Akt1 Activation Can Augment Hypoxia-Inducible Factor-1α Expression by Increasing Protein Translation through a Mammalian Target of Rapamycin–Independent Pathway

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Abstract

The phosphoinositide 3-kinase (PI3K)/Akt pathway is commonly activated in cancer; therefore, we investigated its role in hypoxia-inducible factor-1α (HIF-1α) regulation. Inhibition of PI3K in U87MG glioblastoma cells, which have activated PI3K/Akt activity secondary to phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mutation, with LY294002 blunted the induction of HIF-1α protein and its targets *vascular endothelial growth factor* and *glut1* mRNA in response to hypoxia. Introduction of wild-type PTEN into these cells also blunted HIF-1α induction in response to hypoxia and decreased HIF-1α accumulation in the presence of the proteasomal inhibitor MG132. Akt small interfering RNA (siRNA) also decreased HIF-1α induction under hypoxia and its accumulation in normoxia in the presence of dimethyloxallyl glycine, a prolyl hydroxylase inhibitor that prevents HIF-1α degradation. Metabolic labeling studies showed that Akt siRNA decreased HIF-1α translation in normoxia in the presence of dimethyloxallyl glycine and in hypoxia. Inhibition of mammalian target of rapamycin (mTOR) with rapamycin (10-100 nmol/L) had no significant effect on HIF-1α induction in a variety of cell lines, a finding that was confirmed using mTOR siRNA. Furthermore, neither mTOR siRNA nor rapamycin decreased HIF-1α translation as determined by metabolic labeling studies. Therefore, our results indicate that Akt can augment HIF-1α expression by increasing its translation under both normoxic and hypoxic conditions; however, the pathway we are investigating seems to be rapamycin insensitive and mTOR independent. These observations, which were made on cells grown in standard tissue culture medium (10% serum), were confirmed in PC3 prostate carcinoma cells. We did find that rapamycin could decrease HIF-1α expression when cells were cultured in low serum, but this seems to represent a different pathway. (Mol Cancer Res 2006;4(7):471–9)

Introduction

The phosphoinositide 3-kinase (PI3K)/Akt pathway is commonly activated in human cancers. One means of activating this pathway is through loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN), also known as MMAC-1 or TEP-1. This protein functions primarily as a lipid phosphatase to dephosphorylate the D-3 position of phosphoinositide phosphates, such as $PI(3,4,5)P_3$, to convert them to $PI(4,5)P_2$ (1). The PTEN tumor suppressor gene product therefore acts as an antagonist of PI3K signaling. Its loss leads to increased PI3K/Akt activity, which has been implicated in cell proliferation, adhesion, migration, invasion, and apoptosis (2-4). PTEN mutations are frequently found in melanomas, glioblastomas, and cancers of the endometrium and prostate (1, 5-7). The PI3K/Akt pathway may also be activated by amplification or mutation of the PI3K subunits, which sometimes occurs in glioblastomas and cancers of the breast and ovary (8-11). Akt overexpression has been reported in cancers of the ovary, breast, and thyroid (12, 13).

PI3K signaling has also been implicated in the regulation of hypoxia-inducible factor-1α (HIF-1α) by numerous groups (14-23). HIF-1 is a master transcription factor consisting of two subunits, the α subunit, which is induced by hypoxia, and the β subunit, which is expressed constitutively. HIF-1α binds to HIF-1β to transactivate target genes, including *vascular endothelial growth factor* (*VEGF*) and *glut1*, and various glycolytic enzymes that help cells adapt to hypoxia (24). HIF-1α has also been shown to be induced by numerous stimuli other than hypoxia, including insulin, insulin-like growth factors, epidermal growth factor, heavy metals, and HER-2/*neu* activation (18, 25-29). Introduction of wild-type PTEN into

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human glioblastoma cells has been shown to inhibit the hypoxia-induced up-regulation of HIF-1 target genes and expression of a luciferase reporter containing HIF-1-binding sites in the promoter (23). Conversely, activated Akt increased HIF-dependent transcription in a luciferase reporter assay. Another group found that the PI3K inhibitor LY294002 inhibited basal as well as growth factor– and hypoxia-induced expression of HIF-1α in human prostate carcinoma cells and that dominant-negative Akt blocked HIF-1-dependent gene transcription in a luciferase reporter assay (21). LY294002 also blocks HIF-1α induction in response to insulin, interleukin-1β, and epidermal growth factor (16, 19). The PI3K/Akt pathway has also been shown to be involved in the induction of HIF-1α by various metals, including vanadate, arsenite, and nickel (26-28).

Although a link between the PI3K/Akt pathway and HIF-1α has been proposed by numerous groups, others have suggested that Akt is not necessary for the induction of HIF-1α under hypoxia (30, 31). Because Akt activation is a common feature of human cancers and controversy persists regarding the role of Akt in HIF-1α regulation, we investigated the potential link between Akt activity and HIF-1α expression in greater detail using a variety of approaches to activate or block the PI3K/Akt pathway and then assessed the effects of these manipulations of HIF-1α expression. We also examined the effect of the mammalian target of rapamycin (mTOR) HIF-1α expression using small interfering RNA (siRNA) and the mTOR inhibitor rapamycin.

Results

U87MG glioblastoma cells were used to determine the effect of inhibition of the PI3K pathway on HIF-1α expression. Cells were treated with the PI3K inhibitor LY294002. This significantly blunted HIF-1α induction in response to both hypoxia and the hypoxia mimetic $CoCl_2$ (Fig. 1A). To show that attenuated HIF-1 target gene expression after inhibition of the PI3K pathway has biological significance, we examined the expression of *VEGF* and *glut1* mRNA, both well-known HIF-1 targets. Treatment with LY294002 resulted in a decrease in *VEGF* mRNA under normoxic conditions and decreased induction of this transcript under hypoxic conditions (Fig. 1B, compare *lanes 1* and *2* and *lanes 3* and *4*). A similar pattern was seen with *glut1* mRNA. The mRNA for HIF-1α itself was not decreased by the drug, indicating that the inhibition of HIF-1α protein expression seen in Fig. 1A was not due to suppression of HIF-1α mRNA expression. Similarly, activity of the *VEGF* promoter, which contains a HIF-1-binding element, was inhibited by LY294002 in U87MG cells (Fig. 1C).

