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D-Glucosamine down-regulates HIF-1α through inhibition of protein translation in DU145 prostate cancer cells

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ABSTRACT

D-Glucosamine has been reported to inhibit proliferation of cancer cells in culture and *in vivo*. In this study we report a novel response to D-glucosamine involving the translation regulation of hypoxia inducible factor (HIF)-1α expression. D-Glucosamine caused a decreased expression of HIF-1α under normoxic and hypoxic conditions without affecting HIF-1α mRNA expression in DU145 prostate cancer cells. D-Glucosamine inhibited HIF-1α accumulation induced by proteasome inhibitor MG132 and prolyl hydroxylase inhibitor DMOG suggesting D-glucosamine reduces HIF-1α protein expression through proteasome-independent pathway. Metabolic labeling assays indicated that D-glucosamine inhibits translation of HIF-1α protein. In addition, D-glucosamine inhibited HIF-1α expression induced by serum stimulation in parallel with inhibition of p70S6K suggesting D-glucosamine inhibits growth factor-induced HIF-1α expression, at least in part, through p70S6K inhibition. Taken together, these results suggest that D-glucosamine inhibits HIF-1α expression through inhibiting protein translation and provide new insight into a potential mechanism of the anticancer properties of D-glucosamine.

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Introduction

Hypoxia inducible factor 1 (HIF-1) is a transcription factor composed of two subunits, HIF-1α and HIF-1β. The HIF-1α subunit is degraded rapidly and continuously by the proteasome under normoxic conditions and stabilized under hypoxic conditions, while HIF-1β is constitutively expressed [1–3]. The proteasomal degradation of HIF-1α occurs through protein hydroxylation on proline residues 402 and 564 by specific HIF-prolyl hydroxylases in the presence of iron and oxygen. The hydroxylated protein then interacts with von Hippel–Lindau protein which functions as an E3 ubiquitin ligase [4,5]. Under hypoxia, HIF-1α accumulates and translocates to the nucleus where it heterodimerizes with HIF-1β and activates the transcription of more than 40 genes important for adaptation and survival under hypoxia [5]. In addition, oxygen-independent signaling pathways activated by some growth factors and cytokines can induce HIF-1α accumulation by increasing the rate of protein synthesis [6–8]. Thus, the steady state of HIF-1α protein expression is controlled by the balance of degradation and synthesis.

HIF-1α plays important roles in tumor progression and angiogenesis *in vivo*. Overexpression of HIF-1α has been demonstrated in more than 70% of human cancers and their metastases compared to their adjacent normal tissue, including breast, prostate, brain, lung, and head and neck cancers [9]. Furthermore, HIF-1α overexpression is associated with treatment failure and patient mortality in several types of cancer [1]. Therefore, the development of cancer therapeutics targeting HIF-1 activity appears to be attractive.

D-Glucosamine, an amino monosaccharide which is widely taken as a dietary supplement to relieve discomfort of osteoarthritis-related joint pain, has been reported as an inhibitor of tumor growth *in vitro* and *in vivo* [10–12]. Previously, we have reported D-glucosamine inhibits p70S6K/RPS6 (Ribosomal protein S6 kinase/Ribosomal protein S6) signaling, an important regulator of protein translation, in cancer cells [12]. p70S6K is a well known target of mTOR [13]. mTOR/p70S6K signaling play important role in increase of HIF-1α protein translation stimulated by growth factors, cytokines, and other signaling molecules [6,7,14]. Given the links between D-glucosamine and p70S6K, and p70S6K and HIF-1α protein synthesis, we were interested in testing experimentally if D-glucosamine modulated HIF-1α expression.

Here, we report a novel potential mechanism mediating the antitumor activity of D-glucosamine. Our findings demonstrate that D-glucosamine inhibits HIF-1α expression by translation inhibition rather than activation of proteasome-dependent degradation pathway. We further show that D-glucosamine inhibits serum induced HIF-1α expression in association with inhibition of p70S6K.

