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A_3 Adenosine Receptors Modulate Hypoxia-Inducible Factor-1α Expression in Human A375 Melanoma Cells

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Abstract

Hypoxia-inducible factor-1 (HIF-1) is a key regulator of genes crucial to many aspects of cancer biology. The purine nucleoside, adenosine, accumulates within many tissues under hypoxic conditions, including that of tumors. Because the levels of both HIF-1 and adenosine are elevated within the hypoxic environment of solid tumors, we investigated whether adenosine may regulate HIF-1. Here we show that, under hypoxic conditions (< 2% O_2), adenosine upregulates HIF-1α protein expression in a dose-dependent and time-dependent manner, exclusively through the A_3 receptor subtype. The response to adenosine was generated at the cell surface because the inhibition of A_3 receptor expression, by using small interfering RNA, abolished nucleoside effects. A_3 receptor stimulation in hypoxia also increases angiopoietin-2 (Ang-2) protein accumulation through the induction of HIF-1α. In particular, we found that A_3 receptor stimulation activates p44/p42 and p38 mitogen-activated protein kinases, which are required for A_3-induced increase of HIF-1α and Ang-2. Collectively, these results suggest a cooperation between hypoxic and adenosine signals that ultimately may lead to the increase in HIF-1–mediated effects in cancer cells.

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Introduction

Adenosine is a ubiquitous autacoid that accumulates to high levels in hypoxic tissues as a result of ATP breakdown [1–4]. 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 [5], suggesting a role for this autacoid in tumor growth. However, it was only recently that adenosine has been shown to be a crucial factor in determining the cell progression pathway, either in the apoptotic state or the cytostatic state [6,7].

Adenosine modulates a variety of cellular functions through occupancy of four cell surface G-protein–coupled receptors, named A_1, A_{2A}, A_{2B}, and A_3 [8,9]. In particular, adenosine was found to exert its effects on cell proliferation, clone formation ability, UV resistance, and cell death mainly through the A_3 subtype [10–13], which is highly expressed in tumor cells [14–18]. These findings confirm recent data indicating that A_3 receptor overexpression may be a good candidate as a tumor cell marker [18,19]. Adenosine also plays a role in the promotion of angiogenesis [20,21]. The regulation of expression of the angiogenic vascular endothelial growth factor (VEGF) through adenosine receptors has been demonstrated in several cell types [22–25].

Hypoxia-inducible factor (HIF)-1 is a transcription factor that functions as a master regulator of oxygen homeostasis [26]. HIF-1 is a heterodimer composed of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit [27]. HIF-1α and HIF-1β mRNA are constantly expressed under normoxic and hypoxic conditions [28]. During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation [29,30]. However, aberrant HIF-1α expression could be caused by mutations in the *HIF-1α* gene itself or its

Abbreviations: Act-D, actinomycin D; Ang-2, angiopoietin-2; CHX, cycloheximide; Cl-IB-MECA, N^6(3-iodobenzyl)2-chloroadenosine-5′*N*-methyluronamide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; HIF-1, hypoxia-inducible factor-1; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MRE 3008F20, 5*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine; MRE 2029F20, *N*-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; RT-PCR, reverse transcription polymerase chain reaction; SCH 58261, 7-(2-phenylethyl)2-(2-furyl)pyrazolo[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine; siRNA, small interfering RNA; $siRNA_{A3}$, small interfering RNA targeting A_3 receptor mRNA; $siRNA_{HIF\text{-}1\alpha}$, small interfering RNA targeting HIF-1α mRNA; VEGF, vascular endothelial growth factor

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regulating pathways, leading to hypoxia-independent expression of HIF-1α [31,32].

A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis [33,34]. Immunohistochemical analyses have shown that HIF-1α is present in higher levels in human tumors than in normal tissues [35]. In particular, the levels of HIF-1 activity in cells are correlated with tumorigenicity and angiogenesis in nude mice [36]. Tumor cells lacking HIF-1 expression are markedly impaired in their growth and vascularization [37–40]. Therefore, because HIF-1α expression and activity appear central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target [26,40,41].

Production of adenosine in hypoxia has not yet been related to HIF-1α. 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. Furthermore, as HIF-1α plays a key role in inducing angiogenesis [20–25], we have also studied the role of adenosine in mediating the production of VEGF and angiopoietin-2 (Ang-2) in hypoxic cells.

Materials and Methods

Cell Lines, Reagents, and Antibodies

NCTC 2544 keratinocytes, and A375 melanoma, HT29 colon carcinoma, MCF-7 breast carcinoma, OVCAR-3 ovary carcinoma, U2OS osteosarcoma, and U87MG glioblastoma human cells were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). Tissue culture media and growth supplements were obtained from Bio-Whittaker (Bergamo, Italy). The GasPak Pouch System was obtained from Becton Dickinson (Milan, Italy). Anti–HIF-1α and anti–HIF-1β antibodies were obtained from Transduction Laboratories (BD; Milan, Italy). U0126 [inhibitor of mitogen-activated protein kinase kinase (MEK)-1 and MEK-2], SB202190 (inhibitor of p38 MAP kinase), anti–ACTIVE mitogen-activated protein kinase (MAPK), and anti–ERK 1/2 (pAb) were from Promega (Milan, Italy). Lamin A, phospho-p38, and p38 MAP kinase antibodies were from Cell Signaling Technology (Celbio; Milan, Italy). Anti–adenosine A_{2A} receptor (pAb) was from Santa Cruz Biotechnology (RBA; Milan, Italy). Anti–adenosine A_3 receptor (polyAb) was from Aviva Antibody Corporation (RBA; Milan, Italy). Unless otherwise noted, all other chemicals were purchased from Sigma (Milan, Italy).

Cell Culture and Hypoxia Treatment

Cells were maintained in DMEM (A375, HT-29, and MCF-7), EMEM (NCTC 2544) or RPMI 1640 (OVCAR-3, U87MG, and U2OS) 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. Cells were passaged two or three times weekly at a ratio between 1:5 and 1:10. Hypoxic exposure was performed using the BBL GasPak pouch System (Becton Dickinson) that reduces the oxygen concentration at less than 2% within 2 hours of incubation at 37°C.

RNA Interference

To generate four small interfering RNA targeting A_3 receptor mRNA ($siRNA_{A3}$), eight oligonucleotides were synthesized and annealed, according to the manufacturer's instructions (*Silencer* siRNA Construction Kit; Ambion, Milan, Italy) and as previously described [42]. 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 positions 337, 679, 1009, and 1356 downstream of the start codon of A_3 receptor mRNA sequence (L20463), respectively.