As an alternate approach to inhibit the PI3K pathway, we transduced U87MG cells with recombinant adenovirus expressing either wild-type or phosphatase-dead PTEN. Previous work from our laboratory showed that this approach was highly effective in decreasing the level of phosphorylated Akt levels in U87MG and other cell lines (32). Similar to our results using the inhibitor LY294002, expression of wild-type PTEN significantly blunted the HIF-1α response to both $CoCl_2$ and hypoxia compared with controls treated either without virus or

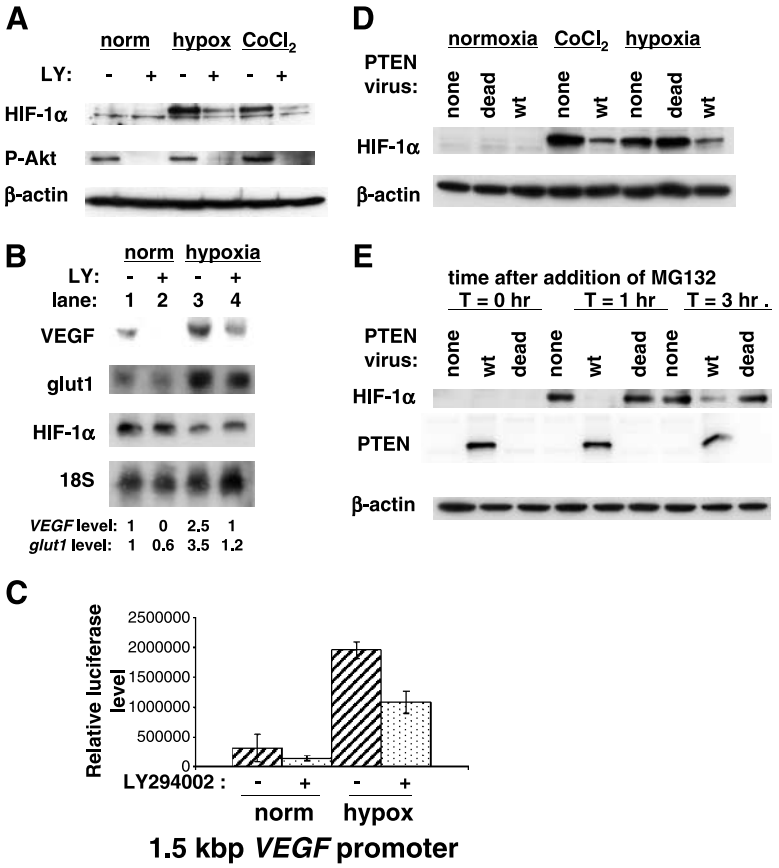

FIGURE 1. PI3K pathway regulates expression of HIF-1α protein and HIF-1α mRNA targets. **A.** U87MG cells were treated with LY294002 (10 μmol/L) for 16 hours and then exposed to hypoxia (0.2% O_2) or $CoCl_2$ (150 mmol/L). Three hours later, cells were harvested and Western blotting was done. **B.** U87MG cells were treated with LY294002 for 16 hours and then exposed to hypoxia (0.2% O_2) or kept in normoxia. Eight hours later, cells were harvested for RNA and Northern blotting was done. VEGF and glut1 levels (*bottom*) indicate ratio of *VEGF* mRNA or *glut1* densitometric quantitation to 18S quantitation. **C.** U87MG cells were cotransfected with a β-galactosidase-expressing plasmid along with a plasmid containing the 1.5-kbp VEGF promoter upstream of a luciferase reporter gene. Twenty-four hours after transfection, cells were treated with LY294002 and exposed to normoxia or hypoxia (0.2% O_2) for another 16 hours. Cells were then collected and assayed for luciferase and β-galactosidase activity. Y axis, normalized luciferase levels (ratio of luciferase to β-galactosidase readings). Columns, mean of three independent transfections; bars, SD. **D.** U87MG cells were transduced with adenovirus expressing wild-type (*wt*) PTEN or phosphatase-dead PTEN at a multiplicity of infection of 10. Thirty hours after infection, cells were exposed to hypoxia (0.2% O_2) or $CoCl_2$ (150 mmol/L). Three hours later, cells were harvested and Western blotting was done. **E.** Cells were infected with wild-type or phosphatase-dead PTEN-expressing adenovirus. Thirty hours after infection, cells were treated with proteasomal inhibitor MG132. Cells were harvested at different intervals following the addition of MG132 and Western blotting was done.

