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AMP-Dependent Protein Kinase Alpha 2 Isoform Promotes Hypoxia-Induced VEGF Expression in Human Glioblastoma

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ABSTRACT
Tumor cells respond to hypoxic stress by upregulating a variety of genes involved in glucose uptake, glycolysis, and angiogenesis, all essential to maintaining nutrient availability and intracellular ATP levels. Adenosine monophosphate-dependent kinase (AMPK) is a key sensor for cellular homeostasis and is highly sensitive to changes in AMP:ATP ratios. The two catalytic AMPK alpha isoforms (AMPKα1, AMPKα2) were investigated with respect to their expression, cellular distribution, and contribution to VEGF expression under hypoxic stress in human U373 glioblastoma cells. Quantitative real-time PCR analysis showed AMPKα1 mRNA to be constitutively expressed in normoxia and hypoxia, whereas AMPKα2 mRNA levels were low in normoxia and significantly induced in hypoxia. Fluorescent immunohistochemistry showed that AMPKα2 protein redistributed to the nucleus under hypoxia, whereas AMPKα1 remained distributed throughout the cell. The AMPK chemical inhibitor, 5-iodotubericidin, effectively repressed the hypoxic induction of VEGF mRNA levels and hypoxia inducible factor-1 dependent transcription. AMPKα2 repression with RNA interference reduced hypoxia-induced VEGF mRNA and HIF-1 transcription, whereas AMPKα1 repression did not. Human glioblastoma cell lines U118 and U138 also showed hypoxia-induction of AMPKα2 as well as VEGF. Immunohistochemistry analysis of human astrocytoma/glioma samples revealed AMPKα2 present in high grade gliomas within hypoxic pseudopalisading microenvironments. These data suggest that prolonged hypoxia promotes the expression and functional activation of AMPKα2 and VEGF production in glioma cell lines and glioblastoma multiform tumors, thus contributing to tumor survival and angiogenesis in high grade human gliomas.

INTRODUCTION

Hypoxia plays a critical role in regulating tumor growth and angiogenesis. The cellular response to hypoxia is adaptive and necessary to maintain the minimal energy levels required for cell survival. Once a tumor reaches a volume greater than a few mm^3, regions of hypoxia begin to occur and neovascularization is essential for tumor survival (Folkman et al., 1989). Angiogenesis, the growth of new blood vessels from pre-existing blood vessels to supply the tumor with oxygen and nutrients, is regulated by a number of angiogenic factors (Claffey et al., 1996; Dvorak, 2000). An important angiogenic factor is vascular endothelial growth factor-A (VEGF), which is over expressed in response to environmental hypoxia (Denko et al., 2003; Michiels, 2004). Human glioblastomas are among the most highly vascularized tumors and express high levels of VEGF (Kaur et al., 2004). A primary mechanism of increased VEGF expression is through the activation of hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor (Semenza, 2000). Under normoxia, HIF-1α protein levels are regulated by ubiquitination and rapid proteosomal degradation (Maxwell et al., 2001; Maxwell and Ratcliffe, 2002). In hypoxic conditions, HIF-1α degradation is inhibited; HIF-1α is stabilized, translocates from the cytoplasm to the nucleus, and dimerizes with HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT). This complex associates with coactivators, such as p300/CREB, to induce transcription of genes containing hypoxia responsive elements (HREs), such as VEGF (Fedele et al., 2002; Lee et al., 2004).

AMP-dependent kinase or AMP-activated kinase (AMPK) is a heterotrimeric protein composed of a catalytic alpha subunit, and two regulatory subunits, beta and gamma. The alpha subunit contains a serine/threonine protein kinase catalytic domain (Carling, 2004). AMPK has been identified as a primary sensor of cellular energy change by responding to increases in AMP:ATP ratios, concurrent with hypoxia or nutrient depletion (Hardie, 1999; Hardie and Carling, 1997). AMPK is activated by the increase in AMP and in turn activates catabolic pathways and provides general repression of anabolic pathways by direct phosphorylation of downstream substrates, resulting in cellular protection from metabolic or nutritional stress (Hardie et al., 2003). Previous reports have shown that AMPK is activated in hypoxia (Frederich et al., 2005; Lee et al., 2003; Marsin et al., 2002; Nagata et al., 2003).