Materials and methods

Cell lines and reagents. DU145 cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in DMEM medium containing 10%

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fetal bovine serum (Hyclone), penicillin and streptomycin. For cell culture under hypoxia, cells were incubated in a chamber containing 1% oxygen, 5% CO_2, and 94% nitrogen at 37 °C. D-Glucosamine, protein synthesis inhibitor cycloheximide (CHX) and trichloroacetic acid (TCA) were obtained from Sigma. The proteasome inhibitor MG132 was purchased from Calbiochem. Prolyl hydroxylase inhibitor *N*-(methoxyoxoacetyl)-glycin methyl ester (DMOG) was obtained from Cayman Chemical.

Immunoblotting. Anti-HIF-1α (BD Biosciences), anti-HIF-1β (Santa Cruz Biotechnology), anti-phospho-p70S6K (Thr421/Ser424) (Cell Signaling Technology), anti-phospho-RPS6 (Ser235/236) (Cell Signaling Technology) antibodies were used at a dilution of 1:1000. Anti-actin antibody (Sigma) was used at a dilution of 1:5000. Western blotting were performed as described previously [12]. Immunoblotting was detected by enhanced chemiluminescence (Amersham Biosciences). The membrane was exposed to X-ray film or analyzed with LAS 3000 (Fujifilm Co.) image analyzer using MultiGauge software.

RT-PCR. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen). RT-PCR reactions were performed as described previously [15]. The RT-PCR products were separated on agarose gels and visualized by ethidium bromide staining under ultraviolet transillumination. The primer sequences were as follows: (forward primer) 5′-CTC AAA GTC GGA CAG CCT CA-3′ and (reverse primer) 5′-CCC TGC AGT AGG TTT CTG CT-3′ for HIF-1α, (forward primer) 5′-CGT CTT CAC CAC CAT GGA GA-3′ and (reverse primer) 5′-CGG CCA TCA CGC CAC AGT TT-3′ for GAPDH.

Transient transfection and luciferase assay. The hypoxia response element (HRE) driving firefly luciferase expression plasmid pGL2-TK-HRE was a kind gift from Dr. Giovanni Melillo (National Cancer Institute, Frederick, Maryland). DU145 cells were seeded in a 24 well-plate at a density of 5×10^4 cells/well the day before transfection. The cells were transiently cotransfected with pGL2-TK-HRE plasmids and pRL-TK plasmids (Promega), a renilla luciferase expression plasmid, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were cultured 20 h, followed by incubation with D-glucosamine for 6 h. The cells were analyzed with the Dual-Glo luciferase assay system (Promega). The relative luciferase activity was determined by the ratio of firefly/renilla luciferase activity.

Metabolic labeling, immunoprecipitation, and TCA precipitation. DU145 cells were seeded in 60 mm culture dishes at a density of 4×10^5 cells/dish. After overnight incubation, the cells were washed with PBS and treated with D-glucosamine in methionine-free DMEM. After 2 h, ^{35}S-methionine (GE Healthcare life Sciences) was added to a final concentration of 200 μCi/ml. The cells were incubated for 1 h, and harvested. Equal amount of the extracted protein were subjected to immunoprecipitation using anti-HIF-1α antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed and separated by SDS–PAGE. The gel was dried and exposed to X-ray film. To examine general protein synthesis, radioactivity incorporated into TCA precipitable material in cell lysates extracted from metabolic labeled cells were measured by liquid scintillation analyzer (Packard instrument Co.).

Quantitation of VEGF production. Media were collected from 1.5×10^5 cells in 6-well culture plates and centrifuged at 800 rpm for 4 min at room temperature to remove cellular debris and then stored at −70 °C. VEGF in the medium was measured

