

# Caffeine Inhibits Adenosine-Induced Accumulation of Hypoxia-Inducible Factor-1 $\alpha$ , Vascular Endothelial Growth Factor, and Interleukin-8 Expression in Hypoxic Human Colon Cancer Cells

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## ABSTRACT

Frequent coffee consumption has been associated with a reduced risk of colorectal cancer in a number of case-control studies. Coffee is a leading source of methylxanthines, such as caffeine. The induction of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) is an essential feature of tumor angiogenesis, and the hypoxia-inducible factor-1 (HIF-1) transcription factor is known to be a key regulator of this process. In this study, we investigated the effects of caffeine on HIF-1 protein accumulation and on VEGF and IL-8 expression in the human colon cancer cell line HT29 under hypoxic conditions. Our results show that caffeine significantly inhibits adenosine-induced HIF-1 $\alpha$  protein accumulation in cancer cells. We show that HIF-1 $\alpha$  and VEGF are increased through A<sub>3</sub> adenosine receptor stimulation, whereas the effects on IL-8 are mediated via the A<sub>2B</sub> subtype. Pretreatment of cells with caffeine signif-

icantly reduces adenosine-induced VEGF promoter activity and VEGF and IL-8 expression. The mechanism of caffeine seems to involve the inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and Akt, leading to a marked decrease in adenosine-induced HIF-1 $\alpha$  accumulation, VEGF transcriptional activation, and VEGF and IL-8 protein accumulation. From a functional perspective, we observe that caffeine also significantly inhibits the A<sub>3</sub> receptor-stimulated cell migration of colon cancer cells. Conditioned media prepared from colon cells treated with an adenosine analog increased human umbilical vein endothelial cell migration. These data provide evidence that adenosine could modulate the migration of colon cancer cells by an HIF-1 $\alpha$ /VEGF/IL-8-dependent mechanism and that caffeine has the potential to inhibit colon cancer cell growth.

Coffee and tea are the most commonly consumed beverages in the world (Fredholm, 1999). Results of epidemiological studies have not resolved whether coffee consumption is re-

lated to colorectal cancer risk. A report by the World Cancer Research Fund concluded that the available evidence was not sufficient to draw any firm conclusions about a decreased risk of colorectal cancer associated with coffee consumption (World Cancer Research Fund/American Institute for Cancer Research, 1997). However, some researchers contend that a link between high consumption of coffee and a low incidence

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**ABBREVIATIONS:** IL-8, interleukin-8; Cl-IB-MECA, *N*<sup>6</sup>(3-iodobenzyl)2-chloroadenosine-5'-*N*-methyluronamide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; 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; MRE 3008F20, 5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3e]1,2,4-triazolo[1,5c] pyrimidine; HIF-1, hypoxia-inducible factor-1; HUVEC, human umbilical vein endothelial cell; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; siRNA, small interfering RNA; siRNA<sub>A2B</sub>, small interfering RNA that targets A<sub>2B</sub> receptor mRNA; siRNA<sub>A3</sub>, small interfering RNA that targets A<sub>3</sub> receptor mRNA; VEGF, vascular endothelial growth factor; ZM 241385, 4-[2-[7-amino-2-[furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol; ERK1/2, extracellular signal-regulated kinase 1/2; Ab, antibody; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; PBS, phosphate-buffered saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NECA, 5'-*N*-ethylcarboxamidoadenosine; DMSO, dimethyl sulfoxide; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; SH5, D-3-deoxy-2-*O*-methyl-*myo* inositol 1-[(*R*)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]; B64, 4-[3-(2-furan-2-yl-8-alkyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl)ureido]benzenesulfonic acid derivative.

of colorectal cancer has been firmly established (Ekbom, 1999; Woolcott et al., 2002).

Coffee is a leading source of methylxanthines, such as caffeine. A cup of coffee contains approximately 100 mg of caffeine (Fredholm, 1999); thus, caffeine can be found in micromolar concentrations in the human circulation as a result of dietary intake or pharmacological use.

Most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Clinical and experimental evidence suggests that tumor hypoxia is associated with a more aggressive phenotype (Hockel and Vaupel, 2001). 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 (Melillo, 2004).

Interleukin-8 (IL-8), originally discovered as a chemotactic factor for leukocytes, has been shown recently to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic, and motogenic factor (Xie, 2001). Although it is constitutively detected in human cancer tissues and established cell lines, IL-8 expression is regulated by various tumor microenvironment factors, such as hypoxia, acidosis, nitric oxide, and cell density. Furthermore, hypoxia is a potent stimulator of vascular endothelial growth factor (VEGF) expression, a key proangiogenic factor, and this induction is believed to be mediated primarily through hypoxia-inducible factor-1 (HIF-1) (Maxwell et al., 1997).

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-1 $\alpha$  subunit and a constitutively expressed HIF-1 $\beta$  subunit. A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis (Giaccia et al., 2003; Semenza, 2003). Immunohistochemical analyses have shown that HIF-1 $\alpha$  is present in higher levels in human tumors than in normal tissues (Zhong et al., 1999). HIF-1 is a potent activator of angiogenesis and invasion through its up-regulation of target genes critical for these functions (Carmeliet et al., 1998; Kung et al., 2000; Ratcliffe et al., 2000). Such genes share the presence of hypoxia response elements, which contain binding sites for HIF-1 (Semenza, 2003). Therefore, because HIF-1 $\alpha$  expression and activity seem central to tumor growth and progression, HIF-1 inhibition becomes an appropriate anticancer target (Maxwell et al., 1997; Kung et al., 2000; Giaccia et al., 2003; Semenza, 2003).

It is interesting that VEGF is overexpressed not only in advanced colon cancers but also in premalignant colonic adenomas (Wong, 1999). The factors that may contribute to this enhanced VEGF expression are not defined fully.

Although the mechanism of the possible protective effect of coffee or its products is unclear, potential protective effects could include antagonistic effects of the adenosine receptors, named A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> (Fredholm et al., 2001). These receptors belong to the P1 subclass of the purinergic family of G protein-coupled receptors, which are activated by adenosine. Adenosine is an ubiquitous autacoid that accumulates to high levels in hypoxic tissues as a result of ATP breakdown (Fredholm et al., 2001). This nucleoside has been involved in the regulation of the cellular response to hypoxia. It is recognized that significant levels of adenosine are present in the extracellular fluid of solid tumors (Fredholm et al., 2001), suggesting a role for this autacoid in tumor growth. In par-

ticular, the A<sub>3</sub> subtype is highly expressed in tumor cells (Gessi et al., 2001, 2002; Merighi et al., 2001) and is able to significantly up-regulate the expression of HIF-1 in hypoxic tumors (Merighi et al., 2005a, 2006), suggesting that A<sub>3</sub> receptor overexpression may be a good candidate as a tumor cell marker (Gessi et al., 2004; Madi et al., 2004). Adenosine also plays a role in the promotion of angiogenesis (Montesinos et al., 2004). Regulation of expression of VEGF through adenosine receptors has been demonstrated in different cell types (Feoktistov et al., 2002, 2003, 2004; Leibovich et al., 2002). The aim of this study is to determine whether caffeine may regulate HIF-1 $\alpha$ , VEGF, and IL-8 in colon cancer cells during hypoxia.

## Materials and Methods

**Cell Lines, Reagents, and Antibodies.** HT29 human tumor colon cells were obtained from American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells (HUVEC), tissue culture media and growth supplements were obtained from Lonza Bioscience (Bergamo, Italy). Antiadenosine A<sub>2B</sub> and antiadenosine A<sub>3</sub> receptor antibodies (pAb) were from Alpha Diagnostic (Milano, Italy). Human anti-HIF-1 $\alpha$  and human anti-HIF1 $\beta$  antibodies (mAb) were obtained from BD Transduction Laboratories (Milan, Italy). Anti-human vascular endothelial growth factor (VEGF) antibody was developed in goat using recombinant human VEGF<sub>165</sub> as immunogen. U0126 (inhibitor of MEK-1 and MEK-2), SB202190 (inhibitor of p38 MAP kinase), human anti-ACTIVEMAPK, and human anti-ERK1/2 antibodies (pAb) were from Promega (Milan, Italy). SH5 (inhibitor of Akt) was from Vinci-Biochem (Florence, Italy). Human phospho-p38 and human p38 MAP kinase antibodies were from Cell Signaling Technology (Milan, Italy). P11w, a firefly luciferase reporter plasmid, comprising the 5'-flanking -985 to -939 base pairs of the human VEGF gene that include an HIF-1-binding site, and p11m, the mutated version of p11w containing a nonfunctional HIF-1-binding site (Forsythe et al., 1996), were obtained from the American Type Culture Collection. BriteLite Ultra-High Sensitivity Luminescence Reporter Gene Assay System kit was obtained from PerkinElmer Life and Analytical Sciences (Milan, Italy). Eugene 6 transfection reagent was purchased from Roche Molecular Biochemicals (Milan, Italy). ZM 241385 and [<sup>3</sup>H]ZM 241385 (specific activity, 17 Ci/mmol) were obtained from Tocris Cookson Ltd. (Bristol, UK). MRE 2029F20, MRE 3008F20, and B64 were synthesized by Dr. Pier Giovanni Baraldi (Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy). [<sup>3</sup>H]MRE 2029F20 (specific activity, 123 Ci/mmol) and [<sup>3</sup>H]MRE 3008F20 (specific activity, 67 Ci/mmol) were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). [<sup>3</sup>H]DPCPX (specific activity, 120 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Adenosine A<sub>2B</sub> and A<sub>3</sub> receptor small interfering RNAs (siRNAs) were from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise noted, all other chemicals were purchased from Sigma (Milan, Italy).

