Effect of Low Glutamine/Glucose on Hypoxia-Induced Elevation of Hypoxia-Inducible Factor-1α in Human Pancreatic Cancer MiaPaCa-2 and Human Prostatic Cancer DU-145 Cells

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Abstract

Purpose and Experimental Design: Tumor microenvironment is characterized by regions of fluctuating and chronic hypoxia, low extracellular pH, and nutrient depletion. Although it is well known that hypoxia stimulates the accumulation of hypoxia-inducible factor-1α (HIF-1α), the role of low extracellular pH and nutrient depletion on hypoxia up-regulation of HIF-1α is not well known. In this study, human pancreatic cancer MiaPaCa-2 and human prostatic cancer DU-145 cells were exposed to hypoxia in the presence or absence of glucose, glutamine, and/or pyruvate.

Results: We observed that low glucose and low glutamine, but not low pyruvate, effectively suppressed the elevation of HIF-1α level during hypoxia (0.1-1% oxygen). Deprivation of glutamine or glucose inhibited the accumulation of HIF-1α in the presence of MG-132, a protease inhibitor, regardless of oxygen tensions. Data from reverse transcription-PCR analysis revealed that the levels of HIF-1α mRNA were not significantly changed at different concentrations of glutamine or glucose under hypoxia. The amount of HIF-1α suppression was proportional to protein synthesis inhibition.

Conclusions: Our data suggest that glutamine or glucose deprivation inhibits the accumulation of HIF-1α under hypoxic conditions by disrupting translational processes rather than transcriptional or proteasomal degradation processes.

It is well known that severe architectural and functional abnormalities are commonly observed in the capillary network that develops during tumor growth (1). Tumor vessels are tortuous and highly irregular, have arterial venous shunts and blind ends, and lack smooth muscles. These abnormalities cause an insufficient blood supply and development of a pathophysiologic tumor microenvironment. Studies with the micropore chamber sampling procedure (2) and tumor-isolated preparations (3) reveal differences in the constituents of serum (vascular compartment) compared with interstitial fluid (interstitial compartment). Vascular and interstitial compartments are two major compartments of the extracellular space of solid tumors. The tumor interstitial compartment is characterized by low oxygen tensions (hypoxia; ref. 4), low glucose concentrations (5), high lactate concentrations (6, 7), and low extracellular pH (8). These characteristic features induce several stress-inducible proteins, such as glucose-regulated proteins (9, 10), oxygen-regulated proteins (11–13), heat shock proteins (14), erythropoietin (15), vascular endothelial growth factor (VEGF; ref. 16), and basic fibroblast growth factor (17).

Angiogenesis is essential for the growth, progression, and metastasis of a tumor (18). Angiogenic factors include acidic fibroblast growth factor, basic fibroblast growth factor, angiogenin, platelet-derived growth factor, VEGF, transforming growth factor-α, and transforming growth factor-β. Among these angiogenic factors, VEGF is a prominent mediator of angiogenesis (19, 20). Previous studies showed that the expression of VEGF is responsive to tumor environmental factors, including hypoxia (16, 21), and insufficient vascular delivery of nutrients, such as glucose and amino acids (22–25). It is well known that hypoxia-inducible factor-1α (HIF-1α), a transcription factor, plays an important role in the regulation of VEGF gene expression (26). The level of HIF-1α is elevated under hypoxic conditions (27). The mechanism of hypoxia-mediated stabilization of HIF-1α has been substantially studied (28). Under normoxic conditions, protein von Hippel-Lindau, a component of an E3 ubiquitin protein-ligase complex, targets HIF-1α for ubiquitination and proteasomal degradation (29). The interaction between protein von Hippel-Lindau and HIF-1α depends on the hydroxylation of proline residues (Pro^{402} and Pro^{564}) in the oxygen-dependent degradation domain of HIF-1α (30, 31). These hydroxylations are mediated by HIF-specific prolyl hydroxylases in the presence of iron and oxygen (32, 33). Under hypoxic conditions, the oxygen-requiring process of hydroxylation is prevented and the loss of protein von Hippel-Lindau function leads to stabilization of HIF-1α. HIF-1α dimerizes with HIF-1β (aryl hydrocarbon receptor nuclear translocator), which is constitutively expressed. The

