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Adenosine modulates vascular endothelial growth factor expression via hypoxia-inducible factor-1 in human glioblastoma cells

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Act-D, actinomycin-D
Cl-IB-MECA, N^6(3-iodobenzyl)2-chloroadenosine-5′N-methyluronamide
GBM, glioblastoma multiforme
HIF-1, hypoxia-inducible factor-1
MAPK, mitogen-activated protein kinase
MEK, mitogen-activated protein kinase kinase

ABSTRACT

Hypoxia appears to induce a program which shifts the cellular phenotype toward an increase in extracellular adenosine. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of genes crucial to many aspects of cancer biology. Since in gliomas there is a strong correlation between HIF-1α expression, tumor grade and tumor vascularization, the aim of this study was to investigate whether adenosine may regulate HIF-1 in human glioblastoma cell lines. The results indicate that in the human hypoxic A172 and U87MG glioblastoma cell lines adenosine up-regulates HIF-1α protein expression via the A_3 receptor subtype. In particular, we investigated the effect of A_3 receptor antagonists on HIF-1 and vascular endothelial growth factor (VEGF) expression. We found that A_3 antagonists inhibit adenosine-induced HIF-1α and VEGF protein accumulation in the hypoxic cells. Investigations in the molecular mechanism showed that A_3 receptor stimulation activates p44/p42 and p38 MAPKs that are required for A_3-induced increase of HIF-1α and VEGF. Further studies are required to demonstrate the in vivo relevance of these observations with regard to the proposed role for adenosine as a key element in hypoxia and in tumors.

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MRE 3008F20, 5N-(4-methoxyphenyl-carbamoyl) amino-8-propyl-2-(2-furyl)-pyrazolo -[4,3e]1,2,4-triazolo [1,5c] pyrimidine
MRE 3005F20, 5-[[(4-pyridyl)amino] carbonyl]amino-8-methyl-2-(2-furyl) -pyrazolo[4,3-e]1,2,4-triazolo[1,5 -c]pyrimidine hydrochloride
RT-PCR, reverse transcription-PCR
siRNA, small interfering RNA
$siRNA_{A3}$, small interfering RNA that targets A_3 receptor mRNA
$siRNA_{HIF\text{-}1\alpha}$, small interfering RNA that targets HIF-1α mRNA
VEGF, vascular endothelial growth factor

1. Introduction

Glial tumors are the most common primary brain malignancies. Glioblastoma multiforme (GBM) (Grade IV astrocytoma) accounts for 80% of malignant astrocytomas and is marked by an extremely poor prognosis: half of all patients die within 1 year of diagnosis [1]. Key histopathological features of GBM, such as necrosis and endothelial proliferation, distinguish these tumors from lower-grade astrocytic tumors that have a much better prognosis [2]. Glioblastomas, like other solid tumors, have extensive areas of hypoxia and necrosis. The presence of hypoxia in tumors plays a negative role in patient prognosis because it promotes a more malignant phenotype [3]. Hypoxic tumor cells are resistant to conventional chemotherapy and radiotherapy. It is therefore rational to target the hypoxic regions of tumors or disrupt events initiated by hypoxia [4].

Hypoxia-inducible factor-1 (HIF-1) is one of the master regulators that orchestrate the cellular responses to hypoxia. It is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1α and -1β mRNAs are constantly expressed under normoxic and hypoxic conditions. The unique feature of HIF-1 is the regulation of HIF-1α expression: it increases as the cellular O_2 concentration is decreased. During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation [5]. A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis [6,7].

Immunohistochemical analyses have shown that HIF-1α is present in higher levels in human tumors than in normal tissues [8]. Most important to gliomagenesis, HIF-1 is a potent activator of angiogenesis and invasion through its up-regulation of target genes critical for these functions [9–12]. Such genes share the presence of hypoxia response elements (HRE), which contain binding sites for HIF-1 [7]. Activation of the HIF-1 pathway is a common feature of gliomas and may explain the intense vascular hyperplasia often seen in GBM. While HIF activation strongly promotes angiogenesis, the emerging vasculature is often abnormal, leading to a vicious cycle that causes further hypoxia and HIF up-regulation [13]. Therefore, since HIF-1α expression and activity appear central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target [6,7,11,12].

Adenosine is an ubiquitous autacoid that accumulates to high levels in hypoxic tissues as a result of ATP breakdown [14]. Therefore, this nucleoside could be involved in the regulation of the cellular response to hypoxia. In particular, it is recognized that significant levels of adenosine are present in the extracellular fluid of solid tumors [15], suggesting a role for this autacoid in tumor growth. However, it is only recently that adenosine has been shown to be a crucial factor in determining the cell progression pathway, either in the apoptotic or cytostatic state [16]. Adenosine modulates a variety of cellular functions via occupancy of four cell surface G protein-coupled receptors, named A_1, A_{2A}, A_{2B} and A_3 [17]. In particular, adenosine was found to exert its effects on cell proliferation, on clone formation ability, on UV resistance and on cell death mainly through the A_3 subtype [18–21], highly expressed in tumor cells [22–25]. Recent data indicate that A_3 receptor overexpression may be a good candidate as a tumor cell marker [26,27]. Adenosine also plays a role in the promotion of angiogenesis [28]. Regulation of expression of the angiogenic vascular endothelial growth factor (VEGF) via adenosine receptors has been demonstrated in several cell types [29–32].

Production of adenosine in hypoxia has recently been related to HIF-1α in different human tumor cell lines [33]. Furthermore, hypoxia causes the accumulation of extracellular adenosine via regulation of enzymes involved in adenosine metabolism, a process in which a contribution of HIF-1 has been described [34]. The aim of this study is to determine whether or not extracellular adenosine might serve as an endogenous, physiological regulator of HIF-1α in hypoxia in GBM cells, as recently suggested [35]. Furthermore, as HIF-1α plays a key role in inducing angiogenesis, we have also studied the role of adenosine in mediating the production of VEGF in hypoxic GBM cells.

2. Materials and methods

2.1. Cell lines, reagents and antibodies

U87MG and A172 GBM human cells were obtained from American Tissue Culture Collection (ATCC). Tissue culture media and growth supplements were obtained from Cambrex (Bergamo, Italy). Anti-HIF-1α and -1β antibodies (mAb) were obtained from Transduction Laboratories (Milan, Italy). U0126 (inhibitor of MEK-1 and -2), SB202190 (inhibitor of p38 MAP kinase), Anti-ACTIVE®MAPK and anti-ERK 1/2 (pAb) were from Promega (Milan, Italy). SH5 (inhibitor of Akt) was from Vinci-Biochem (Florence, Italy). Phospho-p38 and p38 MAP Kinase antibodies were from Cell Signaling Technology (Milan, Italy). Anti-Adenosine A_{2A} receptor (pAb) was from Santa Cruz Biotechnology (Milan, Italy). Anti-Adenosine A_3 receptor (polyAb) was from Aviva Antibody Corporation (Milan, Italy). P11w, a firefly luciferase reporter plasmid, comprising the 5′-flanking −985 to −939 base pairs of the human VEGF gene that include a hypoxia-inducible factor-1 (HIF-1)-binding site, and p11m, the mutated version of p11w containing a nonfunctional HIF-1-binding site [36] were obtained from the ATCC. BriteLite Ultra-High Sensitivity Luminescence Reporter Gene Assay System kit was obtained from Perkin–Elmer (Milan, Italy). Fugene 6 transfection reagent was purchased from Roche Molecular Biochemicals (Milan, Italy). Unless otherwise noted, all other chemicals were purchased from Sigma (Milan, Italy).

