

Hypoxia Inhibits Paclitaxel-Induced Apoptosis through Adenosine-Mediated Phosphorylation of Bad in Glioblastoma Cells

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Received October 17, 2006; accepted March 30, 2007

ABSTRACT

Solid tumors contain hypoxic cells that are resistant to radiotherapy and chemotherapy. The resistance in glioblastoma has been linked to the expression of antiapoptotic Bcl-2 family members. In this study, we found that in human glioblastoma cells hypoxia induces the phosphorylation of the Bcl-2 family protein Bad, thus protecting hypoxic cells from paclitaxel-induced apoptosis. Akt activation is required for the hypoxia-induced protection. In contrast, the extracellular signal-regulated kinase 1/2 activities have only a partial effect, being able to modulate Bad phosphorylation but not paclitaxel-induced apoptosis in hypoxia. We also demonstrated that the degrada-

tion of adenosine with adenosine deaminase, the knockdown of A₃ adenosine receptor expression by gene silencing, and the blockade of this receptor through A₃ receptor antagonists blocked the hypoxia-induced phosphorylation of Bad and the prolonged cell survival after treatment with paclitaxel in hypoxia. Thus, the adenosinergic signaling may be an essential component in the hypoxia survival pathway. These results suggest that hypoxia-induced chemoresistance of human glioblastoma cells may occur in a novel mechanism involving activation of adenosine-A₃ receptor-Akt pathway, which mediates Bad inactivation and favors cell survival.

Solid tumors contain areas of hypoxia. Adaptation of tumor cells to a hypoxic environment results in aggressive and metastatic cancer phenotype, which is associated with resistance to radiation and chemotherapy and a poor treatment outcome (Harris, 2002).

Glioblastoma multiforme (GBM), the most common subtype of malignant brain tumors in adult humans (Holland, 2001), is highly invasive, angiogenic, and incurable: half of all patients die within 1 year of diagnosis (Kemper et al., 2004). Taxanes are antineoplastic agents that promote mi-

cro-tubule assembly and stabilization and have been shown in preclinical and clinical studies to exhibit activity against a wide range of tumors (Chamberlain and Kormanik, 1995). Paclitaxel, the most widely studied taxane, has demonstrated modest activity with minimal toxicity in patients with recurrent primary brain tumors (Rosenthal et al., 2000), although it has shown a greater effectiveness for the treatment of high-grade gliomas (Glantz et al., 1999). A review of published studies evaluating paclitaxel alone or in combination with other chemotherapeutic agents suggests that paclitaxel alone is not highly active against newly diagnosed or recurrent GBM. Future investigations should be directed at evaluating paclitaxel-based chemotherapy regimens in selected brain tumor types and determining the importance of

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.031849.

ABBREVIATIONS: GBM, glioblastoma multiforme; ADA, adenosine deaminase; Cl-IB-MECA, *N*⁶(3-iodobenzyl)2-chloroadenosine-5'-*N*-methyluronamide; ERK, extracellular signal-regulated kinase; HIF-1 α , hypoxia-inducible factor-1 α ; MEK, mitogen-activated protein kinase kinase; MRE 3008F20, 5*N*-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3e]1,2,4-triazolo[1,5c] pyrimidine; siRNA, small interfering RNA; siRNA_{A₃}, small interfering RNA that targets A₃ receptor mRNA; siRNA_{Bad}, small interfering RNA that targets Bad; siRNA_{HIF-1 α} , siRNA targeting the transcription factor hypoxia-inducible factor-1 α ; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance; PI3K, phosphatidylinositol-3-OH kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; SH-5, D-3-deoxy-2-*O*-methyl-myoinositol 1-[(*R*)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate].

other proposed mechanisms of action of paclitaxel (e.g., inhibition of angiogenesis and tumor invasion) (Guensberg et al., 2002).

Resistance to chemotherapy in glioblastoma has been linked to the expression of antiapoptotic Bcl-2 family members (Adams and Cory, 1998). Proteins in the Bcl-2 family are key regulators of apoptosis that may either promote or inhibit cell death (Danial and Korsmeyer, 2004). The family can be separated into three groups based on function and sequence homology: prosurvival members, including Bcl-2, Bcl-xL; multidomain proapoptotic proteins, including Bax and Bak; and "BH3 only" proteins, like Bad. Interaction between death-promoting and death-suppressing Bcl-2 family members have led to a model in which the ratio of proapoptotic and antiapoptotic proteins controls cell fate. Heterodimerization between prosurvival Bcl-2 family members and proapoptotic family members regulates apoptosis (Bonni et al., 1999). Bad, a proapoptotic member of the Bcl-2 family, binds and antagonizes prosurvival Bcl-xL and induces apoptosis. Bad protein contains 23 serines and 10 threonines within 204 amino acids, and among them, serines 112, 136, and 155 have been identified as phosphorylation sites (Datta et al., 1997). Phosphorylation of these serines inhibits binding of Bad to prosurvival protein Bcl-xL on the outer membrane of mitochondria, and Bad changes subcellular distribution from the mitochondria to cytosol where it binds the 14-3-3 protein. Thus, Bad regulates apoptosis from the cell cytoplasm and must be expressed or delivered into the cytosol to be used as a death promoter.

Recent findings showed that hypoxia increases antiapoptotic potentials in tumor cells regulating the molecules involved in the apoptosis signaling pathways (Harris, 2002). These effects of hypoxia make tumor cells resistant to various cancer therapies and facilitate the survival of tumor cells (Vaupel et al., 2001; Shannon et al., 2003). Thus, tumor cell responses to hypoxia are important for tumor progression and tumor therapy.

The increased concentration of adenosine and its receptor subtype A₃ has been observed in hypoxia and in solid tumors (Blay et al., 1997; Merighi et al., 2003b), but the role of the adenosine axis in apoptosis regulation in glioblastoma cells has not been investigated. Here, we report the role of this nucleoside in hypoxia-induced chemoresistance to paclitaxel treatment.

Materials and Methods

Chemicals and Reagents. GBM cells were obtained from American Type Culture Collection (Manassas, VA). Tissue culture media and growth supplements were obtained from Lonza Bioscience (Bergamo, Italy). Anti-ACTIVE mitogen-activated protein kinase (Thr183/Tyr185) and anti-ERK1/2 (pAb) were from Promega (Milano, Italy). Anti-adenosine A₃ receptor antibody was from Aviva Systems Biology (Milano, Italy). Phospho-Akt (Ser473), phospho-Bad (Ser112), Bad, phospho-Bcl-2 (Ser70), Bcl-xL, Bax, and Bak antibodies were from Cell Signaling Technology (Milano, Italy). Unless otherwise noted, all other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Hypoxia Treatment. U87MG and A172 GBM cells were maintained in minimal essential medium and Dulbecco's modified Eagle's medium, respectively, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) at 37°C in 5% CO₂/95% air (normoxia). For hypoxic

conditions, cells were placed for the indicated times in a modular incubator chamber and flushed with a gas mixture containing 1% O₂, 5% CO₂, and balance N₂ (MiniGalaxy; RSBiotech, Irvine, Scotland).

Trypan Blue Exclusion. Cells were collected and stained with 0.4% trypan blue for 5 min at room temperature before being examined under the microscope. The number of viable cells was determined by trypan blue exclusion. The dead cells that stained blue were scored positive and counted against the total number of cells to determine the percentage of cell death.

ATPLite Assay. The intracellular ATP concentration was determined with a luminescent ATP detection kit (ATPLite-M; PerkinElmer Life Sciences, Milano, Italy) according to the manufacturer's directions. Light units generated by ATP in each sample were normalized to control (solution with known ATP concentration) and expressed as the absolute ATP levels.

Flow Cytometry Analysis of the Cell Cycle. U87MG and A172 GBM-adherent cells were trypsinized, mixed with floating cells, washed with PBS, and permeabilized by a 70% (v/v) ethanol/PBS solution at 4°C for at least 24 h. Cells were washed with PBS, and DNA was stained with a PBS solution, containing 20 µg/ml propidium iodide and 100 µg/ml Rnase, at room temperature for 30 min. Cells were analyzed with an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA), and the content of DNA was evaluated by the Expo program (Beckman Coulter). Cell distribution among cell cycle phases and the percentage of apoptotic cells were evaluated as described previously (Merighi et al., 2002). In brief, the cell cycle distribution is shown as the percentage of cells containing 2n (G₀/G₁ phases), 4n (G₂ and M phases), 4n > x > 2n DNA amount (S phase) judged by propidium iodide staining. The apoptotic population is the percentage of cells with DNA content less than 2n.