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TABLE 1. qRT-PCR Primers

Target	Forward Primer (5′ – 3′)	Reverse Primer (5′ – 3′)
huCyclophilinA	CTGGACCCAACACAAATGGTT	CCACAATATTCATGCCTTCTTTCA
huAMPKα1	AGGAGAGCTATTTGATTATATCTGTAAGAATG	ACACCAGAAAGGATCTGTTGGAA
huAMPKα2	CGGCTCTTTCAGCAGATTCTGT	ATCGGCTATCTTGGCATTCATG
huVEGF	CGAGGGCCTGGAGTGTGT	GGCCTTGGTGAGGTTTGATC

Repression of total AMPKα with chemical inhibitors or dominant negative isoform over expression represses HIF-1 dependent transcription (Hwang et al., 2004; Lee et al., 2003). However, there are two distinct AMPK alpha isoforms—AMPKα1 and AMPKα2. Although highly homologous, it is becoming clear that AMPKα1 and AMPKα2 have exclusive functions. Mouse genetic deletion studies have shown that AMPKα2-null mice demonstrate glucose intolerance and reduced insulin sensitivity, while the AMPKα1-null mice do not show any appreciable alterations (Viollet et al., 2003a; Viollet et al., 2003b). However, the potential roles for the selective AMPK alpha isoform regulation and activity in response to hypoxia have not been clearly evaluated. The studies performed here investigated the role of AMPK in the response to hypoxia in U373 glioma cells and whether the catalytic alpha isoforms of AMPK show differential gene expression or functional roles in this response. RNAi technology targeting AMPK alpha isoforms selectively repressed protein levels and isoform specific activities. These studies indicate that AMPKα2, but not AMPKα1, is selectively induced in hypoxic conditions and significantly contributes to VEGF expression in human glioma cells as well as in high grade glioblastoma tumors.

MATERIALS AND METHODS

Cell Line and Culture Conditions, Antibodies, and Chemical Effectors

Human glioma cell lines, U373, U118, U138, and U87, were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, and penstrep (100 ug/ml). Cells were cultured either under normoxic conditions (5% CO2, 21% O2, 74% N2) or hypoxic conditions (5% CO2, 2% O2, 93% N2) as determined previously to be effective for maximal activation of VEGF expression without cytotoxicity through 48 h treatment (Claffey and Robinson, 1996; Shih et al., 1999). Antibodies used for immunoblots: AMPKα1 and AMPKα2 were from US Biological (Swampscott, MA), purchased p-AMPK(Thr172) and phospho-p70S6K(Thr389) from Cell Signaling Technology (Beverly, MA), and β-actin from Abcam (Cambridge, MA). Chemical effectors AICAR (5-Aminoimidazole-4-carboxamide 1-b-D-ribofuranoside) and 5-iodotubericidin were purchased from Sigma-Aldrich (St. Louis, MO).

Northern Blot Analysis

Total RNA was extracted using RNeasy RNA extraction kit (Qiagen, Chatsworth, CA). Northern blot was performed as described previously (Claffey et al., 1998; Hong et al., 2003). Hybridization was carried out overnight at 65°C with [α^{32}P]dCTP-labeled human VEGF 165 Acc I/Nco I fragment (823 base pairs) and an [α^{32}P] dCTP-labeled human Glut-1 probe BamH I/BamH I fragment (2473 base pairs) (ATCC, Rockville, MD). A ribosome-associated protein cDNA probe, 36B4, was used as a loading control. Blots were washed at high stringency (1% SDS, 1X SSC at 60°C) and exposed to Kodak MR film. Quantification was determined by densitometry using ImageQuant Software.