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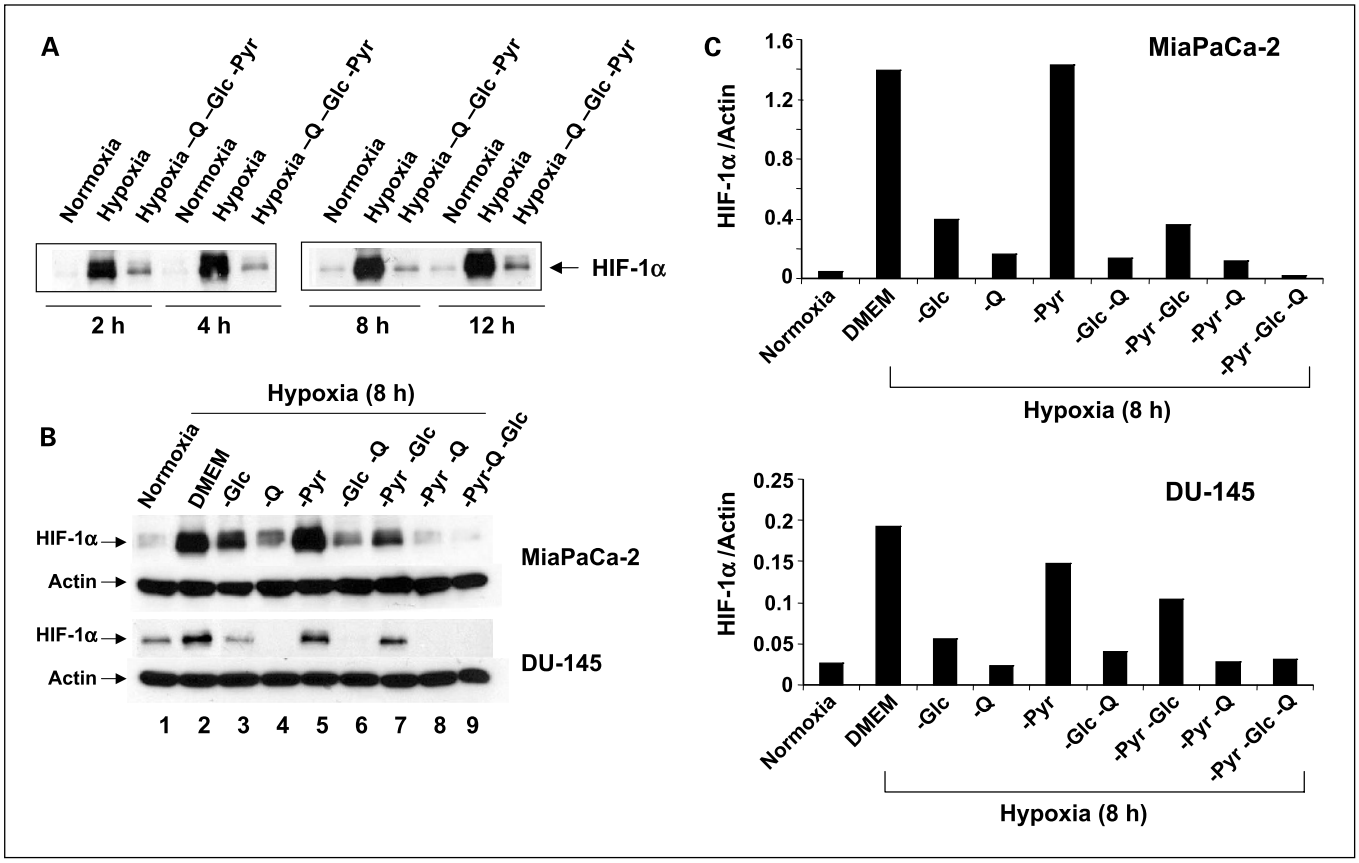

Fig. 1. Effect of glucose, glutamine, or pyruvate deprivation on hypoxia-induced HIF-1α elevation. *A*, MiaPaCa-2 cells were exposed to hypoxia (0.1% O_2) for various times (2-12 hours) in the presence or absence of glucose (*Glc*), glutamine (*Q*), and pyruvate (*Pyr*). Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-HIF-1α antibody. *B*, MiaPaCa-2 or DU-145 cells were exposed to hypoxia for 8 hours in the presence or absence of glucose, glutamine, and/or pyruvate. Cell lysates were subjected to immunoblotting for HIF-1α. Actin was shown as an internal standard. *C*, quantitative measurement of HIF-1α level. Immunoblots in *B* were analyzed with a densitometer. The ratio of the area integration of absorbance of HIF-1α to that of actin is plotted.

dimer then recognizes a specific element, such as hypoxia response element, in the promoters of hypoxia-responsive target genes involved in anaerobic metabolism, angiogenesis, cell survival, and erythropoiesis (34). HIF-1α transactivation also involves the physical interaction with p300/CBP (35, 36), two coactivators required for the transcriptional activity of a variety of transcription factors (37, 38).