**Cell Culture.** HT29 human tumor colon cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and L-glutamine (2 mM) at 37°C in 5% CO<sub>2</sub>/95% air. HUVEC used in this study were from passages 2 to 7.

**Establishment of Hypoxic Culture Condition.** For hypoxic conditions, cells were placed for the indicated times in a modular incubator chamber and flushed with a gas mixture containing 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub> (MiniGalaxy, RSBiotech, Irvine, Scotland). Maintenance of the desired O<sub>2</sub> concentration was constantly monitored during incubation using a microprocessor-based oxygen controller.

**Caffeine Treatment of Cancer Cells.** Exponentially growing cells (70–80% confluence) in complete medium were pretreated for

1 h with different concentrations of caffeine, followed by continual incubation in normal culturing conditions or exposure to hypoxia (1% O<sub>2</sub>) for indicated time intervals according to the purpose of the experiment.

**Membrane Preparation.** For membrane preparation, the culture medium was removed. The cells were washed with PBS and scraped off of T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl and 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland), and the cell suspension was centrifuged for 10 min at 1000g. The supernatant was then centrifuged again for 30 min at 100,000g, and the membrane pellet was frozen at  $-80^{\circ}\text{C}$  until the use in competition binding experiments.

**Competition Binding Experiments at A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> Adenosine Receptors.** Binding of [<sup>3</sup>H]DPCPX to A<sub>1</sub> receptors expressed in HT29 cells was performed for 120 min at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 nM [<sup>3</sup>H]DPCPX, diluted membranes (100  $\mu\text{g}$  of protein per assay), and caffeine. Nonspecific binding was determined in the presence of 1  $\mu\text{M}$  DPCPX and was always  $\leq 10\%$  of the total binding. Binding of 1 nM [<sup>3</sup>H]ZM 241385 to human A<sub>2A</sub> expressed in HT29 membranes (100  $\mu\text{g}$  of protein per assay) was performed using 50 mM Tris-HCl buffer, 10 mM MgCl<sub>2</sub> pH 7.4, and different concentrations of caffeine for an incubation time of 60 min at 4°C. Nonspecific binding was determined in the presence of 1  $\mu\text{M}$  ZM 241385 and was approximately 20% of total binding. Competition experiments to human A<sub>2B</sub> expressed in HT29 membranes were performed using 3 nM [<sup>3</sup>H]MRE 2029F20 for an incubation time of 60 min at 4°C. Nonspecific binding was defined as binding in the presence of 1  $\mu\text{M}$  MRE 2029F20 and was 25% of total binding. Binding of [<sup>3</sup>H]MRE 3008F20 to human A<sub>3</sub> expressed in HT29 membranes was carried out in 50 mM Tris-HCl buffer, 10 mM MgCl<sub>2</sub>, and 1 mM EDTA, pH 7.4, containing 1 nM [<sup>3</sup>H]MRE 3008F20, membranes (100  $\mu\text{g}$  of protein per assay), and caffeine for 120 min at 4°C. Nonspecific binding was defined as binding in the presence of 1  $\mu\text{M}$  MRE 3008F20 and was approximately 25 to 30% of total binding. Eight different concentrations of caffeine were studied.

**Measurement of cAMP Levels.** HT29 cells in exponential growth were exposed to drugs for 2 h. After the incubation, the HT29 cells were collected, washed three times in ice-cold PBS, lysed, and centrifuged. The supernatants were assayed for cAMP determination using an R&D cAMP assay kit following the manufacturer's instructions (Parameter kit; R&D Systems, Minneapolis, MN).

**Conditioned Medium.** To obtain conditioned medium from N<sup>6</sup>(3-iodobenzyl)2-chloroadenosine-5'-N-methyluronamide (Cl-IB-MECA)-treated HT29 human tumor colon cells, we plated 10<sup>6</sup> HT29 cells in a 10-cm diameter plate containing RPMI 1640 medium with 10% fetal bovine serum. After 24 h, the medium of these cells was replaced with fresh growth medium containing Cl-IB-MECA (0 or 100 nM). The plates were then incubated under normoxic or hypoxic conditions. After 1 day of incubation, conditioned medium was removed and centrifuged at 4000g for 20 min at 4°C through an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA) to remove any trace of Cl-IB-MECA. The molecular mass cutoff of the filters was 5 kDa, and the molecular mass of Cl-IB-MECA is 0.544 kDa. The flow-through containing excess Cl-IB-MECA was discarded, and the retentate was collected. Furthermore, to exclude that Cl-IB-MECA itself may have an inhibitory effect on the migration assay, we treated HUVECs directly with Cl-IB-MECA 100 nM, which was insufficient to modulate HUVEC migration. The final filter retentate was concentrated 40-fold for use in the migration and proliferation assays.

**JAM Test.** This assay measures cell death by quantifying the amount of fragmented DNA, as described previously (Merighi et al., 2005b). Target cells were labeled with 1  $\mu\text{Ci}/\text{ml}$  [<sup>3</sup>H]thymidine for 20 h in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), L-glutamine (2 mM). The cells were then washed and treated with new unlabeled medium containing caffeine for 24 h. At the end of the incubation period, the

cells were trypsinized, dispensed in 4 wells of a 96-well plate, and filtered through Whatman GF/C glass-fiber filters using a Micro-Mate 196 cell harvester (PerkinElmer). The filter-bound radioactivity was counted on a TopCount Microplate Scintillation Counter (efficiency 57%) with MicroScint-20 (both from PerkinElmer Life and Analytical Sciences). The amount of apoptotic and necrotic cells, measured as the loss of radioactivity associated with the loss of fragmented and degraded DNA, was detected by filtration and subsequent washing with a MicroMate 196 cell harvester followed by quantification with a TopCount Microplate Scintillation Counter. The percentage of cell death is expressed as  $100 \times (\text{dpm}_{(\text{U})} - \text{dpm}_{(\text{T})}) / \text{dpm}_{(\text{U})}$ , where  $\text{dpm}_{(\text{U})}$  is the radioactivity of untreated cells, and  $\text{dpm}_{(\text{T})}$  is the radioactivity of treated cells (Merighi et al., 2005b).

**MTS Assay.** The MTS assay was performed to determine colon cell viability and proliferation according to the manufacturer's protocol from the CellTiter 96 Aqueous One Solution (Promega) cell proliferation assay, as described previously (Merighi et al., 2005b). Cells (10<sup>5</sup>) were plated in 24-multiwell plates; 500  $\mu\text{l}$  of complete medium was added to each well with different concentrations of caffeine. The cells were then incubated for 24 h. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 570 nm. For each experiment, four individual wells of each drug concentration were prepared. Each experiment was repeated three times.

**Migration Assay.** Cell migration was performed with the Transwell system (Chemicon International, Temecula, CA), which allows cells to migrate through 8- $\mu\text{m}$  pore size polycarbonate membrane. In brief, cells were trypsinized, washed, and resuspended in serum-free Dulbecco's modified Eagle's medium ( $5 \times 10^5$  cells/ml). This suspension (300  $\mu\text{l}$ ) was added to the upper chamber of Transwells. The lower chamber was filled with 500  $\mu\text{l}$  of conditioned medium. After the incubation (6–24 h), filters were removed, and cells remaining on the upper surface of the membrane (i.e., that had not migrated through the filter) were removed with a cotton swab. Then, membranes were washed with PBS, and cells present beneath the membrane were fixed with ice-cold methanol for 15 min and stained with the Cell Stain Solution (QCM Colorimetric Cell Migration Assay; Chemicon International). The stained insert was transferred to a well containing the extraction buffer. The dye mixture was transferred to a 96-well microtiter plate suitable for colorimetric measurement. Analysis was performed on three wells for each condition, and each experiment was repeated three times.

**Western Blot Analysis.** Whole-cell lysates, prepared as described previously (Merighi et al., 2005b), were resolved on a 10% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as described previously (Merighi et al., 2005a) with anti-HIF-1 $\alpha$  (1:250 dilution) and anti-HIF-1 $\beta$  antibodies (1:1000 dilution) in 5% nonfat dry milk in PBS/0.1% Tween 20 overnight at 4°C. Aliquots of total protein sample (50  $\mu\text{g}$ ) 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 phosphorylated Akt (Ser473) (1:1000 dilution). The protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Membranes 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 (GE Healthcare). The membranes were then stripped and reprobed with antitubulin antibodies (1:250) to ensure equal protein loading.

**Densitometry Analysis.** The intensity of each band in immunoblot assay was quantified using molecular analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA). Mean densitometry data from independent experiments were normalized to results in cells in the control. The data were presented as the mean  $\pm$  S.E. and were analyzed by the Student's test.



**Treatment of Cells with siRNA.** HT29 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 RNAiFect Transfection Kit (QIAGEN, Valencia, CA). Cells were cultured in complete media, and at 48 h, total proteins were isolated for Western blot analysis of A<sub>2B</sub> and A<sub>3</sub> receptor protein. A nonspecific random control ribonucleotide sense strand (5'-ACU CUA UCU GCA CGC UGA CdTdT-3') and antisense strand (5'-dTdT UGA GAU AGA CGU GCG ACU G-3') were used under identical conditions (Merighi et al., 2005b).

