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Interleukin 6 Enhances Glycolysis through Expression of the Glycolytic Enzymes Hexokinase 2 and 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase-3

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Abstract

Enhanced glycolysis is important for oncogenesis and for the survival and proliferation of cancer cells in the tumor microenvironment. Recent studies have also shown that proinflammatory cytokine signaling, such as that mediated by nuclear factor κB and signal transducer and activator of transcription 3 (STAT3), is important for the generation of inflammation-associated tumors. However, the link between inflammation and enhanced glycolysis has not been identified. In the present study, we found that the proinflammatory cytokine interleukin (IL)-6 enhanced glycolysis in mouse embryonic fibroblasts and human cell lines. Moreover, STAT3 activated by IL-6 enhanced the expression of the glycolytic enzymes hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3). Ectopic expression of PFKFB3 enhanced glycolysis, suggesting that the IL-6-STAT3 pathway enhances glycolysis through the induction of these enzymes. Our findings may provide a novel mechanism for inflammation-associated oncogenesis.

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Key words: interleukin 6, STAT3, glycolysis, hexokinase 2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3

Introduction

Cancer cells preferentially utilize aerobic glycolysis for energy production, and their increased dependency on glycolysis for ATP synthesis instead of oxidative phosphorylation in the presence of oxygen is known as the Warburg effect[1,2]. This phenomenon may confer a selective advantage for the survival and proliferation of cancer cells in the tumor microenvironment[3,4]. Recently, we demonstrated that the loss of tumor-suppressor p53 functions enhances the DNA-binding and transcriptional activities of the transcription factor nuclear factor-κB (NF-κB) via activation of inhibitor of κB kinase (IKK) α and β[5]. Furthermore, we found that activated NF-κB enhances the expression of the glucose transporter, type 3 (GLUT3), and the rate of aerobic glycolysis[5]. The IKK-NF-κB pathway is a critical regulator of innate and adaptive immune

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responses, inflammatory responses, and oncogenesis[6-8]. Moreover, oncogenic Ras-induced cell transformation and acceleration of aerobic glycolysis in p53-deficient ($p53^{-/-}$) mouse embryonic fibroblasts (MEFs) were suppressed in the absence of p65/NF-κB expression, and were recovered by GLUT3 expression[5]. In addition, in the absence of p53, we found that the positive feedback regulation by which glycolysis enhances the IKK-NF-κB signaling pathway through *O*-linked glycosylation of IKKβ further enhances glucose metabolism[9]. These results suggest that, in addition to the survival and proliferation of cancer cells, enhanced glycolysis plays an integral role in oncogenesis.

Inflammation arises through a complex set of interactions among soluble inflammatory mediators, including the proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, produced by macrophages, and the process leads to both recovery from infection and healing[10]. Numerous clinical and epidemiological studies have suggested that chronic infection, inflammation, and cancer are closely linked[11,12]. Recent genetic analyses using genetically modified mice have clearly demonstrated roles for proinflammatory cytokine signal pathways in inflammation-associated tumors. For example, conditional inactivation of IKKβ, a signal mediator for inflammatory cytokines, in intestinal epithelial cells markedly decreases the tumor multiplicity of colitis-associated cancer[13]. Moreover, IL-6-null ($Il6^{-/-}$) mice and mice with conditional deletion of IL-6 gene in bone marrow derived cells show reduced tumor incidence in colitis-associated cancer[14]. Similar findings have been observed for mice with conditional deletion of the transcription factor signal transducer and activator of transcription 3 (STAT3), a critical mediator of IL-6 signaling[14]. These results suggest that the tissue microenvironment is a powerful regulator of oncogenesis and tumor proliferation and that proinflammatory cytokine signals induced by inflammation are important regulators of these processes. Moreover, these observations and the above-described role of enhanced glucose metabolism in oncogenesis raise the possibility that the tissue microenvironment in inflammation enhances glucose metabolism.

In the present study, we analyzed the roles of inflammatory mediators in glucose metabolism. We found that the IL-6-STAT3 pathway enhanced glycolysis and the expression of the glycolytic enzymes hexokinase 2 (HK2) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3). Our results suggest a role for enhanced glucose metabolism in oncogenesis in the inflammation-induced tissue microenvironment.

