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Original Articles

NFκB up-regulation of glucose transporter 3 is essential for hyperactive mammalian target of rapamycin-induced aerobic glycolysis and tumor growth

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ABSTRACT

Accumulating evidence indicates that mammalian target of rapamycin (mTOR) exerts a crucial role in aerobic glycolysis and tumorigenesis, but the underlying mechanisms remain largely obscure. Results from Tsc1- or Tsc2-null mouse embryonic fibroblasts (MEFs) and human cancer cell lines consistently indicate that the expression of glucose transporter 3 (Glut3) is dramatically up-regulated by mTOR. The rapamycin-sensitive mTOR complex 1 (mTORC1), but not the rapamycin-insensitive mTOR complex 2 (mTORC2), was involved in the regulation of Glut3 expression. Moreover, mTORC1 enhances Glut3 expression through the activation of the IKK/NFκB pathway. Depletion of Glut3 led to the suppression of aerobic glycolysis, the inhibition of cell proliferation and colony formation, and the attenuation of the tumorigenic potential of the cells with aberrantly hyper-activated mTORC1 signaling in nude mice. We conclude that Glut3 is a downstream target of mTORC1, and it is critical for oncogenic mTORC1-mediated aerobic glycolysis and tumorigenesis. Hence Glut3 may be a potential target for therapy against cancers caused by the aberrantly activated mTORC1 signaling.

Introduction

Unlike normal cells, tumor cells preferentially metabolize glucose through glycolysis even in the presence of sufficient oxygen, which is known as aerobic glycolysis or the Warburg effect [1,2]. Even though aerobic glycolysis is inefficient for energy production, it is believed to offer a selective advantage for the proliferation and survival of cancer cells. Currently, although the mechanism of aerobic glycolysis still remains largely obscure, the measurement of aerobic glycolysis in patients has been used clinically to diagnose cancers and monitor cancer progression in response to treatment [1,3]. Glycolytic inhibitors have also been tested for cancer treatment in clinical trials, such as 2-deoxyglucose (2-DG) and dichloroacetate (DCA) [4,5].

An extremely higher rate (up to 200-fold) of glycolysis is always detected in tumor cells, which is believed to compensate for the inefficiency on generating energy. This enhancement of glucose metabolism requires an accelerated glucose uptake into tumor cells. A family of glucose transporter proteins (Gluts) facilitates the glucose transport across the plasma membranes of mammalian cells in a tissue-specific manner. So far, 14 different Glut isoforms have been identified. These Gluts are divided into three subclasses (class I, class II, and class III) based on sequence similarities [6]. Class I Glut isoforms which have been well-characterized comprise Glut1-Glut4. Among this family, Glut1, Glut3, and Glut4 are high-affinity transporters, whereas Glut2 is a low-affinity transporter [7]. Under normal physiological conditions, Glut1 is ubiquitously expressed at low level, Glut4 is mainly expressed in adipose and muscle, and Glut3 is expressed primarily in neurons [8]. Most impressively, Glut3 has at least a 5-fold greater transporting capacity than Glut1 or Glut4

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[8]. Over-expression of Glut1 was observed in a variety of tumors, and its function in tumor development has been intensively investigated [4,7]. However, there is very limited research on Glut3. A recent study showed that Glut3 expression is correlated with poor survival in a broad range of tumor types [9], suggesting that many cancers may dysregulate Glut3 to drive tumor growth. However, the precise mechanisms leading to the up-regulation of Glut3 in tumor cells and the significance of Glut3 in tumor development are largely unknown.