A375 cells were plated in six-well plates and grown to 50% to 70% confluence before transfection. Transfection of siRNA was performed at a concentration of 100 nM using the RNAiFect Transfection Kit (Qiagen, Milan, Italy). To quantify cell transfection efficiency, we used siRNA FITC (Qiagen). After 24 hours of transfection, cells were trypsinized and resuspended in PBS for flow cytometry analysis. Fluorescence obtained from siRNA–FITC–transfected cells was compared to autofluorescence generated by untransfected control. A nonspecific control ribonucleotide sense strand (5′-ACU CUA UCU GCA CGC UGA CdTdT-3′) and an antisense strand (5′-dTdT UGA GAU AGA CGU GCG ACU G-3′) were used under identical conditions.

Small interfering RNA targeting HIF-1α mRNA ($siRNA_{HIF\text{-}1\alpha}$) were synthesized and experiments were performed as previously described [43]. The sequences target nt 1378 to 1398 of the human HIF-1α mRNA (accession no. AF304431.1).

Flow Cytometry Analysis

A375 adherent cells were trypsinized, mixed with floating cells, washed with PBS, and permeabilized in 70% (vol/vol) ethanol/PBS solution at 4°C for at least 24 hours. The cells were washed with PBS and the DNA was stained with a PBS solution, containing 20 μg/ml propidium iodide and 100 μg/ml RNAse, at room temperature for 30 minutes. Cells were analysed with an EPICS XL flow cytometer (Beckman Coulter, Miami, FL) and the content of DNA was evaluated. Cell distribution among cell cycle phases and the percentage of apoptotic cells were evaluated as previously described [10]. Briefly, the cell cycle distribution is shown as the percentage of cells containing $2n$ (G_0/G_1 phases), $4n$ (G_2 and M phases), $4n > x > 2n$ DNA amounts (S phase) judged by propidium iodide staining. Apoptotic population is the percentage of cells with DNA content lower than $2n$.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Experiments

Total cytoplasmic RNA was extracted by the guanidinium thiocyanate phenol method, as previously described [18]. Quantitative real-time RT-PCR assay [18] of HIF-1α and A_3 mRNA transcripts was carried out using gene-specific double fluorescently labeled TaqMan MGB probe (minor groove binder) in an 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 real-time RT-PCR of the *HIF-1α* gene, the assays-on-demand gene expression product accession no. NM 019 058 was used (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-labeled with VIC (Applied Biosystems).

Western Blot Analysis

A375, HT29, MCF-7, NCTC 2544, OVCAR-3, U2OS, and U87MG cells were treated with adenosine or adenosine analogues and exposed to normoxia and hypoxia for different times (2–24 hours). Western blot analysis was performed as previously described [10]. Membranes were incubated with antibodies against HIF-1α (1:250 dilution) and HIF-1β (1:1000 dilution) 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 A_3 receptor (1 μg/ml dilution). Specific reactions were revealed with the enhanced chemiluminescence Western blot analysis detection reagent (Amersham Corp., Arlington Heights, IL). The membranes were then stripped and reprobed with tubulin (1:250) to ensure equal protein loading.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of VEGF and Ang-2 protein secreted by the cells in the medium were determined by an ELISA kit (R&D Systems, Milan, Italy). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of adenosine analogues for 24 or 48 hours in hypoxia. The medium was collected, and 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 ± SD from three independent experiments.

Statistical Analysis

All values in the figures and text are expressed as mean ± SE of n observations (with $n \geq 3$). Data sets were examined by analysis of variance (ANOVA) and Dunnett's test (when required). A P value less than .05 was considered statistically significant.

Results

Adenosine Induces HIF-1α Protein Accumulation in Hypoxia

We have evaluated the biologic effect produced by prolonged oxygen deprivation in the human A375 melanoma cell line. Viability and proliferation of A375 cells exposed to hypoxia for 24 hours were assessed by analyzing the percentage of apoptotic cells and the distribution among the different phases of the cell cycle. We employed flow cytometry and DNA staining by propidium iodide for discrimination of cells in apoptosis, in G_0/G_1, S, and G_2/M phases. The results indicated that hypoxia did not promote significant cell death while interfering with proliferation by arresting melanoma cells in the G_0/G_1 phase and reducing the number of cells in the S and G_2/M phases (Figure 1). These data were confirmed by using trypan blue exclusion, cell counts, and [^{3}H]thymidine incorporation assay (data not shown).

Exposure of human melanoma A375 cells to hypoxia led to HIF-1α protein expression (Figure 2*A*). To study the effect of adenosine on the transcription factor HIF-1, A375 cells were treated for 4 hours in hypoxic conditions with increasing concentrations of the nucleoside. Hypoxia alone increased HIF-1α over normoxia. Adenosine further increased HIF-1α protein expression in a time-dependent and dose-dependent manner, with an $EC_{50} = 2.1 \pm 0.2$ μM and a maximal increase of 2.6 ± 0.2–fold at 100 μM (Figure 2*B*). We did not observe any modulation of HIF-1β protein.

The family of adenosine receptors consists of four subtypes of G-protein–coupled receptors, designated A_1, A_{2A}, A_{2B}, and A_3 [8,9]. We have previously demonstrated that all four adenosine receptors are expressed in A375 cells [15]. To evaluate the functional role of adenosine receptor subtypes on HIF-1α protein expression under hypoxic conditions, we tested the effect of adenosine in combination with DPCPX (1,3-dipropyl-8-cyclopentylxanthine; an A_1

