Enhanced Glycogen Synthase Kinase-3β Activity Mediates Hypoxia-induced Apoptosis of Vascular Smooth Muscle Cells and is Prevented by Glucose Transport and Metabolism

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Running Title: GSK-3 mediates hypoxia-induced apoptosis
Hypoxia triggers apoptosis in a number of different cell types largely through a mitochondrial cell death pathway which can be largely abrogated by enhanced glucose metabolism. The purpose of the current study was to identify intracellular signaling mechanisms that mediate hypoxia-induced apoptosis and are regulated by glucose metabolism. Hypoxia-induced apoptosis in vascular smooth muscle cells and COS-7 cells was accompanied by a significant reduction in Akt and glycogen synthase kinase-3 (GSK-3) phosphorylation resulting in increased GSK-3 activity. Morphologic features of apoptosis, as well as caspase 3 and 9 activation, were prevented by GSK-3 inhibition with either LiCl or SB216763. Phosphorylation of Akt and GSK-3 was enhanced by glucose metabolism or overexpression of the glucose transporter, GLUT1, and was prevented by glycolytic inhibition. These findings indicate that GSK-3 is an important mediator of hypoxia-induced apoptosis and that GSK-3 mediated apoptotic effects occur via activation of the mitochondrial death pathway. Moreover, the results suggest that the prevention of hypoxia-mediated apoptosis by enhanced glucose transport and metabolism results, in part, from inhibition of GSK-3 activation.

Key Words: GSK-3, apoptosis, hypoxia, vascular smooth muscle
Introduction

Many cellular stresses are known to induce apoptosis. In most cells, it appears that this type of programmed cell death takes place via activation of the mitochondrial death pathway, the so-called intrinsic pathway (1). For example, UV irradiation, growth factor withdrawal, hypoxia, and reactive oxygen species trigger release of cytochrome c and other mitochondrial factors into the cytoplasm which then associate with and activate the complex of apaf1 and procaspase 9. Activated caspase 9 in turn cleaves and activates downstream caspases leading to apoptosis. We and others have shown that hypoxia as well as hypoxia followed by reoxygenation can trigger cytochrome c release and apoptosis (2, 3, 4). Although release of cytochrome c and other proapoptotic factors could be due to generalized disruption of mitochondrial function and mitochondrial depolarization during hypoxia, our studies suggest that cytochrome c release is specifically regulated and occurs before mitochondrial failure (4,5). However, the specific upstream signaling mechanisms by which hypoxia causes cytochrome c release remain unidentified.

Glycogen synthase kinase-3 (GSK-3) has been shown to participate in apoptosis in several cell types and is known to be an upstream regulator of programmed cell death (6). GSK-3 is a serine-threonine kinase ubiquitously expressed in most cell types. Two isoforms of GSK-3 have been identified and have a high degree of amino acid homology (7, 8). The two isoforms appear to function in a similar manner in most cell systems, however, the β isoform appears to be the predominant isoform in adult cells. GSK-3 has been demonstrated to induce caspase 3 activation and activate the proapoptotic tumor suppressor gene, p53 (9, 10). It also has been suggested that GSK-3 promotes activation and translocation of the proapoptotic Bcl-2 family member, Bax (11, 12) which, upon aggregation and mitochondrial localization, induces
cytochrome c release. Phosphorylation at serine 21/9 (α/β isoforms respectively) of GSK-3 inhibits its activity and reduces apoptosis (13). Akt is one of the critical regulators of GSK-3, and phosphorylation and inactivation of GSK-3 may mediate some of the antiapoptotic effects of Akt.

Enhanced glucose transport and metabolism are known to prevent hypoxia-induced apoptosis in a variety of cell types including cardiac myocytes and vascular smooth muscle cells through effects on the mitochondrial pathway. Glucose uptake and glycolysis as well as overexpression of the facilitative glucose transporter, GLUT1, have been shown to inhibit cytochrome c release and downstream caspase activation during hypoxia (2, 3). Similar studies have suggested that glucose metabolism is critical for prevention of apoptosis due to other causes, such as growth factor withdrawal. For example, Thompson’s laboratory has found that withdrawal of lymphocyte growth and survival factors led to cellular atrophy and apoptosis accompanied by reduction in GLUT1 expression, glucose uptake, ATP levels and mitochondrial potential (14). In this system, apoptosis could be accelerated by glucose depletion (14) and could be prevented via Akt stimulated increases in glucose uptake, GLUT1 expression and glucose metabolism (15). Finally, Hall et. al. demonstrated that GSK3β-induced apoptosis could be attenuated by GLUT1 overexpression in cultured vascular smooth muscle cells (16).

