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Glucose is necessary for stabilization of hypoxia-inducible factor-1α under hypoxia: Contribution of the pentose phosphate pathway to this stabilization

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ABSTRACT

In this study, we observed that low glucose or fructose reduces the increase in hypoxia-inducible factor-1α (HIF-1α) protein under hypoxic conditions. 6-Aminonicotinamide (6-AN), an inhibitor of the pentose phosphate pathway (PPP), also inhibited the increase of HIF-1α protein under hypoxic conditions, while the reduced protein levels of HIF-1α by low glucose were apparently recovered by the addition of MG-132 or NADPH. Moreover, siRNA for glucose-6-phosphate dehydrogenase, which produces NADPH, reduced the increase in HIF-1α protein. On the other hand, cobalt-induced expression of HIF-1α protein was not affected by low glucose or 6-AN under normoxic conditions. In conclusion, glucose metabolism through the PPP, but not in glycolysis, plays an important role in the stabilization of HIF-1α protein under hypoxic conditions.

1. Introduction

Under chronic hypoxia, cells switch from aerobic to anaerobic metabolism to meet the energy requirements for survival [1]. Unlike aerobic cells, hypoxic cells are less able to use ATP produced through oxidative phosphorylation in mitochondria, and must rely primarily on the catabolism of glucose via glycolysis for their energy needs [2]. Because glycolysis is considerably less efficient than oxidative phosphorylation in harvesting energy from glucose, a hypoxic cell must increase the rate of glucose uptake and glycolysis to meet its energy demands.

During hypoxia, glucose transporter (GLUT) 1 [3,4], 3 [4], and 4 [5], and some glycolytic enzymes [6], are induced depending upon hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, the latter known as aryl hydrocarbon receptor nuclear translocator (ARNT) [7,8]. Although ARNT protein is readily found in cells under normoxic conditions, HIF-1α is virtually undetectable under normal conditions, because it is degraded through prolyl hydroxylation, which permits the binding of von Hippel-Lindau protein (pVHL), a component of E_3 ubiquitin ligase [9,10]; however, HIF-1α evades prolyl hydroxylation and is stabilized under hypoxic conditions. It has been well documented that cobalt mimics hypoxia by causing the stabilization of HIF-1α. Because a HIF-specific prolyl-hydroxylase has an iron binding-center and the iron can be replaced by cobalt at this site, hydroxylase activity results in inactivation [11]; thus, cobalt appears to stabilize HIF-1α via different mechanisms from during hypoxia.

We have previously found that HIF-1α signal transduction during hypoxia was mediated by NADPH-cytochrome P-450 reductase (NPR) [12], although the mechanism by which NPR induces the stabilization of HIF-1α under hypoxic conditions is still unclear. On the other hand, NADPH as the substrate of NPR, is provided mainly through the metabolism of glucose via the pentose phosphate pathway (PPP). In this pathway glucose-6-phosphate dehydrogenase (G6PD) is a rate-limiting enzyme, and produces NADPH by catalytic action. Interestingly, hypoxia is reported to stimulate the expression and activity of G6PD in rat pheochromocytoma PC12 cells [13]. Thus, glucose, especially its metabolism through the PPP, may be involved in the hypoxic responses of cells, including the stabilization of HIF-1α.

Abbreviations: HIF-1, hypoxia-inducible factor; GLUTs, glucose transporters; HMCs, human mesangial cells; PPP, pentose phosphate pathway; 6-AN, 6-aminonicotinamide; ARNT, aryl hydrocarbon receptor nuclear translocator; NPR, NADPH-cytochrome P-450 reductase; G6PD, glucose-6-phosphate dehydrogenase; VEGF, vascular endothelial growth factor; EPO, erythropoietin

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Recently, we found that a high concentration (30 mM) of glucose induces a time-dependent increase in vascular endothelial growth factor (VEGF) mRNA, and stabilizes HIF-1α protein in human mesangial cells (HMCs) [14]; however, the role of glucose in the stabilization and activation of HIF-1 is still unclear. In this study, we examined whether glucose controls the stabilization of HIF-1α during hypoxia in HMCs and human hepatoma cell line Hep3B. It was also examined which pathway of the glycolysis or the PPP is involved in HIF-1α stabilization under hypoxic conditions by using fructose instead of glucose or 6-aminonicotinamide (6-AN), an inhibitor of the PPP [15]. Since fructose is not metabolized through the PPP, it is thought to be a suitable tool for studying the contribution of the PPP in hypoxia-induced HIF-1α stabilization. Moreover, it was established that the hypoxia-induced stabilization of HIF-1α was suppressed in cells transfected siRNA of G6PD. Here we show that using for HMCs and Hep3B cells, glucose metabolism through the PPP is especially important for the stabilization of HIF-1α under hypoxic conditions.

2. Materials and methods

2.1. Materials

MCDB131 medium, Dulbecco's modified Eagle's medium (DMEM), MG-132, and anti-β-actin IgG were purchased from Sigma (St. Louis, MO). Penicillin and streptomycin were from Meiji (Tokyo, Japan). 6-AN was from Aldrich (Milwaukee, WI). Glucose and fructose were from Wako Pure Chemical (Osaka, Japan). NADPH and $NADP^+$ were from Oriental Yeast Co. (Tokyo, Japan). Isogen was from Nippon Gene (Toyama, Japan). ReverTra Ace and ReverTra Ace (qPCR RT) were from Toyobo (Osaka, Japan). Ampli Taq and SYBR Green Universal PCR Master Mix were from Applied Biosystems (Foster City, CA). Anti-HIF-1α antibody and anti-NPR antibody were prepared as described previously [12]. Anti-G6PD antibody was from Bethyl Laboratories (Montgomery, TX). Lipofectamin RNAiMAX was from Invitrogen (Carlsbad, CA). siRNA for G6PD was from NAGASE & Co., LTD. (Kobe, Japan).