Materials and Methods

Cell Culture and Measurement of Glucose Consumption and Lactate Production

Wild-type, $p53^{-/-}$ and $p65^{-/-}$ MEFs were prepared as previously described[5]. TIG3 cells and HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum and 50 μg/mL kanamycin. Glucose consumption and lactate production were analyzed as previously described[5]. The glucose level was determined with a glucose assay kit (Sigma-Aldrich, St Louis, MO, USA). Recombinant human IL-6 (PeproTech, Rocky Hill, NJ), human TNF-α (PeproTech), human IL-1β (PeproTech), soluble IL-6 receptor (sIL-6R; PeproTech), lipopolysaccharide (LPS) (Sigma-Aldrich), and 12-O-tetradecanoyl phorbol-13-acetate (TPA; Sigma-Aldrich) were used for this assay. The lactate level was determined using an F-kit L-lactic acid (Roche, Mannheim, Germany). The STAT3 inhibitor cucurbitacin I was purchased from Calbiochem (San Diego, CA, USA).

Quantitative Real-time Polymerase Chain Reaction Analysis

Total RNA was extracted with a Fast Pure RNA Kit (TaKaRa Bio, Shiga, Japan). cDNA was prepared using a PrimeScript RT Reagent Kit (TaKaRa Bio). Quantitative real-time polymerase chain reaction (PCR) analysis was performed using a TaqMan Probe Mix (Applied Biosystems, Foster City, CA, USA) and a StepOne™ Real-Time PCR system

(Applied Biosystems) with the following conditions: initial incubation at 95℃ for 20 seconds, followed by 40 cycles of 95℃ for 1 second, and 60℃ for 20 seconds. The primer and probe sets used were predesigned primer/probe sets as follows: β-actin, Mm00607939_s1, Hs03023880_g1; GLUT1, Mm 00441473_m1; GLUT3, Mm00441483_m1; HK1, Mm 00439344_m1; HK2, Mm00443385_m1, Hs01034064_g 1 ; PFKFB2, Mm00435575_m1; PFKFB3, Mm00504650_ m1, Hs00190079_m1; phosphoglucose isomerase (GPI), Mm02026122_g1; aldolase A (ALDOA), Mm 00833172_g1; triose phosphate isomerase (TPI) 1, Mm 00833691 _ g 1 ; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mm 99998915 _ g 1 ; phosphoglycerate mutase (PGM) 1, Mm02526975_g1; phosphoglycerate kinase (PGK) 1, Mm01225301_m1; enolase (ENO) 1, Mm01619597_g1; pyruvate kinase M (PKM) 2, Mm00834102_gH; haptoglobin, Hs 00978377_m1. Data were calculated as the mRNA expression levels relative to the β-actin mRNA expression levels according to the manufacturer's protocol.

RNA Interference and DNA Transfection

Retroviral vectors encoding a short-hairpin RNA (shRNA) against mouse STAT3 were constructed by cloning a suitable oligonucleotide sequence (5'-GCAGCAGCTGAACAACATG-3') into pSUPER. retro.puro (OligoEngine, Seattle, WA, USA). The *p65* shRNA-expressing retrovirus vector was described previously[5]. For virus production, 293T cells were transfected with 2 μg of ecotropic helper retrovirus plasmid plus 2 μg of each pSUPER.retro.puro vector. Culture supernatants were collected at 36 to 48 hours posttransfection and filtered. Target cells were infected with the filtered viral supernatants in the presence of 8 μg/mL polybrene for 24 hours, followed by exchange of the medium. Infected cells were selected using puromycin (2 μg/mL) for 3 days. Hemagglutinin (HA)-tagged mouse HK2 and Flag-tagged mouse PFKFB3 complementary (c) DNAs were obtained by reverse transcriptase (RT)-PCR and cloned into pCDNA3 vector (Invitrogen, Carlsbad, CA, USA). One million cells were transfected with 6 μg of each vectorsusing Lipofectamine 2000 (Invitrogen).

Antibodies and Immunoblot Analysis

Antibodies against STAT3 (Cell Signaling Technology, Beverly, MA, USA; 1 : 500), β-actin (Sigma-Aldrich; 1 : 1,000), phospho-Stat3 (Tyr 705/p-Stat3; Cell Signaling Technology; 1 : 500), HK2 (Cell Signaling Technology; 1 : 500), PFKFB3 (Abgent, San Diego, CA, USA; 1 : 500), HA (Covance, Berkeley, CA, USA; 1 : 1,000), and Flag (Sigma-Aldrich; 1 : 1,000) were used for immunoblotting analyses. Cells were lysed in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM NaF, 1 mM orthovanadate, 0.1 mM dithiothreitol, and a protease inhibitor cocktail). The immunoblotting analyses were performed as previously described[5].