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) (PI3K/AKT/mTOR) signaling pathway is frequently hyperactivated in human cancers, via gain-of-function mutations of proto-oncogenes such as EGFR, PI3K, and AKT, or loss-of-function mutations of tumor suppressors, including PTEN, LKB1, and the tuberous sclerosis complex 1 and 2 (TSC1/2) [10,11]. mTOR, a highly conserved serine/threonine protein kinase, is the key effector in this pathway via integrating multiple inputs, such as growth factor signaling and nutrient status to orchestrate a number of cellular processes, including cell growth, proliferation, differentiation, and survival [12,13]. As two upstream regulators of mTOR, TSC1 and TSC2 have distinct characteristics. These two proteins form a functional complex negatively regulating the activity of mTOR [14]. Aberrant activation of mTOR due to the loss of function of the TSC1/TSC2 complex is the major cause of tuberous sclerosis complex (TSC), a benign tumor syndrome that affects multiple organs [15,16]. mTOR can associate with different binding partners to form two functional complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is sensitive to rapamycin and activated by growth factor stimulation via PI3K/AKT pathway, whereas mTORC2 is rapamycin-insensitive, and its upstream regulatory signaling pathways remain poorly characterized [10]. Recently, the pivotal role of mTOR in glycolysis during tumorigenesis has been established, but the precise mechanisms still require further elucidation.

Our study demonstrates that mTORC1 positively regulates the expression of Glut3 through the activation of IKK/NFκB signaling. The knockdown of Glut3 results in reduction of aerobic glycolysis, inhibition of cell proliferation, suppression of colony formation, and delay of tumor growth. Since Glut3 is pivotal for hyperactive mTORC1-induced glycolysis and tumorigenesis, we suggest that Glut3 can serve as a potential target for the treatment of cancers associated with dysregulated mTOR signaling.

Materials and methods

Reagents, plasmids, and antibodies

Rapamycin and BAY 11–7082 were purchased from Sigma. WYE-354 was from Selleck Chemicals. Lipofectamine 2000 and 4–12% Bis-Tris Nu-PAGE gels were from Life Technologies. pNFκB-TA-Luc was from Clontech. pBabe Ha-RasV12 was from Addgene. Full-length mouse p65 cDNA and mouse Glut3 cDNA were obtained by PCR from mouse embryonic fibroblasts (MEFs) cDNA pools and cloned into a pLXIN retroviral vector. pLL3.7-GFP lentiviral vector and the pLXIN vector have been described previously [11]. Glut1 (ab652), Glut3 (ab41525), LaminB1 (ab133741) and Ki-67 (ab16667) antibodies were from Abcam. TSC2 (sc893) and β-actin (sc47778) antibodies were from Santa Cruz. TSC1 (#6935), S6 (#2217), p-S6 (Ser235/236) (#4857), AKT1 (#2967), p-AKT(ser473) (#4060), IKKα (#2682), IKKβ (#2370), p-IKKα/β (Ser176/180) (#2697), p65 (#8242), p-p65 (Ser536) (3033), mTOR (#2983), Raptor (#2280) and Rictor (#2114) antibodies were from Cell Signaling.

Cell cultures and treatments

All the mouse embryonic fibroblasts (MEFs) and rat uterine leiomyoma-derived Tsc2-null ELT3 cells used in this study have been described previously [11,17–19]. PT67 packaging cells were purchased from Clontech. HEK 293T and human glioblastoma cell line U87 cells were obtained from the ATCC. ELT3 cells were maintained and propagated in DMEM/F12 (1:1) with 10% FBS. The other cells were cultured in DMEM with 10% FBS. All cells were incubated at 37 °C in the presence of 5% CO_2. Production of retroviruses and subsequent generation of stable gene expression cell lines have been described previously [17]. Cells were harvested 24 h after final feeding at a confluence of approximately 80–90%, unless indicated differently. For drug treatment, cells were plated in 6-well plates at 30–40% density 24 h prior to treatment. The DMSO stocks of the agents used, including BAY 11–7082 and WYE-354 were diluted to appropriate concentrations with the cell culture medium. Rapamycin was diluted with ethanol.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol (Life Technologies) according to the protocol provided by the manufacturer. RNA was converted to cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa). qRT-PCR was performed using Power SYBR® Master Mix (Life Technologies) according to the manufacturer's protocol. The primer sequences are listed in Supplementary Table S1.