2.2. Cell culture and hypoxia treatment

U87MG and A172 GBM cells were maintained in RPMI1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) at 37 °C in 5% CO_2/95% air. For hypoxic conditions, cells were placed for the indicated times 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).

2.3. Western blot analysis

Whole cell lysates, prepared as described previously [37], were resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as described previously using antibody against HIF-1α (1:250 dilution) and HIF-1β (1:1000 dilution) in 5% non-fat dry milk in PBS/0.1% Tween-20 overnight at 4 °C. Aliquots of total protein sample (50 μg) were analyzed using antibodies specific for phosphorylated (Thr183/Tyr185) or total p44/p42 MAPK (1:5000 dilution), phosphorylated (Thr180/Tyr182) or total p38 MAPK (1:1000 dilution) and for A_{2A} or A_3 receptor (1 μg/ml dilution). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against mouse and rabbit IgG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Amersham Corp., Arlington Heights, Ill.). The membranes were then stripped and reprobed with tubulin (1:250) to ensure equal protein loading.

2.4. Densitometry analysis

The intensity of each band in immunoblot assay was quantified using molecular analyst/PC densitometry software (Bio-Rad). Mean densitometry data from independent experiments were normalized to result in cells in the control. The data were presented as the mean ± S.E., and analyzed by the Student's *t*-test.

2.5. Small interfering RNA (siRNA) design

To generate a small interfering RNA that targets A_3 receptor mRNA ($siRNA_{A3}$), eight oligonucleotides consisting of ribonucleosides, except for the presence of 2′-deoxyribonucleosides at the 3' end, were synthesized and annealed, according to the manufacturer's instructions (Silencer™ siRNA Construction Kit, Ambion) and as previously described [37]. Target sequences were aligned to the human genome database in a BLAST search to ensure sequences without significant homology to other genes. The target sequences for oligo-1, oligo-2, oligo-3 and oligo-4 are localized at position 337, 679, 1009 and 1356 bases downstream of the start codon of A_3 receptor mRNA sequence (L20463), respectively. siRNA targeting HIF-1α ($siRNA_{HIF\text{-}1\alpha}$) were synthesized and experiments were performed as previously described [38]. The sequences target nt 1378–1398 of the human HIF-1α mRNA (accession no. AF304431.1).

2.6. Treatment of cells with siRNA

GBM cells were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of siRNA was performed at a concentration of 100 nM using RNAiFect™ Transfection Kit (Qiagen). 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.

2.7. Real-time RT-PCR experiments

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method, as previously described [37]. Quantitative real-time RT-PCR assay [37] of human HIF-1α, VEGF and A_3 mRNA transcripts was carried out using gene-specific double fluorescently labelled TaqMan MGB probe (minor groove binder) in a ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington Cheshire, UK). The following primer and probe sequences were used for real-time RT-PCR: A_3 forward primer, 5′-ATG CCT TTG GCC ATT GTT G-3′; A_3 reverse primer, 5′-ACA ATC CAC TTC TAC AGC TGC CT-3′; A_3 MGB probe, 5′-FAM-TCA GCC TGG GCA TC-TAMRA-3′; for the realtime RT-PCR of the HIF-1α and VEGF genes the assays-on-demand™ Gene expression Product Accession Nos. NM019058 and Hs00173626_m1 were used, respectively (Applied Biosystems, Monza, Italy). The fluorescent reporter FAM and the quencher TAMRA are 6-carboxy fluorescein and 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine, respectively. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kit was used, and the probe was fluorescent-labelled with VIC™ (Applied Biosystems, Monza, Italy).

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGF protein secreted by the cells in the medium were determined by a VEGF ELISA kit (R&D Systems). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of adenosine analogues in hypoxia. The medium was collected, and VEGF protein concentrations were measured by ELISA according to the manufacturer's instructions. The results were normalized to the number of cells per plate. The data were presented as mean ± S.D. from three independent experiments.

2.9. Transient transfection and luciferase reporter assays

GBM cells were prepared for transfection by seeding them into 24-well plates (30,000 cells/well) in 0.5 ml of standard growth medium. After an overnight culture, the cells were transfected with 100 ng of p11w or p11m. Transfections were performed with 1.2 μl of Fugene 6 per well. The cells were then treated with drugs or the solvent vehicle only, then incubated under hypoxic (1% O_2) or normoxic conditions. The cells were then prepared for the luciferase reporter assay, according to the manufacturer's instructions. Briefly, the cells were lysed at ambient temperature for 2 min with 200 μl of 1× lysis buffer. The extracts were assayed for plamids (p11w and p11m) and control (*Renilla*) luciferase activities with a Perkin–Elmer luminometer. Samples were normalized for transfection efficiency based on the *Renilla* luciferase activity.

2.10. Statistical analysis

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

3. Results

3.1. Adenosine increases HIF-1α expression in human GBM cells

Initial experiments were performed to determine the expression of HIF-1α in the human GBM U87MG and A172 cells. Both cell lines expressed high levels of HIF-1α under hypoxic culture conditions. Treatment of the cells with adenosine for 5 h in hypoxia resulted in a dose-dependent increase of HIF-1α protein levels (Fig. 1A). The concentrations of adenosine required for 50% increase of HIF-1α in U87MG and A172 cells were 17 ± 1 and 14 ± 2 μM, respectively. In the presence of 50 μM adenosine, HIF-1α protein levels increased significantly at 2 h and were stable at 5 h and thereafter, up to 48 h of hypoxic treatment (Fig. 1B). We did not observe any modulation of HIF-1β protein. In particular, adenosine did not induce HIF-1α protein accumulation in normoxia (data not shown).