For the current studies, we hypothesized that hypoxia induces apoptosis through downregulation of the PI3K/Akt pathway releasing the inhibition of GSK-3 and promoting apoptosis via a mitochondrial pathway. We further hypothesized that glucose prevents apoptosis through mechanisms involving the inhibition of GSK-3 and promotion of Akt activity. The results presented here demonstrate that activation of GSK-3β mediates hypoxia-induced apoptosis and
that the protective effect of glucose attenuates the activation of GSK-3β and promotes phosphatidylinositol 3'-Kinase(PI3K)/Akt-mediated cell survival.
**Materials**

Polyclonal antibodies for phospho Akt\textsubscript{Ser473}, phospho GSK-3\textsubscript{α/β}\textsubscript{Ser9/21}, and total Akt were obtained from Cell Signaling Technology (Beverly, MA). The antibody for total GSK-3\textsubscript{α/β}, phosphoglycogen synthase peptide-2 substrate and caspase 9 activity substrate were from Upstate Biotechnology (Lake Placid, NY). The antibody for GLUT1 was a gift from Dr. Christin Carter-Su (University of Michigan). SB216763 was obtained from GlaxoSmithKline (Research Triangle Park, NC). LY294002 and caspase 3 activity substrate were obtained from Calbiochem (San Diego, CA). Oxyrase was purchased from Oxyrase, Inc (Ashland, OH). All other reagents were from Sigma (St. Louis, MO).

**Hypoxia**

A7r5 cells (ATCC #CRL-1444) or COS-7 cells were grown in 60 mm Falcon culture dishes (Fisher Scientific) to 80% confluence. Cells were washed twice with serum- and glucose-free media and placed in serum-free media with or without glucose (5 mM). Cells were subjected to hypoxia in a Plexiglas chamber superfused with 95% N\textsubscript{2}-5% CO\textsubscript{2} at 37°C as previously reported (2, 3). Oxyrase (6% v/v), a mixture of bacterial monoxygenases and dioxygenases, was added to the media of hypoxia samples to reduce the oxygen tension to <5 mmHg. Normoxic samples were placed in serum-free medium in a conventional incubator with 95% air/5% CO\textsubscript{2} at 37°C for equivalent time points. After hypoxia, cells were placed on ice and rinsed twice with ice cold PBS. Cell lysis buffer [Tris HCl pH7.5 (10 mM), NaCl (150 mM), Triton X-100 (1%), sodium dodecyl sulfate (0.1%), sodium orthovanadate (1 mM), sodium fluoride (50 mM), phenylmethanesulfonylfluoride (PMSF) (1 mM), leupeptin (10 µg/ml), aprotonin (10 µg/ml), pepstatin A (10 µg/ml), okadaic acid (1 µM)] was added to each dish and whole cell lysates were
collected by cell scraping. Lysates were incubated on ice for 15 min followed by sonication for 5 sec at 40% duty cycle (Sonicator Vibracell, Sonics and Materials, CT) and centrifuged at 14,000 rpm for 10 min at 4°C to clear cellular debris. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce).

**Immunoblotting**

Whole cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with TBS-Tween20 and 5% milk for 1 hour and placed in primary antibody in TBS-T 5% milk overnight at 4°C. After three 10 min 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 enhanced chemiluminescence reaction, and exposed to autoradiography.