Western Blot Analysis. Whole-cell lysates, prepared as described previously, were resolved on a 15% SDS gel and transferred onto the nitrocellulose membrane. Western blot analyses were performed as described previously (Merighi et al., 2005b) using antibody for P-Bcl-2, P-Bad or total Bad, Bcl-xL, Bax, Bak, phosphorylated or total ERK-1/ERK-2 mitogen-activated protein kinase, P-Akt, A₃ receptor, tubulin. Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

RNA Interference. U87MG cells expressing high levels of endogenous Bad were transfected with Bad siRNA from Cell Signaling Technology, according to siRNA transfection protocol from the manufacturer (QIAGEN, Valencia, CA). To generate a small interfering RNA that targets A₃ receptor mRNA (siRNA_{A3}), eight oligonucleotides consisting of ribonucleosides, except for the presence of 2'-deoxyribonucleosides at the 3' end, were synthesized and annealed according to the recommendations of Elbashir et al. (2001), to the manufacturer's instructions (Silencer siRNA Construction Kit; Ambion, Austin, TX), and as described previously (Merighi et al., 2005b). For oligonucleotide-1: sense, 5'-GCU UAC CGU CAG AUA CAA GUU-3'; antisense, 5'-CUU GUA UCU GAC GGU AAG CUU-3'. For oligonucleotide-2: sense, 5'-GAC GGC UAA GUC CUU GUU UUU-3'; antisense, 5'-AAA CAA GGA CUU AGC CGU CUU-3'. For oligo-3: sense, 5'-ACA CUU GAG GGC CUG UAU GUU-3'; antisense, 5'-CAU ACA GGC CCU CAA GUG UUU-3'. For oligonucleotide-4: sense, 5'-CCU GCU CUC GGA GGA UGC CUU-3'; antisense, 5'-GGC AUC CUC CGA GAG CAG GUU-3'. 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 oligonucleotides 1, 2, 3, and 4 are localized at positions 337, 679, 1009, and 1356 bases downstream of the start codon of A₃ receptor mRNA sequence (Lys20463), respectively. siRNA targeting the transcription factor hypoxia-inducible factor-1α (HIF-1α) (siRNA_{HIF-1α}) were synthesized, and experiments were performed as described previously (Merighi et al., 2005a). The sequences target nucleotides 1378 to 1398 of the human HIF-1α mRNA (accession no. AF304431.1).

Treatment of Cells with siRNA. The 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). Cells were cultured in complete media and at 24, 48, and 72 h total RNA was isolated for real-time RT-PCR analysis of A₃ receptor mRNA and for Western blot analysis of A₃ receptor protein. As control, cells were exposed to RNAiFect Transfection reagent with scramble siRNA. To quantify cell transfection efficiency, we used fluorescein isothiocyanate-labeled siRNA (QIAGEN). After 24 h of transfection, cells were trypsinized and resuspended in PBS for flow cytometry analysis. Fluorescence obtained from fluorescein isothiocyanate-siRNA-transfected cells was compared with autofluorescence generated by untransfected control.

Real-Time RT-PCR Experiments. Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method, as described previously (Merighi et al., 2005b). Quantitative real-time RT-PCR assay (Merighi et al., 2005b) of A₃ mRNA transcript 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: A₃ forward primer, 5'-ATG CCT TTG GCC ATT GTT G-3'; A₃ reverse primer, 5'-ACA ATC CAC TTC TAC AGC TGC CT-3'; A₃ MGB probe, 5'-FAM-TCA GCC TGG GCA TC-TAMRA-3' (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 fluorescence-labeled with VIC (Applied Biosystems, Monza, Italy).

High-Pressure Liquid Chromatographic-Fluorometric Assay. Adenosine was measured by high-performance liquid chromatography assay using fluorometric detection (Andresen et al., 1999). Cells (100,000) were plated in 24-multiwell plates; 0.5 ml of complete medium was added to each well. Cells were incubated at 37°C for 24 h. Then the medium was removed, and 0.5 ml of Krebs' solution was added to the cultures. After 3 to 6 h of incubation, the extracellular fluid was collected for adenosine analysis.

Each 200- μ l sample received 10 μ l of 0.5 M acetate buffer, 10 μ l of 1 μ M internal standard, and 10 μ l of 50% aqueous chloroacetaldehyde. The samples were then incubated at 80°C for 1 h. After incubation, a total of 80 μ l of this solution was injected into a high-performance liquid chromatography system (4.6 \times 250 mm C₁₈ reverse-phase column with 5 μ m particle size). The mobile phase was 10 mM citrate buffer with 4.5% acetonitrile and was run isocratically at 1 ml/min. Fluorescence detection was achieved at an excitation wavelength of 275 nm and an emission wavelength of 420 nm using a fluorescence detector. The ratio of the area under the adenosine peaks to the area under the internal standard peak was compared with a standard curve. As internal standard, adenine 9- β -D-arabino-furanoside was used.

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

Results

Effects of Hypoxia on GBM Cell Survival. The effects of hypoxia (1% O₂) for 24 h on cell viability of two human GBM cell lines, A172 and U87MG, were examined. Hypoxia did not promote apoptosis above basal levels (<4%) in any of the cell lines. There was no difference in the transition through S phase of the cells cultured for 24 h in normoxia compared with those in hypoxia or in the total cell number at

24 h. In addition, there was no impact of hypoxia on the intracellular ATP level in the two cell lines.

Hypoxia Protects GBM Cells from Apoptosis Induced by Paclitaxel. GBM cells were incubated with or without increasing concentrations of the chemotherapeutic agent paclitaxel for 24 to 48 h under either hypoxic or normoxic conditions. Apoptosis was assessed as the percentage of cells with a DNA content lower than that of cells in the G₁-G₀ phase, as analyzed by flow cytometry (Fig. 1A). The induction of apoptosis induced by paclitaxel was significantly reduced in hypoxia compared with normoxia (Fig. 1B). The effect was more pronounced in U87MG cells than in A172 cells.

Effects of Hypoxia on the Level of Bcl-2 Family Proteins. We investigated the effect of oxygen deprivation on protein levels of some members of the Bcl-2 protein family. When the human GBM cells were cultured for up to 24 h in hypoxia, there were no major changes in the levels of the antiapoptotic proteins Bcl-2 and Bcl-xL. No changes were observed in the levels of the proapoptotic proteins Bad, Bax, and Bak. However, there was a slight but reproducible increase in Bad phosphorylation (Fig. 1C).

Hypoxia Induces Bad Phosphorylation in Association with Increased Survival of Human Hypoxic GBM Cells. Then we better characterized the effects of hypoxia on phosphorylation of Bad. To test it, U87MG and A172 GBM cells were treated in normoxia and in hypoxia for 6 and 24 h. Results indicate that hypoxia stimulates Bad phosphorylation at Ser112 (Fig. 1D). The effect was first observed at 6 h and was maintained at 24 h.

Paclitaxel and Bad Phosphorylation. We have evaluated the effects of paclitaxel on Bad phosphorylation levels in normoxia and in hypoxia. The results show that in normoxia paclitaxel did not affect Bad phosphorylation. On the contrary, Bad phosphorylated levels were significantly down-regulated after treatment with 15 ng/ml paclitaxel for 24 h in hypoxic GBM cells (Fig. 1E). These results indicate that paclitaxel induces apoptosis in normoxia through a mechanism that is essentially P-Bad-independent, whereas paclitaxel-induced apoptosis in GBM hypoxic cancer cells may occur in a mechanism involving Bad signaling.

To confirm this, an RNA interference approach was used. We used siRNA-targeting Bad expression to silence Bad in U87MG cells. Results show that the Bad siRNA can potently and specifically reduce Bad expression by more than 90%, whereas the control with scramble siRNA had no effect (Fig. 1F). Hypoxia had no additional survival effect in hypoxic cells transfected with Bad siRNA and treated with paclitaxel compared with normoxic values (Fig. 1G).

Akt and MEK1/2 Inhibitors Block Hypoxia-Induced Bad Phosphorylation and Promote Apoptosis. To investigate how paclitaxel decreases Bad phosphorylated levels in hypoxic GBM cells, we have evaluated the involvement of Akt and p44/p42 phosphorylation. In particular, glioblastoma cells were treated with paclitaxel (15 ng/ml) for 24 h, and then phosphorylation of Akt, p44, and p42 was evaluated. By these experiments, it is possible to observe that p-Akt seemed to be inhibited by paclitaxel, whereas p-p44 and p-p42 were not affected by paclitaxel treatment (Fig. 2A).