As mentioned previously, the tumor microenvironment is characterized by low oxygen tensions as well as nutrient deprivation, such as glucose and amino acids (39, 40). This is exacerbated by the fact that cancer cell energy metabolism deviates significantly from that of normal tissues. Cancer cells often exhibit greatly increased rates of glucose utilization (high aerobic glycolytic rates) and glutamine consumption and produce high levels of lactate and pyruvate (22, 41). It is therefore very important to determine whether nutrient deprivation affects hypoxia-induced stabilization of HIF-1α. In this report, we observed that low glucose and low glutamine, but not low pyruvate, effectively suppress the elevation of HIF-1α level during hypoxia.

Materials and Methods

Cell culture and hypoxia treatment. Human pancreatic cancer MiaPaCa-2 and prostate cancer DU-145 cells were cultured in DMEM (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone, Logan, UT). Petri dishes/flasks containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. Before the experiments, cells were grown to ~80% confluence in 60 or 100 mm tissue culture dishes. For hypoxia treatment, Petri dishes were incubated in a hypoxic chamber (Forma Scientific, Marietta, OH) with a 94-94.9:5:0.1-1 mixture of $N_2/CO_2/O_2$. It is difficult to accurately measure oxygen concentration in unstirred systems above monolayers. Thus, we incubated medium in the hypoxic chamber overnight before use. In addition, Petri dishes containing cells were put on a shaker for 15 minutes after medium was replaced in the hypoxic chamber. The oxygen level is monitored by an oxygen meter, which is equipped with OXELP oxygen electrode (World Precision Instruments, Sarasota, FL). This electrode has a 2 mm diameter tip and can accurately measure dissolved oxygen concentrations (0.1-100%) with 0.1 ppm resolution. The oxygen level is maintained at either 1% or 0.1%.

Glucose, glutamine, or pyruvate deprivation. Cells were rinsed thrice with PBS. Cells were then exposed to glucose-free, glutamine-free, or pyruvate-free DMEM with 10% dialyzed fetal bovine serum (Invitrogen, Carlsbad, CA).

Drug treatment. MG-132, a protease inhibitor, was obtained from EMD Biosciences (San Diego, CA). Drug treatment was accomplished by aspirating the medium from the cells and replacing it with drug-containing medium.

Protein extracts and PAGE. Cells were lysed with 1× Laemmli lysis buffer [2.4 mol/L glycerol, 0.14 mol/L Tris (pH 6.8), 0.21 mol/L SDS, 0.3 mmol/L bromophenol blue] and boiled for 10 minutes. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1× lysis buffer

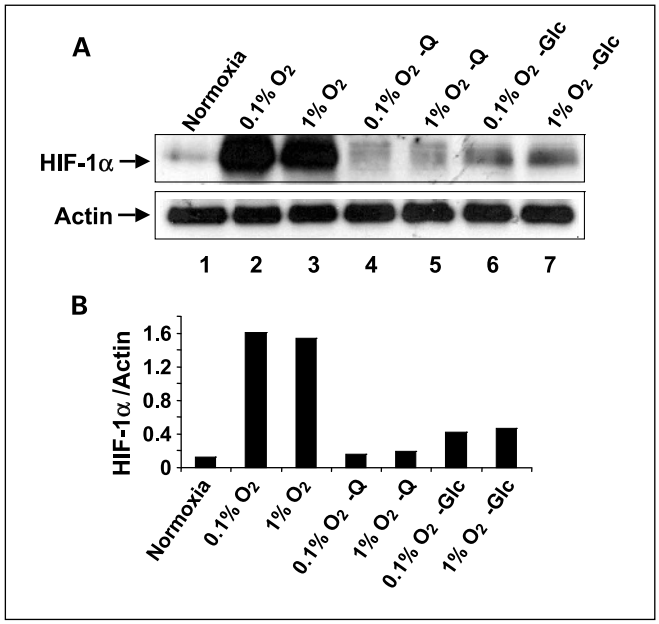

Fig. 2. Effect of glutamine or glucose deprivation on 0.1% or 1% O_2 hypoxia-induced accumulation of HIF-1α. *A*, MiaPaCa-2 cells were exposed to hypoxia (0.1% or 1% O_2) for 8 hours in the presence or absence of glucose or glutamine. Equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-HIF-1α antibody. *B*, quantitative measurement of HIF-1α level. Immunoblots in *A* were analyzed with a densitometer. The ratio of the area integration of absorbance of HIF-1α to that of actin is plotted.

containing 1.28 mol/L β-mercaptoethanol, and equal amounts of protein were loaded on 8% to 12% SDS-polyacrylamide gels. SDS-PAGE analysis was done according to Laemmli (42) using a Hoefer gel apparatus.