Results

To analyze whether inflammatory mediators could enhance glucose metabolism, we measured the glucose consumption of wild-type MEFs stimulated by the proinflammatory cytokines TNF-α, IL-1β, and IL-6 and the inflammation inducers LPS and TPA. As shown in **Figure 1a**, IL-6 stimulation significantly enhanced glucose consumption. In contrast, the other stimulations showed only weak enhancement effects. The extracellular lactate levels were increased in IL-6-treated MEF cultures (**Fig. 1b**), indicating an enhancement of the glycolytic flux. Previously, we demonstrated that NF-κB enhances glycolysis through expression of the glucose transporter GLUT3 in $p53^{-/-}$ MEFs[5]. More recently, it has been shown that the maintenance of NF-κB activity in tumors requires Stat3[15], suggesting the possibility that IL-6 enhanced glycolysis is mediated by NF-κB. In contrast, IL-6-induced enhancement of glucose consumption was not affected in *NF-κB*/$p65^{-/-}$ MEFs or $p53^{-/-}$ MEFs (**Fig. 1c** and **d**). In addition, glucose consumption in $p53^{-/-}$ MEFs was suppressed by p65 knockdown using RNA interference (RNAi) (**Fig. 1d**), as previously described[5]. However, the enhancement rate of glucose consumption was not affected by p65 knockdown (1.27-fold induction by IL-6 in *GFP* shRNA-expressing cells vs. 1.26-fold induction by IL-6 in *p65* shRNA-expressing cells).