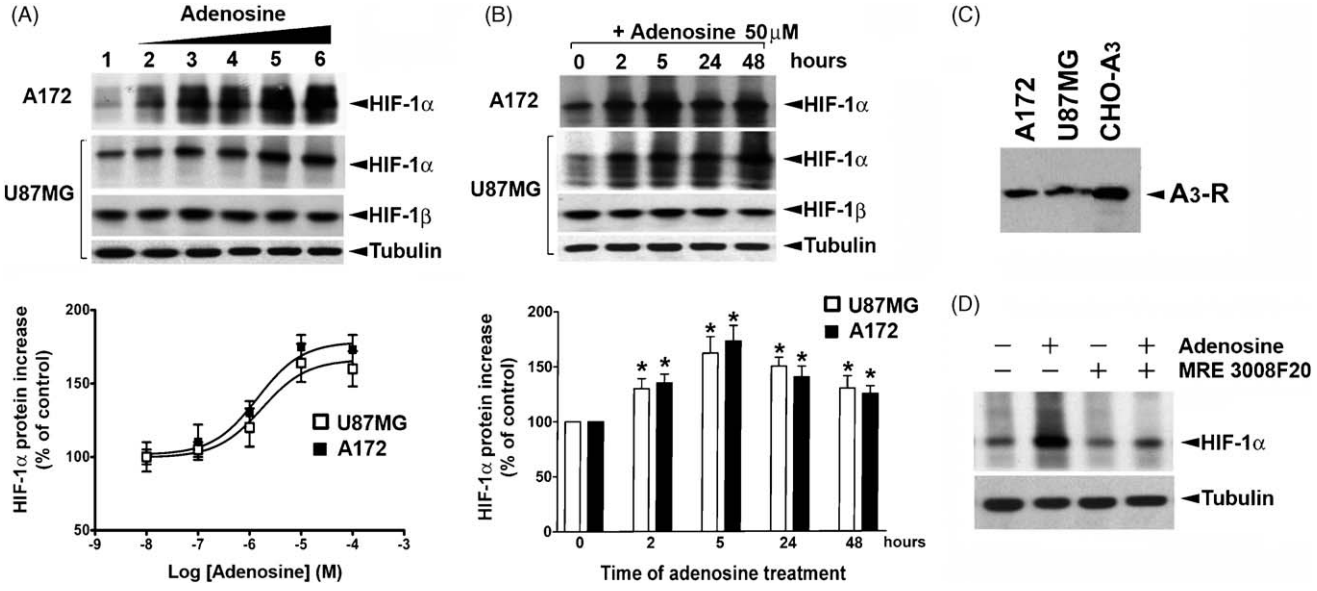

Fig. 1 – Induction of HIF-1α expression by adenosine. (A) Western blot analysis for HIF-1α and -1β levels of 35 μg total protein lysates from A172 and U87MG cells treated without (line-1, control) or with adenosine 10 nM (line-2), 100 nM (line-3), 1 μM (line-4), 10 μM (line-5) and 100 μM (line-6) in hypoxia for 5 h. The mean densitometry data were normalized to the result obtained in hypoxic cells in the absence of adenosine (control). Plots are mean ± S.E. values (n = 3). (B) Induction of HIF-1 expression by adenosine (50 μM): time course. The mean densitometry data from three independent experiments were normalized to the result obtained in cells in the absence of adenosine after 2 h of hypoxia (control). Plots are mean ± S.E. values (n = 3). $^*P < 0.01$ compared with the control. (C) A_3 receptor (A_3-R) expression in A172, U87MG, and chinese hamster ovary cells transfected with the human A_3 adenosine receptor (CHO-A_3) cells by Western blot. One representative experiment of 3 is shown. (D) A172 cells were treated without (line-1, control) or with adenosine 100 μM (lines 2 and 4) and exposed to MRE 3008F20 100 nM (lines 3 and 4) in hypoxia for 4 h.

The family of adenosine receptors consists of four subtypes of G protein-coupled receptors, designated A_1, A_{2A}, A_{2B} and A_3 [17]. U87MG and A172 cells express A_3 adenosine receptors (Fig. 1C). To evaluate whether A_3 receptors may have a functional role on HIF-1α protein expression under hypoxic conditions, we tested the effect of adenosine in combination with MRE 3008F20 (a selective A_3 receptor antagonist) [18]. When utilized alone under hypoxic conditions, the MRE 3008F20 compound has no effect on hypoxia-induced HIF-1α expression (Fig. 1D). MRE 3008F20 100 nM abrogated the adenosine-induced increase of HIF-1α protein expression (Fig. 1D). These results indicate that adenosine may increase HIF-1α protein expression via A_3 receptors.

3.2. A_3 adenosine receptor induces HIF-1α protein accumulation in hypoxia

To verify the involvement of A_3 receptors in the modulation of HIF-1α protein expression, we treated GBM cells with the high affinity A_3 receptor agonist Cl-IB-MECA [39]. A_3 adenosine receptor stimulation did not promote HIF-1α protein accumulation in normoxia (Fig. 2A, lines 2–5), while under hypoxic conditions HIF-1α protein expression was increased in a time-dependent manner (Fig. 2A, lines 7–10). As already observed with adenosine, Cl-IB-MECA did not modify HIF-1β expression in normoxia or in hypoxia (Fig. 2A). Further experiments found that Cl-IB-MECA was a very potent agonist, having an EC_{50} of 11.0 ± 0.3 and 9.3 ± 0.5 nM, for U87MG and A172 cells, respectively (Fig. 2B), consistent with an effect via adenosine A_3 receptors.

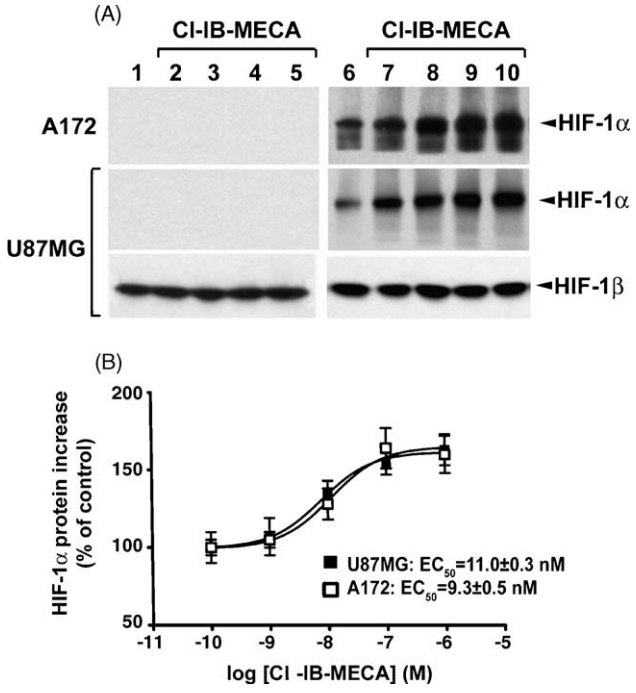

Fig. 2 – (A) Modulation of HIF-1α expression by A_3 receptor stimulation: time course. A172 and U87MG cells were cultured in normoxia (lines 1–5) or under hypoxic conditions (lines 6–10) for 4 h without Cl-IB-MECA (lines 1 and 6), or with Cl-IB-MECA 100 nM for 4, 6, 24 and 48 h (lines 2–7, 3–8, 4–9, 5–10, respectively). Whole cellular protein extracts were prepared and subjected to immunoblot assay. The blot was then stripped and used to determine HIF-1β expression. (B) Induction of HIF-1α expression by A_3 receptor stimulation: dose response. A172 and U87MG cells were treated without or with increasing concentrations of Cl-IB-MECA in hypoxia for 4 h. The mean densitometry data from three independent experiments were normalized to the result obtained in cells in the absence of Cl-IB-MECA (control). Plots are mean ± S.E. values ($n = 3$).

3.3. A_3 receptor antagonists prevent HIF-1α protein expression induced by A_3 receptor stimulation

The potency of the A_3 receptor antagonist MRE 3008F20 was quantified in U87MG and A172 cells. In U87MG cells, increasing concentrations of MRE 3008F20 (0.1–10 nM) were able to inhibit HIF-1α protein accumulation induced by a submaximal dose (10 nM) of Cl-IB-MECA with an IC_{50} of 0.63 ± 0.03 nM (Fig. 3). Analogously, in A172 cells, MRE 3008F20 10 nM effectively inhibited the HIF-1α induction induced by Cl-IB-MECA 10 nM (Fig. 3B). In addition, other selective A_3 antagonists (a series of substituted pyrazolotriazolopyrimidine derivatives) [40] also prevented Cl-IB-MECA action (data not shown).