Next, we determined whether these signaling pathways protect hypoxic cells from apoptosis induced by paclitaxel. We used the Akt inhibitor SH-5 (10 μ M) and the MEK1/2

inhibitor U0126 (10 μ M), which were able to block in GBM cells Akt and p44/p42 phosphorylation, respectively (Fig. 2B). U87MG and A172 cells cultured under normoxic or hypoxic conditions were incubated with paclitaxel alone and in the presence of SH-5 or U0126 (10 μ M for 5 h), the cells were collected, and phosphorylation of Bad was evaluated. Results indicate that SH-5 and U0126 can block hypoxia-induced Ser112 Bad phosphorylation (Fig. 2, C and D). Then, apoptosis was analyzed. As shown in Fig. 2E, the cell-protective effect against apoptosis induced by paclitaxel under hypoxic

conditions was completely reversed by SH-5, whereas U0126 did not produce any significant effect on paclitaxel-induced apoptosis. These data indicate a role for the Akt signaling in the protection from paclitaxel-induced apoptosis.

The Role of Adenosine. To study the involvement of the adenosinergic system, we treated GBM cells with 3 IU/ml adenosine deaminase (ADA), a purine catabolic enzyme that degrades adenosine. In these experimental conditions, Bad-phosphorylated levels found in hypoxia were not different from those of cells cultured in normoxia (Fig. 3, A and B).

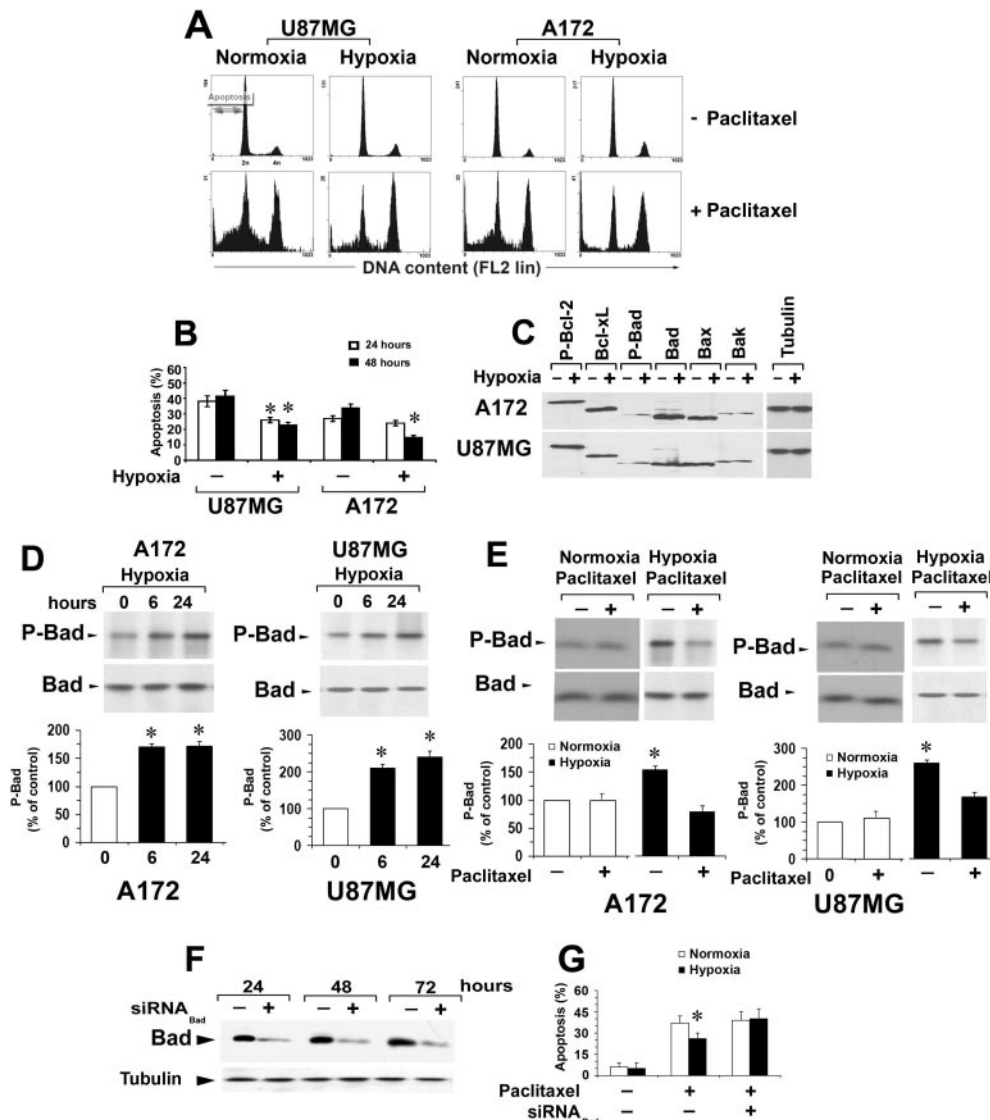


Fig. 1. Hypoxia induces Bad phosphorylation in association with suppression of apoptosis in human GBM cancer cells. **A**, U87MG and A172 cells were treated with 15 ng/ml paclitaxel in normoxia or in hypoxia. Samples were harvested and analyzed by flow cytometry. Histograms are generated plotting in abscissa the phosphatidylinositol-derived fluorescence (FL2 channel, linear scale). The amount of apoptotic cells –sub2n– is calculated by gating events with fluorescence less than those of cells in G₀/G₁ cell cycle phase (the first pick). Thus, apoptosis is reported in the following histogram as a percentage of more than 10,000 cells, doublets, and cellular debris-free. **B**, data represent the mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (drug vehicle-treated cells). Analysis was by ANOVA followed by Dunnett's test. **C**, effect of hypoxia on protein levels of Bcl-2 family protein. A172 and U87MG cells were incubated for 6 h under normoxic (–) or hypoxic (+) conditions, and lysates were immunoblotted for P-Bcl-2, Bcl-xL, P-Bad, Bad, Bax, and Bak. Tubulin shows equal loading protein. **D**, A172 and U87MG cells were incubated for 0, 6, and 24 h in hypoxia, and lysates were immunoblotted for P-Bad protein. Densitometric quantification for P-Bad is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (0 h of hypoxia); analysis was by ANOVA followed by Dunnett's test. **E**, A172 and U87MG cells were incubated without (–) and with (+) 15 ng/ml paclitaxel in normoxia and in hypoxia for 24 h and lysates were immunoblotted for P-Bad protein. Densitometric quantification for P-Bad is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (normoxia = 0 h hypoxia); analysis was by ANOVA followed by Dunnett's test. **F**, Bad silencing by siRNA transfection; Western blot analysis of protein extracts from U87MG cells treated with scramble (–) or with siRNA_{Bad} (+) and cultured for 24, 48, and 72 h. Tubulin shows equal loading protein. **G**, the effect of the siRNA_{Bad} on apoptosis induced by 15 ng/ml paclitaxel. U87MG cells were incubated in normoxia or in hypoxia. The data are the mean \pm S.E. of four independent experiments. *, $P < 0.01$ compared with the control (absence of paclitaxel). Analysis was by ANOVA followed by Dunnett's test.

Similar results were obtained in serum-free medium (data not shown). Then we treated the cells with 15 ng/ml paclitaxel in the absence and in the presence of ADA. Paclitaxel, in the presence of ADA, induced apoptosis in hypoxia and in normoxia (Fig. 3C). To confirm the effects of adenosine, U87MG and A172 GBM cells were treated in normoxia for 24 h with increasing concentrations of adenosine (0.1–100 μ M). Results indicate that adenosine stimulates serine phosphorylation of Bad (Fig. 3, D–G). U87MG and A172 cells express the A₃ adenosine receptor subtype (Merighi et al., 2006). To evaluate whether A₃ receptors may have a functional role on Bad phosphorylation, we tested the effect of adenosine in combination with MRE 3008F20 (a selective A₃ receptor antagonist) (Merighi et al., 2002). MRE 3008F20 100 nM abrogated the adenosine-induced increase of Bad phosphorylation (Fig. 3, H and I). These findings suggest that hypoxia-induced survival and chemoresistance may occur in a mechanism probably, at least in part, involving adenosine-ergic system via A₃ receptors.

