slightly higher in TR21 than in E cells (39±4 and 49±5 μM in E and TR 21 cells, respectively) (Fig. 9A). The NECA effect was strongly reduced by PSB 36 and SCH 442416, selective antagonists of A_1 and A_{2A} ARs, respectively, suggesting the involvement of A_1 and A_{2A} subtypes, and reduced to a lesser extent by PSB 603, selective antagonist of A_{2B} (Fig. 9A). The effect of AR siRNAs was also tested. A_1AR siRNA was the most potent at reducing NECA-induced stimulation of NO levels, followed by A_{2A} and A_{2B}AR siRNAs (Fig. 9B). siRNA of HIF-1α greatly reduced NECA-stimulated NO increase, suggesting that ARs were acting through HIF-1α modulation (Fig. 9C). After 48 and 72 h posttransfection with siRNA targeting each AR and HIF-1, protein levels were significantly reduced (Fig. 10A); the specificity of a given siRNA to the other AR subtypes is also shown in Fig. 10B. We therefore evaluated HIF-1α accumulation after incubation of E and TR21 MCs with NECA under hypoxia (2 h). NECA stimulated HIF-1α accumulation in both cell types, confirming the involvement of this transcription factor (Fig. 11).

ARs stimulate VEGF secretion in MCs

We tested VEGF production by MCs after treatment with NECA under hypoxia (24 h). NECA increased VEGF levels in a dose-dependent fashion by 314±32% and 330±35% in E and A cells, respectively. TR21 cells presented a lower basal level of VEGF (222±26 pg/ml in TR21 vs 404±52 pg/ml in E cells), and NECA showed a lower affinity for stimulation of VEGF secretion in TR21with respect to E cells (EC_{50} 245± 26, 480±50 nM in E and TR21, respectively). The NECA effect was strongly reduced by PSB603 (300 nM), suggesting the involvement of A_{2B}AR, and to a lesser extent by the A_{2A} antagonist SCH442416 (25 nM), but not by PSB36 (50 nM) the A_1 antagonist (Fig. 12A). The effect of AR siRNAs was also

tested. A_{2B}AR siRNA produced the most potent reduction of NECA-induced stimulation of VEGF, followed by A_{2A}AR siRNA, while A_1 siRNA has no effect (Fig. 12B). Treatment with nitric oxide synthase antagonist L-NG-Nitroarginine methyl ester (L-NAME 150 μM) did not reduce NECA-stimulated increase in VEGF, suggesting that ARs were not acting through NO production (data not shown). The effect of NECA on VEGF was also observed on mRNA (2.6± 0.2-fold increase) (Fig. 12C).

Discussion

Human reproduction is a complex process prone to failure, and several mechanisms, including angiogenesis, inflammatory and immune-related processes, have been considered as possible mediators of SA. Recently, it has been reported that aberrant maternal inflammation associated with SA is closely linked to deficient placental perfusion (Renaud et al., 2011). Emerging evidence also suggests that Ado, a proangiogenic nucleoside and a sensor of overactive immunity and inflammation, may be involved in determining pregnancy outcome (Hitoglou et al., 2004; Blackburn et al., 1999; Perni et al., 2009). In order to elucidate which ARs may affect the success of this event, we evaluated their expression in TR21, chromosomal abnormalities that very often end in abortion and may therefore represent a good model for elucidating the mechanism regulating miscarriage. Our findings show, for the first time, a reduction in A_1, A_{2B} and A_3 ARs in CV biopsies obtained from TR21, in comparison to those from euploid pregnancies as evaluated by both western blotting and saturation binding experiments. The A_{2B} antibody reveals a band of 50 kDa, substantially higher than theoretical molecular weight of this receptor subtype. As it has been previously demonstrated that 50–55 kDa immunoreactivity detected in many tissues may not represent the A_{2B}AR we evaluated its specificity in transfected and untransfected Chinese hamster ovary (CHO) cells (Linden et al., 1999). Our data show that the A_{2B} antibody was specific for A_{2B}ARs, according to literature data (von Versen-Höynck et al., 2009) and revealed a decrease in receptor density in TR21 CV. In order to quantify the affinity and density of ARs, saturation binding studies were performed. In CV, the ARs affinities were in the nanomolar range and the receptor densities of A_1, A_{2B} and A_3 subtypes were decreased in TR21 samples. Even though DPCPX and ZM 241385 used as A_1 and A_{2A} radiolabeled ligands can also bind A_{2B}ARs, in the range of concentrations investigated we detected a single class of high affinity binding sites. Similar results have been observed in a recent paper by Varani in human synoviocytes expressing all four ARs (Varani et al., 2010).We therefore went on to further investigate ARs expression in CV MCs, which are routinely withdrawn for genotype analysis. Accordingly, we found, also for the first time, a reduction of A_1 and A_{2B} ARs in A MCs, whilst A_3AR was expressed at very low levels in both E and A samples; this suggests that it may be more relevant in other cell types e.g. in preeclampsia (PE), A_3AR expression has been found to be upregulated in trophoblasts, where it regulates MMP-2/9 expression (Kim et al., 2008). As a whole, these data support the hypothesis that both A_1 and A_{2B} AR gene products

may positively regulate normal pregnancy. Indeed, A_1AR has been reported to play an essential role in protecting the embryo against hypoxia and intrauterine stress (Wendler et al., 2007; Rivkees and Wendler, 2011; Buscariollo et al., 2011). As for A_{2B}ARs, it is known to be involved in increasing angiogenesis through modulation of VEGF, and its genetic loss has recently been found to increase platelet aggregation, suggesting it has a beneficial effect in vascular injury (Grant et al., 1999; Feoktistov et al., 2002; Yang et al., 2010). A_{2B}AR is also involved in chorionic vasoconstriction,with pathophysiological implications for PE and vascular diseases (Donoso et al., 2005). Since TR21 placentae are known to feature trophoblastic hypoplasia and hypovascularity, we investigated the potential role played by ARs in NO and VEGF regulation in both TR21 and normal pregnancies (Pipitone et al., 2003); previously, the four ARs had been linked to the angiogenic actions of Ado in endothelial cells, smooth muscle, fibroblasts, monocytes, macrophages, mast and foam cells, all of which are recognized as important sources of proangiogenic factors (George et al., 2010; Gessi et al., 2010; Wendler et al., 2007; Clark et al., 2007). We found slightly higher NO content in MCs from TR21 pregnancies than in E cells; A_1 followed by A_{2A} ARs were shown to increase NO production in an HIF-1- dependent fashion,with A_{2B}AR only playing a minor role, confirming previous reports (Lima et al., 2010; Vàsquez et al., 2004). In contrast, A_{2B}AR appeared to be the main subtype involved in VEGF secretion, as indicated by the affinity of NECA and the antagonizing effect mediated by both specific blockers and siRNA of ARs. A_{2A}AR also contributed to VEGF secretion according to literature data (Fredholm et al., 2011). We found reduced VEGF in TR21 with respect to E cells; this was reversed by NECA stimulation, albeit with a lower affinity. These low levels of VEGF production, in addition to the higher NO content, have previously been reported in TR21 stem cells, and may be attributed to the fact that the candidate Down syndrome region gene lies on chromosome 21 and encodes a negative regulator of VEGF–calcineurin signaling (Tranquilli et al., 2003; Salvolini et al., 2011; Baek et al., 2009). Furthermore, NO production, the main vasodilator in pregnancy, has been suggested as a compensatory response for restoring proangiogenic conditions in hypoxic MCs.

As a whole, our data show that the Ado transduction cascade is disturbed in TR21 by two major anomalies, namely downregulation and reduced expression of A_{2B} and A_1 ARs. Such anomalies may negatively affect pregnancy to varying degrees; based on the literature, as well as our results, pregnancy can be interpreted as a vascular phenomenon whose destiny depends, among other factors, on the degree of disruption of each of the two ARs, which may be implicated in a range of complications, including SA, fetal malformation, fetal growth

restriction and preeclampsia (Vesce et al., 2002; 2001; 1997). Accordingly, stimulation of ARs, particularly A_1 and A_{2B}, may turn out to improve fetoplacental perfusion by increasing NO and VEGF. Our results also suggest that A_1ARs and A_{2B}ARs may be useful as biomarkers to provide an early indication of SA risk, and, last but by no means least, lay the foundations for future studies investigating the molecular causes of miscarriage (Gessi et al., 2012).

Figures Legend

Figure 5: Expression levels of A_1, A_{2A}, A_{2B}, A_3AR proteins in E and TR21 CV. Representative Western blot analyses of ARs in CV biopsies from women with E (line 2) and TR (line 3) pregnancies at 12 weeks of gestation. CHO cells transfected with the different ARs were loaded as positive control (line 1). Histograms represent % decrease with respect to E pregnancies. Densitometric quantification of Western blots is the mean±SE values (N=4 for each group) ∗P<0.01 vs E CV. (A); Saturation curves of [^{3}H]DPCPX, [^{3}H]ZM 241385, [^{3}H] MRE 2029 F20 and [^{3}H]MRE 3008 F20 binding to A_1, A_{2A}, A_{2B}, A_3ARs in CV biopsies from E (N=13) and TR21 pregnancies (N=10) (B).Specific (■) and nonspecific equilibrium binding (▲) were determined as described in the methods. Each value represents the mean±SEM of experiments performed in duplicate.

Figure 6: Immunophenotyping by flow cytometry analysis of purified CVMCs. Cell surface expression of MSC (CD13, CD73, CD90 and CD105), myeloid (CD14, CD45) and endothelial (CD31, CD34) markers is reported. The number of positive cell is reported in each diagram as % of total gated cells (> 10,000 events were analyzed). As negative control, cells were also stained with isotype-matched irrelevant antibody fluorescence (Irr.-FITC and Irr.PE). A representative sample is reported (N = 3).

Figure 7: Expression levels of A_1, A_{2A}, A_{2B} AR and CD73 target genes in E and TR21 CVMCs. Histograms showing the content of AR (A) and CD73 (B) mRNA in E (N = 13, black) and TR21 (N = 8, white) MCs. Data were expressed as percentage of β-actin expression. *P < 0.01, vs. the corresponding receptor in E cells.

Figure 8: Expression levels of A_1, A_{2A}, A_{2B}, A_3AR and CD73 proteins in E and TR21 CVMCs. Representative Western blot analyses of ARs and CD73 in MC cells from euploid and TR21 pregnancies at 12 weeks of gestation. Histograms represent % decrease with respect to E pregnancies. Densitometric quantification of Western blots is the mean ± SE values (N = 3 for each group). *P < 0.01 vs E CV.

Figure 9: NECA-stimulated NO secretion in E (white) and TR21 cells (black). NO levels in MC treated with 1 μM NECA in the absence and presence of 50 nM PSB 36, 25 nM SCH 44216 and 300 nM PSB 603 (A) and with specific siRNAs of A_1, A_{2A}, and A_{2B} ARs (B).

Effect of HIF-1α siRNA on NO secretion (C). *P < 0.05 vs. the corresponding control (E and A cells without NECA) and **P < 0.05 vs. the corresponding NECA (N = 5 for each group).

Figure 10: ARs and HIF-1α silencing by siRNA transfection in MC. Western blot analysis using anti A_1, A_{2A}, A_{2B} and HIF-1 polyclonal antibodies of protein extracts from MC treated with siRNA and cultured for 24, 48 and 72 h (A). Specificity of Ado receptors siRNAs (B). Western blot analysis using anti A_1, A_{2A}, and A_{2B} receptor polyclonal antibodies of protein extracts from MC transfected with control ribonucleotides (ctr.) or with siRNA of each AR subtype and cultured for 72 h. Tubulin shows equal loading protein.

Figure 11: Effect of NECA on HIF-1α accumulation in E and TR21 cells. Stimulation of HIF-1α accumulation by NECA (B,D) in CVMC E (A,B) and TR21 (C,D) by means of immunofluorescence analysis; bar graph data (E), expressed as mean ± SE percentage of total HIF-1α staining (N = 3 for each group); *P < 0.05 vs. the corresponding control (E and A cells without NECA). Figure shows 1 representative experiment.

Figure 12: NECA-stimulated VEGF secretion in E (white) and TR21 cells (black). VEGF levels in CV-derived MCs treated with 1 μM NECA in the absence and in the presence of 50 nM PSB 36, 25 nM SCH 44216 and 300 nM PSB 603 (A), and with specific siRNAs of A_1, A_{2A}, and A_{2B} ARs (B). Induction of VEGF mRNA by NECA in healthy (white) and TR21 (black) cells (C); *P < 0.05 vs. the corresponding control (E and A cells without NECA) and **P < 0.05 vs. the corresponding NECA (N = 5 for each group).

Supplemental Figure 1: Specificity of the A_{2B} antibody. Full-length Western blot showing all band of A_{2B}AR in untransfected CHO cells (negative control), CHO cells transfected with A_{2B}AR (positive control) and in CV from E and TR21 pregnancies

Figure 5

A

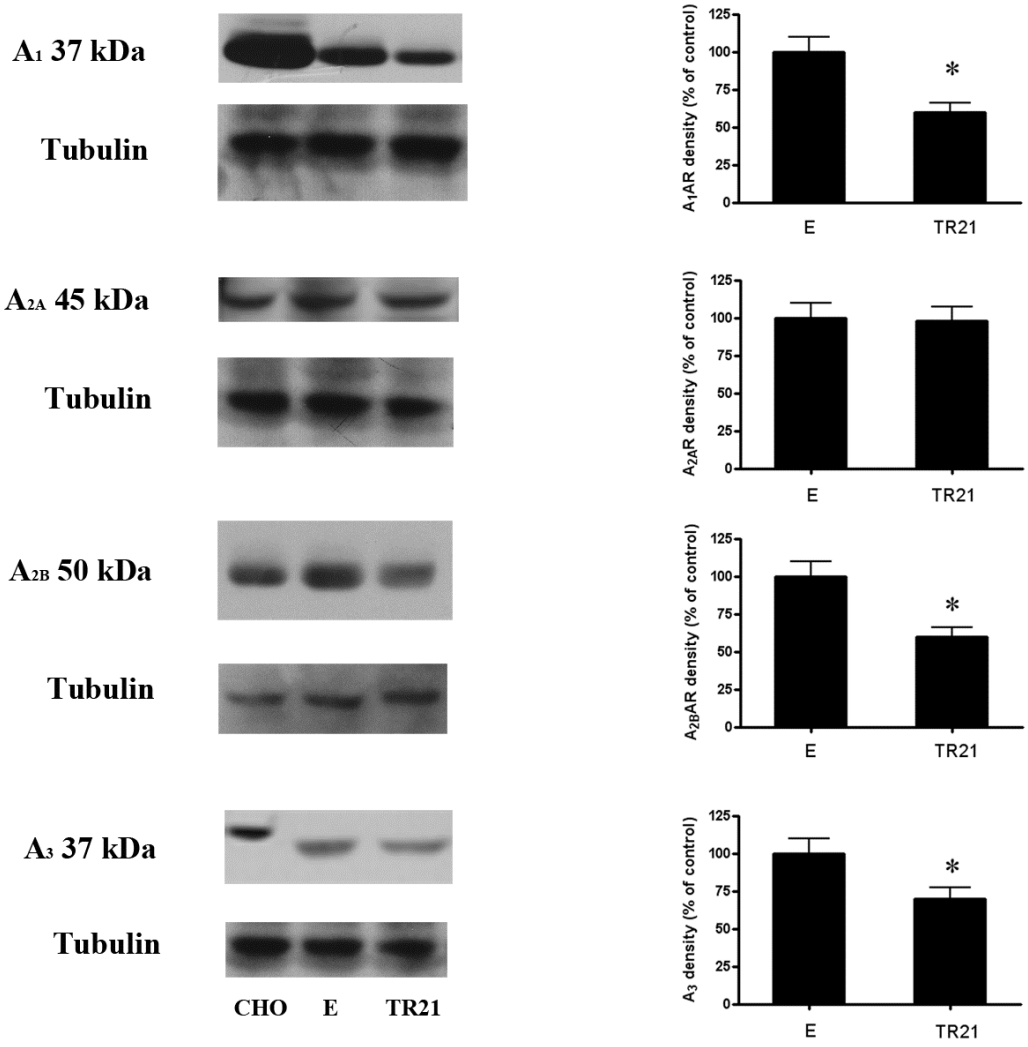
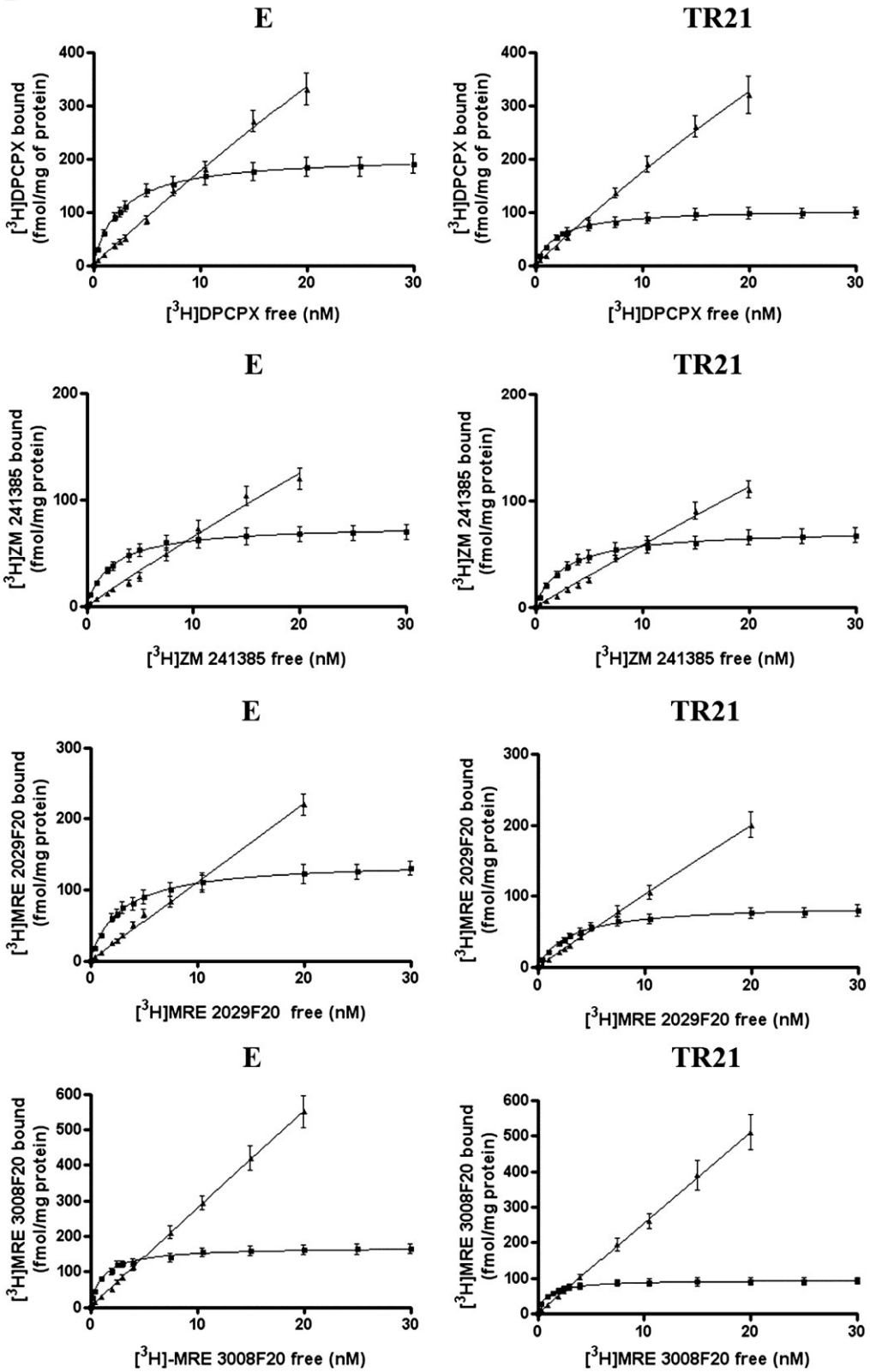

A1 37 kDa
Tubulin
A2A 45 kDa
Tubulin
A2B 50 kDa
Tubulin
A3 37 kDa
Tubulin
CHO
E
TR21
A1AR density (% of control)
A2AR density (% of control)
A2BAR density (% of control)
A3 density (% of control)
125
100
75
50
25
0
*
E
TR21

Figure 5

B


E
TR21
[3H]DPCPX bound (fmol/mg of protein)
[3H]DPCPX free (nM)
E
TR21
[3H]ZM 241385 bound (fmol/mg protein)
[3H]ZM 241385 free (nM)
E
TR21
[3H]MRE 2029F20 bound (fmol/mg protein)
[3H]MRE 2029F20 free (nM)
E
TR21
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[3H]MRE 3008F20 free (nM)