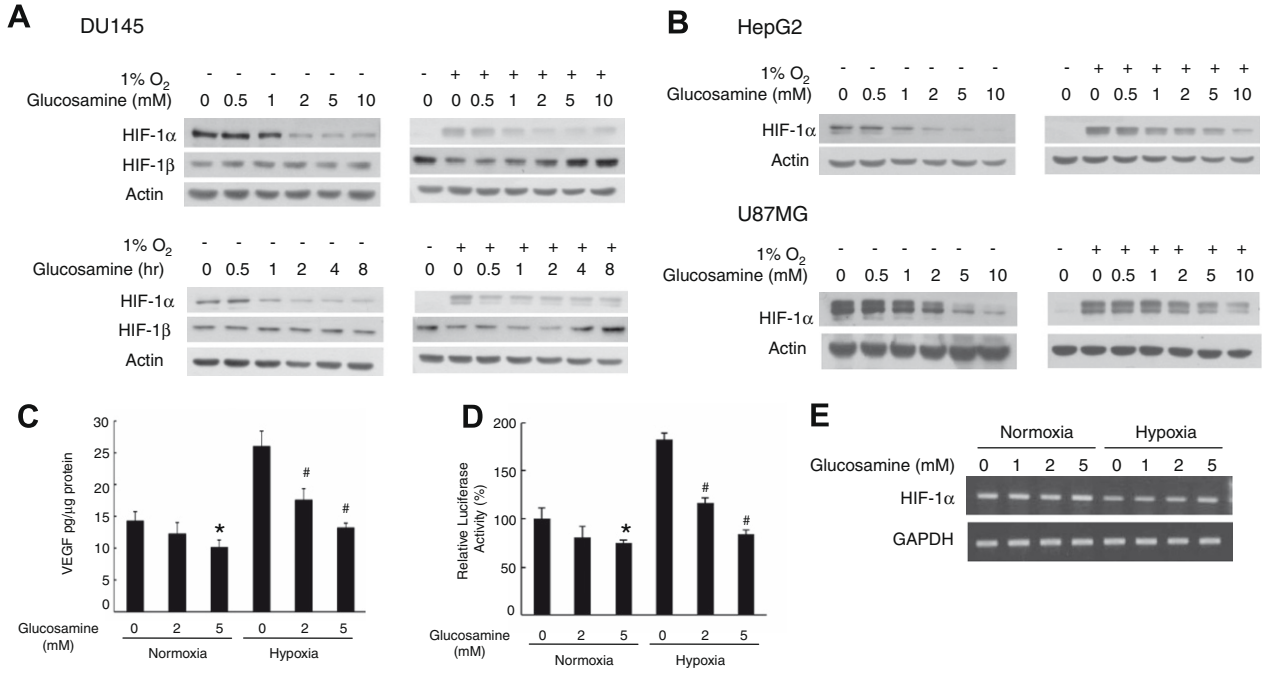

Fig. 1. (A) D-Glucosamine inhibits HIF-1α protein expression in dose- and time-dependent manners. DU145 cells were treated with various concentrations of D-glucosamine for 6 h (upper) or treated with 5 mM D-glucosamine for indicated times (lower) under normoxic and hypoxic conditions, and cell lysates were collected for Western blot analysis. (B) D-Glucosamine inhibits HIF-1α protein expression in HepG2 and U87MG cells. The cells were treated with D-glucosamine for 6 h under normoxic and hypoxic conditions. Cell lysates were subjected to Western blot analysis. (C) DU145 cells were seeded at 1.5×10^5 cells/well on 6-well culture plate. After 24 h, the cells were treated with D-glucosamine for 10 h under normoxic and hypoxic conditions. The VEGF protein levels in the culture medium were analyzed by ELISA as described in Materials and methods. *indicates a significant difference from the vehicle treated group under normoxia ($p < 0.05$). #indicates a significant difference from vehicle treated group under hypoxia ($p < 0.05$). (D) DU145 cells transiently cotransfected with pGL2-TK-HRE plasmids and pRL-TK plasmids were treated with vehicle and D-glucosamine under normoxic or hypoxic condition for 6 h. The luciferase activity was assayed using the Dual-Glo luciferase assay system. The relative luciferase activity was determined by the ratio of firefly/renilla luciferase activity and normalized to the value of control. *indicates a significant difference from the vehicle treated group under normoxia ($p < 0.05$). #indicates a significant difference from vehicle treated group under hypoxia ($p < 0.05$). (E) DU145 cells were treated with the indicated concentrations of D-glucosamine for 6 h under normoxic and hypoxic conditions, and HIF-1α mRNA levels were determined by RT-PCR.

by using the Quantikine human VEGF ELISA kit from R & D Systems according to the manufacturer's instruction.