3.4. A_3 receptor gene silencing prevents the adenosine–HIF-1α pathway

To confirm the role of A_3 receptor stimulation in HIF-1α signaling pathway in vitro, we tried to knockdown A_3 receptor expression using small interfering-(si)-RNA leading to a transient knockdown of the A_3 receptor gene ($siRNA_{A3}$). We designed four siRNAs from the human A_3 receptor gene sequence. Although there was a difference in silencing ability, all of the siRNAs were able to suppress endogenous A_3 receptor mRNA and protein expression in human A172 cells (Fig. 4). After 24, 48 and 72 h post-transfection, A_3 receptor mRNA and protein levels were significantly reduced in $siRNA_{A3}$-treated cells (Fig. 4A and B). Neither mock transfection nor transfection with a siRNA targeted to an irrelevant mRNA inhibited A_3 receptor protein expression. To confirm the specificity of the $siRNA_{A3}$-mediated silencing of A_3 receptor, we investigated the expression of A_{2A} receptor protein in $siRNA_{A3}$-treated cells. Fig. 4B shows that treatment of A172 cells with $siRNA_{A3}$ reduced the expression of A_3 protein but had no effect on the expression of A_{2A} receptor. At 72 h from the $siRNA_{A3}$ transfection, A172 cells were exposed to increasing concentrations of Cl-IB-MECA (10–100 nM) for 5 h in hypoxia. As control, A172 cells were exposed to scramble siRNA. When utilized alone under hypoxic conditions, $siRNA_{A3}$ has no effect on hypoxia-induced HIF-1α expression. We found that the inhibition of A_3 receptor expression is sufficient to block Cl-IB-MECA-induced HIF-1α accumulation (Fig. 4C).

3.5. A_3 receptor stimulation did not alter the mRNA of HIF-1α

To examine whether the increase of HIF-1α protein levels by A_3 receptor stimulation was caused by an increase in its

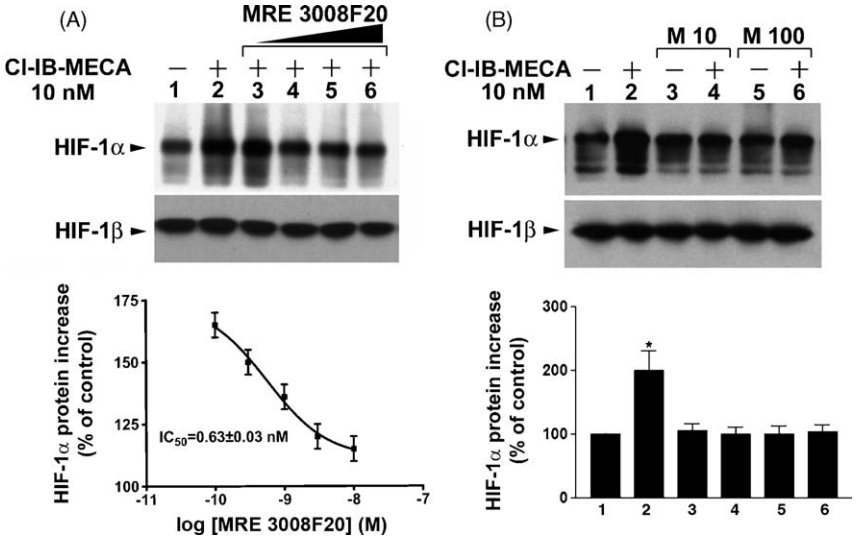

Fig. 3 – Effect of A_3 receptor antagonist MRE 3008F20. (A) U87MG cells were treated in hypoxia for 4 h without (line-1) or with Cl-IB-MECA 10 nM (lines 2–6) and MRE 3008F20 0.3 nM (line-3), 1 nM (line-4), 3 nM (line-5) and 10 nM (line-6). The mean densitometry data from three independent experiments were normalized to the result obtained in cells in the absence of Cl-IB-MECA (control). Plots are mean ± S.E. values ($n = 3$). (B) A172 cells were treated in hypoxia for 4 h without (−) or with Cl-IB-MECA 10 nM (+) and MRE 3008F20 10 nM (lines 3 and 4) and MRE 3008F20 100 nM (lines 5 and 6). The mean densitometry data from independent experiments (one of which is shown here) were normalized to the result obtained in cells in the absence of Cl-IB-MECA (line 1). Plots are mean ± S.E. values ($n = 3$); $^*P < 0.01$ compared with the control.

transcription, HIF-1α mRNA amount was quantified by real-time RT-PCR experiments. Activation of A_3 receptors in hypoxic GBM cells with 10–100–1000 nM Cl-IB-MECA for 1 h produced, respectively, a 1.12 ± 0.12-, 1.08 ± 0.13- and 1.15 ± 0.15-fold change of HIF-1α mRNA accumulation with respect to the corresponding untreated cells, suggesting that A_3 receptor stimulation does not regulate HIF-1α mRNA transcription. Similar results were obtained after 2, 3 and 4 h of hypoxia.

3.6. *Inhibition of transcriptional activity attenuated A_3 receptor-evoked HIF-1α accumulation*

We used actinomycin-D to check for a transcriptional activity in facilitating the A_3 receptor response. Actinomycin-D, a universal inhibitor of mRNA synthesis, dose-dependently attenuated HIF-1α accumulation in response to Cl-IB-MECA. Inhibition was evident at a concentration of 50 ng/ml and more pronounced at 100–200 ng/ml (Fig. 5).

After return of hypoxic A172 cultures to normoxia the levels of HIF-1α protein decreased very rapidly. In particular, a decrease in HIF-1α protein could be seen, in the absence and in the presence of Cl-IB-MECA with unchanged degradation rate (data not shown).

3.7. *The main intracellular signaling pathways sustained by A_3 receptors during HIF-1α accumulation in hypoxia*

A_3 adenosine receptor was assessed for its ability to activate Akt, p38 and p44/p42 kinases in hypoxic GBM U87MG and A172 cells. Cells were treated with increasing concentrations of Cl-IB-MECA for 1–2–3–4 h in hypoxia. We could observe that the phosphorylation of these kinases occurs at early time points following A_3 receptor activation (Fig. 6A). Furthermore, results indicate that Cl-IB-MECA can potently activate Akt in a dose-dependent manner in hypoxic GBM cells (Fig. 6B). Similar results are reported for p38 and p44/p42 (Fig. 6B). To determine whether Akt and MAPK pathways were required for HIF-1α protein increase induced by A_3 receptor activation, A172 cells were pretreated with SH5, an Akt inhibitor, with U0126, which is a potent inhibitor of MEK1/2, or with the inhibitor of p38 MAPK, SB202190 [37]. Cells were then exposed to Cl-IB-MECA 100 nM for 4 h in hypoxia. As shown in Fig. 6C and D, both U0126 (1–10 μM) and SB202190 (1–10 μM) were able to inhibit Cl-IB-MECA-induced increase of HIF-1α protein expression, while SH-5 (1–10 μM) did not.

3.8. *Modulation of VEGF expression*

VEGF was detectable in the media from normoxic U87MG and A172 cells and was significantly increased by 24 and 48 h of hypoxia in A172 cells, by 48 h of hypoxia in U87MG (Table 1). The VEGF concentrations (mean ± S.E., $n = 3$) from cells cultured in normoxia and in hypoxia are reported in Table 1. The highest value of secreted VEGF was found in U87MG cells.

Table 1 – VEGF-A protein expression levels in A172 and U87MG human glioblastoma cell lines

Hours hypoxia	A172	U87MG
0	6.9 ± 0.8	110 ± 8
24	15.8 ± 1.9	100 ± 12
48	36.9 ± 4.5	232 ± 18

VEGF-A concentrations in cell supernatants (pg/ml/10^6 cells) were measured by ELISA.