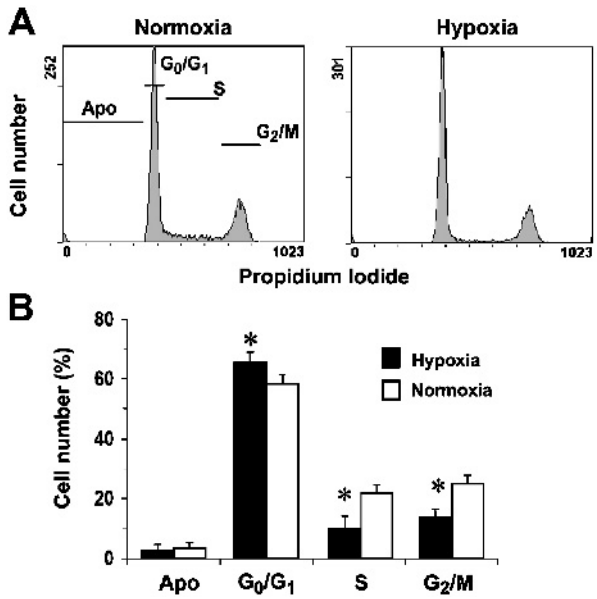

Figure 1. *Apoptosis and cell cycle analysis of A375 cells cultured in normoxia or hypoxia for 24 hours. (A) Representative flow cytometric analysis of cell cycle using propidium iodide for DNA staining; shown is the pattern of A375 cells being in apoptosis and in the G_0/G_1, S, and G_2/M phases during normoxia and hypoxia. Apoptotic cells (Apo) with subdiploid DNA content are reported. (B) The quantitative analysis of subdiploids and cells in the G_0/G_1, S, and G_2/M phases is given in the graph. Plots are mean ± SE values (n = 3). *P < .01 compared with normoxia.*

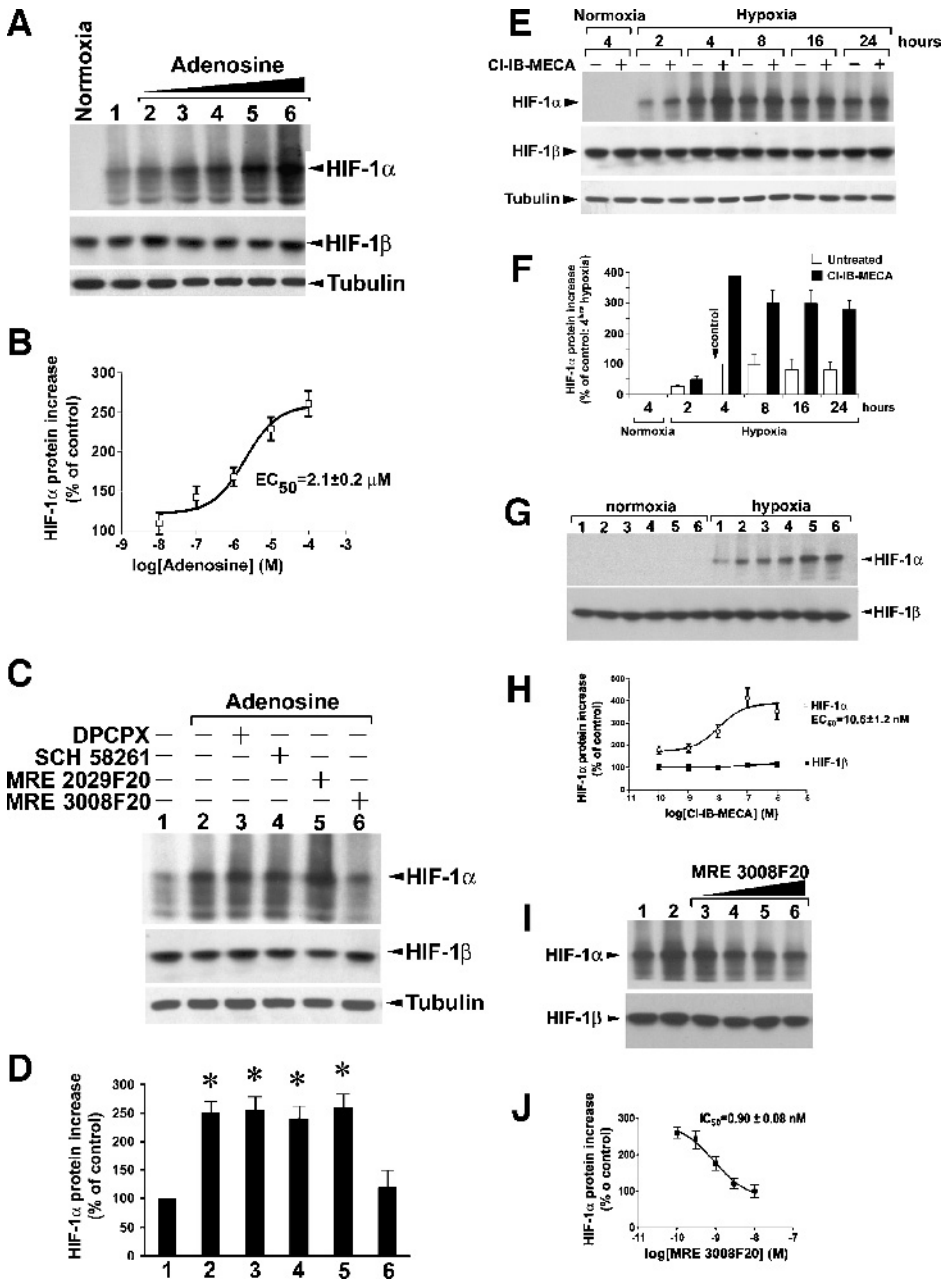

Figure 2. *Induction of HIF-1α expression by adenosine and A_3 receptors. (A) A375 cells were treated without (lane 1, control) or with adenosine at concentrations of 10 nM (lane 2), 100 nM (lane 3), 1 μM (lane 4), 10 μM (lane 5), and 100 μM (lane 6) in hypoxia for 4 hours. (B) The mean densitometry data were normalized to the result obtained in hypoxic cells in the absence of adenosine (control). Plots are mean ± SE values (n = 3). (C) A375 cells were treated without (lane 1, control) or with 100 μM adenosine (lanes 2–6), and exposed to 100 nM DPCPX (lane 3), 100 nM SCH 58261 (lane 4), 100 nM MRE 2029F20 (lane 5), or 100 nM MRE 3008F20 (lane 6) in hypoxia for 4 hours. (D) The mean densitometry data from five independent experiments were normalized to the result obtained in cells in the absence of adenosine (control). Plots are mean ± SE values (n = 5). *P < .01 compared with the control. (E) Induction of HIF-1 expression by A_3 receptor stimulation: time course. Cl-IB-MECA was 100 nM. (F) 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 hours of hypoxia (control). Plots are mean ± SE values (n = 3). *P < .01 compared with the control. (G) Induction of HIF-1α expression by A_3 receptor stimulation: dose response. A375 cells were treated without (lane 1) or with Cl-IB-MECA at concentrations of 0.1 nM (lane 2), 1 nM (lane 3), 10 nM (lane 4), 100 nM (lane 5), and 1 μM (lane 6) in normoxia and hypoxia for 4 hours. (H) The mean densitometry data from 12 independent experiments were normalized to the result obtained in cells in the absence of Cl-IB-MECA (control). Plots are mean ± SE values (n = 3). (I) Effect of A_3 receptor antagonist, MRE 3008F20. A375 cells were treated in hypoxia for 4 hours without (lane 1) or with 10 nM Cl-IB-MECA (lanes 2–6) and MRE 3008F20 at concentrations of 0.3 nM (lane 3), 1 nM (lane 4), 3 nM (lane 5), and 10 nM (lane 6). (J) 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 ± SE values (n = 3).*