**Enzyme-Linked Immunosorbent Assay.** The levels of VEGF and IL-8 protein secreted by the cells in the medium were determined by a VEGF and an IL-8 enzyme-linked immunosorbent assay kit (R&D Systems). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of adenosine analogs in hypoxia. The medium was collected, and VEGF and IL-8 protein concentrations were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions. The results were normalized to the number of cells per plate. The data were presented as mean  $\pm$  S.D. from three independent experiments.

**Transient Transfection and Luciferase Reporter Assays.** HT29 human tumor colon 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  $\mu$ l of Fugene 6 per well. The cells were then treated with drugs or the solvent vehicle only, then incubated under hypoxic (1% O<sub>2</sub>) or normoxic conditions. The cells were then prepared for the luciferase-reporter assay according to the manufacturer's instructions. In brief, the cells were lysed at ambient temperature for 2 min with 200  $\mu$ l of 1 $\times$  lysis buffer. The extracts were assayed for plasmids (p11w and p11m) and control (*Renilla reniformis*) luciferase activities with a PerkinElmer Life and Analytical Sciences luminometer. Samples were normalized for transfection efficiency based on the *R. reniformis* luciferase activity.

**Statistical Analysis.** Competition binding experiments were analyzed with LIGAND (Merighi et al., 2001), which performs weighted, nonlinear, least-squares curve-fitting program. All values in the figures and text are expressed as mean  $\pm$  S.E. of *n* observations (with *n*  $\geq$  3). Data sets were examined by analysis of variance and Dunnett's test (when required). A *P* value less than 0.05 was considered statistically significant.

## Results

**Caffeine Inhibits Adenosine-Induced HIF-1 $\alpha$  Protein Accumulation in Human Colon Cancer Cells.** HIF-1 $\alpha$  protein is undetectable in human HT29 colon cancer cells cultured under normoxic conditions, whereas it is present in hypoxia (Fig. 1A). Adenosine (10 and 100  $\mu$ M) is able to increase HIF-1 $\alpha$  protein accumulation in HT29 hypoxic colon cancer cells (Fig. 1A). The presence of adenosine receptors was recently investigated in HT29 cells, which express all four adenosine receptor subtypes. In particular, A<sub>1</sub> receptors are present with 32  $\pm$  4 fmol/mg of protein, A<sub>2A</sub> receptors with 49  $\pm$  4 fmol/mg of protein, A<sub>2B</sub> receptors with 52  $\pm$  4 fmol/mg of protein, and A<sub>3</sub> receptors with 257  $\pm$  22 fmol/mg of protein (Gessi et al., 2007). To evaluate whether A<sub>3</sub> receptors may have a functional role in HIF-1 $\alpha$  protein expression under hypoxic conditions, we tested the effect of increasing concentrations (10–1000 nM) of the high-affinity A<sub>3</sub> receptor agonist Cl-IB-MECA (Table 1) (Merighi et al., 2005b). A<sub>3</sub> adenosine receptor stimulation promoted HIF-1 $\alpha$  protein accumulation under hypoxic conditions, whereas it did not modify HIF-1 $\beta$  expression in normoxia or in hypoxia (Fig.

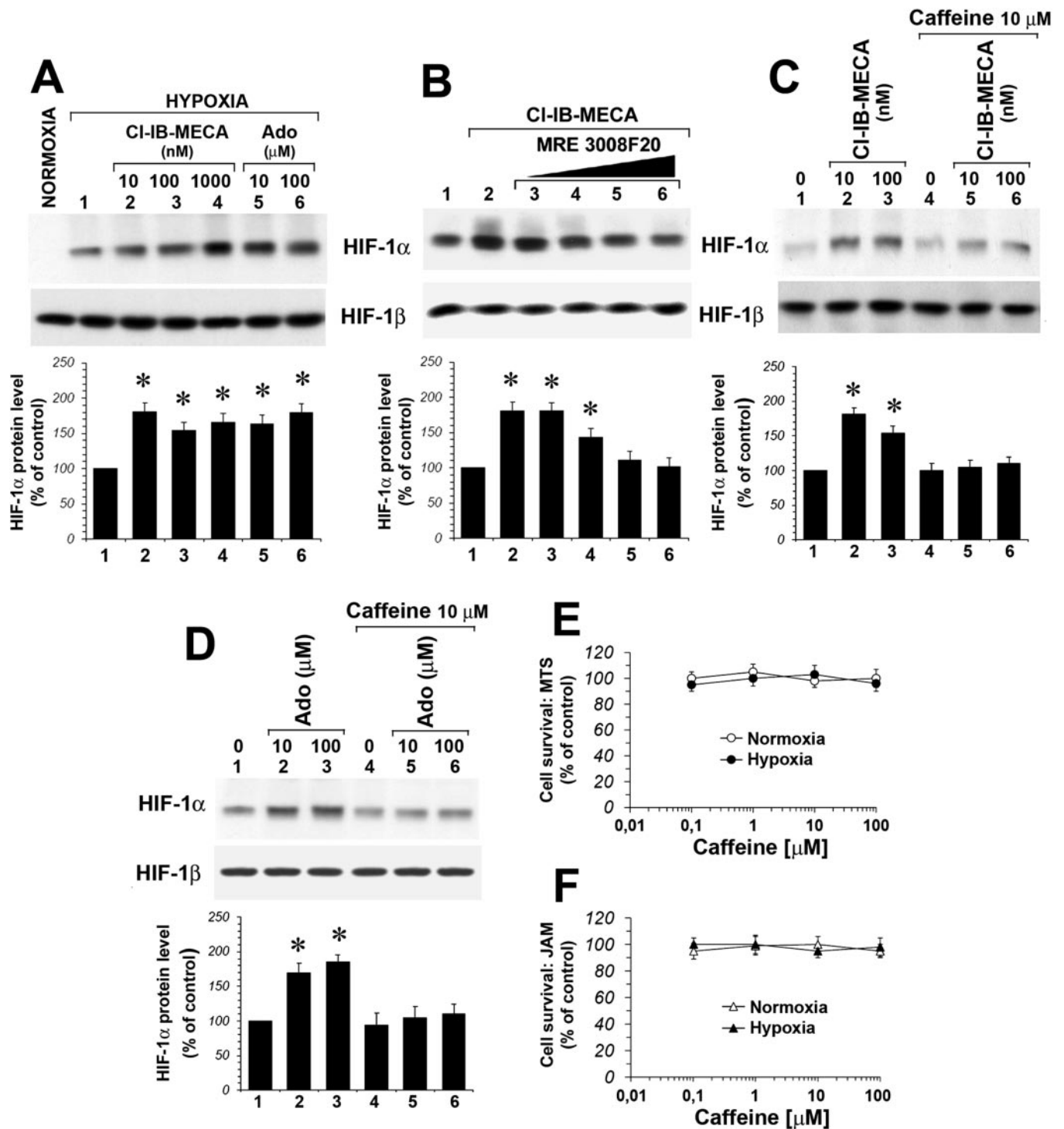
1A). To confirm that A<sub>3</sub> receptors have a functional role in HIF-1 $\alpha$  protein expression under hypoxic conditions, we tested the effect of the high-affinity and selective A<sub>3</sub> receptor antagonist MRE 3008F20 (Table 1) (Varani et al., 2000). MRE 3008F20 (0.1–10 nM) is able to decrease the induction of HIF-1 $\alpha$  expression under hypoxic conditions obtained through Cl-IB-MECA 10 nM (Fig. 1B). These results indicate that adenosine increases HIF-1 $\alpha$  protein expression via A<sub>3</sub> receptors. We next asked whether caffeine, an adenosine receptor antagonist (Fredholm et al., 1999), inhibits adenosine-induced HIF-1 $\alpha$  protein expression in hypoxia. In HT29 cells, 10  $\mu$ M caffeine was able to inhibit HIF-1 $\alpha$  protein accumulation induced by 10 to 100 nM Cl-IB-MECA (Fig. 1C). Furthermore, we observed that pretreatment of HT29 cells with 10  $\mu$ M caffeine abrogated 10 and 100  $\mu$ M adenosine-induced HIF-1 $\alpha$  protein accumulation (Fig. 1D). To rule out the possibility of a cytotoxic effect on HIF-1 $\alpha$  protein suppression by caffeine, cell viability assay using MTS was done. No obvious changes in cell viability were observed in HT29 cells after being challenged with different concentrations of caffeine (0.1–100  $\mu$ M) under both normoxic and hypoxic conditions for 24 h (Fig. 1E), indicating that the inhibition of HIF-1 $\alpha$  protein expression by caffeine was not ascribed to nonspecific tumor cell toxicity. To confirm these results, we analyzed the effect of caffeine on cell survival by the JAM test. HT29 cells, previously labeled with [<sup>3</sup>H]thymidine, were treated for 24 h with increasing concentrations of caffeine (0.1–100  $\mu$ M). Caffeine did not induce cell death, as shown in Fig. 1F.

**Caffeine Inhibits Adenosine-Induced Phosphorylation of Akt, ERK1/2, and p38 MAPK.** HT29 cells were cultured in the absence and in the presence of adenosine analogs for 0.5 to 4 h in hypoxia. We found that exposure to the A<sub>3</sub> receptor agonist Cl-IB-MECA (1–100 nM) and to the nonselective adenosine analog NECA (0.1–1  $\mu$ M) (Table 1) resulted in a sustained increase in the phosphorylated p38 and in a transient increase in Akt and ERK1/2 phosphorylation levels in colon cells (Fig. 2A). We observed that the phosphorylation of p38 kinases occurs at early time points after A<sub>3</sub> receptor activation (Fig. 2A).