Furthermore, we have quantified P-Bad levels under normal and hypoxic conditions in the presence of both paclitaxel and adenosine. GBM A172 cells were treated with 15 ng/ml paclitaxel and with adenosine 10 μ M alone and in combination. Then P-Bad levels were analyzed. The results show that adenosine is able to significantly increase P-Bad levels in normoxia, whereas in hypoxia, the effect is of a minor extent, probably because basal levels of P-Bad in hypoxia are higher than in normoxia (Fig. 3, J and K). Furthermore, we found that whereas paclitaxel alone did not affect P-Bad levels in normoxia, it inhibited P-Bad levels in hypoxia. The effect of paclitaxel on P-Bad levels in hypoxia was reduced when A172

cells were cultured in the presence of both paclitaxel and adenosine; in particular, adenosine was able to reduce the inhibition of P-Bad levels induced by paclitaxel in hypoxia, confirming the hypothesis that adenosine increases P-Bad levels under hypoxia, which protects from paclitaxel-induced apoptosis. Further experiments were performed to evaluate whether the increase in adenosine-promoted P-Bad levels are inhibited by SH-5; GBM cells were culture with adenosine 10 μ M and with SH-5 10 μ M, alone and in combination, and then P-Bad levels were evaluated. We found that the increase in adenosine-promoted P-Bad levels were inhibited by SH-5, confirming the involvement of Akt in the phosphorylation of Bad induced by adenosine (Fig. 3, L and M).

A₃ Receptors and Bad Phosphorylation. To verify the involvement of A₃ receptors in Bad phosphorylation, GBM cells were cultured in normoxia and in hypoxia with increasing concentrations of the high-affinity agonist Cl-IB-MECA (Merighi et al., 2006). Results indicate that Cl-IB-MECA increased phosphorylation of Bad with an EC₅₀ value of 0.8 ± 0.1 nM (Fig. 4, A and B).

We evaluated the effect of A₃ receptor stimulation on Akt and ERK1/2 phosphorylation in hypoxia. We performed a time course of pAkt, pp44/pp42, and P-Bad in response to Cl-IB-MECA. We added Cl-IB-MECA to GBM cells (at the concentration of 100 nM, at which we have the maximal stimulation of P-Bad), and then we evaluated the increase in pAkt, pp44/pp42, and in P-Bad after 5, 15, 30, 60, and 120 min. The results indicate that the increase in pAkt (maximal after 15 min of Cl-IB-MECA treatment) preceded the increase in P-Bad (maximal after 60 min of Cl-IB-MECA treatment) (Fig. 4C). Next, we treated GBM cells with increasing

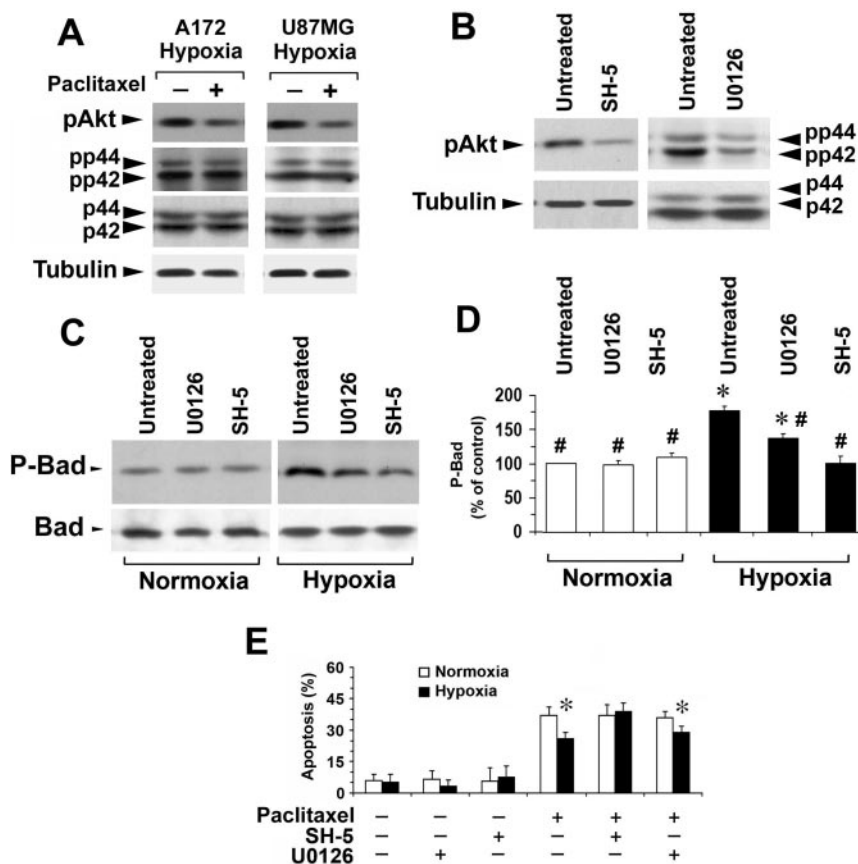


Fig. 2. A, the effect of 15 ng/ml paclitaxel on Akt, p44, and p42 phosphorylation levels in A172 and U87MG cells in normoxia and in hypoxia for 24 h. Tubulin and p44/p42 total levels show equal loading protein. B, the effect of the Akt inhibitor SH-5 and the MEK inhibitor 10 μ M U0126 on pAkt and pp44/pp42 levels, respectively. Tubulin and p44/p42 total levels show equal loading protein. C, Western blot analysis of phosphorylated and total Bad in A172 cells treated under normoxic or hypoxic conditions with 10 μ M U0126 and 10 μ M SH-5. D, densitometric quantification for P-Bad is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (normoxia-untreated = 0 h hypoxia); #, $P < 0.01$ compared with the control (hypoxia-untreated); analysis was by ANOVA followed by Dunnett's test. E, the effect on apoptosis of SH-5 and U0126, alone and in combination with 15 ng/ml paclitaxel, is shown. A172 cells were incubated under normoxic or hypoxic conditions (48 h). The data are the mean \pm S.E. of four independent experiments. *, $P < 0.01$ compared with the normoxic value. Analysis was by ANOVA followed by Dunnett's test.

concentrations of Cl-IB-MECA (0.1–100 nM) for 5 h. Results indicate that A₃ receptors activated Akt in a concentration-dependent manner (Fig. 4, D and E). Similar results are reported for ERK1/2 phosphorylation levels (Fig. 4D).

Furthermore, the selective A₃ receptor antagonist MRE 3008F20 (100 nM) abrogated the Cl-IB-MECA-induced increase of Akt and ERK1/2 activation (Fig. 4D). Therefore, we hypothesized that the A₃ adenosine receptor subtype may be responsible for Cl-IB-MECA-mediated increase of Akt and ERK1/2 in hypoxic GBM cells.

To characterize the phosphorylation of Akt induced by A₃ receptor stimulation, we used LY294002 as inhibitor of the phosphatidylinositol-3-OH kinase (PI3K). Pretreatment of GBM cells with 1 μ M LY294002 for 30 min impaired Cl-IB-MECA activation of Akt and Bad phosphorylation (Fig. 4E). These data suggest that the A₃ adenosine receptor signals through a pathway including PI3K-Akt.