Statistical analysis. Data represents mean ± SD from three independent experiments. Statistical analysis was performed by Student's *t* test at a significance level of $P < 0.05$.

Results

D-Glucosamine reduces the expression of HIF-1α

We investigated the effects of D-glucosamine on HIF-1α protein expression in DU145 prostate cancer cells. As shown in Fig. 1A, D-glucosamine reduced the expression of HIF-1α protein. This inhibition was dose- and time-dependent, and observed under both normoxic and hypoxic conditions. Similar results were obtained after treatment of HepG2 hepatoblastoma and U87MG glioma cells with D-glucosamine (Fig. 1B).

To examine whether the reduction of HIF-1α protein expression by D-glucosamine affects VEGF production, we measured VEGF levels in conditioned media from D-glucosamine treated DU145 cells using ELISA. In accordance with decreased HIF-1α expression, D-glucosamine decreased basal and hypoxia-induced VEGF expression in a dose-dependent manner (Fig. 1C).

To determine whether the decrease in VEGF by D-glucosamine treatment was a direct effect on HIF-1α, we determined the HIF-1 transcriptional activity using a reporter gene assay. We transiently transfected DU145 cells with a construct containing luciferase gene under the control of the hypoxia responsive element (HRE) from the inducible nitric oxide synthase promoter. As shown in Fig. 1D, D-glucosamine treatment reduced the transcriptional activity of HIF-1 in a dose-dependent manner both under normoxic and hypoxic conditions. Collectively, these data suggest that D-glucosamine inhibits VEGF production through reducing HIF-1α expression level.

Next, to determine whether inhibition of HIF-1α protein levels by D-glucosamine was caused by a decrease in its mRNA level, HIF-1α mRNA levels were measured by RT-PCR. D-Glucosamine treatment did not have a significant effect on HIF-1α mRNA levels in DU145 cells (Fig. 1E). This result suggests that D-glucosamine inhibits HIF-1α protein expression not through the modulation of its mRNA level, indicating that posttranscriptional mechanism exists for D-glucosamine action on HIF-1α expression.

D-Glucosamine does not affect HIF-1α degradation but decreases HIF-1α protein translation

To determine the process that might be involved in HIF-1α inhibition by D-glucosamine, we examined the effect of D-glucosamine on HIF-1α posttranscriptional regulation. First, the effects of D-glucosamine on HIF-1α protein stability were examined by using an