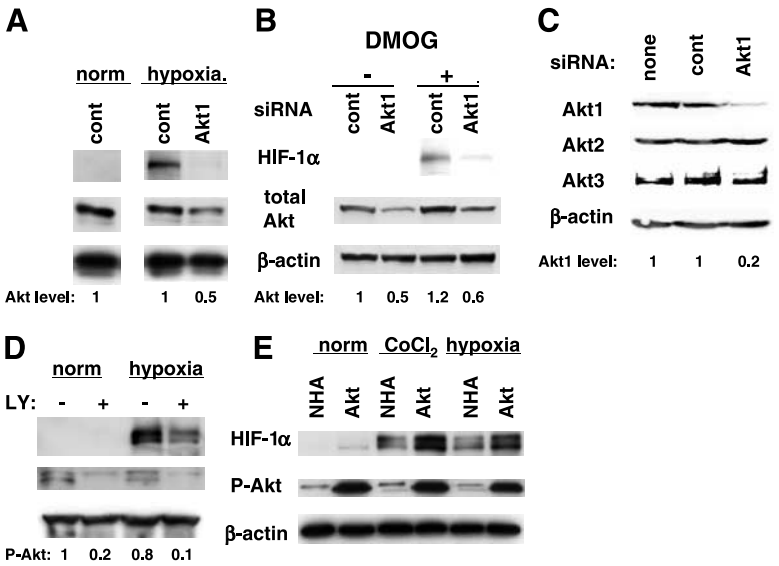

FIGURE 2. Akt regulates HIF-1α expression in U87MG glioblastoma cells and immortalized human astrocytes. **A** to **C.** U87MG cells were transfected with Akt1 or control (GFP) siRNA. Thirty hours after transfection, cells were exposed to hypoxia (0.2% O_2) or DMOG. Three hours later, cells were harvested and Western blotting was done for various proteins. **A** and **B.** Total Akt antibody was used. **C.** Antibodies directed against Akt1, Akt2, and Akt3 were used. Akt level refers to ratio of densitometric quantitation of Akt to β-actin band. **D.** NHA were treated with LY294002 (10 μmol/L) for 16 hours and then exposed to hypoxia (0.2% O_2). Three hours later, cells were harvested and Western blotting was done. **E.** Both NHA and NHA/Akt cells, which overexpress Akt, were exposed to hypoxia (0.2% O_2) or $CoCl_2$ (150 mmol/L). Three hours later, cells were harvested and Western blotting was done.

with control virus expressing phosphatase-dead PTEN (Fig. 1D). To determine the effect of PI3K inhibition on HIF-1α levels under normoxic conditions, PTEN adenovirus-transduced cells were treated with MG132, a proteasomal inhibitor. This was necessary because under normoxic conditions very little HIF-1α is detectable because of rapid degradation by the proteasomal pathway (33, 34). Within 1 hour of adding the drug, HIF-1α accumulation was seen in U87MG cells treated either without virus or with control (phosphatase-dead PTEN) virus (Fig. 1E). However, HIF-1α accumulation was severely diminished in cells treated with virus expressing with wild-type PTEN. Because MG132 inhibits HIF-1α degradation, the observation that PTEN decreased HIF-1α accumulation strongly suggests that it interferes with HIF-1α synthesis (gene transcription and/or protein translation) rather than its degradation.

To test whether the effects of LY294002 and PTEN on HIF-1α expression in U87MG cells seen in Fig. 1 were mediated through Akt, we used siRNA to inhibit Akt1 expression. Even a 50% decrease in total Akt blunted HIF-1α induction in response to hypoxia (Fig. 2A). Similar experiments were done under normoxia with cells treated with the prolyl hydroxylase inhibitor dimethyloxallyl glycine (DMOG), which prevents HIF-1α degradation (35). Here as well, reduced Akt expression led to decreased accumulation of HIF-1α (Fig. 2B). These results support the results obtained using the proteasomal inhibitor MG132 in Fig. 1 and support the notion that Akt1 inhibition interferes with HIF-1α synthesis. The prolyl hydroxylase inhibitor DMOG, although not specific for HIF-1α, should affect the stability of far fewer proteins than a proteasomal inhibitor, such as MG132. The siRNA we designed was chosen to target Akt1 specifically and not Akt2 or Akt3. To confirm that this was the case, we did Western blotting using lysates from U87MG cells transfected with Akt1 siRNA and then probed with antibodies specific for each of the three Akt proteins. Figure 2C shows that Akt1 siRNA decreased Akt1 but not Akt2 or Akt3 expression.

Inhibition of the PI3K/Akt pathway clearly attenuated HIF-1 induction in U87MG glioblastoma cells. We next asked whether there was an effect on nontransformed cells. These experiments were done using an immortalized normal human astrocyte line (NHA) that has been described previously (36, 37). Pretreatment of these cells with LY294002 blunted the hypoxic induction of HIF-1α (Fig. 2D). Conversely, NHA/Akt cells, which were derived from NHA cells and express constitutively active myristoylated Akt, displayed more robust induction of HIF-1α than did the parental NHA line in response to both hypoxia and $CoCl_2$ (Fig. 2E).

Several studies have suggested that the mTOR inhibitor rapamycin can decrease HIF-1α expression (18, 21, 38-40). However, we found that treatment of U87MG cells with 10 nmol/L of the drug had no effect on HIF-1α expression in response to either $CoCl_2$ or hypoxia (Fig. 3A). This dose of rapamycin was effective in inhibiting mTOR as confirmed by phosphorylation of $p70^{S6K}$, which is an established downstream target of mTOR. These experiments were repeated using a range of rapamycin doses (5-100 nmol/L) under normoxia in the presence of DMOG to inhibit HIF-1α degradation or under hypoxic conditions (Fig. 3B). The lowest dose of rapamycin tested (5 nmol/L) inhibited phosphorylation of both $p70^{S6K}$ and S6, another downstream target of mTOR. However, no significant inhibition of HIF-1α induction and no dose response to rapamycin between 5 and 100 nmol/L were observed. As an alternative, nonpharmacologic means of showing that mTOR is not essential for expression of HIF-1α, we again used siRNA gene silencing. This siRNA effectively knocked down mTOR protein expression and S6 phosphorylation; however, there was no effect on the induction of HIF-1α in response to hypoxia (Fig. 3C). The results of this experiment also suggest that hypoxia itself decreased S6 phosphorylation. To investigate this further, U87MG cells were subjected to a range of O_2 concentrations. Figure 3D shows that concomitant with the dose-dependent increase in HIF-1α expression there is a dose-dependent decrease in S6 phosphorylation with decreasing pO_2.