To further demonstrate that the A₃ receptor is required

for the accumulation of P-Bad in response to Cl-IB-MECA, A172 cells were transfected with siRNA_{A3} for degradation. We designed four siRNAs from the human A₃ receptor gene sequence. Although there was a difference in silencing ability, all of the siRNAs were able to suppress endogenous A₃ receptor mRNA and protein expression in human A172 cells (Fig. 5). As shown in Fig. 5, A and B, after 24, 48, and 72 h after transfection, A₃ receptor mRNA and protein levels were significantly reduced in siRNA_{A3}-treated cells. Neither mock transfection nor transfection with a scramble siRNA inhibited A₃ receptor protein expression. At 48 h from the siRNA_{A3} transfection, A172 cells were exposed to increasing concentrations of Cl-IB-MECA (10–100 nM) for 24 h. As control, A172 cells were exposed to scramble siRNA. We found that the inhibition of A₃ receptor expression was sufficient to block Cl-IB-MECA-induced P-Bad accumulation (Fig. 5, D and E). Furthermore, the A₃ receptor-specific antagonist MRE 3008F20 potently inhib-

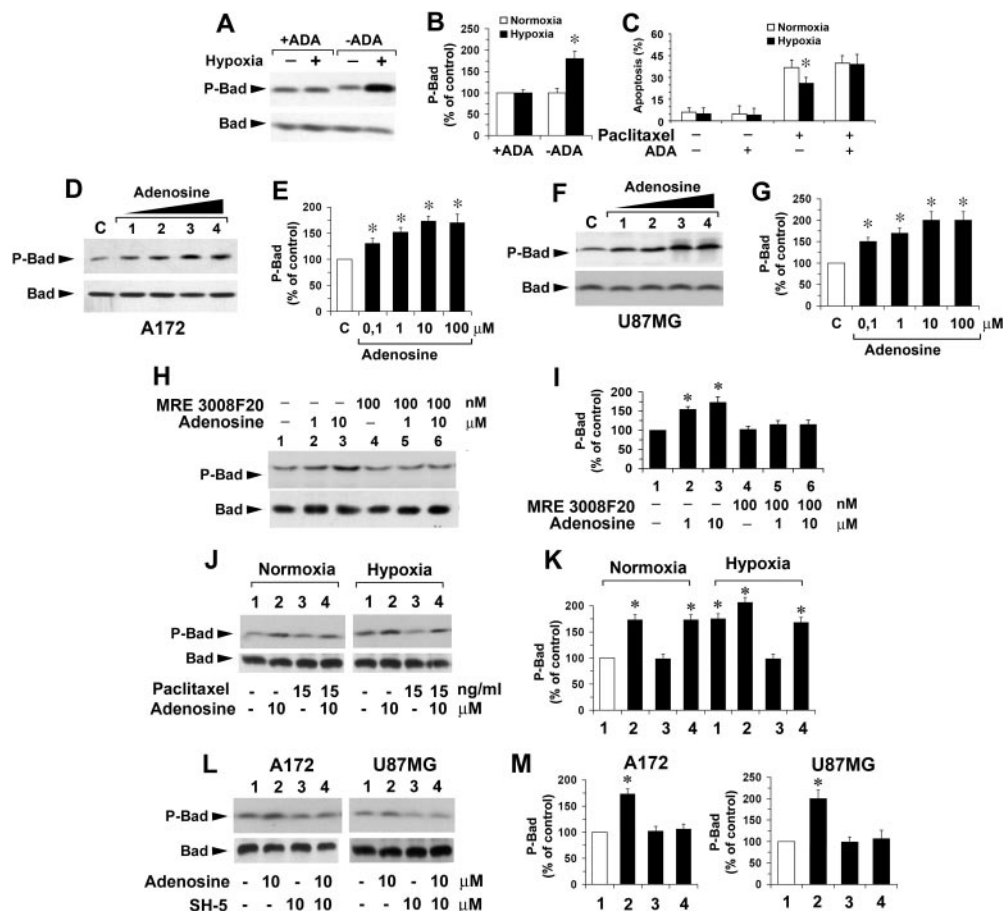


Fig. 3. A, Western blot analysis of phosphorylated and total Bad in A172 cells treated under normoxic or hypoxic conditions with and without ADA 3 IU/ml. The cells were incubated for 6 h. B, densitometric quantification for P-Bad is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (normoxia); analysis was by ANOVA followed by Dunnett's test. C, effect on apoptosis of ADA alone and in combination with 15 ng/ml paclitaxel. A172 cells were incubated under normoxia or hypoxia for 48 h. The data are the mean \pm S.E. of four independent experiments. *, $P < 0.01$ compared with the normoxic value. Analysis was by ANOVA followed by Dunnett's test. D to G, effect of 0.1 μ M (1), 1 μ M (2), 10 μ M (3), and 100 μ M Adenosine (4) on P-Bad levels in A172 (D and E) and U87MG (F and G) cells. H, effect of the A₃ receptor antagonist MRE 3008F20 100 nM alone and in combination with Adenosine on P-Bad protein levels. I, the mean densitometry data for P-Bad Western blot from independent experiments (one of which is shown here) were normalized to the result obtained in untreated cells (lane 1). Plots are mean \pm S.E. values ($n = 4$); *, $P < 0.01$ compared with the normoxic value. Analysis was by ANOVA followed by Dunnett's test. J, A172 cells were treated with 15 ng/ml paclitaxel and Adenosine 10 μ M, alone and in combination, for 24 h, and lysates were immunoblotted for P-Bad protein. K, densitometric quantification for P-Bad is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (normoxia = 0 h hypoxia); analysis was by ANOVA followed by Dunnett's test. L, A172 cells were treated with 10 μ M Adenosine, 10 μ M SH-5, alone and in combination, for 6 h, and lysates were immunoblotted for P-Bad protein. M, densitometric quantification for P-Bad is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (normoxia = 0 h hypoxia); analysis was by ANOVA followed by Dunnett's test.

ited hypoxia-induced Bad phosphorylation in a concentration-dependent manner (Fig. 5, F and G). Furthermore, we evaluated whether siRNA_{A3} inhibits hypoxia-induced Bad phosphorylation. We found that in the presence of siRNA_{A3} hypoxia did not increase Bad phosphorylation levels (Fig. 5, H and I).

A₃ Receptors and Apoptosis. At 48 h from siRNA_{A3} transfection, U87MG cells were exposed to paclitaxel 7.5, 15, and 30 ng/ml for 24 h in hypoxia. As control, U87MG cells were exposed to scramble siRNA. We found that the inhibition of A₃ receptor expression was sufficient to block the protection from the apoptosis induced by paclitaxel in hypoxia (Fig. 6).

To further investigate the effect of A₃ receptor blockade on apoptosis, the cells were incubated with or without paclitaxel (7.5, 15, and 30 ng/ml) for 24 h under either hypoxic and normoxic conditions in the presence or absence of MRE 3008F20 (10–100 nM). Treatment of the cells growing under normoxic conditions with 15 ng/ml paclitaxel increased apoptosis by 34%. As already observed, the induction of apoptosis by paclitaxel was significantly reduced (by 32%) in cells incubated under hypoxic conditions. On the contrary, the incubation of GBM cells with both 100 nM MRE 3008F20 and

15 ng/ml paclitaxel for 24 h under hypoxic conditions increased apoptosis to as much as 39% (Fig. 6). Similar results were obtained for paclitaxel 7.5 and 30 ng/ml (Fig. 6). MRE 3008F20 (10 nM) reversed only partially the protection of hypoxia from the apoptosis induced by paclitaxel (Fig. 6).

The Role of HIF-1 α . To investigate a possible role for the HIF-1 α subunit in Bad phosphorylation and in the hypoxic protection from the apoptosis induced by paclitaxel, we have performed a series of experiments in the presence of siRNA-HIF-1 α . HIF-1 α protein was knocked down with siRNA at 72 h after siRNA_{HIF-1 α} transfection (Fig. 7, A and B). The results show that even if HIF-1 α subunit was knocked down, hypoxia was able to protect glioblastoma cells from the apoptosis induced by paclitaxel and CI-IB-MECA was also able to increase Bad phosphorylation (Fig. 7, C–E).

Analytical Determination of Adenosine Release from U87MG Cells. We measured adenosine concentrations in media collected from cells exposed to hypoxia for 3 and 6 h. We found that 3 h of hypoxia induced an increase in adenosine concentrations from 30 ± 3 nM in normoxia to 66 ± 4 nM in hypoxia (Fig. 8). Similar results were observed after 6 h of hypoxia treatment (Fig. 8).