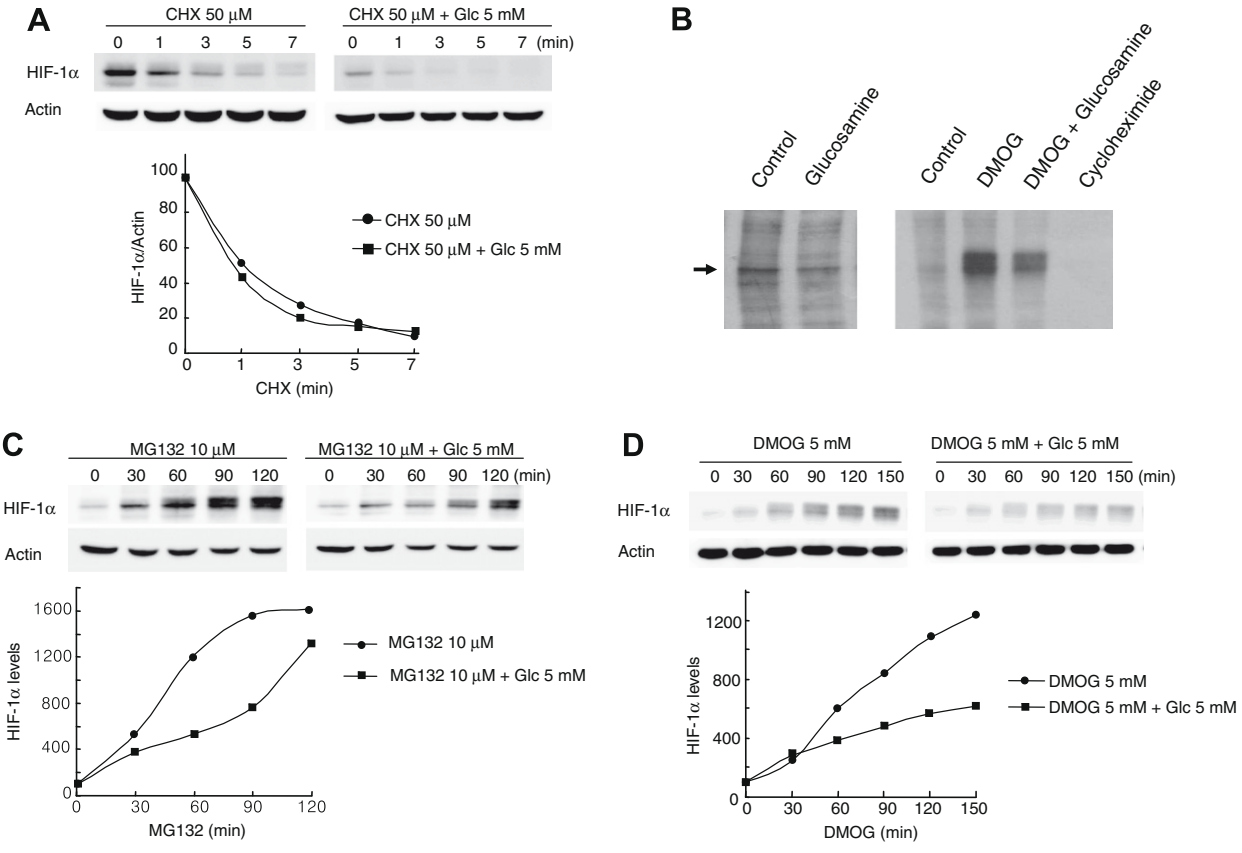

Fig. 2. D-Glucosamine does not affect HIF-1α degradation but inhibits HIF-1α protein translation. (A) DU145 cells were pretreated with vehicle or 5 mM D-glucosamine for 2 h and CHX was added for indicated times. Equal amounts of protein from each sample were resolved by SDS–PAGE, and Western blot was performed as described in Materials and methods. Lower panel shows quantification of the HIF-1α signal by LAS 3000 image analyzer following normalization to actin levels. HIF-1α levels from CHX untreated cells are arbitrarily given the value of 100%. (B) Metabolic labeling was performed in normoxic DU145 cells as described in Materials and methods. (Left) DU145 cells were pretreated with 5 mM D-glucosamine for 2 h, and ^{35}S-methionine was added to medium for 1 h. (Right) DU145 cells were pretreated with 5 mM D-glucosamine for 2 h, and ^{35}S-methionine was added to medium for 1 h. DMOG (5 mM) or CHX (50 μM) were co-treated with ^{35}S-methionine at indicated samples. (C and D) DU145 cells were pretreated with 5 mM D-glucosamine for 30 min and 10 μM MG132 (C) or 5 mM DMOG (D) was added for indicated times. Equal amounts of protein from each sample were resolved by SDS–PAGE, and Western blot was performed. Lower panels show quantification of the HIF-1α signal by LAS 3000 image analyzer following normalization to actin levels. HIF-1α levels from MG132 or DMOG untreated cells are arbitrarily given the value of 100%.

inhibitor of protein translation, cycloheximide (CHX) (Fig. 2A). Untreated and D-glucosamine treated DU145 cells were exposed to CHX for various times and HIF-1α protein levels were determined by Western blot. After exposure to CHX, HIF-1α levels were reduced in both untreated and D-glucosamine treated cells. Although the HIF-1α levels were different at zero time point, the degradation rates of HIF-1α protein were very similar in the absence or presence of D-glucosamine. This result suggests that D-glucosamine does not appear to affect the stability of HIF-1α protein.