Furthermore, 10  $\mu$ M caffeine was able to block the increase in the phosphorylation of p38 kinase mediated by A<sub>3</sub> receptor stimulation in hypoxic HT29 cells (Fig. 2B). Similar results are reported for Akt and ERK1/2 phosphorylation in HT29 colon cancer cells (Fig. 2B). These data suggest that caffeine acts as an adenosine receptor antagonist.

**The Site of Action of Caffeine.** To investigate whether caffeine interacts with signaling molecules downstream of adenosine receptors such as Akt, mitogen-activated protein kinases, or p38, we treated HT29 cells with caffeine (1–10  $\mu$ M) for 4 h in hypoxia, and then we evaluated the effects of caffeine treatment on the kinases under study. Figure 3A shows that caffeine, at these concentrations, did not interact with the signaling molecules investigated because the phosphorylation levels of Akt, ERK1/2, and p38 were unchanged after caffeine treatment. Furthermore, we demonstrated that SH5, an Akt inhibitor, SB202190, an inhibitor of p38 MAPK, and U0126, which is a potent inhibitor of MEK1/2, are selective at a concentration of 10  $\mu$ M, as shown in Fig. 3A.

To consider whether caffeine-dependent alterations in cAMP levels could be influencing the results obtained, we evaluated potential cAMP modulations in colon cells treated



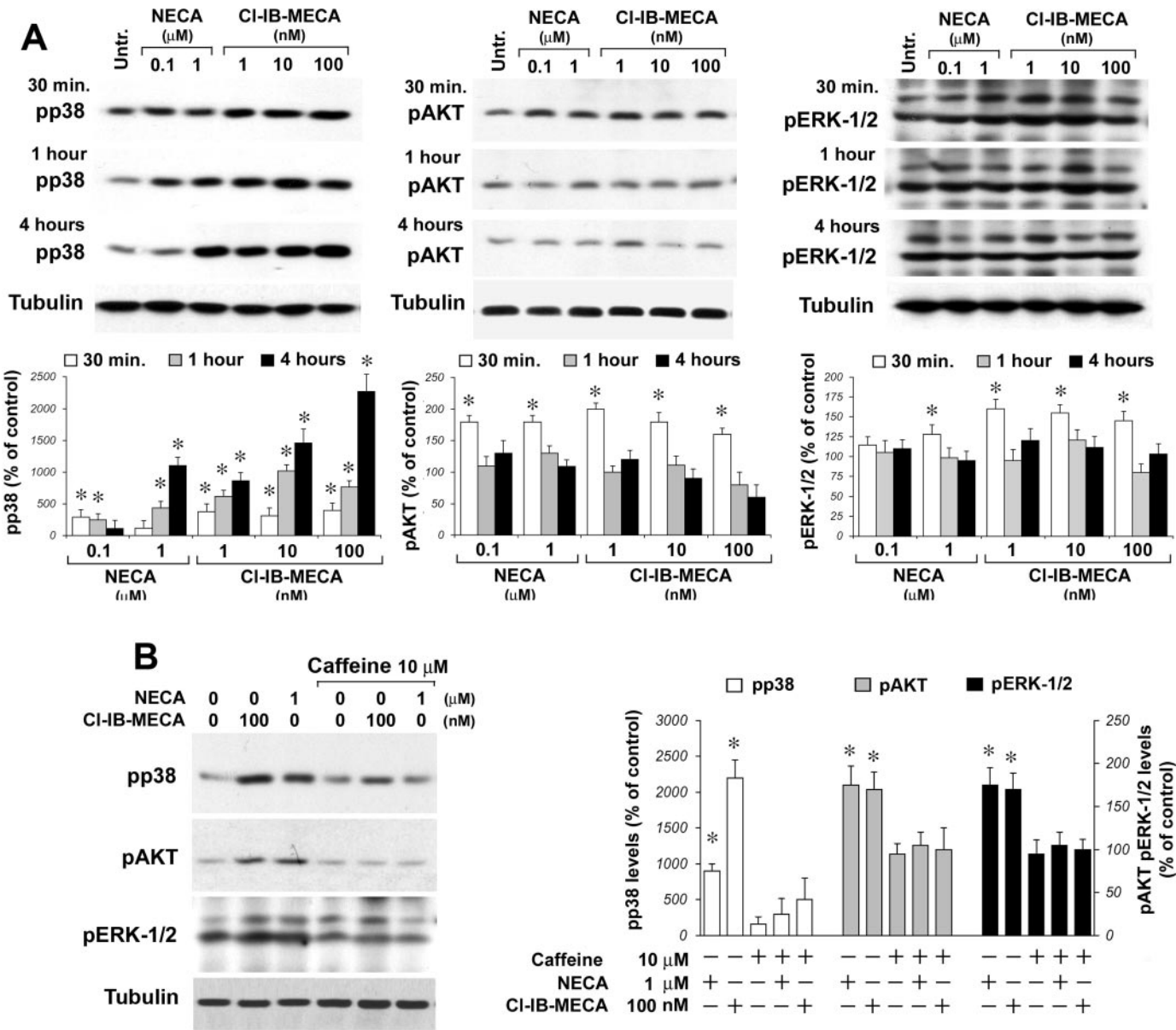
**Fig. 1.** Modulation of HIF-1 $\alpha$  expression by adenosine. **A**, Western blot analysis for HIF-1 $\alpha$  and HIF-1 $\beta$  levels of 35  $\mu$ g of total protein lysates from HT29 cells treated in normoxia or in hypoxia (1% O<sub>2</sub>, 4 h) without or with the selective A<sub>3</sub> agonist CI-IB-MECA 10, 100, and 1000 nM, and adenosine 10 and 100  $\mu$ M. **B**, effect of the selective A<sub>3</sub> antagonist MRE 3008F20. HT29 cells were treated in hypoxia (1% O<sub>2</sub>, 4 h) without (lane 1) or with CI-IB-MECA 10 nM (lanes 2–6) and MRE 3008F20 0.1 nM (lane 3), 1 nM (lane 4), 3 nM (lane 5), and 10 nM (lane 6). **C**, effect of caffeine on HIF-1 $\alpha$  expression induced by CI-IB-MECA. Western blot analysis for HIF-1 $\alpha$  and HIF-1 $\beta$  levels. HT29 cells were treated in hypoxia (1% O<sub>2</sub>, 4 h) without (lane 1) or with 10 nM CI-IB-MECA (lanes 2 and 5), 100 nM CI-IB-MECA (lanes 3 and 6), and 10  $\mu$ M caffeine (lanes 4–6). **D**, effect of caffeine on HIF-1 $\alpha$  expression induced by adenosine. Western blot analysis for HIF-1 $\alpha$  and HIF-1 $\beta$  levels. HT29 cells were treated in hypoxia (1% O<sub>2</sub>, 4 h) without (lane 1) or with 10  $\mu$ M adenosine (lanes 2 and 5), 100  $\mu$ M adenosine (lanes 3, 6), and 10  $\mu$ M caffeine (lanes 4–6). The mean densitometry data from independent experiments (one of which is shown here) were normalized to the result obtained in hypoxic cells in the absence of drug treatment (control). Plots are mean  $\pm$  S.E. values ( $n = 3$ ). \*,  $P < 0.01$  compared with the control. **E** and **F**, HT29 cells were treated with increasing concentrations of caffeine (0.1–100  $\mu$ M) for 24 h under both normoxic and hypoxic conditions, and cell viability was assayed by an MTS test (**E**) and a JAM test (**F**). In MTS, the cell growth is expressed as a percentage of the OD measured on untreated cells (control) assumed as 100% of cell viability. Ordinate reports means of four different OD quantifications with standard error (vertical bar). In JAM test, percentage of cell survival is reported in ordinate with standard error (vertical bar). Values represent means ( $\pm$  S.E.M.) of four separate quantifications in the same experiment. During the experiment, cells treated with the solvent DMSO served as controls.

with caffeine. HT29 cells were exposed to 2 h of hypoxia alone and in the presence of caffeine (1–10  $\mu$ M). Hypoxia significantly increased cAMP levels from  $10 \pm 1$  to  $25 \pm 2$  pmol/ $10^6$  cells. The incubation with caffeine in hypoxia did not modu-

late cAMP levels in these cells (Fig. 3B). As positive control, we show that the stimulation of adenylate cyclase with 1 to 10  $\mu$ M forskolin increased cAMP levels up to 5-fold with respect to hypoxic control (Fig. 3B).

TABLE 1  
Binding affinity of agonists and antagonists at A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> adenosine receptor subtypes  
K<sub>i</sub> values are shown with S.E.M. or 95% confidence intervals in parentheses.