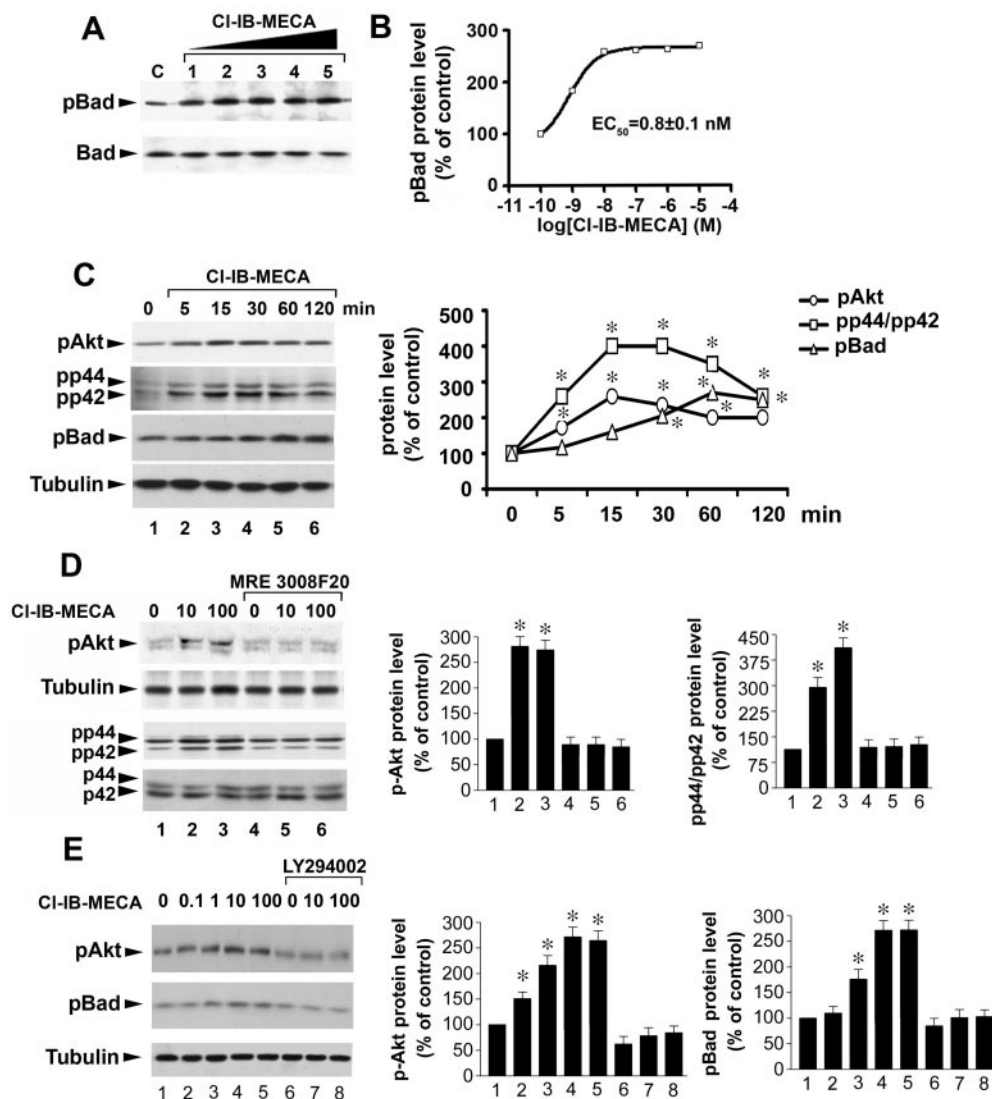


Fig. 4. A and B, Western blot analysis of phosphorylated and total Bad in A172 (A) and in U87MG (B) cells treated under normoxic conditions with CI-IB-MECA (C = absence of CI-IB-MECA; from 1 to 5 is 0.1–1, 10, 100, and 1000 nM concentration CI-IB-MECA). Cells were incubated for 6 h, and lysates were immunoblotted for P-Bad and Bad protein. Densitometric quantification for P-Bad is reported in B; plots are mean \pm S.E. values ($n = 3$); C, time-dependent effects of CI-IB-MECA on Akt and Bad phosphorylation in A172 cells. A172 cells were incubated at 37°C with dimethyl sulfoxide (lane 1) or 100 nM CI-IB-MECA for 5, 15, 30, 60, or 120 min. Densitometric analysis of Akt and Bad phosphorylated isoforms is reported. The unstimulated control (0 min, CI-IB-MECA) was set to 100%. *, $P < 0.05$ with respect to unstimulated control; analysis was by ANOVA followed by Dunnett's test. D, the effect of CI-IB-MECA and of MRE 3008F20 on Akt, p44, and p42 phosphorylation levels in A172 cells. Densitometric quantification is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (0, untreated cells); analysis was by ANOVA followed by Dunnett's test; E, effect of increasing concentrations of CI-IB-MECA and of the PI3K inhibitor 1 μ M LY294002 on pAkt and pBad. Densitometric quantification is reported; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (0, untreated cells); analysis was by ANOVA followed by Dunnett's test.

Discussion

In this study, we have shown that under hypoxic conditions, human glioblastoma cells were slightly resistant to apoptosis induced by paclitaxel compared with normoxic values. The resistance correlated with the increase in the phosphorylation of Bad, a proapoptotic member of the Bcl-2 family. In particular, in cells in which Bad was silenced, the effect of paclitaxel on apoptosis was not significantly different between normoxic and hypoxic conditions. The siRNA against Bad did not affect paclitaxel-induced apoptosis under normoxia, even if, with Bad eliminated, the balance was tilted toward the antiapoptotic Bcl family members. However, the evaluation of the effects of paclitaxel on Bad phosphorylation levels in nor-

moxia and in hypoxia indicates that paclitaxel induces apoptosis in normoxia through a mechanism that is essentially P-Bad-independent, whereas in GBM hypoxic cancer cells, paclitaxel-induced apoptosis may occur in a mechanism involving Bad signaling. Thus, we investigated how paclitaxel decreases P-Bad levels. We evaluated the involvement of Akt and p44/p42 phosphorylation and found that, whereas p44/p42 kinases were not modulated by the chemotherapeutic drug, Akt activation was inhibited by paclitaxel. Moreover, in cells in which Akt signaling was blocked, we observed similar levels of Bad phosphorylation and similar values of apoptosis induced by paclitaxel between normoxic and hypoxic conditions. Therefore, it has been shown previously that Akt may promote cell survival