receptor antagonist), SCH 58261 [7-(2-phenylethyl)2-(2-furyl)pyrazolo[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine; a selective A_{2A} receptor antagonist], MRE 2029F20 [*N*-benzo[1,3]-dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide; a selective A_{2B} receptor antagonist], and MRE 3008F20 [5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine; a selective A_3 receptor antagonist] [15,44,45]. Although the A_1, A_{2A}, and A_{2B} receptor antagonists were not able to prevent

adenosine-induced HIF-1α protein accumulation, the A_3 receptor antagonist, MRE 3008F20, abrogated the adenosine-induced increase of HIF-1α protein expression (Figure 2, *C* and *D*). Furthermore, HIF-1β expression was unaffected by synthetic adenosine receptor antagonists. These results indicate that adenosine may increase HIF-1α protein expression through A_3 receptors.

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 different tumor cell lines with the selective A_3 receptor agonist, Cl-IB-MECA [N^6(3-iodobenzyl)2-chloroadenosine-5′*N*-methyluronamide] [46]. Here, we show the results obtained on the A375 melanoma cell line. We performed a time course experiment in which A375 cells were exposed to 100 nM Cl-IB-MECA for 2 to 24 hours. A_3 adenosine receptor stimulation did not promote HIF-1α protein accumulation in normoxia, whereas under hypoxic conditions, HIF-1α protein expression was increased in a time-dependent manner (Figure 2, *E* and *F*). As already observed with adenosine, Cl-IB-MECA did not modify HIF-1β expression in normoxia or hypoxia. Further experiments found that Cl-IB-MECA was a very potent agonist, having an EC_{50} of 10.6 ± 1.2 nM (Figure 2, *G* and *H*), consistent with an effect through adenosine A_3 receptors. The potency of the A_3 receptor antagonist, MRE 3008F20, was quantified. Increasing concentrations of MRE 3008F20 (0.3–30 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 0.90 ± 0.08 nM (Figure 2, *I*–*J*). Other selective A_3 antagonists (a series of substituted pyrazolotriazolopyrimidine derivatives) [47] also blocked Cl-IB-MECA–induced HIF-1α accumulation (data not shown).

Finally, to further demonstrate that the A_3 receptor is required for HIF-1α protein accumulation in response to adenosine, A375 cells were transfected with small interfering RNA that target A_3 receptor mRNA ($siRNA_{A3}$) for degradation. The transfection efficiency of A375 cells, determined by an siRNA control labeled with fluorescein, was monitored (86.5%; Figure 3*A*). After 24, 48, and 72 hours posttransfection, A_3 receptor mRNA and protein levels were significantly reduced in $siRNA_{A3}$-treated cells (Figure 3, *B*–*D*). 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 (Figure 3*C*). Figure 3*C* demonstrates that treatment of A375 cells with $siRNA_{A3}$ reduced the expression of A_3 protein but had no effect on the expression of A_{2A} receptor. At 72 hours from the $siRNA_{A3}$ transfection, A375 cells were exposed to increasing concentrations of Cl-IB-MECA (1–100 nM) for 4 hours in hypoxia. We found that the inhibition of A_3 receptor expression is sufficient to block Cl-IB-MECA–induced HIF-1α accumulation (Figure 3, *E* and *F*).

To determine whether the effect of A_3 receptor stimulation on HIF-1α expression was a general phenomenon, we assessed the ability of Cl-IB-MECA to induce HIF-1α levels in a variety of cell lines expressing A_3 adenosine receptors [7]. After 4 hours of hypoxia under Cl-IB-MECA treatment, we were able to detect a significant increase of HIF-1α protein expression in human keratinocytes (NCTC 2544) and in different human tumor cells (HT29 colon carcinoma,