	A <sub>1</sub>	A <sub>2A</sub>	A <sub>2B</sub>	A <sub>3</sub>	Reference
NECA	14 (6.4–29)	20 (12–35)		6.2 (3.5–11)	Fredholm et al., 2001
NECA			260 $\pm$ 30		Varani et al., 2005
CI-IB-MECA	115 (114–116)	2100 (1700–2500)	>100,000 (from a cAMP assay)	11 (9.4–13)	Fredholm et al., 2001
MRE 3008F20	1120 $\pm$ 130	165 $\pm$ 18	1500 $\pm$ 165	0.9 $\pm$ 0.1	Varani et al., 2005
MRE 2029F20	200 $\pm$ 25	>1000	3.2 $\pm$ 0.3	>1000	Varani et al., 2005
B64	708 (598–838)	495 (402–608)	34 (26–45)	3.7 (3.2–4.3)	Baraldi et al., 2002



**Fig. 2.** p38, Akt, and ERK1/2 phosphorylation in hypoxic colon HT29 cancer cells. A, pp38, pAkt, and pERK1/2 MAPK phosphoprotein levels under the selective A<sub>3</sub> agonist CI-IB-MECA and the adenosine receptor agonist NECA treatment in hypoxia (1% O<sub>2</sub>): dose- and time-relation effect. The mean densitometry data from independent experiments were normalized to the results obtained in cells in the absence of CI-IB-MECA or NECA (lane 0, Untreated). Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control. B, effect of caffeine on pp38, pAkt, and pERK1/2 MAPK phosphoprotein levels under CI-IB-MECA and NECA treatment in hypoxia. The mean densitometry data from independent experiments were normalized to the results obtained in cells in the absence of CI-IB-MECA or NECA (lane 0, Untreated). Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control.



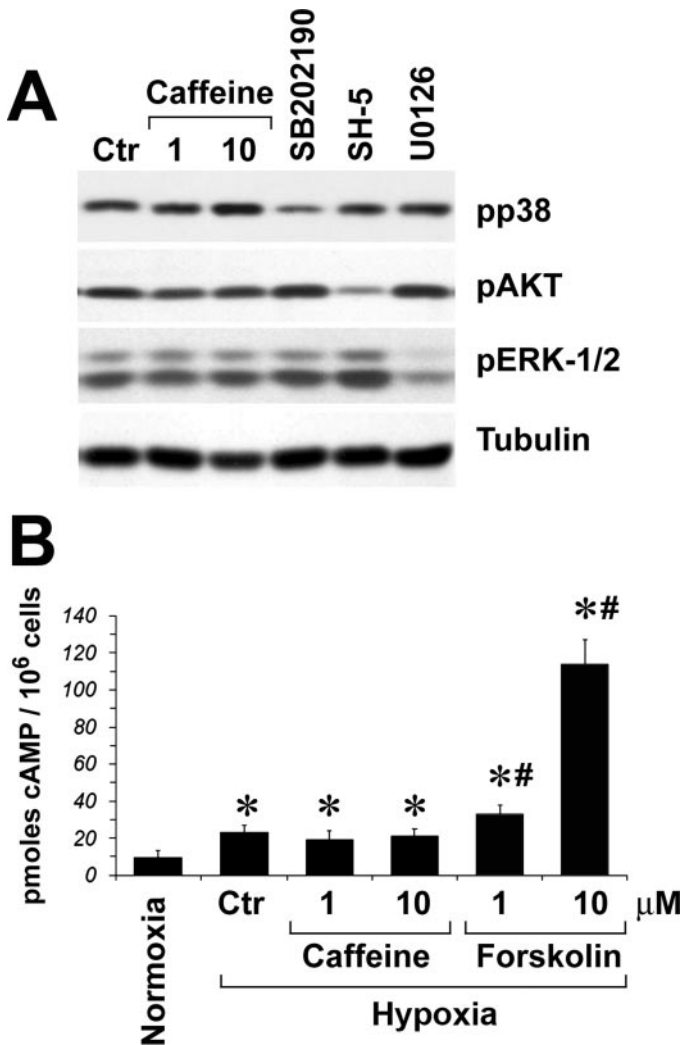
To better address the site of action of caffeine in the inhibitory effects of adenosine-induced responses in hypoxic colon cancer cell cultures, we performed a series of competition binding assays to human adenosine receptors in HT29 cells. Table 2 reports the affinity values versus A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> adenosine receptor subtypes, expressed as the inhibitory binding constant ( $K_i$ ) of caffeine. The results were obtained through [<sup>3</sup>H]DPCPX, [<sup>3</sup>H]ZM 241385, [<sup>3</sup>H]MRE 2029F20, and [<sup>3</sup>H]MRE 3008F20 competition binding experiments per-

formed for A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> in HT29 membranes, respectively. We found that caffeine has affinity in the micromolar range versus all adenosine receptor subtypes, confirming that this antagonist interferes with ligand binding to purinergic receptors.

**Caffeine Inhibits Adenosine-Induced HIF-1 $\alpha$  Protein Accumulation via Blocking of Akt, ERK1/2, and p38 MAPK Phosphorylation.** To determine whether Akt and MAPK pathways were required for HIF-1 $\alpha$  protein increase induced by A<sub>3</sub> receptor activation, HT29 cells were pretreated with SH5, SB202190, or U0126. The cells were then exposed to 100 nM Cl-IB-MECA for 4 h in hypoxia. As shown in Fig. 4, SH5 (10  $\mu$ M), SB202190 (10  $\mu$ M), and U0126 (10  $\mu$ M) were able to inhibit Cl-IB-MECA-induced increase of HIF-1 $\alpha$  protein expression.

**Caffeine Inhibits Adenosine-Induced VEGF Expression.** The effects of A<sub>3</sub> receptor stimulation through the agonist Cl-IB-MECA on secreted VEGF levels in HT29 colon cells were determined under hypoxic conditions. Cl-IB-MECA (10 nM) increased VEGF levels after 48 h of hypoxia in HT29 cells (Fig. 5A). To determine the concentration of caffeine required to inhibit adenosine-regulated VEGF protein increase under hypoxia, HT29 cells were treated with caffeine. VEGF levels were analyzed after 48 h of hypoxia. Complete abrogation of VEGF accumulation induced by 10 nM Cl-IB-MECA was observed with 10  $\mu$ M caffeine (Fig. 5A), at which HIF-1 $\alpha$  accumulation induced by A<sub>3</sub> receptor stimulation was also inhibited (Fig. 1C). To define the adenosine receptor subtype involved, HT29 cells were treated with Cl-IB-MECA in combination with the A<sub>2B</sub> antagonist MRE 2029F20 or with the A<sub>3</sub> receptor antagonist MRE 3008F20 (Table 1) (Varani et al., 2000). When used alone under hypoxic conditions, MRE 2029F20 and MRE 3008F20 had no effect on VEGF protein levels analyzed after 48 h of hypoxia (Fig. 5A). Complete abrogation of VEGF accumulation induced by 10 nM Cl-IB-MECA was seen with MRE 3008F20 10 nM, whereas the antagonist MRE 2029F20 (10 nM) did not block the Cl-IB-MECA effect (Fig. 5A), pointing to a role for the A<sub>3</sub> receptor. To evaluate whether a different A<sub>3</sub> receptor antagonist with affinity also for A<sub>2B</sub> receptors was able to modulate VEGF levels induced by Cl-IB-MECA, HT29 cells were treated with the A<sub>2B</sub>-A<sub>3</sub> receptor antagonist B64 (compound 44 in Baraldi et al., 2002) (Table 1). When used alone under hypoxic conditions, the B64 compound had no effect on VEGF protein levels analyzed after 48 h of hypoxia (Fig. 5A). Complete abrogation of VEGF accumulation induced by 10 nM Cl-IB-MECA was seen at a concentration of 10 nM B64 adenosine receptor antagonist (Fig. 5A), indicating the involvement of the A<sub>3</sub> receptor.

To investigate whether the MAPK pathway was involved in the expression of A<sub>3</sub>-induced VEGF protein, HT29 cells were cultured in hypoxia for 48 h after the addition of the MEK1/2 inhibitor U0126, the AKT inhibitor SH5, or the



**Fig. 3.** Caffeine signaling in HT29 cells. A, pp38, pAkt, and pERK1/2 MAPK phosphoprotein levels under caffeine (1–10  $\mu$ M) treatment in hypoxia (1% O<sub>2</sub>, 4 h). The effect of SH5, an Akt inhibitor, SB202190, inhibitor of p38 MAPK, and U0126, inhibitor of MEK1/2, at the concentration of 10  $\mu$ M is shown. Inhibitors were added to the cells 30 min before hypoxia. B, cAMP levels in normoxia and upon treatment of HT29 cells with caffeine (1–10  $\mu$ M) or forskolin (1–10  $\mu$ M), or no drug (Ctr) for 2 h in hypoxia. Results shown are mean  $\pm$  S.E. values ( $n = 3$ );  $P < 0.01$  compared with the control (normoxia); #,  $P < 0.01$  compared with the control (untreated hypoxic cells).

TABLE 2

Inhibition of [<sup>3</sup>H]DPCPX, [<sup>3</sup>H]ZM 241385, [<sup>3</sup>H]MRE 2029F20, and [<sup>3</sup>H]MRE 3008F20 binding by caffeine at A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> adenosine receptors expressed in human HT29 cells, respectively

Data are expressed as the mean  $\pm$  S.E.M.  $K_i$  value represents the concentration of drug able to displace 50% of the radioligand.