We next examined the effect of D-glucosamine on HIF-1α protein translation. DU145 cells were labeled with ^{35}S-methionine in the presence or absence of D-glucosamine for 60 min (Fig. 2B). HIF-1α protein synthesis was higher in the untreated cells compared with the D-glucosamine treated cells. This was further confirmed by using prolyl hydroxylase inhibitor DMOG. We expected if D-glucosamine inhibits HIF-1α protein translation, the treatment of D-glucosamine would decrease the accumulation of newly synthesized HIF-1α protein even in the presence of DMOG since prolyl hydroxylation is important in degradation of HIF-1α under normoxic condition. As expected, DMOG induced accumulation of newly synthesized HIF-1α protein whereas the cells treated with D-glucosamine showed lower levels of DMOG induced accumulation of HIF-1α protein. Together with the result of the CHX experiment, these results indicate that D-glucosamine inhibits HIF-1α protein synthesis rather than enhancing its degradation. This was further confirmed when cells were treated with proteasome inhibitor MG132 in the presence or absence of D-glucosamine and HIF-1α protein levels were analyzed at various time points (Fig. 2C). MG132 treatment resulted in elevated HIF-1α protein levels in both untreated and D-glucosamine treated cells. However, the accumulation rate of HIF-1α protein in D-glucosamine treated cells was much slower compared to that in the untreated cells. Consistent with this result, D-glucosamine treatment reduced the accumulation rate of HIF-1α protein induced by DMOG (Fig. 2D). Overall, these experiments indicate that D-glucosamine does not affect HIF-1α protein degradation, but indeed decrease HIF-1α translation. To determine whether D-glucosamine acts as a general protein translation inhibitor, we examined general protein synthesis (Fig. 3A and B). Treatment of D-glucosamine did not reduce general protein translation, but indeed moderately increased protein synthesis. These results suggest that D-glucosamine reduces HIF-1α protein synthesis not through the inhibition of global protein synthesis.

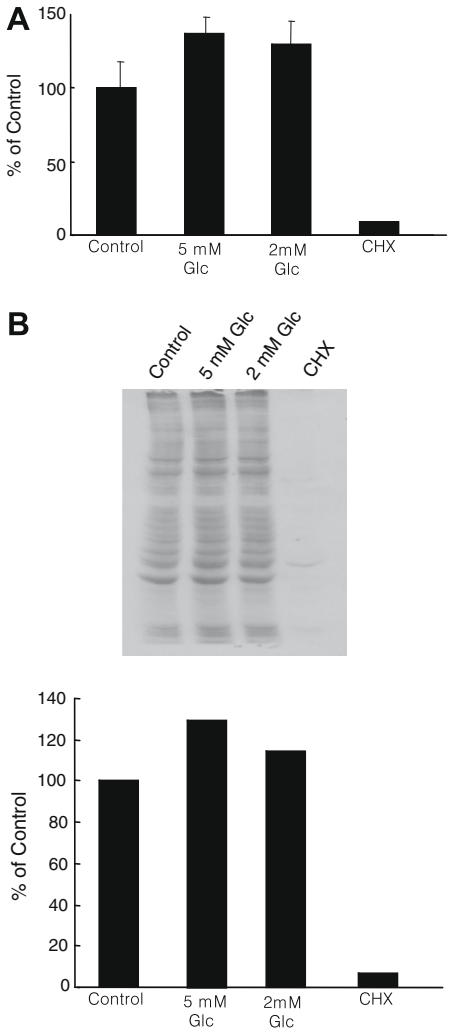

Fig. 3. Effect of D-glucosamine on global protein synthesis. DU145 cells were pretreated with indicated concentrations of D-glucosamine for 2 h, and ^{35}S-methionine was added to medium for 1 h. (A) Cells were harvested after labeling and radioactivity incorporated into TCA precipitable material was measured. Protein synthesis levels are shown as a percentage of the value obtain in the absence of D-glucosamine. (B) Cells were harvested, and cell lysates were electrophoresed in SDS–PAGE and incorporated radioactivity was detected by autoradiography. Lower panel shows quantification of signal densities of each lane.