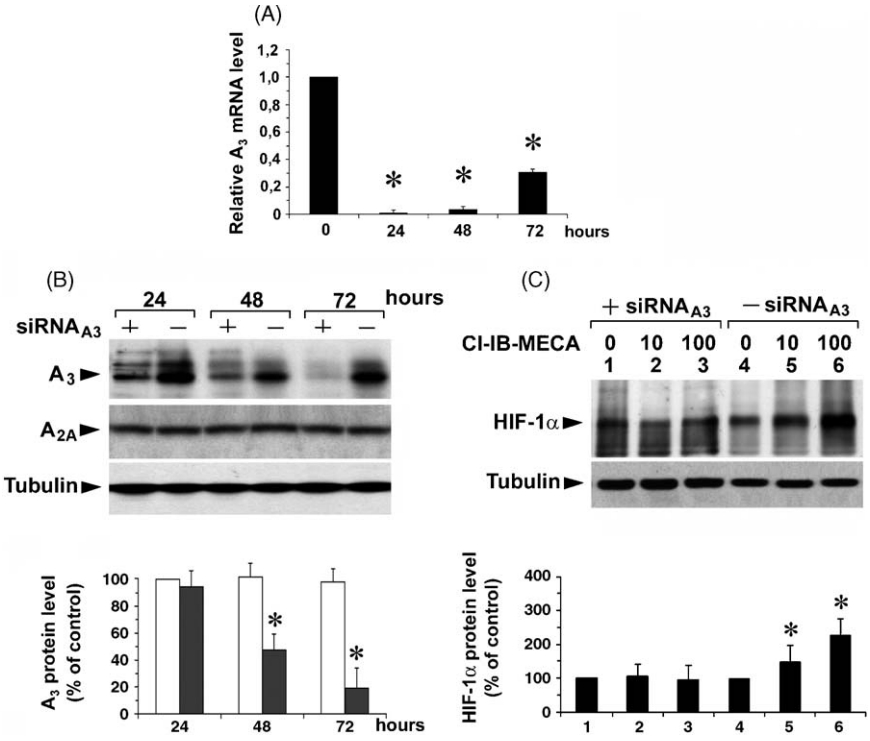

Fig. 4 – A_3 receptor silencing by siRNA transfection. (A) Relative A_3 receptor mRNA quantification, related to β-actin mRNA, by real-time RT-PCR. A172 cells were transfected with $siRNA_{A3}$ by RNAiFect™ Transfection reagent and cultured for 24, 48 and 72 h. Plots are mean ± S.E. values ($n = 3$); $^*P < 0.01$ compared with the control (time = 0). (B) Western blot analysis using an anti-A_3 receptor polyclonal antibody, an anti-A_{2A} receptor polyclonal antibody, of protein extracts from A172 cells treated with scramble (−) or with $siRNA_{A3}$ (+) and cultured for 24, 48 and 72 h. Tubulin shows equal loading protein. Densitometric quantification of A_3 receptor Western blot; plots are mean ± S.E. values ($n = 5$); $^*P < 0.01$ compared with the control (24 h scramble-transfected cells). (C) Western blot analysis of protein extracts from A172 cells treated with scramble (−$siRNA_{A3}$) or $siRNA_{A3}$ (+$siRNA_{A3}$) for 72 h and cultured without (lines 1 and 4) or with Cl-IB-MECA 10 nM (lines 2 and 5) and 100 nM (lines 3 and 6) for 4 h in hypoxia. Tubulin shows equal loading protein. Densitometric quantification of HIF-1α western blot presented in the panel; plots are mean ± S.E. values ($n = 5$); $^*P < 0.01$ compared with the control (lines 1 and 4).

The effects of adenosine on secreted VEGF levels were determined under normoxic and hypoxic conditions. Adenosine 100 μM increased VEGF levels after 48 h of treatment in normoxia as well as in hypoxia. In particular, we found that the concentration of VEGF protein in the medium was increased slightly up to 18% in normoxia ($P < 0.01$, $n = 3$) in both glioblastoma cell lines. In hypoxia the increase was up to 24% and 48% in U87MG and A172 cells, respectively ($P < 0.01$, $n = 3$). To determine the concentration of MRE 3008F20 required to inhibit adenosine-regulated VEGF protein increase under hypoxia, U87MG and A172 cells were treated with different concentrations of the A_3 receptor antagonist MRE 3008F20. When utilized alone under hypoxic conditions, the MRE 3008F20 compound had no effect on VEGF protein increase induced in hypoxia. VEGF levels were analyzed after 48 h of hypoxia. Reduction in VEGF levels was seen at a concentration of 10 nM of A_3 receptor antagonist at which HIF-1α accumulation induced by A_3 receptor stimulation was also inhibited. Complete abrogation of VEGF accumulation induced by adenosine 100 μM was observed with MRE 3008F20 100 nM (Fig. 7A). To determine whether the inhibition of VEGF accumulation was specific to MRE 3008F20 or it was a feature shared by other A_3 receptor antagonists as well, A172 cells were treated with other selective A_3 antagonists (a series of substituted pyrazolotriazolopyrimidine derivatives) [40]. These antagonists (100 nM) also inhibited VEGF levels increased by adenosine (100 μM) in hypoxia (data not shown). To determine whether A_3 receptor stimulation in hypoxia results in a further increase in VEGF regulated gene expression, the level of VEGF protein in U87MG and in A172 cells after exposure to the A_3 agonist Cl-IB-MECA was analyzed. Treatment of cells with increasing concentrations of Cl-IB-MECA significantly modulated VEGF levels, when compared with VEGF levels in untreated hypoxic cells. In particular, Cl-IB-MECA 500 nM was able to increase VEGF levels up to 81 and 73% respect to untreated hypoxic cells, in U87MG and A172 cells, respectively. Fig. 7A and B shows the results obtained in A172 cells. Similar results were obtained for U87MG cells (data not shown).

3.9. *HIF-1α knockdown prevents the A_3 receptor-induced increase of VEGF*

In order to determine if the increased levels of VEGF seen in hypoxia after A_3 receptor stimulation were a result of the raised levels of HIF-1α, $siRNA_{HIF\text{-}1\alpha}$ were directly transfected

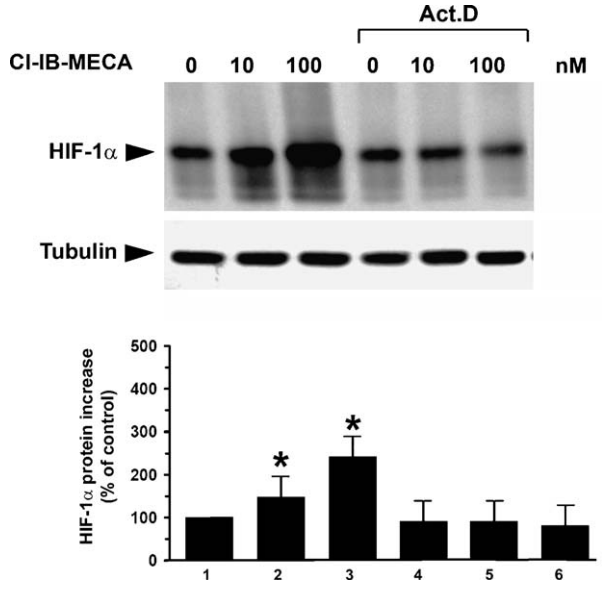

Fig. 5 – A_3 receptor stimulation induces HIF-1α accumulation through a transcription-dependent pathway. A172 cells were pretreated with actinomycin-D (Act.D) (100 ng/ml) for 30 min and then exposed to 4 h hypoxia without (0) or with Cl-IB-MECA 10 and 100 nM. The mean densitometry data from three independent experiments were normalized to the result obtained in cells in the absence of Cl-IB-MECA after 4 h of hypoxia (control = line-l). Plots are mean ± S.E. values (n = 3); $^*P < 0.01$ compared with the control.

into GBM cells 48 h prior to exposure to Cl-IB-MECA (500 nM) for 48 h in hypoxia. When HIF-1α protein was knocked down with siRNA (at 72 h post $siRNA_{HIF\text{-}1\alpha}$ transfection, Fig. 7C and D), VEGF protein increase induced by A_3 receptor stimulation was prevented (Fig. 7E).