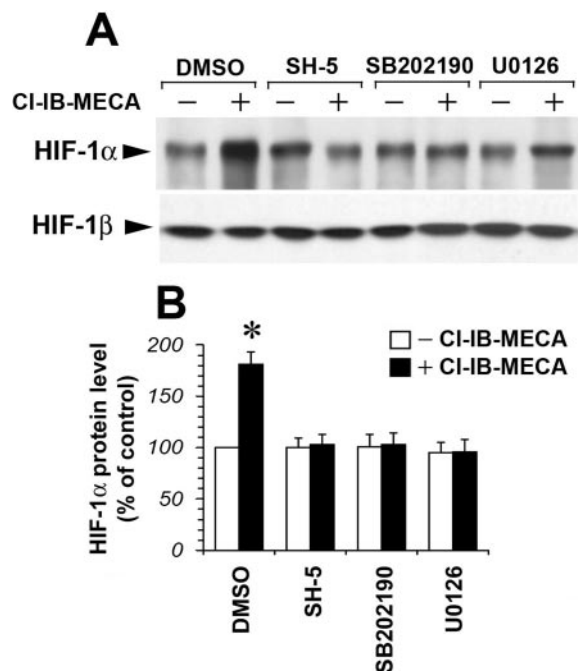
	[ <sup>3</sup> H]DPCPX	[ <sup>3</sup> H]ZM 241385	[ <sup>3</sup> H]MRE 2029F20	[ <sup>3</sup> H]MRE 3008F20
	$\mu$ M			
Caffeine $K_i$	45 $\pm$ 5	18 $\pm$ 3	10 $\pm$ 1	13 $\pm$ 2

inhibitor of p38 MAPK, SB202190, 30 min before the treatment of 10 nM Cl-IB-MECA. U0126, SH5, and SB202190 (10  $\mu$ M) significantly inhibited the VEGF protein levels induced by 10 nM Cl-IB-MECA (Fig. 5A).

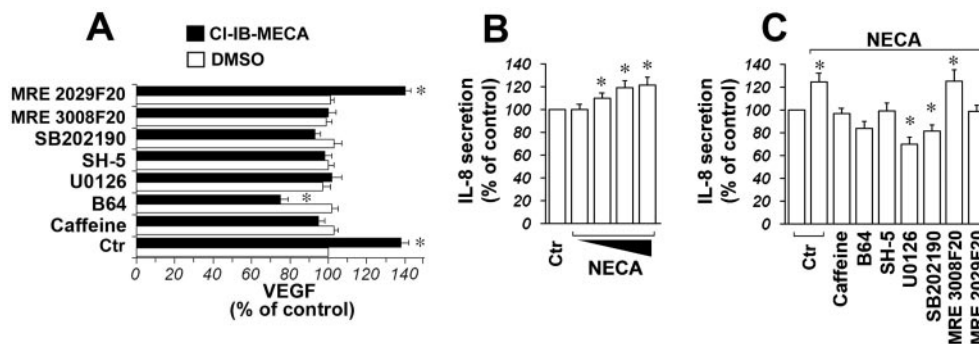
**Caffeine Inhibits Adenosine-Induced IL-8 Expression.** Figure 5B shows that stimulation of adenosine receptors in HT29 cells with increasing concentrations of NECA (0.01–10  $\mu$ M) for 24 h of hypoxia induces secretion of IL-8. The relatively low potency of NECA agrees with previous reports of  $A_{2B}$  receptor-mediated IL-8 production (Feoktistov et al., 2003). To better define the adenosine receptor subtype involved, HT29 cells were treated with 1  $\mu$ M NECA in com-

ination with the  $A_{2B}$  antagonist MRE 2029F20 or with the  $A_3$  receptor antagonist MRE 3008F20 (Table 1) (Varani et al., 2000). When used alone under hypoxic conditions, MRE 2029F20 and MRE 3008F20 had no effect on IL-8 protein levels analyzed after 24 h of hypoxia (data not shown). Complete abrogation of IL-8 accumulation induced by 1  $\mu$ M NECA was seen with 10 nM MRE 2029F20, whereas the antagonist MRE 3008F20 (10 nM) did not block the NECA effect (Fig. 5C), pointing to a role for the  $A_{2B}$  receptor. Furthermore, to evaluate whether a different  $A_{2B}$  receptor antagonist with affinity also for  $A_3$  receptors was able to modulate IL-8 levels induced by NECA, HT29 cells were treated with the  $A_{2B}$ - $A_3$  receptor antagonist B64 (Table 1). Complete abrogation of IL-8 accumulation induced by 1  $\mu$ M NECA was seen at a concentration of 10 nM B64 adenosine receptor antagonist (Fig. 5C), indicating the involvement of the  $A_{2B}$  receptor. Based on these results, we have chosen the incubation of 24 h in hypoxia with 1  $\mu$ M NECA in further studies to analyze the effect of caffeine and the signaling pathways involved in adenosine-induced IL-8 production. Complete abrogation of IL-8 accumulation induced by 1  $\mu$ M NECA was observed with 10  $\mu$ M caffeine (Fig. 5C). Furthermore, we evaluated a potential role of Akt, ERK 1/2, and p38 MAPK kinase in NECA-induced synthesis of IL-8. As shown in Fig. 5C, 10  $\mu$ M SH5, 10  $\mu$ M U0126, and 10  $\mu$ M SB202190 completely blocked NECA-induced production of IL-8.

**$A_3$  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 interacts with the HIF-1 pathway to up-regulate VEGF transcription, we used two luciferase reporters described previously. 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-base pair mutation that prevents HIF-1 binding (Forsythe et al., 1996). We transfected HT29 colon cells with these reporters and treated the cells with adenosine for different times in hypoxia. As shown in Fig. 6A, hypoxia increased luciferase activity of the p11w reporter in HT29 cells in a time-dependent manner. The maximum increase in p11w reporter activity is present at 72 h of hypoxia. Hypoxia also



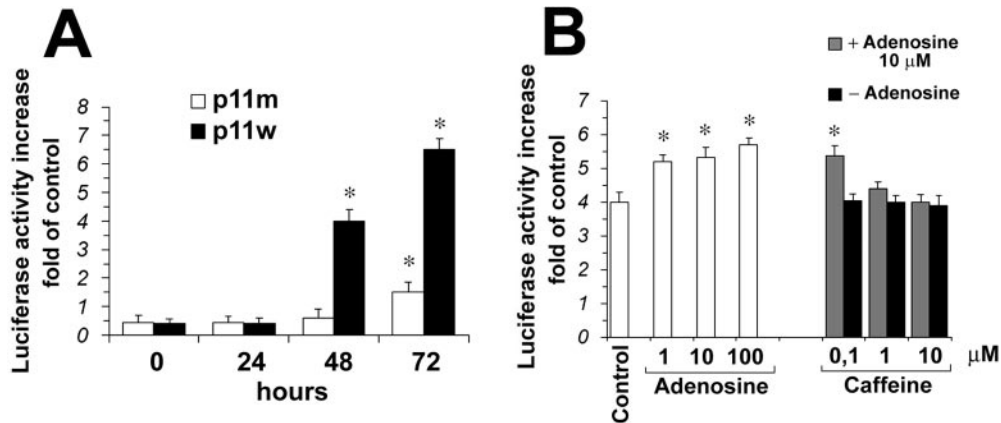
**Fig. 4.** Signaling pathway. A, HT29 cells were pretreated 30 min with or without SH5, an Akt inhibitor, SB202190, inhibitor of p38 MAPK, and U0126, inhibitor of MEK1/2, at the concentration of 10  $\mu$ M and then exposed to the selective  $A_3$  agonist Cl-IB-MECA 100 nM (+) for 4 h in hypoxia (1%  $O_2$ ). The mean densitometry data from independent experiments (one of which is shown here) were normalized to the results obtained in hypoxic cells in the absence of Cl-IB-MECA (lane 1). Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control.



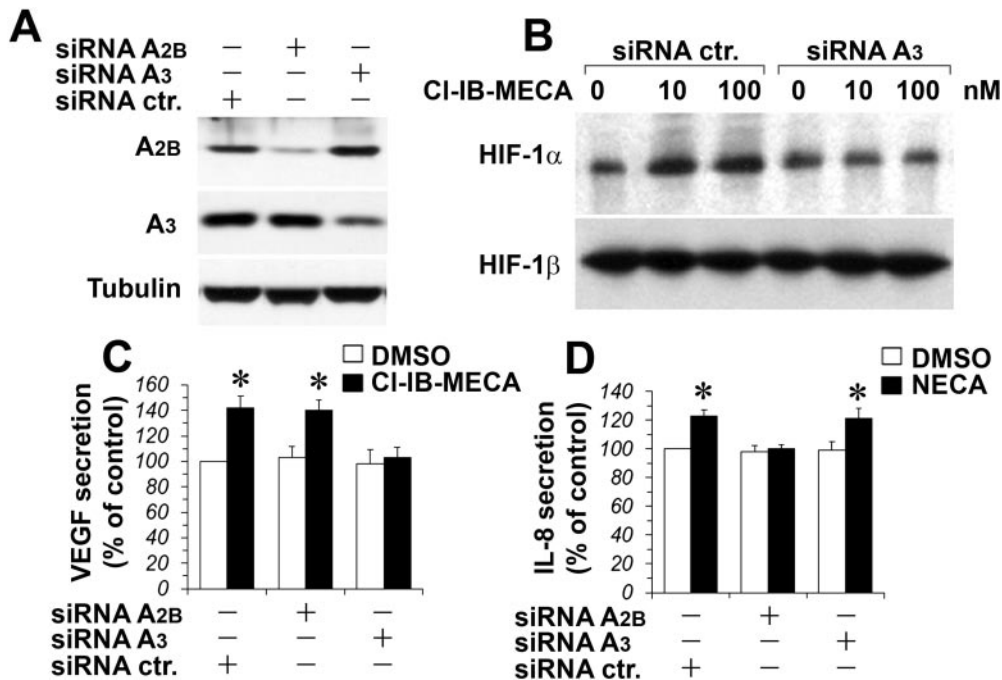
**Fig. 5.** Effect of adenosine receptor stimulation on VEGF and IL-8 expression in hypoxic (1%  $O_2$ ) cells. A, VEGF release into culture media of HT29 cells cultured 48 h in the absence and in the presence of the selective  $A_3$  agonist Cl-IB-MECA (10 nM), caffeine (10  $\mu$ M), the  $A_{2B}$ - $A_3$  antagonist B64 (10 nM), U0126 (10  $\mu$ M), SH5 (10  $\mu$ M), SB 202190 (10  $\mu$ M), the selective  $A_{2B}$  antagonist MRE 2029F20 (10 nM), and the selective  $A_3$  antagonist MRE 3008F20 (10 nM); the inhibitors were added 30 min before Cl-IB-MECA, and then the cells were exposed to hypoxia. Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (untreated hypoxic cells). B, effect of the adenosine receptor agonist NECA (0.01, 0.1, 1, and 10  $\mu$ M) on IL-8 expression in hypoxic HT29 cells cultured 24 h. C, effect of 1  $\mu$ M NECA on IL-8 expression in hypoxic HT29 cells cultured 24 h in the absence and in the presence of 10  $\mu$ M caffeine, 10 nM B64, 10  $\mu$ M SH5, 10  $\mu$ M U0126, 10  $\mu$ M SB 202190, 10 nM MRE 2029F20, and 10 nM MRE 3008F20. Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (untreated hypoxic cells).