D-Glucosamine inhibits serum induced expression of HIF-1α

Several growth factors stimulate HIF-1α expression through increasing HIF-1α translation in normoxic condition [6–8,16]. A prediction is that D-glucosamine as an inhibitor of HIF-1α translation might inhibit HIF-1α protein expression induced by growth factors. Whether this was the case, we examined the effect of D-glucosamine on the expression of HIF-1α induced by serum in DU145 cells (Fig. 4A). The serum treatment induced significant increase in HIF-1α in normoxic condition. However the serum did not induce HIF-1α expression in DU145 cells co-treated with D-glucosamine.

We previously reported that D-glucosamine inhibits p70S6K signaling [12]. To examine whether this effect of D-glucosamine was related to the decrease in HIF-1α level, the phosphorylation state of p70S6K and RPS6 were examined. Coinciding with inhibition of serum induced HIF-1α expression, D-glucosamine inhibited serum induced phosphorylation of p70S6K and its downstream target RPS6. Consistently, it has been reported that p70S6K plays a role in growth factor-induced HIF-1α expression [6,7,14]. Taken together, these results suggest that D-glucosamine inhibits HIF-1α expression through p70S6K inhibition, and D-glucosamine has the potential to inhibit HIF-1α expression which is induced by growth factors. Upon examination of VEGF production in cells treated with serum and D-glucosamine revealed that D-glucosamine inhibited serum induced VEGF production (Fig. 4B). These results suggest that D-glucosamine inhibits growth factor-induced VEGF expression through the inhibition of HIF-1α protein expression.

Discussion

Although D-glucosamine has been reported to inhibit many types of cancer cells *in vivo* and *in vitro*, the anticancer mechanism remains poorly understood [10–12]. In this report, we demonstrate for the first time that D-glucosamine reduces HIF-1α level by inhibition of protein translation. This novel finding provides a new insight into the potential mechanism of the anticancer properties of D-glucosamine.

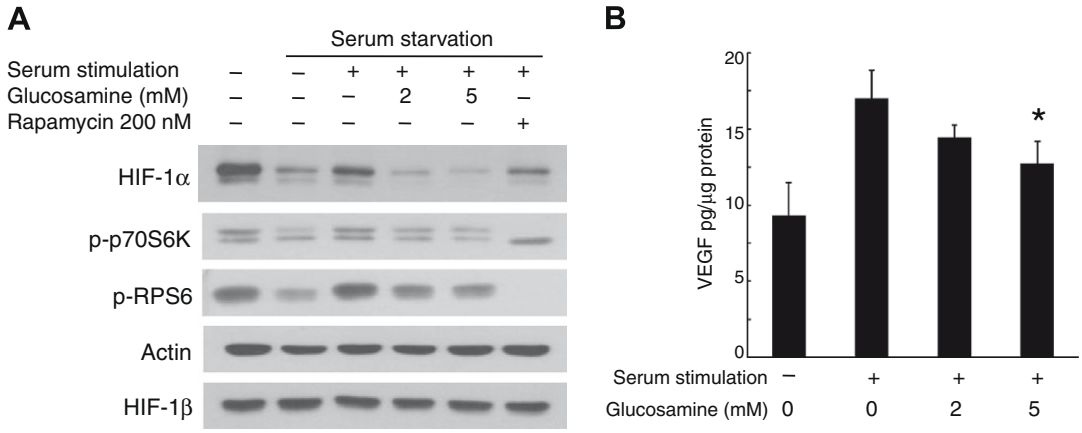

Fig. 4. D-Glucosamine inhibits serum induced HIF-1α expression. (A) DU145 cells were cultured in serum-free medium for 24 h followed by incubation in the absence or presence of 10% serum and D-glucosamine for 10 h. The cell lysates were subjected for Western blot analysis. (B) DU145 cells were seeded at 1.5×10^5 cells/well on 6-well culture plate and cultured in serum-free medium. After 24 h, the cells were treated with 10% serum and D-glucosamine for 10 h. The VEGF protein levels in the culture medium were analyzed by ELISA as described in Materials and methods. * indicates a significant difference from the group treated with only serum ($p < 0.05$).