Figure 6

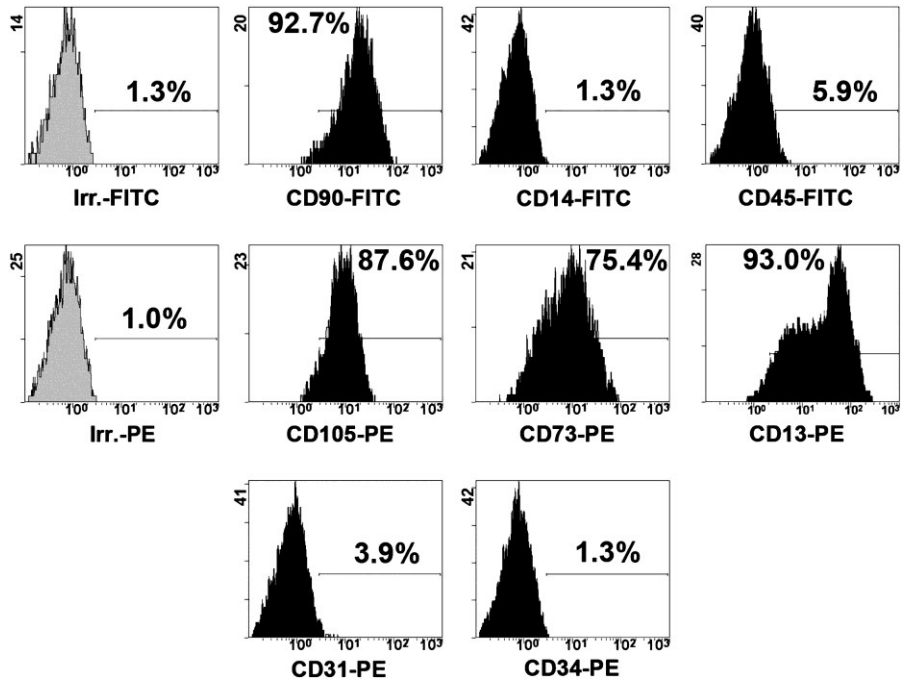


Figure 7

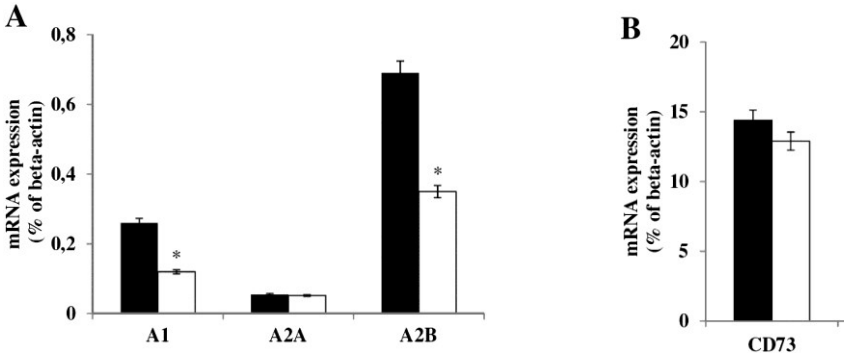


Figure 8

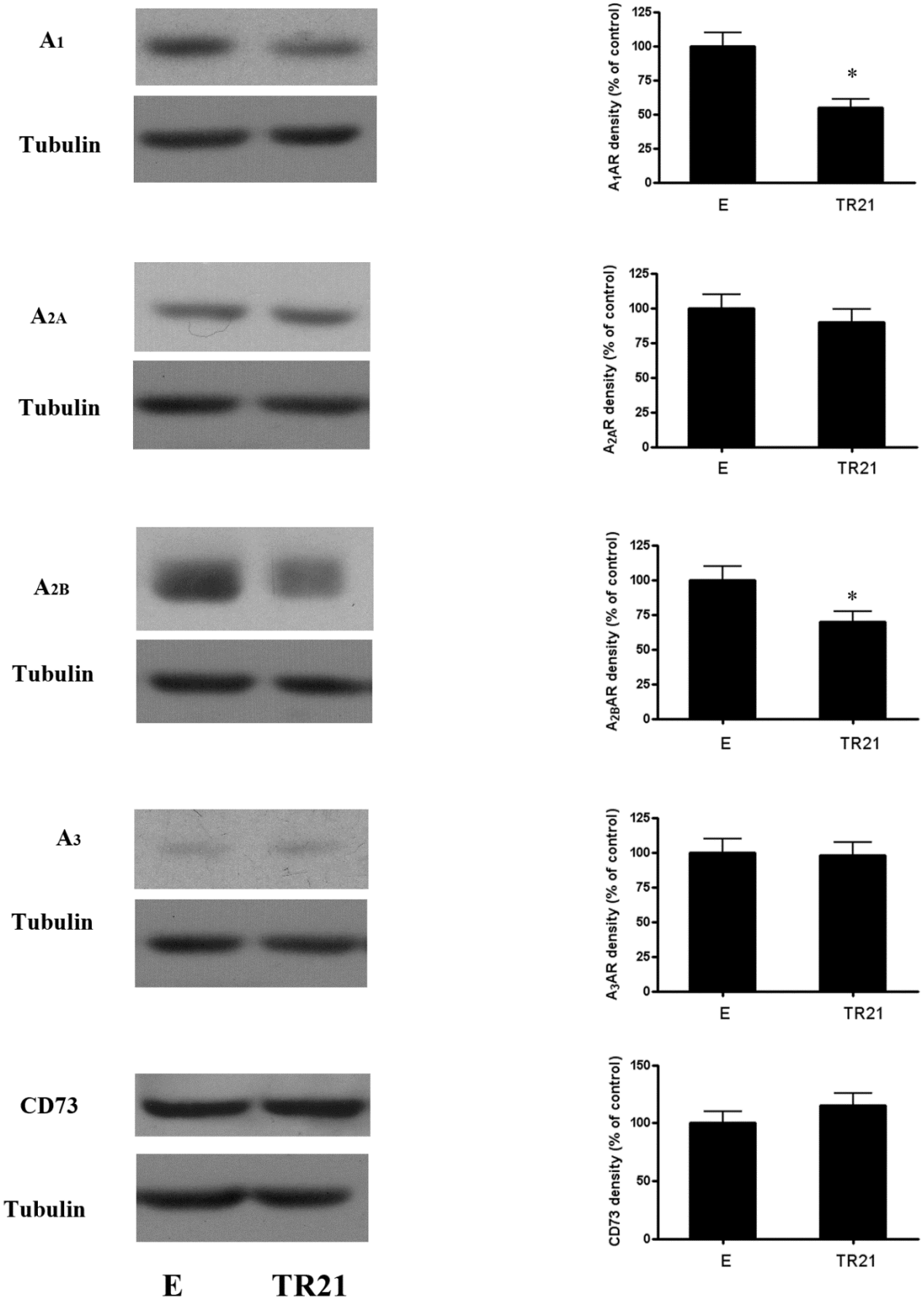

A1
Tubulin
A1AR density (% of control)
125
100
75
50
25
0
*
E
TR21
A2A
Tubulin
A2AR density (% of control)
125
100
75
50
25
0
E
TR21
A2B
Tubulin
A2BAR density (% of control)
125
100
75
50
25
0
*
E
TR21
A3
Tubulin
A3AR density (% of control)
125
100
75
50
25
0
E
TR21
CD73
Tubulin
CD73 density (% of control)
150
100
50
0
E
TR21
E
TR21

Figure 9

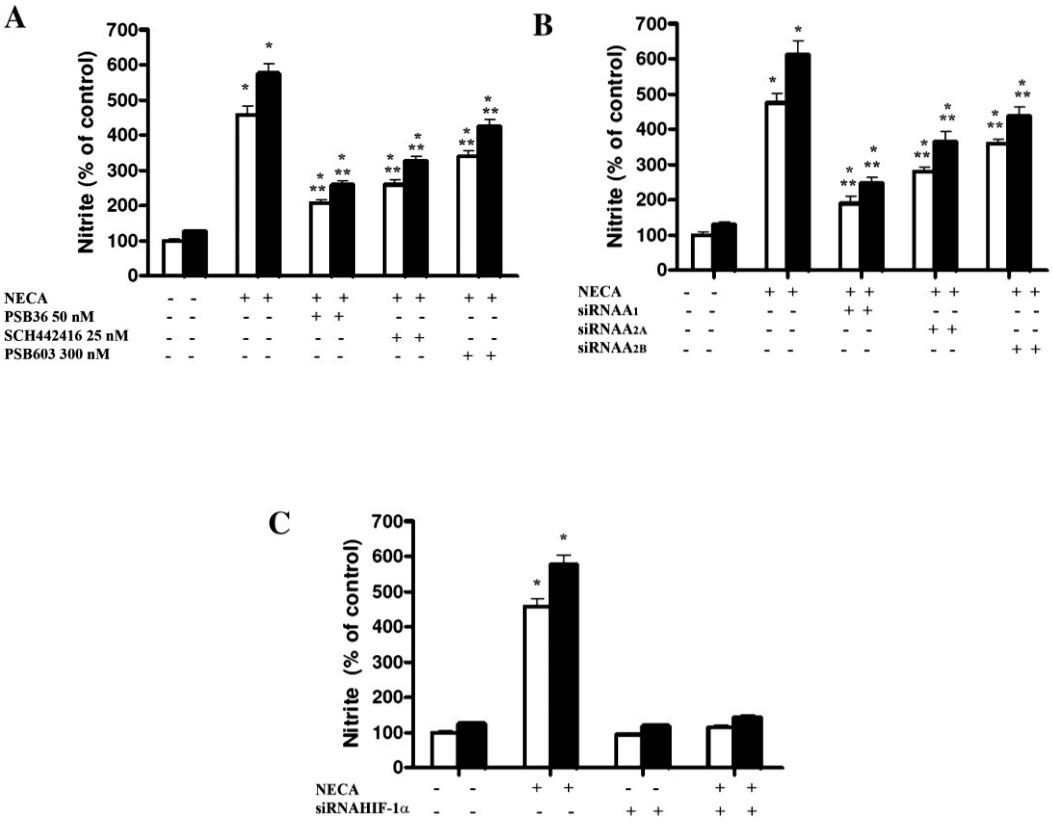
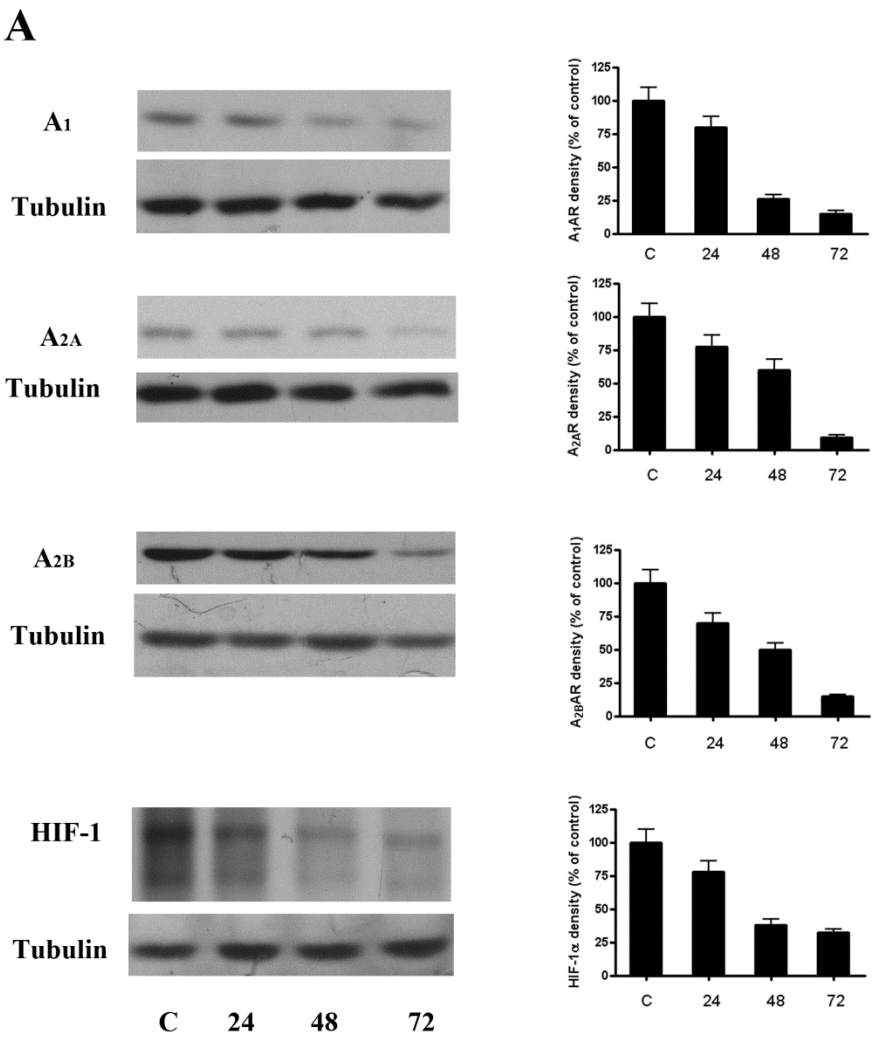

A
Nitrite (% of control)
700
600
500
400
300
200
100
0
NECA
PSB36 50 nM
SCH442416 25 nM
PSB603 300 nM
B
Nitrite (% of control)
NECA
siRNAA1
siRNAA2A
siRNAA2B
C
Nitrite (% of control)
NECA
siRNAHIF-1α

Figure 10


A
A1
Tubulin
A2A
Tubulin
A2B
Tubulin
HIF-1
Tubulin
C 24 48 72
A1AR density (% of control)
A2AR density (% of control)
A2BAR density (% of control)
HIF-1α density (% of control)
125
100
75
50
25
0
C 24 48 72

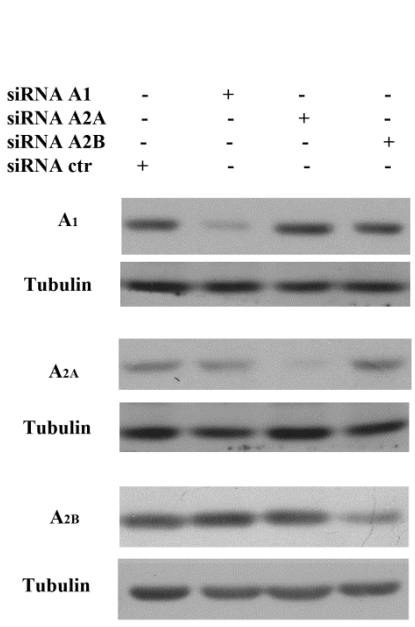
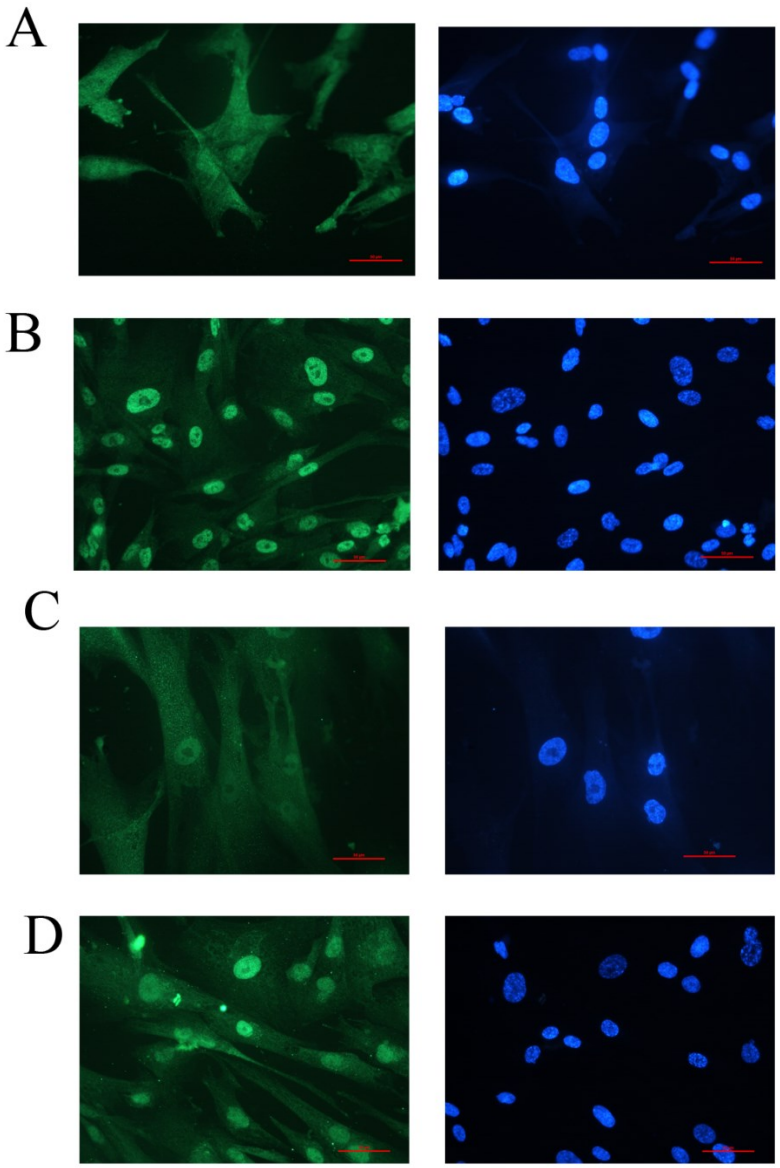

B
siRNA A1 - + - -
siRNA A2A - - + -
siRNA A2B - - - +
siRNA ctr + - - -
A1
Tubulin
A2A
Tubulin
A2B
Tubulin

Figure 11


A
B
C
D

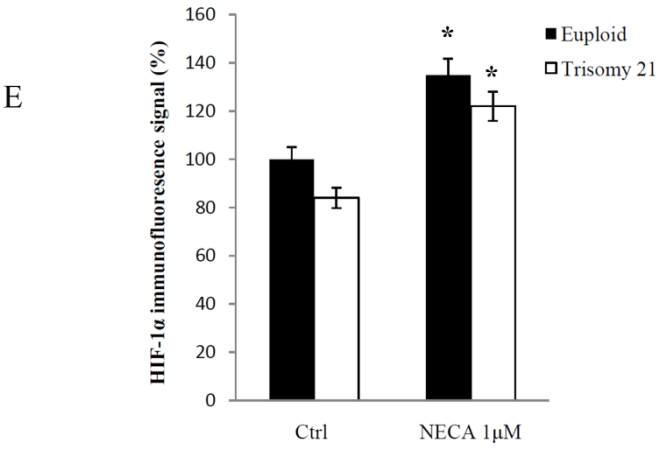
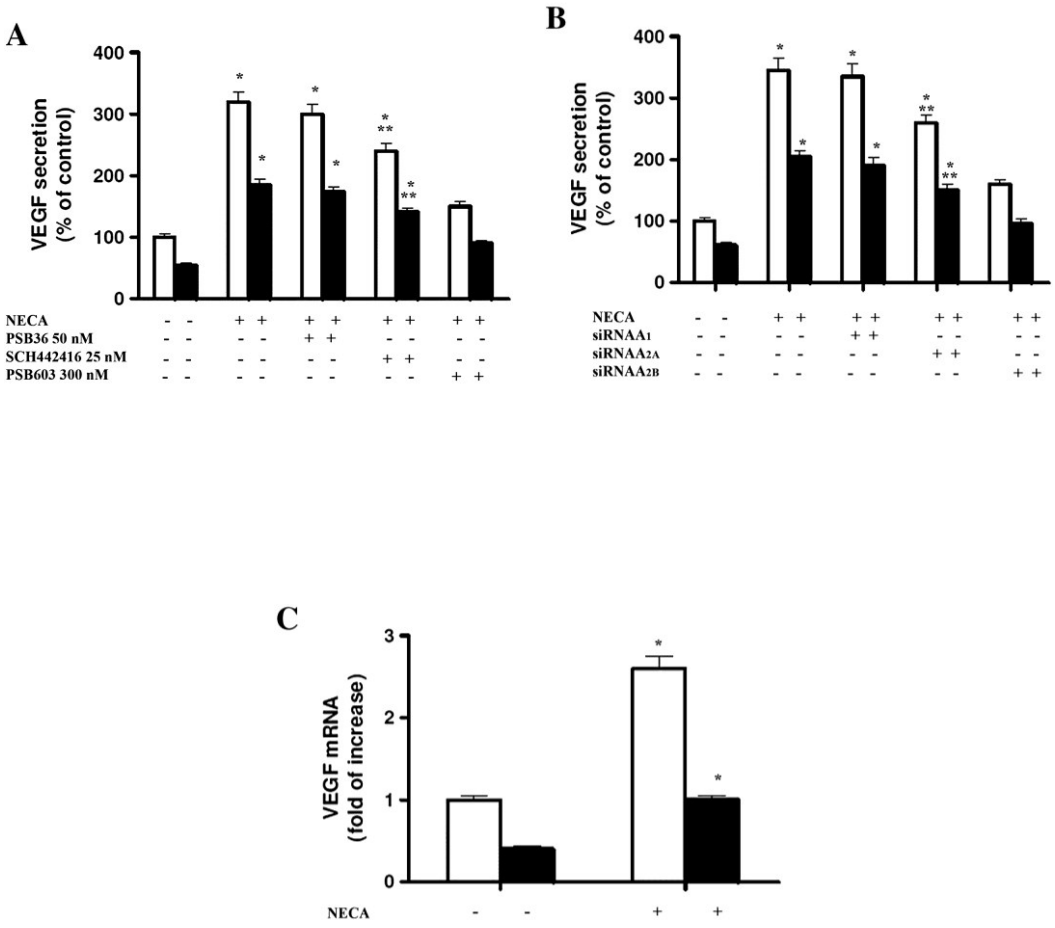
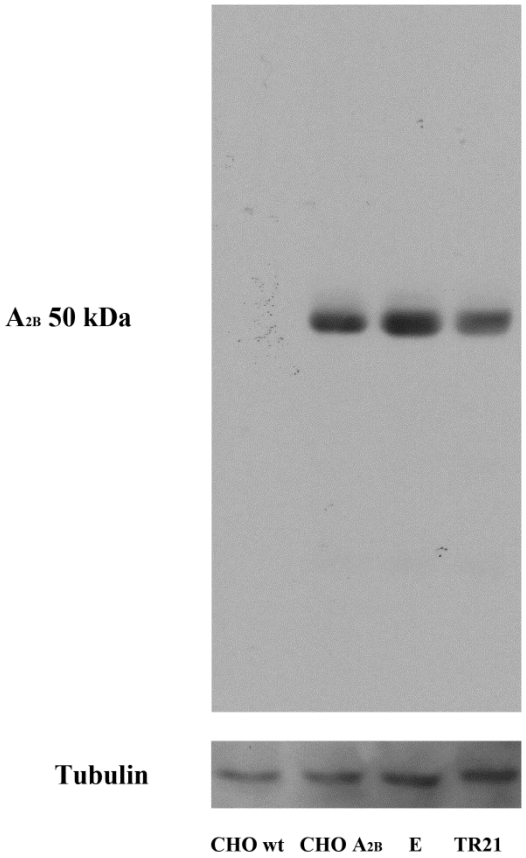

E
HIF-1α immunofluoresence signal (%)
160
140
120
100
80
60
40
20
0
*
*
Euploid
Trisomy 21
Ctrl
NECA 1μM

Figure 12


A
VEGF secretion (% of control)
400
300
200
100
0
NECA
PSB36 50 nM
SCH442416 25 nM
PSB603 300 nM
B
VEGF secretion (% of control)
400
300
200
100
0
NECA
siRNAA1
siRNAA2A
siRNAA2B
C
VEGF mRNA (fold of increase)
3
2
1
0
NECA

Supplemental Figure 1



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A_1 and A_3 ARs inhibit LPS-induced Hypoxia-inducible factor-1 accumulation in murine astrocytes

Introduction

Astrocytes are the predominant cell type in the CNS. They function to maintain normal brain physiology, neuronal homeostasis and plasticity. In addition, astrocytes become reactive following injury to the CNS and serve important modulatory roles during CNS inflammation (Giaume et al., 2007). Inflammation plays a critical role in the pathogenesis of many CNS disorders, such as stroke, ischemia, trauma and neurodegenerative diseases. Several works in the field of stroke and brain ischemia has shown the relevance of the inflammatory response accompanying necrotic brain injury (Wang et al., 2007). Brain ischemia triggers inflammatory responses due to the presence of necrotic cells, generation of reactive oxygen species and production of inflammatory cytokines even within neurons. After cerebral ischemia, astrocytes become a major source of inflammatory mediators such as cytokines, chemokines and iNOS. These factors are involved in cellular degeneration under neuroinflammatory conditions which are associated with pathologies such as ischemia, stroke, CNS injury and neurodegenerative disorders (Vexler et al., 2006). Hypoxia has Janus-faced effects on astrocytes and their ability to support neuronal viability (Vangeinson and Rempe, 2009). For example, hypoxia preconditioning mediates astrocyte-dependent protective effects on neurons (Chu et al., 2010). However, hypoxia in the presence of inflammatory signalling triggers processes in astrocytes, that augment neuronal death (Vangeinson et al., 2008). A principal molecular mechanism, by which cells respond to hypoxia, is the activation of the transcription factor HIF-1α. During hypoxia HIF-1α protein is stabilized and heterodimerizes with HIF-1β to form HIF-1, subsequently regulating the expression of target genes (Semenza, 2002). HIF-1 plays an important role in brain development and hypoxic-ischemic brain injury, and the molecule exhibits both neuroprotective as well as neurotoxic properties depending on the duration of hypoxia, the type of pathological stimuli and the cerebral cell type involved (Sharp and Bernaudin, 2004; Karovic et al., 2007; Fan et al., 2009). A myriad of factors are involved in the regulation of HIF-1α expression (Semenza, 2002). Exposure of macrophages to bacteria or LPS leads to HIF-1 accumulation even under normoxic conditions (Frede et al., 2006; Jantsch et al., 2011). As for glial cells, it has been suggested that LPS increases HIF-1 activity in microglia and oligodendrocytes, whilst no data exist in primary astrocytes (Oh et al., 2008; Yao et al., 2008; 2013).

