Role of GLUT1 in the Mammalian Target of Rapamycin Pathway: Mechanisms of Regulation.

by

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Chapter 1

Introduction

Uptake and metabolism of glucose and other nutrients are highly regulated by intracellular signaling processes which have been studied for over 50 years. Conversely, uptake and metabolism of glucose can affect these and other signaling processes; however, these mechanisms have not been studied as extensively. Understanding how these two processes intertwine in response to physiologic and pathophysiologic stimuli is important in the ultimate cellular responses.

The facilitative glucose transporter GLUT1: Cellular glucose transport is a highly regulated process and is dependent on a variety of signaling events. Glucose enters cells through interaction with the SLC2A gene family of facilitative glucose transporters (GLUTs). This 14-member family of monosaccharide transport proteins is responsible for both basal and agonist-induced cellular uptake of glucose [1]. The facilitative glucose transporter GLUT1 (SLC2A1) was the first to be identified [2]. This 45-55 kDa protein is now known to be ubiquitously expressed and is responsible for basal glucose uptake in a number of tissues, including muscle and some renal cell types [3]. GLUT1 is highly conserved among species [2, 4, 5]. The structure of GLUT1 consists of 12 transmembrane α-helices, and movement of glucose into cells via GLUT1 occurs

down a concentration gradient. Affinity for specific hexoses within the GLUT family of transporters is determined by the formation of hydrogen bonds, and it has been shown that GLUT1 exhibits substrate specificity for glucose and galactose [2, 4]. Transport of glucose into cells via GLUT1 is the rate-limiting step for glucose metabolism in a number of cell types [2, 5] since saturation of GLUT1 transporters occurs at or near physiological glucose concentrations [6]. As glucose is readily metabolized once it enters the cell, the kinetics of GLUT1 have been measured using analogs such as 2-deoxyglucose (2-DOG) and 3-O-methylglucose (3-OMG) that cannot be metabolized [2, 4]. Specific GLUT1 isoform expression experiments in *Xenopus laevis* oocytes, a convenient model for kinetic studies due to low endogenous glucose transport, have shown GLUT1 to have a K_m ranging from 3 to 7 mM for glucose [2, 4, 7]. In these studies, K_m appears to remain static with passive movement of glucose into cells, and therefore V_{max} is increased by increasing the number of functionally active transporters to the cell surface [7-10]. Additionally, it has also been shown that ATP binding to GLUT1 can reduce glucose transport [7, 8]. This suggests that rapid metabolic changes may also trigger acute changes in GLUT1 activity.

Regulation of GLUT1: Changes in metabolic state and oxidative stress, including hypoxia, can affect the expression of GLUT1 [2, 4, 7, 8]. GLUT1 expression can be affected by many signaling pathways, including cAMP, p53, hyperosmolarity, serum, insulin and oncogenes [5]. Multiple studies have described phosphoinositide 3-kinase (PI3K) and Akt as mediators of GLUT1 expression and function [11, 12]. It has been shown that treating cells with

wortmannin, a PI3K inhibitor, can prevent insulin-stimulated increases in GLUT1 at the plasma membrane [13]. A study of cytokine-stimulated glucose uptake demonstrated that Akt can regulate GLUT1 trafficking and enhance cellular glucose flux by preventing internalization of GLUT1 [14]. Several biochemical studies have demonstrated that PI3K signals its downstream effector, Akt, to activate mammalian Target of Rapamycin pathway (mTOR) activity [15-17]. Additional studies have demonstrated a regulatory relationship between mTOR and GLUT1. Zeng et al. [18] reported that incubation of acute myeloid leukemia (AML) cells with rapamycin derivatives results in decreased GLUT1 mRNA expression. Rapamycin, an inhibitor of mTOR, blocks GLUT1 transporter activity in similar myeloid cells [14].

GLUT1 and cell signaling: Although GLUT1 is primarily known for its role in cellular glucose transport, the level of expression of GLUT1 and other transporters has also been linked to downstream signaling events. We and others have generated substantial data demonstrating that glucose uptake and expression of glucose transporters, specifically GLUT1, can modulate a variety of signaling pathways, including c-Jun N-terminal kinase (JNK), glycogen synthase kinase-3 (GSK-3), Hif-1α, and RhoA/Rho kinase pathways in myocardial and vascular smooth muscle cells [19-23]. Upregulated GLUT1 and hexokinase 1 (HK1) can prevent activation of the proapoptotic protein Bax and promote cell survival in conditions of growth factor withdrawal [24, 25]. Microarray analysis of glomerular mesangial cells that overexpress GLUT1 (MCGT1) revealed

increased gene expression of interleukin-6 (IL-6), Hif-1α and vascular endothelial growth factor (VEGF) [26].

GLUT1 in diabetic nephropathy: Diabetic nephropathy, a deadly microvascular complication of chronic diabetes, is the leading cause of end stage renal disease in the US today [27, 28]. The major pathologic alterations of diabetic nephropathy take place in the glomerulus and include glomerular enlargement, podocyte loss, glomerular basement membrane thickening, mesangial matrix expansion, and afferent and efferent arteriolar hyalinosis. [27, 28]. A majority of the extracellular matrix proteins are secreted by mesangial cells, thus mesangial cells play an important role in the evolution of diabetic nephropathy [29]. Enhanced protein kinase C (PKC) isoform and transforming growth factor-β (TGF-β) levels, perhaps due to increased angiotensin II signaling, lead to enhanced synthesis of fibronectin and other extracellular matrix proteins by mesangial cells which drives the mesangial matrix expansion that is a hallmark of early diabetic nephropathy [30]. GLUT1 is the predominant glucose transporter in mesangial cells [31] and has been found to be increased in cultured mesangial cells exposed to high extracellular glucose levels [31] and in glomeruli from rodent models of diabetic nephropathy [27, 32-34]. Increased GLUT1 expression and glucose uptake by mesangial cells are considered to play major roles in the progression of diabetic nephropathy [28, 35]. Indeed, overexpression of GLUT1 in mesangial cells in the absence of an elevated extracellular glucose concentration recapitulates some of the features of diabetic nephropathy including increased glomerular expression of TGF-β and VEGF, and

increased extracellular matrix protein synthesis [36]. Mesangial cells exposed to increased extracellular glucose exhibit increased GLUT1 levels and enhanced fibronectin synthesis. The latter can be prevented in cells expressing antisense GLUT1 [37]. Finally, overexpression of GLUT1 in mesangial cells in mice leads to typical features of early diabetic nephropathy in non-diabetic mice [36] and these features can be prevented in diabetic mice which express a GLUT1 antisense construct [37].

mTOR is regulated by multiple cellular signaling pathways: mTOR, the mammalian ortholog of the yeast protein, Target of Rapamycin, is a critical cellular regulator that is conserved across many species [38-40]. The mTOR signaling pathway integrates a large number of environmental changes and cellular stresses, including reactive oxygen species, nutrient depletion, hyperosmotic stress as well as growth factor and cytokine signaling [41]. mTOR functions in two distinct multi-protein complexes, mTORC1 and mTORC2 [42, 43]. In mTORC1, mTOR associates with mLST8/GβL, raptor and PRAS40, and is sensitive to inhibition by rapamycin; while in the mTORC2 complex, mTOR interacts with mLST8/GβL, rictor and Sin1, and is rapamycin-insensitive, at least acutely [38, 43, 44] (Figure 1). mTORC1 is widely known as a key regulator of cellular growth and proliferation [38], while mTORC2 has recently been identified as being responsible for phosphorylating Akt (Ser473) [43]. Rheb, a GTP-binding protein modulated by the GTPase Tuberous Sclerosis Complex 2 (TSC 2), regulates mTOR activity [45, 46]. When bound to GTP, Rheb activates mTOR activity [45, 46]. The mechanism by which this occurs has recently been

characterized. FKBP38, a mitochondrial protein, appears to be an endogenous mTOR inhibitor that does not readily bind Rheb-GDP, yet has a strong affinity for Rheb-GTP [47, 48]. Increased concentrations of Rheb-GTP lead to competitive disinhibition of mTOR [47, 48]. The function of each of the mTORC1 component proteins is currently a focus of study by many investigators; however, it is known that loss of raptor eliminates mTORC1 activity [49].

The cellular targets of mTORC1 activity that enable cellular growth and proliferation via increases in translation and transcription are S6 kinase and 4E-binding proteins (4E-BPs) [39, 45]. To date, only mTORC1 has been reported to phosphorylate S6K at T389, and therefore phosphorylation of S6K at this residue is regarded as an indicator of mTORC1 activity [18, 38, 42, 44, 45, 50-60]. Functionally, S6K not only phosphorylates ribosomal protein S6, but also alters its interaction with the eIF3 translation initiation complex [50]. Activation of eIF3 leads to general increases in translation [55, 61]. 4E-BPs are repressors of translation that are also targets of mTORC1. Phosphorylation and resultant inactivation of 4E-BPs as well as phosphorylation and activation of S6K activity by mTORC1 facilitate cellular growth and proliferation.

mTOR in diabetic nephropathy: While searching for a mechanism for growth arrest-specific 6 (Gas6)-mediated glomerular hypertrophy, Nagai et al. [62] discovered significant increases in S6K and 4E-BP-1 phosphorylation in glomerular lysates from diabetic animals suggesting that mTOR may play a role in diabetic nephropathy. A later study discovered that rapamycin treatment could not only inhibit enhanced S6K phosphorylation in diabetic animals, but could also

prevent renal enlargement [63]. In another report by Yang, et al. [60], rapamycin treatment before the onset of diabetes in rats led to reduced albuminuria and histopathological changes. Previously observed increases in S6K, TGF-β1 and VEGF levels were also abrogated [51, 60].

Dissertation Objectives

The goal of this dissertation is to study the relationship between GLUT1 expression, glucose uptake and the mTOR pathway. I specifically sought to test the hypothesis that the mTOR pathway can activate GLUT1 expression and glucose uptake and is subject to feedback by GLUT1 expression and GLUT1-mediated glucose uptake to increase mTORC1 activity. This hypothesis was tested by first examining the nature of the relationship between the canonical mTOR pathway and GLUT1 expression. I determined whether GSK-3, TSC2 and mTOR can regulate GLUT1 and glucose uptake. The next set of experiments explored a reciprocal relationship between GLUT1 and mTOR. I examined the effects of GLUT1 expression on mTOR/S6K activity and identified a mechanism of regulation of the mTOR/S6K pathway by GLUT1. These studies are the first to establish a direct relationship between the mTOR pathway and GLUT1. The following two specific aims were developed to address the objectives of this dissertation (Figure 2).

Specific Aim 1: To determine whether the mTOR pathway plays a role in the regulation of GLUT1 expression and GLUT1-mediated glucose.

Chapter 2 outlines a mechanism by which the mTOR pathway regulates GLUT1 expression and glucose uptake.

Specific Aim 2: To determine whether GLUT1 and GLUT1-mediated glucose uptake regulate S6 kinase signaling via a mTOR dependent pathway. Chapter 3 describes the effect of GLUT1 overexpression on the mTOR pathway and details a mechanism for mTOR activation by GLUT1.

Figures

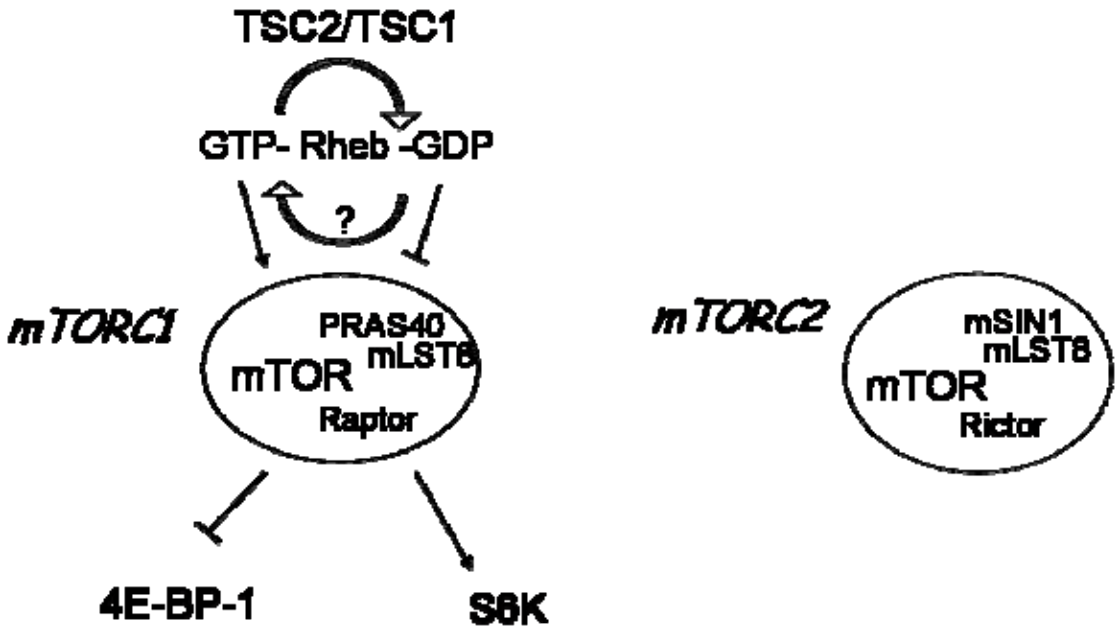

Figure 1. mTOR is active in one of two complexes.
mTOR functions in two distinct multi-protein complexes, mTORC1 and mTORC2. In mTORC1, mTOR associates with mLST8/GβL, raptor and PRAS40, and is sensitive to inhibition by rapamycin; while in the mTORC2 complex, mTOR interacts with mLST8/GβL, rictor and Sin1, and is rapamycin-insensitive, at least acutely.

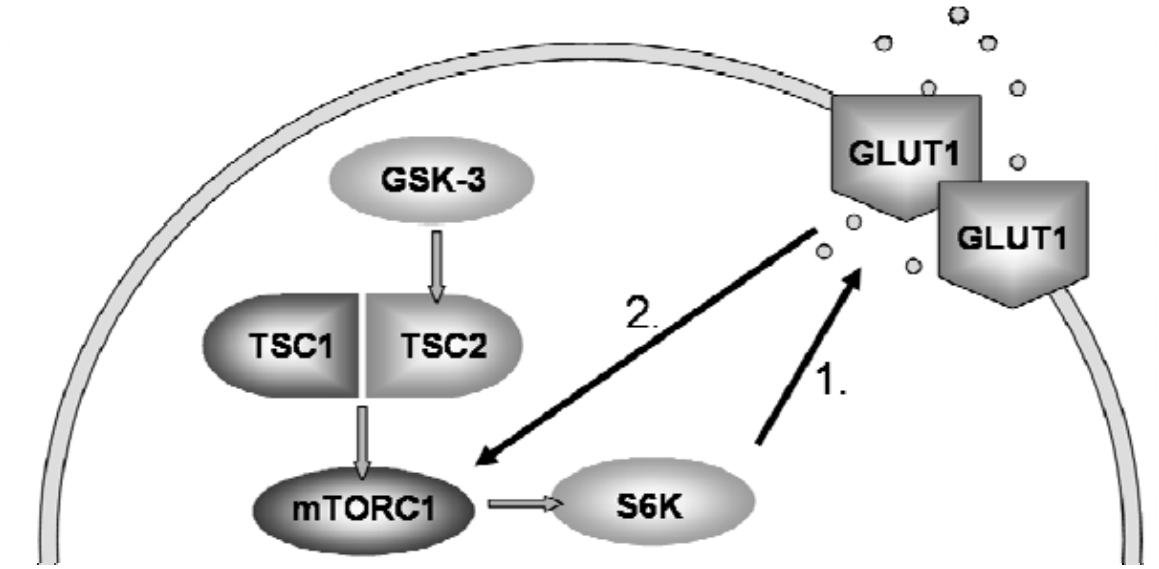

Figure 2. Specific Aims.
Specific Aim 1: To determine whether the mTOR pathway plays a role in the regulation of GLUT1 expression and GLUT1-mediated glucose.
Specific Aim 2: To determine whether GLUT1 and GLUT1-mediated glucose uptake regulate S6 kinase signaling via a mTOR dependent pathway.