stimulated activity of the p11m reporter but to a minor extent (Fig. 6A). Incubation of the cells for 48 h under hypoxic conditions with adenosine resulted in a dose-dependent increase in p11w reporter activity. As shown in Fig. 6B, increasing concentrations of adenosine (1–100  $\mu$ M) up-regulated the p11w reporter up to 41% with respect to untreated hypoxic HT29 cells. In particular, the increase induced by 10  $\mu$ M adenosine at 48 h of hypoxia is blocked by 1 to 10  $\mu$ M caffeine (Fig. 6B).



**Fig. 6.** Effect of hypoxia (1%  $O_2$ ) and adenosine on HIF-1-dependent VEGF reporter activity. HT29 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 hypoxia response element unable to bind HIF-1 (p11m). A, transfected cells were incubated under hypoxia for 24, 48, and 72 h. \*,  $P < 0.01$  compared with the control (time 0 from the transfection). B, HT29 cells were transfected with p11w for 48 h under hypoxia with adenosine (1–100  $\mu$ M). The effect of 10  $\mu$ M adenosine in combination with caffeine (0.1–10  $\mu$ M) is shown. Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (48 h from the transfection with p11w in the absence of adenosine).



**Fig. 7.** A<sub>2B</sub> and A<sub>3</sub> receptor expression silencing by siRNA transfection. A, Western blot analysis using an anti-A<sub>2B</sub> and an anti-A<sub>3</sub> receptor polyclonal antibody of protein extracts from HT29 cells transfected with control (ctr) ribonucleotides or with siRNA<sub>A2B</sub> or siRNA<sub>A3</sub> and cultured for 48 h. Tubulin shows equal protein loading. B, Western blot analysis using an anti-HIF-1 $\alpha$  monoclonal antibody of protein extracts from HT29 cells transfected with control ribonucleotides or siRNA<sub>A3</sub> for 48 h and cultured with the selective A<sub>3</sub> agonist CI-IB-MECA (0–100 nM) for 4 h in hypoxia (1%  $O_2$ ). HIF-1 $\beta$  shows equal protein loading. C, VEGF release into culture media of HT29 cells transfected with control (ctr) ribonucleotides or with siRNA<sub>A2B</sub> or siRNA<sub>A3</sub> and cultured 48 h in hypoxia (1%  $O_2$ ) in the absence and in the presence of 10 nM CI-IB-MECA. Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (DMSO-treated siRNA-ctr transfected hypoxic cells). D, IL-8 release into culture media of HT29 cells transfected with control (ctr) ribonucleotides or with siRNA<sub>A2B</sub> or siRNA<sub>A3</sub> and cultured for 24 h in hypoxia (1%  $O_2$ ) in the absence and in the presence of the adenosine receptor agonist NECA (1  $\mu$ M). Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (DMSO-treated siRNA-ctr transfected hypoxic cells).

48 h in complete media, and then total proteins were isolated for Western blot analysis of  $A_{2B}$  and  $A_3$  receptor protein. As expected,  $A_{2B}$  and  $A_3$  receptor protein expression were strongly reduced in siRNA $_{A_{2B}}$ - and siRNA $_{A_3}$ -treated cells, respectively (Fig. 7A). To confirm the specificity of the siRNA $_{A_3}$ -mediated silencing of  $A_3$  receptor, we investigated the expression of  $A_{2B}$  receptor protein in siRNA $_{A_3}$ -treated cells (Fig. 7A). Figure 7A demonstrates that treatment of HT29 cells with siRNA $_{A_3}$  reduced the expression of  $A_3$  protein but had no effect on the expression of  $A_{2B}$  receptor. Similar results were obtained when HT29 cells transfected with siRNA $_{A_{2B}}$  were analyzed for the expression of the  $A_3$  receptor (Fig. 7A).

Therefore, at 48 h from the siRNA $_{A_3}$  transfection, HT29 cells were exposed to increasing concentrations of the  $A_3$  adenosine receptor agonist Cl-IB-MECA (10–100 nM) for 4 h in hypoxia. We found that the inhibition of  $A_3$  receptor expression is sufficient to block Cl-IB-MECA-induced HIF-1 $\alpha$  accumulation (Fig. 7B). Furthermore, HT29 cells were transfected with siRNA $_{A_3}$  and exposed to 10 nM Cl-IB-MECA to evaluate VEGF levels after 48 h of hypoxia. Complete abrogation of VEGF accumulation induced by Cl-IB-MECA 10 nM was observed when the  $A_3$  receptor was knocked down in colon cells (Fig. 7C). Likewise, to confirm the role of  $A_{2B}$  receptors in the regulation of IL-8 expression, HT29 cells transfected with siRNA $_{A_{2B}}$  were treated with 1  $\mu$ M NECA and IL-8 protein levels were measured after 24 h of hypoxia. We found that the inhibition of  $A_{2B}$  receptor expression is sufficient to block NECA-induced IL-8 accumulation (Fig. 7D).

**Effect of Caffeine on Cell Migration of HT29 Cells.** Recent studies have shown the possible role of HIF-1 $\alpha$  in the regulation of colon carcinoma cell invasion (Krishnamachary et al., 2003). To investigate whether caffeine can inhibit cancer cell migration, an in vitro cell migration assay was done. We examined whether hypoxic condition enhances cell migration of HT29 cells and whether caffeine can suppress tumor migration. Our results show that exposure to hypoxia for 6 to 24 h in the presence of Cl-IB-MECA 100 nM significantly stimulated migration of HT29 cells under serum-free conditions (Fig. 8A). The stimulatory effect of Cl-IB-MECA-

induced migration of HT29 cells was completely abrogated by pretreatment with 10  $\mu$ M caffeine. These results indicated that caffeine suppressed the Cl-IB-MECA-stimulated migration of HT29 cells.

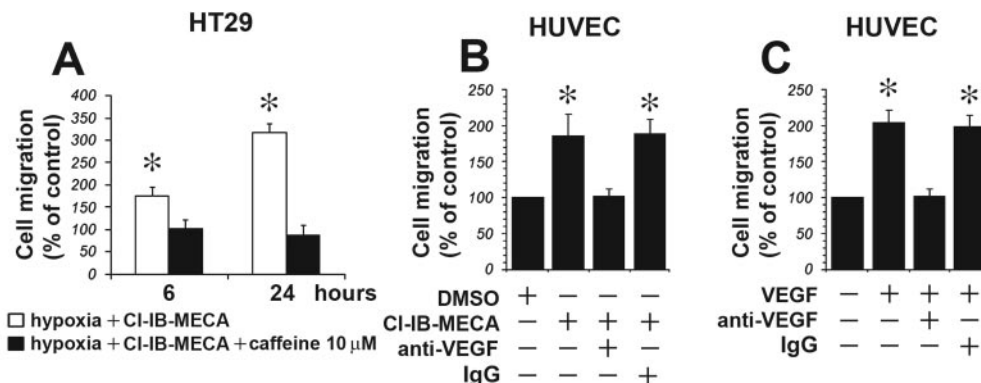
**The Conditioned Medium of Colon Cancer Cells and the Migration of HUVECs.** To determine the functional importance of Cl-IB-MECA-induced increases in VEGF expression, we evaluated the effects of conditioned medium from Cl-IB-MECA-treated colon cells on the migration of HUVECs. Conditioned medium was obtained from the supernatants of colon cells treated with or without 100 nM Cl-IB-MECA for 48 h in hypoxia. We prepared three batches of conditioned media for three independent HUVEC migration experiments. HUVECs were incubated for 6 h with endothelial basal medium or conditioned medium. The conditioned medium from Cl-IB-MECA-treated HT29 colon cells significantly enhanced HUVEC migration compared with the control conditioned medium from untreated colon cells (Fig. 8B). This effect was completely abrogated when conditioned medium from Cl-IB-MECA-stimulated colon cells was preincubated with anti-VEGF neutralizing antibodies, whereas 1  $\mu$ g/ml nonspecific goat IgG failed to block the conditioned medium effect (Fig. 8B).