2.2. Cell culture and treatments

HMCs obtained from Cambrex Co. (Walkersville, MD) were maintained in MCDB131 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Hep3B cells were obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Hep3B cells were maintained in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. After incubating in medium containing 1% FBS for 24 h, the cells were incubated under hypoxic conditions (1% O_2, 5% CO_2). Normal glucose (5.6 mM) media were prepared with the addition of 4.5 g/l glucose to low glucose media (0 mM) supplemented with 1% FBS, 3.84 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in DMEM. The cells were incubated in low glucose or normal glucose media for 6 h before exposure to hypoxia.

2.3. Western blot analysis

Cells were washed with phosphate-buffered saline and collected by centrifugation in ice-cold phosphate-buffered saline. Nuclear extracts from cells were prepared by the method performed as previously described [8]. Whole cell lysates were prepared with 20 mM Tris–HCl (pH 7.4) containing 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 10 μM MG-132, and 100 μM PMSF. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with a 7.5% or 10% gel, and 10–20% gradient gel. Immunoblot analysis was performed with antibodies against HIF-1α and NPR which had been prepared previously [12], G6PD (1:1000), and β-actin (1:5000). Blotted proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG and enhanced chemiluminescence Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

2.4. Isolation of total RNA and reverse transcription-PCR

Total RNA (1 μg) extracted from HMCs or Hep3B cells with Isogen was transcribed into cDNA using ReverTra Ace in 10 μl (total volume) according to the manufacturer's directions as follows: incubation for 10 min at 30 °C and 40 min at 42 °C, followed by heating for 10 min at 70 °C to stop the reaction. PCR with 5 pmol of each primer, 1.0 U of Ampli Taq, and the cDNA was performed for 10 min at 94 °C and then 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 2.5 min at 72 °C. The primers for GLUT1 (Accession No. NM_006516) were 5′-ACA GGC AGC TGG ATG AGA CT-3′ (sense) and 5′-CAT AGC CAC CTC CTG GGA TA-3′ (antisense), and those for GLUT3 (NM_006931) were 5′-CCC AGA TCT TTG GTC TGG AA-3′ (sense) and 5′-CAC AGC ATT GAT CCC AGA GA-3′ (antisense). The primers for HIF-1α (NM_001530) were 5′-CAG CTA TTT GCG TGT GAG GA-3′ (sense) and 5′-CCA AGC AGG TCA TAG GTG GT-3′ (antisense), and those for β-action (NM_001101) were 5′-GAT CAT TGC TCC TCC TGA GC-3′ (sense) and 5′-CAC CTT CAC CGT TCC AGT TT-3′ (antisense). The primers for erythropoietin (EPO) were performed as previously described [12].

2.5. NADPH assay

Hep3B cells (2×10^5 cells) were incubated in low glucose or normal glucose media for 6 h before exposure to hypoxia. After hypoxia for 6 h, the cells were washed with phosphate-buffered saline and collected by centrifugation in ice-cold phosphate-buffered saline. NADPH concentrations in cells were measured with using EnzyChrom NADPH assay kit (BioAssay Systems, Hayward, CA).

2.6. Transfection of siRNA for G6PD

siRNA (20 pmol) for G6PD was transfected by Lipofectamin RNAiMAX (1.7 μl) into Hep3B cells (1.5×10^5) during incubation at 37 °C for 6 h. After cultured for 48 h in normal glucose media, cells transfected with or without siRNA ($G6PD^-$ or control) were incubated for 6 h under normoxic or hypoxic conditions. The siRNA for G6PD (Accession No. NM_000402.3) were 5′-GCC UCA UCC UGG ACG UCU U-3′ (sense) and 5′-AAG ACG UCC AGG AUG AGG CGC-3′ (antisense).

Total RNA (1 μg) extracted from control or $G6PD^-$ cells with Isogen was transcribed into cDNA using ReverTra Ace (qPCR RT) in 10 μl (total volume) according to the manufacturer's directions as follows: incubation for 15 min at 37 °C, followed by heating for 5 min at 98 °C to stop the reaction. Real-time PCR was performed on a 7500 FAST Real-Time PCR System (Applied Biosystems). The cDNA, 4 pmol of each primer and 10 μl of SYBR Green Universal PCR Master Mix were amplified as follows: 10 min at 95 °C and then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Target gene expression was normalized to the endogenous control gene-actin. The primers for G6PD (Accession No. NM_000402) were 5′-GCA AAC AGA GTG AGC CCT TC-3′ (sense) and 5′-GGC CAG CCA CAT AGG AGT T-3′ (antisense), and those for β-action (NM_001101) were 5′-CCA ACC GCG AGA AGA TGA-3′ (sense) and 5′-CCA GAG GCG TAC AGG GAT AG-3′ (antisense) (NAGASE & Co., LTD).