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Chapter 2

A GSK-3, TSC2, mTOR Pathway Regulates Glucose Uptake and GLUT1 Glucose Transporter Expression

Abstract

Glucose transport is a highly regulated process and is dependent on a variety of signaling events. Glycogen synthase kinase-3 (GSK-3) has been implicated in various aspects of the regulation of glucose transport, but the mechanisms by which GSK-3 activity affects glucose uptake have not been well defined. We report that basal glycogen synthase kinase-3 (GSK-3) activity regulates glucose transport in several cell types. Chronic inhibition of basal GSK-3 activity (8–24 h) in several cell types, including vascular smooth muscle cells, resulted in an approximately two-fold increase in glucose uptake due to a similar increase in protein expression of the facilitative glucose transporter 1 (GLUT1). Conversely, expression of a constitutively active form of GSK-3β resulted in at least a twofold decrease in GLUT1 expression and glucose uptake. Since GSK-3 can inhibit mammalian target of rapamycin (mTOR) signaling via phosphorylation of the tuberous sclerosis complex subunit 2 (TSC2) tumor suppressor, we investigated whether chronic GSK-3 effects on glucose uptake and GLUT1 expression depended on TSC2 phosphorylation and TSC inhibition of mTOR. We found that absence of functional TSC2 resulted in a 1.5-to 3-fold

increase in glucose uptake and GLUT1 expression in multiple cell types. These increases in glucose uptake and GLUT1 levels were prevented by inhibition of mTOR with rapamycin. GSK-3 inhibition had no effect on glucose uptake or GLUT1 expression in TSC2 mutant cells, indicating that GSK-3 effects on GLUT1 and glucose uptake were mediated by a TSC2/mTOR-dependent pathway. The effect of GSK-3 inhibition on GLUT1 expression and glucose uptake was restored in TSC2 mutant cells by transfection of a wild-type TSC2 vector, but not by a TSC2 construct with mutated GSK-3 phosphorylation sites. Thus, TSC2 and rapamycin-sensitive mTOR function downstream of GSK-3 to modulate effects of GSK-3 on glucose uptake and GLUT1 expression. GSK-3 therefore suppresses glucose uptake via TSC2 and mTOR and may serve to match energy substrate utilization to cellular growth.

Introduction

The movement of glucose in and out of cells is mediated by facilitative glucose transporters in almost all mammalian cells. There are at least 13 glucose transporter isoforms differentially expressed in mammalian tissues [1], eight of which have been found to participate in glucose uptake. The first member of this family, GLUT1, is expressed in most cells and plays a role in basal glucose uptake in many tissues [2], but may be regulated by insulin and other hormonal factors [3, 4]. Glucose transporter expression and localization is regulated in a variety of ways depending on the cell type and the stimulus involved. The signaling pathways involved in acute insulin stimulated GLUT4

translocation have been the subject of many studies over the past 2 decades. However, relatively few studies have examined the signaling systems involved in long-term regulation of GLUT1 mediated glucose uptake in non-insulin responsive tissues [5-8], and none of these previous reports have investigated the role of a signaling pathway that is an important regulator of cellular growth and metabolism, namely the pathway that includes glycogen synthase kinase-3 (GSK-3), Tuberous Sclerosis Complex (TSC) and mammalian Target of Rapamycin (mTOR).

GSK-3 is an important signaling molecule that is ubiquitously expressed and has been implicated in the regulation of glucose transport and metabolism. Originally identified as a key regulator of glycogen synthase activity, GSK-3 is now known to function as an important signaling molecule in several pathways and is involved in regulating gene transcription, protein translation and apoptosis, as well as hexose metabolism [9]. Two distinct isoforms of GSK-3 (α/β isoforms) that are products of two independent genes [10] have been identified. GSK-3 is known to function downstream of phosphotidylinositol 3 kinase (PI3K) and Akt [11]. There is a paucity of reports about regulation of GLUT1 mediated glucose uptake by GSK-3 and the few studies that have been performed have produced different findings in different cell systems [12, 13].

Recently, the TSC/ mTOR signaling pathway has been shown to be downstream of GSK-3 [14]. The Tuberous Sclerosis Complex (TSC) is comprised of two subunits, TSC1 and TSC2. Normal copies of each protein are needed for proper function of the complex [15] and mutations in either of the

genes encoding the proteins, have been found to cause tuberous sclerosis [16, 17]. Various studies have implicated TSC in several different cellular functions, the most important of which seems to be growth regulation [18]. Studies in Drosophila melanogaster have shown that mutations in the dTSC1 and dTSC2 genes lead to increased cell size. Mutations in TSC genes result in the constitutive activation of S6 kinase, which subsequently enhances protein translation [19-27]. The carboxy-terminus of TSC2 displays GTPase-Activating Protein (GAP) activity and has been shown to interact with the GTPase Rheb directly [28-30]. Inactivation of TSC2 represses GAP activity and allows Rheb-GTP to accumulate. Rheb-GTP activates the protein kinase activity of mTOR, which in turn phosphorylates and activates S6 kinase, leading to enhanced translation [28-30]. Conversely, active TSC2 reduces Rheb-GTP accumulation [28-30] and reduces downstream mTOR and S6K activation.

Since the tuberous sclerosis complex (TSC) is implicated in both the insulin signaling pathway as a substrate for Akt and in the growth regulation pathway as an upstream regulator of mTOR and S6 kinase, we hypothesized that GSK-3 acts via TSC2, as a negative growth regulator, to reduce glucose uptake by suppressing expression of GLUT1. In this report we present data showing that GSK-3 is a negative regulator of basal glucose uptake and GLUT1 expression and that GSK-3 exerts its inhibitory effects through a TSC2 and mTOR dependent pathway.

Materials and Methods

Materials and Cell Lines: Rat LEF cell lines were derived from spontaneous renal tubular tumors in Long Evans Eker rats [16]. Cells from these rats have an inactivating germline mutation in the TSC2 gene [31, 32]. The embryonic fibroblast cells, EEF4 (EEF126-4) and EEF8 (EEF126-8), were derived from passages 12 to 14 of primary explants of embryos from a single heterozygous mating of Eker rats [17, 33]. The retrovirus constructs containing TSC2 and EGFP genes were previously reported [27]. TSC2-3A is a TSC2 mutant in which AMP kinase and GSK-3 activating phosphorylation sites, S1337, S1341 and S1345, have been changed to alanines [14]. The A7r5 rat vascular smooth muscle cell line was obtained from the American Type Culture Collection (CRL-1444). The GLUT1 antibody was a gift from Dr. Christin Carter-Su (University of Michigan). The anti β-tubulin antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies for phosphorylated GSK-3α/ β Ser21/9, and total Akt were obtained from Cell Signaling Technology (Beverly, MA). Rapamycin, a mTOR inhibitor, was obtained from Sigma (St. Louis, MO). The GSK-3 inhibitor, SB216763, was from BIOMOL International (Plymouth Meeting, PA). The GSK-3β inhibitor, Glycogen Synthase Kinase-3β Inhibitor II, was obtained from Calbiochem (San Diego, CA).

^{3}H-2-deoxyglucose (2-DOG) and ^{3}H-3-O-methyl-glucose (3-OMG) Uptakes: EEF or LEF cells were plated in six-well plates in Dulbecco's Modified Eagles Medium (DMEM)/F12 medium with 10% fetal calf serum (FCS). A7r5 cells were plated on 60-mm plates in DMEM with 10% FCS. Cells were grown to

~80% confluence prior to treatment and 2-DOG uptake analysis. 2-DOG uptakes were performed as previously described, [34, 35]. Slight protocol modifications were made in the 2-DOG protocol to accommodate for rapid equilibration when using 3-OMG. Briefly, plates were washed once with Krebs Ringer Phosphate buffer (KRP) (in mmol/l: 128 NaCl, 5.2 KCl, 1.3 CaCl2, 2.6 MgSO4, and 10 Na2HPO4), and then incubated with KRP buffer supplemented with 1% BSA for 10 minutes to one hour at 37°C. Drugs were added to the KRP buffer during the one hour incubation period for various time points as noted. The KRP buffer was removed and replaced with 0.1 mM unlabeled 2-DOG (Sigma) and 0.5μCi/ml ^{3}H-2-DOG (Perkin Elmer, Waltham, MA) or ^{3}H 3-OMG (Sigma Aldrich) in KRP buffer + 1% (wt/vol) bovine serum albumin (BSA) ± 20 nM cytochalasin B, an inhibitor of glucose transport, at 37°C for 5 min, or room temperature for 6 seconds, respectively. We have found that 2-DOG uptake is linear for at least 10 min in EEF, LEF (not shown) and A7r5 cell lines [34]. The plates were subsequently washed twice for 5 minutes each time with cold KRP solution containing 200μM phloretin to quench 2-DOG uptake. The samples were then lysed in buffer (EEF and LEF cell lines in Tris-HCl, pH7.0, 10 mM, NaCl 150mM, Triton X-100 1% and SDS 1%, and the A7r5 cell line in 0.1% SDS). A portion of each sample was used for determination of protein concentration by a bicinchoninic acid assay (Pierce; Rockford, IL), and the rest was utilized for scintillation counting. 2-DOG uptake was expressed as nmol/minute/milligram protein after correction for nonspecific uptake in the presence of cytochalasin B.

Infection Studies: Retroviral infections were performed as previously described [14, 27]. HEK293 cells at ~50-80% confluence were transfected with a retroviral vector pPGS-CMV-CITE-Neo expressing either wild-type TSC2, mutant TSC2 or enhanced green fluorescent protein (EGFP) genes. Forty-eight hours after transfection, the media of transfected cells was harvested and passed through a 0.45 μM filter. Polybrene (5 μg/ml) was added to the viral solution before the mixture was added to the medium of freshly split EEF8 cells. The infection was repeated two more times at 12 and 24 hours. Cells were ready to assay 16 to 24 hours after the final infection.

A non-phosphorylatable GSK-3 β adenoviral construct (GSK-3 β S9A) was a gift from Dr. Morris Birnbaum (University of Pennsylvania School of Medicine). A7r5 cells were cultured at a density of 2 x 104 cells per dish in OptiMEM media for 2 hours at 37°C and a multiplicity of infection (MOI) of 1000 was used to infect the cells. After a two hour incubation period, OptiMEM media was replaced with DMEM + 10% FCS and the cells were returned to 37°C for varying time points.

Immunoblotting: Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBS-Tween20 (TBS-T) and 5% milk for 1 hour and placed in primary antibody in TBS-T 5% milk overnight at 4°C [34]. After three 10 minute washes in TBS-T, the appropriate horseradish peroxidase-linked secondary antibody was then added in TBS-T 5% milk and incubated at room temperature for 1 hour. Membranes were then washed 3 times (10 min each) in TBS-T, subjected to an enhanced chemiluminescence reaction (ECL), and exposed to autoradiography

film. After autoradiography, the films were scanned and quantified using NIH ImageJ.

Animals: Adult, male, Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Aortic lysates were prepared similarly as described previously [36].

Statistics: Data were expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc analysis, or by Student's T-test. Data were considered significant at $p < 0.05$.

Results

GSK-3 regulates acute and long term GLUT1 expression and 2-DOG uptake.

Acute and 24 hour inhibition of GSK-3 with either LiCl (20mM) or SB216763 (10μM) in A7r5 cells resulted in a progressive increase in GLUT1 expression and 2-DOG uptake (Figure 1). Similarly, GSK-3 inhibition for 24 and 48 hours in rat aortic explants resulted in increased GLUT1 protein expression (Figure 2). Moreover, adenoviral infection of A7r5 cells with a constitutively active form of GSK-3 β (AdGSK3 β S9A) for 24 hours resulted in a decrease in GLUT1 expression and 2-DOG uptake compared to values in cells infected with the vector control adenovirus (Figure 3).

Inactivation of the TSC2 gene results in increased 2-DOG uptake, GLUT1 expression and S6K phosphorylation.

Glucose uptake in TSC2 mutant cell lines was substantially higher than in the respective control cells (Figure 4A). Conversely, 2-DOG uptake was reduced in mutant cell lines acutely infected with a wild-type TSC2 expressing retrovirus compared with those infected with a control virus, consistent with the observation that wild-type TSC2 constitutively inhibits glucose uptake (Figure 4B). To determine whether the increased rate of 2-DOG uptake corresponded to glucose transporter expression, we examined the levels of GLUT1 in TSC2 mutant cells. Introduction of the wild-type retroviral TSC2 vector into both TSC2 mutant cell lines caused a ~2-fold reduction in GLUT1 protein expression (Figure 5A and B) and a similar reduction in GLUT1 mRNA levels in the LEF+TSC2 cells (Figure 5C). Expression of functional TSC2 in LEF cells also led to reduced phosphorylation on threonine 389 of S6K (Figure 6).

Glycogen Synthase Kinase-3 (GSK-3) regulates glucose uptake in TSC2 positive cells.

GSK-3 phosphorylates TSC2 to enhance TSC complex stability and hence TSC inhibition of mTOR [14]. Therefore, we assessed whether GSK-3 is upstream of TSC2 in the regulation of glucose uptake by testing the effect of a GSK-3 inhibitor on LEF cells lacking functional TSC2. Uptakes of 3-OMG also were performed in these cells to ensure that the effects of GSK-3 and TSC were on GLUT1 mediated transport and not on hexokinase activity, since 2-DOG is

phosphorylated by hexokinases. Treatment of TSC2 mutant LEF cells with the GSK-3 inhibitor SB216763 (10μM) for 24 hours did not affect GLUT1 expression or 2-DOG or 3-OMG uptake. However, SB216763 treatment of LEF+TSC2 cells led to a significant increase in GLUT1 protein and glucose uptake (Figure 7). To confirm that GSK-3 is upstream of TSC2, TSC2 mutant (EEF8) cells were infected with a wild-type TSC2 construct or a TSC2 construct mutated at GSK-3 phosphorylation sites [14]. 2-DOG uptake was reduced in cells infected with the wild-type, but not with the mutant, TSC2 construct (Figure 8). Together, these data indicate that GSK-3 phosphorylation of TSC2 plays a critical role in stimulating TSC2-mediated suppression of glucose uptake and that essentially all of the GSK-3 effect on glucose uptake is mediated via TSC2 in this cell system. Despite the role of GSK-3 in glucose uptake in these cells and the potential role of GSK-3 in mediating glucose uptake in response to insulin signaling [3], insulin (100 nM) failed to increase glucose uptake at any time point between 15 minutes and 2 hr in either TSC2-positive or TSC2 mutant cells (data not shown).