**GSK-3 Activity Assay**

A7r5 cells were collected by cell scraping into an activity buffer comprised of HEPES pH 7.5 (50 mM), EGTA (1 mM), EDTA (1 mM) β-glycerophosphate (10 mM), sodium pyrophosphate (5 mM), potassium chloride (100 mM), Triton X-100 (0.5%), benzamidine (1 mM), PMSF (0.1 mM), aprotonin (10 µg/ml), vanadate (0.5 mM), and dithiothreitol (1 mM). Whole cell homogenates were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants were transferred to fresh microfuge tubes. Protein concentrations were determined by BCA protein assay (Pierce) and 10 µg samples were added to 5 µl of 5x reaction buffer [HEPES (250 mM), MgCl₂ (50 mM) and 2.5 mg/ml phosphoglycogen synthase peptide-2 substrate (Upstate
Biotechnology) and preincubated for 2 min at 30°C. The reaction was initiated by the addition of 250 μM ATP and 2 μCi/10 μl ³²P-γ-ATP (Amersham) and incubated at 30°C for 15 min. Addition of 200 mM EDTA + 5 mM ATP terminated the reaction and samples were transferred to Whatman P81 filter paper and washed 3x in 100 mM phosphoric acid followed by a final rinse in 95% ETOH. Filter paper was allowed to air dry and radioactivity was assessed by scintillation counting.

*Apoptosis quantitation*

Morphologic analysis of VSMC and COS-7 cell apoptosis was performed using propidium iodide staining. Apoptotic cells were identified based on nuclear condensation and/or fragmentation, as previously reported (3-5). Briefly, cells were grown in 60 mm culture dishes and subjected to hypoxia (6 hr) in the presence of specified chemical agents. After hypoxic treatment, cells were washed with PBS, permeabilized with 30% methanol in PBS (v/v), and stained with propidium iodide (100 nM) in PBS. Apoptotic and nonapoptotic cells were counted in multiple randomly selected fields and data were presented as percent apoptotic cells.

*Caspase 3 and 9 Activity Assays*

Caspase 3 and 9 activities were assessed *via* a colorimetric assay utilizing specific substrates. After treatment, the cells were washed once with ice cold PBS and collected by trypsinization followed by centrifugation. The cellular pellet was resuspended in cell lysis buffer and incubated on ice for 10 min. Lysates were centrifuged for 5 min at 13,000 rpm and the supernatants were transferred to microfuge tubes containing caspase activity assay buffer [HEPES pH 7.4 (50 mM), NaCl (100 mM), CHAPS (0.1%), DTT (10 mM), EDTA (0.1 mM),
glycerol (10%). DTT (10 mM) was added immediately prior to use of assay buffer. To each sample, caspase substrate (2 mM) was added and incubated for 60 min at 37°C. Samples were read at 405 nm in an EL-312e Bio-Kinetics microplate reader from Bio-Tek Instruments.

**Statistical Analysis**

Data were expressed as mean ± SEM unless otherwise indicated in the figure legends. Analysis of variance followed by Bonferroni’s post hoc analysis was used and differences were regarded as statistically significant at a P value <0.05.
RESULTS

**Hypoxia causes apoptosis in COS-7 cells** -

To confirm that hypoxia causes apoptosis in COS-7 cells, we subjected these cells to 6 h of hypoxia without reoxygenation. Typical morphological features of apoptosis were found in 13.0 ± 1.1% of hypoxic COS-7 cells as compared to 1.65 ± 0.2% of normoxic cells (p < 0.01). Moreover, hypoxia was found to induce caspase 3 and 9 activities in these cells. (see Fig. 3B and D respectively). These findings are similar to those previously reported in vascular smooth muscle cells and cardiac myocytes exposed to hypoxia (2, 3).

**Hypoxia decreases Akt and GSK-3β phosphorylation and increase in GSK-3 activity** -

To test the hypothesis that hypoxia-induced apoptosis is mediated in part via activation of GSK-3, A7r5 and COS-7 cells were subjected to 6 hr of hypoxia in serum- and glucose-free media. Hypoxia caused a significant decrease in phosphorylation of both Akt Ser473 (not shown) and GSK-3β Ser9 (normoxia 115 ± 13.8 arbitrary units, hypoxia 18.73 ± 4.9 arbitrary units). (Fig. 1A-B) In accordance with the known inhibition of GSK-3β by phosphorylation, hypoxia caused an increase in GSK-3 activity (normoxia 100%, hypoxia 153 ± 19.4%) as measured by an *in vitro* kinase activity assay. (Fig. 1C).

**GSK-3 Inhibition prevents hypoxia-induced apoptosis via a mitochondrial pathway** -

Preincubation with either of two specific inhibitors of GSK-3 reduced hypoxia-induced apoptosis in A7r5 VSMCs (Fig. 2 A-B) and in COS-7 cells (not shown) by over 50%. In addition, the selective GSK-3 inhibitor, SB216763, reduced caspase 3 and caspase 9 activities in hypoxic cells to levels similar to those of normoxic cells. (Fig. 3 A-B) The effect of GSK-3 inhibition on
caspase 9 activity suggests that GSK-3 mediates its proapoptotic effects during hypoxia via a mitochondrial death pathway.