Ado, is a signalling molecule that has been shown to dampen hypoxia-induced inflammation in many models of disease (Poth et al., 2013) and plays a neuromodulatory role in the CNS

(Fredholm, 2012). Activation of ARs in glial cells seems to mediate many protective effects on the surrounding neurons after hypoxic insult (Daré et al., 2007). In particular, Ado released by astrocytes, through A_1ARs stimulation, contributes to hypoxia-induced modulation of synaptic transmission, inhibits reactive astrogliosis and regulates glutamate uptake (Pascual et al., 2005; Martin et al., 2007; Wu et al., 2011). In addition, A_1 and A_3 subtypes protect astrocytes from hypoxic damage, by reducing ATP release and apoptosis (Björklund et al., 2008) and A_3AR activation inhibits inflammatory cell migration (Choi et al., 2011). It is well recognized that Ado affects HIF-1 induction in peripheral inflammatory cells like tumor cells (Merighi et al., 2005, Gessi et al., 2008), macrophages (De Ponti et al., 2007, Ramanathan et al., 2007; Ernens et al., 2010) and foam cells (Gessi et al., 2010) by activating different receptor subtypes. However no data are available concerning the regulation of HIF-1 by Ado in primary astrocytes.

Astrocyte

Work over the past decade indicates that astrocytes play multiple active roles in acute and chronic neuronal diseases such as seizure, stroke, and ischemia (Kimelberg and Nedergaard, 2010). Unlike microglia and oligodendrocytes, astrocytes form physically coupled networks mediated by gap junctions, which, among other functions, facilitate intercellular transmission of Ca^{2+} signaling and exchange of cytosolic contents, and display oscillations in ion permeability through astrocytic networks. Although astrocytes are typically immune labeled by glial fibrillary acidic protein (GFAP), GFAP immunoreactivity labels only major branches and processes of astrocytes. The actual territory occupied by an astrocyte is much larger than that revealed by GFAP immunostaining. Of note, each astrocyte forms a non-overlapping territory or domain (Iadecola and Nedergaard, 2007), which collectively resemble a lattice framework, appearing crystalline in nature. Although the implications of this organization are not fully understood, it becomes lost when astrocytes transition to reactive states (Oberheim et al., 2008). In addition, astrocytes have extensive contacts with both synapses and cerebral blood vessels, and control the increase in blood flow evoked by synaptic activity. The astrocyte-mediated blood flow increase is fundamental to the blood oxygen-level-dependent (BOLD) signal (Iadecola and Nedergaard, 2007). It is estimated that a single astrocyte can enwrap 140,000 synapses and 4 to 6 neuronal somata, and can contact 300 to 600 neuronal

dendrites in rodents (Bushong et al., 2002; Gao and Ji, 2010; Oberheim et al., 2009). A close contact with neurons and synapses makes it possible for astrocytes not only to support and nourish neurons but also to regulate the external chemical environment during synaptic transmission. Recently, it has been shown that receptor mediated increases in astrocytic Ca^{2+} can modulate neural network activity by active uptake of extracellular K^+ (Wang et al., 2012). Because the extracellular concentration of K^+ is an important determinant of the resting membrane potential and thereby of neuronal activity, active uptake of K^+ represents a simple yet powerful tool for rapid modulation of neural networks (Ji et al., 2013)

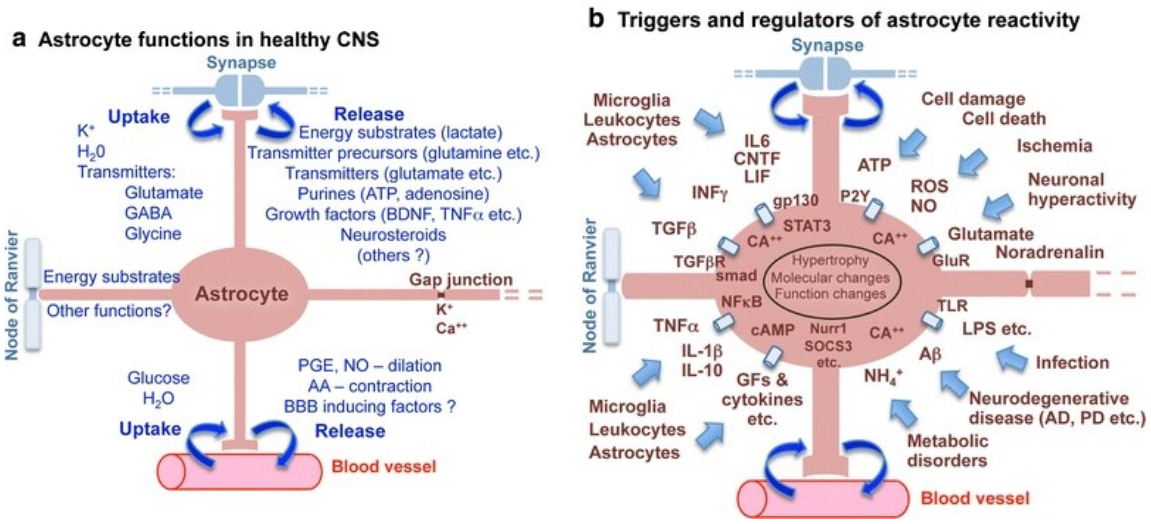


Figure 13:Schematic representations that summarize, a) astrocyte functions in healthy CNS, and b) triggers and molecular regulators of reactive astrogliosis (Sofroniew and Vinters, Acta Neuropathol;119:7-35; 2010)

Biochemical and structural changes of astrocytes during neuroinflammation represent a physiological response to CNS injury to minimize and repair the initial damage. Nevertheless, sustained inflammatory responses might be driven by positive feedback loops between microglia and astrocytes under conditions of severe and/or prolonged brain insults, thus providing detrimental signals that can compromise astrocytic and neuronal functions and lead to chronic neuroinflammation (Glass et al., 2010; Sofroniew and Vinters, 2010). Early stages of disease are associated with the activation of common inflammatory pathways, involving microglia and astrocytes activation, and the release of pro-inflammatory cytokines and other inflammatory mediators that regulate astrocytic hypertrophy and proliferation (Fig.13). Evidence of neuroinflammatory processes has been found in several neurological disorders, such as ischemia, spinal cord injury (SCI) and psychiatric/mood disorders (Okada et al., 2006; Verkhratsky et al., 2013), as well as in neurodegenerative diseases including Alzheimer's

disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia type 1 (SCA1), Huntington's disease (HD) and multiple sclerosis (MS) (Glass et al., 2010). Inflammatory processes are the hallmark of both acute and chronic neurodegenerative diseases. Glial reaction involves activation of receptors, such as Toll-like receptors (TLR), transcription factors (NF-kB, AP-1, etc.) and signaling molecules (p38MAPK, JNK, JAK/STAT3, etc.) of common inflammatory pathways, as well as alteration of protein expression (GFAP, vimentin, aminoacid transporters, receptors, etc.) and enzymes, like COX-2, NOS, MMPs, etc. (Glass et al., 2010). For instance, TLRs are associated with the activation of microglia and astrocytes by amyloid- and the downstream inflammatory response in AD models. Therefore, molecules that antagonize or modulate sensors (TLR, etc.), transducers (NF-kB,AP-1, etc.) and effectors (TNFα, COX-2, etc.) of the inflammatory response may represent a new prospective for disease-modifying therapies (Colangelo et al., 2014).

Astrocytes: targets for neuroprotection in CNS diseases

Brain ischaemia induces a cascade of events that involve a loss of glucose and oxygen, membrane depolarization, and Glu (glutamate) release, leading to excitotoxicity (Badawi et al., 2012). Brain tissue viability after insult depends in part on astrocyte survival and functioning. In hypoxia and hypoxic related injuries to the brain, astrocytes although moderately resilient to injury undergo changes that compromise their abilities to provide support and can even act as executioners of cellular demise. Astrocytes themselves in in vitro models can survive severe hypoxia for up to 96 hours and anoxia for 12-24 hours (Yu et al., 1989; Kelleher et al., 1993; Sochocka et al., 1994). In Middle Cerebral Artery Occlusion (MCAO) rat models, astrocytes experience morphological changes and swelling as early as 30 minutes after occlusion, followed by an increase in GFAP reactivity by six hours and astrocyte death at 24 hours (Garcia et al., 1993). Astrocytes in regions totally deprived of blood flow undergo rapid necrosis within an hour. Similarly protoplasmic astrocytes in the peri-infarct areas exhibit disrupted cellular processes and cell shrinkage, while fibrous astrocytes seem to form the classic glial scar surrounding the necrotic tissue of the core (Lukaszevicz et al., 2002). The differences between in vivo and in vitro observations regarding astrocyte viability are likely the result of shifts in extracellular ion concentrations and pH. Indeed astrocytes in culture that are exposed to a combination of hypoxia and acidosis die rapidly compared to hypoxia treatment alone (Swanson et al., 1997). While astrocyte survival is important to the ultimate cellular fate of neurons, astrocyte functioning following hypoxia can cause changes in the extracellular environment that will alter neuronal viability.

It is likely that the Janus roles of astrocytes in response to hypoxia will depend on which physiological cues are activated when, and in response to which toxic stimuli. Perhaps astrocytes when activated moderately by sublethal toxic stimuli-including hypoxia, can activate protective cues, while prolonged exposure to hypoxic stimuli will promote the deleterious effects of astrocytes resulting in inflammation, cyotoxic release of molecules and loss of the protective buffering roles. Despite the many adaptive effects of astrocytes in injury and normal physiology, astrocytes are also capable of regulating other less desirable functions that could actually perpetuate damage. Upon injury astrocytes can become highly activated, increase GFAP and induce gliosis. Activated astrocytes will then go through a number of morphological and biochemical changes that can result in the release of toxic mediators, or simply limit their ability to mediate their protective functions. Interestingly, recent evidence in other injury models has begun to suggest ways in which astrocytes can mediate pathology in the CNS. For instance, a mutation in Superoxide dismutase (SOD1), previously linked to ALS, was found to mediate toxicity through astrocytes. Astrocytes with mutated SOD1, were capable of inducing non-cell autonomous death of motor neurons, while expression of mutated SOD1 in neurons was not toxic to the neurons themselves. These researchers found that death was induced through a secreted factor, which induced a BAX dependent cell death pathway in neurons (Nagai et al., 2007). Others have identified mitochondrial dysfunction as the mechanism in astrocytes, downstream of mutated SOD1, which causes motor neuron toxicity. Mitochondria from these astrocytes displayed impaired respiratory function, decreased oxygen consumption, lack of ADP-dependent respiratory control, decreased mitochondrial membrane potential, and increased production of superoxide radicals (Cassina et al., 2008). Astrocytes also play a key role in regulating the blood-brain barrier (BBB) integrity in the cerebral vasculature. They are also capable of releasing Ca^{2+} waves which are stimulated by ATP (Arcuina et al., 2002). Increases in Ca^{2+} in the astrocyte cell body are followed by Ca^{2+} increases in astrocyte endfeet and subsequent dilation of small arterioles in stimulated slice preparations (Zonta et al., 2003). As blood flow to the brain is reduced in stroke and other ischemic injuries, astrocytes are able to communicate these changes to neurons (Vangeinson and Rempe,2009).

Hypoxia Inducible Factor

Initially identified as a regulator of erythropoietin (EPO) production, HIF is recognized as a key modulator of the transcriptional response to hypoxic stress. Besides its adaptive function in cellular stress responses, recent work has also revealed important roles for HIF in both physiological and pathological processes (Majmudar et al., 2010).

HIFs are obligate heterodimers consisting of an O_2-labile α subunit and a stable β subunit. Mammals possess three isoforms of HIFα, of which HIF-1α and HIF-2α (also known as EPAS1) are the most structurally similar and best characterized. HIF-3α (or IPAS) exists as multiple splice variants, some of which inhibit HIF-1α and HIF-2α activity in a dominant negative fashion (Kaelin and Ratcliffe, 2008). HIF-1α is expressed ubiquitously in all cells, whereas HIF-2α and HIF-3α are selectively expressed in certain tissues, including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage (Bertout et al., 2008).

HIF-1

HIF-1 is a heterodimetric complex consisting of a constitutively expressed β subunit (also known as the aryl hydrocarbon receptor nuclear translocation, ARNT) and a hypoxia inducible subunit, α subunit (Wang et al., 2002). Both subunits belong to the basic helix-loop-helix (bHLH)- PER-ARNT-SIM (PAS) protein family. The bHLH and PAS motifs are required for dimerization between HIF-1α and HIF-1β. HIF-1α possesses a unique oxygen-dependent degradation domain (ODDD), which mediates oxygen-regulated stability, and two transactivation domains, N-terminal (N-TAD) and C-terminal (C-TAD) (Pugh et al., 1997; Ruas et al., 2002). Under hypoxia, HIF-1α is stabilized and translocated to the nucleus where it dimerizes with HIF-1β. The activated HIF-1 complex subsequently binds to hypoxic response elements (HREs) in the regulatory regions of genes and recruits co-transactivation factors such as CBP/p300 (CREB (cAMP-response element-binding protein)-binding protein/E1A-binding protein]. It induces transcription of more than a hundred genes with various functions. The activity of HIF-1 is mainly controlled through regulating the protein level of HIF-1α (Ke and Costa, 2006). Although the transcription and the synthesis of HIF-1α are constitutive, its protein degradation and transcriptional activity are regulated by post-translational modifications on different amino acid residues in different domains. HIF-1α protein can be hydroxylated. Prolyl hydroxylation determines HIF-1α degradation in

normoxia. HIF-1α is hydroxylated on the prolyl residues 402 and 564 within the ODDD (Hewitson et al., 2004). The hydroxylation is mediated by a family of prolyl hydroxylases, namely PHD1, PHD2, and PHD3 (Srinivas et al., 1999), among which PHD2 has the highest specificity for hydroxylation of HIF-1α (Hewitson et al., 2004). These PHD enzymes are 2-oxoglutarate-dependent dioxygenases and require oxygen as well as Fe^{2+} as cofactors (Ivan et al., 2001; Jaakkola et al., 2001). Hydroxylation promotes interaction of HIF-1α with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Maxwell et al., 1999; Ohh et al., 2000). HIF-1α is thereby poly-ubiquitinated and degraded by the 26S proteasome (Salceda and Caro, 1997; Min et al., 2002). At the transcriptional level, oxygen availability enables hydroxylation of the asparagine residue 803 in the C-TAD of HIF-1α by factor inhibiting HIF (FIH-1), which blocks the association of HIF-1α with transcriptional co-activators CBP/ p300 and thus inhibits its transcriptional activity (Lando et al., 2002). HIF-1α can be acetylated on lysine residue 532 in the ODDD domain. Acetylation may affect HIF-1α hydroxylation and ubiquitination. K532 acetylation by the ARrest-Defective-1 (ARD-1) enhances the interaction between HIF-1α and pVHL and, thus, leads to increased ubiquitination and concomitant proteasomal degradation (Jeong et al., 2002). Direct phosphorylation on threonine 796 by MAPK has been identified as a modification able to increase HIF-1 activity (Gradin et al., 2002). One mechanism to explain the increased transcriptional activity is that HIF-1β binds preferentially to the phosphorylated form of HIF-1α (Suzuki et al., 2001). Moreover, it has been reported that phosphorylation at T796 in HIF-1α increases the affinity of the interaction between HIF-1α and the transcriptional co-activator CBP/p300 (Lancaster et al., 2004). Consistent with this, it is found that phosphorylation of T796 prevents the hydroxylation of N803 by FIH (Dimova et al., 2009). Then, it has been suggested that direct phosphorylation of HIF-1α by MAPK is not correlated with its transcriptional activity, but that phosphorylation of the HIF-1α co-activator p300 by MAPK increases the interaction between the HIF-1α C-TAD and p300 (Brahimi-Horn et al., 2005). Inhibition of these phosphorylation sites has been shown to impair both transcriptional activity and the nuclear localization of HIF-1α. In addition, phosphorylation of HIF-1α by glycogen synthase kinase (GSK)-3β may target HIF-1α for proteasomal degradation (Dimova et al., 2009). HIF-1α can be oxidized. Hydroxyl radical and hydrogen peroxide can destabilize HIF-1α protein in both normoxic and hypoxic conditions (Huang et al., 1996; Liu et al., 2004). The oxidized HIF-1α protein might be recognized and degraded by the ubiquitin-independent 20S proteasomal pathway, which primarily degrades cellular oxidized proteins under oxidative stress conditions (Shringarpure et al., 2003). Therefore, in addition to the 26S proteasomal pathway, 20S proteasomal pathway may play an important role in the degradation of HIF-1α in ischemic conditions (Shi,

2009). Moreover, studies have revealed that S-nitrosation stabilizes HIF-1α protein and S-nitrosation of cysteine 800 of HIF-1α promotes its interaction with CBP/p300, thus enhancing HIF-1α activation (Yasinska et al., 2003). Sumoylation of HIF-1α has been reported to regulate its protein level and transcriptional activity. Increasing the activity of RWD-containing sumoylation enhancer (RSUME) could promote the sumoylation of HIF-1α by Ubc9 SUMO conjugase and stabilize HIF-1α during hypoxia (Carbia-Nagashima et al., 2007). However, desumoylation by SUMO-Specific Protease 1 (SENP-1) has been shown to be essential for the stabilization of HIF-1α (Cheng et al., 2007). Sumoylation has also been found to work with ubiquitination to reduce HIF-1 protein level and transcriptional activity (Cheng et al., 2007; Berta et al., 2007; Zhang et al., 2011)

HIF-2

Shortly after the cloning of HIF-1α, a closely related protein, HIF-2α was identified and cloned (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997). HIF-2α shares 48% amino acid sequence identity with HIF-1α and accordingly shares a number of structural and biochemical similarities with HIF-1α (for instance, heterodimerization with HIF-1β and binding HREs). In contrast to ubiquitously expressed HIF-1α, HIF-2α is predominantly expressed in the lung, endothelium, and carotid body (Ema et al., 1997; Tian et al.,1998; Ke and Costa, 2006).

HIF-3

HIF-3α, which was discovered later, is also expressed in a variety of tissues, dimerizes with HIF-1β, and binds to HREs (Gu et al., 1998). In addition, a splice variant of HIF-3α, inhibitory PAS (IPAS), which is predominantly expressed in the Purkinje cells of the cerebellum and corneal epithelium, was subsequently discovered (Makino et al., 2001). IPAS possesses no endogenous transactivation activity; rather, it interacts with the amino-terminal region of HIF-1α and prevents its DNA binding, acting as a dominant negative regulator of HIF-1 (Makino et al., 2001). However, IPAS can also be induced by hypoxia in the heart and lung, contributing to a negative feedback loop for HIF-1 activity in these tissues (Makino et al., 2002; Ke and Costa, 2006).

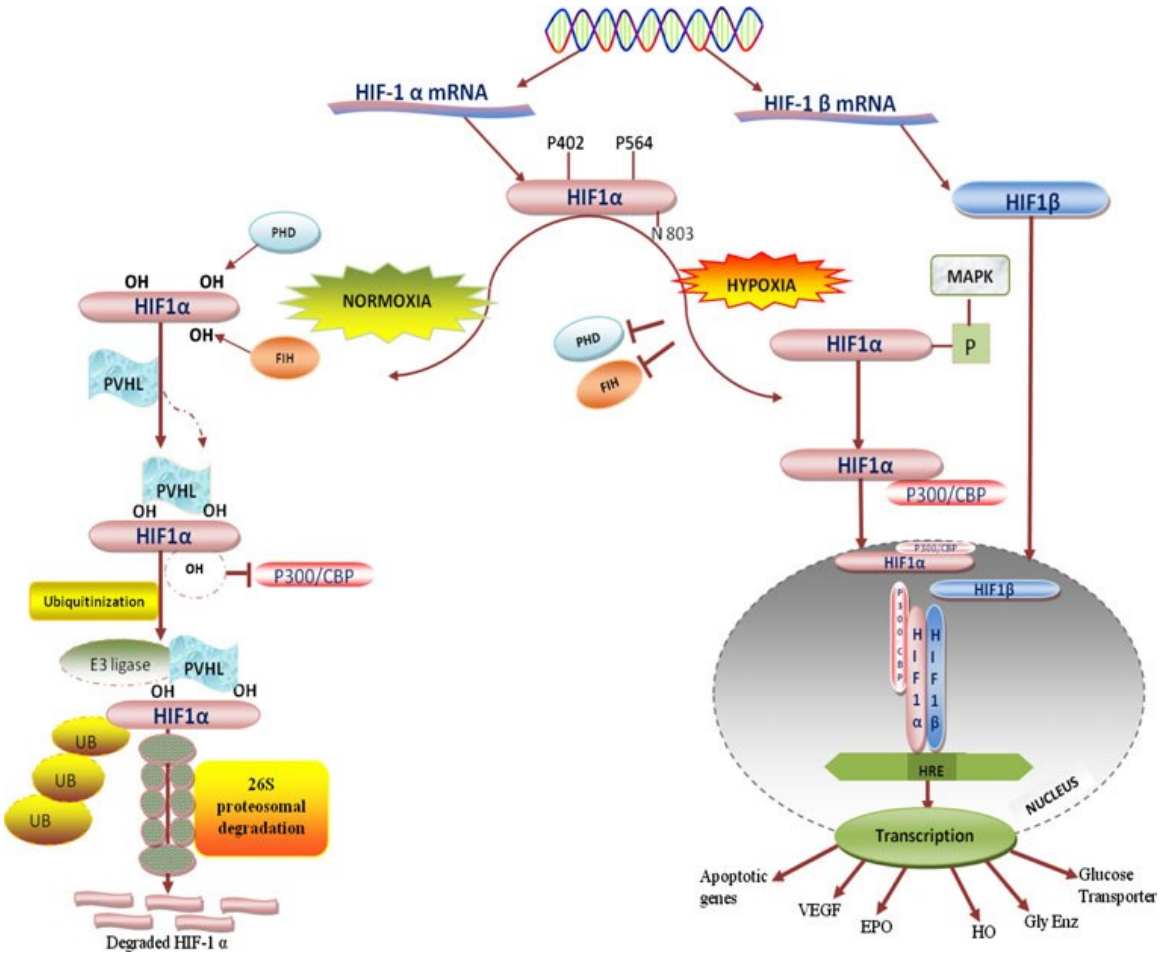


Figure 14: A systematic representation of HIF regulation under normoxic and hypoxic conditions (Singh et al., Cell Mol Neurobiol;32:491-507, 2012).

Recently, there have been extensive efforts to explore the beneficial role of HIF in several neurological diseases such as cerebral stroke and AD. In response to lower oxygen concentration, HIF-1α accumulates and triggers the expression of several genes which initiate angiogenesis, erythropoiesis, vascular tone maintenance, mitochondrial function, cell survival following ischemic/hypoxic injury. There is growing evidence which shows that activation of HIF-1α offer protection in case of cerebral ischemia (Singh et al., 2012).