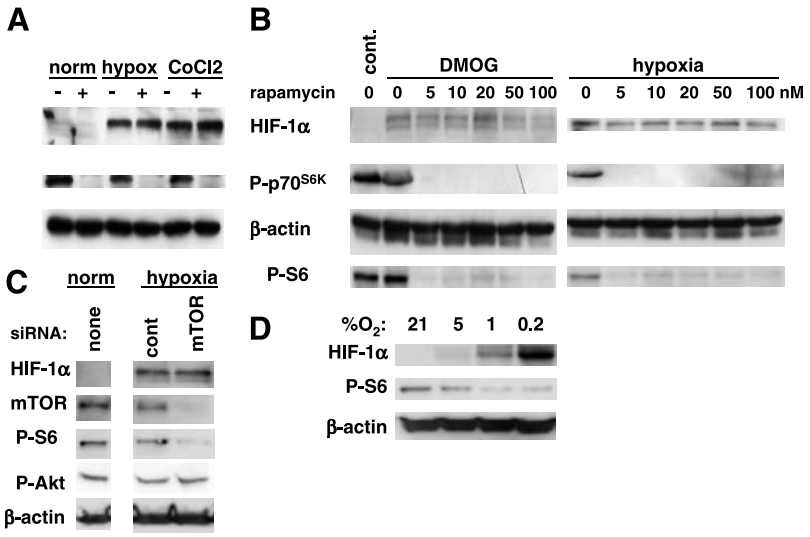

FIGURE 3. Inhibition of mTOR by rapamycin or siRNA does not alter HIF-1α expression. **A.** U87MG cells were treated with mTOR inhibitor rapamycin (10 nmol/L) for 16 hours and then subsequently exposed to hypoxia (0.2% O_2) or $CoCl_2$ (150 mmol/L). Three hours later, cells were harvested and Western blotting was done. **B.** U87MG cells were exposed to varying concentrations of rapamycin for 16 hours and then exposed to the prolyl hydroxylase inhibitor DMOG or hypoxia (0.2% O_2). Three hours later, the cells were harvested and Western blotting was done. **C.** U87MG cells were transfected with mTOR siRNA or control (GFP) siRNA. **D.** U87MG cells were exposed to different concentrations of O_2. Three hours later, samples were harvested for protein and Western blotting was done.

The data in Figs. 1E and 2B suggest that the effect PI3K/Akt inhibition has on HIF-1α levels is mediated by decreased synthesis rather than decreased stability. To directly investigate the possibility of an effect on protein translation, we did metabolic labeling analysis. U87MG cells were pulse labeled by [^{35}S]methionine/cysteine incorporation and then subjected to hypoxia (0.2% O_2) followed by immunoprecipitation of HIF-1α. ^{35}S-incorporated HIF-1α was clearly visible as a band. Cells treated with Akt1 siRNA showed decreased HIF-1α compared with cells treated with control siRNA or no siRNA (Fig. 4A). We verified that the siRNA was effective in decreasing Akt expression by resolving the lysates on a SDS-PAGE gel and immunoblotting for total Akt (40% decrease in Fig. 4A). Immunoblotting for β-actin confirmed that equal amounts of protein were present in lysates used for immunoprecipitation. This result indicates that down-regulation of Akt leads to decreased HIF-1α translation under hypoxia. Next, we determined whether Akt1 siRNA altered HIF-1α translation under normoxia. These experiments were done with DMOG added to stabilize HIF-1α. Our results showed that under normoxia Akt1 down-regulation also led to a decrease in HIF-1α translation (Fig. 4B). In contrast to the effects seen with Akt1 siRNA, rapamycin did not alter translation of HIF-1α, although the drug was clearly effective in decreasing phosphorylation of S6. This result was seen under both hypoxic and normoxic conditions (Fig. 4C). An obvious question that arises with these studies is whether hypoxia itself decreases HIF-1α translation. We tried to address this by exposing cells to either 5% or 0.2% O_2 or normoxia and performing the immunoprecipitations for HIF-1α. DMOG was added to the cells under normoxia to stabilize HIF-1α; otherwise, no HIF-1α would be recovered. Figure 4D shows that there is no appreciable difference in the level of newly synthesized HIF-1α in 5% versus 0.2% O_2. There may be a slight decrease in the amount of immunoprecipitated HIF-1α between normoxia and 5% O_2; however, this is certainly not dramatic and could be due to greater stability of the protein in DMOG than in 5% O_2.

To generalize our findings, we did similar experiments on other cell lines with either wild-type or mutant PTEN status. LY294002 blunted HIF-1α induction in response to hypoxia in U251MG glioblastoma cells (Fig. 5A), but 100 nmol/L rapamycin had no discernable effect on HIF-1α expression, although it did decrease the level of phosphorylated $p70^{S6K}$ (Fig. 5B). Rapamycin was used at two different concentrations, 10 and 100 nmol/L, in DU145 prostate carcinoma cells, SQ20B head and neck squamous cell carcinoma cells, and LN18 glioblastoma cells. These concentrations of rapamycin inhibited S6 phosphorylation, but the drug had no effect on the hypoxic induction of HIF-1α (Fig. 5C). It is interesting to note that in DU145 and LN18 hypoxia itself decreased S6 phosphorylation to such an extent that the addition of rapamycin had little further effect. However, in SQ20B, hypoxia caused only a slight decrease in S6 phosphorylation, and rapamycin led to a much greater decrease.

Further experiments were done on PC3 prostate carcinoma cells, which contain mutant PTEN. Treatment of PC3 cells with Akt1 siRNA decreased Akt protein expression by 60% and abolished the induction of HIF-1α in response to hypoxia (Fig. 6A). In contrast, mTOR siRNA decreased mTOR expression (by 70%) and S6 phosphorylation but had no effect on HIF-1α induction. We did ^{35}S-amino acid labeling studies on PC3 cells subjected to hypoxia. When Akt1 siRNA was used, the amount of newly translated HIF-1α was reduced (Fig. 6B). However, rapamycin had no effect on HIF-1α translation (Fig. 6C).