Western blot analysis. Proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membrane. The membranes were incubated with 8% (w/v) skim milk in PBS containing 0.1% (v/v) Tween 20 for blocking and then reacted with mouse monoclonal anti-HIF-1α antibody (BD Biosciences, San Diego, CA) or mouse anti-actin antibody (Sigma, St. Louis, MO). Horseradish peroxidase–conjugated anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (Amersham Bioscience, Arlington Heights, IL). Quantitation of X-ray film was carried out by scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA) using area integration.

RNA extraction and reverse transcription-PCR. Total RNA from the MiaPaCa-2 cells was extracted using TRIzol (Life Technologies) according to the manufacturer's protocol. For each reverse transcription-PCR reaction, total RNA (1 μg) was used with Novagen One-Step RT-PCR kit (EMD Biosciences). The following sense and antisense primers were used: HIF-1α primers (5′-GCGGCGCGAACGACAAGAAAAAG-3′ and 5′-GAATGTGGCCTGTGCAGTGCAATAC-3′, respectively), actin primers (5′-AAGATGACCCAGATCATGTTTGAG-3′ and 5′-AGGAGGAGCAATGATCTTGATCTT-3′, respectively), and glyceraldehyde 3-phosphate dehydrogenase primers (5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′, respectively).

Determination of total protein synthesis. Effect of glucose or glutamine deprivation on protein synthesis during normoxia or hypoxia was investigated by plating MiaPaCa-2 cells (2×10^5) into 60 mm culture plates. After the cells were grown to ~80% confluence, the medium was replaced with complete medium, glucose-free medium, or glutamine-free medium containing 8 μCi/mL Tran ^{35}S-label, which contains mixtures of [^{35}S]L-methionine, [^{35}S]L-cysteine, [^{35}S]L-methionine sulfone, and [^{35}S]L-cysteic acid (MP Biomedicals, Irvine, CA). After the plates were incubated at 37°C for a period of time, the cells were washed thrice with cold PBS for 3 minutes and twice with cold 10% trichloroacetic acid for 5 minutes. Trichloroacetic acid–insoluble proteins were solubilized with 5 mL of 0.25 N NaOH; then, the solubilized sample (0.25 mL) was added to Packard Scint-A (10 mL). Radioactivity was determined with a Beckman LS-7500 scintillation counter. The protein content was estimated by the Lowry method (43).