RNA Interference

siRNA oligos were obtained from Dharmacon (Lafayette, CO) and were used to specifically target either AMPKα1 or AMPKα2 subunits of the AMPK heterotrimer. RNAi transfections were performed with Oligofectamine (Invitrogen) in OptiMEM according to manufacturer's protocols at various concentrations. Post-transfection, cells were allowed to recover overnight and refed 24 h before treatment with normoxic or hypoxic conditions. RNAi experiments were performed at least three times to assure representative results.

SDS-PAGE and Immunoblots

Cytoplasmic extracts were obtained as described previously (Claffey et al., 1998). Protein extracts were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed using various primary antibodies and horseradish peroxidase-conjugated species appropriate IgG secondary antibodies diluted in blocking buffer according to manufacturer's protocols. Blots were developed using ECL reagents.

VEGF ELISA Assay

VEGF capture ELISAs were performed as described previously (Shih et al., 1999). Culture supernatants in triplicate were collected from cell culture wells and cleared by centrifugation. The samples were buffered with Tris-HCl pH 7.5 to a final concentration of 1 mM prior to analysis.

Quantitative Real-Time PCR

Cellular total RNA was harvested using RNeasy kit (Qiagen) and reverse transcribed with random hexamer primers using PowerScript RT Strips (BD Biosciences, Palo Alto, CA). Quantitative PCR primers were designed using ABI Primer Express software for use with a SYBER Green detection kit (Qiagen). Sequences of qRT-PCR primers are in Table 1.

Samples in duplicate or triplicate were run on an ABI 7900 thermocycler for 40 cycles, and results were ana-

lyzed using GraphPad software using a mathematical model described by Lui and Saint (Liu and Saint, 2002). Results were normalized to an internal control gene, huCyclophilin A.

Luciferase Assay

Cells were transfected with siRNA oligos, as described above, along with a 5X HIF-1 promoter element luciferase reporter (Claffey et al., 1998), luciferase control vector, or CMV driven beta-galactosidase vector, and treated with either normoxia or hypoxia for 24 h. Cells were harvested with a passive lysis buffer from assay kit, and luciferase activity was measured according to supplier's instructions (Promega, Madison, WI). Luciferase activity was normalized to beta-galactosidase expression as determined with a similar kit (Promega).

Immunofluorescence

Cells were grown on coverslips prior to exposure to normoxia or hypoxia. Cells were washed with PBS and fixed with 10% formalin in PBS. Cells were incubated in 0.01% Triton X-100 detergent for 3 min, washed in PBS, and blocked in 3% BSA/PBS at RT for 1 h. Primary antibodies were added at a 1:50 dilution (5 ug/ml) in 3% BSA/PBS and incubated at RT for 1 h. Cells were washed and a secondary antibody at a 1:800 dilution was added and incubated for 30 min at RT. Finally, coverslips were washed thoroughly with PBS and mounted onto slides with PBS:Glycerol.

Human Glioma Sample Immunohistochemistry

Paraffin sections were received from Tom Mikkelsen at the Hermelin Brain Tumor Center at Henry Ford Hospital in Detroit, Michigan. Slides were processed after antigen retrieval in sodium citrate buffer pH 6.0 for 20 min at 95°C. Slides were blocked with PowerBlock (Biogenex, San Ramon, CA) for 10 min; and a 1:200 dilution primary antibody, anti-AMPKα1 or AMPKα2 (US Biological), was incubated overnight at 4°C. Biotinolyated secondary was incubated for 30 min at RT and slides were developed using ABC (Vector Laboratories, Burlingame, CA) and DAB (Electron Microscopy Sciences, Hatfield, PA) reagents. Methyl green was added as a counter stain. Slides were imaged using white light microscopy on a Zeiss Axioplan microscope and quantified using ImagePro Plus software (MediaCybernetics, Silver Spring, MD).

Statistical Analysis

Data from individual experiments were represented as mean ± standard error unless otherwise stated. Statistical comparison of groups was performed using a 2-tailed Student's *t*-test with appropriate tests for equal variances. Statistical significance was defined and indicated as $P < 0.05$ (*) or $P < 0.01$ (**).