Glucose uptake in the absence of TSC2 is partly mediated by a rapamycin-sensitive mTOR-dependent pathway.

TSC inhibits mTOR through the small G protein Rheb [28-30]. Inactivation of TSC or deletion of TSC2 is associated with an increase in mTOR signaling [28-30]. To determine whether mTOR activation is required for the enhanced GLUT1 expression and glucose uptake seen in TSC2 mutant cells, we treated cells with rapamycin to inhibit mTOR signaling. Exposure to rapamycin was

restricted to 8 instead of 24 hours to eliminate possible effects on mTORC2 activity or other nonspecific effects. After treatment with 20nM rapamycin for 8 hours GLUT1 protein expression was significantly reduced compared to that in vehicle-treated cells, but was only slightly and statistically insignificantly reduced in TSC2-positive cells (Figure 9A). The effects on 2-DOG uptake paralleled the changes in GLUT1, with rapamycin reducing uptake only in the TSC2-positive cells (Figure 9B).

Discussion

In the current report, GSK-3 was found to reduce glucose uptake by suppressing GLUT1 expression. We also found that chronic GSK-3 inhibition enhanced glucose uptake and GLUT1 expression in TSC2-expressing cells but not in cells lacking functional TSC2. In addition, mutation of AMPK and GSK-3 phosphorylation sites on TSC2 abrogated the effect of GSK-3 on glucose uptake. Finally, the increase in GLUT1 expression found in TSC2 mutant cells was prevented by the mTOR inhibitor, rapamycin. Thus, the effects of GSK-3 on glucose uptake and GLUT1 were dependent on TSC2, while TSC2 effects on GLUT1 were mediated by mTOR. The chronic changes in glucose uptake and GLUT1 expression were independent of acute insulin signaling in the cells we studied.

In contrast to the literature on participation of GSK-3 in insulin signaling, there have been few studies on the role of GSK-3 in the regulation of glucose uptake in non-insulin stimulated conditions or in cell types in which glucose

uptake is not insulin-sensitive. In one of the few reports, Nikoulina et al. [12], found that inhibition of GSK-3 for 4 days increased both basal and insulin-stimulated glucose uptake in skeletal muscle without changing expression of either GLUT1 or GLUT4. Similarly, Bentley et al. [5] showed that IL3 stimulated translocation of GLUT1 to the cell surface of mast cells and Wieman et al. [13] found that this IL3 effect on GLUT1 translocation was via a PI3K/Akt/GSK-3 dependent mechanism that was not mTOR dependent. Interestingly however, these latter investigators found that glucose uptake was reduced by mTOR inhibition without changes in GLUT1 cell surface levels suggesting that mTOR enhanced the intrinsic activity of GLUT1 transporters [13]. Our results, in different cells, under different conditions, and with different timepoints, contrast with those of these previous studies. Our findings strongly support participation of the GSK-3/TSC2/mTOR pathway in the chronic regulation of basal GLUT1 and glucose uptake in several cell types. We did not directly examine GSK-3/TSC2/mTOR effects on cell surface GLUT1 expression, yet the observed effects on total GLUT1 expression were equal to or greater than the effects on glucose uptake suggesting that regulation of translocation, if present, was modest. Nonetheless, this aspect of GLUT1 regulation was not monitored in our studies and would need to be confirmed with cell surface localization studies. By comparison, the study of Wieman, et al. [13], evaluated a FLAG-tagged transfected GLUT1, and did not consider GSK-3 or mTOR effects on total cellular GLUT1 expression.

Previous studies on GLUT1 regulation have included evidence that cytokines, such as IL3, can stimulate GLUT1-mediated glucose transport in mast cells via a PI3K-dependent mechanism [5]. In addition, a number of investigators have shown that cellular metabolism and ATP availability play a significant role in regulating GLUT1 expression and function through both direct and indirect mechanisms. Another report suggests that direct interaction of ATP with GLUT1-specific peptide sequences can lead to conformational changes that modulate transport of glucose and inhibit degradation of GLUT1 [7, 8, 37]. Moreover, studies in skeletal muscle have shown that expression of a constitutively active form of AMP kinase (AMPK) results in increased GLUT1 and GLUT4 protein levels [38]. Direct regulation of GLUT1 by availability of energy substrates has been reported by multiple groups. Blodgett, et al. [6] have identified GLUT1 domains that undergo conformational changes that may prolong protein longevity and alter glucose transport after direct interaction with ATP. Other investigators have demonstrated that energy depletion and osmotic stress can activate AMPK that in turn leads to concordant increases in glucose transport and GLUT1 expression [38, 39]. Further experimentation will be necessary to ascertain whether AMPK participates in GSK-3/TSC2 regulation of GLUT1.

mTOR functions in one of two protein complexes, the rapamycin sensitive mTOR complex 1 (mTORC1) and the rapamycin insensitive mTOR complex 2 (mTORC2) [26, 40, 41]. mTORC1 is comprised of mTOR, raptor and mLST8, while mTORC2 consists of mTOR, rictor, mLST8 and Sin-1 [26, 41]. Although mTORC2 has been identified as PDK2, responsible for activation of Akt by

phosphorylation at serine 473 [40, 41], whether mTORC2 plays a significant role in regulation of expression and function of glucose transporters is not known. It appears that TSC2 effects on GLUT1 expression and glucose uptake are dependent on inhibition of mTORC1 since the mTORC1 inhibitor, rapamycin, reversed the effects of TSC2 mutation or abrogation.

It appears that the chronic regulatory effects of GSK-3 on GLUT1 expression and glucose uptake are mediated via TSC2. Indeed, the effects of GSK-3 were entirely abrogated in the rat fibroblast system in the absence of TSC2 as well as when a TSC2 molecule with mutations at 3 of the 4 GSK-3 phosphorylation sites was expressed. Although, to our knowledge, this is the first report to show GSK-3 signaling via TSC2 and mTOR effects on GLUT1 gene expression and glucose uptake, Kaelin's group has reported that Hif-1α levels were enhanced through an mTOR-dependent mechanism in cells derived from TSC2 -/- mouse embryo fibroblasts [20]. Hif-1α then subsequently enhanced the transcription of a number of genes, including GLUT1. Although we did not measure Hif-1α levels in these studies, it seems unlikely that it played a role in GLUT1 expression in the cell systems in our report as there was no stimulus for an increase in Hif-1α levels.

GSK-3/TSC2/mTOR regulation of glucose transport likely serves to couple cellular growth with substrate uptake. Cell growth induced by growth factors is mediated in part via activation of the PI3K/Akt pathway. Akt in turn phosphorylates and inactivates both GSK-3β and TSC2. As we have shown in this study, this inactivation of GSK-3β and TSC2 results in increased GLUT1

levels, and enhanced glucose uptake through these high affinity transporters. Conversely, when the PI3 kinase/Akt pathway is inactivated upon withdrawal of growth factors, GSK-3 and TSC2 would be activated resulting in TSC2-mediated inhibition of Rheb which in turn would inactivate mTOR, leading to reduced GLUT1 levels as well as reduced glucose uptake. This regulatory process would help match fuel supply to metabolic requirements and would prevent increases in intracellular glucose concentration which could result in glucose toxicity. However, there are hints that this regulatory pathway could be active in a less homeostatic relationship. Both enhanced mTOR activity [42, 43] and increased GLUT1 expression [44] have been implicated in the pathogenesis of diabetic complications, especially nephropathy. Since upregulation of mTOR leads to enhanced GLUT1 expression, it is possible that mTOR plays a pathogenic role by stimulating GLUT1 expression and glucose uptake into susceptible glomerular cells in the kidney. Further experimentation will be needed to test this possibility.

The data herein suggest that TSC2 is an important negative regulator of GLUT1 expression and glucose uptake and is likely to be active in many cell types. Inactivating mutations in TSC2 are likely to lead to enhanced GLUT1 expression and basal glucose uptake in a non-insulin sensitive manner. Since GLUT1 expression is enhanced in many neoplasms and can lead to increased growth and reduced apoptosis [45], one may speculate that inactivation of TSC2 may contribute to abnormal cell growth and hamartomas via this mechanism.

Figures

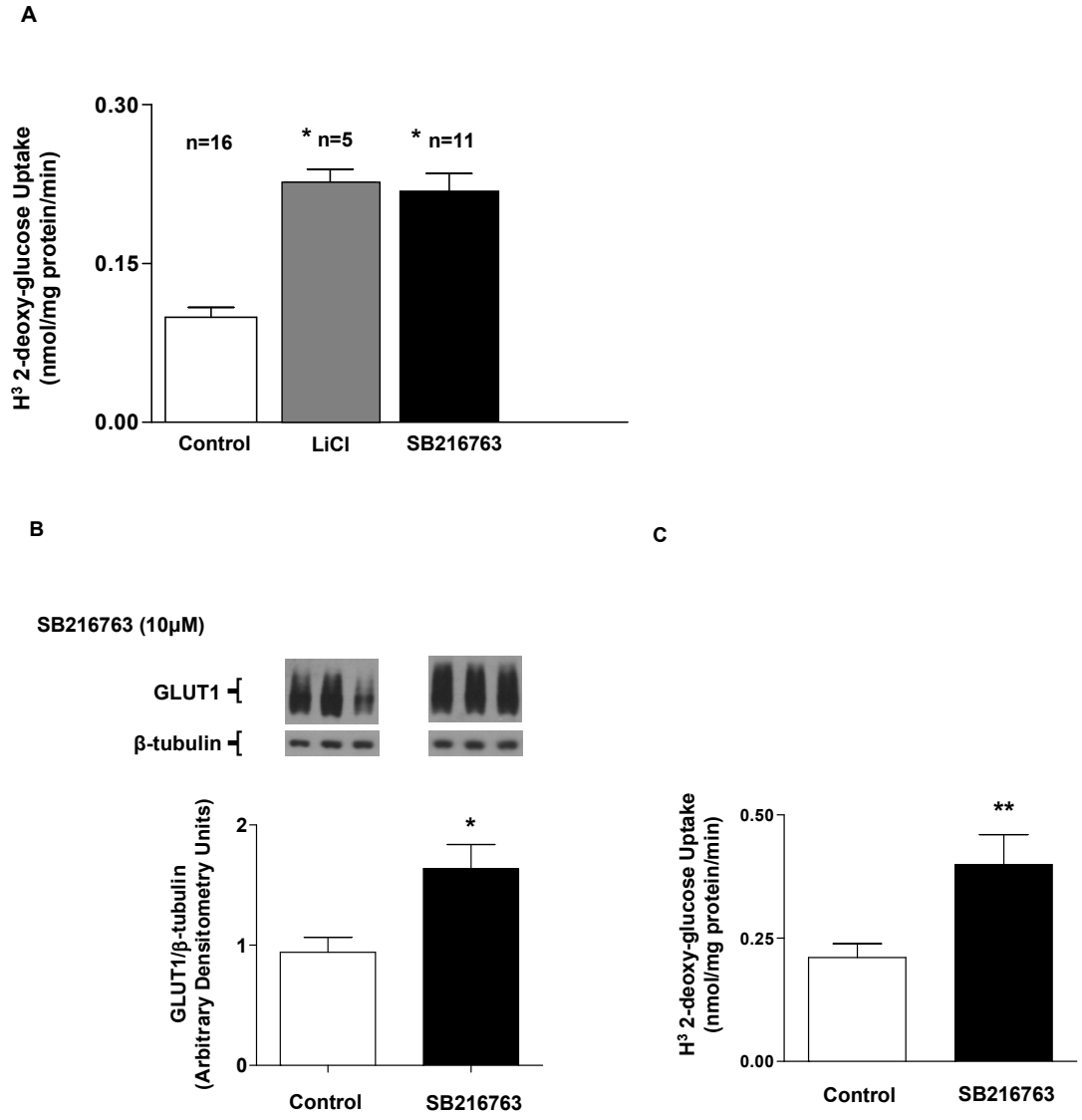

Figure 1. Inhibition of GSK-3 increased glucose uptake and GLUT1 expression. Acute inhibition (30 minutes) of GSK-3 activity with lithium chloride (20mM) or SB216763 (10uM) caused increased 2-DOG uptake in A7r5 cells (*$p<0.05$ vs. control) (A). More chronic GSK-3 inhibition (24hrs) induced GLUT1 protein expression (n=6, *$p<0.05$ vs. control) (B) as well as 2-DOG uptake (n=8, **$p<0.01$) (C).

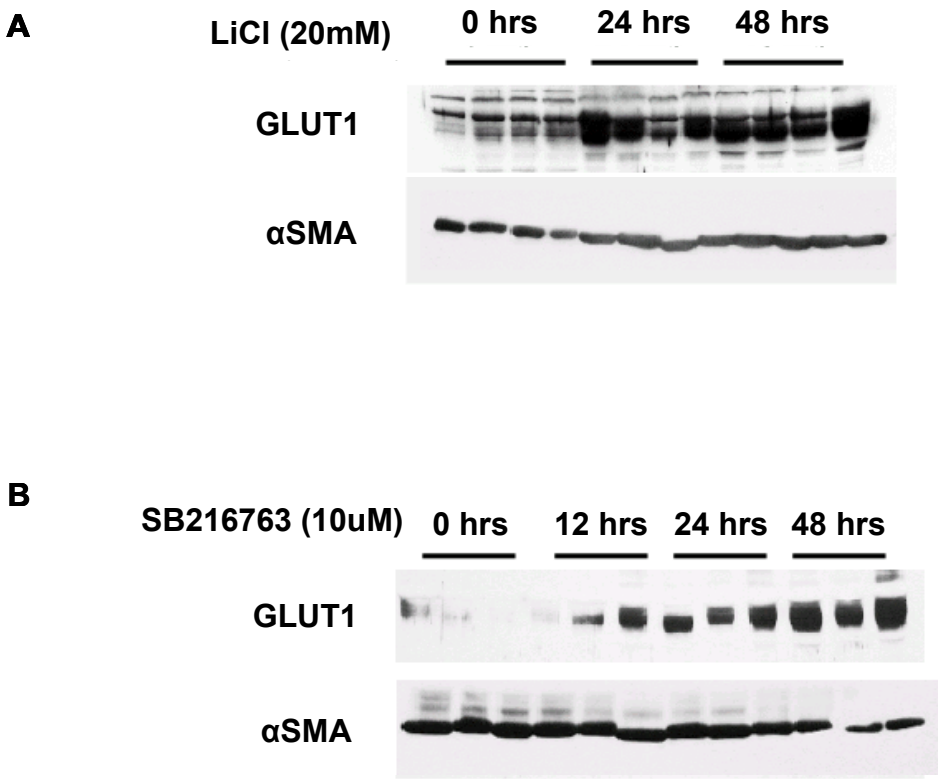

Figure 2. GSK-3 inhibition increased GLUT1 expression in aortic explants. GSK-3 inhibition with 20mM lithium chloride (A) or 10μM SB216763 (B) in rat aortic explants resulted in increased GLUT1 protein expression.