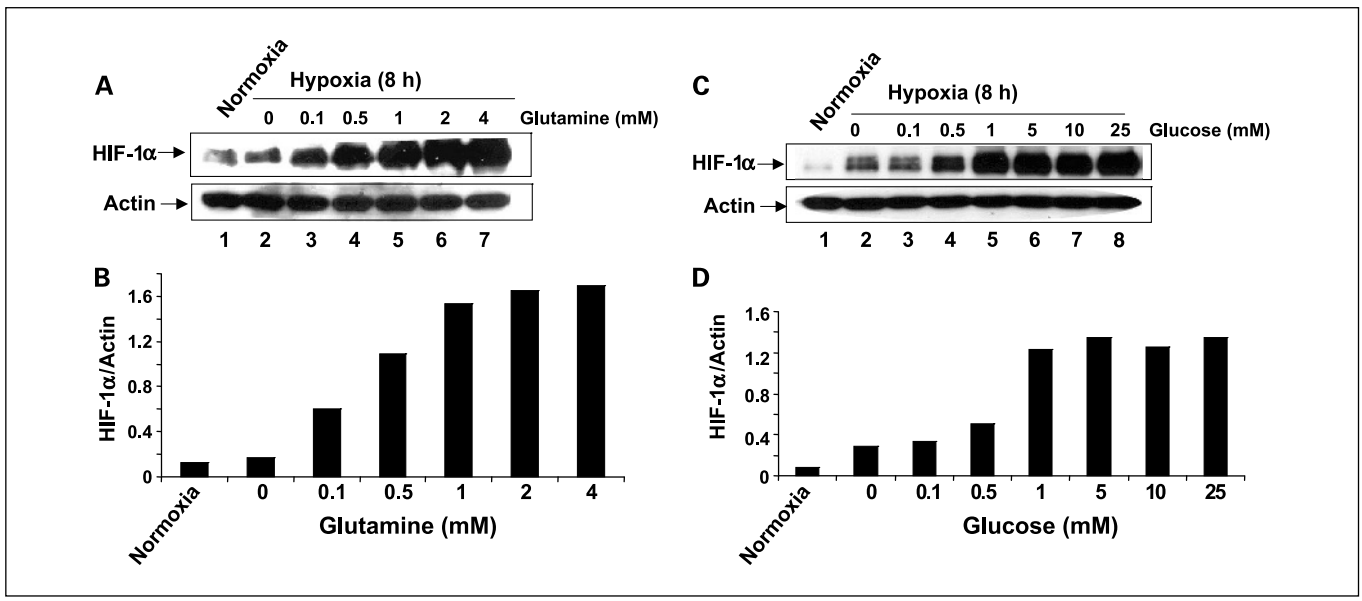

Fig. 3. Effect of various concentrations of glutamine (*A* and *B*) or glucose (*C* and *D*) on hypoxia-induced HIF-1α elevation. MiaPaCa-2 cells were exposed to hypoxia (0.1% O_2) for 8 hours in the presence of various concentrations of glutamine or glucose. *A* and *C*, equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-HIF-1α antibody. Actin was shown as an internal standard. *B* and *D*, quantitative measurement of HIF-1α level. Immunoblots in *A* and *C* were analyzed with a densitometer. The ratio of the area integration of absorbance of HIF-1α to that of actin is plotted.

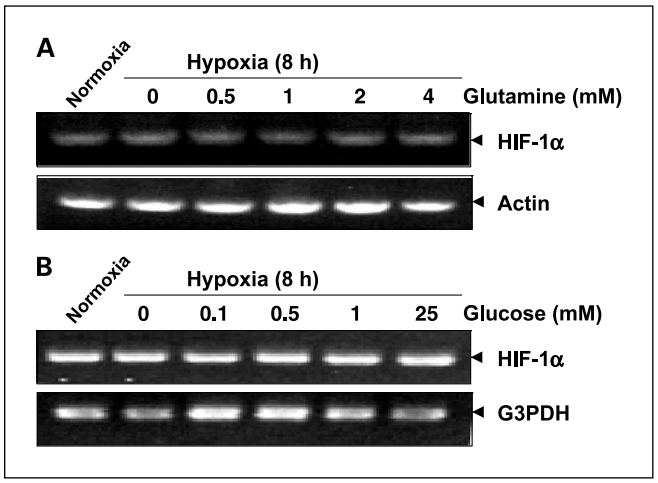

Fig. 4. Effect of various concentrations of glutamine (*A*) or glucose (*B*) on HIF-1α mRNA during hypoxia. MiaPaCa-2 cells were exposed to hypoxia (0.1% O_2) for 8 hours in the presence of various concentrations of glutamine or glucose. Reverse transcription-PCR analysis was done for detecting HIF-1α mRNA expression.

Results

Glucose deprivation and/or glutamine deprivation, but not pyruvate deprivation, effectively suppresses an increase in hypoxia-inducible factor-1α protein level during hypoxia. To examine whether nutrient deprivation affects hypoxia-induced stabilization of HIF-1α, MiaPaCa-2 or DU-145 cells were exposed to hypoxia in the presence or absence of glucose, glutamine, and/or pyruvate. Figure 1 shows that the level of HIF-1α was elevated under hypoxic conditions in both cell lines. The elevation of HIF-1α was significantly suppressed by deprivation of glucose, glutamine, and pyruvate (Fig. 1A). The lack of glucose, glutamine, and pyruvate affected HIF-1α expression after as little as 2 hours of incubation. To investigate which component contributes the most effectively, each component was systematically removed from DMEM and the level of HIF-1α was determined during 8 hours of hypoxia. Figure 1B reveals that hypoxia-induced elevation of HIF-1α was suppressed by glutamine or glucose deprivation in both cell lines. Data from densitometer analysis show that hypoxia-elevated HIF-1α was inhibited by 72% or 89% during incubation with glucose-free or glutamine-free medium, respectively, in MiaPaCa-2 cells (Fig. 1C). Similar results were observed in DU-145 cells. Glutamine deprivation, compared with glucose deprivation, caused more effective inhibition of HIF-1α protein level. Unlike glucose and glutamine deprivation, pyruvate deprivation did not effectively suppress the hypoxia-elevated HIF-1α level. Figure 2 shows that the level of HIF-1α was elevated by 0.1% or 1% oxygen tensions. Glutamine or glucose deprivation inhibited the elevation of HIF-1α regardless of oxygen tensions. Figure 3 reveals that the inhibition of hypoxia-increased HIF-1α level by deprivation of either glutamine or glucose was dependent on their concentrations. Data from densitometer analysis show that a significant suppression of HIF-1α level occurred at a glucose/glutamine concentration of ≤0.5 mmol/L (Fig. 3B and D). Hypoxia-elevated HIF-1α in complete medium was suppressed by 35% or 63% in the presence of 0.5 mmol/L glutamine or 0.5 mmol/L glucose, respectively.