The experiments discussed above, which showed no effect of rapamycin on HIF-1α expression, were all done under standard (10%) serum conditions. In reviewing the published reports, we noticed that some of the articles showing a suppressive effect of rapamycin on HIF-1α expression used low serum for their experiments (15, 18, 21). Therefore, we repeated our experiments with low serum to see whether this made a difference. Figure 7A shows that in 0.1% serum the same dose of rapamycin that previously showed no effect on HIF-1α expression in 10% serum in U87MG glioblastoma cells now led to 50% decreased HIF-1α expression (compare *lanes 5* and *6*). In 2% serum, there was a slight effect of rapamycin on HIF-1α induction (compare *lanes 2* and *3*). In PC3 prostate carcinoma cells, no effect of rapamycin was seen in 10% serum,

but under 0.1% serum there was an 80% inhibition of HIF-1α induction with hypoxia (compare *lanes 10* and *11*). Likewise, with LN229 glioblastoma cells, there was an inhibitory effect on HIF-1α expression in 0.1% serum (*lanes 15* and *16*) but not in 10% serum (*lanes 13* and *14*). Therefore, rapamycin has differing effects on HIF-1α expression depending on the serum concentration.

Discussion

Numerous reports indicate that the PI3K/Akt pathway is involved in the regulation of HIF-1α expression (14-23). However, the importance of the PI3K/Akt pathway in the hypoxic induction of HIF-1α has been questioned by others (30, 31). Because of this controversy, we investigated this issue in greater detail. We started with U87MG glioblastoma cells in which the PI3K/Akt pathway is basally activated as a consequence of PTEN loss. Treatment of U87MG cells with the PI3K inhibitor LY294002 attenuated the hypoxic induction

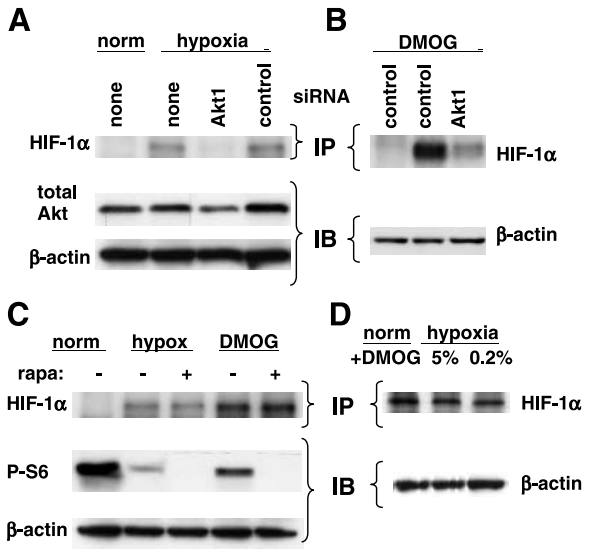

FIGURE 4. Akt regulates translation of HIF-1α independent of rapamycin. **A** to **D.** Pulse-labeling experiments to measure the translation of HIF-1α. **A.** U87MG cells were transfected with Akt siRNA or control (GFP) siRNA. Thirty hours after transfection, medium was replaced with [^{35}S]methionine/cysteine-free medium. One hour later, [^{35}S]methionine/cysteine was added to the medium, and cells were exposed to hypoxia (0.2% O_2). Three hours later, cells were harvested and lysates were immunoprecipitated with a HIF-1α antibody. **B.** Similar to **A**, except that following the addition of [^{35}S]methionine/cysteine, cells were kept in normoxia and DMOG was added to medium. **C.** U87MG cells were pretreated with rapamycin (10 nmol/L) for 2 hours and the medium was changed to [^{35}S]methionine/cysteine-free medium with rapamycin added. Thirty minutes later, [^{35}S]methionine/cysteine was added and cells were exposed to hypoxia (0.2% O_2). Three hours later, cells were harvested and lysates were immunoprecipitated with a HIF-1α antibody. **D.** U87MG cells were seeded and 24 hours later, medium was replaced with [^{35}S]methionine/cysteine-free medium. Thirty minutes later, [^{35}S]methionine/cysteine was added to dishes that were exposed to DMOG or hypoxia (5% or 0.2% O_2). Three hours later, cells were harvested and lysates were immunoprecipitated with a HIF-1α antibody. **A** to **D.** Immunoprecipitated proteins were electrophoresed, gel was vacuum dried, and autoradiography was done. Gel electrophoresis was also done using lysates obtained before immunoprecipitation. Equal amount of proteins were run on SDS-PAGE gel and Western blotting was done for β-actin to confirm that lysates contained equal amounts of protein and for P-S6 to confirm that rapamycin was effective in inhibiting mTOR.

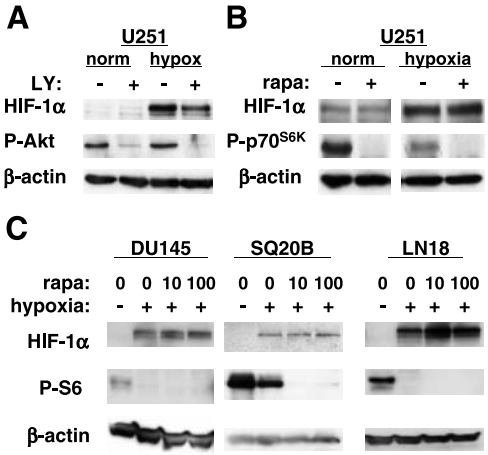

FIGURE 5. Effect of PI3K inhibition and rapamycin on HIF-1α expression in other cell lines Various cell lines (U251MG, DU145, SQ20B, and LN18) were treated with drugs LY294002 (10 μmol/L) or rapamycin (10 nmol/L) for 16 hours and then exposed to hypoxia (0.2% O_2). Three hours later, cells were harvested and Western blotting was done.

