EXPERIMENTAL CELL RESEARCH 316 (2010) 1179–1189

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Research Article

High glucose concentrations attenuate hypoxia-inducible factor-1α expression and signaling in non-tumor cells

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ARTICLE INFORMATION

Article Chronology:
Received 22 September 2009
Revised version received
15 January 2010
Accepted 16 February 2010
Available online 23 February 2010

Keywords:
Prolyl hydroxylase
Glutamine
Tumor cells
Non-tumor cells

ABSTRACT

Hypoxia-inducible factor (HIF) is the major transcription factor mediating adaption to hypoxia e.g. by enhancing glycolysis. In tumor cells, high glucose concentrations are known to increase HIF-1α expression even under normoxia, presumably by enhancing the concentration of tricarboxylic acid cycle intermediates, while reactions of non-tumor cells are not well defined. Therefore, we analyzed cellular responses to different glucose concentrations in respect to HIF activation comparing tumor to non-tumor cells. Using cells derived from non-tumor origin, we show that HIF-1α accumulation was higher under low compared to high glucose concentrations. Low glucose allowed mRNA expression of HIF-1 target genes like adrenomedullin. Transfection of C_2C_{12} cells with a HIF-1α oxygen-dependent degradation domaine-GFP fusion protein revealed that prolyl hydroxylase (PHD) activity is impaired at low glucose concentrations, thus stabilizing the fusion protein. Mechanistic considerations suggested that neither O_2 redistribution nor an altered redox state explains impaired PHD activity in the absence of glucose. In order to affect PHD activity, glucose needs to be metabolized. Amino acids present in the medium also diminished HIF-1α expression, while the addition of fatty acids did not. This suggests that glucose or amino acid metabolism increases oxoglutarate concentrations, which enhances PHD activity in non-tumor cells. Tumor cells deprived of glutamine showed HIF-1α accumulation in the absence of glucose, proposing that enhanced glutaminolysis observed in many tumors enables these cells to compensate reduced oxoglutarate production in the absence of glucose.

Introduction

A hallmark of malignant cancer cells is the increased uptake of glucose even under normoxic conditions, an effect known as "aerobic glycolysis" or the Warburg effect [1]. Tumor cells continue to catabolize glucose at a high rate and produce considerable amounts of lactate, even when oxygen is available for mitochondrial respiration [2]. A high glycolytic activity is achieved by upregulation of glucose transporters, glucose metabolizing enzymes and pyruvate dehydrogenase kinase, which attenuates

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Abbreviations: ADM, adrenomedullin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DMNQ, 2,3-dimethoxy 1,4-naphthoquinone; DMOG, dimethyloxalylglycine; GLUT-1, glucose transporter; HIF, hypoxia-inducible factor; ODD, oxygen-degadation domaine; PHD, prolyl hydroxylase; ROS, reactive oxygen species; TCA, tricarboxylic acid

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doi:10.1016/j.yexcr.2010.02.019

pyruvate consumption in the tricarboxylic acid (TCA) cycle [3]. Affected genes are targets of the hypoxia-inducible transcription factor (HIF). Indeed, HIF is upregulated in many cancer cells and it was shown that HIF is one of the most prominent factors accounting for the Warburg effect [3].

HIF is a heterodimer of an α- and β-subunit, which both are members of the family of basic-helix-loop-helix (bHLH)/ PER, ARNT, SIM (PAS) transcription factors [4]. Abundance and activity of HIF-1α is limited under ambient oxygen concentrations, while the HIF-1β protein is constantly expressed [4]. The oxygen sensors that control the abundance of the HIF-1α protein are prolyl hydroxylases named PHD1, PHD2 and PHD3 [4]. In the presence of oxygen, these PHDs catalyze the Fe(II)-dependent hydroxylation of specific prolyl residues within the oxygen-dependent degradation (ODD) domains of HIF-α subunits [4]. Once hydroxylated, the von Hippel-Lindau (pVHL) tumor suppressor protein, which is the recognition component of an E3 ubiquitin ligase complex, binds to the HIF-α subunits and thereby links prolyl hydroxylation to ubiquitination and proteasomal degradation [4]. In response to oxygen deprivation, PHD activity decreases. In turn, the α-subunits are stabilized and heterodimerize in the nucleus with the β-subunit [4]. Apart from oxygen deprivation, an increase in TCA cycle intermediates, oxidative stress, or iron deficiency attenuate PHD activity and thus, increase abundance of the HIF-α proteins [4]. When HIF is stabilized, the dimer specifically binds to hypoxia response elements (HREs) to induce target gene expression [4]. Besides increasing angiogenesis and erythropoesis, HIF also affects the energy metabolism of non-malignant tissue [5,6].