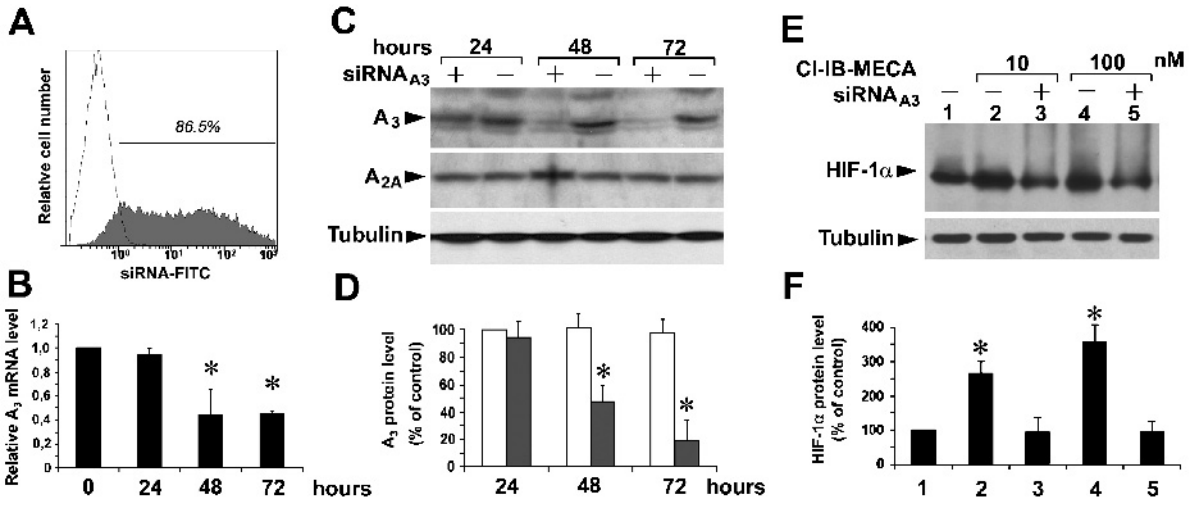

Figure 3. *A_3 receptor expression silencing by siRNA transfection. (A) Analysis of siRNA transfection efficiency in A375 cells. Representative flow chromatograms of siRNA–FITC accumulation (gray filled area) in A375 cells transfected with siRNA–FITC. Unfilled area shows A375 cells transfected with RNAiFect Transfection reagent without siRNA–FITC. Fluorescence was quantified by flow cytometry 5 hours posttransfection. (B) Relative A_3 receptor mRNA quantification related to β-actin mRNA by real-time RT-PCR. A375 cells were transfected with $siRNA_{A3}$ by the RNAiFect Transfection reagent and cultured for 24, 48, and 72 hours. Plots are mean ± SE values (n = 3). *P < .01 compared with the control (time 0). (C) Western blot analysis using an anti–A_3 receptor polyclonal antibody and an anti–A_{2A} receptor polyclonal antibody of protein extracts from A375 cells treated with scramble (–) or $siRNA_{A3}$ (+) and cultured for 24, 48, and 72 hours. Tubulin shows equal loading protein. (D) Densitometric quantification of A_3 receptor Western blot analysis; plots are mean ± SE values (n = 5). *P < .01 compared with the control (scramble transfected cells). (E) Western blot analysis using an anti–HIF-1α monoclonal antibody of protein extracts from A375 cells treated with scramble (–) or $siRNA_{A3}$ (+) for 72 hours and cultured without (lane 1) or with Cl-IB-MECA at concentrations of 10 nM (lanes 2 and 3) and 100 nM (lanes 4 and 5) for 4 hours in hypoxia. Tubulin shows equal loading protein. (F) Densitometric quantification of HIF-1α Western blot analysis presented in panel E; plots are mean ± SE values (n = 5). *P < .01 compared with the control (lane 1).*

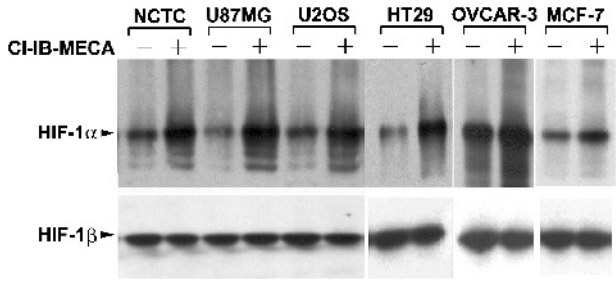

Figure 4. *A_3 receptor stimulation induces HIF-1α accumulation in various human cell lines under hypoxia. NCTC 2544 keratinocytes, and U87MG glioblastoma, U2OS osteosarcoma, HT29 colon carcinoma, OVCAR-3 ovarian carcinoma, and MCF-7 breast carcinoma cells were treated without (–) or with (+) 100 nM Cl-IB-MECA in hypoxia for 4 hours. Cellular extracts were prepared and subjected to immunoblot assay using an anti–HIF-1α monoclonal antibody. The blot was then stripped and used to determine HIF-1β expression using an anti–HIF-1β monoclonal antibody.*

MCF-7 breast carcinoma, OVCAR-3 ovarian carcinoma, U87MG glioblastoma, and U2OS osteosarcoma; Figure 4).

A_3 Receptor Mediates HIF-1α Accumulation through a Transcription-Independent and Translation-Dependent Pathway

We investigated by real-time RT-PCR experiments if transcription of HIF-1α mRNA was modulated. Activation of A_3 receptors in hypoxic melanoma cells with 10/100/1000 nM Cl-IB-MECA produced, respectively, a 1.13 ± 0.10–, 1.25 ± 0.15–, and 1.19 ± 0.13–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. To confirm this hypothesis, A375 cells were pretreated with 10 μg/ml actinomycin D (Act-D) to inhibit *de novo* gene transcription. We found that A_3 receptor stimulation was able to increase HIF-1α protein expression also in the presence of Act-D (Figure 5, *A* and *B*).

Next, we determined if A_3 receptor induces HIF-1α expression through a translation-dependent pathway. In cells exposed to 1 μM cycloheximide (CHX), 100 nM Cl-IB-MECA failed to increase HIF-1α levels within 6 hours (Figure 5, *C* and *D*).

These results suggest that A_3 receptor activation increases HIF-1α protein levels through a transcription-independent and translation-dependent pathway.

After the return of hypoxic A375 cultures to normoxia, the levels of HIF-1α protein decreased very rapidly (Figure 5*E*). 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 (Figure 5*F*). These results indicate that A_3 receptor activation may not be able to significantly prevent HIF-1α degradation in normoxic conditions.

Main Intracellular Signaling Pathways Sustained by A_3 Receptors during HIF-1α Accumulation in Hypoxia

To determine whether the MAPK pathway was required for HIF-1α protein increase induced by A_3 receptor activation, A375 cells were pretreated with U0126, which is a potent inhibitor of MEK1/2 [48], or with the inhibitor of p38 MAPK, SB202190 [49]. Cells were then exposed to 100 nM Cl-IB-MECA for 4 hours in hypoxia. As shown in Figure 6*A*, both U0126 (10 μM) and SB202190 (10 μM) were able to inhibit the Cl-IB-MECA–induced increase of HIF-1α protein expression. These results suggest that p44/p42 and