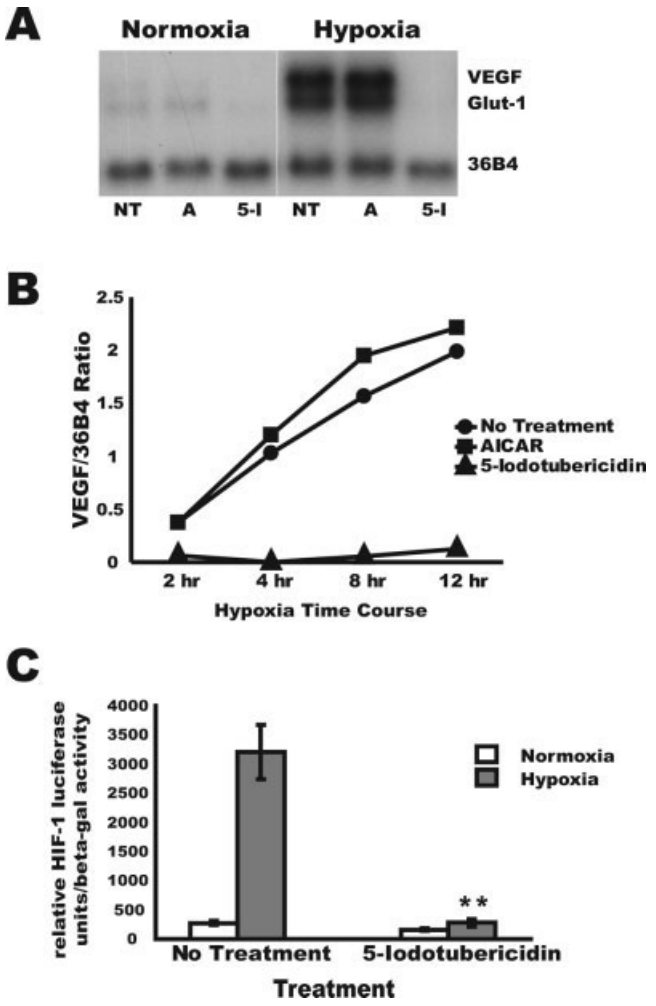

Fig. 1. Chemical modulators of AMPK affect hypoxia induced VEGF expression. U373 cells incubated in Normoxia or Hypoxia treated with 0.5 mM AICAR (A), 10 μM 5-iodotubercidin (5-I), or no treatment (NT). **A**: VEGF, Glut-1, and 36B4 mRNA expression determined by northern blot. **B**: Hypoxia time course of VEGF mRNA normalized to 36B4 mRNA expression. **C**: HIF-1 dependent luciferase expression normalized to beta-galactosidase transfection control from cellular lysates. (**$P \leq 0.01$.)

RESULTS

Chemical Modulators of AMPK Affect Hypoxia Induced VEGF Expression

To determine if AMPK has a role in the regulation of the hypoxia-inducible gene, VEGF, the AMPK activating agent, 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), and an AMPK inhibitor, 5-iodotubericidin, were used to treat human U373 glioblastoma cells under normoxic and hypoxic conditions. Cells were treated with AICAR, 5-iodotubericidin, or no treatment control and exposed to hypoxia for 2, 4, 8, and 12 h. The treated cells were harvested for total RNA and northern blots performed to analyze VEGF and Glut-1 mRNA expression levels, Fig. 1A. No treatment controls showed a large increase in VEGF mRNA in hypoxia as compared to normoxia at 12 h. The addition of AICAR (0.5 mM) showed increases over the hypoxic induction of VEGF and Glut-1