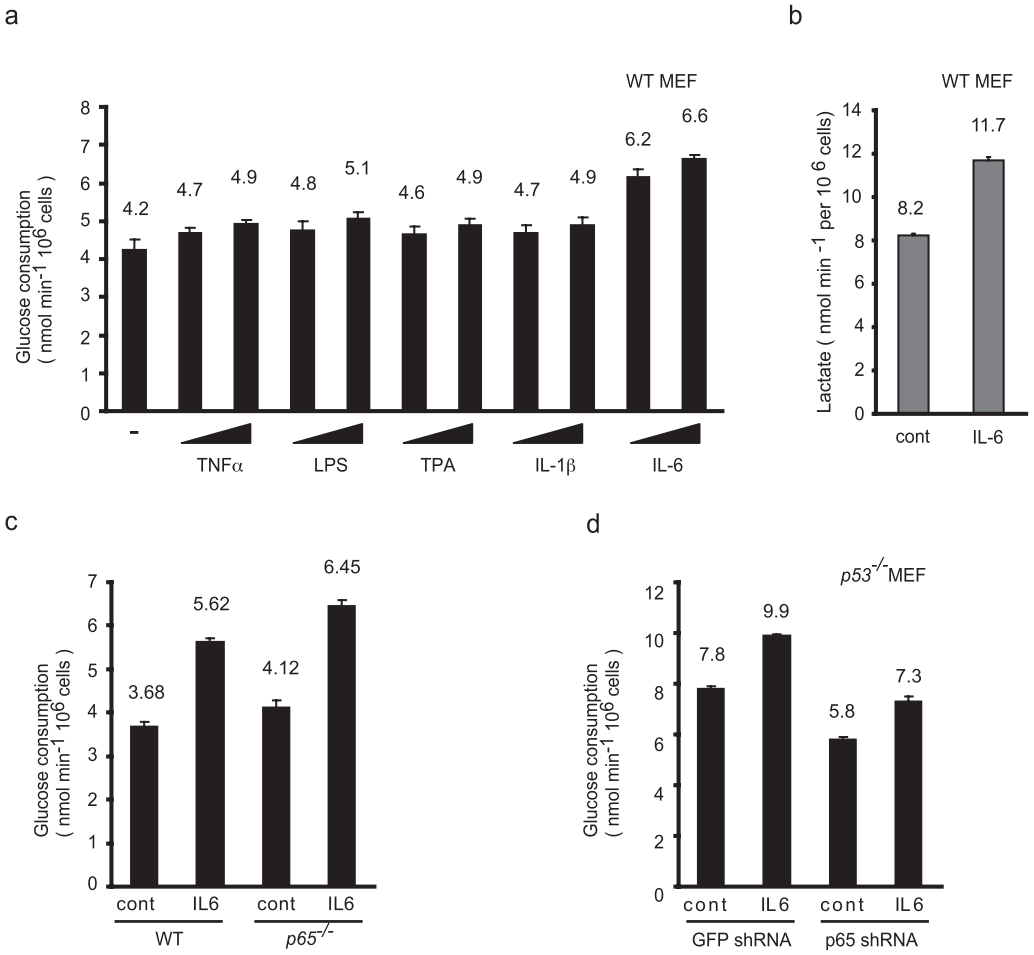

Fig. 1 IL-6 enhances glucose consumption. **a**: Wild-type MEFs were treated with TNFα (0.3 or 0.6 μg/mL), LPS (10 or 20 μg/mL), TPA (0.1 or 0.2 μg/mL), IL-1β (1.0 or 2.0 ng/mL), or IL-6 (0.4 or 0.8 μg/mL) plus sIL-6R (0.4 or 0.8 μg/mL) for 16 hours. The cells were collected and analyzed for their glucose consumption. **b**: Lactate production was measured after 16 hours of stimulation with IL-6 (0.8 μg/mL) plus sIL-6R (0.8 μg/mL). **c**: Wild-type and *p65*$^{-/-}$ MEFs were treated with 0.8 μg/mL each of IL-6 and sIL-6R for 16 hours, and the glucose consumption was measured. **d**: *p53*$^{-/-}$ MEFs were infected with a *p65* shRNA-expressing retrovirus. The cells were treated with 0.8 μg/mL each of IL-6 and sIL-6R for 16 hours and then evaluated for glucose consumption. Data represent the means ± SD from 3 independent experiments. Mean values are also indicated.

Next, we analyzed the expression of glucose transporters and glycolytic enzymes in IL-6-stimulated wild-type MEFs. As shown in **Figure 2a**, the mRNA expression of GLUT1, but not of GLUT3, and all analyzed glycolytic enzymes were enhanced by IL-6. These findings suggest that IL-6 directly activates the expressions of these genes or that de novo synthesized proteins induced by IL-6 and/or the enhanced glucose metabolism activate these genes. IL-6 transduces its signals through the receptor component gp130 and Janus kinases (JAKs), resulting in activation of the preexisting transcription factor Stat3[16]. Therefore, to analyze whether IL-6 directly activates these genes, we performed the same experiment in the presence of the protein synthesis inhibitor cycloheximide. Interestingly, the mRNA expression of only HK2 and PFKFB3 was significantly activated by IL-6 in the presence of cycloheximide **(Fig. 2b)**. Indeed, the protein expressions of HK2 and PFKFB3 were also induced by IL-6 stimulation in the absence of cycloheximide CHX **(Fig. 2c)**. These results suggest that IL-6 enhances glucose metabolism through activation of *HK2* and *PFKFB3* gene expressions. To