of HIF-1α as well as the induction of *VEGF* mRNA and *VEGF* promoter activity in response to hypoxia. Therefore, Akt activation augments the expression of HIF-1α and its target gene *VEGF* under hypoxia in this cell line. Furthermore, wild-type PTEN prevented HIF-1α accumulation, and inhibition of Akt with siRNA blunted the hypoxic induction of HIF-1α. We saw a similar effect with LY294002 in U251 MG cells and with Akt siRNA in PC3 prostate carcinoma cells. All three of these cell lines have PI3K/Akt activation as a result of PTEN loss. Our data with U87MG and PC3 cells show that Akt siRNA led to decreased HIF-1α translation under both normoxia and hypoxia. However, the effect is difficult to detect under normoxia unless HIF-1α degradation is pharmacologically inhibited with either a proteasomal inhibitor, such as MG132, or a prolyl hydroxylase inhibitor, such as DMOG. Hypoxia itself seemed to have a minimal, if any, effect on HIF-1α translation compared with that seen under normoxic conditions (Fig. 4D), which is consistent with what others have reported (41, 42). This is in contrast to the translation of many other proteins, which are suppressed by hypoxia (43, 44). From our data, we can conclude that activation of the PI3K/Akt pathway is responsible for increasing HIF-1α translation under both normoxia and hypoxia in these cells. LY294002 also blunted HIF-1α induction in NHA cells, which are immortalized astrocytes but are nontransformed. Therefore, the PI3K/Akt pathway can modulate HIF-1α expression even in a cell line in which this pathway is not activated secondary to genetic alterations. The PI3K/Akt pathway is not *required* for HIF-1α induction under hypoxia. However, Akt contributes to HIF-1α translation and can certainly amplify the induction of HIF-1α under hypoxia in many cell lines.

We found that rapamycin inhibited phosphorylation of the downstream targets $p70^{S6K}$ and S6 at 5 to 10 nmol/L. However, doses as high as 100 nmol/L had no significant effect on HIF-1α accumulation, either under normoxia (in the presence of DMOG), in hypoxia, or with $CoCl_2$ (Figs. 3B and C and 5B

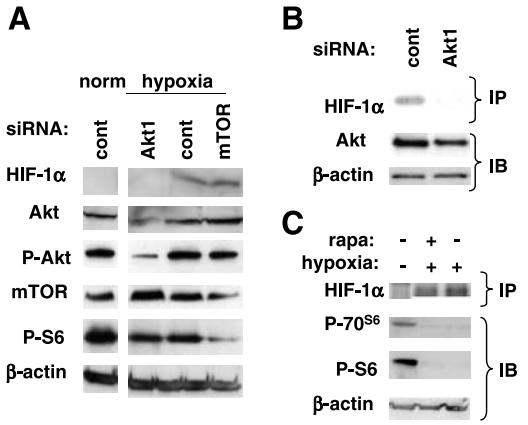

FIGURE 6. Effect of Akt siRNA or mTOR inhibition on HIF-1α expression in PC3 cells. **A.** PC3 cells were transfected with Akt1 siRNA, control (GFP) siRNA, or mTOR siRNA. Thirty hours after transfection, cells were exposed to hypoxia (0.2% O_2). Three hours later, cells were harvested for protein and Western blotting was done. **B.** PC3 cells were transfected with Akt1 siRNA or control (GFP) siRNA. Thirty hours after transfection, medium was replaced with [^{35}S]methionine/cysteine-free medium. One hour later, [^{35}S]methionine/cysteine was added to the medium and cells were exposed to hypoxia (0.2% O_2). Three hours later, cells were harvested and lysates were immunoprecipitated with a HIF-1α antibody. Immunoprecipitated proteins were electrophoresed, gel was vacuum dried, and autoradiography was done. Gel electrophoresis was also done using lysates obtained before immunoprecipitation. Equal amount of proteins were run on SDS-PAGE gel, and Western blotting was done for β-actin to confirm that lysates contained equal amounts of protein and for Akt to confirm that the siRNA was effective in down-regulating this protein. **C.** Similar to **B**, except that no siRNA was used, and cells were treated with rapamycin (10 nmol/L) 2 hours before addition of [^{35}S]methihionine/cysteine-free medium containing [^{35}S]methionine/cysteine and cells were exposed to hypoxia (0.2% O_2).

and C) under standard tissue culture conditions (10% serum). We used a variety of different cell lines with both PTEN loss (PC3, U251 MG, and U87MG) and wild-type PTEN (LN18 and DU145). As a nonpharmacologic approach, we used mTOR siRNA and found that the expression of HIF-1α under hypoxia or in normoxia in the presence of DMOG was not altered. Neither rapamycin nor mTOR siRNA altered HIF-1α translation as measured by metabolic labeling. Therefore, our results indicate that Akt can increase HIF-1α translation through a mTOR-*independent* pathway.

We found that rapamycin could inhibit HIF-1α expression, under low serum conditions, most strikingly in 0.1% serum. Several groups have shown an inhibitory effect of rapamycin on HIF-1α expression (21, 38-40). In contrast, other groups have reported that PI3K/Akt inhibition, but *not* rapamycin, decreases HIF-1α-mediated promoter transactivation in response to nickel compounds (28) or hypoxia (45). The simplest explanation for these seemingly contradictory results is that there are two possible pathways downstream of Akt that can modulate HIF-1α expression (Fig. 7B). Our results strongly suggest that under normal serum conditions there is a pathway that involves increased HIF-1α translation that is *independent* of mTOR. However, under low serum conditions, a rapamycin-sensitive pathway that can modulate HIF-1α expression seems to be unmasked. Hudson et al. found that overexpression of mTOR in PC3 cultured in 2% or 0.1% serum led to increased HIF-1α expression (15). The Tsc1/Tsc2 tuberous sclerosis complex is part of the Akt/mTOR pathway. Loss of Tsc2 leads to increased levels of HIF-1α, especially under low serum conditions (14). The increased level of HIF-1α in Tsc2$^{-/-}$ cells compared with Tsc$^{+/+}$ cells was not evident at 3 hours but could only be seen after *prolonged* hypoxia (≥8 hours) and could be decreased by rapamycin (46). The mechanism by which rapamycin regulates HIF-1α expression under low serum conditions was not explored in our study. However, other studies have implicated altered HIF-1α stability in PC3 cells overexpressing mTOR (15) and altered HIF-1α translation in renal cell carcinoma cells with VHL loss (40).