Effect of glutamine or glucose deprivation on hypoxia-inducible factor-1α mRNA level during hypoxia. To determine whether the decreased level of HIF-1α by glutamine or glucose deprivation occurred at the transcriptional level, MiaPaCa-2 cells were exposed to hypoxia in the presence of various concentrations of glutamine or glucose and then subjected to reverse transcription-PCR analysis. Figure 4 shows that hypoxia did not alter the level of HIF-1α mRNA. The levels of HIF-1α mRNA were not significantly changed at different concentrations of glutamine or glucose under hypoxia.

Effect of glutamine or glucose deprivation on proteasomal degradation of hypoxia-inducible factor-1α during hypoxia. To examine whether the effect of glutamine or glucose deprivation occurs on proteasome-mediated degradation of HIF-1α, MiaPaCa-2 cells were exposed to glutamine-free or glucose-free medium in the presence or absence with MG-132, a proteosomal inhibitor, under normoxic or hypoxic conditions (Fig. 5). Treatment with MG-132 elevated the level of HIF-1α in both

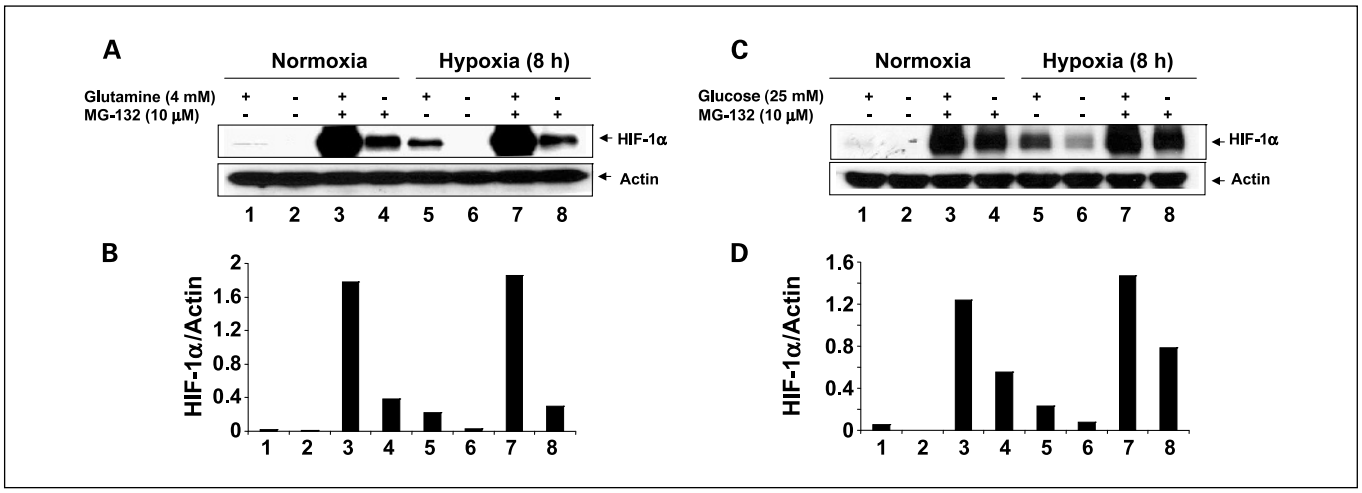

Fig. 5. Effect of combining treatment with MG-132 and low glutamine/glucose on hypoxia-induced HIF-1α elevation. MiaPaCa-2 cells were exposed to normoxia or hypoxia (0.1% O_2) for 8 hours in combination with MG-132 and glutamine (*A* and *B*) or glucose (*C* and *D*). *A* and *C*, equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-HIF-1α antibody. Actin was shown as an internal standard. *B* and *D*, quantitative measurement of HIF-1α level. Immunoblots in *A* and *C* were analyzed with a densitometer. The ratio of the area integration of absorbance of HIF-1α to that of actin is plotted.