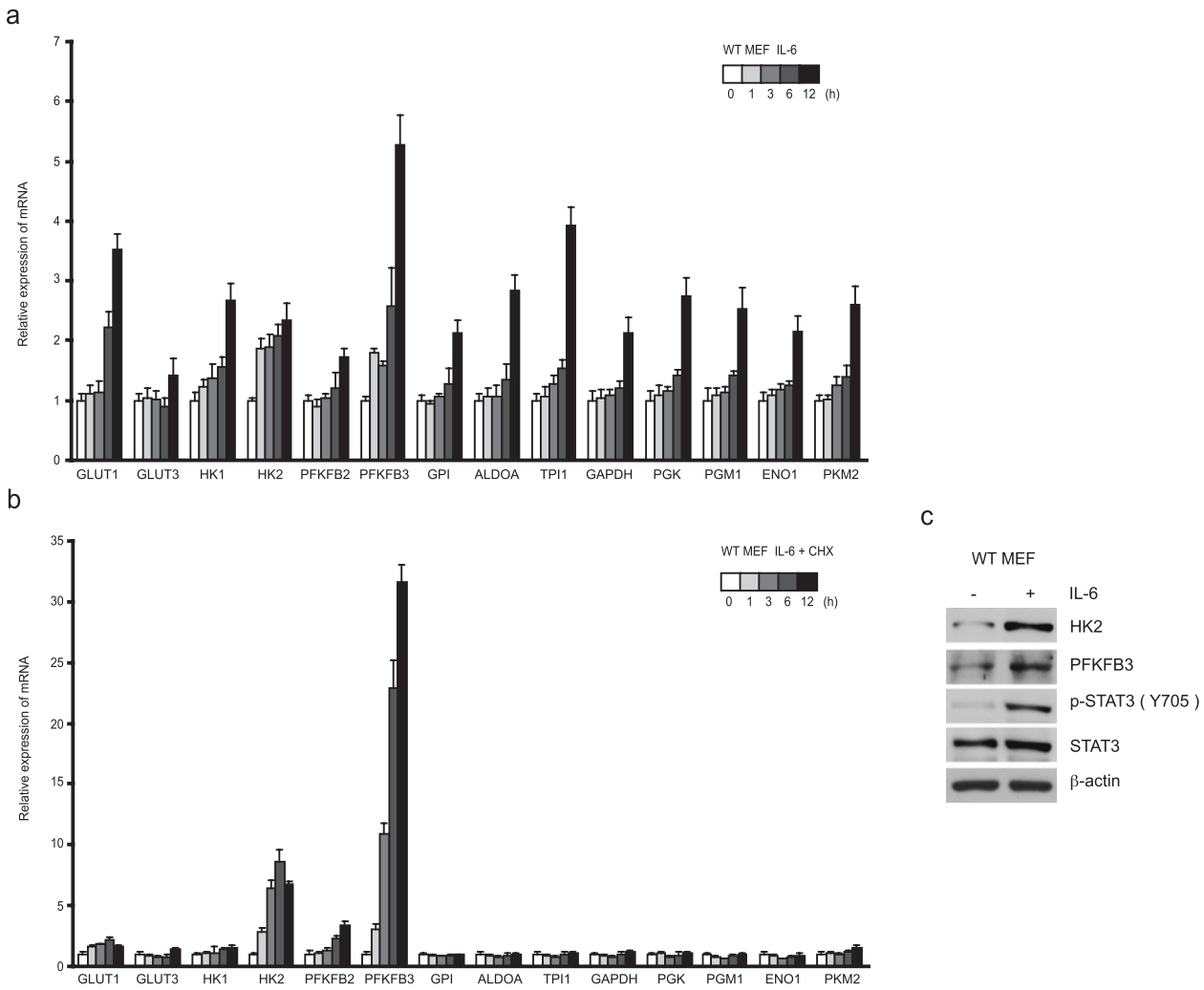

Fig. 2 IL-6 enhances the mRNA expressions of HK2 and PFKFB3. **a, b**: Wild-type MEFs were treated with 0.8 μg/mL each of IL-6 and sIL-6R in the absence (a) or presence (b) of 100 μg/mL cycloheximide (CHX) for the indicated times. The gene expressions of glucose transporters and glycolytic enzymes were examined by with quantitative real-time PCR. Data represent the means ± SD from three 3 independent experiments. The components evaluated were glucose transporters (GLUT1 and GLUT3) and the following glycolytic enzymes: HKs, hexokinases (HK1 and HK2); phosphofructokinases PFKs (PFKFB2 and PFKFB3); phosphoglucose isomerase (GPI); aldolase A (ALDOA); triose phosphate isomerase (TPI); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); phosphoglycerate mutase (PGM); phosphoglycerate kinase (PGK); enolase 1 (ENO1); and pyruvate kinase M (PKM). **c**: Wild-type MEFs were treated with IL-6 and sIL-6R for 16 hours as described for (A). The cells were collected and analyzed with immunoblotting for their expression levels of HK2, PFKFB3, and the activated form of STAT3 (p-STAT3) by immunoblotting.

analyze whether the activation of these gene expressions is mediated by STAT3 or other signals such as mitogen-activated protein MAP kinases[16], we generated Stat3 STAT3 knockdown MEFs by expressing a shRNA for STAT3 **(Fig. 3a)**. As a result, we found that, the IL-6-enhanced glucose consumption was clearly suppressed in STAT3 knockdown MEFs **(Fig. 3b)** and that the IL-6-induced mRNA expression of *HK2* and *PFKFB3* in the presence of cycloheximide was also suppressed **(Fig. 3c** and **d)**.

Glucose consumption and the expressions of *HK2* and *PFKFB3* in the presence of cycloheximide were also enhanced in human diploid fibroblast TIG3 cells **(Fig. 4a** and **b)** and human hepatoma HepG2 cells **(Fig. 4c** and **d)**, which are widely utilized to analyze the expressions of acute phase proteins induced by IL-6[17]. Moreover, cucurbitacin I, a specific inhibitor of the JAK2/STAT3 pathway, inhibited the expressions of *HK2* and *PFKFB3* in HepG2 cells **(Fig.**