HIF-Regulated genes: Neuroprotection or Inflammation

Vascular Tone

During cerebral ischemia, the disturbance in vascular tone is one of the major consequences, which results in neuronal cell death. The iNOS is one such gene which is regulated by HIF-1 and helps the cells to cope up with these adverse conditions (Singh et al., 2012). NO, serves as a signaling molecule and participates in a plethora of biological functions. In the mammalian brain, NO has been defined as an atypical neurotransmitter (Boehning and Snyder, 2003), being secreted by neurons and having direct effects on their electrical activity. More specifically, it has been postulated that NO serves as a retrograde messenger between the post- and the presynaptic elements in the most abundant excitatory synapses. It has been largely assumed that a neuronal Ca^{2+}/calmodulin-regulated NO synthase (nNOS) consists in the principal isoform producing the NO that acts at the synapse (Arancio et al., 1996; Micheva et al., 2003). Another Ca^{2+}/calmodulin-regulated NOS isoform is expressed primarily by endothelial cells, eNOS and is known to take part in blood flow regulation, although it has been proposed that NO produced by the cerebral vasculature also affects nearby neurons (Garthwaite et al., 2006). A third, "inducible" iNOS is expressed by glia cells, and acts in a Ca^{2+}-independent manner (Aktan, 2004; Alderton et al., 2001). The involvement of this isoform in modulating neuronal activity has been largely ignored, as the prevailing viewpoint maintains that this enzyme is not detectable under normal conditions, but rather it is up-regulated by gene induction following detrimental events such as ischemia or inflammation (Aktan, 2004; Saha and Pahan, 2006), where NO participates in the regulation of inflammatory processes. Several studies over the years have pointed to a weak constitutive expression of iNOS in various brain regions, and raised the possibility of astrocytic-derived NO participating in physiological brain processes (Buskila et al., 2005; Chan et al., 2001, 2003). iNOS is the only isoform whose activity is not dependent on the elevation of intracellular Ca^{2+} (Amitai, 2010).

Vascular Endothelial Growth Factor

The human and rodent VEGF genes (Liu et al., 1995) and their activation are triggered by the HIF-1α binding (Forsythe et al., 1996). During cerebral ischemia, the damaged tissue tries to increase oxygen delivery by the induction of angiogenesis via VEGF. This hypothesis is supported by the fact that there is an increase in the number of microvessels in the infarct area

of brain at various survival times in stroke patients as compared with normal hemisphere (Krupinski et al., 1994). The VEGF and its receptors are upregulated in the brain by HIF-1α and 2α during 6 to 24-h post-ischemia (Singh et al., 2012).

Glycolytic Enzymes

Since ischemic disturbance in cerebral blood flow results in high oxygen and glucose starvation. Glucose is the only source of ATP generation by the neuronal cells in brain and needs to be supplied to meet its required amount. Hence, to overcome this unfavorable situation the neuronal cells have to fulfill the glucose demand by increasing the activity of glycolytic enzymes and Glucose transport (GLUTs), which enhance the concentration of glucose and thereby ameliorate neuroprotection. GLUT and glycolytic flow as a result of HIF-1α activation by hypoxia has been linked to cell survival (Lawrence et al., 1996). It has been shown that there is an HIF-1α associated increase in GLUT1 expression, which starts from 7.5 h and last up to 24 h after permanent MCAO (Bergeron et al., 1999). The study also provided evidence that there is an increased expression of HIF-1α dependent glycolytic enzymes in cingulate/retrosplenial cortex following permanent MCAO (Singh et al., 2012).

A_{2B}ARs

Previous studies had identified transcriptionally regulated alterations of A_{2B}ARs expression during hypoxia-elicited inflammation. These studies demonstrated selective induction of the A_{2B}ARs following exposure of human vascular endothelia to ambient hypoxia. Additional studies investigating the promoter activity, functional chromatin binding, and HIF-1 loss-of-function studies demonstrated a critical role of HIF-1 in mediating hypoxia-associated induction of the A_{2B}ARs (Kong et al., 2006). Other works demonstrated HIF-1–dependent induction of the A_{2B}ARs during myocardial ischemia (Eltzschig et al., 2012). This study indicated that increases in A_{2B}ARs following exposure to inflammatory stimuli involved alterations in mRNA stability (Eckle et al., 2014)

Effect of adenosine in astrocytes

Ado can modulate astrocyte functions in many ways (Fig. 15). Astrocytes are endowed with all the known subtypes of ARs and they control metabolism of carbohydrates, astrogliosis and the release of neuroactive substances (Ciccarelli et al., 2001). Not all effects are receptor

mediated. For example, Ado (and inosine)-mediated reduction of cell death in glucose deprived astrocyte cultures (Shin et al., 2002), appears to be due to intracellular formation of ribose-1-phosphate, which is able to fuel the intracellular ATP production (Jurkowitz et al., 1998). By contrast, Ado-mediated rapid glycogenolysis followed by increased glycogen synthesis in primary astrocytes (Allaman et al., 2003), seems to be mediated by the A_{2B}AR and involves transcriptional changes of genes encoding proteins regulating energy metabolism. Astrocytes respond to traumatic and chemical brain injury with morphological and biochemical changes, including reactive gliosis and astrocytic swelling, which are presumably independent processes (Aschner et al., 1998). Reactive gliosis consists in phenotypic alterations, increased size and number of astrocytes. This process involves the up-regulation of enzymes and cytoskeletal proteins, e.g., GFAP and vimentin, as well as increased levels of TGF-β, nerve growth factor (NGF), basic-fibroblast growth factor (bFGF) and growth factor receptors, e.g., truncated forms of trkB. The elevated level of Ado that follows brain injury could critically modulate astrogliosis, as suggested by the increased number of GFAP-positive cells after microinjection of the Ado analogue 5′-(N-cyclopropyl)-carboxamidoadenosine into rat cortex (Hindley et al., 1994). This effect was abolished by coinfusion of the A_{2A}AR antagonist 1,3-dipropyl-7- methylxanthine (DPMX), indicating a role for A_{2A}ARs in regulating astrogliosis via Ado. In agreement, the A_{2A}AR antagonist 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH58261) reduced the formation of reactive astrocytes induced by bFGF in astrocytes in vitro (Brambilla et al., 2003). Ado was also reported to stimulate “stellation”, i.e., astrocytic hypertrophy, in primary cultures, via activation of ARs and tyrosine dephosphorylation (Abe and Saito, 1998). Besides A_2ARs, A_3ARs may also mediate astrogliosis (Abbracchio et al., 1998), whereas A_1ARs inhibit astrocyte proliferation (Ciccarelli et al., 1994). Reactive gliosis is generally regarded as beneficial, because it involves the production of neurotrophins and it isolates the damaged tissue. However, its prolonged effects may enhance the production of toxic factors, such as NO and arachidonic acid metabolites (Neary et al., 1996). Indeed, astrogliosis is also observed in neurodegenerative diseases. Paradoxically, Ado at high concentration can induce astrocyte cell death by apoptosis in vitro, via mechanisms involving both the activation of the A_3AR and Ado internalization and metabolism (Di Iorio et al., 2002). The capability of high levels of Ado to induce apoptosis has been interpreted as a possible mechanism to limit excessive cell proliferation due to extensive reactive gliosis (Ciccarelli et al., 2001). This pathway may become a promising target for the development of therapeutic strategies to treat neurodegenerative conditions, such as Parkinson's disease. In some circumstances, e.g., ischemia and traumatic brain injury, the challenging insults cause

astrocytic swelling, consisting in volume changes in the absence of GFAP accumulation, increases in extracellular K^+ concentration, and loss of L-glutamate, D-aspartate and taurine. Astrocytes produce a variety of factors that act as key mediators of immunity and inflammation. These include cytokines, chemokines, proteases, protease inhibitors, adhesion molecules and extracellular matrix (Dong and Benveniste, 2001). There is currently a great interest in investigating a possible role of Ado in the regulation of these factors. A recent study (Wittendorp et al., 2004) has demonstrated that stimulation of primary mouse astrocytes with the specific A_3AR agonist CL-IB-MECA induces the release of the chemokine CCL2 through a G_i protein-independent pathway. Thus, Ado acting on the A_3AR appears to modulate the level of a chemokine that can exert both neuroprotective and detrimental effects. Ado is able to enhance the production of other important neuroprotective substances, such as NGF, TGF-β1 and S100 β, in cultured astrocytes via activation of A_1ARs (Ciccarelli et al., 1999, Schwaninger et al., 1997). The increase in IL-6 synthesis and secretion induced by Ado, instead, is inhibited by the nonselective ARs antagonist 8-(p-sulfophenyl) theophylline, but not by specific A_1 or A_{2A} antagonists (Schwaninger et al., 1997). Furthermore, signalling via A_{2B}ARs is influenced by interleukins in parallel with the generation of NGF (Fredholm and Altiok, 1994). Thus, Ado may augment the neuroprotective effects of astrocytes both via changes of the astrocyte metabolism and through pathways mediated by different ARs subtypes. However, high levels of Ado caused by prolonged or exaggerated exposure to pathological conditions may also exacerbate degenerative processes, leading to both astrocyte death and damage of neurons. It should be remembered that astrocytes are an important component of the BBB and are directly involved in the control of the BBB permeability via release of mediators. The factors participating in this regulation are for the most part unknown and Ado itself is a candidate. Experiments performed with an experimental BBB in vitro model have not supported the hypothesis of a major role of ARs subtypes in the regulation of the BBB permeability. Instead, they have suggested a role of the ENTs in the transport of Ado derivatives in astrocytes, providing a possible target for delivering synthetic compounds to the brain (Schaddelee et al., 2003; Daré et al., 2007).

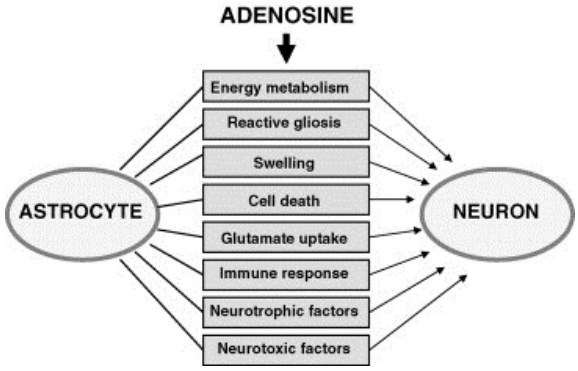


Figure 15: Ado modulates a variety of astrocyte functions
(Daré et al., Physiology & Behavior;92:15-20; 2007)

Possible role of HIF, Astrocyte and Adenosine in neuroprotection

HIF-1 promotes the production of Ado, because of binding to the CD73 promoter and subsequent induction of CD73 transcript and protein levels (Eltzschig et al., 2004; Thompson et al., 2004). As such, hypoxia and inflammation are associated with increased production of Ado from its precursor molecules, thereby shifting the balance from proinflammatory ATP signaling towards anti-inflammatory Ado signaling (Poth et al., 2013). Ado binds to the presynaptic A_1AR and lead to a decrease in Ca^{2+} influx. This further decreases the release of Glu and excitation of the NMDA receptors, thus preventing cellular damage caused by the subsequent increases in Ca^{2+} influx (Monopoli et al., 1998; Wardas, 2002). A study by Batti et al. (2010) has shown that the stabilization of HIF-1α through prolyl hydroxylase inhibition protected against Glu-induced damage in the hippocampus of the rat ischaemic brain mainly through Ado accumulation in response to hypoxia. A review article by Vangeison and Rempe (2009) clearly describes how hypoxia and HIF-1 can regulate various proteins, including connexin 43, CD73 and the ENT-1, which ultimately leads to enhanced Ado levels. Of these, both CD73 and ENT-1 have been shown to be regulated by HIF-1 in intestinal epithelia (Synnestvedt et al., 2002) and endothelial cells (Eltzschig et al., 2005) respectively. CD73 and ENT-1 are expressed in astrocytes (Vangeison and Rempe, 2009); therefore it is possible that HIF-1 can regulate their activity and increase Ado in astrocytes. In fact, it has been shown that Ado has a direct protective effect by reducing glucose deprivation-induced death on rat primary astrocytes (Shin et al., 2002) and an indirect action by decreasing human astroglioma cells damage following oxygen deprivation, through the preservation of ATP levels (Bjorklund et al., 2008; Badawi et al., 2012).

Aim of the thesis

Astrocytes perform several functions that are essential for normal neuronal activity. Accordingly, they can influence neuronal survival during ischemia (Annunziato et al., 2013). Many clinically relevant diseases are characterized by significant tissue hypoxia (Semenza, 2012; 2011; Eltzschig and Carmeliet, 2011). For example, ischemic tissue injury - such as that occurring in the context of myocardial infarction (Eckle et al., 2008; 2012), AKI (Grenz et al., 2012), or stroke (Iadecola and Anrather, 2011) - is characterized by a profound increase in tissue hypoxia due to lack of arterial supply with oxygen from the blood (Eltzschig and Collard, 2004; Poth et al., 2013). In the present study, we discussed the modulation of HIF-1α by LPS in presence and in absence of hypoxia in primary murine astrocytes and then we examined whether Ado and Ado analogues could modulate HIF-1α accumulation and transcription in primary astrocytes activated by LPS in normoxic and hypoxic conditions. In the end the effects of hypoxia, LPS and Ado on the HIF-1α target genes, considering the main genes involved in hypoxia, ischemia, and inflammation have been studied.

Materials and Methods

Drugs and materials

A_1, A_{2B}, A_3 ARs siRNAs, HIF-1α siRNA and anti-A_{2B} and A_3 ARs rabbit polyclonal antibodies were from Santa Cruz DBA (Milano, Italy). RNAiFect Transfection Kit was purchased from Qia-gen (Milano, Italy). Rabbit polyclonal antibody against A_1AR was obtained from Alpha Diagnostic, Vinci Biochem (Firenze, Italy). Rabbit polyclonal anti-HIF-1α was from Cayman, Vinci Biochem(Firenze, Italy). D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate] (SH5) was from Enzo-Life, Vinci Biochem (Firenze, Italy). 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and rabbit polyclonal anti-ERK1/2 were from Promega (Milano,Italy). Anti-beta actin and echinomycin were from Millipore (Milano, Italy). Anti-GFAP and anti-CD11b antibodies were from Becton Dickin-son (Milano, Italy). Rabbit monoclonal anti-phospho-p44/42MAPK (Erk1/2) (Thr202/Tyr204), rabbit polyclonal phospho-Akt(Ser473), Akt and iNOS were from Cell Signaling, Euroclone (Milano, Italy). The Assays-on-demandTMGene expression Products for HIF-1 (Mm00468869_m1), GLUT-1 (Mm00441480 _m1),GLUT-3 (Mm00441483_m1), GAPDH (Mm99999915_g1), hexokinase 2 (HK2) (Mm00443385_m1), VEGF (Mm00437304_m1), iNOS (Mm00440485_m1), A_1(Mm01308023_m1), A_{2B}(Mm00839292_m1) and A_3(Mm00802076_m1) were purchased from Life Technologies Italia (Monza, Italy). PSB 36, Cl-IB-MECA and BAY 60-6583 were purchased fromTocris, Space Import-Export (Milano, Italy). Unless otherwisenoted, all other reagents were purchased from Sigma (Milano,Italy).

Animals

One-day-old Balb/c mice were obtained from Charles River (Calco, Italy). Animal care procedures were in accordance with the guidelines of the European Council directives (86/609/EEC) and were approved by the local Animal Care and Ethics Committee.

Primary astrocyte cultures

Briefly, after anaesthesia (Zoletil 100, 30 mg·kg-1, Virbac Laboratories, France) and decapitation, forebrains from newborn Balb/c mice were excised, meninges were removed and tissue was dissociated mechanically in 0.25% Trypsin-EDTA solution (Gibco, Life Technologies Italia, Monza, Italy). Cells were re-suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, then plated on poly-L-lysine-coated (5 $mg \cdot mL^{-1}$) 75 cm^2 flasks

(Falcon; Euroclone, Milano, Italy). The culture medium was changed every 2-3 days. Astrocytes were obtained by treating the mixed cultures with cytosine arabinoside (AraC) for 4 days (10 μM) as previously described (Saura et al., 2005; Hamby et al., 2006). Cells were grown in a humidified environment containing 5% CO_2 at a constant temperature of 37°C. Astrocyte cultures were characterized by immunostaining with GFAP and the absence of microglial cells was confirmed using Mac-1 anti-CD11b antibody. LPS, a cell wall component of Gram negative bacteria, is a potent activator of glia. Hence, astrocytes were treated with 1 $\mu g \cdot mL^{-1}$ LPS under normoxia and hypoxia after exposure to Ado or receptor agonists for 30 min. All treatments with ARs agonists were performed in the presence of ADA.

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).

Immunofluorescence analysis

For HIF-1α detection cells were treated with Ado and LPS for 4 hours, under normoxia and hypoxia. Astrocytes were washed two times with PBS, fixed in 10% paraformaldehyde for 10 min, permeabilized in a PBS solution containing 0.1% of Triton X-100 and incubated for 30 min with PBS plus 5% goat serum and 0.5% bovine serum albumin. The cells were then incubated O.N. at 4°C in a humidified chamber with anti-HIF-1α Ab solutions (1:50) containing 0.5% of goat serum and 0.5% of bovine serum albumin in PBS. Excessive antibody was washed away with PBS and rabbit antibodies were detected with fluorescein isothiocyanate- labeled goat anti-rabbit IgG. Coverslips were stained with 4',6- diamino-2-phenyl-indole (DAPI), mounted in DABCO glycerol- PBS and observed on Nikon fluorescent microscope (Eclipse 50i) as previously described (Gessi et al., 2012). Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Milan, Italy). Levels of normoxic and hypoxic HIF-1 in astrocytes were the basis for calculation of the additional LPS-mediated increase and for the inhibition played by Ado. The mean intensity of each cell was obtained from the cells pixels that had a higher intensity than that of the mean background intensity. A mean of 150 cells was analyzed for each condition at 40X magnification, at fixed time exposure.

Western blotting

Whole cell lysates from murine astrocytes were prepared as described previously (Gessi et al., 2010), whilst nuclear extracts were obtained by using the Nuclear Extract Kit (Active Motif, Vinci Biochem, Firenze, Italy) according to the manufacturer's recommendations. The protein concentration was determined using a protein assay kit (Bio-Rad, Milano, Italy). Equivalent amounts of protein (40 μg) were subjected to electrophoresis on sodium dodecyl sulfate-acrylamide gel. The gel was then electroblotted onto a nitrocellulose membrane. The membranes were probed with anti-A_1 antibody (1:1000 dilution), anti-A_3 antibody (1:1000 dilution), anti-HIF-1α (1:1000 dilution), anti-iNOS (1:1000 dilution) and anti-A_{2B} (1:1000 dilution). Filters were washed and incubated for 1h at room temperature with peroxidase-conjugated secondary antibodies against rabbit IgG (1:2000 dilution). For detection of phosphorylated proteins antibodies specific for phosphorylated or total p44/p42 MAPK (1:1000 dilution), and phosphorylated or total Akt (1:1000 dilution) were used. Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Perkin Elmer, Milano, Italy). Actin (1:1000) was used to ensure equal protein loading. Please note that to better visualize the HIF-1 modulation by LPS and Ado in normoxia, the exposure times of western blots were higher for normoxia with respect to hypoxia.

Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad, Milano, Italy). Mean densitometry data from independent experiments were normalized to the results in control cells.

Real-time RT-PCR

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assays of HIF-1, VEGF, GLUT1, GLUT3, HK2, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), iNOS and A_{2B} mRNA were carried out using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) in a MX3000P Stratagene Real-Time PCR System (M-Medical, Milano, Italy) (Gessi et al., 2010). For the real-time RT-PCR of the reference gene the endogenous control mouse β-actin kit was used, and the probe was fluorescent-labeled with VIC™ (Life Technologies Italia, Monza, Italy). Reactions were normalized to β-actin mRNA within the same sample using the $\Delta\Delta^{CT}$ method.

small interfering RNA (siRNA) treatment

Astrocytes were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of siRNA-A_1, siRNA-A_3, siRNA-A_{2B} receptors and siRNA-HIF-1α, was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit for 72 hours. Cells were cultured in complete media, and then RNA and total proteins were isolated at 24, 48 and 72 h; after that realtime RT-PCR and western blot analysis of A_1, A_3, A_{2B} ARs and HIF-1α proteins were performed. A randomly chosen non-specific siRNA was used under identical conditions as control (Merighi et al., 2012).

Nitrite assay

NOS activity was assessed indirectly by measuring nitrite (NO_2^-) accumulation in the cell culture media using a colorimetric kit (Merck Chemicals, Nottingham, UK). At the end of the treatment period, the nitrite concentration in the conditioned media was determined according to a modified Griess method (Merighi et al., 2012). Briefly, the NADH-dependent enzyme nitrate reductase was used to convert the nitrate to nitrite prior to quantification of the absorbance, measured at 540 nm by a spectrophotometric microplate reader (Multiskan Ascent, Labsystems, Sweden). Sodium nitrite was used as the standard compound.

MTS Assay

The MTS assay was performed to determine astrocytes viability and proliferation according to the manufacturer's protocol from the CellTiter 96 AQueous One Solution (Promega) cell proliferation assay. Cells were plated in 24-multiwell plates; 500 µl of complete medium was added to each well with LPS, Ado and Ado + LPS.The cells were then incubated for 4, 8 and 16 h in normoxia andhypoxia. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read ona spectrophotometer at 490 nm. Experiment was repeated three times.

Statistical analysis

All values in the figures and text are expressed as mean ± standard error (SE) of N observation (with $N \geq 4$). N is equal to the number of mice from which the cells were derived. 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

LPS increases the expression of HIF-1α

A kinetic analysis of HIF-1α modulation by LPS was performed in murine astrocytes. In normoxia LPS significantly increases HIF-1α accumulation starting after 4 hours (Fig. 16A). In hypoxia HIF-1α protein induction could be slightly seen after a 2h incubation, and maximal induction was attained between 4 and 8h. Treatment of astrocytes with LPS enhanced hypoxia-induced HIF-1α accumulation starting after 2h and peaking at 4h (Fig. 16B). LPS at concentrations from 0.01 to 1 μg/ml significantly increased the expression of HIF-1α in a dose-dependent manner in normoxia and hypoxia (Fig. 16C,D, respectively).

Ado inhibits LPS-induced HIF-1α accumulation

In normoxia Ado reduced LPS-induced HIF-1α accumulation in a dose dependent way (Fig. 17A). The effect of the nucleoside was mimicked by the A_1 agonist N(6)-cyclohexylAdo (CHA) and the A_3 agonist Cl-IB-MECA in a dose-dependent manner, suggesting the involvement of A_1 and A_3 AR subtypes (Fig. 17B-C). The inhibitory effect induced by Ado, CHA and Cl-IB-MECA was also observed when LPS was incubated under hypoxic conditions (Fig. 17D-F). 2-p-(2- Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoAdo hydrochloride hydrate (CGS 21680) decreased it only at 1 μM and BAY 60-6583 did not affect its level in both normoxic and hypoxic conditions (data not shown). Furthermore Ado did not significantly affect HIF-1α levels in hypoxia-exposed cells (data not shown). HIF-1α subunit is a pivotal transcription factor, that requires nuclear translocation for activity. We therefore analyzed the nuclear translocation of HIF-1α protein after treatment with LPS without and with Ado under normoxia and hypoxia by immunofluorescence. As demonstrated by figure 18, LPS displayed an increase in the intensity of the nuclear HIF-1α immunofluorescence in normoxia and the effect was reduced by the nucleoside. Similar results were observed in hypoxic conditions (Fig. 19). This inhibitory effect was also observed by CHA and Cl-IB-MECA in nuclear extract prepared from primary astrocytes incubated with HIF-1α antibody in both normoxic and hypoxic conditions (Fig. 20A,B, respectively). To confirm the involvement of A_1 and A_3ARs in this response, astrocytes were treated with 10 nM PSB 36 and 1 μM MRS 1523, A_1 and A_3 antagonists, respectively, before addition of 30 nM CHA and 100 nM Cl-IB-MECA in normoxia and hypoxia. As shown in figure 19, the inhibitory effect induced by CHA and Cl-IB-MECA was antagonized by PSB36

and MRS 1523, respectively, both in normoxia (A) and hypoxia (B). Antagonists alone did not modified HIF-1α basal levels (data not shown). As the effect of Ado agonists on LPS-mediated HIF-1α increase in normoxic and hypoxic astrocytes were strictly similar, the inhibitory effect of Ado was evaluated in primary astrocytes exposed to A_1 and A_3 siRNAs under hypoxia. Silencing of primary astrocytes for 72 h reduced mRNA levels of A_1 (0.30±0.03) and A_3 AR subtypes (0.25±0.03) in comparison to control cells transfected with non-specific random control ribonucleotides (siRNA scrambled, siRNActr) (1.0±0.1) and strongly reduced protein expression (Suppl. Fig. 2). In this condition both siRNAs of A_1 and A_3 ARs partially reverted the nucleoside inhibition of LPS mediated HIF-1α protein accumulation, suggesting the involvement of A_1 and A_3 subtypes in this effect (Fig. 20C and D, respectively).