Often, it is assumed that Akt must exert its effect on HIF-1α by altering its translation via mTOR (18, 21). However, this is difficult to reconcile with the observations that (*a*) Akt activation is associated with *increased* HIF-1α translation even under hypoxia and (*b*) hypoxia suppresses the mTOR signaling pathway. Our data show that (*a*) is true in U87MG and PC3 cells because down-regulating Akt1 using siRNA led to decreased HIF-1α translation. Our data and that of other groups suggest that (*b*) is also true. We found that p70^{S6K} and S6 phosphorylation were suppressed by hypoxia. Decreased phosphorylation of components of the mTOR signaling pathway, including S6, p70^{S6K}, 4E-BP1, and mTOR itself, in response to hypoxia has been shown by others (14, 46, 47). Kaper et al. have recently proposed that loss of PTEN leads to activation of the PI3K/Akt/mTOR pathway and attenuates the hypoxia-induced inhibition of mTOR activity (48). This may be true, but we found that U87MG and PC3 cells, both of which have PTEN mutation, still showed significant decrease in S6 phosphorylation in response to hypoxia. The mTOR signaling cascade is responsible for regulating cap-dependent mRNA translation (49, 50) by phosphorylating p70^{S6K} and the translational repressor 4E-BP1, allowing the formation of functional eIF-4F complexes, which promotes translational initiation of 5′ cap (7-methyl GTP) mRNA (50). Given that the mTOR signaling pathway is suppressed by hypoxia, it seems inconsistent that this pathway would be responsible for mediating increased HIF-1α translation by Akt under low O_2 concentrations. Clearly, not all RNAs are translated through the mTOR pathway under all conditions because RNAs have been identified whose translation is unaffected or even induced following rapamycin treatment (51). In particular, HIF-1α has been reported to have an internal ribosomal entry site, allowing for cap-independent translation (41), which is a potential explanation for its translation being rapamycin insensitive under normal serum conditions in our studies.

In summary, we have shown that activation of Akt leads to increased HIF-1α expression in tumor cells through increased HIF-1α protein translation under both hypoxic and normoxic conditions and that this can occur through a mTOR-independent pathway. Of note, others have also suggested that there is a pathway downstream of PI3K but independent of mTOR that can regulate protein translation (52).

Materials and Methods

Tissue Culture and Reagents

U87MG, U251MG, LN18, PC3, SQ20B, DU145, NHA, and NHA/Akt cells were cultured in DMEM (4,500 mg/L glucose;

Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and grown in an incubator with 5% CO_2 and 21% O_2 or under hypoxic conditions as described below. NHA are immortalized normal human astrocytes, and NHA/Akt have myristoylated Akt introduced into these cells. The details by which NHA and NHA/Akt cells were created has been described previously (36).

Northern Blot Analysis

Total RNA was isolated with RNazol (Life Technologies) using the manufacturer's directions. RNAs (10-15 μg) were denatured with formaldehyde and formamide and run on a 0.9% agarose gel containing formaldehyde. RNA was transferred by capillary action in 20× SSC [1× SSC is 0.15 mol/L NaCl, 0.15 mol/L sodium citrate (pH 7)] to a Duralon-UV membrane (Stratagene, La Jolla, CA) and UV cross-linked before hybridization. Labeling of radioactive probes was done using [^{32}P]dCTP and a Prime-It kit (Stratagene) using the manufacturer's directions. Hybridization was carried out at 65°C, after which the membranes were washed with 0.1× SSC, 0.1% SDS at 65°C. Autoradiography was carried out at −80°C with intensifying screens. A 200-bp VEGF cDNA fragment was excised with *Eco*RI from the pGEMh204 plasmid (gift from B. Berse, Boston University School of Medicine, Boston, MA). A 2.47-kbp human *glut1* cDNA fragment (from Dr. M. Birnbaum, University of Pennsylvania, Philadelphia, PA) was used to make radioactive probes. To verify equal loading between lanes, all gels were stained with ethidium bromide and the membranes were probed with a DNA fragment of the 18S rRNA.

Protein Extraction and Western Blot Analysis

For protein isolation, cells were trypsinized and washed once in PBS. The pellets were then solubilized in 1× sample lysis buffer [0.3-0.5 mL; 2% SDS, 60 mmol/L Tris (pH 6.8)], boiled for 5 minutes, and passed repeatedly through a 26-gauge needle. Samples were centrifuged at 10,000 × *g* and the supernatants were retained. Protein concentrations were determined using a BCA Protein Assay kit (Pierce, Rockford, IL).

For Western blotting, equal amounts of total protein were run in each lane of an SDS-PAGE gel (12% acrylamide). Each protein sample was mixed with an equal volume of 2× Laemmli buffer and boiled at 95°C for 5 minutes before loading onto the gel. After completion of gel electrophoresis, protein was transferred to a Hybond nitrocellulose membrane (Amersham, Pharmacia, Piscataway, NJ) over 1 hour using a blotting apparatus. For detection of the phosphorylated form of Akt protein, we used a monoclonal antibody that recognized phosphorylated Ser^{473} on Akt (New England Biolabs, Ipswich, MA) followed by a goat anti-mouse antibody (Amersham). As a loading control, the blot was reprobed with an anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) at a 1:1,000 dilution followed by a goat anti-mouse antibody (Bio-Rad, Hercules, CA) at a dilution of 1:500. The anti-HIF-1α antibody (Labvision, Fremont, CA) was used at 1:1,000 dilution followed by a secondary goat anti-mouse antibody (1:2,000). The following antibodies were supplied by Cell Signaling (Danvers, MA) and used at a 1:1,000 dilution: total Akt, Akt2, Akt3, phosphorylated Akt (Ser^{473}) mTOR, phosphorylated S6, and phosphorylated p70^{S6K}. For all of these, the secondary antibody used was goat anti-rabbit (Amersham). A mouse monoclonal antibody against Akt1 was also used (Cell Signaling). For this, the secondary antibody used was goat anti-mouse (Amersham).