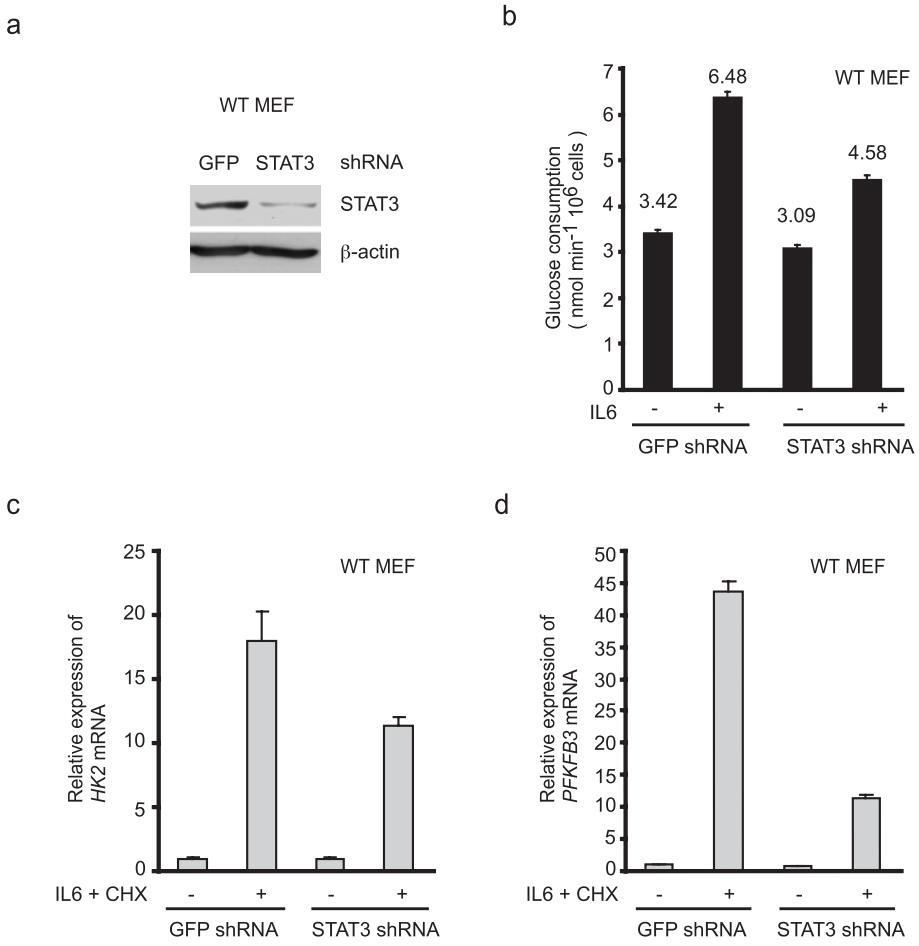

Fig. 3 The IL-6-induced enhancement of glucose consumption and the expressions of glycolytic enzymes are mediated by STAT3. **a**: Wild-type MEFs were infected with a control green fluorescent protein or STAT3 shRNA-expressing retrovirus. The expression levels of STAT3 were analyzed with immunoblotting. **b**: Cells were treated with 0.8 μg/mL each of IL-6 and sIL-6R for 16 hours. The cells were collected and analyzed for glucose consumption. **c, d**: Cells were treated with 0.8 μg/mL each of IL-6 and sIL-6R with 100 μg/mL cycloheximide (CHX) for 6 hours. Expression of *HK2* (c) and *PFKFB3* (d) genes was measured with quantitative real-time PCR. The data in (b) to (d) represent the means ± SD from 3 independent experiments.

4e). These results indicated that the effects of the IL-6-STAT3 pathway on glycolysis were not limited to MEFs. Finally, we analyzed whether the enhanced expressions of HK2 or PFKFB3 or both are sufficient for the enhancement of glucose consumption. As shown in **Figure 4f** and **g**, transient expression of PFKFB3 in $p53^{-/-}$ MEFs clearly enhanced glucose consumption, suggesting that IL-6 enhances glucose metabolism through the expression of glycolytic enzyme (s).

Discussion

The roles of IL-6 in glucose metabolism have been analyzed in the contexts of insulin sensitivity[18] and gluconeogenesis in the liver[19]. In the present study, we have demonstrated for the first time that the IL-6-STAT3 pathway enhances glycolysis through the enhanced expressions of glycolytic enzymes. Therefore, our results present new insights into the roles of IL-6 in glucose metabolism. In contrast, the effects of stimulation with TNF-α, IL-1, LPS, and TPA were relatively weak. These stimulations,