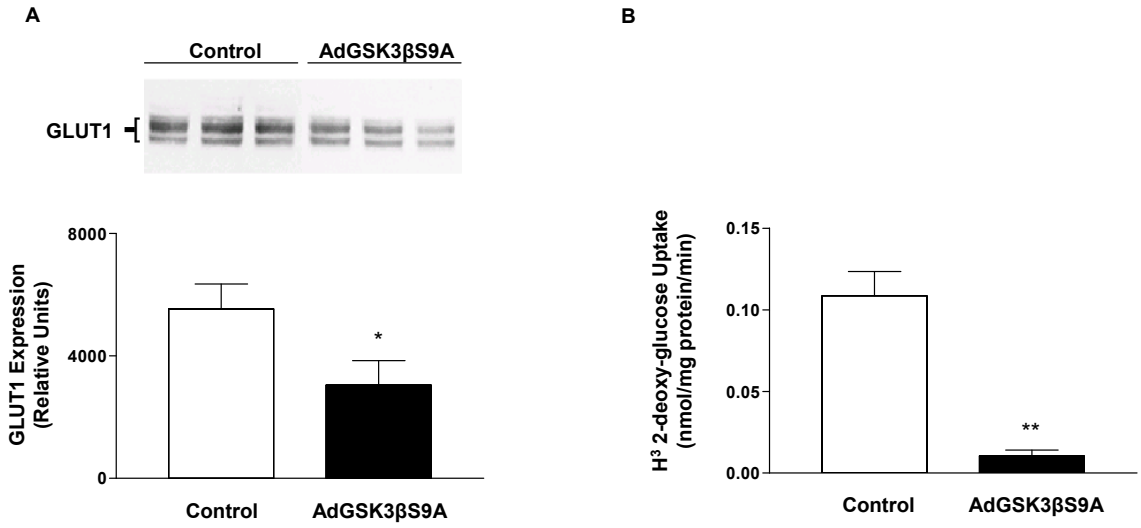

Figure 3: Constitutively active GSK-3β decreased GLUT1 expression and glucose uptake. Adenoviral infection with a constitutively active GSK-3β for 48 hours decreased GLUT1 expression (A) and 2-DOG uptake (B) in VSMCs (n=7, *p<0.05; **p<0.01 vs. control).

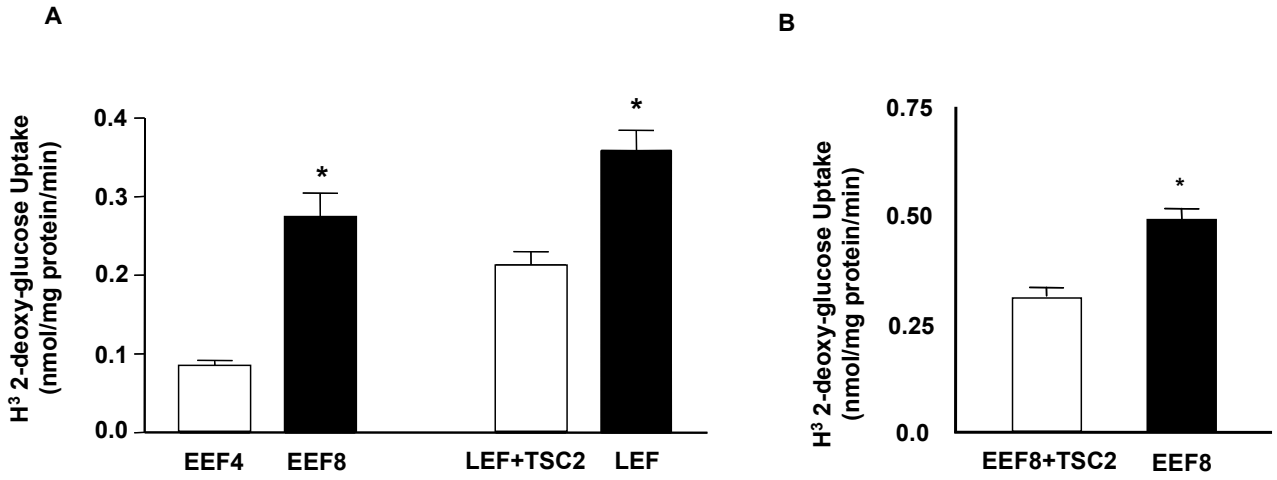

Figure 4. Loss of functional TSC2 leads to increased glucose uptake. 2-DOG uptake was increased in embryonic fibroblast cells (EEF8) and renal cells (LEF) lacking functional TSC2 compared to wild-type cells (EEF4) or TSC2 mutant cells in which a wild-type TSC2 construct was stably expressed (LEF+TSC2) (n=12, *$p<0.05$) (A). Similarly, 2-DOG uptake was increased in TSC2 mutant cells acutely infected with a retrovirus expressing wild type TSC2 (EEF8+TSC2, n=14) vs. the same cells infected with a control vector (EEF8, n=11) for 24hrs (*$p<0.05$ vs. TSC2 infected cells) (B).

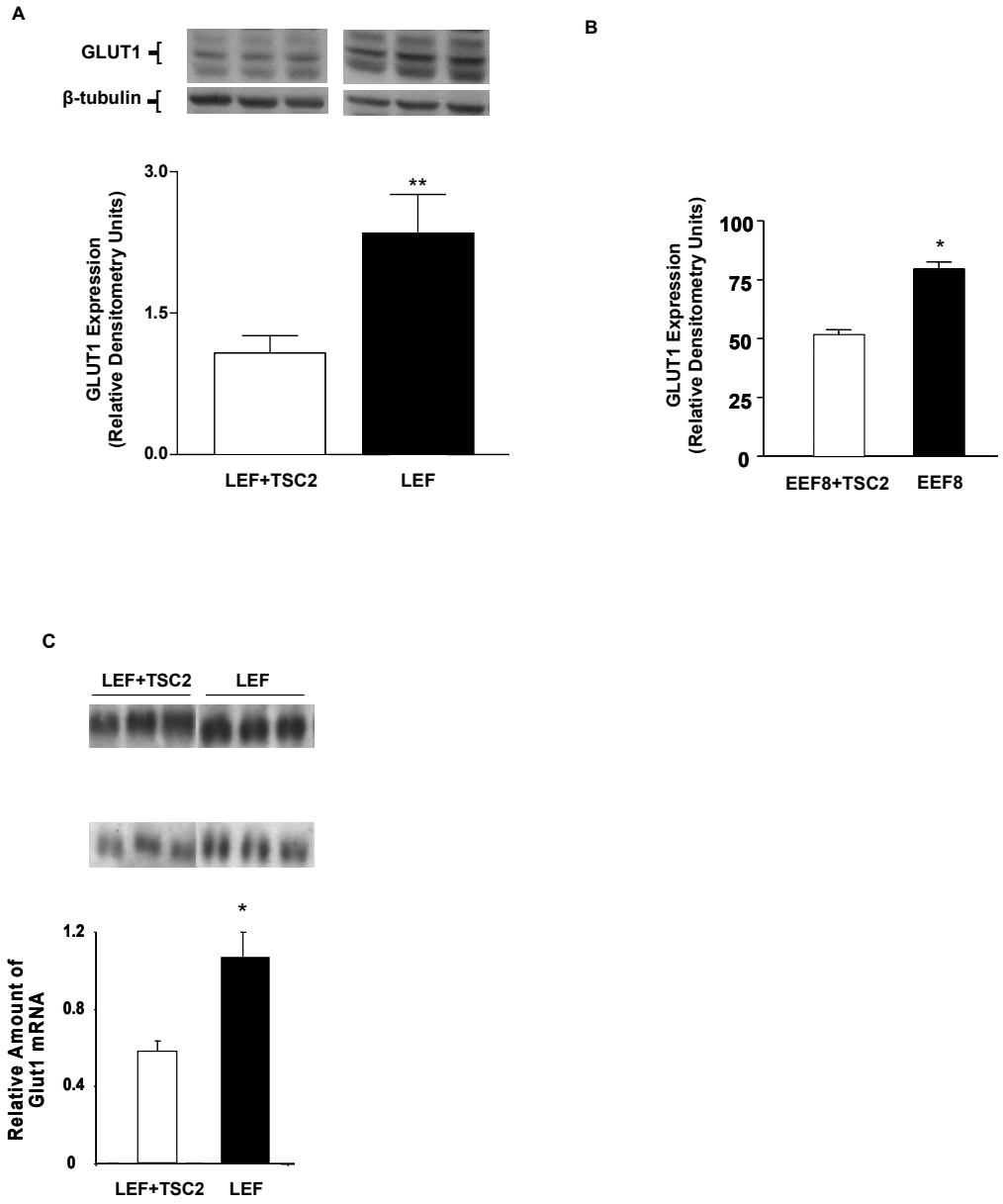

Figure 5. Loss of functional TSC2 leads to increased GLUT1 expression. GLUT1 protein expression was increased in the absence of functional TSC2 in LEF cells (n=12, **p<0.01) (A) and in EEF8 cells (n=3, *p<0.05) (B) when compared to cells with stable (LEF) or transient (EEF8) expression of a wild-type TSC2 construct. GLUT1 RNA expression was also increased in cells that do not express functional TSC2 (n=6, *p<0.05 vs. LEF + TSC2) (C).

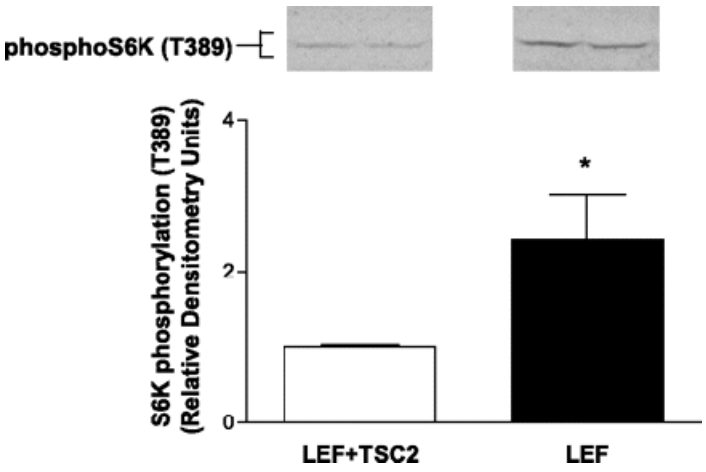

Figure 6. Loss of functional TSC2 leads to increased S6K phosphorylation. LEF cells that lack functional TSC2 exhibit increased S6K phosphorylation on mTORC1-specific residue Thr389 (n=8, *p<0.05 vs. LEF + TSC2).

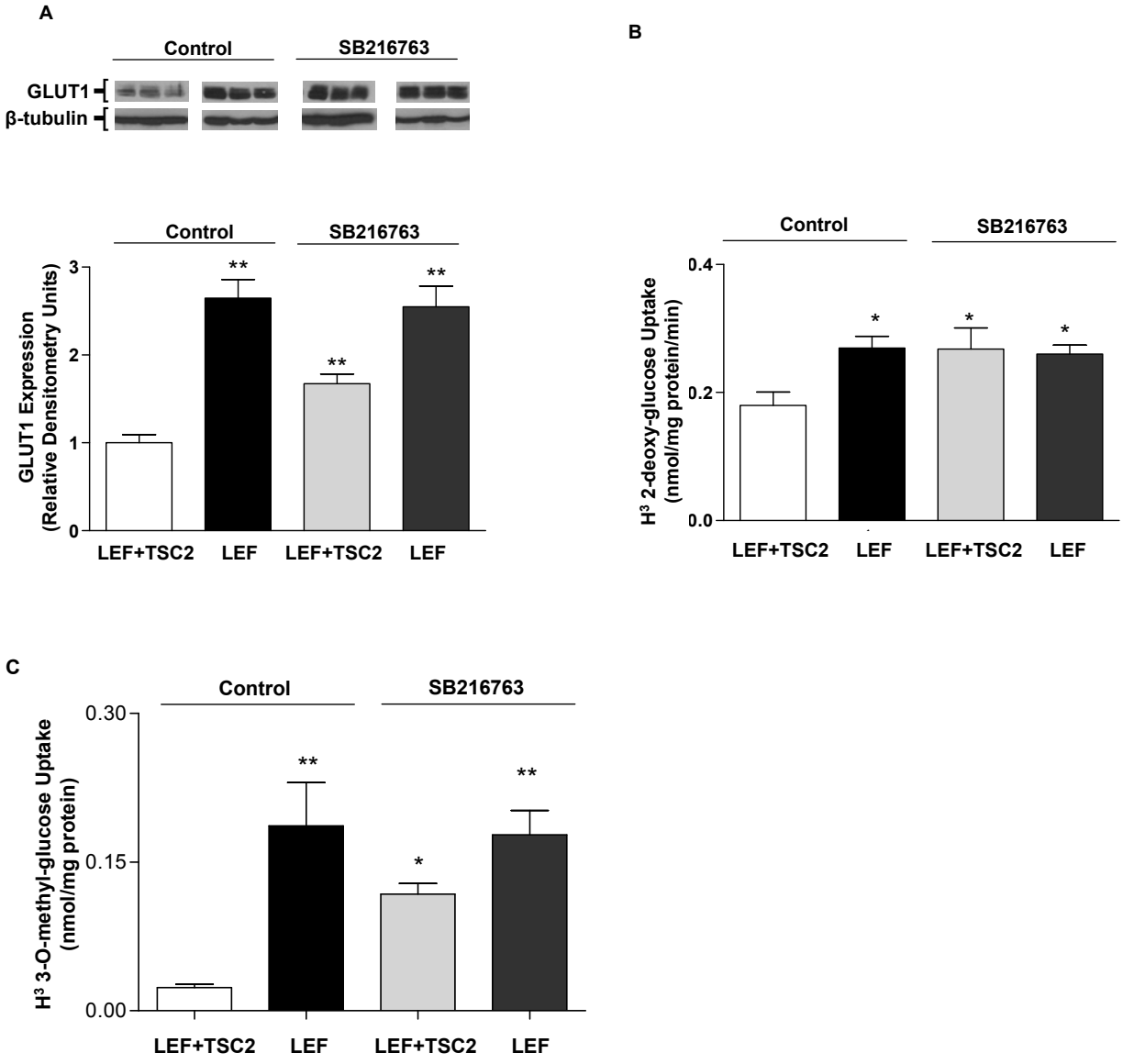

Figure 7. Functional TSC2 is required for GSK-3 inhibition-mediated increases in GLUT1 and glucose uptake. GSK-3 inhibition (24 hrs) with SB216763 (10uM) increased GLUT1 expression (n=9, **p<0.01; vs. LEF+TSC2 Control) (A), increased 2-DOG uptake (n=12, *p<0.05 vs. LEF+TSC2 Control) (B), and increased 3-OMG uptake (n=8, **p<0.01, *p<0.05 vs. LEF+TSC2 Control) (C) only in the presence of functional TSC2.