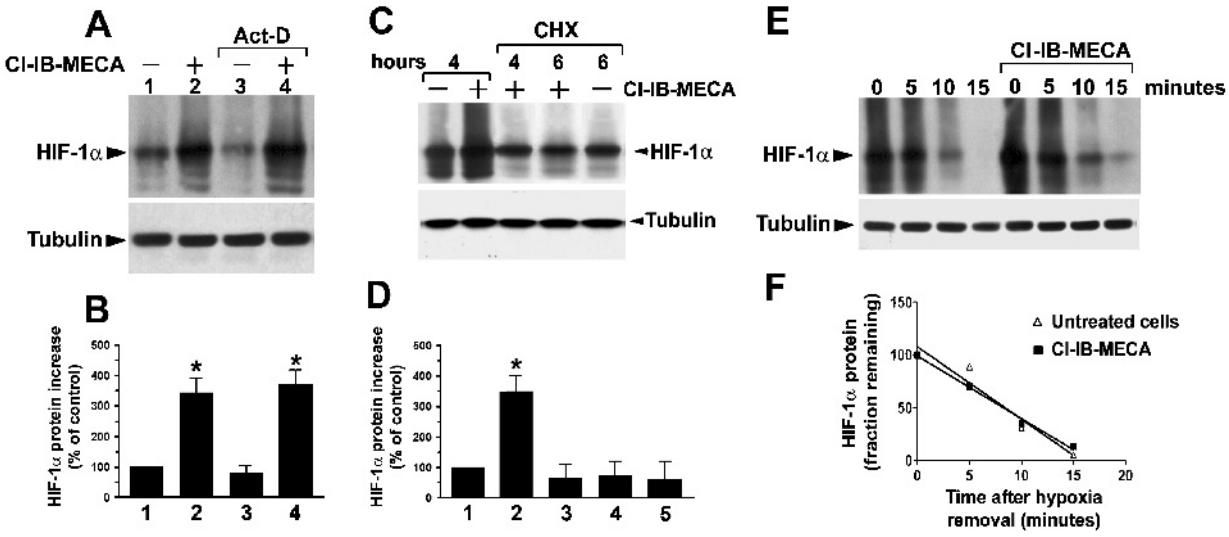

Figure 5. *A_3 receptor stimulation induces HIF-1α accumulation through a transcription-independent and transduction-dependent pathway. (A) A375 cells were pretreated with Act-D (10 μg/ml) for 30 minutes and then exposed to 4 hours of hypoxia without (–) or with (+) 100 nM Cl-IB-MECA. (B) The mean densitometry data from three independent experiments were normalized to the result obtained in cells in the absence of Cl-IB-MECA after 4 hours of hypoxia (control, lane 1). Plots are mean ± SE values (n = 3). *P < .01 compared with the control. (C) HIF-1α accumulation was induced by the exposure of A375 cells to 100 nM Cl-IB-MECA (+) for 4 or 6 hours in the absence (lanes 1 and 2) or in the presence (lanes 3 and 5) of 1 μM CHX. (D) 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 hours of hypoxia (control, lane 1). Plots are mean ± SE values (n = 3). *P < .01 compared with the control. (E) A375 cells were incubated in hypoxia in the absence or in the presence of 100 nM Cl-IB-MECA. After 4 hours, melanoma cells were exposed to normoxia and a time course of HIF-1α disappearance was performed at 0, 5, 10, and 15 minutes. (F) The mean densitometry data from three independent experiments were normalized to the result obtained at time 0 (control). The fraction of remaining HIF-1α is indicated.*

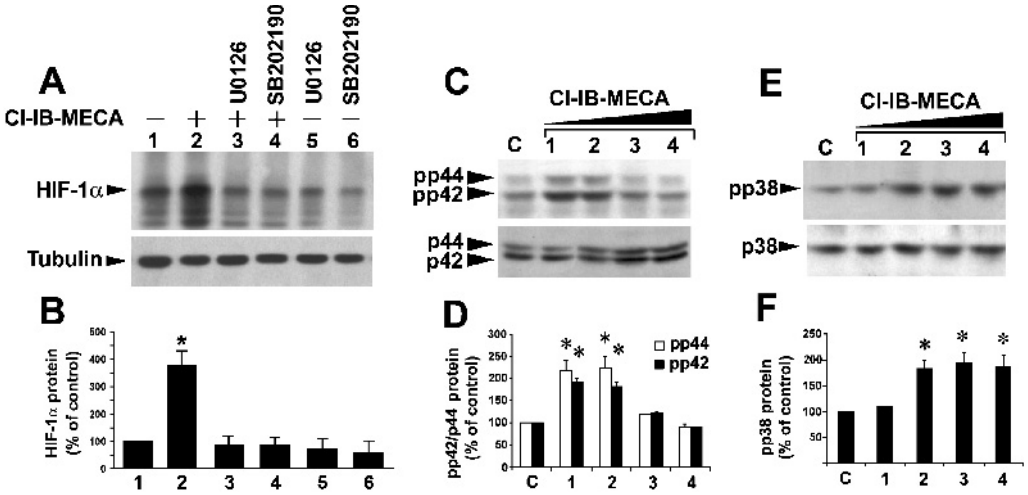

Figure 6. *Role of p38, p44, and p42 MAPKs in A_3 signalling. (A) A375 cells were pretreated with or without 10 μM U0126 or 10 μM SB202190, and then exposed to 100 nM Cl-IB-MECA (+) for 4 hours in hypoxia. (B) The mean densitometry data from three independent experiments were normalized to the results obtained in hypoxic cells in the absence of Cl-IB-MECA (lane 1). Plots are mean ± SE values (n = 3). *P < .01 compared with the control. (C) pp44 and pp42 MAPK protein levels under A_3 receptor stimulation: Cl-IB-MECA at concentrations of 0 nM (lane C), 10 nM (lane 1), 100 nM (lane 2), 500 nM (lane 3), and 1000 nM (lane 4) was added to A375 cells for 4 hours in hypoxia. (D) The mean densitometry data from three independent experiments were normalized to the results obtained in cells in the absence of Cl-IB-MECA (lane C). Phosphorylation data are shown. Plots are mean ± SE values (n = 3). *P < .01 compared with the control. (E) pp38 protein levels under A_3 receptor stimulation: Cl-IB-MECA at concentrations of 0 nM (lane C), 10 nM (lane 1), 100 nM (lane 2), 500 nM (lane 3), and 1000 nM (lane 4) nM was added for 4 hours in hypoxia to A375 cells. (F) The mean densitometry data from three independent experiments were normalized to the result obtained in cells in the absence of Cl-IB-MECA (lane C). Phosphorylation data are shown. Plots are mean ± SE values (n = 3); *P < .01 compared with the control.*

p38 MAPK activity was required for the HIF-1α expression increase induced by A_3 receptor activation. Furthermore, to confirm that p44/p42 and p38 MAPK belong to the signaling pathways utilized by A_3 receptor stimulation, we also investigated endogenous p44/p42 and p38 MAPK activation levels in response to A_3 receptor agonist treatment. p44/p42 kinase phosphorylation status was maximal following the treatment of 100 nM Cl-IB-MECA (Figure 6*D*), whereas the phosphorylation of p38 MAPK increased in a dose-dependent manner (Figure 6*F*).