Glucose metabolism promotes GSK-3β and Akt phosphorylation during hypoxia-

To determine whether glucose metabolism affects GSK-3 activity, we supplemented the media of A7r5 cells with 5 mM glucose during hypoxia. The presence of glucose completely prevented the hypoxia-associated reduction in GSK-3β Ser9 phosphorylation (Fig. 4A-B), consistent with prevention of the associated increase in GSK-3 activity. The presence of glucose also completely prevented the hypoxia-induced decline in AktSer473 phosphorylation. (Fig. 5A-B) Similar effects of glucose on GSK-3 and Akt phosphorylation were observed in COS-7 cells. (Fig.4 C-D and Fig. 5C-D). The effects of glucose on GSK-3 phosphorylation were very similar to those seen with insulin. In normoxic A7r5 cells, exposure to 100 nM insulin for 15 minutes induced a 3.5 ± 0.13-fold increase in GSK-3 phosphorylation (data not shown).

PI3-K inhibition attenuates the effects of glucose on Akt and GSK-3β phosphorylation-

Given the parallel changes in GSK-3β and Akt phosphorylation in hypoxia, it appears likely that hypoxia and glucose affect signaling to Akt and GSK-3β via a PI3K-dependent pathway. Therefore, VSMCs were preincubated with LY294002, a PI3K inhibitor and GSK-3β and Akt phosphorylation during hypoxia in the presence and absence of glucose were determined. Preincubation with LY294002 (2 μM) reversed the glucose effects on GSK-3 phosphorylation. (Fig 6A-B), confirming that glucose restores PI3K/Akt signaling during hypoxia.
Glycolytic inhibition prevents the glucose-mediated preservation of Akt and GSK-3β phosphorylation during hypoxia -

We have previously demonstrated that glucose mediated prevention of apoptosis is dependent on glycolytic metabolism. To determine if the effects on GSK-3β and Akt were similarly dependent on glycolysis, A7r5 VSMCs were exposed to either of the glycolytic inhibitors, 2-deoxyglucose (2-DOG) or 5-thio-glucose (5-TG) in the presence of 5 mM glucose during hypoxia. Glycolytic inhibition prevented the glucose-mediated effects on GSK-3β and Akt phosphorylation. (Fig. 7) In contrast to glucose, none of the alternate energy substrates, pyruvate (5 mM), lactate (4 mM), or propionate (4 mM) prevented hypoxia-induced reduction in GSK-3β phosphorylation. (Fig. 8)

GLUT1 overexpression maintains GSK-3β and Akt phosphorylation during hypoxia-

Glucose transporter overexpression prevents apoptosis in a number of different settings by enhancing glucose metabolic flux (3, 15, 16). Therefore, the effects of GLUT1 overexpression on hypoxia-induced GSK-3β and Akt phosphorylation was assessed in A7r5 VSMCs. GLUT1-overexpressing cells had enhanced GSK-3β and Akt phosphorylation during normoxia, when compared to control cells in the presence of 5 mM glucose. In addition, GLUT1 overexpression completely prevented the hypoxia-induced reduction in GSK-3β and Akt phosphorylation. (Fig. 9A-C)
Discussion

GSK-3 is a ubiquitously expressed enzyme that participates in the regulation of many fundamental cellular processes including Wnt signaling during development, carbohydrate metabolism throughout the life of the cell, and apoptosis (7). Only over the past few years has the role of GSK-3 in apoptosis been appreciated. Several recent reports have suggested that increased GSK-3 activity promotes apoptosis via activation of the mitochondrial cell death pathway (11, 17). Our studies have extended and clarified the role and regulation of GSK-3 in hypoxia-induced apoptosis. We have shown that inhibition of GSK-3 with either LiCl or SB216763 markedly attenuates hypoxia-induced apoptosis. This inhibition of GSK-3 during hypoxia prevents caspase 9 activation indicating that GSK-3 exerts its effects at the mitochondrion, or more proximally. In addition, we have shown that glucose-mediated prevention of apoptosis during hypoxia appears to be due, at least in part, to the inhibition of GSK-3 by Akt, the activity of which is enhanced by glucose metabolism. Finally, we have demonstrated that overexpression of the facilitative glucose transporter, GLUT1, enhances these effects of glucose uptake and metabolism, confirming the critical role of plasma membrane expression of these transporters in preventing hypoxia-induced apoptosis.