Mechanisms involved in the LPS-induced HIF-1α accumulation and effect of Ado

We elucidated the mechanism underlying the enhanced accumulation of HIF-1α protein by LPS under normoxia and hypoxia before to evaluate the inhibitory effect exerted by Ado. To investigate transcriptional modulation of HIF-1α by LPS, cells were incubated with actinomycin D (ActD). As shown in figure 21A the effect of LPS on HIF-1α protein accumulation was completely abrogated after incubation with the inhibitor of transcription, both in normoxia (A) and hypoxia (B). As shown in figure 21C, quantification of mRNA revealed a significant increase of HIF-1α expression by LPS under normoxic and hypoxic conditions reaching a peak at 4h, whilst hypoxia by itself did not affect mRNA level. Furthermore HIF-1α mRNA levels stimulated by LPS were decreased following Ado treatment both in normoxia and hypoxia, whilst Ado alone did not affect mRNA levels (Fig. 21D). Finally, primary astrocytes were pre incubated for 4h under hypoxic conditions in the absence and in the presence of LPS and LPS plus Ado, followed by reoxygenation for up to 30 min. Our results show that after the return of hypoxic astrocytes to normoxia, the levels of HIF-1α protein decreased very rapidly. In particular, LPS in the absence and in the presence of Ado did not alter the degradation rate of the HIF-1α protein, suggesting that a reduced or increased degradation of HIF-1 after reoxygenation was not affected by both LPS and Ado, respectively (Fig. 21E).

Signalling pathways involved in the LPS-induced HIF-1α accumulation and effect of Ado

To examine whether MAPK and Akt pathways were involved in the inhibitory effect mediated by Ado on LPS-induced HIF-1α accumulation, primary astrocytes were pretreated

with U0126 and SH5, inhibitors of MEK1/2, and Akt respectively, before exposure to LPS in normoxia and hypoxia. As shown in figure 22, U0126 and SH5 strongly reduced the LPS-mediated HIF-1α stimulation, suggesting the involvement of ERK1/2 and Akt in this effect both in normoxia (A) and hypoxia (C). To confirm these data, phosphorylation of ERK and Akt was assessed following normoxia and hypoxia in the absence and in the presence of LPS, Ado and Ado with LPS. LPS increased ERK1/2 and Akt phosphorylation in normoxia (Fig. 22B) and hypoxia (Fig. 22D) and Ado inhibited this effect under both conditions.

Downstream HIF-1α target genes: effect of LPS and Ado

Given the increase in the proportion of nuclear localized HIF-1α after treatment with LPS in both normoxia and hypoxia and the inhibitory effect of Ado, we then evaluated whether the transcriptional activity of HIF-1 was also affected in murine astrocytes. We looked for a series of genes that are known to be regulated by HIF-1, such as those for angiogenesis and cell metabolism. To this aim VEGF, GLUT1, GLUT3, HK2 and GAPDH were quantified under normoxic and hypoxic conditions. In normoxia, LPS did not modulate VEGF, GLUT3 and GAPDH but increased GLUT1 (Fig. 23A) and HK2 (Fig.23C), in a time-dependent way. The involvement of HIF-1 in this effect was assessed by treating cells with echinomycin, an inhibitor which prevents HIF-1 binding to specific target promoter sequences. Furthermore, U0126 and SH5 were able to reduce the LPS mediated GLUT1 and HK2 increase, suggesting the involvement of ERK1/2 and Akt in this effect. Finally, CHA and Cl-IB-MECA inhibited LPS-induced mRNA increase of GLUT1 and HK2 and PSB36 and MRS 1523 reverted this effect (Fig. 23B and D, respectively). Hypoxia by itself induced an increase in VEGF, GLUT1, GLUT3, HK2, GAPDH mRNA expression in astrocytes; this effect was HIF-1 dependent, because their level was reduced following incubation with HIF-1α siRNA and echinomycin, (Fig. 23 E-I). HIF-1α silencing for 72h reduced mRNA expression at 0.32±0.03 in comparison to cells transfected with non-specific siRNActr (1.0±0.1) and also protein level was reduced (Suppl. Fig. 3). However LPS did not further increase the expression of VEGF, GLUT1, GLUT3, HK2, GAPDH in hypoxia, neither CHA and Cl-IB-MECA receptor agonists changed significantly the level of these genes (data not shown). Cell proliferation and survival were not affected by LPS and Ado at any time investigated (4,8,16h) (Suppl. Fig.4). Therefore we evaluated the regulation of genes involved in inflammation like iNOS and A_{2B}ARs. In normoxia iNOS levels were stimulated by LPS in a time-dependent way (Fig. 24A). Hypoxia by itself induced a slight stimulation of iNOS, whilst the increase induced by LPS in concert with hypoxia was strong and time-dependent, starting from 4h and peaking at 8h under hypoxia (Fig. 24B). The effect induced by LPS in normoxia was reduced by HIF-1α

inhibition, incubation with U0126 and SH5 suggesting that iNOS induction was stimulated by LPS through a pathway involving HIF-1α transcription factor, via ERK and Akt activation (Fig.24C). CHA and Cl-IB-MECA reduced iNOS mRNA level stimulated by LPS (Fig. 24C). Antagonism of PSB36 and MRS1523 reversed the CHA and Cl-IB-MECA reduction of iNOS mRNA level stimulated by LPS, confirming the involvement of A_1 and A_3 ARs in this response (Fig. 24C). The effect induced by hypoxia was HIF-1 dependent (Fig. 24D). Furthermore also the effect induced by LPS in hypoxia was reduced by HIF-1α silencing, incubation with echinomycin, U0126 and SH5 suggesting that iNOS induction was stimulated by LPS through a pathway involving HIF-1α transcription factor, via ERK and Akt activation (Fig. 24E). Like in normoxia A_1 and A_3 ARs inhibited this effect (Fig. 24F). Analogous results were obtained for A_{2B}ARs. In normoxia A_{2B}ARs were stimulated following LPS treatment. Hypoxia by itself increased A_{2B}ARs levels but LPS in concert with hypoxia induced a strong increase of A_{2B} mRNA values (Fig. 25A). The increase was reduced following echinomycin, U0126 and SH5 treatment (Fig. 25B) suggesting again a role for HIF-1α, via ERK and Akt activation in the LPS increase. The LPS stimulatory effect on A_{2B} expression was reduced by CHA and Cl-IB-MECA and restored in the presence of PSB36 and MRS 1523 receptor antagonists (Fig. 25B). The increase induced by hypoxia alone was HIF-1 dependent (Fig. 25C). Furthermore also the effect induced by LPS in hypoxia, on A_{2B}AR levels involved the activation of HIF-1α transcription factor, via ERK and Akt pathways (Fig. 25D). Like in normoxia A_1 and A_3 ARs inhibited this effect (Fig. 25E).

Ado-mediated inhibition of LPS-stimulated iNOS and A_{2B} AR expression

We investigated the stimulatory effect induced by LPS on iNOS and A_{2B}ARs protein level and the inhibition exerted by Ado in primary astrocytes. As shown in figure 26A iNOS was not detectable in both normoxia and hypoxia without LPS stimulation and was increased following LPS treatment. The effect of LPS was higher when cells were incubated in hypoxia, and Ado was able to strongly reduce iNOS protein level stimulated by LPS under both normoxia and hypoxia. Accordingly, when nitrite levels were evaluated in the supernatant of murine astrocytes an increase following LPS incubation and a reduction in cells treated with Ado was observed in both conditions (Fig. 26B). Similarly a significant increase in A_{2B}AR protein levels following LPS incubation under normoxic and hypoxic conditions was observed. The effect of LPS was higher in cells incubated in hypoxia and a reduction of this effect in Ado treated cells was detected according to mRNA data (Fig. 26C). The specificity of the bands is demonstrated by their reduction after treatment of primary astrocytes for 48-72

h with siRNA of $A_{2B}AR$ in comparison to control cells transfected with non-specific random control ribonucleotides (Suppl. Fig. 5).

Discussion

HIF-1α is an important protein in the regulation/induction of many genes in the cellular response to hypoxia and a central mediator in inflammatory signalling. In this study, we have provided evidence, for the first time, that Ado is able to decrease HIF-1α expression and protein accumulation induced by LPS in murine astrocytes under both normoxic and hypoxic conditions. By using agonists, antagonists and receptor silencing, we found that A_1 and A_3 ARs were responsible for the Ado-mediated inhibitory effect. The inhibitory effect of Ado on HIF-1α levels was quite surprising, as the nucleoside usually increased HIF-1α levels in peripheral cells, by activating different receptor subtypes, depending on the cellular model investigated. A_{2A}ARs have been linked to HIF-1α accumulation in human and murine macrophages (De Ponti et al., 2007; Ramanathan et al., 2007; Ernens et al., 2010), A_3, A_{2B} ARs in tumor cells and in foam cells (Merighi et al., 2005; Gessi et al., 2008; 2010), A_1 ARs in neonatal rats and mesenchymal cells (Gessi et al., 2012; Wendler et al., 2007). In general, the role of HIF-1 in astrocytes has received much less attention in comparison to neurons, although astrocytes also play critical roles in health and disease. In particular, astrocytes and Ado have a clear role in mediating the protective effects on neurons (Vangeinson and Rempe, 2009). In this regard, selective loss of HIF-1 in astrocytes profoundly inhibits hypoxia-induced neuronal death, whereas loss of HIF-1 function in neurons increased neuronal susceptibility to hypoxia induced damage, suggesting that the adaptive and pathological function of HIF-1 is cell type specific (Vangeinson et al., 2008). So the inhibition of HIF-1 stimulated by LPS in both normoxia and hypoxia may be considered another neuroprotective mechanism operated by Ado against brain injuries. Our data show also, for the first time, that LPS stimulates HIF-1α accumulation in murine astrocytes, through an increase in mRNA expression, in both normoxic and hypoxic conditions. Accordingly, it is known that HIF-1α transcription is detected as an essential mechanism of HIF-1α activation in response to bacterial infection and has been observed previously in human macrophages and in other glial cells (Nizet and Johnson, 2009; Frede et al., 2006; Oh et al., 2008; Yao et al., 2008; 2013). Downstream signaling of LPS leads to the activation of a wide variety of signalling pathway, including activation of the p44/42 MAPK cascade. We observed a significant increase in p44/42 phopshorylation after LPS treatment under normoxia and hypoxia, which was followed by HIF-1α protein accumulation. Activation of p44/42 MAPK seems to be critical for LPS-induced HIF-1α accumulation and for the inhibitory effect exerted by Ado, since

inhibition of the upstream kinase MEK1/2 by U0126 reduced HIF-1α accumulation induced by LPS and Ado inhibited LPS-stimulated ERK phosphorylation. Accordingly, previous results show that LPS increased HIF-1α mRNA expression by activating upstream p44/42 MAPK in human monocytes (Frede et al., 2006). As for the effect of ARs on this pathway, Cl-IB-MECA has been reported to reduce p44/42 MAPK phosphorylation in melanoma cells (Merighi et al., 2005a) and to induce a biphasic effect on ERK1/2 phosphorylation in microglia cells (Hammarberg et al., 2003). Usually all ARs activate the ERK1/2 pathway in CHO cells, anyway nothing is known about ARs signalling in glial cells stimulated by LPS (Schulte and Fredholm, 2002). Another pathway linked to HIF- 1α accumulation is PI3K/Akt (Semenza, 2002). In our system, LPS-induced HIF-1α accumulation was inhibited by SH5 and accordingly an increase in Akt phosphorylation following LPS stimulation was observed. Ado decreased this effect as previously observed in microglial cells where Cl-IB-MECA, through inhibition of Akt pathway, inhibited pro-inflammatory cytokine expression (Lee et al., 2006). A wide variety of genes that regulate major cellular processes, like glicolysis, angiogenesis and inflammation are activated by HIF-1; some of them are known to be regulated by LPS and Ado as well. In normoxic astrocytes LPS did not affect VEGF, GLUT3 and GAPDH expression whilst, through HIF-1 activation, induced an increase in glucose transporter isoform GLUT1 and glycolysis-associated enzyme HK2. Stimulation of A_1, A_3 ARs reduced this effect, possibly to limit glucose consumption by astrocytes during inflammation. In hypoxic astrocytes we found that VEGF, GLUT-1, GLUT-3, HK-2, GAPDH were upregulated by hypoxia in an HIF-1 dependent way, but they were not further increased by LPS, suggesting that the amount of HIF-1 induced by hypoxia alone is probably sufficient to induce these genes very efficiently as already observed by Jantsch and coworkers in myeloid cells (Jantsch et al., 2011). In contrast, as for genes involved in inflammation we found that LPS increased iNOS levels in normoxia, hypoxia by itself had only minor effects on iNOS expression and LPS in concert with hypoxia markedly increased iNOS level, suggesting that hypoxia and HIF-1 are insufficient for a robust induction of iNOS mRNA and protein. Accordingly we did not detect iNOS protein under hypoxia alone, suggesting that iNOS is a TLR-induced, inflammatory HIF-1α-dependent gene (Jantsch et al., 2011). Whilst glial activation and iNOS expression alone do not always lead to neuronal death, extensive damage has been demonstrated if inflammatory activation of astrocytes was combined with hypoxia (Mander et al., 2005). NO has been implicated in DNA damage, PARP activation and induction of apoptosis (Vangeinson and Rempe, 2009). The results of this study reveal that A_1 and A_3 activation inhibited iNOS induction in astrocytes in both normoxic and hypoxic conditions supporting a protective effect during inflammation and hypoxic injury.

Accordingly, protection from hypoxic damage and reduction of apoptosis by A_1 and A_3 subtypes has been suggested also by Fredholm's group in primary astrocytes (Björklund et al., 2008). Furthermore an inhibitory effect on iNOS induction has been already observed following A_1 and A_3 activation in PC12 and macrophages, respectively supporting their anti-inflammatory role (Pingle et al., 2007; Fishman et al., 2012; Lee et al., 2011). It is well known that hypoxia induces an increase in Ado providing an endogenous signal that dampens excessive inflammation and promotes tissue repair and healing (Poth et al., 2013; Haskò et al., 2008). Several studies have suggested that signaling through A_{2B}ARs may be involved in tissue protection during hypoxia (Poth et al., 2013). The A_{2B}AR has been demonstrated to play a role in both stimulation or inhibition of cytokine release, which can exacerbate or reduce inflammation (Zhong et al., 2004; Vazquéz et al., 2008; Koscsò et al., 2012; Thimm et al., 2013). In astroglial cells, a bi-directional relation between cytokines and A_{2B}ARs has been reported as an important mechanism to modulate the cerebral damage progression (Fredholm and Altiok, 1994). Its expression is decreased by proinflammatory cytokines following acute treatment but is upregulated after chronic cytokines exposure resulting in excessive astrocytic activation (Trincavelli et al., 2004; 2008). We found that A_{2B}AR level was stimulated following LPS activation, raised by hypoxia alone and further increased after LPS stimulation in concert with hypoxia in an HIF-1 dependent way; the HIF-1 dependent modulation of A_{2B}AR in hypoxia was according to previous studies (Poth et al., 2013). The increase induced by LPS both in normoxia and hypoxia was reduced by A_1 and A_3 ARs activation, suggesting a potential mechanism to dampen excessive activation(Gessi et al, 2013).

In summary, our results show for the first time that Ado, through A_1 and A_3 ARs activation, reduces LPS-stimulated HIF-1α mRNA expression and protein accumulation by inhibiting LPS triggered p44/42 MAPK and Akt phosphorylation in normoxic and hypoxic conditions. This leads to an inhibition of genes involved in inflammation like iNOS and A_{2B}ARs, that are stimulated by LPS and further increased by LPS in concert with hypoxia, whilst does not affect angiogenesis and metabolic related genes, with the exception of GLUT1 and HK2 that are stimulated by LPS in normoxia and reduced by A_1 and A_3 ARs activation. These findings add a new molecular pathway activated by Ado in astrocytes to give a reduction of genes involved in inflammation and hypoxic injury that may cohexist in stroke, ischemia and other CNS disorders.

Figures Legend

Figure 16: Murine astrocytes accumulate HIF-1 in response to LPS stimulation. (A and B) Astrocytes were incubated under normoxic (A) and hypoxic (B) conditions for up to 8 h in the presence or absence of LPS (1μg/ml). (C and D) Astrocytes were incubated for 4 h in normoxic (C) and hypoxic (D) conditions in the presence of increasing concentrations of LPS (0.01, 0.1, 1 μg/ml). HIF-1and actin were detected by immunoblotting in whole cell lysates. Actin shows equal loading protein. Densitometric quantification of HIF-1 Western blots is the mean ± SE values (N = 4); cells in the absence of LPS (A and C) and cells in the absence of LPS incubated for 4 h in hypoxia (B and D) were set to 100%(Control). *$P < 0.05$ compared with cells in the absence of LPS.$^{\S}P < 0.05$ compared with cells in the absence of LPS at each time of hypoxia. $^{\#}P < 0.05$ compared with cells in the absence of LPS incubated for 4 h in hypoxia.

Figure 17: Ado, A_1 and A_3 ARs agonists inhibit HIF-1 accumulation stimulated by LPS. (A and D) Cells were pretreated for 30 min with increasing concentrations (1,10, 100 μM) of Ado; (B and E) (1, 10, 100 nM) CHA; (C and F) (1, 10, 100 nM) Cl-IB-MECA, before stimulation with LPS (1 μg/ml) for 4 h in normoxia (A–C) and hypoxia (D–F). The protein levels of HIF-1were determined by Western blotting in whole cell lysates and actin served as control for equal protein loading. Densitometric quantification of HIF-1Western blots is the mean ± SE values (N = 4); cells in the absence of LPS was set to 100% in A and D; cells in the presence of LPS without agonists was set to 100% in B, C, E and F; *$P < 0.05$,$^{\S}P < 0.05$ compared with cells treated with LPS in the absence of Ado or receptor agonists in normoxia and hypoxia, respectively.

Figure 18: Ado inhibits HIF-1 nuclear localization induced by LPS stimulation inmurine astrocytes in normoxia. (A–F) Cells were incubated under normoxia (4 h) in the presence of LPS stimulation (C–F) and Ado 100 μM (E and F). HIF-1nuclear accumulation was evaluated by means of immunofluorescence analysis; (G) bargraph data expressed as mean ± SE percentage of HIF-1staining (N = 4 for each group); *$P < 0.05$ vs cells in the presence of LPS. (B, D and F) The fluorescent dye DAPI was used to stain the nuclei of all astrocytes. Figure shows 1 representative experiment.

Figure 19: Ado inhibits HIF-1 nuclear localization induced by LPS stimulation in murine astrocytes in hypoxia. (A–F) Cells were incubated under hypoxia (4 h) in the presence of LPS

stimulation (C–F) and Ado 100 μM (E and F). HIF-1nuclear accumulation was evaluated by means of immunofluorescence analysis; (G) bargraph data expressed as mean ± SE percentage of HIF-1staining (N = 4 for each group);$^{\S}P < 0.05$ vs cells in the presence of LPS in hypoxia. (B, D and F) The fluorescent dye DAPI was used to stain the nuclei of all astrocytes. Figure shows 1 representative experiment.

Figure 20: Effect of $A_1$and A_3 ARs antagonists and receptor silencing on HIF-1 protein increase stimulated by LPS. (A and B) Effect of CHA (30 nM) and Cl-IB-MECA (100 nM), on HIF-1 protein accumulation induced by LPS under normoxia (A) and hypoxia (4 h) (B) and antagonism by 10 nM PSB 36 and 1 μM MRS 1523. The protein levels of HIF-1were determined by Western blotting in nuclear extracts and actin served as control for equal protein loading. Densitometric quantification of Western blots is the mean ± SE values (N = 4); $^{*}P < 0.05$,$^{\S}P < 0.05$ compared with LPS in normoxia and hypoxia, respectively. (C and D) Ado effect on HIF-1 levels stimulated by LPS under hypoxia in the presence of siRNA of A_1(C) and A_3(D) ARs (lines 4–6) or siRNActr (lines 1–3). The protein levels of HIF-1were determined by Western blotting in nuclear extracts and actin served as control for equal protein loading. Densitometric quantification of Western blots is the mean ± SE values (N = 4);$^{\S}P < 0.05$ compared with LPS in hypoxia;$^{\#}P < 0.05$ compared with cells treated with Ado + LPS in hypoxia. Cells in the absence of LPS were set to 100% (control).

Figure 21: Molecular mechanism involved in the inhibitory effect mediated by Ado on LPS-induced HIF-1 accumulation. (A and B) Cells were treated with ActD (10 mg/ml) before a 4 h incubation with LPS under normoxic (A) and hypoxic (B) conditions. HIF-1 and actin were detected by immunoblotting in whole cell lysates. Densitometric quantification of HIF-1 Western blots is the mean ± SE values (N = 4); (C) total RNA was extracted from cells treated in normoxia (white bars) and in hypoxia (black bars)without and with LPS for 2, 4 and 8 h. Data were expressed as fold of increase vs control arbitrarily fixed as 1 (cells in the absence of LPS in normoxia). $^{*}P < 0.05$ compared with control in normoxia. (D) Total RNA was extracted from cells pretreated with 100 μM Ado before addition of LPS for 4 h in normoxia (white bars) and hypoxia (black bars). Data were expressed as fold of increase vs control arbitrarily fixed as 1 (cells in the absence of LPS). $^{*}P < 0.05$,$^{\S}P < 0.05$ compared with cells in the absence of LPS in normoxia and hypoxia, respectively. (E) Astrocytes were incubated under hypoxic conditions in the absence or presence of LPS and LPS + Ado. After 4 h, cells were exposed to normoxia and a time course of HIF-1 disappearance was performed at 0, 5, 10 and 15 min. The mean densitometry data from four independent experiments were

normalized to the result obtained at time 0 (control). The fraction (%) of remaining HIF-1 is indicated.

Figure 22: Signaling pathways involved in the inhibitory effect mediated by Ado on LPS-induced HIF-1 accumulation. (A and C) Cells were preincubated with the MEK1/2 inhibitor U0126 10μM and the Akt blocker SH5 10μM before addition of LPS in normoxia and hypoxia (4 h), respectively. HIF-1and actin were detected by immunoblotting in whole cell lysates. Actin shows equal loading protein. Densitometric quantification of HIF-1 Western blots is the mean ± SE values (N = 4); cells in the absence of LPS were set to 100%. (B and D) Astrocytes were treated without and with LPS, Ado and Ado + LPS under normoxia and hypoxia (15 min), respectively. Phosphorylated p44/42MAPK, pAkt as well as total MAPK and Akt, were detected by immunoblot. Densitometric quantification of p44/42 MAPK, pAkt Western blots is the mean ± SE values (N = 4). $^{*}P < 0.05$,$^{\S}P < 0.05$ compared with cells in the presence of LPS in normoxia and hypoxia, respectively.