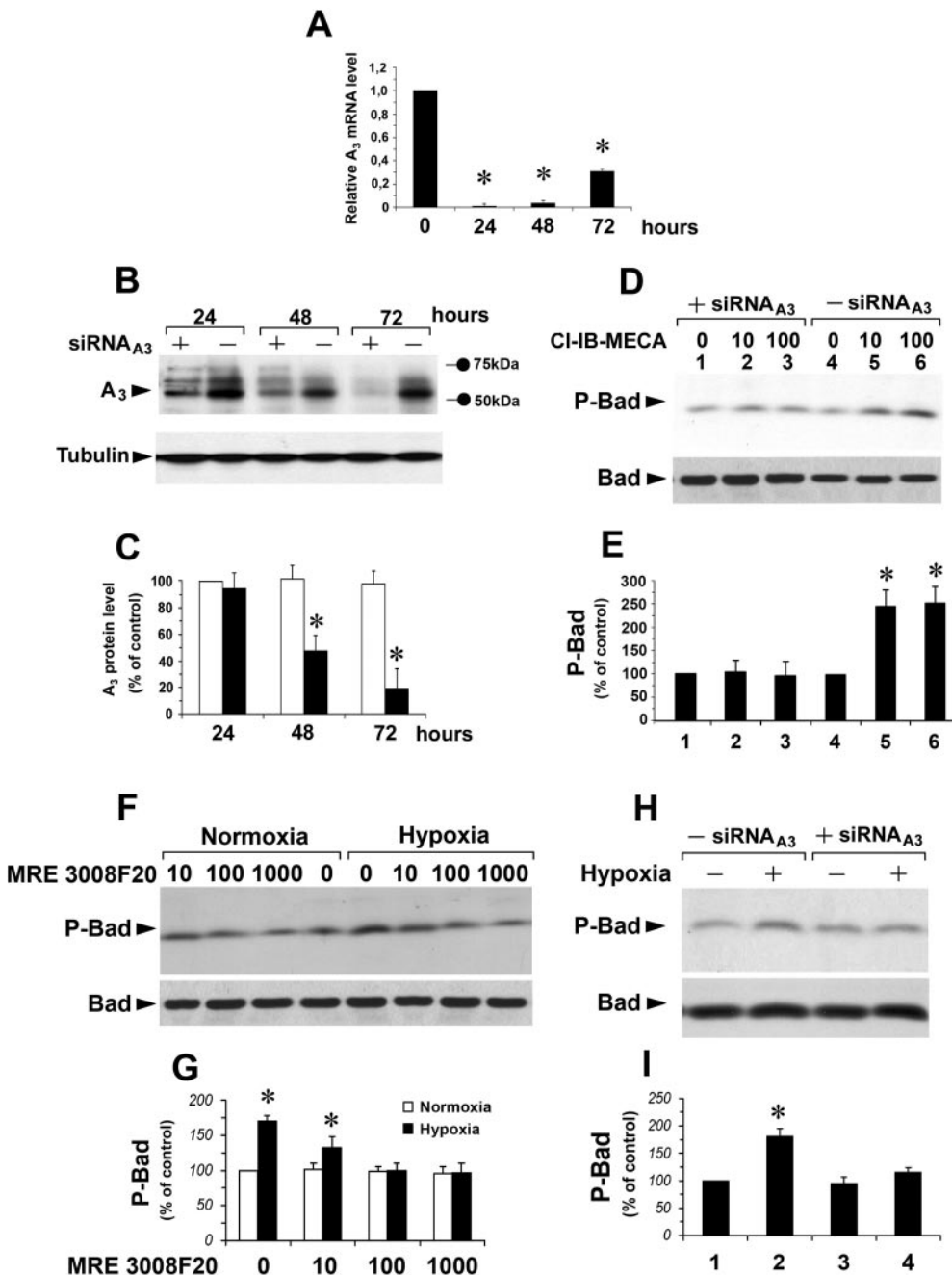


Fig. 5. A₃ receptor silencing by siRNA transfection. **A**, relative A₃ receptor mRNA quantification, related to β -actin mRNA, by real-time RT-PCR. A172 cells were transfected with siRNA_{A3} by RNAiFect transfection reagent and cultured for 24, 48, and 72 h. Plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control (time = 0). Analysis was by ANOVA followed by Dunnett's test. **B**, Western blot analysis using an anti-A₃ receptor polyclonal antibody of protein extracts from A172 cells treated with scramble (-) or with siRNA_{A3} (+) and cultured for 24, 48, and 72 h. Tubulin shows equal loading protein. **C**, densitometric quantification of A₃ receptor Western blot; plots are mean \pm S.E. values ($n = 5$); *, $P < 0.01$ compared with the control (24-h scramble-transfected cells). Analysis was by ANOVA followed by Dunnett's test. **D**, Western blot analysis of protein extracts from A172 cells treated with scramble (- siRNA_{A3}) or siRNA_{A3} (+ siRNA_{A3}) for 72 h and cultured without (lanes 1 and 4) or with 10 nM CI-IB-MECA (lanes 2 and 5) and 100 nM CI-IB-MECA (lanes 3 and 6) for 4 h in hypoxia. Bad shows equal loading protein. **E**, densitometric quantification of P-Bad Western blot presented in **D**; plots are mean \pm S.E. values ($n = 5$); *, $P < 0.01$ compared with the control (lanes 1 and 4). Analysis was by ANOVA followed by Dunnett's test. **F**, effect of A₃ receptor antagonist MRE 3008F20 on Bad phosphorylation in normoxia and in hypoxia. A172 cells were treated for 4 h without (0) or with MRE 3008F20 10, 100, and 1000 nM. **G**, densitometric quantification of P-Bad Western blot presented in **F**; plots are mean \pm S.E. values ($n = 3$); *, $P < 0.01$ compared with the control. Analysis was by ANOVA followed by Dunnett's test. **H**, Western blot analysis of protein extracts from A172 cells treated with scramble (- siRNA_{A3}) or siRNA_{A3} (+ siRNA_{A3}) for 72 h and then cultured in normoxia or in hypoxia for 6 h. Bad shows equal loading protein. **I**, densitometric quantification of P-Bad Western blot presented in **H**; plots are mean \pm S.E. values ($n = 5$); *, $P < 0.01$ compared with the control (lane 1). Analysis was by ANOVA followed by Dunnett's test.

by phosphorylating and inactivating the proapoptotic protein Bad (Datta et al., 1997; Cardone, 1998). Furthermore, it has been reported that Akt inactivation sensitizes human ovarian cancer cells to cisplatin (Hayakawa et al., 2000) and paclitaxel (Alvarez-Tejado et al., 2001; Mabuchi, 2002) and to paclitaxel in vivo (Hu, 2002). In addition, in many cell types, the Akt pathway is sufficient to promote survival, and the pharmacological blockade of Akt can suppress the ability of trophic factors to support survival (del Peso et al., 1997; Yamaguchi and Wang, 2001). These results suggest that paclitaxel-induced apoptosis involves the phosphorylation of Bad via an Akt cascade and that inhibition of this cascade in hypoxia sensitizes glioblastoma cells to paclitaxel.

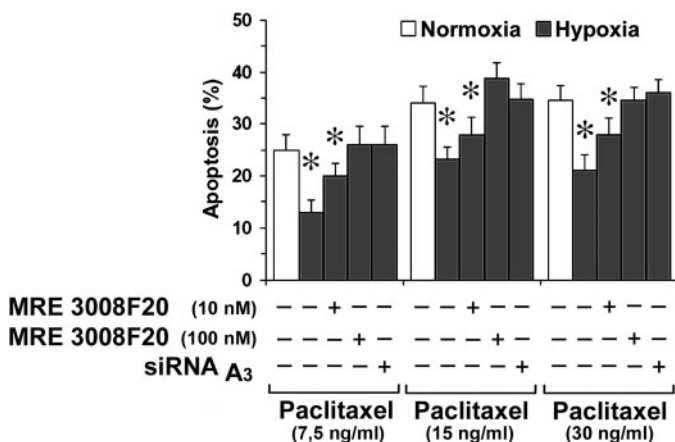


Fig. 6. Apoptosis of U87MG cells treated with 10 or 100 nM MRE 3008F20 or with siRNA_{A3} for 72 h and cultured with 7.5, 15, or 30 ng/ml paclitaxel in normoxia or in hypoxia. Apoptosis was assessed as the percentage of cells with a DNA content lower than that of cells in G₁/G₀. The data are the mean \pm S.E. values ($n = 4$); *, $P < 0.01$ compared with the control. Analysis was by ANOVA followed by Dunnett's test.

Because adenosine concentration is highly increased in hypoxia (Blay et al., 1997), we have investigated the involvement of the adenosinergic system. Thus, we verified that hypoxia increases adenosine concentration in cell culture medium. The concentration of adenosine reached in our in vitro model during hypoxia was relatively modest compared with micromolar concentrations reported in hypoxic tissues in vivo (Blay et al., 1997). However, these data agree with those recently reported in HMEC-1 cells and may be caused by the limited number of cells present in a monolayer culture and by the dilution of adenosine released into a relatively large volume of medium (Ryzhov et al., 2007). Nevertheless, hypoxia-induced Bad phosphorylation in glioblastoma cells was prevented with ADA, suggesting that adenosine was required. How can the nanomolar extracellular adenosine concentration activate adenosine receptors? The activation of adenosine receptors is promoted by the adenosine present next to the cell membrane. Furthermore, adenosine present in the small volume "wetting cell surface membrane" should be considered to be in a dynamic equilibrium binding with receptors. Because the cell is the source of extracellular adenosine (intracellular ATP and additional alternative pathways), adenosine concentration next to the cell membrane should be greater than far to the cell surface. Thus, in the small volume (respect to total cell culture volume) surrounding the cell membrane (1 μ l), we believe that in hypoxia, adenosine may be present in a gradient of concentrations decreasing up to 66 nM far from the cell. We have observed that 1×10^5 cells, seeded in 0.5 ml of medium, produced 33 pmol concentration of adenosine. In the presence of a gradient of concentration, in the volume of 1 μ l near to the cell, a small amount of adenosine (e.g., 10% of total secreted adenosine, that is, 3.3 pmol) should generate a concentration of 3.3 μ M. In conclusion, the flux of adenosine production