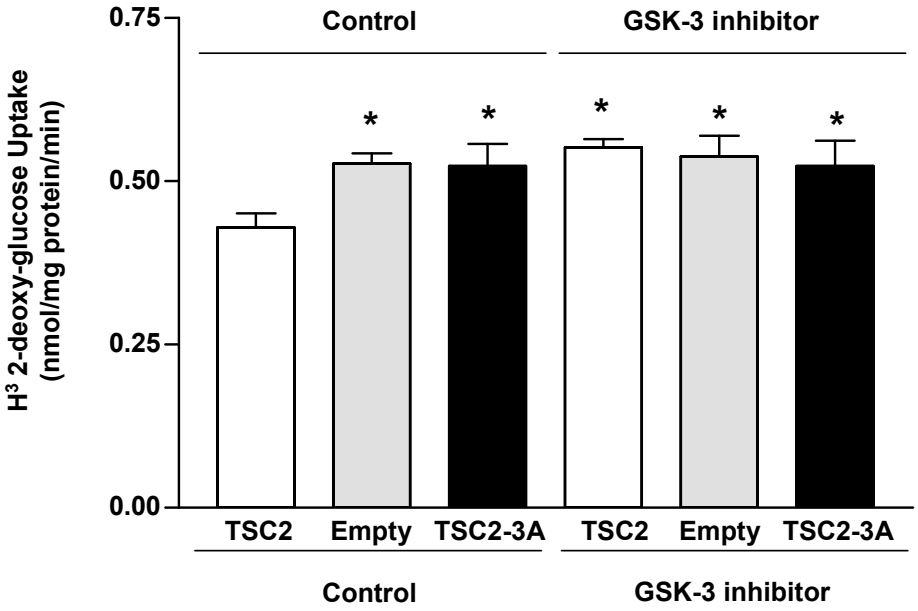

Figure 8. GSK-3 phosphorylation of TSC2 is necessary to inhibit glucose uptake. Wild-type, but not mutant, TSC2 expression resulted in decreased 2-DOG uptake in EEF8 cells infected (24hrs) with either a wild-type TSC2 construct (EEF8 + TSC2), empty control vector (EEF8 + Empty) or a TSC2 construct mutated at three GSK-3 phosphorylation sites (EEF8 + TSC2-3A). GSK-3 inhibition (Glycogen Synthase Kinase-3β Inhibitor II 10nM, 8hrs) abrogated the wild-type TSC2 effect (n=5, *$p<0.05$, vs. TSC2 Control).

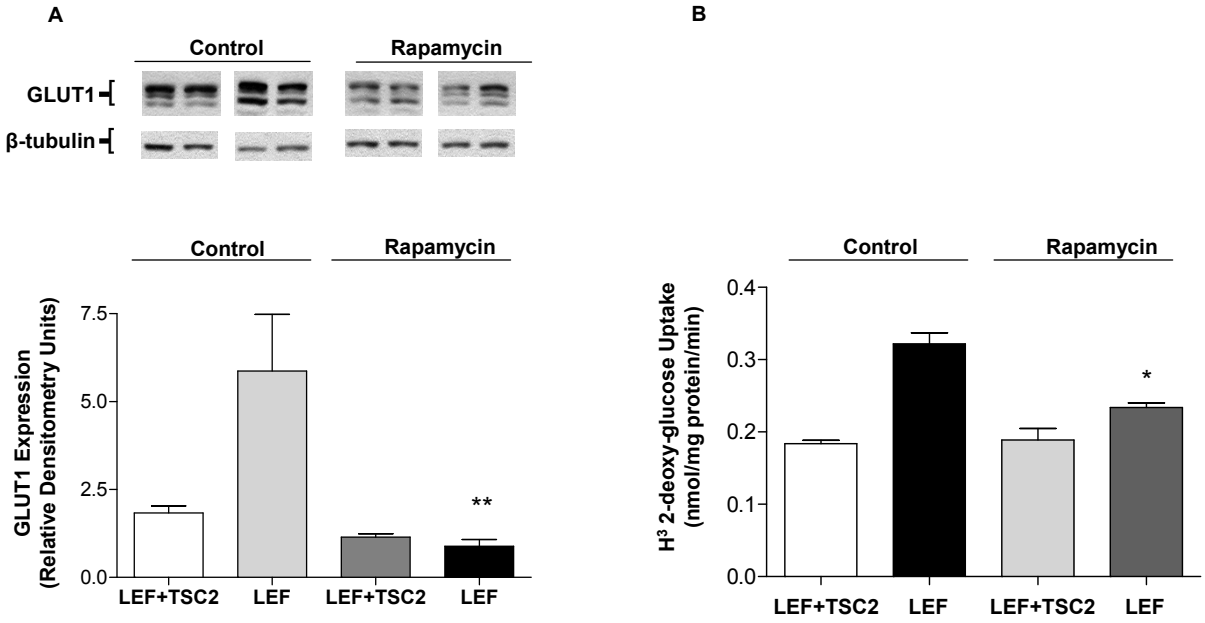

Figure 9. Inhibition of mTOR reduces GLUT1 expression and glucose uptake in cells lacking functional TSC2. Rapamycin treatment (20nM, 8 hrs) resulted in reduced GLUT1 expression (n=3, *p<0.05, vs. LEF Control) (A) and 2-DOG uptake (n=12, *p<0.05 vs. LEF Control) (B) only in cells lacking functional TSC2.

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Chapter 3

GLUT1 Enhances mTOR Activity Independent of TSC2 and AMPK.

Abstract

Enhanced GLUT1 expression in mesangial cells appears to play a role in the development of diabetic nephropathy by increasing several cell signaling pathways resulting in enhanced glomerular matrix accumulation. Similarly, enhanced mammalian target of rapamycin (mTOR) activation has been implicated in mesangial matrix expansion and glomerular hypertrophy in diabetes. We sought to examine whether GLUT1 expression enhanced mTOR activity and, if so, to identify the mechanism. We found that levels of GLUT1 expression and mTOR activation, as evidenced by S6 kinase phosphorylation, changed in tandem in several cell types exposed to elevated levels of extracellular glucose. We then showed that increased GLUT1 expression enhanced mTOR activity by 1.7-2.9-fold in cultured mesangial cells and in glomeruli from GLUT1 transgenic mice. Treatment with the mTOR inhibitor, rapamycin, eliminated the GLUT1 effect on mTOR activation. In cells lacking functional Tuberous Sclerosis Complex (TSC) 2, GLUT1 effects on mTOR activity were not altered, indicating that GLUT1 effects were not mediated by TSC. Similarly, AMP kinase (AMPK) phosphorylation and activity were not

altered by enhanced GLUT1 expression. Conversely, enhanced GLUT1 expression led to a 2.4-fold increase in binding to its activator, Rheb, and a commensurate 2.1-fold decrease in binding of Rheb to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) consistent with mediation of GLUT1 effects by a metabolic effect on GAPDH. Thus, GLUT1 expression may augment mesangial cell growth and matrix protein accumulation via effects on glycolysis and decreased GAPDH interaction with Rheb.

Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease in the United States [1, 2]. Many factors have been implicated in the pathogenesis of diabetic nephropathy; however the mechanisms remain incompletely understood. The earliest manifestations of the disease occur in the kidney glomerulus and, therefore, most attention to mechanisms has focused on altered responses of diabetic glomerular cells, especially mesangial cells. Glomerular expression of the facilitative glucose transporter, GLUT1, is increased in animal models of diabetes [3]. In mesangial cells and in nondiabetic mice, increased GLUT1 expression leads to alterations that are similar to cells exposed to high glucose concentrations and in diabetic mice, including activation of Protein Kinase C (PKC) isoforms and aldose reductase, increased vascular endothelial growth factor production and increased extracellular matrix production [4-6]. Conversely, reduction of GLUT1 expression in mesangial cells exposed to high glucose [7] and in diabetic mice prevents such alterations.

There is increasing evidence that the mammalian target of rapamycin (mTOR) and its downstream effector, p70 S6 kinase 1 (S6K), play significant roles in the pathogenesis of diabetic nephropathy [8]. In animal models of diabetes, there is increased glomerular mTOR activation and S6K phosphorylation [8]. Moreover, treatment of diabetic animals with rapamycin, a mTORC1-specific inhibitor, ameliorates glomerular hypertrophy and mesangial expansion in diabetic animals [8-11]. mTOR functions in two distinct multi-protein complexes, mTORC1 and mTORC2 [12, 13]. In mTORC1, mTOR associates with mLST8/GβL, raptor and PRAS40, and is sensitive to inhibition by rapamycin; while in the mTORC2 complex, mTOR interacts with mLST8/GβL, rictor and Sin1, and is rapamycin-insensitive, at least acutely [13-15]. Rheb, a GTP-binding protein modulated by Tuberous Sclerosis Complex (TSC), regulates mTOR activity [16, 17]. When bound to GTP, Rheb activates mTOR activity [16, 17]. We have previously found that GLUT1 expression is regulated by a GSK-3/TSC2/mTOR pathway, whereby mTORC1 activation enhances GLUT1 expression in several cell types [18]. In the current study, we wished to determine whether GLUT1 expression, conversely, could affect the rapamycin-sensitive mTORC1/S6K pathway. We hypothesized that GLUT1 enhances activation of mTORC1 in mesangial cells, resulting in a feedforward or amplification pathway whereby GLUT1 expression and function are further enhanced, and that this process could play an important role in the pathogenesis of some disease states. We found that GLUT1 expression enhances S6K activity via an mTORC1-mediated pathway but that the activation of mTOR

occurs through an unexpected pathway involving glyceraldehyde 3-phosphate dehydrogenase (GAPDH) interaction with Rheb which is modulated by GLUT1 expression.

Materials and Methods

Cell Lines: HEK293 cells were obtained from American Type Culture Collection (Manassas, VA). Rat glomerular mesangial cell (MC) lines were developed by Dr. Charles Heilig (University of Florida) and have been previously characterized by our groups [4, 19, 20]. The MC lines were grown in RPMI, 20% NuSerum IV and G418. Rat LEF cell lines were derived from spontaneous renal tubular tumors in Long Evans Eker rats [21]. Cells from these rats have an inactivating germline mutation in the TSC2 gene [22, 23]. LEF+TSC2 cells are stably-transfected to express functional TSC2 and have been reported previously [18]. LEF±TSC2 cells were grown in DMEM/F-12 and 10% fetal calf serum ± G418. Appropriate concentrations of mannitol were used in the media to maintain osmolarity in the increased extracellular glucose experiments. Mannitol and 2-deoxy-glucose (2-DOG) were purchased from Sigma Aldrich (St. Louis, MO).

Infection Studies: A GLUT1 adenoviral construct (Ad-GT1),a gift from Dr. Arno Kumagi (University of Michigan) was utilized as previously reported [24]. Cells were cultured in conditioned RPMI media for 24 hours at 37ºC and a multiplicity of infection (MOI) of 5 was used to infect the cells as previously

reported [24]. All adenoviral work was done in accordance with the guidelines of the University of Michigan Institutional Biosafety Committee.

[^{3}H] 2-DOG Uptake: Cells were grown to ~90% confluence prior to treatment and 2-DOG uptake analysis. 2-DOG uptakes were performed as previously described [25]. Briefly, plates were washed once with Krebs Ringer Phosphate buffer (KRP) (in mmol/l: 128 NaCl, 5.2 KCl, 1.3 CaCl2, 2.6 MgSO4, and 10 Na2HPO4), and then incubated with KRP buffer supplemented with 1% BSA for 10 minutes to one hour at 37ºC. Treatments were added to the KRP buffer during the one hour incubation period for various time points as noted. The KRP buffer was removed and replaced with 0.1 mM unlabeled 2-DOG (Sigma Aldrich) and 0.5µCi/ml ^{3}H-2-DOG (Perkin Elmer, Waltham, MA) in KRP buffer + 1% (wt/vol) bovine serum albumin (BSA) ± 20 nM cytochalasin B, an irreversible inhibitor of glucose transport, at 37^{o}C for 5 min. Studies have shown that uptake is linear for at least 10 min in the control MC line [5]. The plates were subsequently washed twice for 5 minutes each time with cold KRP solution containing 200µM phloretin to quench 2-DOG uptake. The samples were then lysed in buffer (Tris-HCl, pH7.0, 10 mM, NaCl 150mM, Triton X-100 1% and SDS 0.1%). A portion of each sample was used for determination of protein concentration by a bicinchoninic acid assay (Pierce; Rockford, IL), and the rest was utilized for scintillation counting. 2-DOG uptake (in nmol/milligram protein/min) was calculated after correction for nonspecific uptake in the presence of cytochalasin B.

Animals: Animals were handled according to the guidelines set forth by the University Committee for the Use and Care of Animals (UCUCA) at the University of Michigan. Transgenic mice that overexpress GLUT1 (GT1S mice) were generated in collaboration with Dr. Charles Heilig [26]. Renal glomeruli were isolated for Western Blot analysis as previously described [27]. Briefly, anesthetized animals were perfused with iron oxide and kidneys were excised, minced and pressed through a nylon filter into a beaker while being rinsed with ice cold PBS. A magnet on the bottom of the beaker was used to collect glomeruli containing iron oxide. The glomeruli were transferred to a microcentrifuge tube and resuspended in protein lysis buffer.

Western Blotting Analysis: Western Blotting was used to measure protein expression. Cellular proteins were separated by SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were blocked with 5% milk in TBS-T for 30 minutes to an hour, incubated in primary antibody overnight at 4ºC and secondary antibody for 1 to 2 hours. After autoradiography, the films were scanned and quantified using NIH ImageJ.

Primary antibodies, with the exception of the GLUT1 antibody, were obtained from Cell Signaling Technology (Beverly, MA), while secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The GLUT1 antibody was a gift from Dr. Christin Carter-Su (University of Michigan).

Immunoprecipitation experiments were performed using Protein A/G agarose beads from Santa Cruz per the manufacturer's recommended protocol.

In brief, 200 to 500ug of protein was incubated with antibody for 1 hour on ice and then incubated with protein A/G beads overnight at 4ºC. The beads were then washed and resuspended in loading buffer before use for Western Blot analysis.

Statistical Analysis: Prism 4 (Graphpad Software, La Jolla, CA) was used for all statistical analysis. Data were expressed as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc analysis, or by Student's t-test. Differences were considered significant at $p < 0.05$.

Results

Increased extracellular glucose increases S6K phosphorylation in rat mesangial cells.

We observed a significant increase in S6K (Thr389) phosphorylation in rat glomerular mesangial cells after incubation in high glucose media (25mM) for 24 hours, suggesting a significant activation of mTOR (Figure 1A). A concurrent increase in GLUT1 protein expression was also detected (Figure 1B), as previously reported [19]. In contrast, incubation of HEK293 cells in 25 mM glucose resulted in a significant decrease in both GLUT1 expression and S6K phosphorylation when compared to levels in cells incubated in 6 mM glucose (Figure 1C, D).

Enhanced GLUT1 expression and glucose uptake augments mTOR activity.