Figure 23: Downstream HIF-1 target genes involved in cell metabolism and angiogenesis in normoxia and hypoxia. (A and C) Cells were incubated in the presence of LPS under normoxia for 4, 8 and 16 h and mRNA levels of GLUT-1 (A) and HK2 (C), were evaluated by real-time RT-PCR. (B and D) Cells were incubated with LPS in normoxia after pretreatment with 20 nM echinomycin, 10 μM U0126, 10 μM SH5, 30 nM CHA, 100 nM Cl-IB-MECA, 10 nM PSB36 and 1 μM MRS 1523 and mRNA levels of GLUT-1 (B) and HK2 (D) were evaluated. Data were expressed as fold of increase vs control (cells without LPS), arbitrarily fixed as 1. $^{*}P < 0.05$ compared with cells without LPS; $^{\#}P < 0.05$ compared with cells with LPS. (E-I) Cells were incubated in normoxia and hypoxia for 8 h in the absence and in the presence of siRNActr, siRNA-HIF-1, 20 nM echinomycin and mRNA levels of VEGF, GLUT-1, GLUT-3, HK2 and GAPDH, were evaluated by real-time RT-PCR. Data of hypoxia were expressed as fold of increase vs normoxia, arbitrarily fixed as 1; data of HIF-1siRNA and echinomycin were expressed as fold of increase vs hypoxia. $^{\S}P < 0.05$ compared with cells in hypoxia.

Figure 24: iNOS is stimulated by LPS in normoxia and hypoxia through a pathway involving HIF, ERK1/2 and Akt and A_1, A_3 ARs inhibit this effect. (A and B) Cells were incubated with LPS in normoxia and hypoxia for 4, 8 and 16 h and mRNA levels of iNOS were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs normoxia, arbitrarily fixed as 1. (C–F) Astrocytes were incubated with LPS in the absence and in the presence of echinomycin, U0126, SH5, CHA, Cl-IB-MECA, PSB36 and MRS1523 in normoxia (C) and

hypoxia (D–F) and mRNA levels of iNOS were evaluated. Data were expressed as fold of increase vs control (cells in the absence of LPS), arbitrarily fixed as 1. In (D) data of hypoxia were expressed as fold of increase vs normoxia, arbitrarily fixed as 1; data of HIF-1siRNA and echinomycin were expressed as fold of increasevs hypoxia. *P < 0.05 compared with cells in normoxia, $^{\#}$P < 0.05 compared with cells treated with LPS in normoxia, §P < 0.05 compared with cells exposed to hypoxia and †P < 0.05 compared with cells exposed to LPS and hypoxia. Mean ± SE values from four experiments are shown.

Figure 25: A_{2B}AR is stimulated by LPS in normoxia and hypoxia through a pathway involving HIF, ERK1/2 and Akt and A_1, A_3 ARs inhibit this effect. (A) Cells were incubated with LPS in normoxia and hypoxia for 4, 8 and 16 h and mRNA levels of A_{2B} were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs normoxia, arbitrarily fixed as 1. (B–E) Astrocytes were incubated with LPS in the absence and in the presence of echinomycin, U0126, SH5, CHA, Cl-IB-MECA, PSB36 and MRS1523 in normoxia (B) and hypoxia (C–E) and mRNA levels of A_{2B} were evaluated. Data were expressed as fold of increase vs control (cells in the absence of LPS), arbitrarily fixed as 1. In (C) data of hypoxia were expressed as fold of increase vs normoxia, arbitrarily fixed as 1; data of HIF-1siRNA and echinomycin were expressed as fold of increase vs hypoxia. *P < 0.05 compared with cells in normoxia, $^{\#}$P < 0.05 compared with cells treated with LPS in normoxia, §P < 0.05 compared with cells exposed to hypoxia and †P < 0.05 compared with cells exposed to LPS and hypoxia. Mean ± SE values from four experiments are shown.

Figure 26: Inhibition by Ado on LPS-stimulated iNOS and A_{2B} protein levels in normoxia and hypoxia. (A and C) Astrocytes were treated with 100 μM Ado before addition of LPS under normoxia and hypoxia. Protein levels of iNOS (A) and A_{2B}(C) were evaluated by means of Western blotting in whole cell lysates, actin shows equal loading protein. Densitometric quantification of iNOS and A_{2B} Western blots is the mean ± SE values (N = 4). (B) Astrocytes were treated with 100 μM Ado before addition of LPS under normoxia and hypoxia and nitrite levels were evaluated by Greiss reaction. Bar graph of colorimetric nitrite assay is the mean ± SE values (N = 4). *P < 0.05, §P < 0.05 compared with cells in the presence of LPS in normoxia and hypoxia, respectively.

Supplemental Figure 2: Western blot analysis using anti A_1 and A_3 ARs polyclonal antibodies of protein extracts from cells treated with siRNA-A_1 and siRNA-A_3 and cultured for 24, 48 and 72 h; cells transfected with siRNActr for 72 h (line 1). Actin shows equal loading protein. *P < 0.05 vs cells transfected with siRNActr for 72 h.

Supplemental Figure 3: Western blot analysis using anti HIF-1 receptor polyclonal antibody of protein extracts from cells treated with siRNA-HIF-1 and cultured for 24, 48 and 72 h; cells transfected with siRNActr for 72 h (line 1). Actin shows equal loading protein. *P < 0.05 vs cells transfected with siRNActr for 72 h.

Supplemental Figure 4: MTS assay. Cell growth is expressed as a percentage of the OD measured in untreated cells (control) assumed as 100% of cell viability. Ordinate reports means of OD quantifications ± SEM (N = 4).

Supplemental Figure 5: Western blot analysis using anti A_{2B}AR polyclonal antibodies of protein extracts from cells treated with siRNA-A_{2B} and cultured for 24, 48 and 72 h; cells transfected with siRNActr for 72 h (line 1). Actin shows equal loading protein. *P < 0.05 vs cells transfected with siRNActr for 72 h.

Figure 16

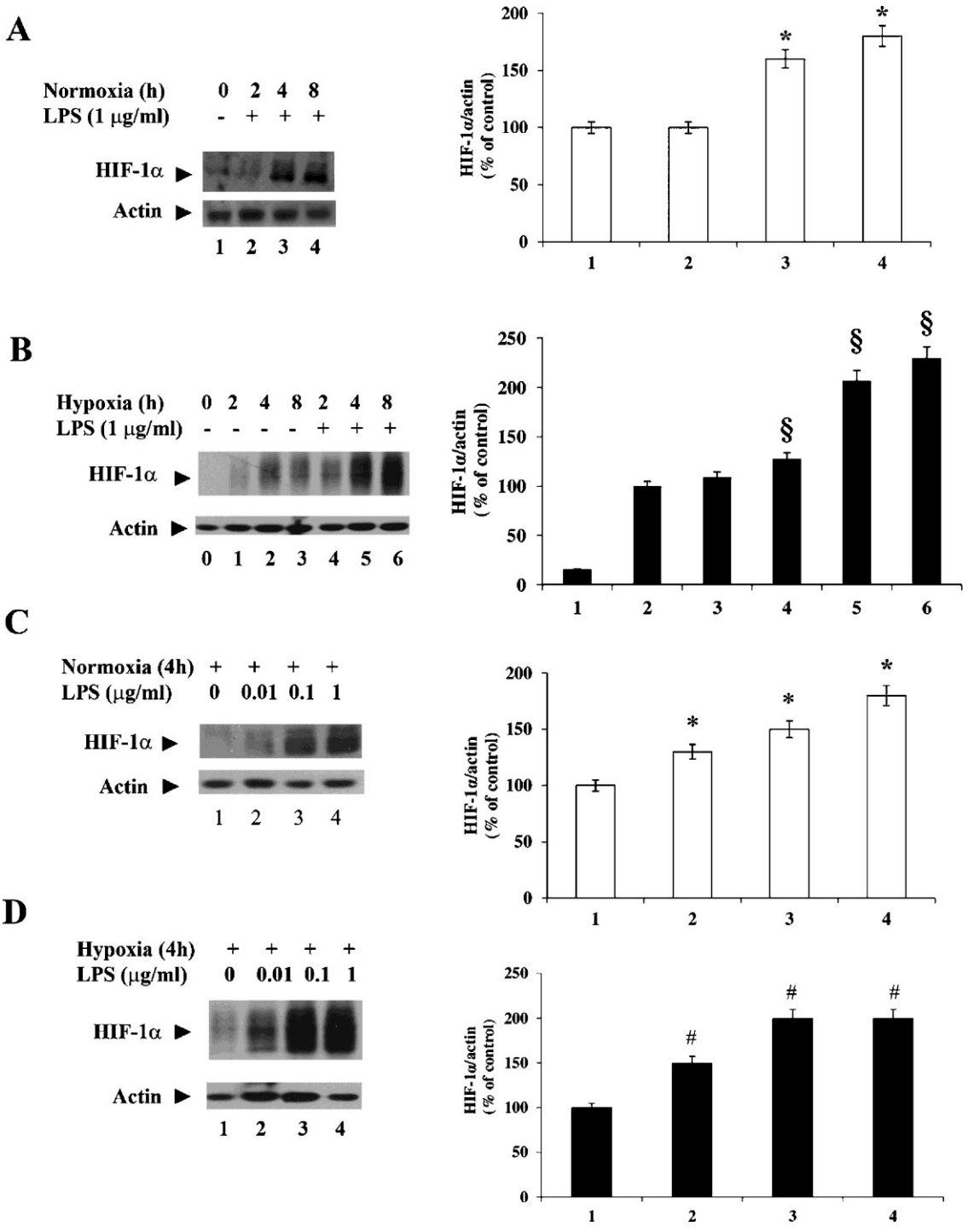

A
Normoxia (h) 0 2 4 8
LPS (1 μg/ml) - + + +
HIF-1α
Actin
1 2 3 4
HIF-1α/actin (% of control)
B
Hypoxia (h) 0 2 4 8 2 4 8
LPS (1 μg/ml) - - - - + + +
HIF-1α
Actin
0 1 2 3 4 5 6
C
Normoxia (4h) + + + +
LPS (μg/ml) 0 0.01 0.1 1
HIF-1α
Actin
1 2 3 4
D
Hypoxia (4h) + + + +
LPS (μg/ml) 0 0.01 0.1 1
HIF-1α
Actin
1 2 3 4

Figure 17

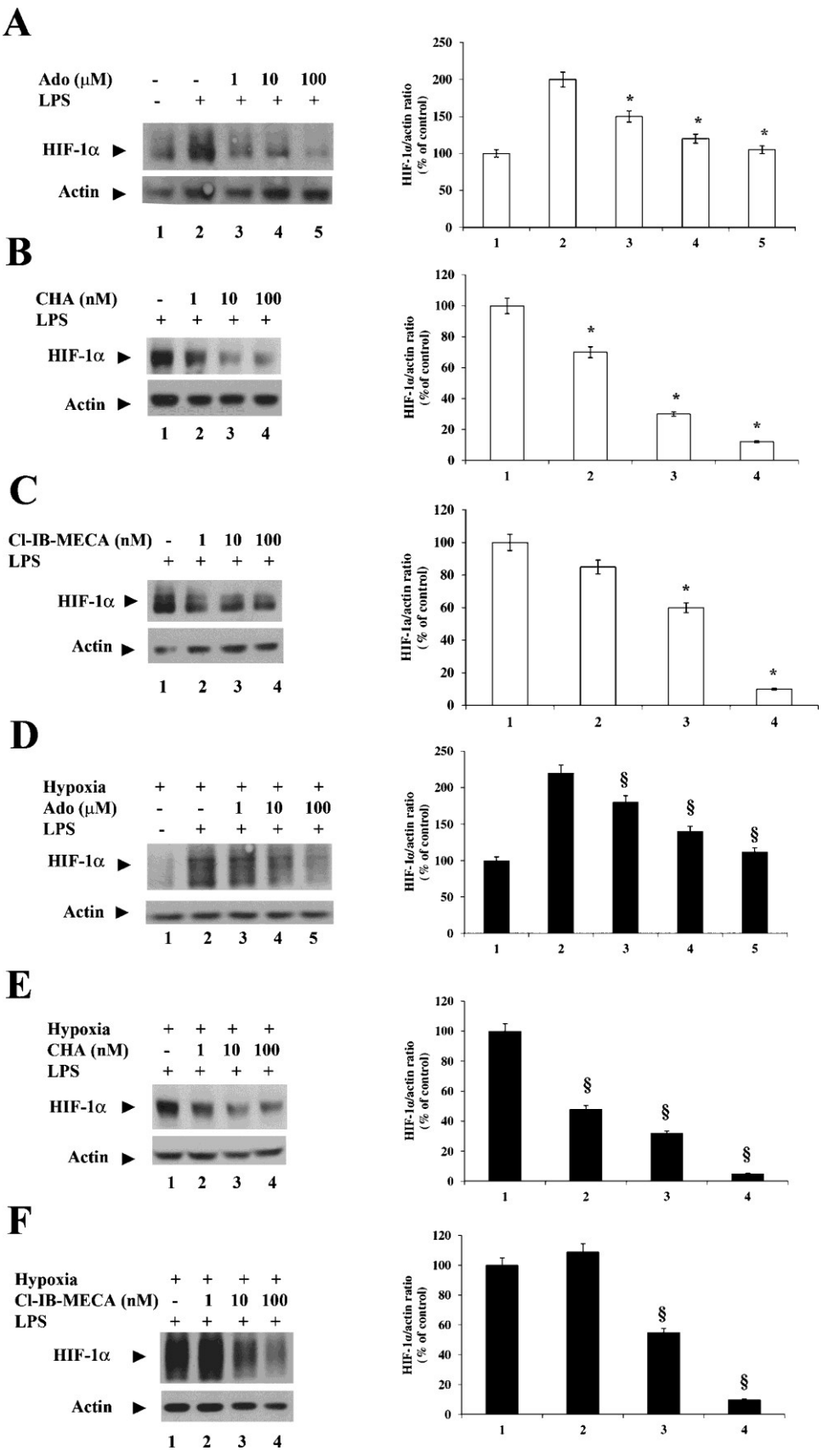

A
Ado (μM) - - 1 10 100
LPS - + + + +
HIF-1α
Actin
1 2 3 4 5
HIF-1α/actin ratio (% of control)
B
CHA (nM) - 1 10 100
LPS + + + +
HIF-1α
Actin
1 2 3 4
HIF-1α/actin ratio (%of control)
C
Cl-IB-MECA (nM) - 1 10 100
LPS + + + +
HIF-1α
Actin
1 2 3 4
HIF-1a/actin ratio (% of control)
D
Hypoxia + + + + +
Ado (μM) - - 1 10 100
LPS - + + + +
HIF-1α
Actin
1 2 3 4 5
HIF-1α/actin ratio (% of control)
E
Hypoxia + + + +
CHA (nM) - 1 10 100
LPS + + + +
HIF-1α
Actin
1 2 3 4
HIF-1α/actin ratio (% of control)
F
Hypoxia + + + +
Cl-IB-MECA (nM) - 1 10 100
LPS + + + +
HIF-1α
Actin
1 2 3 4
HIF-1α/actin ratio (% of control)

Figure 18

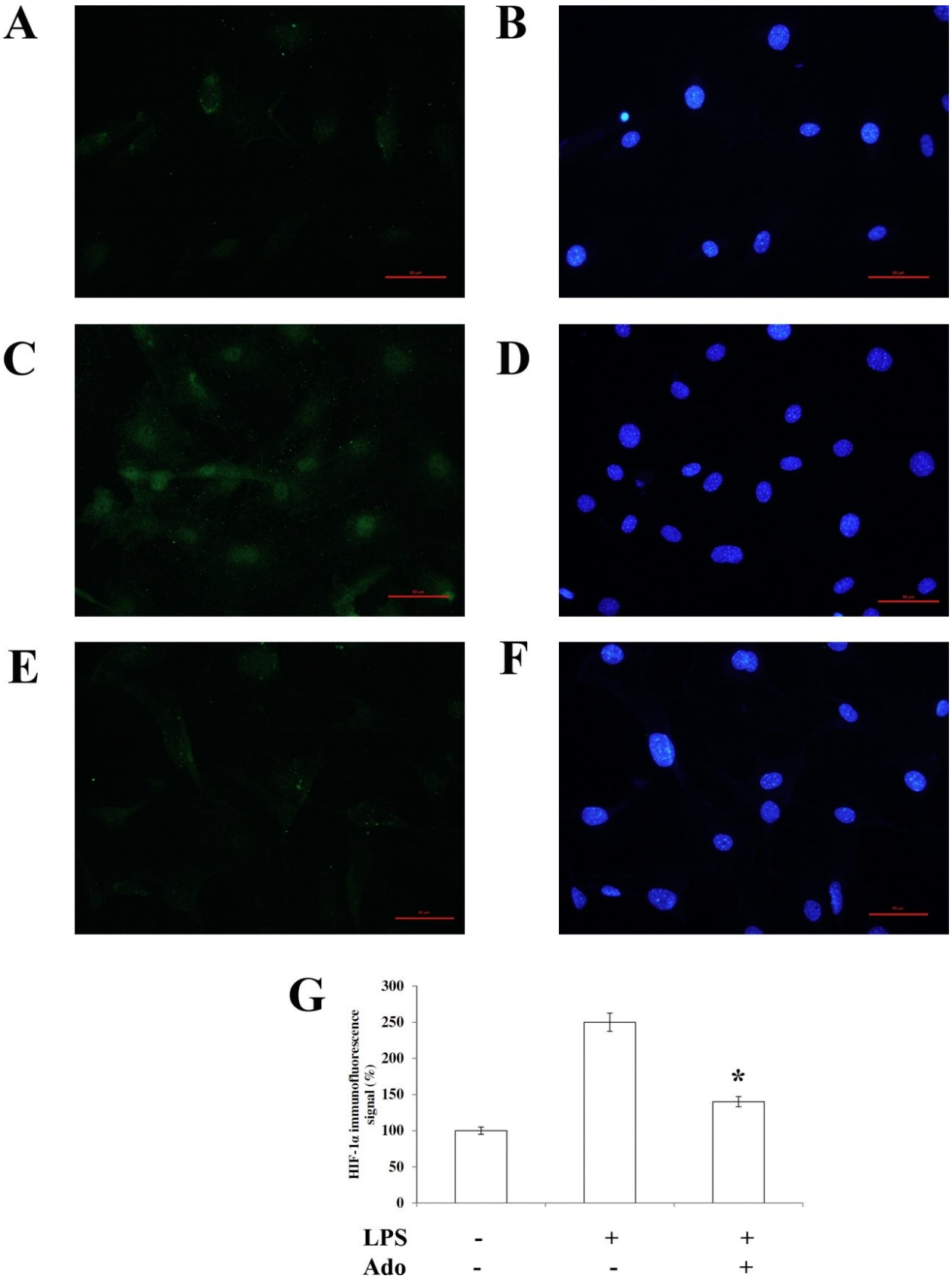

A
B
C
D
E
F
G
HIF-1α immunofluorescence signal (%)
300
250
200
150
100
50
0
*
LPS - + +
Ado - - +

Figure 19

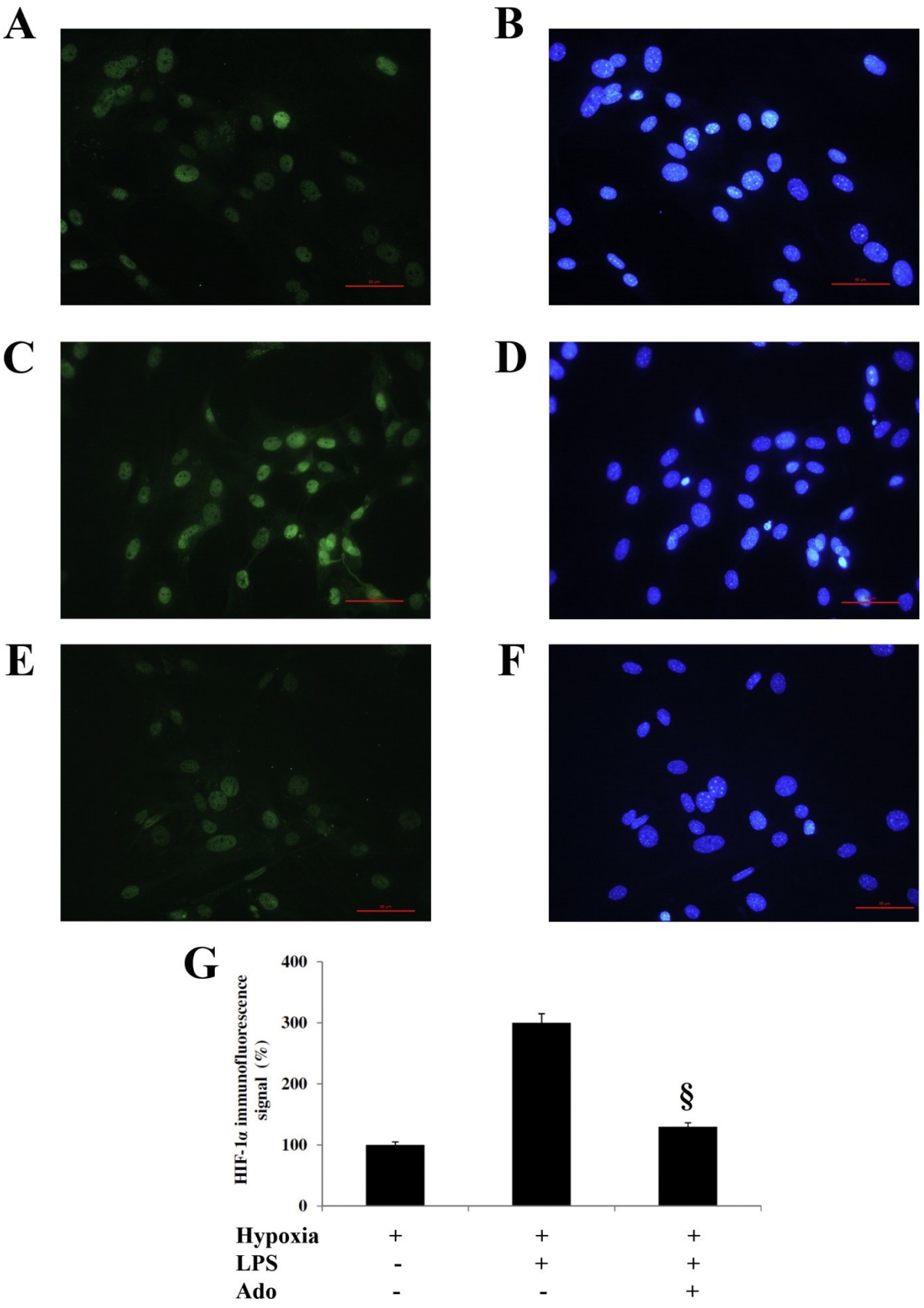

A
B
C
D
E
F
G
HIF-1α immunofluorescence signal (%)
400
300
200
100
0
§
Hypoxia + + +
LPS - + +
Ado - - +