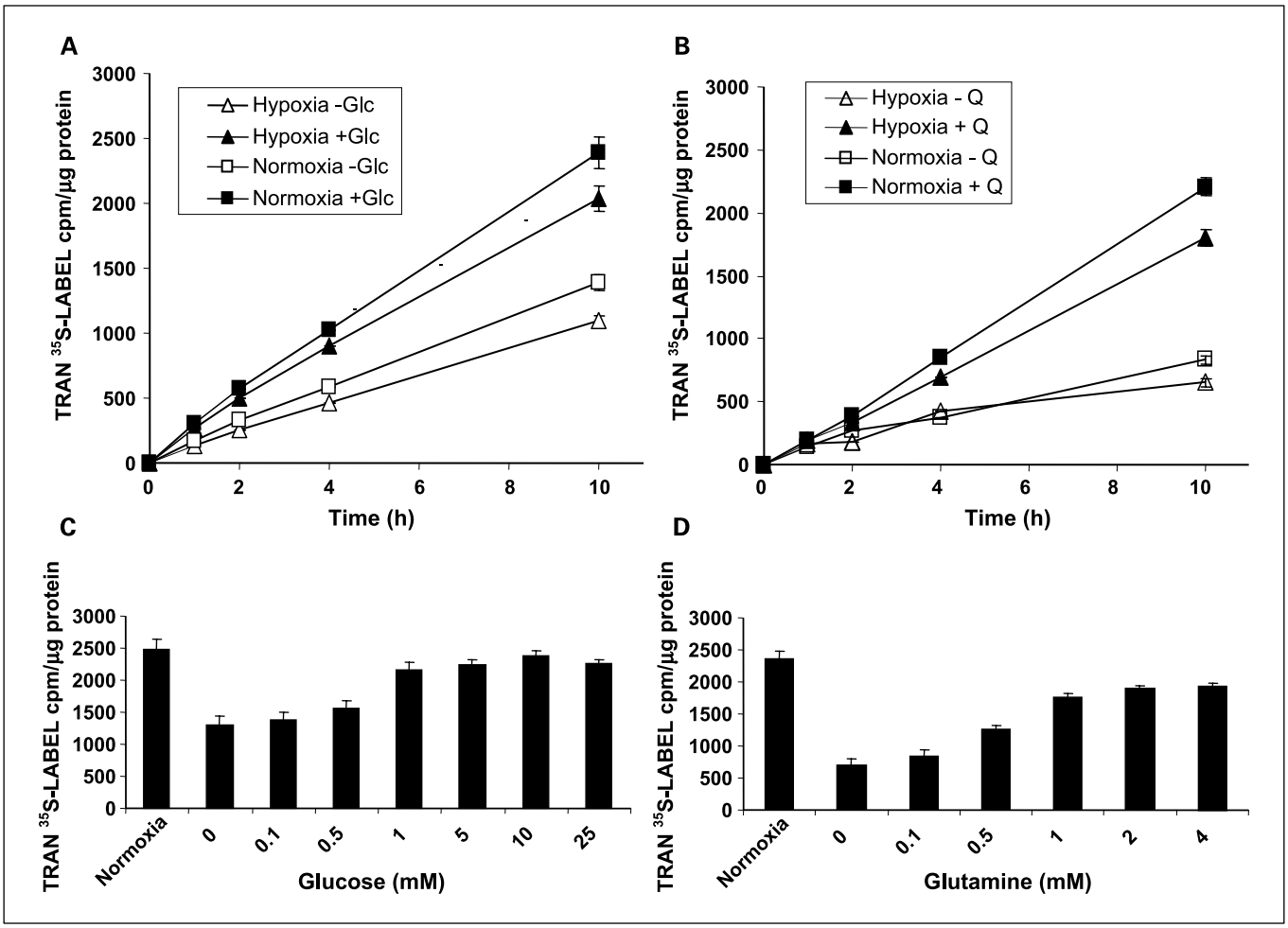

Fig. 6. Effect of glucose (*A* and *C*) or glutamine (*B* and *D*) deprivation on the incorporation of Tran ^{35}S-label into protein during normoxia or hypoxia. *A* and *B*, MiaPaCa-2 cells were labeled various times during hypoxia (0.1% O_2) or normoxia in the presence or absence of glucose/glutamine. *Points,* mean in triplicate; *bars,* SD. *C* and *D*, MiaPaCa-2 cells were labeled for 10 hours during hypoxia (0.1% O_2) or normoxia in the presence of various concentrations of glucose or glutamine. *Columns,* mean in triplicate; *bars,* SD.

conditions. Interestingly, deprivation of glutamine or glucose decreased the level of HIF-1α in the presence of MG-132 regardless of oxygen tensions (Fig. 5A and C; lane 3 versus lane 4 and lane 7 versus lane 8). These results suggest that deprivation of glutamine or glucose under hypoxic conditions does not affect the process of proteasomal degradation of HIF-1α. Accumulation of HIF-1α level after treatment with MG-132 was inhibited by 78% to 84% or 47% to 55% in the absence of glutamine or glucose, respectively (Fig. 5B and D).