In contrast to its effects on migration, Cl-IB-MECA did not significantly modulate the proliferation of HUVECs compared with the untreated cells (data not shown). Likewise, the conditioned medium from Cl-IB-MECA-treated colon cells did not modulate the proliferation of HUVECs.

Finally, we have shown that a commercial VEGF preparation enhances HUVEC migration, but this effect was abrogated when HUVECs were preincubated with the anti-VEGF neutralizing antibodies (Fig. 8C), whereas 1  $\mu$ g/ml nonspecific goat IgG failed to block the VEGF effect.

## Discussion

Because substantial amounts of caffeine are ingested by people drinking coffee, tea, or caffeinated soft drinks, an understanding of the biological effects of caffeine is of considerable importance. The concentrations of caffeine used in



**Fig. 8.** Cell migration of HT29 and HUVECs. A, cell migration of HT29 cells. The cells were cultured for 6 and 24 h at 37°C under hypoxia in the presence of the selective  $A_3$  agonist Cl-IB-MECA (100 nM). The effect of 10  $\mu$ M caffeine is shown. Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (hypoxic untreated cells). B, cell migration of HUVECs incubated for 6 h without (DMSO) or with conditioned medium from Cl-IB-MECA-treated hypoxic HT29 cells (Cl-IB-MECA). The effect of treatment of conditioned medium from Cl-IB-MECA-treated HT29 cells with 1  $\mu$ g/ml anti-VEGF antibodies (anti-VEGF) is shown. Anti-human VEGF was developed in goat using recombinant human VEGF $_{165}$  as immunogen. In this assay, 1  $\mu$ g/ml anti-VEGF antibodies was incubated with conditioned medium from Cl-IB-MECA-treated HT29 cells for 1 h at 22°C. After the preincubation, HUVECs were added to the antigen-antibody mixture. The assay mixture was incubated at 37°C for 6 h. As negative control, 1  $\mu$ g/ml nonspecific goat IgG (IgG) was used. The plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (DMSO-treated HT29 cells). C, cell migration of HUVECs incubated for 6 h without (–) or with VEGF 1  $\mu$ g/ml. The effect of 1  $\mu$ g/ml anti-VEGF antibodies (anti-VEGF) is shown. Plots are mean  $\pm$  S.E. values ( $n = 3$ ); \*,  $P < 0.01$  compared with the control (untreated cells).

this study (10  $\mu$ M) may seem unphysiologically high. In fact, we want to emphasize that even higher concentrations are reached in coffee drinkers (Ekbom, 1999).

To our knowledge, this is the first report examining the *in vitro* effect of caffeine on hypoxic cancer cells. Taken together, our data suggest three potential chemopreventive targets for caffeine: 1) HIF; 2) VEGF and IL-8; and 3) cell migration. In the current study, we have demonstrated that caffeine inhibits the up-regulation of HIF-1 $\alpha$ , VEGF, and IL-8 expression induced by the adenosine receptor agonist CI-IB-MECA in human colon cancer cells exposed to severe hypoxia. In particular, we have shown that HIF-1 $\alpha$  and VEGF are increased through A<sub>3</sub> adenosine receptor stimulation, whereas the effects on IL-8 are mediated via the A<sub>2B</sub> subtype. We have demonstrated previously that, in hypoxic glioblastoma cells, adenosine is able to increase the production of the proangiogenic factor VEGF (Merighi et al., 2006) through the A<sub>3</sub> receptor subtype. Furthermore, our results indicate that, in tumor colon hypoxic cells, adenosine increases VEGF promoter activity via the HIF-1 pathway and that caffeine is able to block this effect. It has been reported, in previous studies, that A<sub>2B</sub> receptors stimulate IL-8 production in normoxic conditions (Zeng et al., 2003). In this study, we found that also in hypoxia, there is a modulation in IL-8 levels mediated by the adenosine receptor agonist NECA. These effects may seem rather modest and were examined only during concomitant hypoxia. However, the aim of this work was to study the effects of caffeine on HIF-1 protein accumulation and on VEGF and IL-8 expression in the human colon cancer cell line HT29 under hypoxic conditions.

The signaling pathways involved are Akt, MEK, and p38 MAPK, having a key role in A<sub>3</sub> receptor ability to enhance HIF-1 $\alpha$  and VEGF protein expression. Moreover, we have shown that Akt, ERK1/2, and p38 MAPK activities were required for the IL-8 expression increase induced by A<sub>2B</sub> receptor activation.

Although caffeine did not interact with signaling molecules downstream of adenosine receptor activation, such as Akt, mitogen-activated protein kinases, p38, or cAMP, we have demonstrated that it interferes with adenosine receptor binding as an antagonist with micromolar affinity. As a consequence, we suggest that caffeine may serve as an antagonist of adenosine receptor activities in hypoxic cells as a means to retard tumorigenesis *in vivo*. In particular, it will be of interest to study paraxanthine in future studies. Paraxanthine is the main metabolite of caffeine in humans, and, at least in some receptor subtypes, it is as potent as the parent compound. As a consequence, when discussing the plasma concentrations of caffeine achieved clinically, one underestimates the amount of adenosine receptor antagonism, because plasma concentrations of paraxanthine can be just as high (Biaggioni et al., 1991).

It has been shown that HIF-1 $\alpha$  overexpression, either as a result of intratumoral hypoxia or genetic alterations, activates the transcription of genes, the protein products of which contribute to the basement membrane invasion of colon cancer cells. In the present study, we have shown that caffeine inhibited the stimulatory effects of the adenosine receptor agonist CI-IB-MECA on the migration ability of hypoxic tumor colon cancer cells (Fig. 8), which could be attributed to its potent inhibitory effects on CI-IB-MECA-

induced HIF-1 $\alpha$  protein accumulation and VEGF expression. Even if these are only “*in vitro*” results that are in accordance with the *in vitro* observation that caffeine inhibits tumor cell motility (Lentini, 1998), they may be indicative of increased tumor migration *in vivo*. However, caffeine was not able even to prevent the effects produced by hypoxia alone. This implies that, under the conditions of the assays, not enough endogenous adenosine was generated to mediate the effects of hypoxia on markers of tumor growth. In our *in vitro* cell model, the effects demonstrated for caffeine are those related to adenosine receptor antagonism.

Furthermore, to determine the functional importance of adenosine-induced increases in VEGF expression, we evaluated the effects of conditioned medium from CI-IB-MECA-treated colon cells on the migration of HUVECs. Our data indicate that the increased VEGF expression produced by CI-IB-MECA-treated colon cancer cells stimulates migration of vascular endothelial cells. The finding that the CI-IB-MECA-stimulated increase in VEGF was blocked by caffeine indicates that strategies aimed at blocking adenosine receptors will not only affect colon cell migration but also will affect surrounding vasculature dependent on tumor-derived VEGF. Although it is well-known that hypoxia stimulates VEGF levels, hypoxia coordinately stimulates IL-8 in tumor cells (Desbaillets et al., 1997), and in tumor xenografts, hypoxic areas of tumors coexpressed VEGF and IL-8. Targeting HIF-1 $\alpha$  is an attractive strategy, with the potential for disrupting multiple pathways crucial for tumor growth. However, recent findings have investigated whether the inhibition of HIF-1 alone is sufficient to block tumor angiogenesis (Mizukami et al., 2005). In particular, it has been demonstrated that HIF-1 $\alpha$  deficiency in cancer cells can inhibit proliferation and overall growth but not angiogenesis. The new finding of these studies is that compensatory pathways can be activated to preserve the tumor angiogenic response. In particular, it has been demonstrated that in the absence of HIF-1, the proangiogenic cytokine IL-8 is induced in a compensatory manner to maintain tumor vascularity. The absence of HIF-1 can therefore stimulate IL-8 on a transcriptional level, and this is further enhanced in hypoxia. Our results provide evidence that an additional role of adenosine in colon tumor progression may be the enhancement of angiogenesis via up-regulation not only of VEGF, A<sub>3</sub>-HIF-1-mediated, but also of IL-8, A<sub>2B</sub>-mediated. It has been suggested that strategies that inhibit HIF-1 $\alpha$  may be most effective when IL-8 is simultaneously targeted. Therefore, we suggest that an A<sub>2B</sub>-A<sub>3</sub> receptor antagonist may be regarded as a target for the development of a new antitumor drug through its ability to inhibit HIF-1 $\alpha$ , VEGF, and IL-8 in the context of tumor hypoxia, a common feature of most invasive cancers.

Although our studies have been performed using tumor cell lines, our finding that caffeine is able to prevent HIF-1 $\alpha$ , VEGF, and IL-8 accumulation induced by adenosine receptor activation provides proof-of-principle that the application of small molecules such as caffeine might be used in chemotherapy to reduce morbidity and mortality associated with neoplastic disease. This possibility was especially compelling because high caffeine intake has been associated with decreased cancer mortality in human populations (Michels et al., 2005; Baker et al., 2006). In this context, further studies are needed to better investigate possible antitumor effects of



caffeine and to clarify the involvement of adenosine in the development of tumors.

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