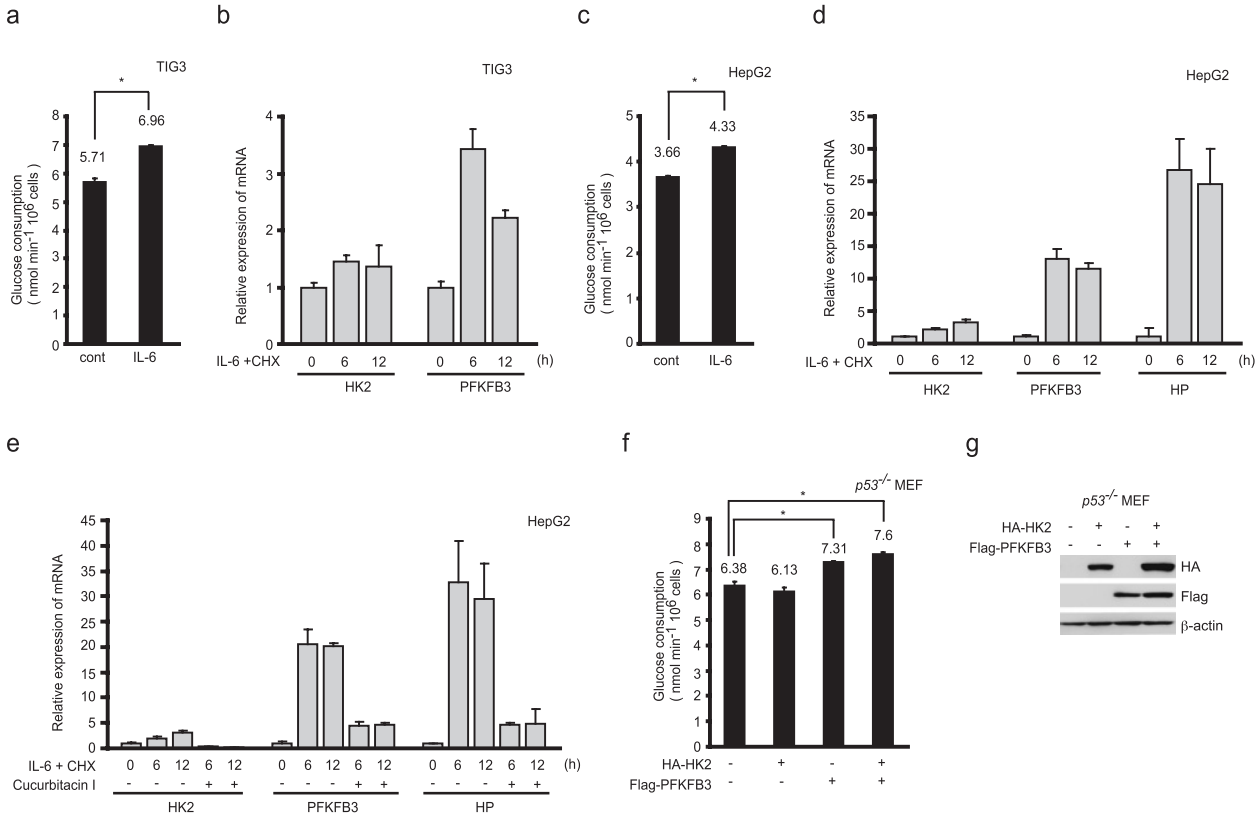

Fig. 4 The IL-6-induced glucose consumption in human cells and the roles of *HK2* and *PFKFB3* in glucose consumption. **a**: TIG3 cells were treated with 0.8 μg/mL each of IL-6 and sIL-6R for 16 hours. The cells were collected and assessed for glucose consumption. *P<0.01 for the indicated comparisons (*t*-test). **b**: TIG3 cells were treated with 0.8 μg/mL each of IL-6 and sIL-6R with 100 μg/mL cycloheximide (CHX) for the indicated times. Expression of the *HK2* and *PFKFB3* genes was measured. **c**: HepG2 cells were treated with IL-6 and sIL-6R as described for (a). The glucose consumption was measured. **d**: HepG2 cells were treated with IL-6 and sIL-6R plus cycloheximide (CHX) as described for (b) for the indicated times. Expression of *HK2, PFKFB, 3* genes was measured, and expression of the *HP* gene (haptoglobin, an acute-phase protein known to be induced by IL-6[17]) as a positive control. Data represent the means ± SD from 3 independent experiments. **e**: HepG2 cells were treated with IL-6, sIL-6R, and cycloheximide (CHX) as described for (b) plus 10 μM cucurbitacin I for the indicated time. Cells were collected and analyzed for expression of HK2 and PFKFB3 with quantitative real-time PCR. **f, g**: $p53^{-/-}$ MEFs were transfected with expression plasmids for HK2 or PFKFB3 or both. After 24 hours, glucose consumption was measured (f). Data represent the means ± SD from 3 independent experiments. * P<0.01 for the indicated comparisons (*t*-test). The expression levels of HA-tagged HK2 and Flag-tagged PFKFB3 were analyzed with immunoblotting (g).

especially that with TNF-α, are known to activate NF-κB[20]. We have previously shown that NF-κB enhances glycolysis in p53-deficient cells[5] and that p53 inhibits the transcriptional activity of NF-κB by directly inhibiting IKKα/β[21]. These results suggest that, in contrast to the IL-6-STAT3 pathway, the effects of NF-κB-mediated inflammatory responses on glycolysis are weak in the presence of p53.