Modulation of VEGF and Ang-2 Expression

To investigate whether induction of HIF-1α expression by A_3 receptor stimulation in hypoxia results in a further increase in HIF-1–regulated gene expression, the level of VEGF protein in A375 cells after exposure to the A_3 agonist, Cl-IB-MECA, was analyzed using ELISA. Treatment of cells with increasing concentrations of Cl-IB-MECA did not significantly modulate VEGF levels, when compared with VEGF levels in untreated hypoxic cells (Figure 7*A*).

On the contrary, treatment of cells with increasing concentrations of Cl-IB-MECA in hypoxia for 24 hours significantly increased Ang-2 levels. In particular, Cl-IB-MECA was able to increase Ang-2 levels up to 35% with respect to untreated hypoxic cells (Figure 7*B*).

HIF-1α Knockdown Prevented the A_3 Receptor–Induced Increase of Ang-2

To determine if the increased levels of Ang-2 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 into A375 cells 48 hours prior to exposure to Cl-IB-MECA (500 nM) for 24 hours in hypoxia. When HIF-1α protein was knocked down with siRNA (at 72 hours post–$siRNA_{HIF\text{-}1\alpha}$ transfection; Figure 7, *C* and *D*), Ang-2 protein increase induced by A_3 receptor stimulation was prevented (Figure 7*E*).

A_3 Receptor Mediates Ang-2 Accumulation in Hypoxia through a MAPK Pathway

The selective A_3 antagonist, MRE 3008F20 (10 nM), inhibited the Cl-IB-MECA–stimulated Ang-2 protein expression (Figure 7*E*). To investigate whether the MAPK pathway was involved in the expression of A_3-induced Ang-2 protein, A375 cells were cultured in hypoxia for 24 hours following the addition of the MEK1/2 inhibitor, U0126, or with the inhibitor of p38 MAPK, SB202190, 30 minutes prior to the treatment of Cl-IB-MECA. As shown in Figure 7*E*, U0126 and SB202190 (10 μM) significantly inhibited the Ang-2 protein levels induced by Cl-IB-MECA.

Discussion

This is the first report that describes the role of adenosine in modulating cellular response during hypoxia in an O_2-sensitive cell.

Hypoxia creates conditions that, on one hand, are conducive to the accumulation of extracellular adenosine and, on the other hand, stabilize HIFs, such as HIF-1α [1–4,29,30].

We demonstrate that adenosine is able to increase HIF-1α protein expression in response to hypoxia in a dose-dependent and time-dependent manner in A375 human melanoma cells, whereas HIF-1β protein levels are not affected.

We have previously demonstrated that all four adenosine receptors are expressed in human melanoma A375 cells [15]. Here, we report that A_3 receptor subtype mediates the observed adenosine effects on HIF-1α regulation in this cell line.

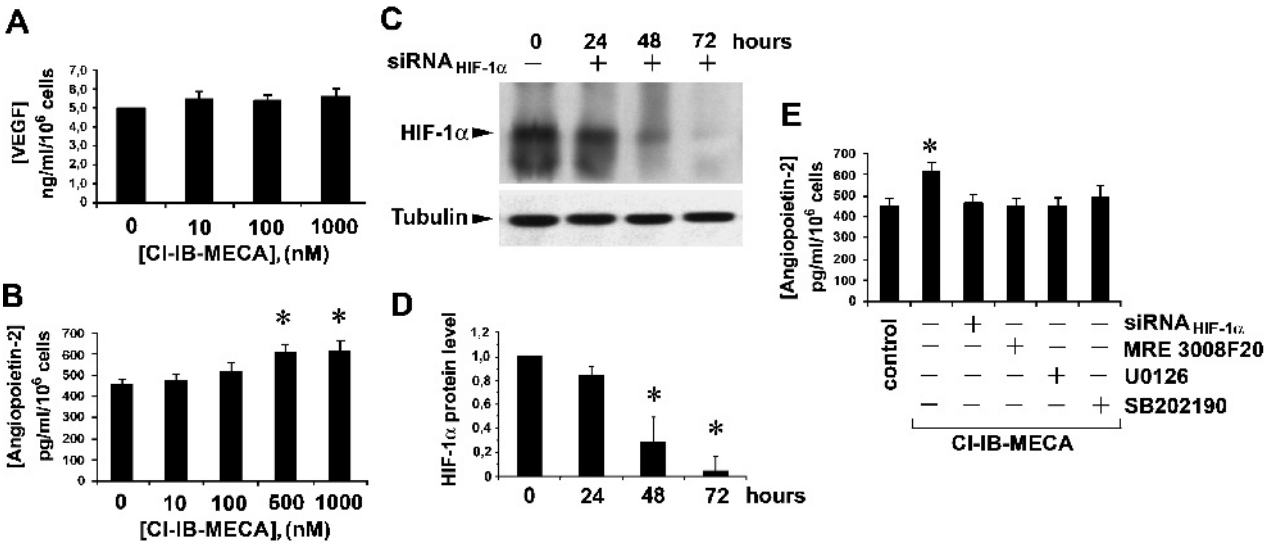

Figure 7. *Effect of A_3 adenosine receptor stimulation on VEGF and Ang-2 expression in hypoxic A375 cells. (A) VEGF release into culture media from A375 cells incubated in the absence and in the presence of increasing concentrations of Cl-IB-MECA for 24 hours in hypoxia (reported as ng/ml per 10^6 cells). (B) Ang-2 release into culture media from A375 cells incubated in the absence and in the presence of increasing concentrations of Cl-IB-MECA for 24 hours in hypoxia (reported as pg/ml per 10^6 cells). Plots are mean ± SE values (n = 5). *P < .01 compared with the control (untreated hypoxic cells). (C) HIF-1α silencing by siRNA transfection. Western blot analysis using an anti–HIF-1α antibody of protein extracts from A375 cells transfected with $siRNA_{HIF-1\alpha}$ by the RNAiFect Transfection reagent and cultured for 24, 48, and 72 hours in hypoxia. Tubulin shows equal loading protein. (D) Densitometric quantification of HIF-1α Western blot analysis. Plots are mean ± SE values (n = 5). *P < .01 compared with the control (time 0). (E) Pharmacologic analysis of A_3 receptors regulating Ang-2 secretion (reported as pg/ml per 10^6 cells). Effects of $siRNA_{HIF-1\alpha}$, 10 nM A_3 receptor antagonist MRE 3008F20, 10 μM U0126, and 10 μM SB202190 on Ang-2 secretion in the presence of 500 nM Cl-IB-MECA.*

The effects of adenosine on HIF-1α protein accumulation are not mediated by A_1, A_{2A}, or A_{2B} receptors. In support of this conclusion, DPCPX, SCH 58261, and MRE 2029F20, adenosine receptor antagonists for A_1, A_{2A}, and A_{2B} receptors, respectively, did not block the stimulatory effect of adenosine on HIF-1α protein increase.