We have found that GSK-3 mediates apoptosis in both vascular smooth muscle cells and COS-7 cells during hypoxia. Other investigators have demonstrated that GSK-3 is critical for progression of apoptosis in a broad range of cell types due to a variety of cellular stresses including growth factor withdrawal, PI3 kinase inhibition, inhibitors of mitochondrial electron transport, heat shock and staurosporine (6, 10, 11, 17, 18, 19, 20). Thus, GSK-3 appears to mediate or potentiate apoptosis due to many stimuli that activate the mitochondrial cell death
pathway. In contrast, apoptosis triggered by death receptors, the so-called extrinsic pathway, is not promoted by GSK-3 and, indeed, may be inhibited by GSK-3 activation (21, 22).

We have previously determined that glucose uptake and metabolism and GLUT1 overexpression result in attenuation of hypoxia-induced apoptosis (2, 3). Although glucose metabolism clearly inhibits the mitochondrial cell death pathway, the mechanism by which glucose metabolism prevents activation of this pathway during hypoxia has not previously been determined. Recently, Hall et al. confirmed that GLUT1 overexpression attenuates vascular smooth muscle cell apoptosis and results in reduced GSK-3 phosphorylation. They also found that increased apoptosis due to overexpression of GSK-3 was markedly reduced in GLUT1 overexpressing cells (16). We have confirmed and extended these findings in hypoxia-induced apoptosis. We have found that GSK-3 is activated during hypoxia and that its inhibition prevents caspase 9 activation and activation of the mitochondrial apoptotic pathway. We have also shown that glucose metabolism, but not that of other substrates, prevents hypoxia-induced apoptosis and that most, but not all, of the glucose protective effect during hypoxia appears to be mediated by its inhibition of GSK-3. In addition we have shown that this effect of glucose metabolism or GLUT1 overexpression during hypoxia is via stimulation of the PI3K/Akt pathway, as inhibition of PI3K dramatically reduces the GSK-3 phosphorylation that is found in cells with enhanced glucose metabolism. Finally, we have found that GLUT1 overexpression enhances PI3K/Akt signaling and maintains GSK-3 in its inactive, phosphorylated state in both normoxic and hypoxic conditions. Although not yet fully assessed, it seems likely that insulin will have very similar effects to glucose in terms of its effects on GSK-3 activity and perhaps on apoptosis during hypoxia. We have shown in our system, as others have shown in other systems, that insulin induces GSK-3 phosphorylation and inactivation. However, we have not yet tested the
effects of insulin during hypoxia. In any case, however, it is certain that glucose effects on both GSK-3 phosphorylation and apoptosis can be independent of insulin, since all of our experiments were performed in serum-free conditions and in the absence of insulin.

The antiapoptotic effects of glucose metabolism and GLUT1 expression occur in a wide variety of cells and conditions, besides those already cited. For example, Moley et al. (23) have found that elevated extracellular glucose levels led to decreased GLUT1 expression which resulted in diminished glucose uptake and intraembryonic free glucose levels despite extracellular elevation of glucose levels. These embryos underwent apoptosis at a 4-fold greater rate and showed increased Bax expression and apoptosis (23). More recently, Moley’s group has found that antisense-GLUT1 expression in murine blastocysts augmented apoptosis in murine blastocysts incubated in normal glucose concentrations. The investigators also found that apoptosis was blocked in Bax -/- mice versus the Bax +/+ and +/- mice. These findings suggest that hyperglycemia, by decreasing glucose transport, acts as a cell death signal to trigger a mitochondrial apoptotic pathway in murine blastocysts (24). Finally, Thompson’s group has found that withdrawal of lymphocyte growth and survival factors led to cellular atrophy and apoptosis accompanied by reduction in GLUT1 expression, glucose uptake, ATP levels and mitochondrial potential (14). These investigators also showed that apoptosis could be accelerated by glucose depletion (14) and could be prevented via Akt stimulated increases in glucose uptake, GLUT1 expression and glucose metabolism (15).