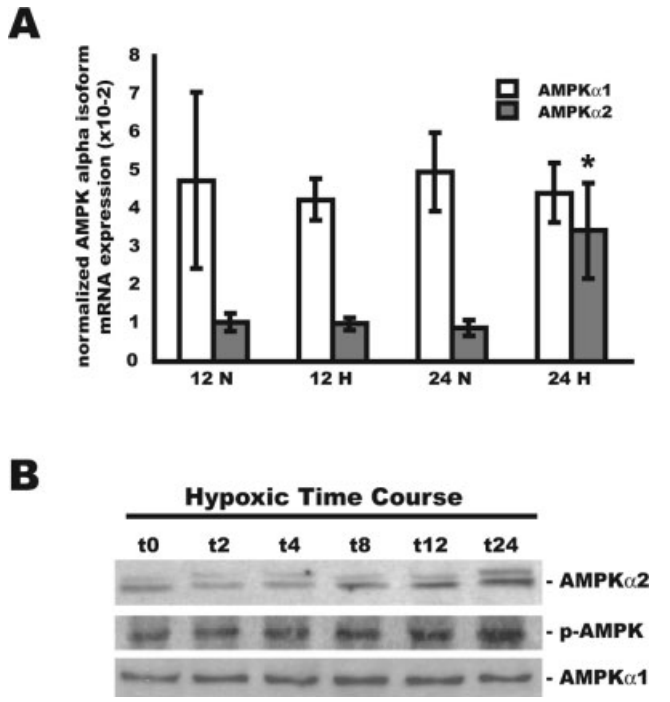

Fig. 2. AMPKα isoforms are differentially expressed under hypoxic conditions. **A**: AMPKα1 (white bars) or AMPKα2 (dark bars) isoform mRNA expression as determined by qRT-PCR from U373 cells incubated in normoxia (N) or hypoxia (H) over 12 and 24 h periods. (*$P \leq 0.05$.) **B**: Immunoblots on cellular lysates exposed to a hypoxia time course detected for AMPKα1, AMPKα2, and p-AMPK.

mRNAs as compared to vehicle control. The addition of 5-iodotubercidin (10 μM) completely repressed the hypoxic expression of VEGF and Glut-1 mRNAs. In time course analysis, 5-iodotubercidin effectively repressed VEGF mRNA levels from 2 to 12 h in hypoxia, Fig. 1B.

To evaluate the potential influence of AMPK on HIF-1 mediated transcription, required for VEGF mRNA induction under hypoxic conditions, an HIF-1 dependent transcriptional assay was performed with the AMPK inhibitor, 5-iodotubericidin. Transcriptional activity was measured using a transiently transfected HIF-1 responsive element driven luciferase reporter normalized to beta-galactosidase transfection baseline control, Fig. 1C. Under hypoxia, there was a significant, 12-fold increase in HIF-1 dependent transcription. This induction was completely inhibited to normoxic levels by AMPK inhibitor (5-iodotubericidin) treatment prior to hypoxic induction. There was no regulation of HIF-1 dependent transcription using AICAR alone in a similar assay (data not shown).

Differential Expression and Subcellular Localization of AMPK Isoforms Under Hypoxic Conditions

In order to determine whether AMPKα isoforms are differentially regulated by hypoxia in human glioblastoma cells, the mRNA expression of the two AMPKα isoforms was determined by quantitative RT-PCR in U373 cells exposed to normoxic or hypoxic conditions. Under normoxic conditions the AMPKα1 isoform was the predominant isoform, showing 2- to 4-fold more mRNA than the AMPKα2 isoform. Exposure of U373 cells to 12 h of hypoxia showed little change in the baseline ratio of AMPKα1 to AMPKα2 mRNA levels. However, over a 24 h hypoxic treatment, a significant 3-fold increase of AMPKα2 mRNA was observed over normoxic controls, Fig. 2A.

A direct analysis of AMPKα isoform protein expression over a time course of hypoxia treatment showed a similar pattern of increasing AMPKα2 protein levels. The levels of phosphorylated AMPK (p-AMPK) also increased with exposure to hypoxia. AMPKα1 protein levels did not significantly change with the hypoxic time course, Fig. 2B.