Figure 20

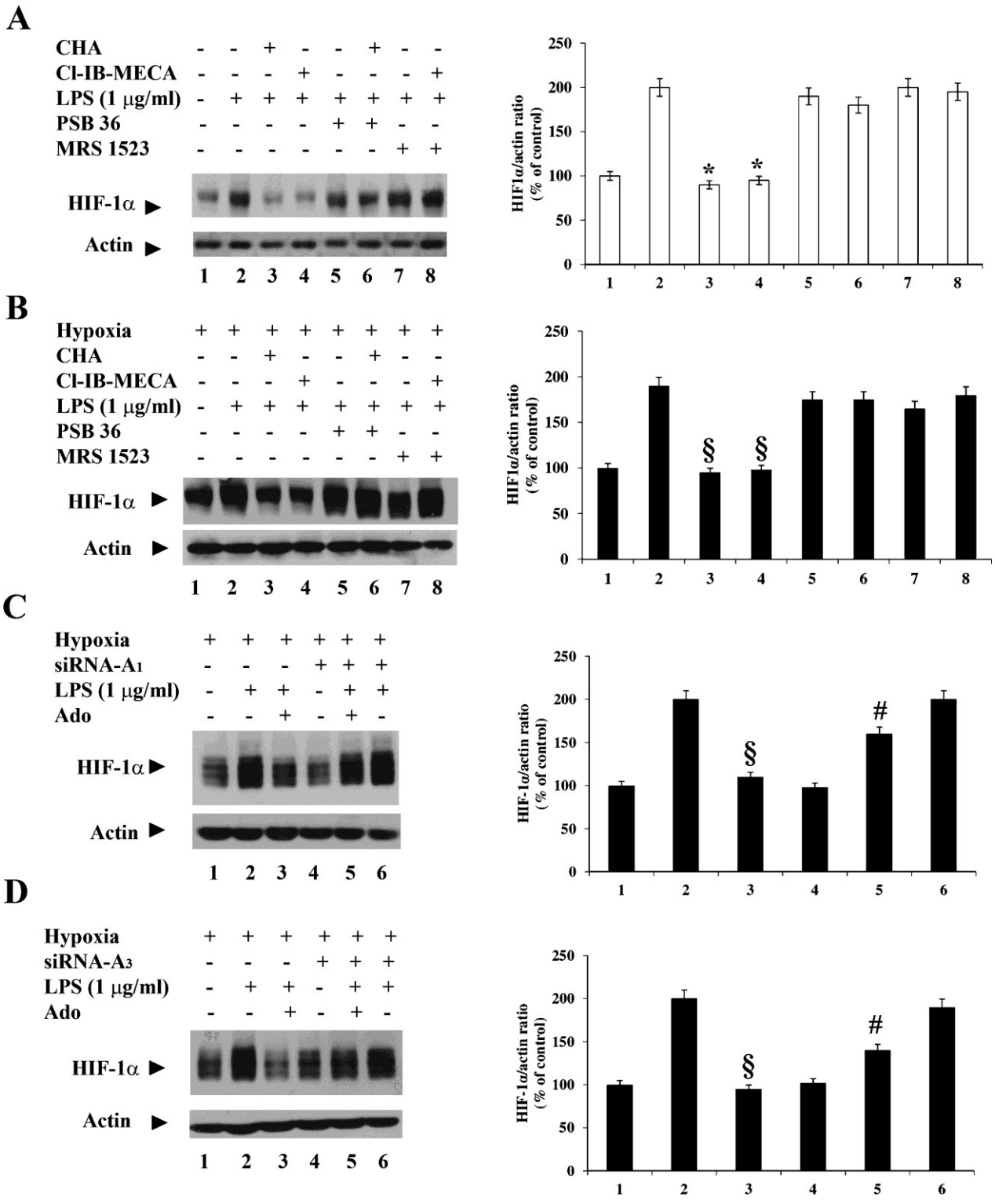

A
CHA - - + - - + - -
Cl-IB-MECA - - - + - - - +
LPS (1 μg/ml) - + + + + + + +
PSB 36 - - - - + + - -
MRS 1523 - - - - - - + +
HIF-1α ▶
Actin ▶
1 2 3 4 5 6 7 8
HIF1α/actin ratio (% of control)
B
Hypoxia + + + + + + + +
CHA - - + - - + - -
Cl-IB-MECA - - - + - - - +
LPS (1 μg/ml) - + + + + + + +
PSB 36 - - - - + + - -
MRS 1523 - - - - - - + +
HIF-1α ▶
Actin ▶
1 2 3 4 5 6 7 8
HIF1α/actin ratio (% of control)
C
Hypoxia + + + + + +
siRNA-A1 - - - + + +
LPS (1 μg/ml) - + + - + +
Ado - - + - + -
HIF-1α ▶
Actin ▶
1 2 3 4 5 6
HIF-1α/actin ratio (% of control)
D
Hypoxia + + + + + +
siRNA-A3 - - - + + +
LPS (1 μg/ml) - + + - + +
Ado - - + - + -
HIF-1α ▶
Actin ▶
1 2 3 4 5 6
HIF-1α/actin ratio (% of control)

Figure 21

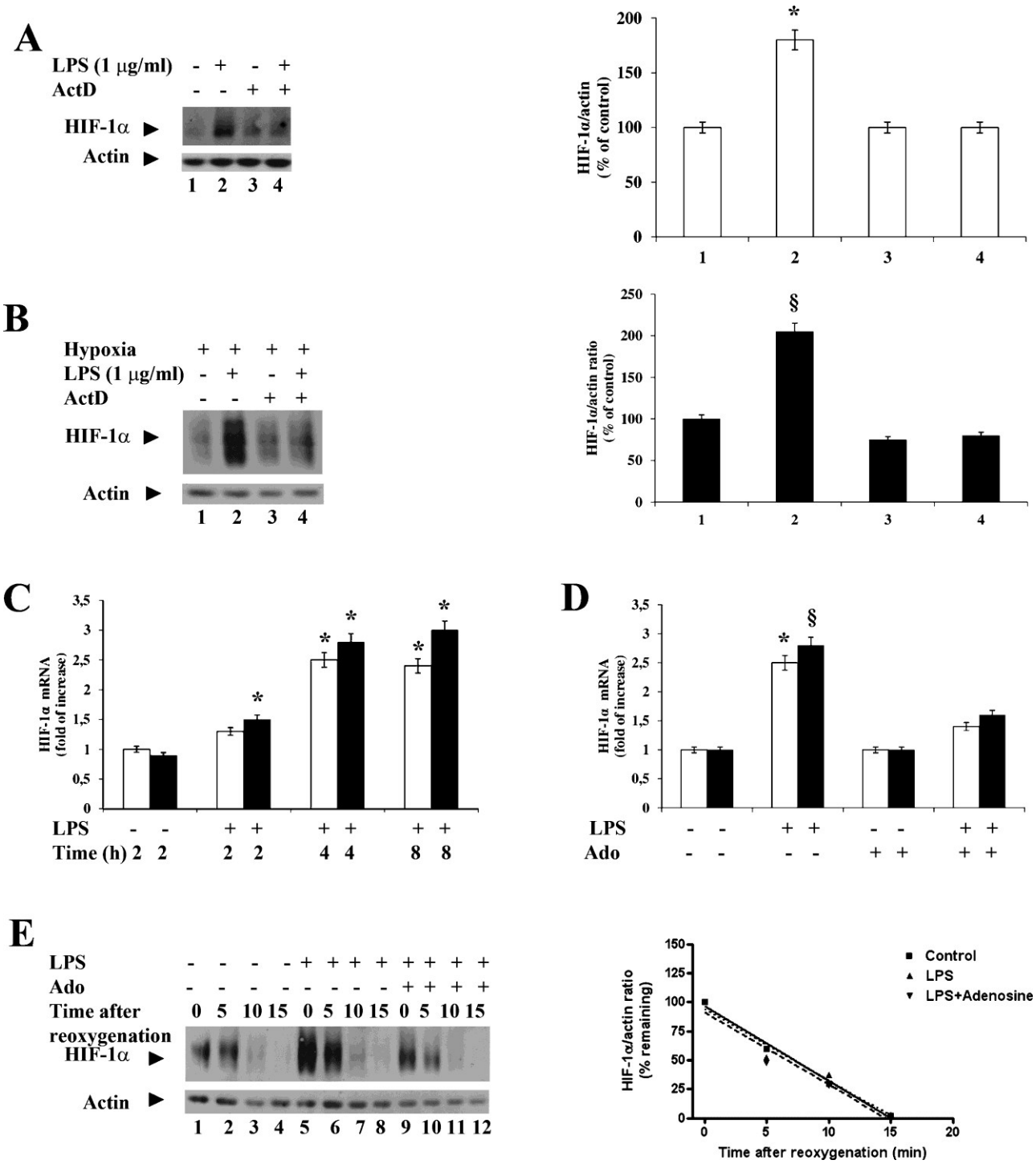

A
LPS (1 μg/ml) - + - +
ActD - - + +
HIF-1α ▶
Actin ▶
1 2 3 4
HIF-1α/actin (% of control)
200
150
100
50
0
*
1 2 3 4
B
Hypoxia + + + +
LPS (1 μg/ml) - + - +
ActD - - + +
HIF-1α ▶
Actin ▶
1 2 3 4
HIF-1α/actin ratio (% of control)
250
200
150
100
50
0
§
1 2 3 4
C
HIF-1α mRNA (fold of increase)
3,5
3
2,5
2
1,5
1
0,5
0
*
LPS - - + + + + + +
Time (h) 2 2 2 2 4 4 8 8
D
HIF-1α mRNA (fold of increase)
3,5
3
2,5
2
1,5
1
0,5
0
* §
LPS - - + + - - + +
Ado - - - - + + + +
E
LPS - - - - + + + + + + + +
Ado - - - - - - - - + + + +
Time after reoxygenation 0 5 10 15 0 5 10 15 0 5 10 15
HIF-1α ▶
Actin ▶
1 2 3 4 5 6 7 8 9 10 11 12
HIF-1α/actin ratio (% remaining)
150
125
100
75
50
25
0
0 5 10 15 20
Time after reoxygenation (min)
Control
LPS
LPS+Adenosine

Figure 22

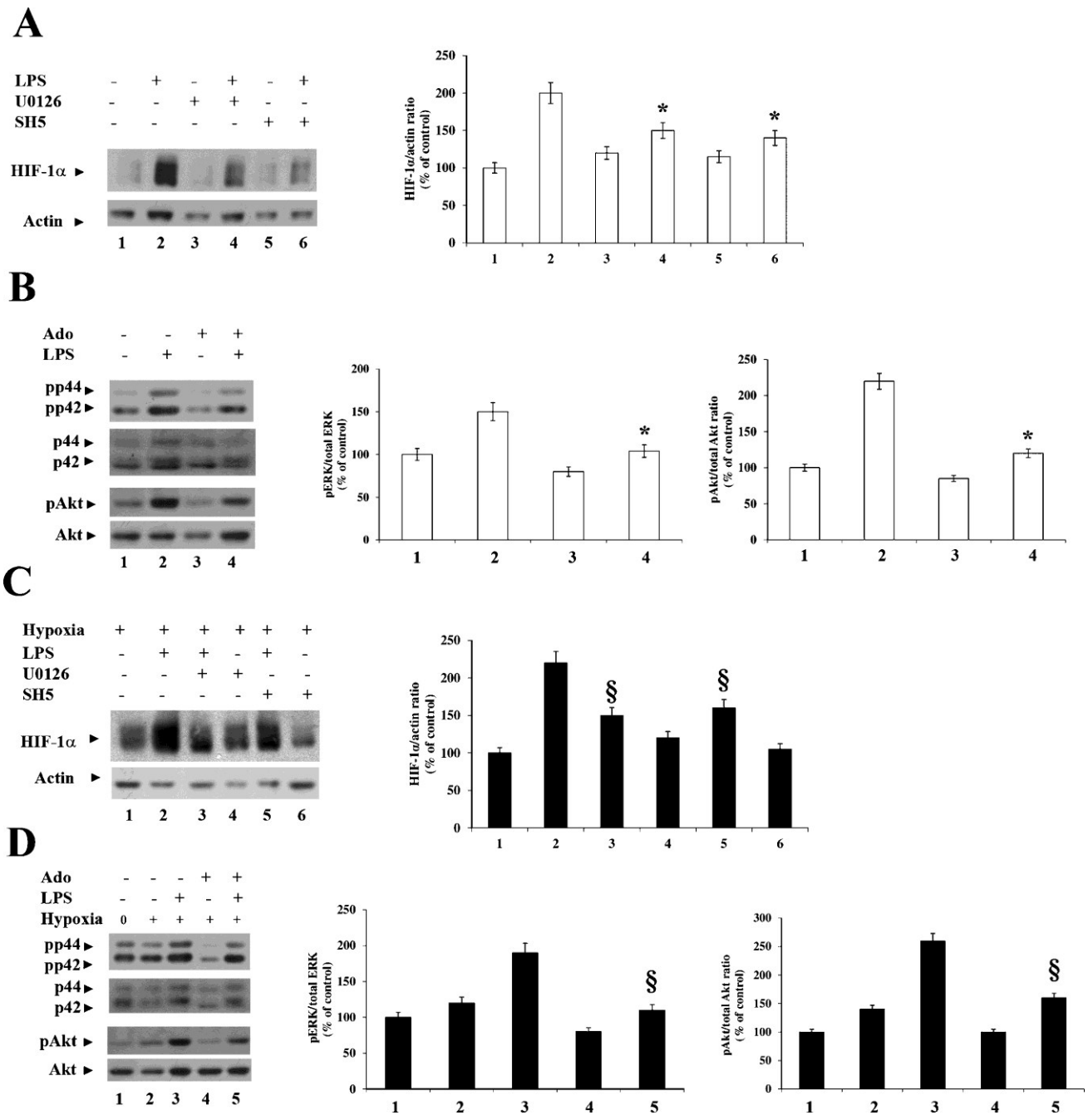

A
LPS
U0126
SH5
HIF-1α ►
Actin ►
1 2 3 4 5 6
HIF-1α/actin ratio (% of control)
B
Ado
LPS
pp44 ►
pp42 ►
p44 ►
p42 ►
pAkt ►
Akt ►
1 2 3 4
pERK/total ERK (% of control)
pAkt/total Akt ratio (% of control)
C
Hypoxia
LPS
U0126
SH5
HIF-1α ►
Actin ►
1 2 3 4 5 6
HIF-1α/actin ratio (% of control)
D
Ado
LPS
Hypoxia
pp44 ►
pp42 ►
p44 ►
p42 ►
pAkt ►
Akt ►
1 2 3 4 5
pERK/total ERK (% of control)
pAkt/total Akt ratio (% of control)

Figure 23

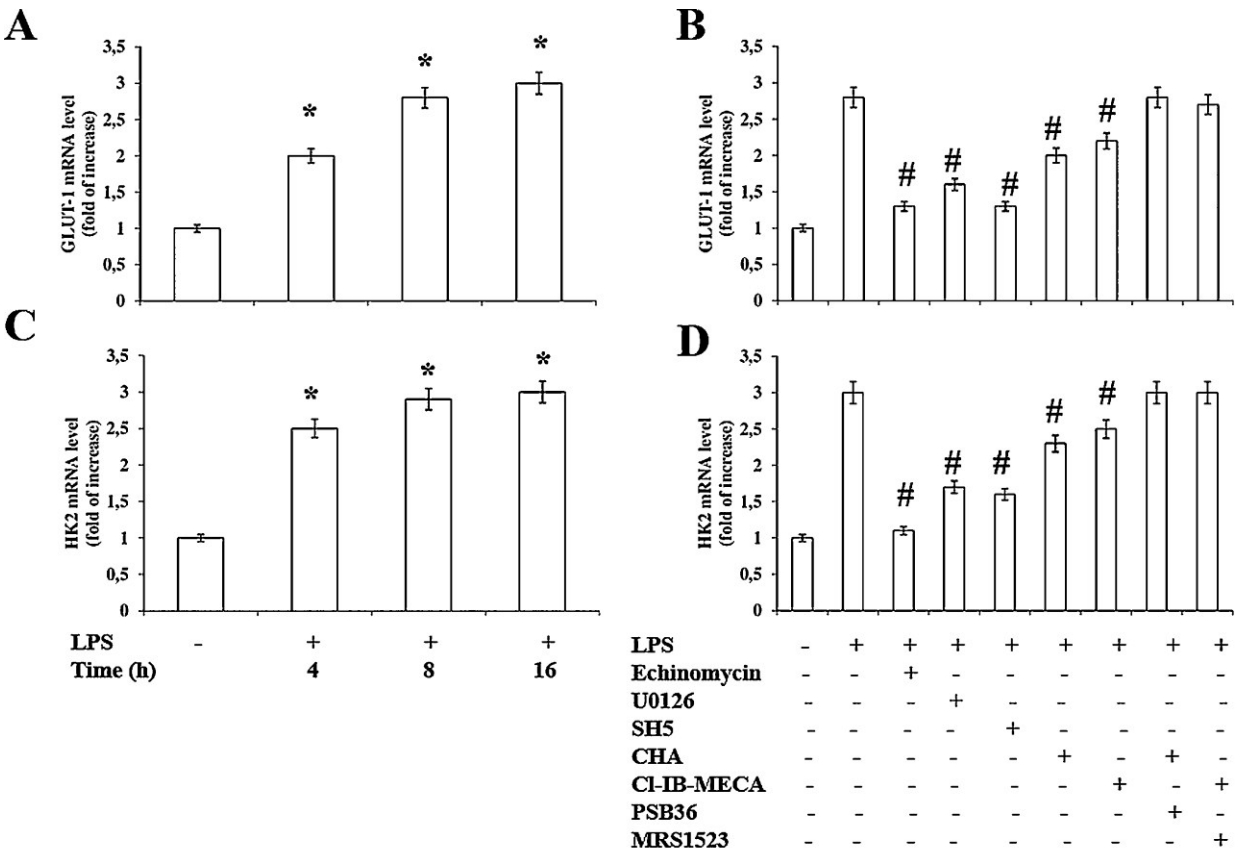

A
B
C
D
GLUT-1 mRNA level (fold of increase)
HK2 mRNA level (fold of increase)
3,5
3
2,5
2
1,5
1
0,5
0
*
#
LPS - + + +
Time (h) 4 8 16
LPS - + + + + + + + +
Echinomycin - - + - - - - - -
U0126 - - - + - - - - -
SH5 - - - - + - - - -
CHA - - - - - + - + -
Cl-IB-MECA - - - - - - + - +
PSB36 - - - - - - - + -
MRS1523 - - - - - - - - +

Figure 23

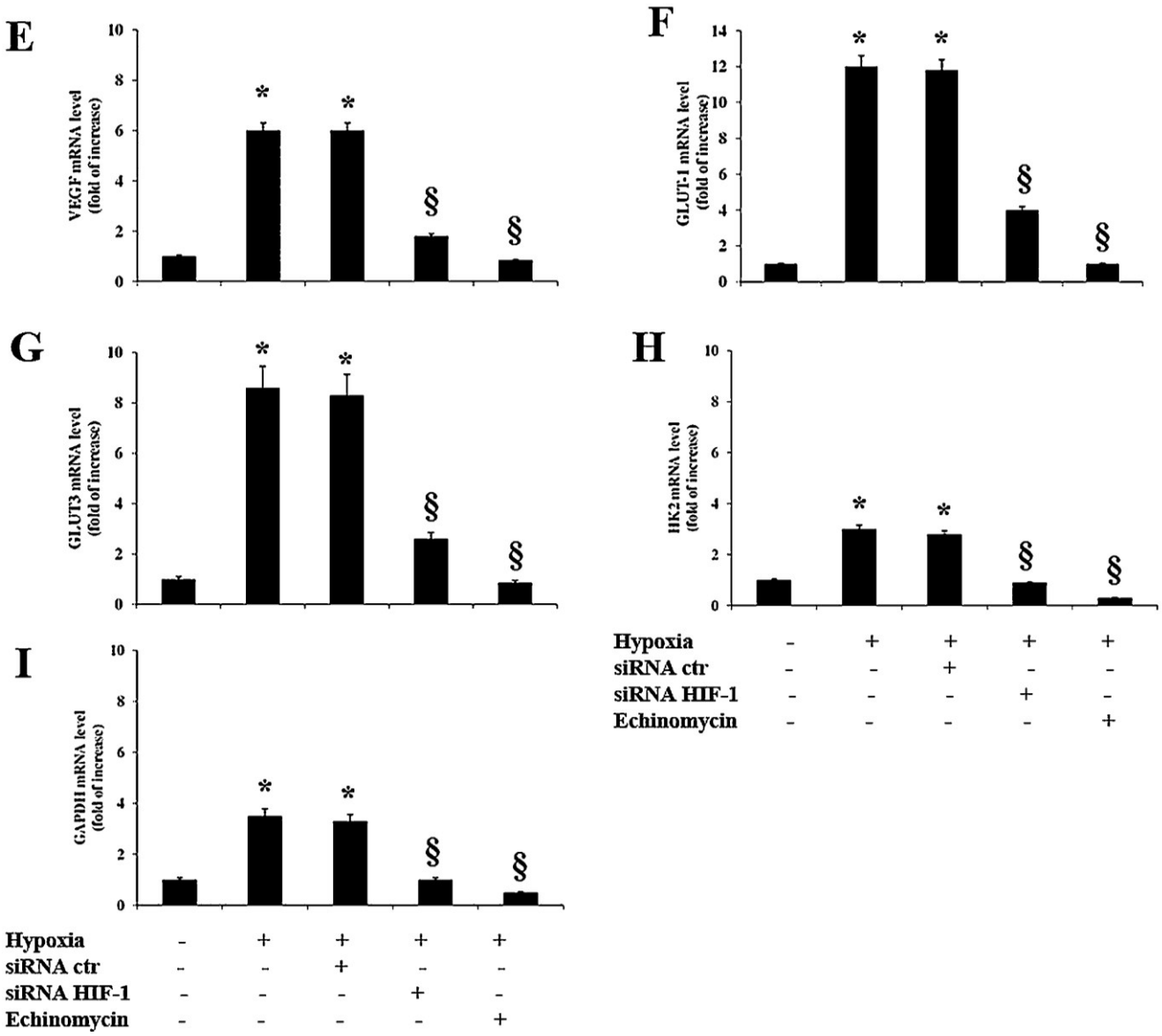

E
VEGF mRNA level (fold of increase)
F
GLUT-1 mRNA level (fold of increase)
G
GLUT3 mRNA level (fold of increase)
H
HK2 mRNA level (fold of increase)
I
GAPDH mRNA level (fold of increase)
Hypoxia - + + + +
siRNA ctr - - + - -
siRNA HIF-1 - - - + -
Echinomycin - - - - +