These latter studies and our own work suggest an important positive feedback mechanism connecting PI3K/Akt signaling with GLUT1 expression and glucose uptake. Akt enhances glucose uptake and GLUT1 expression, but conversely, increased GLUT1 expression and the resultant glucose uptake and metabolism enhance PI3K/Akt signaling, maintaining GSK-3
phosphorylation and keeping GSK-3 inactive. This self-reinforcing, antiapoptotic system maintains cellular integrity and metabolism. However, in cases of severe cellular stress, or withdrawal of critical growth factors, PI3K/Akt signaling is inhibited leading to a reduction in glucose uptake and GLUT1 expression, both of which can rapidly lead to programmed cell death. The mechanisms of the inhibition of the PI3K/Akt signaling pathway during hypoxia and its restoration with glucose are unknown. It is possible that this represents a generic effect of ATP depletion during hypoxia in the absence of glucose and normalization of ATP levels with glucose, thus providing substrate for phosphorylation. However, since GSK-3 inhibition protects cells against apoptosis during hypoxia, it is likely that GSK-3, at least, has residual kinase activity despite hypoxia and the absence of glucose. Moreover, the fact that GLUT1 overexpression induces activation of the PI3K/Akt pathway during normoxia suggests that increased glucose flux directly stimulates PI3K/Akt activity even in the presence of normal ATP levels. It is possible that glucose uptake and metabolism specifically induce activation of the insulin receptor, insulin receptor substrate 1 or PI3K. However, there are no published data to support such an effect. Mechanistic understanding of the interactions of glucose transport and metabolism with cellular signaling pathways should help further elucidate the regulatory role of glucose metabolism in apoptosis as well as during normal cell function.
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Figure 1: Hypoxia increases GSK-3 activity. A7r5 cells were subjected to 6 hrs of hypoxia in the absence of serum and glucose and assessed for GSK-3 activity. A) Total cell lysates (40 µg/lane) were analyzed via immunoblot analysis for GSK-3β Ser9 phosphorylation, B) Quantification of GSK-3β phosphorylation was performed with NIH ImageJ software and data were expressed in densitometric units (± S.E.M.), and C) Total cell lysates were subjected to an *in vitro* GSK-3 activity assay using pGS2, a pseudosubstrate for GSK-3, expressed as percent of normoxic controls. * P<0.05 vs. control samples.

Figure 2: GSK-3 inhibition prevents hypoxia-induced apoptosis. A) Histochemical analysis of PI-stained A7r5 cells. Arrows indicate condensed/fragmented nuclei. Magnification = 400x, B) Percentages of cells with apoptotic nuclear changes in control and hypoxic cells +/- GSK-3 inhibitors LiCl or SB216763. * P<0.01 vs. normoxia control cells. # P<0.01 vs. hypoxia cells without inhibitor.

Figure 3: GSK-3 inhibition reduces caspase 3 and 9 activities. A7r5 (A and B) and COS-7 (C and D) cells were subjected to hypoxia in the presence or absence of SB216763, a GSK-3 inhibitor (1 µM and 10 µM). Total cell lysates were harvested by trypsinization and incubated for 60 min at 37°C in the presence of DEVD-pNA (caspase 3 substrate) or LEHD-pNA (caspase 9 substrate). * P<0.01 vs. normoxia control cells. # P<0.05 vs. hypoxia cells without inhibitor.

Figure 4: The presence of glucose increases GSK phosphorylation in hypoxic A7r5 cells (A, B) and COS-7 cells (C,D). A and C) Phospho-GSK-3β immunoblots. B and D) Quantitation of
phospho-GSK-3β levels. The number of individual samples tested is expressed in parentheses above each bar. * P<0.01 vs. other groups.

Figure 5: The presence of glucose increases Akt phosphorylation in hypoxic A7r5 cells (A, B) and COS-7 cells (C, D). A and C) Immunoblots of AktSer473 phosphorylation. B and D) Quantitation of phospho-AktSer473 levels. The number of individual samples tested is expressed in parentheses above each bar. * P<0.05 vs. other groups.