3.10. *A_3 receptor mediates VEGF accumulation in hypoxia through a MAPK pathway*

The selective A_3 antagonists MRE 3008F20 and MRE 3005F20 10 nM inhibited the Cl-IB-MECA-stimulated VEGF protein expression, in both GBM cells, pointing to the involvement of A_3 receptors in adenosine-induced increase of VEGF production (Fig. 7E). To investigate whether MAPK pathway was involved in the expression of A_3-induced VEGF protein, U87MG and A172 cells were cultured in hypoxia for 24 h following the addition of the MEK1/2 inhibitor U0126 or with the inhibitor of p38 MAPK, SB202190, 30 min prior to the treatment of Cl-IB-MECA 500 nM. U0126 and SB202190 (10 μM) significantly inhibited the VEGF protein levels induced by Cl-IB-MECA 500 nM.

3.11. *A_3 receptors modulate VEGF promoter activity*

HIF-1 is a transcription factor that mediates the effects of hypoxia on VEGF expression by binding to the hypoxia-response element of the VEGF promoter. To examine whether adenosine and A_3 receptor stimulation interact with the HIF-1 pathway to up-regulate VEGF transcription, we used two previously described luciferase reporters. The p11w reporter is regulated by a fragment of the VEGF promoter that includes an HIF-1-binding site. The p11m reporter is identical except for a 3-bp mutation that prevents HIF-1 binding [36]. We transfected GBM cells with these reporters and treated the cells with Cl-IB-MECA for different times in hypoxia. As shown in Fig. 8A, hypoxia increased luciferase activity of the p11w reporter in A172 cells incubated under hypoxia in a time-dependent manner. The maximum increase in p11w reporter activity is present at 48 h of hypoxia. Hypoxia also stimulated activity of the p11m reporter but to a minor extent (Fig. 8A). Incubation of the cells under hypoxic conditions with Cl-IB-MECA resulted in a time- and dose-dependent increase in p11w reporter activity. As shown in Fig. 8B, increasing concentrations of Cl-IB-MECA up-regulated luciferase activity of the p11w reporter up to 58% respect to untreated hypoxic A172 cells. In particular, the increase induced by the A_3 agonist at 48 h of hypoxia seems mediated by A_3 receptors, as indicated by the ability of the A_3 receptor antagonist MRE 3008F20 10 nM to block this increase (Fig. 8C). To investigate whether MAPK pathway was involved in the transcription of A_3-induced VEGF protein, A172 cells were transfected with the p11w reporter, cultured in hypoxia for 48 h following the addition of U0126 or SB202190 and treated with Cl-IB-MECA. As shown in Fig. 8C, U0126 and SB202190 (10 μM) significantly inhibited the activity of p11w reporter induced by Cl-IB-MECA. Similar results were obtained at 72 h of hypoxia (data not shown). Cl-IB-MECA also stimulated activity of the p11m reporter but to a minor extent, underlying the crucial role of an intact HRE box (Fig. 8D). Similar results were obtained in U87MG cells. These results indicate that, in GBM hypoxic cells, A_3 receptor stimulation increases VEGF promoter activity by a MAPK signaling via the HIF-1 pathway. To confirm that VEGF regulation may occur at a transcriptional level, we quantified VEGF RNA by real-time RT-PCR experiments. Incubation of GBM cells in the presence of adenosine 100 μM for 48 h in hypoxia increased mRNA expression of VEGF by 2.8 ± 0.2-fold compared with cells incubated with vehicle.

4. Discussion

The data presented in this study provide the indications that:

1. adenosine can further potentiate the effect of hypoxia on HIF-1 α and VEGF expression in human GBM cells;
2. both are increased via A_3 receptor stimulation;
3. MEK and p38 MAPK may have a key role in A_3 receptor ability to improve HIF-1α and VEGF protein expression.

Our results demonstrate that adenosine receptor-mediated signals induce HIF-1 activation under hypoxic conditions in a receptor subtype-specific manner. A_3 receptors induced HIF-1α protein accumulation in a receptor–ligand-dependent manner in GBM cells. To test whether A_3 agonists affect HIF-1 levels, we analyzed HIF-1α and -1β expression and showed that the A_3 agonist specifically increases HIF-1α accumulation, but not HIF-1β expression in the cancer cells. Overall, the