In order to determine whether GLUT1 expression was responsible for the effects of high glucose incubation on S6K phopshorylation, we examined the effect of short-term GLUT1 overexpression on the mTOR pathway. We infected control rat mesangial cells (MClacZ) with an adenoviral vector to acutely overexpress GLUT1. GLUT1 levels were increased by 2.4-fold and glucose uptake, as determined by uptake of the glucose analog, 2-DOG, was increased 2.3-fold ($p<0.05$) in these cells 24 hours after infection (Figure 2D). S6K phosphorylation was increased 2.9-fold at the same time point (Figure 2A), suggesting that acute GLUT1 increases were sufficient to augment S6K phosphorylation. The GLUT1 effect on S6K phosphorylation was dependent on glucose uptake and metabolism. Cells were incubated with or without 8mM glucose or 8mM mannitol as an osmotic control. Removal of glucose eliminated the increase in S6K phosphorylation in cells that acutely overexpressed GLUT1 (Figure 3). To confirm that the increased S6K phosphorylation was due to enhanced mTOR activity, we examined an additional direct target of mTOR, the transcriptional repressor 4E-BP-1. Phosphorylation of 4E-BP-1 (Thr37/46) was 2.2 times greater in cells acutely overexpressing GLUT1 (Figure 2C) ($p<0.05$). To further confirm that the effect of GLUT1 on S6K is via mTOR, we incubated rat mesangial cells in rapamycin after adenoviral infection. Rapamycin, a specific acute mTOR inhibitor, abrogated the effect of GLUT1 overexpression on S6K phosphorylation (Figure 4).

Chronic augmentation of GLUT1 expression results in persistent mTOR activity in vitro and in vivo.

To determine the chronic effects of increased GLUT1 expression on mesangial cell mTOR activity, we examined a stable GLUT1-overexpressing rat mesangial cell line (MCGT1) which has been systematically analyzed previously [4, 7, 19]. These cells exhibited a 1.8-fold increase in S6K phosphorylation compared to the control-transfected stable cell line (Figure 5A). We also examined GLUT1 transgenic (GT1S) mice which stably overexpress GLUT1 in mesangial cells in the glomerulus [26]. We found a 1.7-fold increase in S6K phosphorylation in renal glomeruli isolated from these animals (Figure 5C).

GLUT1 effects on mTOR activity are independent of TSC2 and AMP kinase (AMPK).

Loss of TSC2 leads to a constitutive increase in mTOR activity [28-30]. To test whether TSC2 mediates the effect of GLUT1 on S6K phosphorylation, we used LEF cells which lack functional TSC2 and exhibit increased S6K phosphorylation when cultured under normal conditions [18] as well as LEF cells that have had TSC function restored by chronic transfection with a wild-type TSC2 cDNA. After adenoviral overexpression of GLUT1, we observed a significant increase in S6K phosphorylation (Figure 6) in both cell lines. Importantly, the augmentation induced by GLUT1 overexpression was similar in each line, although basal S6K phosphorylation was higher in cells without

functional TSC2. Thus, functional TSC2 was unnecessary for GLUT1 effects on mTOR activity in these cells.

We also determined whether AMPK mediates the effects of GLUT1 overexpresion on mTOR activity. Although primary regulation of mTOR by AMPK occurs via TSC2, it has been shown that AMPK can also directly regulate mTORC1 via the mTOR subunit, raptor [31]. We examined AMPK phosphorylation at Thr172, a marker of activated AMPK, in the rat mesangial cells and detected no significant change in phosphorylation after adenoviral GLUT1 overexpression (Figure 7B). Additionally, we looked at phosphorylation of acetyl co-A carboxylase (ACC) (Ser79) a substrate for AMPK, and found no difference between GLUT1-overexpressing and control cells (Figure 7A). Finally, the AMPK inhibitor, Compound C, increased the levels of S6K phosphorylation in both GLUT1 overexpressing and control cells, but the difference between the cell types was not altered by the treatment (Figure 7C). All three findings indicate that the difference in mTOR activity found in GLUT1-overexpressing cells was not due to altered AMPK activity.

GLUT1 effects on mTOR activity are associated with reduced Rheb and GAPDH interaction.

An alternate pathway that has been recently reported to mediate the effect of glucose metabolism on mTOR activity involves glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the mTOR activator and small GTPase, Rheb and mTOR. GAPDH has been found to bind Rheb and sequester it, thus preventing

its activation of mTOR [32]. Exposure of cells to increased extracellular glucose and glycolytic flux leads to dissociation of GAPDH from Rheb and increased interaction of Rheb with mTORC1 promoting its activation [32]. In rat mesangial cells that acutely overexpress GLUT1, we found a 2.1-fold decrease in the amount of GAPDH bound to Rheb in immunoprecipitation experiments (Figure 8A) ($p<0.05$). This change coincided with a 2.4-fold increase in the amount of mTOR associated with Rheb (Figure 8B) ($p<0.01$).

Discussion

In this study, we demonstrate that enhanced GLUT1 expression results in a glucose dependent increase in mTORC1 activity as measured by rapamycin-inhibitable phosphorylation of S6K and 4E-BP-1. This is the first time that a direct relationship has been established between GLUT1 expression, glucose metabolism and the mTOR pathway. We have shown that GLUT1 overexpression via an increase in glucose uptake, without a change in extracellular glucose, results in stimulation of mTOR activity in mesangial cells that is both TSC2- and AMPK-independent and appears to occur due to decreased GAPDH/Rheb binding and increased Rheb/mTOR binding. These findings confirm and extend those of Lee et al. [32] in a different system.

A search by those investigators for binding partners of Rheb revealed a novel interaction with GAPDH [32]. mTOR association with GTP-bound Rheb is necessary for activation and S6K phosphorylation [17]. A thorough investigation revealed that activation of mTOR via GAPDH was both TSC2- and AMPK-

independent. Additionally, the interaction between GAPDH and Rheb occurred regardless of the nucleotide binding status of Rheb. We found that enhanced GLUT1 expression, independent of extracellular glucose levels, induces a very similar process in cultured mesangial cells, suggesting that the mechanism for such enhanced mTOR activation is via effects of GLUT1 on glucose metabolic flux, Indeed, our experiments help explain why the effects of high extracellular glucose were transient in the systems reported by Lee, et al. As we have shown in this current study, HEK293 cells rapidly reduce GLUT1 levels and mTOR activity after being placed in high extracellular glucose concentrations. This presumably protective response does not occur in some cell types, such as the mesangial cells that were the basis of most of our analysis. These cells develop a sustained increase in GLUT1 expression in the presence of high extracellular glucose levels [19] and therefore maintain chronic mTOR activation as shown in our analysis of cells and mouse glomeruli with chronic GLUT1 overexpression.

GLUT1 expression increases in glomeruli and in whole kidney in animal models of type 1 and type 2 diabetes [26]. This increase in GLUT1 expression is associated with a large increase in glucose metabolic flux [3, 33]. Prevention of this increased GLUT1 expression in cultured mesangial cells [7] and in preliminary studies in vivo [34], eliminates many of the pathogenic aspects associated with diabetic glomerulopathy, including the abnormal activation of PKC, aldose reductase and other pathways as well as the enhanced synthesis of extracellular matrix proteins, such as fibronectin. To date, however, the effects of GLUT1 on activation of mTOR signaling have not been examined.

Over the past few years, a number of studies have shown that activation of the mTOR pathway may be important in the development and progression of diabetic kidney disease [1, 3, 35-38]. Nagai et al. [8] have shown that increased phosphorylation of S6K is characteristic of early stages of diabetic nephropathy in animal models. There is a significant increase in mTOR activity shortly after induction of type 1 diabetes by streptozotocin injection in animal models [10] which coincides with significant mesangial and glomerular hypertrophy [8]. Treatment of diabetic rats and mice with the mTOR inhibitor, rapamycin, has been shown to reduce albuminuria, mesangial matrix expansion and glomerular basement membrane thickening [11]. Thus, mTOR activity appears to play an important role in the changes of early diabetic nephropathy and that some of the augmentation of nephropathic changes by GLUT1 occur via its effects on mTOR.

We have previously shown that increased mTOR activity leads to increased expression of GLUT1 and glucose flux. Here, we demonstrate that increased GLUT1 expression in mesangial cells leads to increased glucose flux and mTOR activity, as measured by S6K phosphorylation. Together, this suggests a feedforward mechanism leading towards persistent GLUT1 overexpression and mTOR activation in diabetic glomeruli. Thus, treatments targeting the potetntially pathogenic increase in GLUT1 expression as well as those that inhibit mTOR activation may prove highly effective in reducing the progression of diabetic nephropathy.

Figures

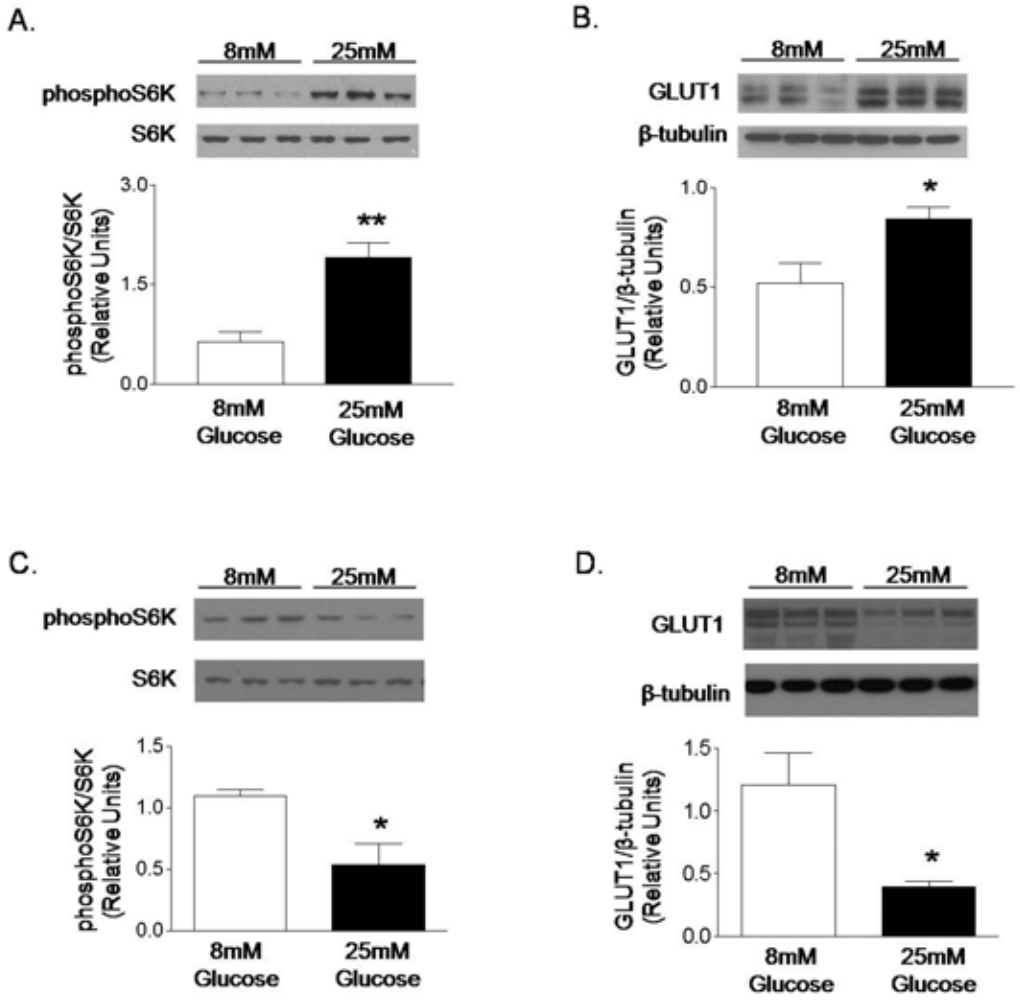

Figure 1. Differential effects of high glucose on mesangial cells and HEK cells. Exposure to high glucose increased S6K(Thr389) phosphorylation (A. n=5, **p<0.01) and GLUT1 protein expression (B. n=5, *p<0.05) in rat mesangial cells when compared to control. In HEK cells, incubation in high glucose for 24 hours decreased S6K(Thr389) phosphorylation (C. n=5, *p<0.05) and GLUT1 expression (D. n=5, *p<0.05).

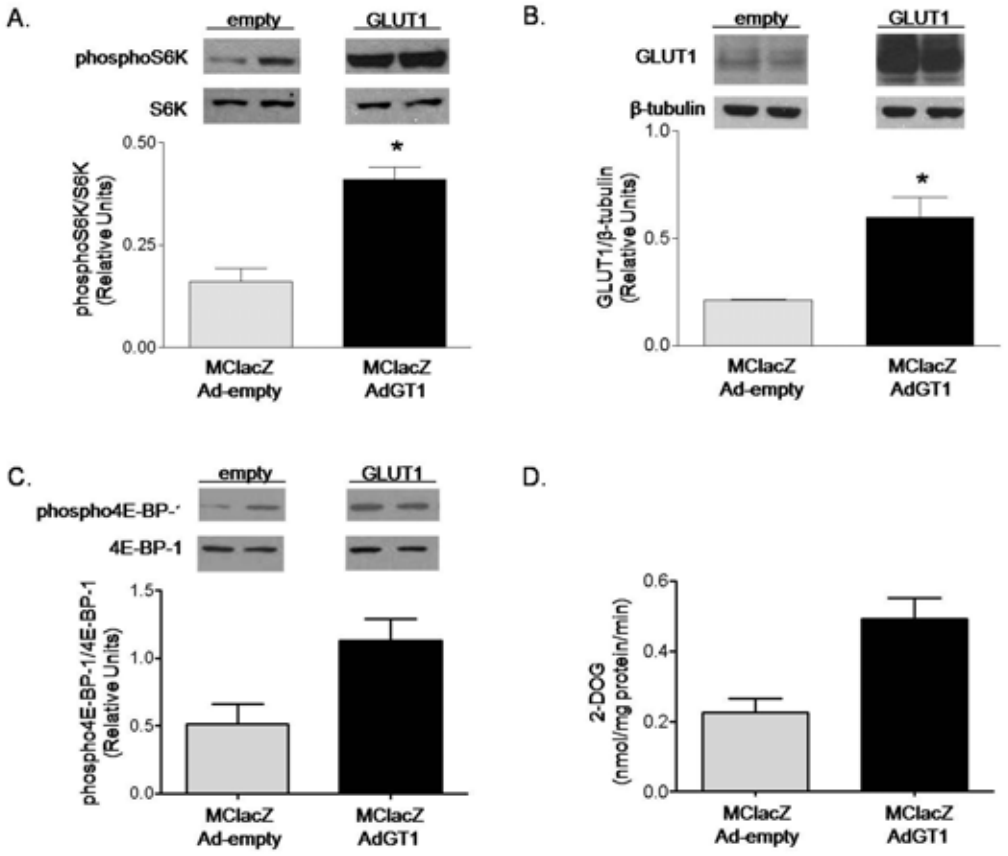

Figure 2. Short-term GLUT1 overexpression increased mTOR activity and glucose uptake. Adenoviral infection of GLUT1 in mesangial cells led to increased S6K(Thr389) phosphorylation (A. n=6, **p<0.01) and a two-fold increase in GLUT1 expression (B. n=6, *p<0.05) after 24 hours. GLUT1 overexpression increased phosphorylation of 4E-BP-1(Thr37/46) (C. n=6, *p<0.05). Acute GLUT1 overexpression increased glucose uptake (D. n=6, **p<0.01; 24hrs post-infection).