In order to determine whether hypoxia affects AMPKα isoform intracellular distribution, isoform-specific fluorescent immunohistochemistry was employed on cells exposed to normoxic or hypoxic conditions for 24 h, Fig. 3. No significant change was observed in the localization of AMPKα1 between normoxia and hypoxia, showing a widespread nuclear and cytoplasmic distribution. AMPKα2 was found to redistribute within the cell from a uniform cytoplasmic/nuclear distribution to a predominantly nuclear localization under hypoxia. These data demonstrate that AMPKα2, but not AMPKα1, is regulated at the mRNA and protein levels by hypoxia in U373 cells.

Inhibition of AMPK Alpha Isoform Expression by RNA Interference

In order to evaluate possible AMPK alpha isoform-specific functions, RNA interference (RNAi) protocols were performed to repress AMPK alpha isoform gene expression and function. Figure 4A shows AMPKα1 mRNA levels determined by quantitative RT-PCR when cells were transfected with either AMPKα1- or AMPKα2-targeted siRNA oligos. Post-transfected samples show that AMPKα1 mRNA levels were inhibited at greater than 90% in both normoxic and hypoxic conditions when treated with AMPKα1 RNAi. AMPKα2 siRNA transfection did not significantly affect AMPKα1 mRNA levels. Conversely, Fig. 4B shows a similar pattern for the AMPKα2 RNAi. AMPKα2 mRNA was repressed greater than 90% in normoxia and hypoxia by AMPKα2 RNAi, and AMPKα1 siRNAs did not significantly affect AMPKα2 mRNA levels.

To determine the effectiveness of the RNAi treatments at the protein level, AMPKα isoforms were evaluated by direct immunoblot of total cell extracts taken 60 h after transfection, Fig. 4C. The AMPKα1 RNAi repressed AMPKα1 protein in normoxia and hypoxia when compared to mock transfected controls. Hypoxia alone greatly increased AMPKα2 protein, as was observed in Fig. 2B. The AMPKα2 siRNA was effective at repressing AMPKα2 protein levels in hypoxia, although the protein levels in normoxia were not significantly affected. These data demonstrate that RNAi against the individual AMPKα isoforms was effective at repressing the targeted AMPKα mRNA and protein in U373 glioblastoma cells.