Figure 24

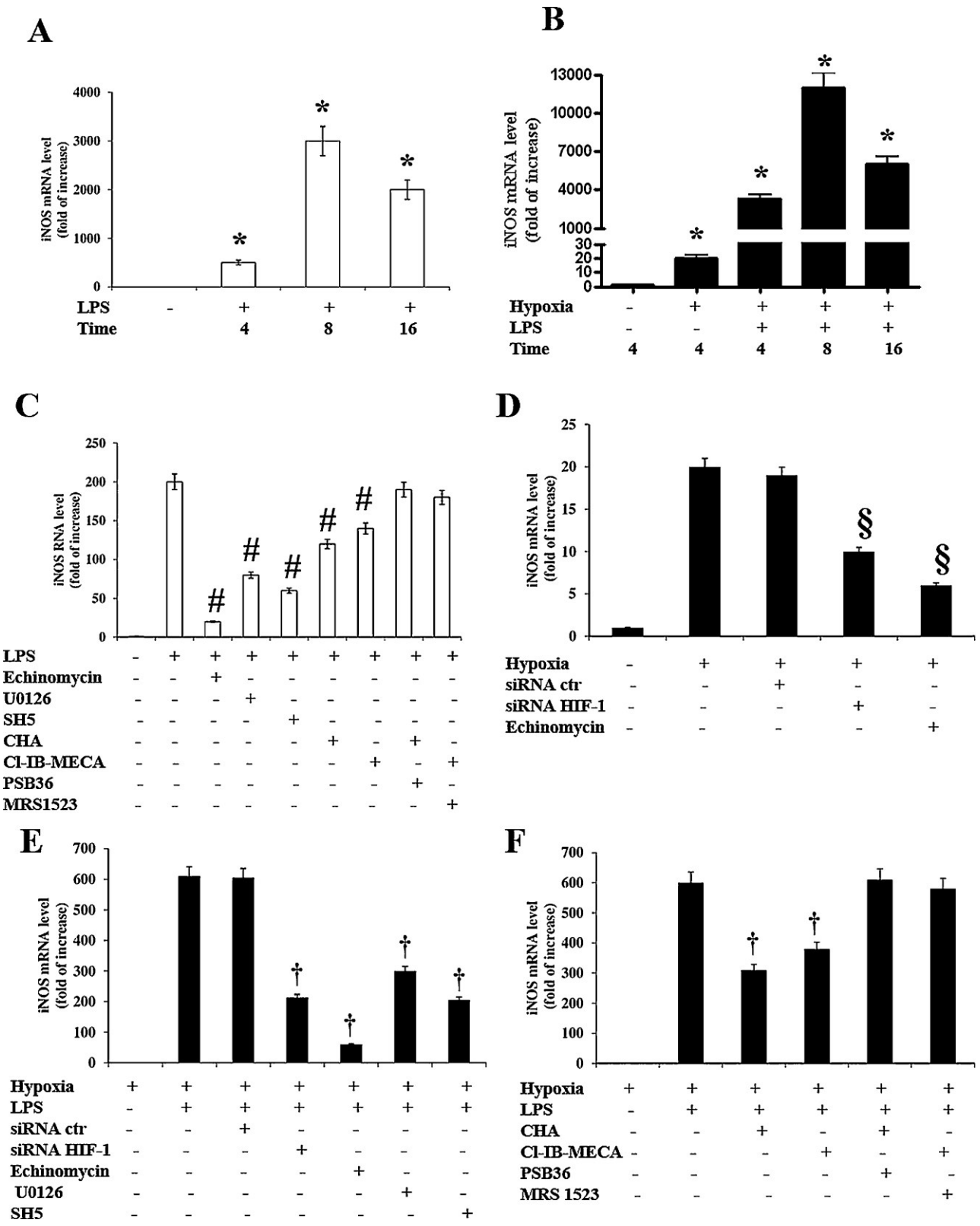

A
iNOS mRNA level (fold of increase)
4000
3000
2000
1000
0
LPS - + + +
Time 4 8 16
B
iNOS mRNA level (fold of increase)
13000
10000
7000
4000
1000
30
20
10
0
Hypoxia - + + + +
LPS - - + + +
Time 4 4 4 8 16
C
iNOS RNA level (fold of increase)
250
200
150
100
50
0
LPS - + + + + + + + +
Echinomycin - - + - - - - - -
U0126 - - - + - - - - -
SH5 - - - - + - - - -
CHA - - - - - + - + -
Cl-IB-MECA - - - - - - + - +
PSB36 - - - - - - - + -
MRS1523 - - - - - - - - +
D
iNOS mRNA level (fold of increase)
25
20
15
10
5
0
Hypoxia - + + + +
siRNA ctr - - + - -
siRNA HIF-1 - - - + -
Echinomycin - - - - +
E
iNOS mRNA level (fold of increase)
700
600
500
400
300
200
100
0
Hypoxia + + + + + + +
LPS - + + + + + +
siRNA ctr - - + - - - -
siRNA HIF-1 - - - + - - -
Echinomycin - - - - + - -
U0126 - - - - - + -
SH5 - - - - - - +
F
iNOS mRNA level (fold of increase)
700
600
500
400
300
200
100
0
Hypoxia + + + + + +
LPS - + + + + +
CHA - - + - + -
Cl-IB-MECA - - - + - +
PSB36 - - - - + -
MRS 1523 - - - - - +

Figure 25

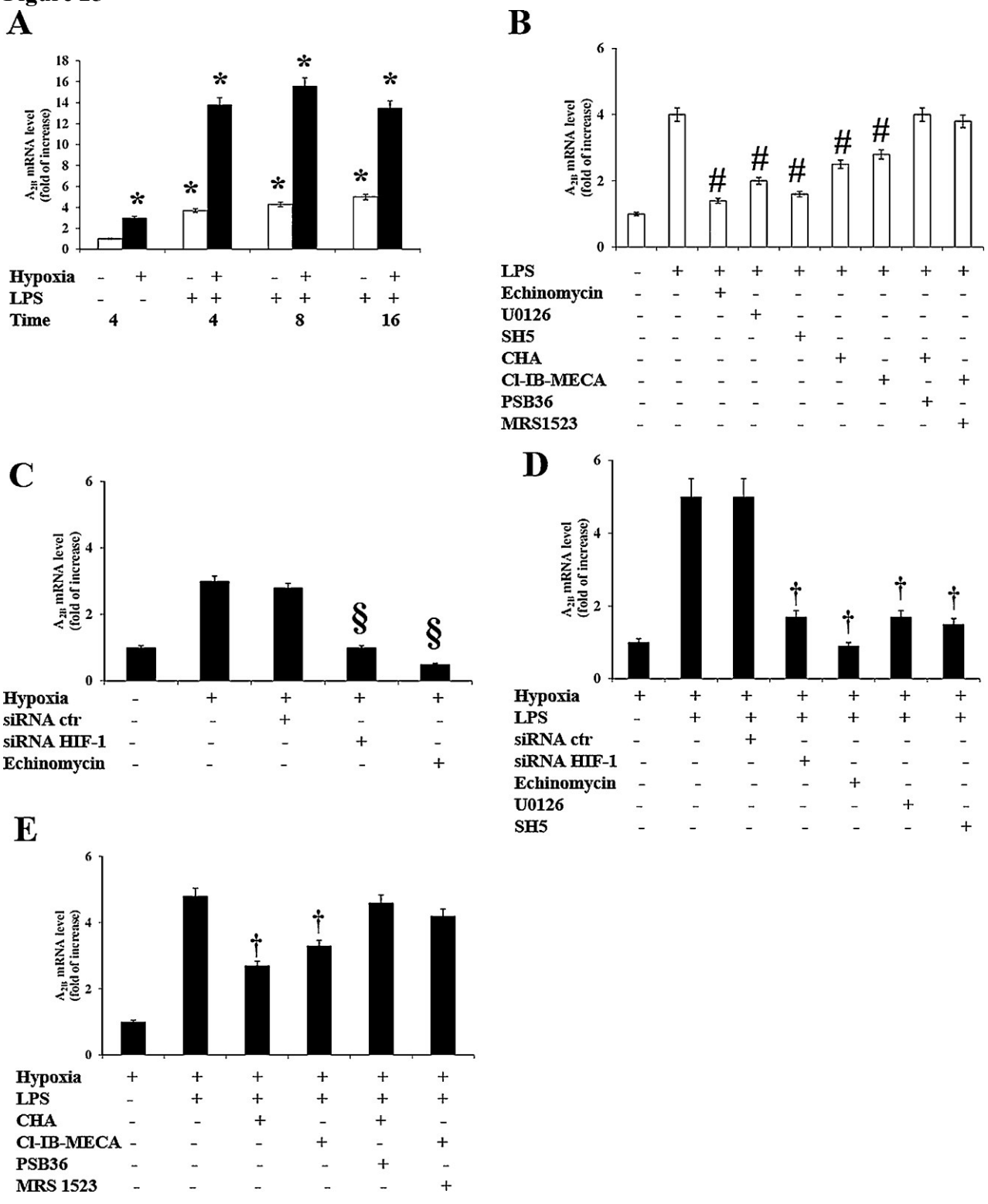
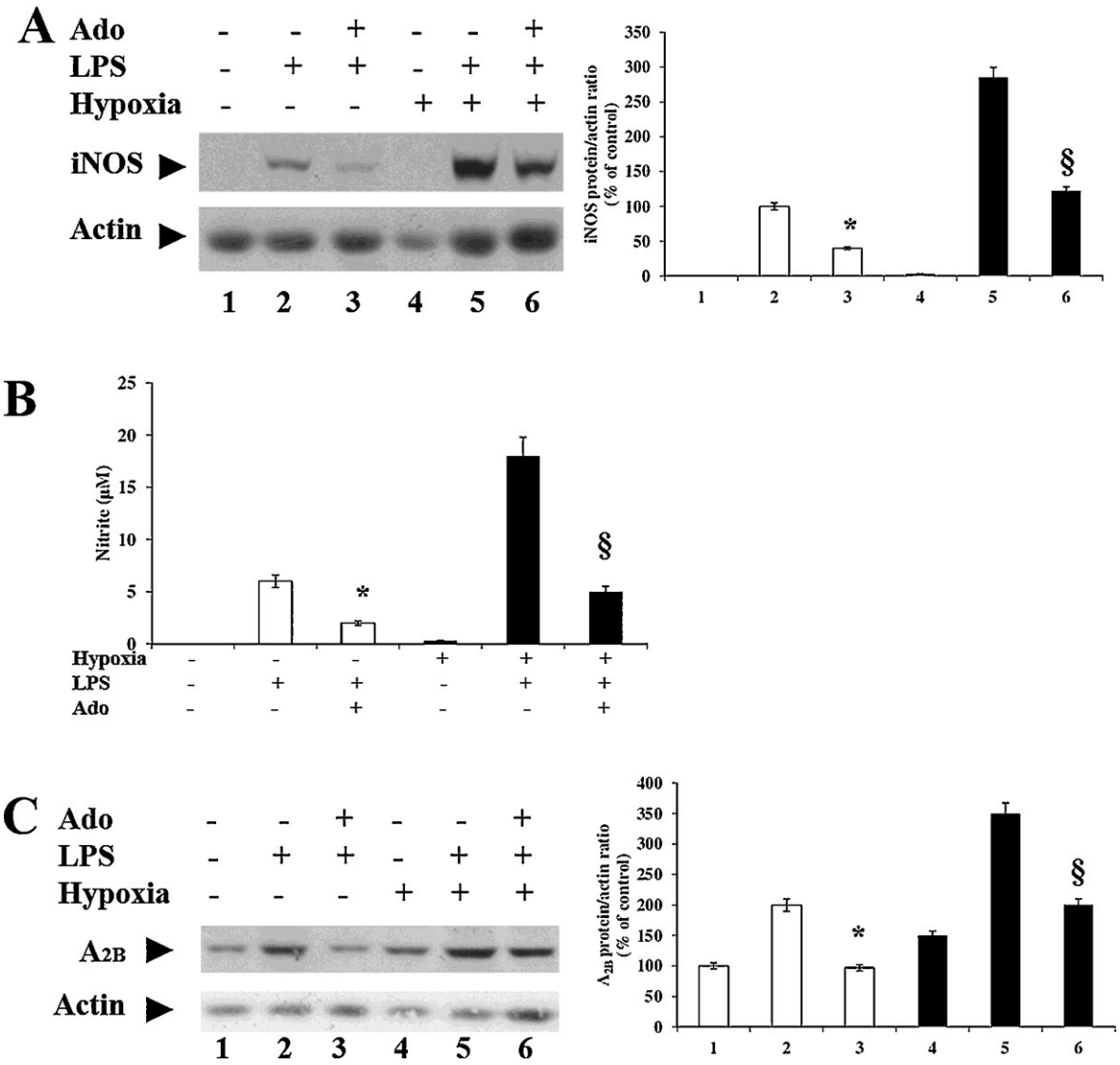
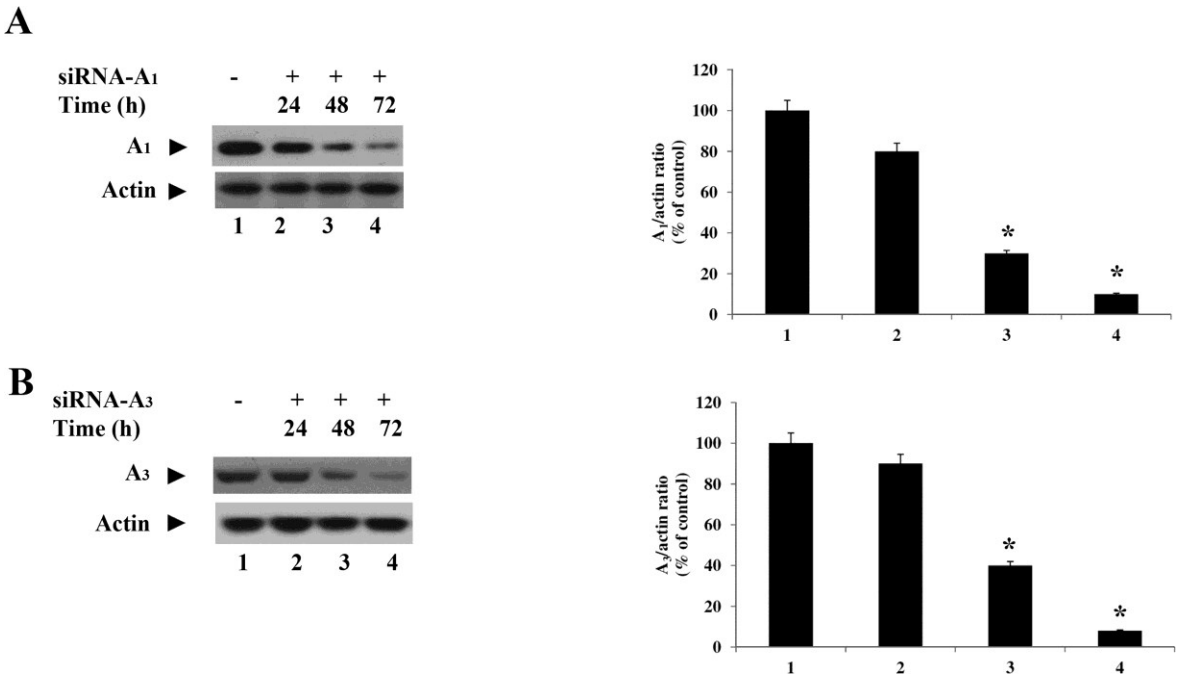

A
A2B mRNA level (fold of increase)
Hypoxia
LPS
Time
4
4
8
16
B
A2B mRNA level (fold of increase)
LPS
Echinomycin
U0126
SH5
CHA
Cl-IB-MECA
PSB36
MRS1523
C
A2B mRNA level (fold of increase)
Hypoxia
siRNA ctr
siRNA HIF-1
Echinomycin
D
A2B mRNA level (fold of increase)
Hypoxia
LPS
siRNA ctr
siRNA HIF-1
Echinomycin
U0126
SH5
E
A2B mRNA level (fold of increase)
Hypoxia
LPS
CHA
Cl-IB-MECA
PSB36
MRS 1523

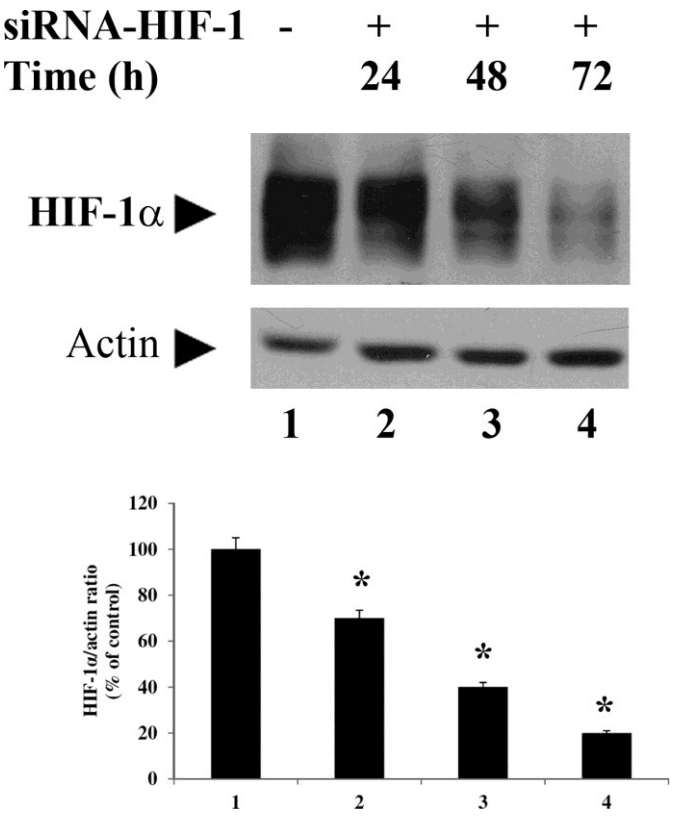
Figure 26


A
Ado - - + - - +
LPS - + + - + +
Hypoxia - - - + + +
iNOS
Actin
1 2 3 4 5 6
iNOS protein/actin ratio (% of control)
350
300
250
200
150
100
50
0
*
§
B
Nitrite (μM)
25
20
15
10
5
0
*
§
Hypoxia - - - + + +
LPS - + + - + +
Ado - - + - - +
C
Ado - - + - - +
LPS - + + - + +
Hypoxia - - - + + +
A2B
Actin
1 2 3 4 5 6
A2B protein/actin ratio (% of control)
400
350
300
250
200
150
100
50
0
*
§

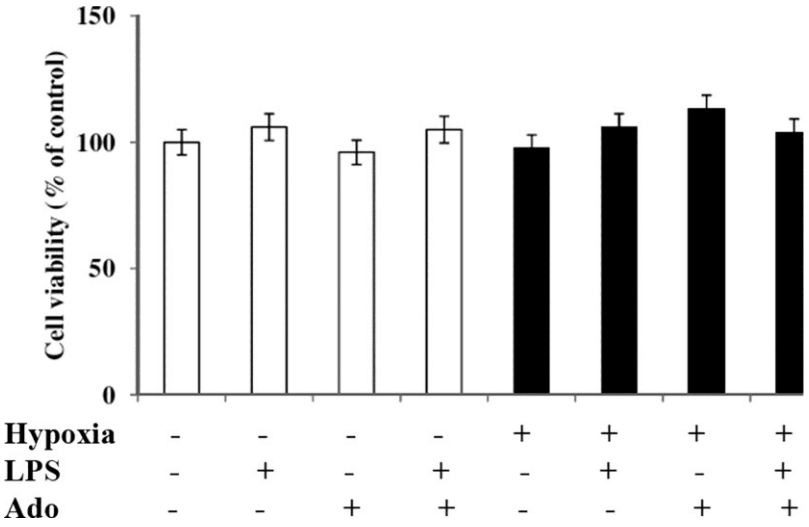
Supplemental Figure 2



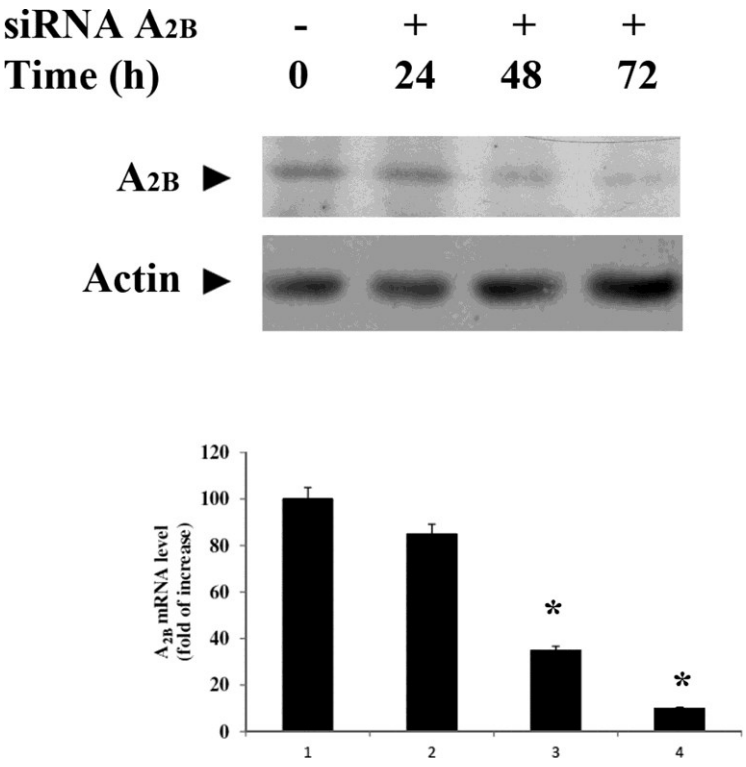
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



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Fazzi D, Merighi S, Gessi S, Varani K., **Stefanelli A**., Mirandola P., Borea P.A (2012). Cannabinoid CB2 receptor attenuates morphine-induced inflammatory responses in activated microglia cells Rimini, 16-19 Settembre 2012, 16° Seminario Nazionale SIF Dottorandi e Assegnisti di Ricerca

Gessi S, Merighi S, **Stefanelli A**, Mirandola P, Bonfatti A, Fini S, Sensi A, Fazzi D, Varani K, Vesce F, Borea PA (2012) "Downregulation of A1 and A2B adenosine receptors in human trisomy 21 mesenchymal cells from first-trimester chorionic villi" Annual Purine club meeting 2012

Gessi S, Merighi S, **Stefanelli A**, Varani K, Borea PA (2012) "Dal caffè una nuova arma per combattere l'aterosclerosi". Ferrara, Ottobre 2012, Festival della Ricerca

Gessi S, Merighi S, **Stefanelli A**, Fazzi D, Varani K, Borea PA (2013) "A1 and A3 adenosine receptors inhibit lps-induced hypoxia-inducible factor-1 accumulation in murine astrocytes". Torino, 23-26 Ottobre 2013, 36° Congresso Nazionale SIF

Merighi S., Gessi S., Varani K., Fazzi D., **Stefanelli A**., Borea P.A. (2013). "Morphine mediates a proinflammatory phenotype via μ-opioid receptor-PKCε-Akt-ERK1/2 signalling pathway in activated microglial cells". Torino, 23-26 Ottobre 2013, 36° Congresso Nazionale SIF

Meetings

2007 Attestato di Partecipazione al X week-end clinico "L'azoospermia" e al I meeting itinerante A.G.E.O.-S.I.d.R "Sessualità e contraccezione nell'adolescenza". (LE

2008 Attestato di partecipazione al convegno: "Bambini ed anziani: la Farmacovigilanza nelle età a maggior rischio" (FE)

2008 Attestato di Partecipazione al convegno: "Pain control in cancer patients". (LE)

2009 Attestato di partecipazione al workshop: "Il Placebo nelle sperimentazioni cliniche: Aspetti farmacologici ed etici" (FE)

2010 Attestato di partecipazione: " Problematiche "difficili"in Reumatologia Clinica VIII Edizione: Farmaci biologici e malattie reumatiche

2012 Attestato di partecipazione: "Convegno Monotematico "Cannabinoidi: presente e futuro" Ferrara

2012 Attestato di partecipazione: "XVI Seminario Nazionale SIF Dottorandi ed Assegnisti di Ricerca" Rimini

2013 Attestato di partecipazione: "Analisi di miRNA: Studi di espressione e "Whole profiling" e "Gene Synthesis, services and Precision TALs from GeneArt" Ferrara

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