We found that the IL-6-STAT3 pathway induce the expression of the glycolytic enzymes HK2 and PFKFB3. HKs catalyze the irreversible first step of the glycolytic pathway, in which glucose is phosphorylated to glucose-6-phosphate[22]. HK2 is highly overexpressed in many cancer cells, and amplification of the *HK2* gene is also observed in cancer cells[22]. PFKFB3 generates fructose-2,6-bisphosphate (F2,6BP), which allosterically activates 6-phosphofructo-1-kinase (PFK1), a master regulator of the glycolytic pathway[23]. It is also known that overexpression of HIF-1α and *myc*, activation of *ras*, and loss of p53, which are usually observed in cancer cells, stimulate glycolysis through the activation of PFKFB3[23]. Therefore, it is possible that the inductions of HK2 and PFKFB3 are sufficient for

enhancement of glycolysis. This notion is supported by the enhancement of glucose consumption by ectopic expression of PFKFB3 (**Fig. 4f**). We also analyzed the effect of PFKFB3 using a stable expression system by retrovirus; however, significant enhancement of glucose consumption was not detected (data not shown). This result suggests that high expression of PFKFB3 alone is not sufficient to prolong high glucose flux. Moreover, ectopic expression of HK2 was not found to enhance glucose consumption (**Fig. 4f**) but marginally enhanced glycolysis in PFKFB3 cDNA-transfected cells (glucose consumption was 7.31 nmol/min in PFKFB3-expressing cells and 7.6 nmol/min in PFKFB3/HK2-coexpressing cells; $p<0.05$; *t*-test). This result suggests that HK2 alone is not sufficient to enhance glycolysis but that HK2 support the function of PFKFB3. As shown in **Figure 2a** and **b**, the mRNA expression levels of *HK2* and *PFKFB3* induced by IL-6 were highly enhanced in the presence of cycloheximide compared with those in the absence of cycloheximide. The glycolytic pathway is regulated by the availability of substrates, allosteric effectors and the activities of metabolic transporters, enzymes, and regulators[3]. Therefore, it is possible that a negative feedback regulatory mechanism evoked by enhanced glycolysis inhibits the STAT3-mediated induction of HK2 and PFKFB3. It is also possible that a negative feedback regulatory mechanism attenuates enhanced glycolysis by ectopic expression of HK2 or PFKFB3 or both. In addition, we found that the mRNA expression of GLUT1 and PFKFB2 were weakly activated by IL-6 in the presence of cycloheximide. Therefore, it is possible that these proteins and other regulators are important for full activation of glycolysis by IL-6 in a long periode of time. However, these issues remain to be clarified. Recently, it has been shown that mitochondrial STAT3 supports Ras-dependent oncogenic transformation of MEFs and that mitochondrial STAT3 sustains altered glycolytic and oxidative phosphorylation activities characteristic of cancer cells[24]. Although the roles of mitochondrial STAT3 in IL-6-activated glycolysis are still unclear, the present results clearly show that nuclear STAT3 plays a role in glycolysis in untransformed cells.

It has previously been shown that STAT3 functions as an oncogene[25], and accumulating evidence supports an important role for STAT3 in oncogenesis[26]. Moreover, recent findings have shown that STAT3 mediates tumor-promoting inflammation and increases tumor cell proliferation, survival, and invasion[27]. In the course of such research, the present results showing that STAT3 enhances glycolysis may be important for understanding the mechanism underlying the roles of STAT3 in oncogenesis. Furthermore, we have recently demonstrated that enhanced glycolysis is important for activated Ras-induced cellular transformation[5], and there is much evidence that enhanced glycolysis confers a significant growth advantage on cancer cells[3,4]. Under conditions of chronic inflammation, the activated STAT3 pathway evoked by IL-6 enhances glycolysis in the surrounding cells. This effect may enhance the susceptibility of these cells to oncogenic transformation and maintain the transformed cells. In addition, it is possible that the inflammatory responses themselves are regulated by enhanced glycolysis. To further analyze the roles of the IL-6-STAT3 pathway in oncogenesis and inflammatory responses, we will determine the effects of this pathway using inflammation-associated cancer models in mice.

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