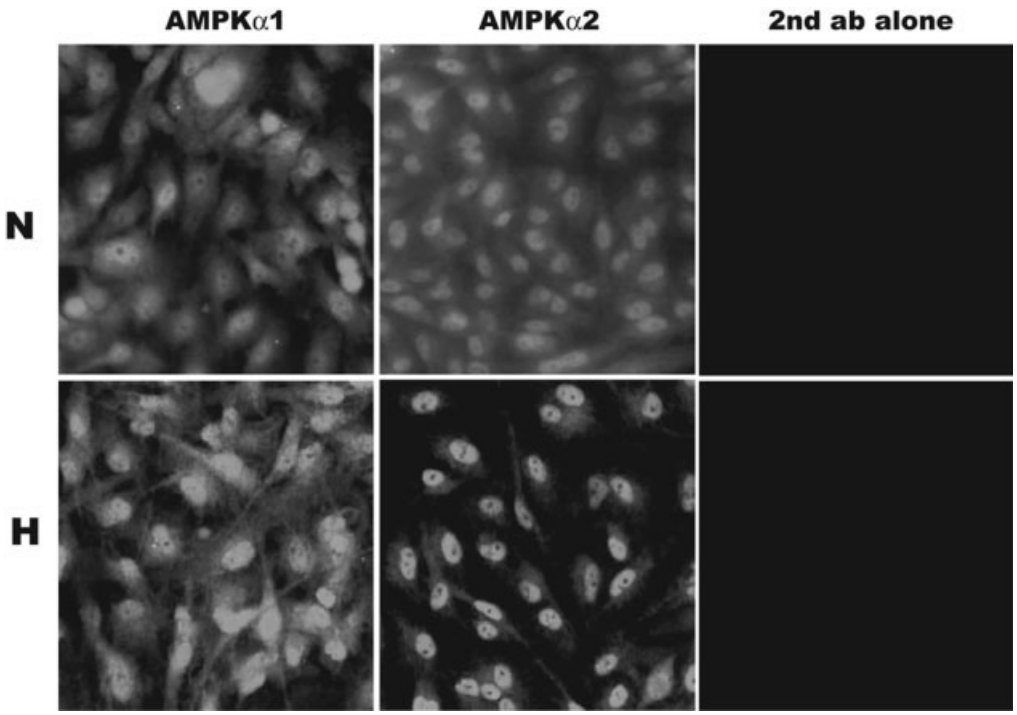

Fig. 3. AMPKα isoforms are differentially localized under hypoxic conditions. AMPKα1 or AMPKα2 protein localization as determined by immunofluorescent histochemistry under normoxic (N) and hypoxic (H) conditions.

The Effect of AMPKα Isoform Repression by RNAi on Hypoxia-Induced AMPK Activation and Downstream Signal Transduction

In order to determine the effect of AMPK alpha isoform repression on functional AMPK protein, we examined the level of total phosphorylated AMPK, which detects both p-AMPKα1 and p-AMPKα2 isoforms at Thr172, an activating phosphorylation site (Woods et al., 2003). Figure 5A shows that hypoxia increased p-AMPK and that application of siRNA targeted to AMPKα1 repressed the total p-AMPK considerably under normoxic and hypoxic conditions. AMPKα2 RNAi also repressed total p-AMPK under normoxia and hypoxia, although to a lesser extent than AMPKα1. Since AMPKα1 mRNA and protein are more abundant in the U373 cells, AMPKα2 repression only partially affects the total amount of p-AMPK protein.

One essential function for AMPK is to repress protein translation by blocking mTOR/S6 kinase pathways through phosphorylation of the TSC1/2 complex (Inoki et al., 2003). To determine whether the mTOR pathway inhibition was selective to either AMPK alpha isoform, levels of phospho-p70S6K were evaluated in AMPK RNAi repressed cells. Hypoxia alone significantly reduced the amount of phospho-p70S6K signal in U373 glioblastoma cells, Fig. 5B. This data supports the observation of Krause et al., 2002, which states that AMPK activation leads to the repression of phospho-p70S6K through modulation of the mTOR pathway. Under normoxic conditions, AMPKα1 and AMPKα2 RNAi resulted in increased levels of phospho-p70S6K. It was found that the RNAi treatment for both AMPKα isoforms increased the phospho-p70S6K in hypoxia, with the repression of AMPKα2 demonstrating a slightly greater increase in phospho-p70S6K. These data indicate that both AMPK alpha isoforms contribute to the repression of the protein translational pathways by repressing active p70S6K under hypoxia. This also confirms that RNAi to AMPK alpha isoforms is effective at altering downstream pathways of AMPK and is sufficient to study the AMPK alpha isoforms independently.

AMPKα2 Selectively Contributes to Hypoxia-Induced VEGF Expression at the mRNA and Protein Levels

In an effort to examine the potential selectivity for the AMPKα isoforms in regulating important hypoxia responsive genes, the hypoxic induction of VEGF under conditions where AMPKα1 or AMPKα2 is repressed with RNAi treatments was examined. Hypoxia responsive U373 cells were transfected with mock or AMPKα isoform specific siRNA oligo pools. To assess RNAi effectiveness for each experiment, the level of AMPKα1 or AMPKα2 specific mRNA repression was determined to be greater than 90% of mock transfected control by qRT-PCR (data not shown). Total cellular RNA was analyzed for VEGF mRNA expression by qRT-PCR, and the level of secreted VEGF in conditioned media from the same cells was determined by ELISA. Figure 6A shows the quantitative levels of VEGF mRNA levels determined for normoxic and hypoxic cells corresponding to mock transfected, AMPKα1 RNAi, or AMPKα2 RNAi treated U373 cells. Only the AMPKα2 RNAi treated cells showed repressed hypoxic-induced VEGF mRNA at a significant level (50% of non-transfected cells), whereas AMPKα1 RNAi had no effect. Similarly, the conditioned media of the AMPKα2 RNAi treated cells showed significant repression of secreted VEGF protein, as

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