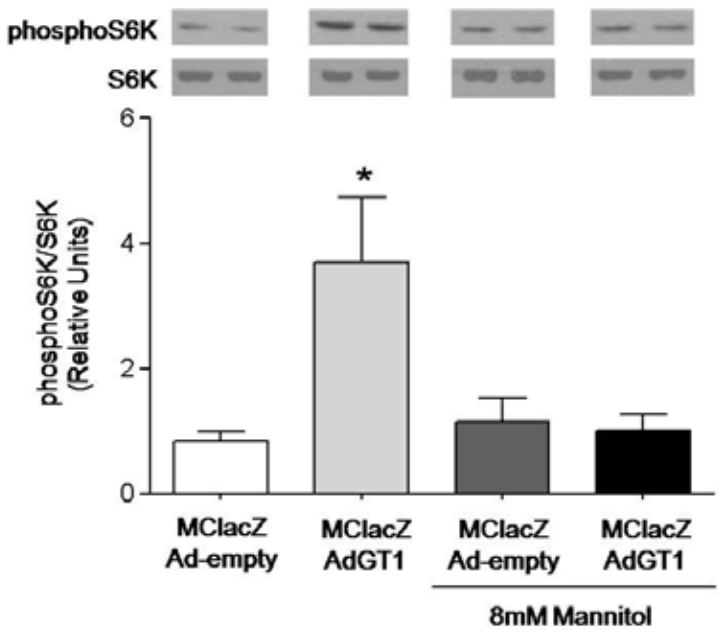

Figure 3. Increased S6K phosphorylation resulting from GLUT1 overexpression was glucose dependent. GLUT1-induced increases in S6K(Thr389) phosphorylation were eliminated when glucose was removed from the media (n=6, *p<0.05; 8mM mannitol 1hr).

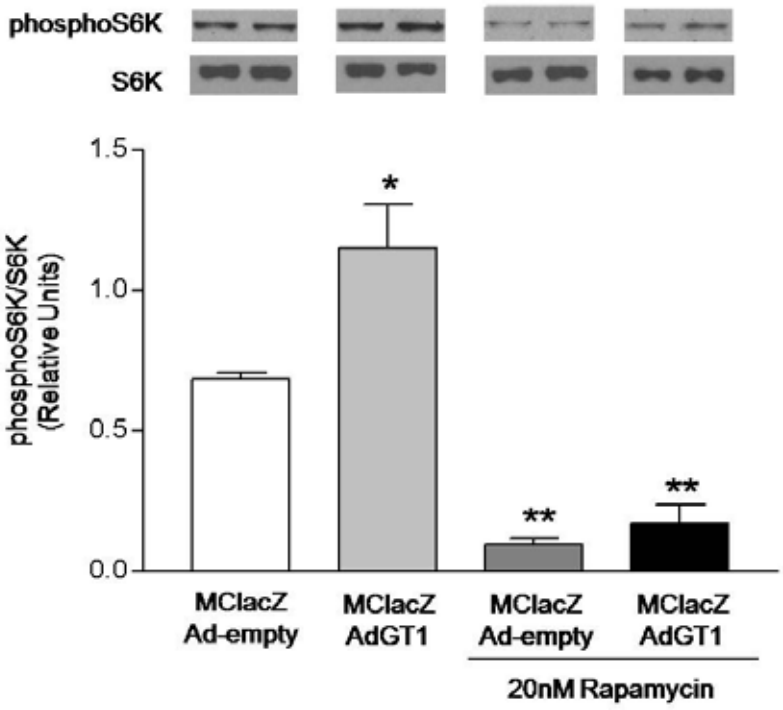

Figure 4. S6K phosphorylation is decreased by rapamycin treatment in rat mesangial cells. Inhibition of mTOR by rapamycin (20nM, 24hrs) decreased S6K(Thr389) phosphorylation in rat mesangial cells (n=3, *p<0.05, **p<0.01).

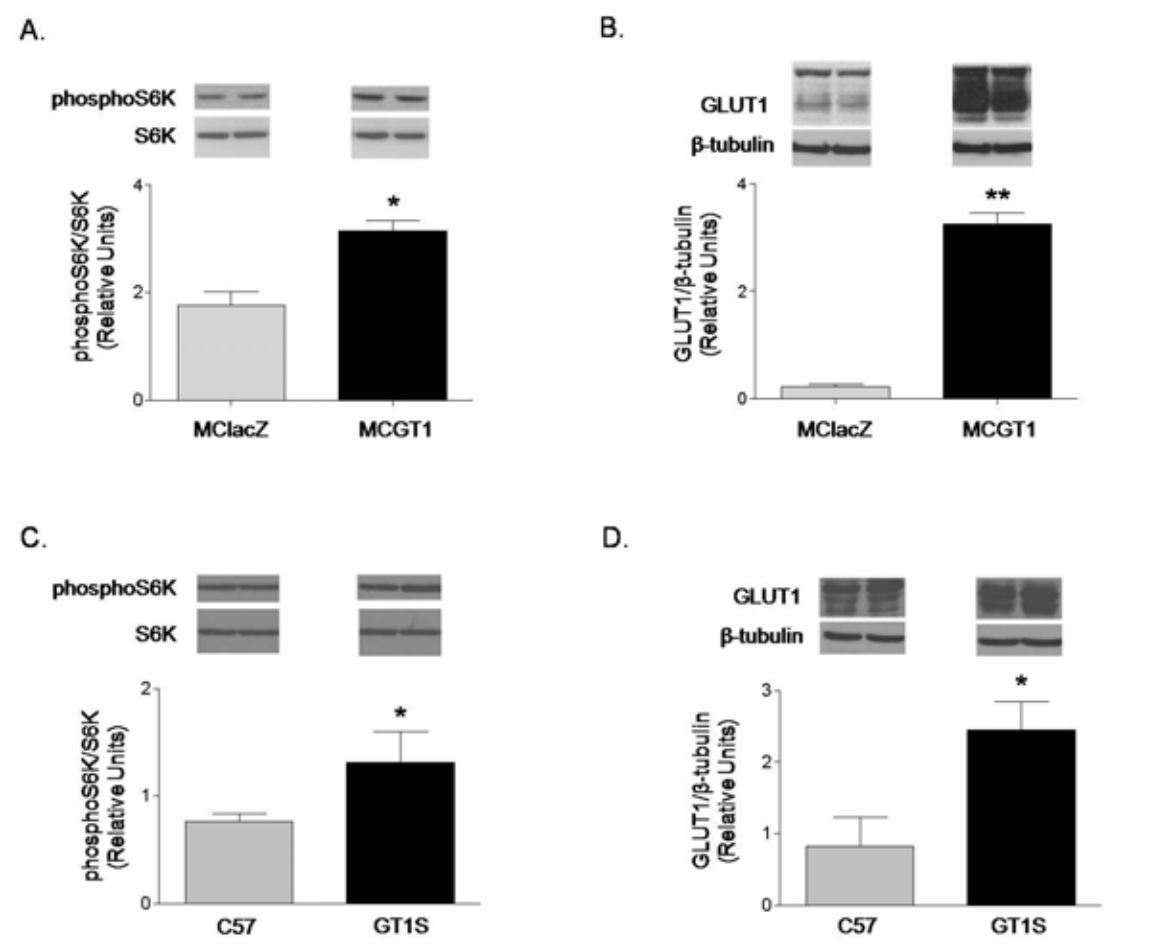

Figure 5. Long-term GLUT1 overexpression results in increased S6K phosphorylation. Chronic GLUT1 overexpression increased S6K (Thr389) phosphorylation in rat mesangial cells (A. n=4, * $p<0.05$) and glomeruli isolated from GLUT1 trangenic mice (C. n=4, *$p<0.05$). GLUT1 protein concentration is indeed elevated in models of GLUT1 overexpression: rat MC (B. n=4, **$p<0.01$) and GLUT1 transgenic mice (D. n=4, *$p<0.05$).

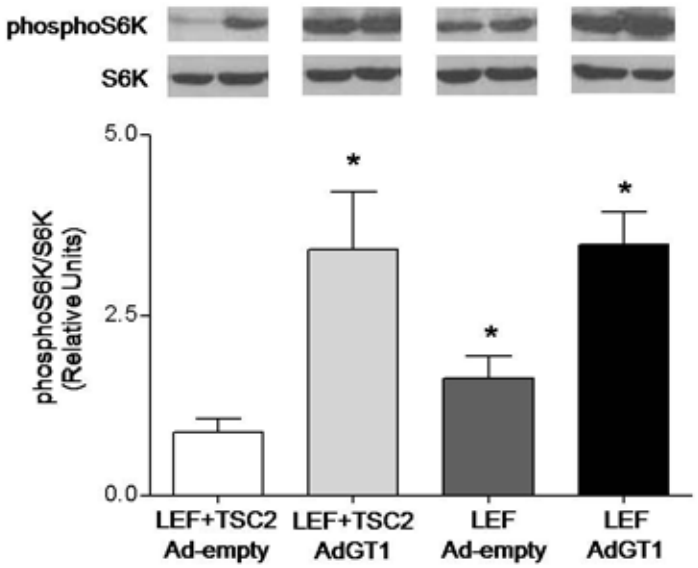

Figure 6. TSC2 is not required for increased S6K phosphorylation by GLUT1 overexpression. Increased GLUT1 expression in kidney-derived LEF cells increased S6K(Thr389) phosphorylation even in the absence of functional TSC2 (n=8, *p<0.05).

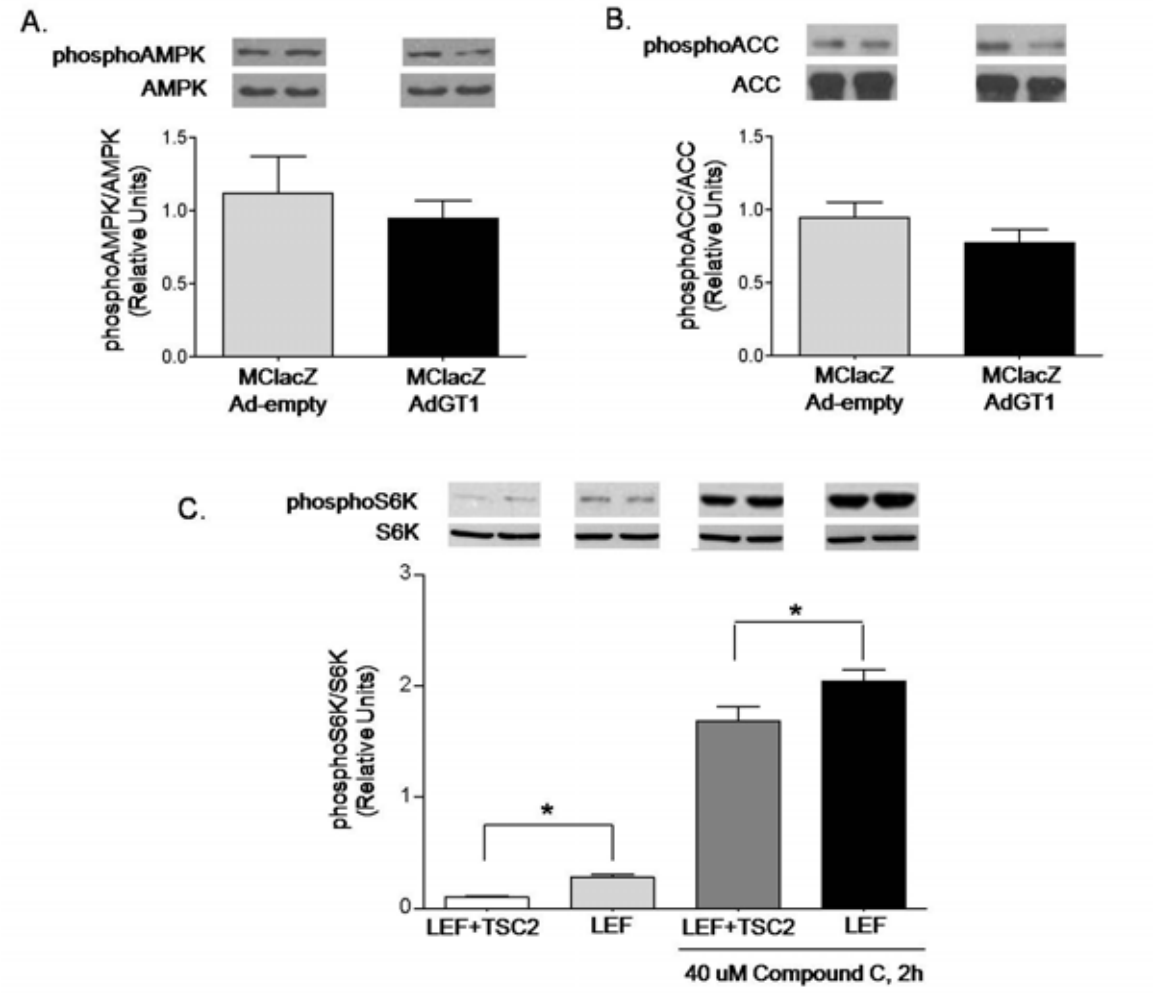

Figure 7. AMPK activity is not increased by GLUT1 overexpression. GLUT1 overexpression in mesangial cells did not significantly decrease AMPK activity as examined by AMPK(Thr172) and acetyl CoA carboxylase(ACC)(Ser79) phosphorylation (A. n=3, B. n=4, p=NS). Additionally, a significant increase between control cells and those that overexpress GLUT1 was maintained after treating cells with an AMPK inhibitor (C. n=6, *p<0.05).

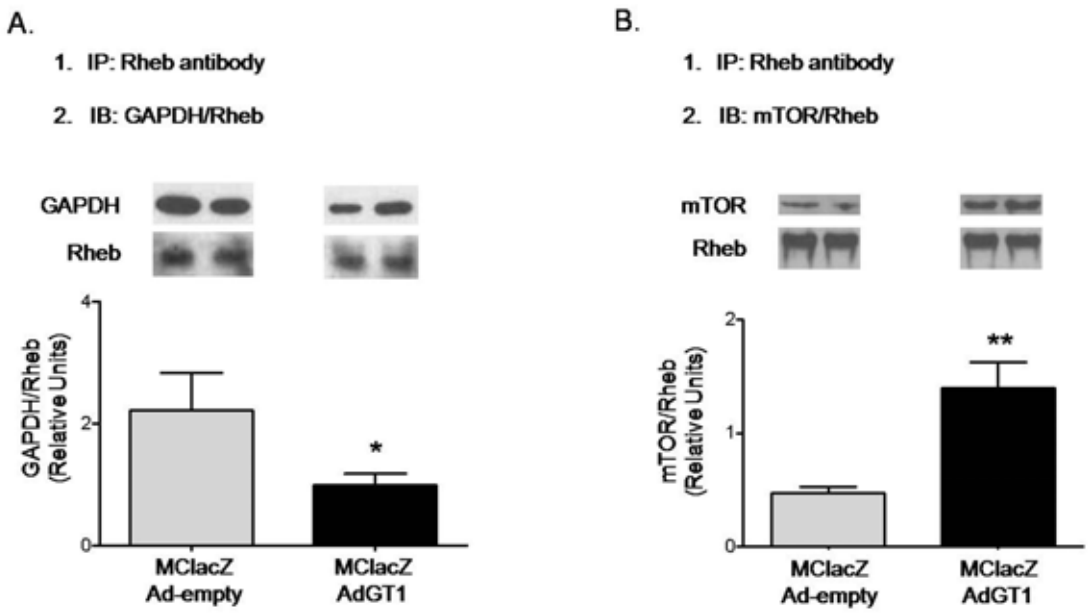

Figure 8. GLUT1 overexpression decreased GAPDH- and increased mTOR-bound Rheb. Increased GLUT1 reduced the amount of GAPDH bound to Rheb (A. n=9, *p<0.05). There was a significant increase in the amount of Rheb bound to mTOR (B. n=7, **p<0.01).