Cell fractionation and western blot

Cytoplasmic and nuclear protein fractions were extracted using a NE-PER Reagent Kit (Pierce) according to the manufacturer's instructions. Western blot analysis of protein expression was performed as described previously [11].

RNA interference

Cells seeded in 12-well plates were transfected with siRNAs using Lipofectamine 2000 following the manufacturer's instructions. All siRNA oligonucleotides were synthesized by GenePharma (Shanghai, China). The siRNA target sequences are listed in Supplementary Table S2.

Reporter constructs and luciferase reporter assay

Mouse Glut3 promoter-intron 1 fragments (from –938 to +1132 bp) were generated by PCR amplification using mouse genomic DNA as template and then cloned into the *Nhe* I/*Hind* III sites of the luciferase reporter plasmid pGL3-basic (Promega). The primer sequences were as follows: forward, 5′-CGGCTAGCTCAGTTCAGTCCATCAGTCT-3′; reverse, 5′-GATGAAGCTTGGCCTGCTACAACCTCT-3′. The potential NFκB binding site on the first intronic region of mouse Glut3 gene was mutated using the Quick Change site-directed mutagenesis kit (Promega). The primer sequences were as follows: forward, 5′-GTATTTGTAAGGAAAACTCCATTTCCACGAAGATTAACTTG-3′; reverse, 5′-CAAGTTAATC TTCGTGGAAATGGAGTTTTCCTTACAAACAT-3′. Cells were cultured in triplicate to 80% confluence in 24-well plates and transfected the promoter constructs (200 ng) in combination with the plasmid pRL-TK (20 ng) as an internal control. Luciferase activity was detected with the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay

Immunoprecipitation experiments with an anti-p65 antibody to detect protein–DNA interactions were performed using a SimpleChIP® Enzymatic Chromatin IP kit (Cell Signaling) according to the manufacturer's protocol. The immunoprecipitated DNA was purified and analyzed by qRT-PCR. The primer sequences used in the analysis of the target regions are as follows: the putative NFκB-binding sites region (PBR) of mouse Glut3 forward, 5′-GTCCTTACTATGGTTGTGAG-3′, reverse, 5′-CACGGTCTCTTAATTTACTT-3′; a nonspecific NFκB-binding region (NBR) of mouse Glut3 forward, 5′-GCAA GCATAATTTTCTCTGTTTC-3′, reverse, 5′-CAAAACACCCATAATGCATAAA-3′. The PBR of human Glut3 forward, 5′-CCGCTTCATCAGTTCTCTTTG-3′, reverse, 5′-CCTCAGT GGCATATGGTTACC-3′; the NBR of human Glut3 forward, 5′-CTAGGCCCTCAACCAAAACC-3′, reverse, 5′-TGCAACCCTTTCAGTACTGGA-3′.

Lentiviral vector production and transduction

pLL3.7-GFP plasmids were digested with *Xho* I and *Hpa* I enzymes, and then followed by insertion of annealed oligonucleotides. The sequences of the oligonucleotides are listed in Supplementary Table S3. HEK 293T cells were transfected with pLL3.7-GFP vector containing either of these sequences together with packaging vectors (pVSVG, pREV, and pMDL). Culture supernatants were collected after 48 h of transfection and then used to infect target cells.

Cell proliferation assay

Cell proliferation was measured using an MTT assay as described previously [11,15]. In brief, cell were seeded into 96-well plates in triplicate at a starting density of 1×10^3 per well and the proliferation was monitored for up to 4 days according to the manufacturer's specifications.

Measurements of glucose consumption and lactate production

Cells were seeded in culture dishes and cultured for 8 h. The culture medium was then changed and cells were incubated for an additional 16 h. Subsequently, the culture medium was collected for determination of glucose concentration and lactate levels using a Glucose assay kit and a Lactate assay kit (Eton Bioscience). Data were normalized to final cell counts. Glucose consumption was calculated as the difference in glucose concentration between fresh medium and supernatant.