Effect of glutamine or glucose deprivation on total protein synthesis during hypoxia. A fundamental question, which remains unanswered, is how glucose/glutamine deprivation suppressed the level of HIF-1α under hypoxic conditions. One possibility is that low glucose/glutamine inhibits translational process. To investigate this possibility, protein synthesis was measured when cells were exposed to glucose-free or glutamine-free medium at various times during normoxia/hypoxia (Fig. 6A and B). Protein synthesis was inhibited by 13% under hypoxic conditions. Glutamine or glucose deprivation inhibited protein synthesis by 62% to 64% or 42% to 46%, respectively, regardless of oxygen tensions. As shown in Fig. 1B, the HIF-1α protein level decreased more at glutamine deprivation than at glucose deprivation. A similar effect was also observed in total protein synthesis. The inhibition of total protein synthesis was more profound with glutamine deprivation than with glucose deprivation (Fig. 6A and B). Inhibition of protein synthesis was inversely proportional to the concentrations of glucose (Fig. 6C) and glutamine (Fig. 6D). Significant decrease in protein synthesis was observed in the presence of 0.5 mmol/L glucose or 0.5 mmol/L glutamine. These results were similar to the inhibition of the accumulation of HIF-1α in the presence of these concentrations of glucose or glutamine (Fig. 3).

Discussion

The major observation reported here is that accumulation of HIF-1α under hypoxic conditions is significantly inhibited by nutrient (glutamine and glucose) depletion in human pancreatic cancer MiaPaCa-2 cells as well as human prostate cancer DU-145 cells. This inhibition is probably due to disruption of the translational pathway (Fig. 6) rather than the transcriptional pathway (Fig. 4) or proteasomal degradation of HIF-1α (Fig. 5). Our data also reveal that glutamine or glucose deprivation alone

does not lead to the accumulation of HIF-1α (Fig. 5). These results seem to be somewhat inconsistent with previous reports demonstrating that hypoxia or ischemia promotes VEGF gene expression by elevating the level of HIF-1α (44–46). However, several researchers reported that a lack of glutamine or glucose alone still stimulates VEGF expression (23–25, 47). Thus, our results suggest that glutamine or glucose deprivation can regulate VEGF gene expression independently of the accumulation of HIF-1α. Recent studies also revealed that glucose or glutamine deprivation activates transcription factors, including NF-κB, through promoting endoplasmic reticulum stress response (23). It is known that NF-κB is also involved in the regulation of VEGF expression (48).

With respect to total energy generation, tumor cells are quite capable of maintaining energy charge (ATP) of the cell at low oxygen tensions by reducing global protein synthesis (49, 50). Unlike hypoxia, intracellular ATP levels are rapidly decreased when exposed to glucose or glutamine deprivation (51–54). Glucose and glutamine are major energy source nutrients in the medium and become rapidly exhausted during the incubation of tumor cells. Glutamine deprivation is associated with cell death by apoptosis independent of energetic failure, whereas glucose deprivation is followed by rapid loss of mitochondrial function with reduction of intracellular ATP and cell death by necrosis (54). Previous studies reported that although total protein synthesis is reduced during hypoxia, HIF-1α synthesis is maintained by initiation via an internal ribosomal entry mechanism (49, 55). In this study, however, the accumulation of HIF-1α is suppressed by glucose or glutamine deprivation even under hypoxic conditions. At the present time, we can only speculate how low glucose or low glutamine inhibits the elevation of HIF-1α during hypoxia. Several studies show that ATP utilization is required for protein synthesis. It is well known that mRNA binding to the ribosome is the rate-limiting step in eukaryotic protein synthesis. This process is catalyzed by the eIF-4 group of initiation factors (56). These factors collectively bind to the m^7GTP-containing cap of mRNA and unwind mRNA secondary structure at the expense of ATP. Thus, we postulate that reduction of ATP generation by glucose or glutamine deprivation is responsible for inhibiting the accumulation of HIF-1α under hypoxic conditions. This model will provide a framework for future studies.

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