HIF not only affects glucose metabolism, but also is under the control of glucose itself. In many cancer cells HIF-1α accumulation is diminished by decreasing glucose concentrations under hypoxia, presumably due to reduced translation [7–10]. At ambient oxygen concentrations, HIF-1α protein expression is slightly elevated in the presence of high glucose concentrations because hydroxylation by PHDs is impaired [8]. In contrast, studying C_2C_{12} cell responses toward ischemia, we constantly noticed a slightly stronger HIF-1α expression in the absence of glucose at 21% O_2 [11]. Additionally, in some primary rat, human or bovine cells *in vitro* hypoxic HIF-1α accumulation is reduced in the presence of high glucose concentrations [12–14] indicating that in non-tumor tissue HIF-1α expression is reduced rather than enhanced by high glucose. Therefore, we investigated the expression of HIF-1α comparing non-tumor to tumor cells, while modulating glucose concentrations. We noticed that in non-tumor cells low compared to high glucose concentrations enhanced HIF-1α expression.

Research design and methods

Cell culture and incubation procedures

If not indicated otherwise, all chemicals were purchased from Sigma (Steinheim, Germany), while cell lines came from ATCC (LGC Promochem, Wesel, Germany). The mouse myoblast cell line C_2C_{12} was cultured as described previously [11]. Te617.T human rhabdomyosarcoma cells were cultured in DMEM with 10% FCS and DU145 in MEM with 10% FCS, 0.1 mM non-essential amino acids, 0.11 mg/ml sodium pyruvate, all supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (from PAA Laboratories, Cölbe, Germany). Primary human blood macrophages were isolated as described [15]. Cells were cultured in a humidified atmosphere to approximately 80% of cell density before used in experiments. Myotubes were differentiated from confluent myoblast cultures by replacement of fetal calf serum with 2% heat inactivated horse serum (PAA Laboratories) for 6 days.

Cells were either incubated in glucose-rich DMEM (25 mM glucose), or glucose-free DMEM (Invitrogen, Karlsruhe, Germany) with 10% FCS or in Krebs Henseleit buffer prepared as described previously [16] with the addition of glucose, amino acid as being present in DMEM, or 0.05 mM palmitic acid as indicated. Dimethyloxalylglycine (DMOG; 1 mM; Alexis Biochemicals, Grünburg, Germany), L-glucose (25 mM) or MG132 (10 μM; Calbiochem, Darmstadt, Germany) was added directly at the beginning of the experiment. Ascorbate (100 μM) was incubated overnight before starting experiments, but was absent during incubations. Hypoxic incubations were performed in a hypoxic workstation with 1% O_2, 94% N_2, 5% CO_2 (Invivo2 400, Ruskinn Technology, Leeds U.K.) or in a hypoxic incubator with 8% O_2, 92% N, 5% CO_2 (Heraeus, Hanau, Germany).

Western blot analysis

Nuclear protein extracts were prepared from 100 mm dishes, except for experiments performed on 60 mm gas permeable dishes (Greiner Bio One, Frickenhausen, Germany), protein was isolated to perform Western analysis as described previously [11]. As primary antibodies, a rabbit polyclonal antibody for mouse HIF-1α (Novus Biologicals Acris, Hiddenhausen, Germany), a monoclonal mouse antibody for human HIF-1α (BD Biosciences, Heidelberg, Germany) and a monoclonal mouse anti-tubulin antibody (Sigma) were used. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Sigma) was used as secondary antibodies. Densitometry was performed using ImageJ software (National Institutes of Health, USA).

Transfection

Cells were grown in culture flasks to 40% confluence and myoblasts were transfected with 1% Tfx™-20 (Promega, Mannheim, Germany) and 0.047 μg/cm^2 of ODD-GFP expression vector DNA as described by Hagen et al. [17]. DU145 cells were transfected with 0.047 μg/cm^2 pcDNA3-VHL.HA expression vector DNA using 1 % TFX™-20. Cells were incubated another 24 h before they were seeded on 100 mm dishes (Greiner Bio One) and grown over night to 80% confluence. Afterward, experiments were performed as described above and cells were lysed for GFP detection using the following buffer: 6.65 M urea, 10% glycerol, 1% SDS, 10 mM Tris, pH 7.4 or used for coimmunoprecipitation. The protein concentration was determined with a commercial protein assay and 100 μg protein were used for Western analysis of GFP expression using the anti-GFP antibody from Santa Cruz Biotechnology (Heidelberg, Germany) and horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody.

Coimmunoprecipitation (Co-IP)

Transfected cells were treated with MG132 to block HIF-1α protein degradation and lysed in 150 μl buffer A (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, protease