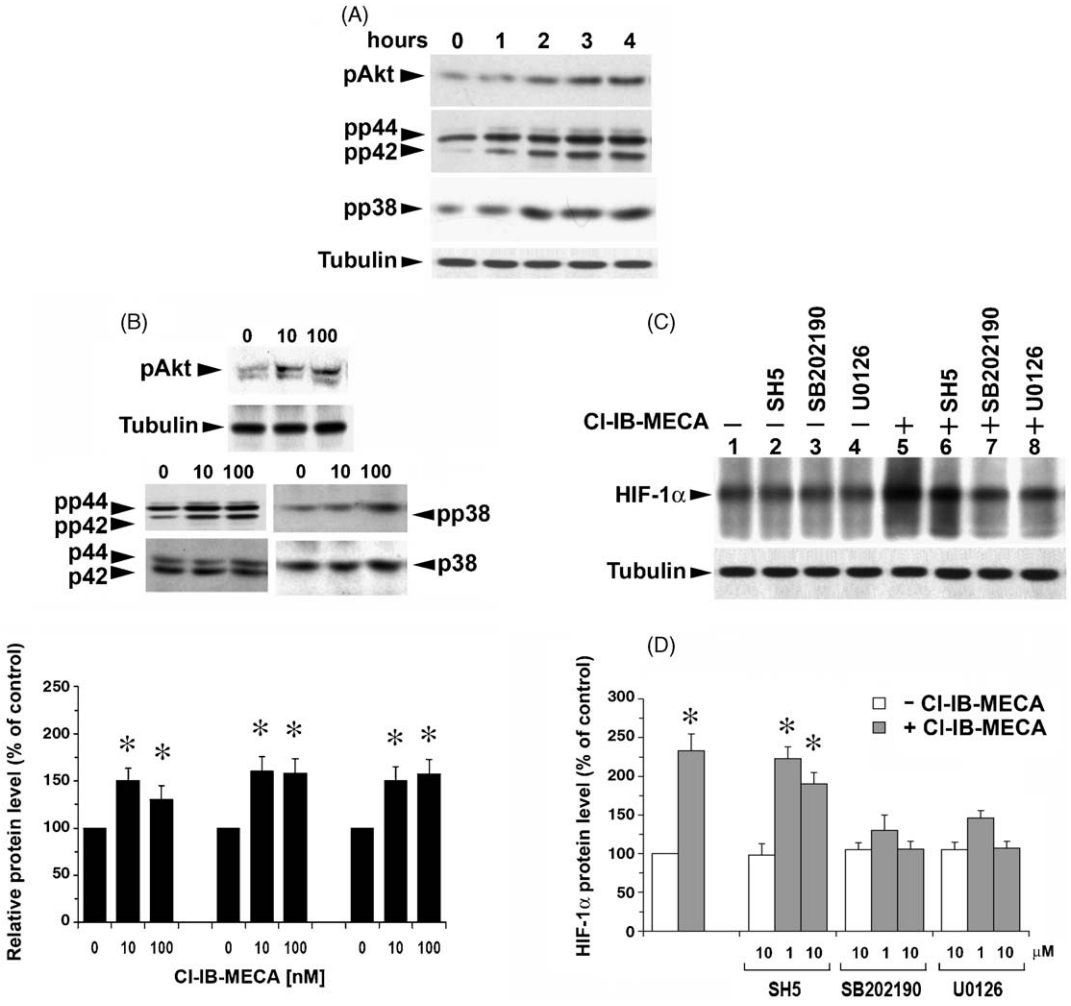

Fig. 6 – Role of Akt, p38, p44 and p42 MAPKs in A_3 signaling. (A) pAkt, pp44 and pp42 MAPK and pp38 protein levels under A_3 receptor stimulation: Cl-IB-MECA 100 nM was added to A172 cells for 1, 2, 3 and 4 h in hypoxia. (B) pAkt, pp38, pp44 and pp42 MAPK protein levels under A_3 receptor stimulation: Cl-IB-MECA 10 and 100 nM was added to A172 cells for 4 h in hypoxia. The mean densitometry data from three independent experiments were normalized to the results obtained in cells in the absence of Cl-IB-MECA (line 0). Plots are mean ± S.E. values ($n = 3$); $^*P < 0.01$ compared with the control. (C) A172 cells were pretreated with or without 10 μM SH5, 10 μM SB202190 or 10 μM U0126 and then exposed to Cl-IB-MECA 100 nM (+) for 4 h in hypoxia. (D) The mean densitometry data from three independent experiments were normalized to the results obtained in hypoxic cells in the absence of Cl-IB-MECA (line 1). Dose-responses for the effects of the inhibitors are shown. Plots are mean ± S.E. values ($n = 3$); $^*P < 0.01$ compared with the control.

results presented in this study demonstrated that, while specific A_3 receptor stimulation up-regulated HIF-1α expression in human GBM cells, A_3 receptor antagonists strongly inhibited the agonist-induced HIF-1α accumulation in hypoxia. Our results indicate that A_3 receptor activation left the mRNA level of HIF-1α unaltered, although HIF-1α protein accumulated. However, blocking transcriptional activity with actinomycin-D, we provide evidence that a transcriptional activity is a prerequisite for HIF-1α accumulation in response to Cl-IB-MECA. At this point, any gene product awaits identification. Furthermore, A_3 receptor activation may not be able to significantly prevent HIF-1α degradation in normoxic conditions. This A_3 behaviour in HIF-1 modulation has been recently observed also in the human melanoma A375 cell line supporting the hypothesis that A_3 receptor may improve HIF-1α stability by the transcription of a panel of unknown genes, directly related to HIF-1α protein stabilization in hypoxia [33].

To gain insight into the potential physiological role of HIF-1α protein accumulation induced in hypoxia by adenosine, the expression of VEGF, a gene product that is induced by hypoxia and regulated by HIF, was determined. VEGF is a critical angiogenic factor and is well established to be increased under hypoxia and at least in part, regulated by HIF [7,41]. Depending on the cell type studied, adenosine has been shown to up-regulate VEGF under normoxic or hypoxic conditions [28–32]. Our results in GBM cells show that adenosine can up-regulate VEGF not only in normoxia, but also under hypoxic conditions. In particular, the increase in VEGF induced by adenosine in hypoxia occurs dependently on HIF-1, as demonstrated with

