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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.

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

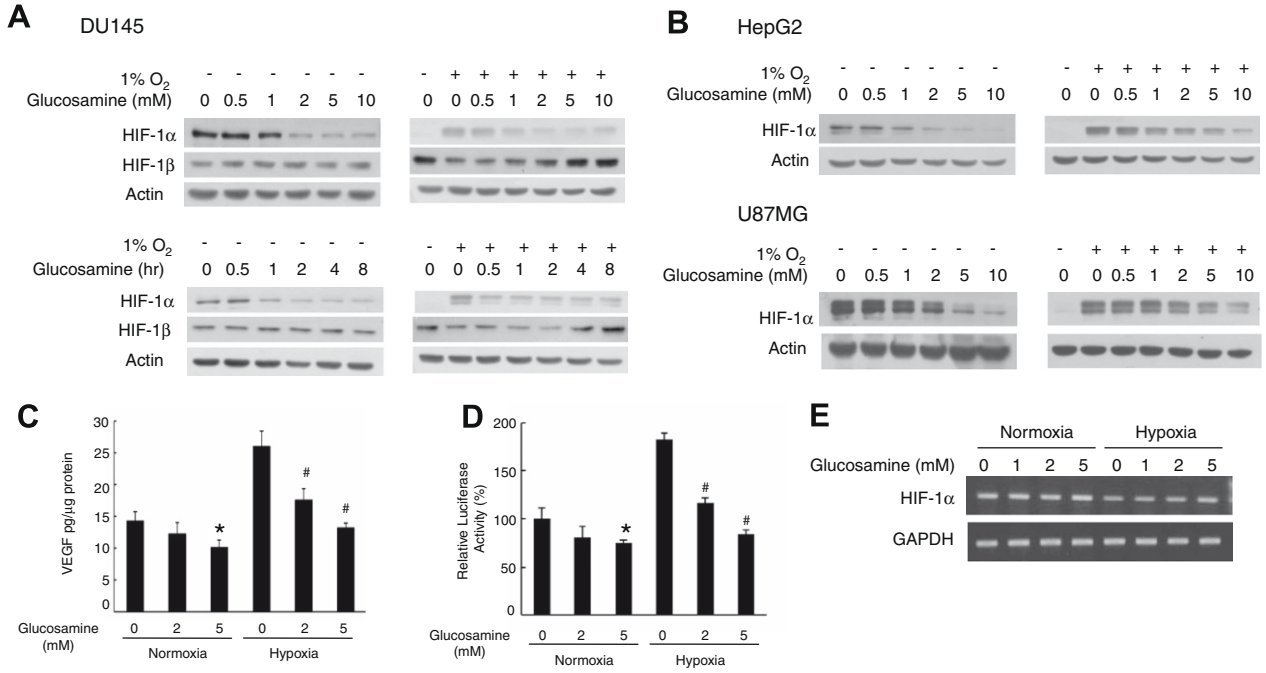

Fig. 1. (A) D-Glucosamine inhibits HIF-1α protein expression in dose- and time-dependent manners. DU145 cells were treated with various concentrations of D-glucosamine for 6 h (upper) or treated with 5 mM D-glucosamine for indicated times (lower) under normoxic and hypoxic conditions, and cell lysates 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.