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Chapter 4

Conclusions

The mTORC1 pathway is responsible for integrating input from a number of cellular signaling pathways in order to maintain cellular growth and proliferation. GLUT1 is an important facilitator of glucose uptake in most mammalian cells. In this dissertation I have provided data to support the hypothesis that the GSK-3, TSC2, mTORC1 pathway regulates GLUT1 expression and resultant glucose uptake and, conversely, that GLUT1 and GLUT1-mediated glucose uptake regulate mTORC1 and S6 kinase activity in a manner that may be important in diabetic nephropathy. Specifically, using biochemical techniques, I have delineated a relationship by which mTORC1 activation augments GLUT1 expression and glucose uptake, which in turn facilitates a GLUT1-mediated glucose-dependent mechanism that enhances activation of the mTORC1 pathway (Figure 1).

Potential upstream molecules involved in mTORC1 regulation of GLUT1: In chapter 2, I have demonstrated that the GSK-3, TSC2, mTORC1 pathway regulates GLUT1 expression and glucose uptake. However, upstream activators remain to be identified. PI3K activation of the mTORC1 pathway is well understood. Binding of the growth factor to its receptor leads to a cascade of phosphorylation events cumulating in the activation of PI3K and subsequent

binding and activation of Akt. Activated Akt phosphorylates and inactivates GSK-3 and TSC2 [1]. A likely candidate for upstream activation of mTOR in our study is growth factor activation of receptor tyrosine kinases leading to stimulation of PI3K and Akt. It is well known that the mTORC1 pathway is activated by insulin and other growth factors, such as EGF. Additionally, it has been shown that TGF-β resulting from increased extracellular glucose activates mTORC1 [2]. The cells studied in this model are known to be sensitive to many growth factors, including IGF, EGF, FGF and TGF-β [3-5]. The responses that we have observed may be part of a general response to growth factor signaling in the cells studied.

The GSK-3, TSC2, mTORC1-mediated regulation of GLUT1 outlined in chapter 2 may indeed be AMPK-dependent as well. Cellular energy and oxygen availability also regulate mTORC1 activity through AMPK's effects on TSC2. Thus, AMPK could regulate TSC2 and mTOR by way of a PI3K- and Akt-independent process. AMPK phosphorylation of TSC2 (Ser1387) is a priming site for phosphorylation by GSK-3 of TSC2 (Ser1383, Ser 1379) [6, 7]. This leads to a system of signal integration that depends on the activation state of AMPK and GSK-3. Together, these signaling proteins can modulate TSC formation and therefore determine mTORC1 activity. The TSC2-3A construct with mutated GSK-3 phosphorylation sites used in chapter 2 is also mutated at the AMPK (Ser1387) phosphorylation site. Thus, the experimental results with the TSC2-3A construct could equally have been due to inhibition of AMPK

phosphorylation of TSC2. However, experiments with GSK-3 inhibitors support a role for GSK-3 in this process. Further specific experiments that independently modulated AMPK activity would need to be performed to establish whether AMPK is also involved in regulation GLUT1 expression.

Mechanisms by which mTORC1 regulates GLUT1: Further avenues of study include identification of the mechanisms by which activated mTORC1 leads to enhanced GLUT1 expression. It is known that mTORC1 promotes protein synthesis via two effectors, S6K1 and 4E-BP-1 [8-10], and mTORC1 is widely known for its role in cellular growth and proliferation via control of translation initiation components [11]. Phosphorylation of S6K by mTORC1 leads to activation of ribosomal protein S6 which then assists in the translation of mRNA [12]. This coincides with mTORC1 phosphorylation of the translational repressor 4E-BP-1, resulting in decreased association of 4E-BP-1 to eIF4E and allowing eIF4E to bind to eIF4G. Increased interaction of eIF4E with eIF4G enables mRNA to bind to the 43S preinitiation complex to enhance initiation of translation [12]. Regulation of elongation factors by mTORC1 may also play a role in GLUT1 expression. mTORC1 is thought to modulate the activity of eukaryotic elongation factor 2 (eEF2) kinase. Phosphorylation of eEF2 kinase and subsequent phosphorylation of eEF2 leads to decreased ribosomal translocation during peptide elongation [13]. Additional experiments examining eIF4E phosphorylation status, or the level of interaction between eIF4E and eIF4G, would serve to confirm that these mechanisms play a role in mTORC1 stimulation of GLUT1 protein expression.

In chapter 2, we found that increased mTORC1 activation was associated with increased GLUT1 mRNA expression as well as increased protein synthesis. Effects of mTORC1 activation on GLUT1 would likely be mediated by enhanced translation; therefore, an explanation for the observed increase in GLUT1 mRNA remains to be identified. It is unknown whether the increased GLUT1 that was observed was due to enhanced transcription, inhibition of degradation or a combination of both processes.

GLUT1 regulation of mTORC1: GLUT1 overexpression plays a role in disease progression in the diabetic kidney [14-16]. The stably-transfected GLUT1 cells used in these experiments has been previously shown to lead to increased expression of pathogenic markers of diabetic nephropathy [17-19]. Many studies have shown that mTORC1 activation plays an integral role in hypertrophy associated with diabetic nephropathy. Research suggests that high glucose-induced Akt signaling and inhibition of AMPK are responsible for increased mTORC1 activity in some cell types [20, 21]; however the precise mechanisms are not fully understood. Considering the results of chapter 3, AMPK was initially hypothesized to be responsible for the observed GLUT1 effects. AMPK has long been considered to be a major energy sensor of the cell since it is activated in response to decreased intracellular ATP and increased intracellular AMP [22]. Barnes et al. [23] have reported that mimicking metabolic stress by activation of AMPK leads to increased GLUT1 expression in rat liver epithelial cells. Phosphorylation of TSC2 by AMPK is an important step in the canonical mTORC1 signaling pathway [22]. Therefore, the increase in S6K

phosphorylation seen in GLUT1-infected LEF cells, which do not express functional TSC2, was surprising. Despite this result, it was possible that AMPK could affect the rapamycin-sensitive mTOR pathway further downstream, bypassing TSC2. Indeed, it has been shown that AMPK can directly phosphorylate raptor at Ser792 in response to energy stress [24]. However, we did not find a significant increase in AMPK activity in our model system. So at least in cultured mesangial cells, the common paradigm of glucose stimulated AMPK-TSC2-dependent activation of mTOR was not observed.

A study by Lee et al. [25] discovered a new mechanism by which glycolytic flux can affect the mTOR pathway. After identifying GAPDH as a Rheb binding protein, the authors demonstrated that brief exposure of HEK293 cells to increased extracellular glucose (25mM, <30 min) led to decreased association of Rheb and GAPDH and increased association between Rheb and mTOR. There was no significant change in total Rheb expression. These cells also exhibited increased phosphorylation of S6K and 4E-BP-1. This effect was observed in TSC1-null cells and in AMPK siRNA experiments. The authors supported their findings by knocking down GAPDH. siRNA knockdown of GAPDH in the absence of hyperglycemia led to a decrease in GAPDH-bound Rheb, and a significant increase in mTOR-bound Rheb. Based on these findings [25] and our results, it appears that increased GLUT1 expression leads to decreased interaction between Rheb and GAPDH due to enhanced glucose flux from the increased numbers of high affinity plasma membrane transporters without any change in extracellular glucose concentration.

Additional experiments to further support our findings were considered, including GAPDH and Rheb overexpression. Overexpression of Rheb leads to increased S6K phosphorylation [26]. It is possible that Rheb overexpression could mimic the GLUT1 effect of increased interaction between Rheb and mTOR by increasing the amount of Rheb available for binding. Assuming that the concentration of mTOR is rate-limiting and maximally occupied by Rheb in cells that overexpress GLUT1, increasing the amount of available Rheb in GLUT1 overexpressing cells should not further activate mTORC1. Another option was overexpression of GAPDH. Specifically, high concentrations of GAPDH could prevent the GLUT1 effect. Phosphorylation of raptor itself plays a major role in regulating mTORC1 signaling in response to changes in nutrient availability and growth factors [27]. Experiments aimed at exploring raptor and mTOR phosphorylation in cells that overexpress GLUT1 could provide evidence of another method of TSC2-independent regulation of mTORC1. Lastly, our laboratory has recently shown that GLUT1 overexpression leads to glucose dependent phosphorylation of Akt (Ser473) [28]. Akt phosphorylation of PRAS40 may play a feedforward role here, which would be worthwhile to explore in future studies.

mTORC1, GLUT1 and diabetic nephropathy: Although many reports have explored the roles of GSK-3 and TSC2 in regulating mTOR [1, 7], this is the first time that this pathway has been shown to lead to increased expression of GLUT1 and enhanced glucose uptake. In some cell types, mTOR has been reported to elicit negative feedback regulation on the PI3K pathway through the

ability of S6K to inhibit IRS-1 and thereby inhibit translocation of the insulin-responsive glucose transporter, GLUT4, to the cell surface [29]. However, in this study, we have demonstrated a feedforward regulatory relationship where mTORC1 activation enhances GLUT1 expression which serves to further increase mTORC1 activity. The initial stimulus for this pathway remains unknown. We can speculate that in the diabetic milieu, increased glycolytic flux may be responsible. In the HEK293 cells, we found a significant increase in S6K phosphorylation after exposure to increased extracellular glucose within 30 minutes that was lost by 6 hours (data not shown). These effects preceded any observed changes in GLUT1 expression. Also, it has been reported that cytoplasmic ATP levels can regulate movement of glucose through GLUT1 via direct interaction. Interaction of ATP with GLUT1 leads to conformational changes to restrict movement glucose flux [30]. This type of acute regulation may be aberrant in pathogenesis-prone cells such as glomerular mesangial cells.

Identifying these types of relationships could lead to improving our understanding of adverse cellular responses to stimuli, such as perturbation of metabolic activity in response to increased glucose availability, and may lead to new therapies aimed at slowing the pathogenesis of diabetic nephropathy. A key compound to examine is rapamycin. Rapamycin has been used clinically as an immunosuppressant for renal transplant patients for many years, yet its efficacy in the treatment of diabetic nephropathy remains to be studied [31]. Based on the successful results of multiple animal studies, mTOR inhibition may prove to

ameliorate the pathogenesis of diabetic nephropathy by eliminating hypertrophy and improving renal function.

Figure

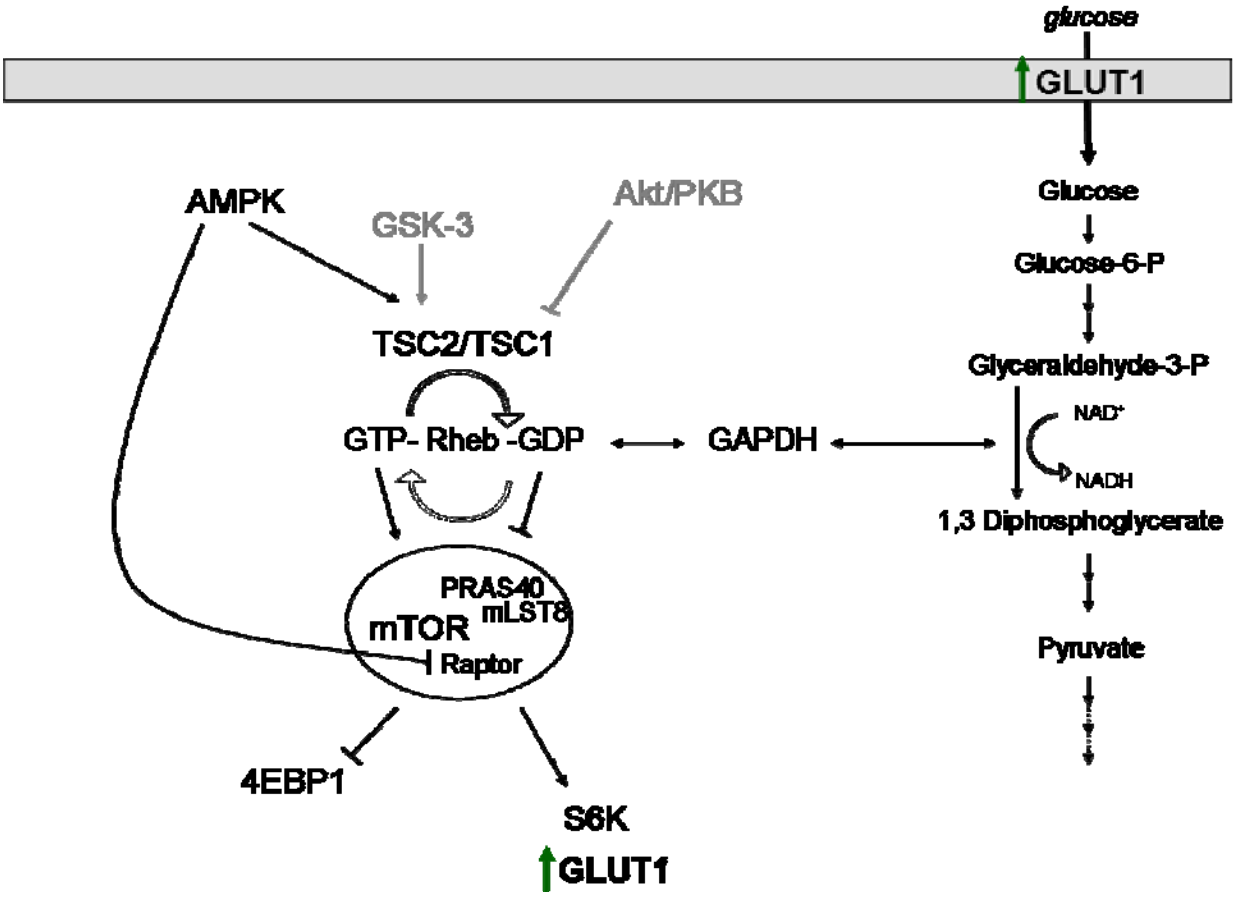

Figure 1. A feedforward mechanism of GLUT1 and mTORC1 regulation. A GSK-3/TSC/mTOR pathway regulates GLUT1 expression and glucose uptake. Increased GLUT1 expression leads to enhanced glucose metabolism and decreased interaction of GAPDH with Rheb. The subsequent increased interaction between mTOR and Rheb increases S6K phosphorylation.

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