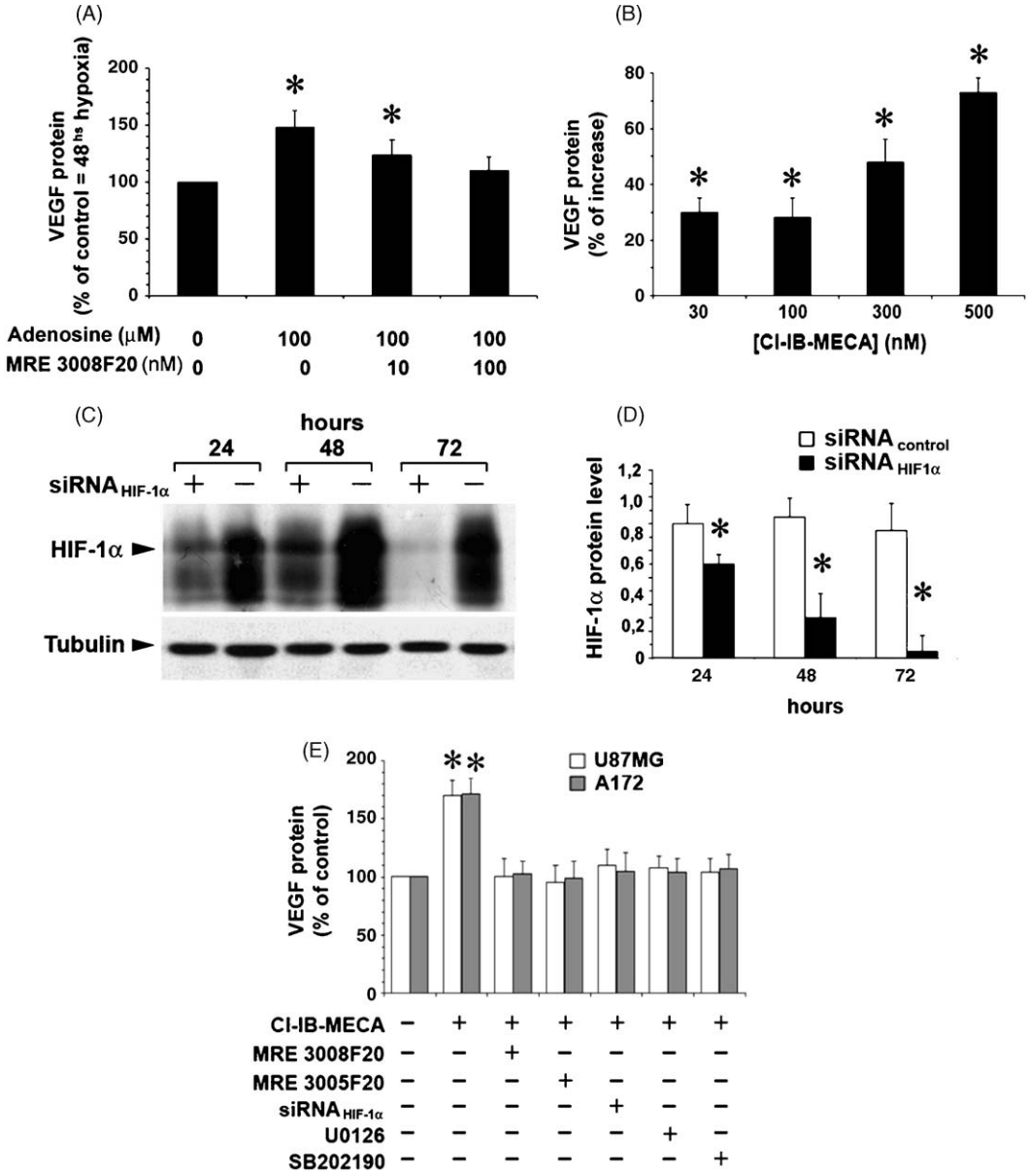

Fig. 7 – Effect of A_3 adenosine receptor stimulation on VEGF expression in hypoxic cells. (A) VEGF release into culture media of A172 cells cultured 48 h in the absence and in the presence of Adenosine 100 μM and the A_3 receptor antagonist MRE 3008F20 10 and 100 nM. Plots are mean ± S.E. values ($n = 3$); $^*P < 0.01$ compared with the control (untreated hypoxic cells). (B) VEGF release into culture media of A172 cells incubated for 48 h in hypoxia with increasing concentrations of Cl-IB-MECA (reported as % of increase respect to untreated hypoxic cells). Plots are mean ± S.E. values ($n = 5$); $^*P < 0.01$ compared with the control (untreated hypoxic cells). (C) HIF-1α silencing by siRNA$_{HIF\text{-}1\alpha}$ transfection. Western blot analysis of protein extracts from A172 cells transfected with siRNA$_{HIF\text{-}1\alpha}$ (+) or with scramble (−) by RNAiFect™ Transfection reagent and cultured for 24, 48 and 72 h in hypoxia. Tubulin shows equal loading protein. (D) Densitometric quantification of HIF-1α Western blot; plots are mean ± S.E. values ($n = 5$); $^*P < 0.01$ compared with the control (time 0). (E) Pharmacological analysis in U87MG and A172 cells of A_3 receptors regulating VEGF secretion (reported as % of VEGF secretion in drug-vehicle-treated cells cultured in hypoxia for 48 h). Effects of 10 nM A_3 receptor antagonist MRE 3008F20, 10 nM MRE 3005F20, siRNA$_{HIF\text{-}1\alpha}$, 10 μM U0126 and 10 μM SB202190 on VEGF secretion in the presence of Cl-IB-MECA 500 nM.

siRNA$_{HIF\text{-}1\alpha}$ We observed an increase in HIF-regulated gene product VEGF at 24 h subsequent to the up-regulation of HIF-1α at 4 h. This apparent discrepancy between the time courses of HIF-1 and VEGF increase after Cl-IB-MECA treatment may reflect the different time requirements for the sequential activation of a transcription factor, the subsequent expression of the secondary response gene and the ultimate changes in level of the corresponding protein.

To understand the mechanism of A_3 receptor-modulated VEGF expression, we found that the A_3 agonist increased the

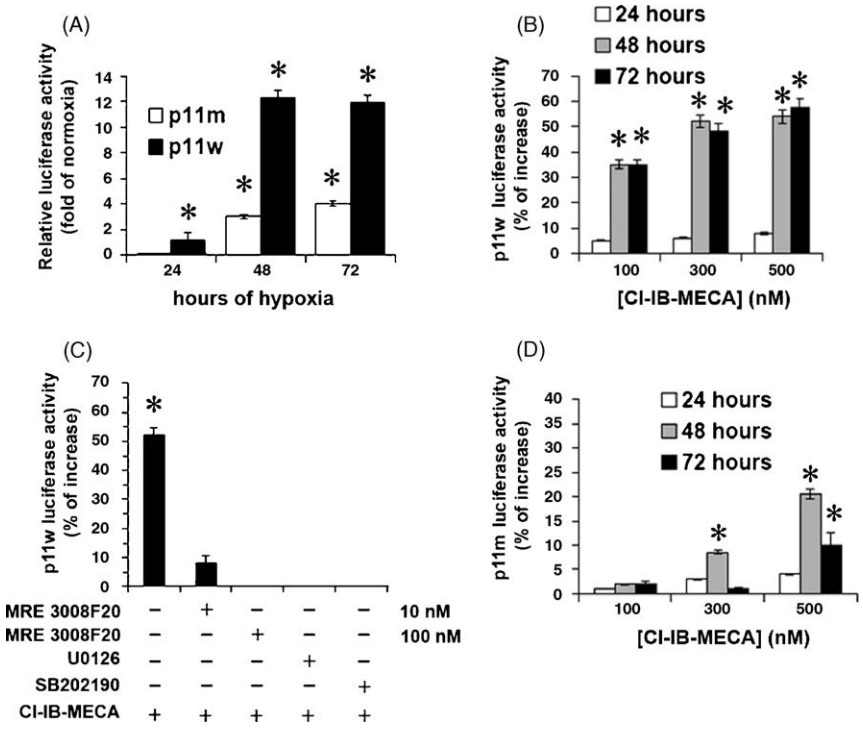

Fig. 8 – Effect of hypoxia and A_3 receptor stimulation on HIF-1-dependent VEGF reporter activity. A172 cells were transfected with plasmids encoding luciferase reporters driven by the VEGF promoter region containing a native HIF-1-binding element (p11w) or a mutated HRE unable to bind HIF-1 (p11m). Cells were incubated under hypoxia for 24, 48 and 72 h with vehicle (A) or with increasing concentrations of Cl-IB-MECA (B and D). Plots are mean ± S.E. values ($n = 3$); $^*P < 0.01$ compared with the control (time 0 from the transfection in the absence of Cl-IB-MECA). (C) Pharmacological analysis of A_3 receptors regulating VEGF transcriptional activation. Effects of 10 and 100 nM A_3 receptor antagonist MRE 3008F20, 10 μM U0126 and 10 μM SB202190 on p11w luciferase activity in the presence of Cl-IB-MECA 500 nM. $^*P < 0.01$ compared with the control (time 0 from the transfection in the absence of Cl-IB-MECA).

VEGF reporter activity with the normal HIF-1 binding site, but not with the mutation of the HIF-1 binding site, suggesting that HIF-1α is the master transcriptional factor involved. Furthermore, A_3 antagonist treatment inhibited the VEGF transcriptional activation through the HIF-1 DNA binding site in the VEGF promoter region.

HIF-1 expression is known to play an important role in VEGF transcriptional activation in response to hypoxia [36]. Thus, A_3 antagonists may inhibit VEGF transcriptional activation through the decrease of HIF-1α expression in cancer cells. HIF-1 is stabilized by hypoxia, and activates the transcription of many genes including VEGF, endothelin-1, and inducible nitric oxide synthase, which are implicated in vasodilation, neovascularization, and tumor metastasis [7]. As a consequence, we suggest that A_3 antagonists may inhibit hypoxic GBM growth at multiple levels by interfering with HIF-1α expression.

In many cancers, the HIF-1 pathway is not only activated by low oxygen tension, it is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways. Increased HIF-1α protein synthesis via the activation of PI3K-Akt-mTOR or MAPK pathways is a common theme accounting for the up-regulation [6,12]. Recent studies demonstrated that MAPK signaling mediates angiogenesis and VEGF expression. We found that p44/p42 and p38 MAPK activities were required for the HIF-1α expression increase induced by A_3 receptor activation, independently on Akt. These data agree with those supporting the ability of A_3 receptor to activate p44/42 and p38 MAPK [14,17].

We suggest that the effect of the A_3 agonist on HIF-1α levels in GBM cell types may be hypoxia specific, because adenosine concentrations are significantly increased in hypoxia [15].

In recent years HIF-1α has emerged as a potentially important therapeutic target for cancer therapy [7]. Strategies suggested for HIF-1α targeting include disruption of the normal coactivational response to hypoxia [42], the use of decoy oligonucleotides [43], and gene therapy approach based on hypoxia response element-regulated gene expression that exploits the presence of hypoxia/anoxia in tumors for the induction of therapeutic genes [44]. In addition to these genetic approaches, pharmacological intervention of HIF is also actively pursued in many laboratories [45–48]. Our study on GBM cells, along with the earlier study on melanoma cells, suggests that a therapeutically feasible approach of targeting HIF is through the use of A_3 receptor antagonists.

Additional studies are needed to determine whether these inhibitors are capable of blocking in vivo the HIF-1α-dependent invasiveness, survival, and angiogenesis of GBM.

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