Plasmid Constructs, Transient Transfections, and siRNA

Construction of the luciferase reporters with the 1.5-kb VEGF promoter (53) and the glut1 promoter have been described previously (54). Transfections were done using Fugene (Roche, Nutley, NJ) according to the manufacturer's

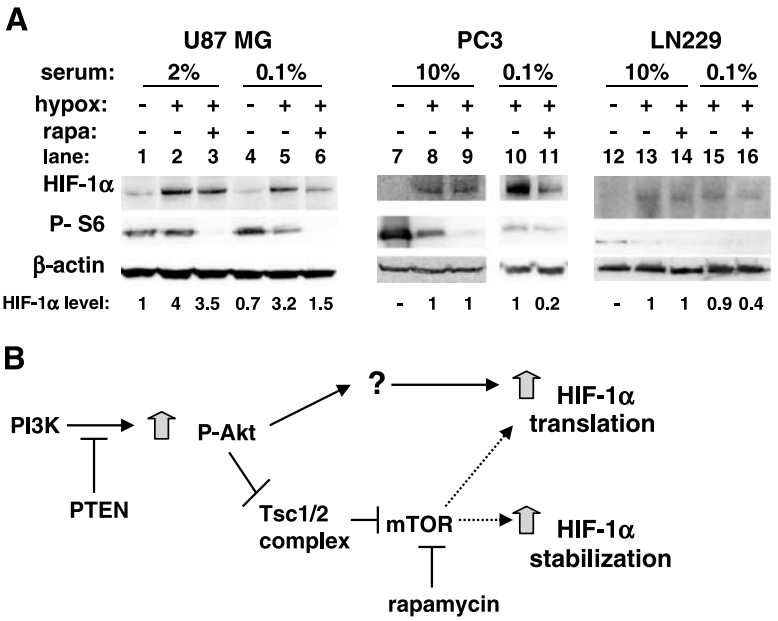

FIGURE 7. Rapamycin decreases hypoxic induction of HIF-1α in low serum. **A.** U87MG, PC3, or LN229 cells were seeded in standard medium (10% serum). The next day, medium was replaced with medium containing low serum (2% or 0.1%) with or without rapamycin (10 nmol/L). Sixteen hours later, cells were subjected to hypoxia (0.2% O_2). Three hours later, cells were harvested for protein and Western blotting was done. HIF-1α level refers to ratio of HIF-1α densitometric intensity to β-actin intensity. **B.** Overview of the PI3K/Akt/mTOR pathway. Akt activation can increase HIF-1α protein translation via a mTOR-independent pathway. However, HIF-1α expression can also be modulated by mTOR, particularly under low serum conditions. This occurs by either altered translation or stability (*dotted arrows*).

instructions. Briefly, cells were split into 60 mm dishes so that 24 hours later they were ~50% confluent. At this time, each dish was transfected using 6 μL Fugene and 2 μg reporter plasmid and, to control for transfection efficiency, 1 μg pSV-β-galactosidase (Promega, Madison, WI). Cells were harvested by removing the medium, washing twice with PBS, and directly adding 100 μL lysis buffer per dish. Of this lysate, 80 μL were used for luciferase determinations and 10 μL were used for β-galactosidase assays. These determinations were done using the LucLite kit (Perkin Elmer, Wellesley, MA) and the β-galactosidase Enzyme Assay System (Promega). Luciferase readings were done on a TopCount Microplate Scintillation and Luminescence Counter (Perkin Elmer).

siRNA (RNA interference) was prepared by Dharmacon Research (Lafayette, CO). The targeted sequence used to silence transcription of Akt1 was 5′-GGAGGGUUGGCUGCACAAA-3′. For mTOR, the RNA interference sequence was 5′-CCCUGCCUUUGUCAUGCCU-3′. The RNA interference targeting sequence for green fluorescent protein (5′-GGCTACGTC-CAGGAGCGCACC-3′) mRNA was used as a negative control. RNA interference was transfected into cells by Oligofectamine (Invitrogen) according to the manufacturer's instructions. For both Akt1 and mTOR siRNA, 600 pmol/60 mm dish was used.

Adenovirus

The creation and use of wild-type and phosphatase-dead PTEN adenovirus has been described previously (32).

Translation Studies

A total of 3×10^5 cells were plated in a 60 mm dish, and 24 hours later, the cells were either transfected with siRNA [green fluorescent protein (GFP) or Akt] or rapamycin (10 nmol/L). After 24 to 48 hours of the respective treatments, cell culture medium was replaced with [^{35}S]methionine/cysteine-free DMEM containing 5% serum for 30 minutes. Thereafter, [^{35}S]methionine/cysteine was added to a final concentration of 0.2 mCi/mL, and the cells were pulse labeled for 2 hours under hypoxia or in the presence of DMOG and then harvested. An equal amount of protein extract was precleared with 60 μL protein A-Sepharose for 1 hour and thereafter immunoprecipitated with anti-HIF-1α antibody H1α67 at 4°C for overnight. Immunoprecipitates were isolated with protein A, and the beads were washed four times with buffer. Finally, beads were resuspended in 1× SDS-PAGE loading buffer [50 μL; 0.06 mol/L Tris-HCl (pH 8.0), 1.71% SDS, 6% glycerol, 0.1 mol/L DTT, 0.002% bromophenol blue] and boiled at 95°C. The released proteins were separated on 12% SDS-PAGE gel and autoradiographed.

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