The conclusion that the effects of adenosine on HIF-1α accumulation are mediated through A_3 receptors is supported by the observation that the stimulatory effects of this nucleoside on HIF-1α protein are mimicked by the A_3 receptor agonist, Cl-IB-MECA, and inhibited by A_3 receptor antagonists. In particular, the potencies of these drugs are in agreement with their inhibitory equilibrium binding constant (K_i) observed in binding experiments for the adenosine A_3 receptor [15]. Furthermore, the inhibition of A_3 receptor expression at the mRNA and protein levels is sufficient to block A_3 receptor–induced HIF-1α protein accumulation. Therefore, our results indicate that the cell surface A_3 adenosine receptor transduces extracellular hypoxic signals into the cell interior.

A_3 adenosine receptor stimulation had no effect on HIF-1α mRNA accumulation, as observed by real-time RT-PCR experiments. Accordingly, Act-D experiments indicate that the A_3 receptor does not regulate HIF-1α protein expression through a transcription-dependent mechanism. The lack of adenosine effect on HIF-1α at the transcriptional level is not surprising, in view of the fact that hypoxic regulation of HIF-1α is primarily determined by the stabilization of HIF-1α protein [50]. In addition, we have obtained evidence that A_3 adenosine receptors modulate HIF-1α protein levels through a translation-dependent pathway while not affecting HIF-1α oxygen-dependent degradation. Our data suggest that A_3 adenosine receptor activation does not increase the half-life of HIF-1α protein whereas it may increase the rate of HIF-1α protein synthesis, in a manner similar to the effect of various growth factors [51,52]. Nevertheless, we cannot exclude the possibility that A_3 adenosine receptor regulates, in hypoxia, the translation of a protein, which inhibits HIF-1α degradation.

Phosphorylation and dephosphorylation activities have been suggested to be critical in the signaling pathway leading to HIF-1 activation. Several reports demonstrated that hypoxia induces the phosphorylation of HIF-1α by p44/p42 and p38 MAPKs, which increases both HIF-1α nuclear localization and transcriptional activity [52,53]. In addition, adenosine, through A_{2A} receptors, has been shown to directly enhance MAPK activity in A375 human melanoma cells [10], but also in nonhuman cell lines stably transfected with the human A_3 receptor [54–56]. In the present study, we observed that p44/p42 and p38 MAPKs are necessary to increase HIF-1α levels, but also that these kinases are included in the molecular signaling pathways generated by A_3 receptor stimulation. Based on these data, we suggest that adenosine, through A_3 receptors, is able to increase the levels of HIF-1α through p44/p42 and p38 MAPK pathways. In our previous study on A375 melanoma cells, A_3 adenosine receptor stimulation decreased MAPK activity through the inhibition of Raf by a PI3K–Akt pathway [57]. However, the experimental conditions were quite different, being those experiments performed in normoxia and with high concentrations (micromolar) of the A_3 receptor agonist. Now, the concentration of the agonist is nanomolar and the cells are cultured in hypoxic conditions. These data may explain the

biphasic effect of Cl-IB-MECA on p42/p44 phosphorylation levels. Actually, further studies are needed to evaluate the role of p44/p42 and p38 MAPK in reduced turnover, increased life, and transduction of HIF-1α proteins in hypoxia.

Adenosine A_3 receptors are expressed in many different cell types [7]. To determine whether their effect on HIF-1α expression was not limited to the A375 cells, we assessed the ability of Cl-IB-MECA to induce HIF-1α levels in a variety of cell lines expressing A_3 adenosine receptors [7]. We detected a significant increase in HIF-1α protein expression through A_3 receptors in human keratinocytes (NCTC 2544) and in different human tumor cells (HT29 colon carcinoma, MCF-7 breast carcinoma, OVCAR-3 ovarian carcinoma, U87MG glioblastoma, and U2OS osteosarcoma).

Finally, we decided to look further into the physiological relevance of the A_3 receptor stimulation in hypoxia. HIF-1α is overexpressed in tumors and is involved in key aspects of tumor biology, such as invasion, altered energy metabolism, and angiogenesis [26]. VEGF and Ang-2 are key elements controlling angiogenesis and are increased in tumors [58]. Adenosine also plays a role in the promotion of angiogenesis [20,21,59]. The regulation of expression of the angiogenic VEGF through adenosine receptors has been demonstrated in several cell types [22–25]. However, in our experimental model, there was no effect on VEGF protein levels following the modulation of HIF-1α induced by A_3 receptor stimulation, indicating that these cells do not respond to A_3 receptor stimulation with an increase in VEGF and/or that this degree of stimulation is not enough to alter VEGF protein levels. In particular, the effects of adenosine on VEGF expression demonstrated in previous works [22–24] are mediated by A_{2B} adenosine receptors. In addition, VEGF is coregulated not only by the HIF system, but also by the AP-1 family of transcription factors possibly mediated by calcium ions [60]. On the contrary, we demonstrated for the first time that Ang-2 protein expression was increased by A_3 adenosine receptor stimulation through a MEK–HIF-1α signaling cascade. Furthermore, these data confirm that activation of A_3 receptors upregulates the expression of Ang-2, as previously reported [23].

It is recognized that the inhibition of HIF-1 activity represents a novel therapeutic approach to cancer therapy, especially in combination with angiogenesis inhibitors, which would further increase intratumoral hypoxia and thus provide an even greater therapeutic window for use of an HIF inhibitor [57,61]. Recent studies indicate that pharmacologic inhibition of HIF-1α, and particularly of HIF-regulated genes important for cancer cell survival, may be more advantageous than HIF gene inactivation therapeutic approaches [62,63]. Recently, we have demonstrated the mechanisms of the antiproliferative action of A_3 receptor stimulation in A375 normoxic melanoma cells [57]. Now, given the ability of A_3 adenosine receptor antagonists to block HIF-1α and Ang-2 protein expression accumulation in hypoxia, our data may indicate a new approach for the treatment of cancer, based on the cooperation between hypoxic and adenosine signals, that ultimately may lead to the increase in HIF-1–mediated effects in cancer cells.

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