HIF-1 plays critical roles in cancer biology, including immortalization, vascularization, metabolic reprogramming, invasion/metastasis, and treatment failure [1,17]. A growing number of anticancer agents which inhibit HIF-1 activity have been discovered and developed. The mechanism of action of these agents involve reducing HIF-1α mRNA or protein levels, or HIF-1-mediated transactivation of the target genes. Several agents that reduce HIF-1α protein expression do so by decreasing the rate of HIF-1α synthesis and/or increasing the rate of HIF-1α degradation [1,17].

The steady state of HIF-1α is controlled by the balance of degradation and synthesis of the protein. We found in this study, D-glucosamine inhibited accumulation of HIF-1α without affecting HIF-1α mRNA levels and the half-life of HIF-1α protein. D-Glucosamine was found to decrease HIF-1α protein synthesis, as shown by the reduction of *de novo* HIF-1α protein synthesis in metabolic labeling assays and decrease in the rate of HIF-1α accumulation in the presence of proteasome or prolyl hydroxylase inhibitors. Several investigators reported that D-glucosamine inhibited protein synthesis in cancer cells [10,18]. However, in our experiments, D-glucosamine did not inhibit general protein synthesis suggesting D-glucosamine would decrease the translation of a subset of cellular mRNAs including HIF-1α rather than reducing HIF-1α protein levels through inhibition of global protein synthesis in DU145 cells. The discrepancy of D-glucosamine's effects on the global protein synthesis between previous reports [10,18] and our results would be caused by the differences in the cellular context. Further evaluation is needed to clarify the effects of D-glucosamine on protein synthesis regulation.

A variety of stimuli, such as growth factors, cytokines and hormones can induce HIF-1α protein synthesis via activation of the PI3K/AKT/mTOR, and MAPK pathways [6–8,16]. Previously, we reported D-glucosamine inhibited p70S6K, a downstream target of AKT/mTOR signaling, in various cancer cells [12]. Moreover, it has been reported that p70S6K plays a role in HIF-1α expression [6,7,14] and p70S6K activate translation of mRNA containing 5′-terminal oligopyrimidine tract (TOP) sequences in their 5′-UTR [19,20]. Interestingly, it is known that 5′-UTR of HIF-1α mRNA contains 5′-TOP sequences [8,21]. We have demonstrated, in this report, D-glucosamine inhibits HIF-1α expression induced by serum stimulation coinciding with the inhibition of p70S6K. In contrast, D-glucosamine did not inhibit IGF-1 induced HIF-1α expression in PC-3 cells in which D-glucosamine did not inhibit p70S6K (Data not shown). During the preparation of this report we also found that D-glucosamine reduces expression of $p21^{WAF1}$ protein (Supplementary Fig. S1). Gaben et al. suggested that rapamycin, an inhibitor of mTOR/p70S6K signaling, selectively inhibits mitogen-induced p21 expression at the translational level [22]. Therefore, these results suggest that D-glucosamine can inhibit growth factor-induced HIF-1α expression through p70S6K inhibition. Further experiments are required to fully elucidate the association of p70S6K inhibition and HIF-1α inhibition induced by D-glucosamine.

Nowadays, D-glucosamine is widely taken as a dietary supplement to relieve discomfort of osteoarthritis-related joint pain, thus it is very interesting if D-glucosamine can exert chemopreventive effect in cancer. We demonstrate here for the first time that D-glucosamine inhibits HIF-1α expression by inhibiting protein translation, and further show that D-glucosamine inhibits HIF-1α expression induced by serum stimulation. This novel finding provides new insight into the potential mechanism of the anticancer properties of D-glucosamine. Molecular targeting of HIF-1α by D-glucosamine would be an attractive strategy for chemoprevention and/or treatment of cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.129.

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