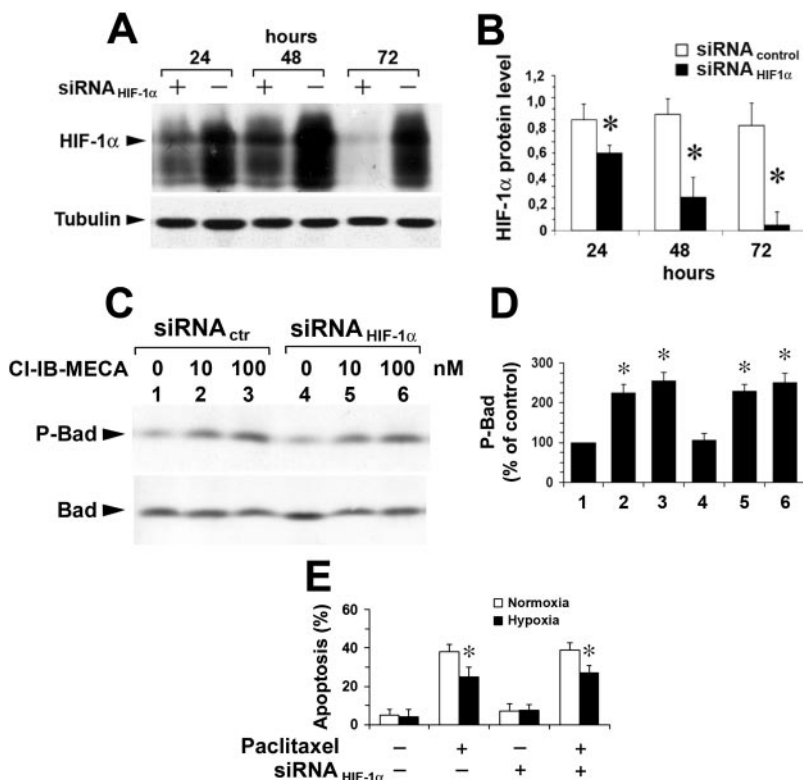


Fig. 7. A, HIF-1 α silencing by siRNA_{HIF-1 α} transfection. A, Western blot analysis of protein extracts from A172 cells transfected with siRNA_{HIF-1 α} (+) or with scramble (-) by RNAiFect transfection reagent and cultured for 24, 48, and 72 h in hypoxia. Tubulin shows equal loading protein. B, densitometric quantification of HIF-1 α Western blot; plots are mean \pm S.E. values ($n = 5$); *, $P < 0.01$ compared with the control (time 0). C, Western blot analysis of protein extracts from A172 cells treated with scramble (- siRNA_{HIF-1 α}) or siRNA_{HIF-1 α} (+ siRNA_{HIF-1 α}) for 72 h and cultured with 0, 10, and 100 nM CI-IB-MECA for 6 h in hypoxia. Bad shows equal loading protein. D, densitometric quantification of P-Bad Western blot presented in C; plots are mean \pm S.E. values ($n = 5$); *, $P < 0.01$ compared with the control (lane 1). Analysis was by ANOVA followed by Dunnett's test. E, the effect of the siRNA_{HIF-1 α} on apoptosis induced by 15 ng/ml paclitaxel. U87MG cells were incubated in normoxia or in hypoxia. The data are the mean \pm S.E. of four independent experiments. *, $P < 0.01$ compared with the control (absence of paclitaxel). Analysis was by ANOVA followed by Dunnett's test.

should generate a concentration range of adenosine able to activate adenosine receptors.

We also demonstrated the following: 1) adenosine increased P-Bad levels under hypoxia through Akt activation, which is protected from paclitaxel-induced apoptosis; 2) adenosine induced the phosphorylation of Bad through its A_3 receptor subtype activation; 3) A_3 receptor activation promoted Akt phosphorylation through PI3K activation; and 4) the pharmacological blockade of the A_3 receptor with an antagonist or the biochemical down-regulation of the receptor with specific siRNA abolished the increase in Bad phosphorylation induced by adenosine (Fig. 9).

Because Bad-phosphorylated levels are increased by adenosine, we suggest that adenosine signaling could be targeted in conjunction with paclitaxel to improve sensitivity of glioma cells when cultured under hypoxia. This hypothesis has been examined in detail treating the cells with multiple concentrations of paclitaxel and the A_3 receptor antagonist MRE 3008F20 under normoxia and hypoxia. The significance of these data are that the blockade of the A_3 receptor signaling was able to sensitize hypoxic glioblastoma cells to the effect of paclitaxel. These findings are consistent with previous findings in other cell lines, indicating that combination treatment with A_3 receptor antagonists plus paclitaxel is

more effective than treatment with paclitaxel alone (Merighi et al., 2003a).

One of these mechanisms of resistance to paclitaxel promoted by adenosine in glioblastoma cells might be the induction of Akt signaling by adenosine through A_3 adenosine receptor activation. In particular, the role of Akt in A_3 receptor signaling pathway is consistent with a consolidated literature, showing the involvement of Akt downstream from the A_3 receptor in different cell models (Gao et al., 2001; Schulte and Fredholm, 2002, 2003; Merighi et al., 2003b, 2005b).

Furthermore, with hypoxia as the central issue under study and the prior links between adenosine, hypoxia, and HIF-1 (Merighi et al., 2005a, 2006) and links between hypoxia, HIF-1, and Bad (Erler et al., 2004), we have investigated a possible role for the HIF-1 α subunit in Bad phosphorylation and in the hypoxic protection from the apoptosis induced by paclitaxel. The results indicate that the effect of hypoxia on Bad phosphorylation and on cell survival were mediated through the A_3 receptor in a manner independent of HIF-1 α . The role of HIF-1 α in contributing to resistance or enhancing cell death still is controversial. Our results are consistent with recent reports showing antiapoptotic effects of hypoxia, which are HIF-1-independent. Blocking HIF-1 activity through overexpression of an HIF-1 α dominant-negative mutant did not influence the protection induced by hypoxia of glucose starvation-induced apoptosis in HepG2 (Suzuki et al., 2005). Hypoxia-induced radioresistance is similar in HIF-1 α -deficient mouse embryonic fibroblasts than in wild-type cells (Erler et al., 2004). Hypoxia-induced drug resistance in human colon carcinoma cells occurs via down-regulation of Bid and Bax through HIF-1 α -dependent and -independent mechanisms (Erler et al., 2004). Hypoxia protects HepG2 cells against etoposide-induced apoptosis via an HIF-1-independent pathway (Piret et al., 2006). Another transcription factor, activator protein-1, was reported to have a potential role in the hypoxia-induced protection against apoptosis (Piret et al., 2006). Together, all of these data underscore the idea that hypoxia could mediate its antiapoptotic role via different transcription factors depending on the cellular context and proapoptotic stimuli, pointing to alternative hypoxia-induced survival pathways. Further investigations will reveal new molecules involved in regulating cell viability in hypoxic GBM cells after paclitaxel treatment.

In conclusion, our results and other reports on Bad phosphorylation in tissue culture models reveal an intricate network of signaling pathways (hypoxia-adenosine- A_3 receptor) that control Bad phosphorylation. The effects that are detected in our cellular model *in vitro* are biologically modest. However, it has been demonstrated that the role of Bad phosphorylation in tumors *in vivo* may be more prominent. It was reported that prostate tumors have increased Bad levels probably because of proliferation advantages that phosphorylated Bad gives to cancer cells (Royuela et al., 2001). Indeed, involvement of Bad in stimulating survival, glycolysis, and progression through the cell cycle was reported recently (Maslyar et al., 2001; Danial et al., 2003; Janumyan et al., 2003; Seo et al., 2004). Prompted by these observations, it is of interest to test whether adenosine receptor antagonists may sensitize gliomas to cytotoxic treatments *in vivo* by preventing Bad phosphorylation.

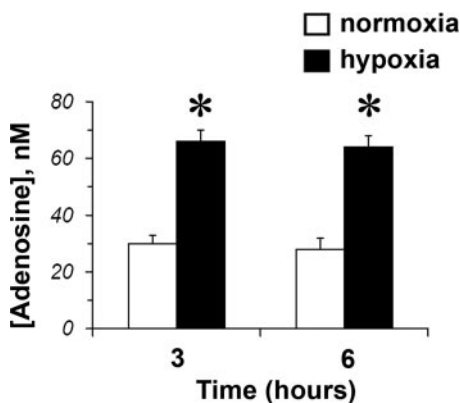


Fig. 8. Effect of hypoxia on release of adenosine from U87MG cells. Cells were incubated under hypoxia for 3 and 6 h. The data are the mean \pm S.E. of three independent experiments. *, $P < 0.01$ compared with the control normoxia. Analysis was by ANOVA followed by Dunnett's test.

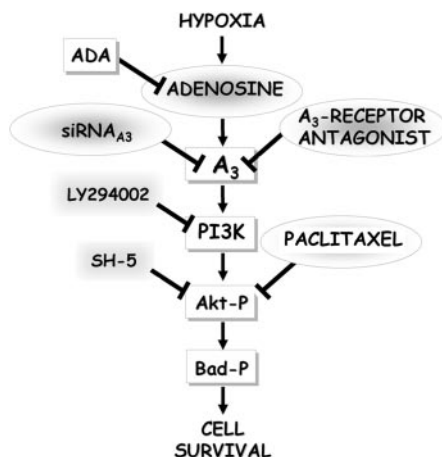


Fig. 9. Proposed model of hypoxia survival signaling. Hypoxia increases adenosine, which, through A_3 receptor stimulation, induces Akt activation that phosphorylates Bad and promotes cell survival.

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