Figure 6: PI3K inhibition prevents glucose-enhanced GSK-3 phosphorylation in hypoxic A7r5 cells. Cells were preincubated in the presence of absence of LY294002 (2 µM), a PI3K inhibitor, for 30 min prior to hypoxic challenge. A) Phospho-GSK-3 immunoblot. B) Quantitation of phospho GSK-3β levels. The number of individual samples tested is expressed in parentheses above each bar. * P<0.01 vs. normoxia + glucose samples; # P<0.05 vs. hypoxia + glucose samples.

Figure 7: Glucose enhanced GSK3 phosphorylation is dependent on glycolysis. Phospho-GSK-3 and total GSK-3 immunoblots of A7r5 cells subjected to hypoxia in the presence or absence of glucose (5 mM) or 5 mM glucose + either of the glycolytic inhibitors 2-deoxy-glucose (2-DOG; 5 mM) or 5-thio-glucose (5-TG; 5 mM).

Figure 8: Alternative energy substrates do not affect GSK-3 phosphorylation in hypoxia. Phospho-GSK-3 immunoblot of A7r5 cells in the presence of either glucose (5 mM), pyruvate (5 mM), lactate (4 mM) or propionate (4 mM).
Figure 9: **Overexpression of GLUT1 in A7r5 VSMCs increases GSK-3 and Akt phosphorylation in hypoxia.** A) Phospho-GSK-3 and phospho-Akt immunoblots. B) Quantitation of B) phospho-GSK-3β and C) Akt levels. The number of individual samples tested is expressed in parentheses above each bar. * P<0.01 vs. normoxia + glucose samples; # P<0.05 vs. hypoxia + glucose samples.
Figure 1

A. Hypoxia - - - + + +

phospho GSK-3β

GSK-3β

B. Hypoxia - +

phospho GSK-3β (Arbitrary Units)

(4)

(4)

C. CPM (% control)

Control Hypoxia

(3) (8)
Figure 2

A. normoxia

B. hypoxia

C. hypoxia + LiCl

Hypoxia - + + +
LiCl (20mM) - - + -
SB216763 (10μM) - - - +
Figure 3
**Figure 4**

A. Hypoxia
Glucose (5 mM)
phospho GSK3β

B. Hypoxia
Glucose (5 mM)
phospho GSK3β

C. Hypoxia
Glucose (5 mM)
phospho GSK3β

D. Hypoxia
Glucose (5 mM)
phospho GSK3β

Figure 4
Figure 5

A.

Hypoxia
Glucose (5 mM)
phospho Akt<sub>Ser473</sub>

B.

|            | phospho Akt<sub>Ser473</sub> (Arbitrary Units) |
|------------|-----------------------------------------------|
| Hypoxia    |                                               |
| Glucose    |                                               |
|            | 0                                             |
| +          | +                                             |
| +          | 0                                             |
| +          | +                                             |

C.

Hypoxia
Glucose (5 mM)
phospho Akt<sub>Ser473</sub>

D.

|            | phospho Akt<sub>Ser473</sub> (Arbitrary Units) |
|------------|-----------------------------------------------|
| Hypoxia    |                                               |
| Glucose    |                                               |
|            | 0                                             |
| +          | +                                             |
| +          | 0                                             |
| +          | +                                             |

* Significant differences (p < 0.05)
A.

|                | Hypoxia | Glucose | LY |
|----------------|---------|---------|----|
|                |         |         |    |
| phosho GSK-3β  |         |         |    |

B.

```
| Hypoxia | Glucose (5 mM) | phosho GSK-3β (Arbitrary Units) |
|---------|----------------|----------------------------------|
|         |                | (8)                              |
|         | +              | (4)                              |
|         | +              | (8)                              |
|         | +              | *                                 |
|         | +              | #                                 |
|         | +              | *                                 |
|         | +              | *                                 |
|         | +              | #                                 |
|         | +              | *                                 |
|         | +              | *                                 |
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Figure 6
Figure 7

| Hypoxia | Glucose | 2DOG | 5TG |
|---------|---------|------|------|
| -       | -       | +    | +    |
| +       | +       | +    | +    |

Phospho GSK-3α/β

Total GSK-3α/β
Figure 9

A. 

|        | Vector Control | GLUT1 |
|--------|----------------|-------|
| Hypoxia| -              | -     |
|        | +              | +     |
|        | -              | +     |

phospho Akt_{Ser473}  
phospho GSK3β

B. 

![Bar graph showing phospho GSK-3β levels under hypoxic conditions with Vector Control and GLUT1 expression.](image)

C. 

![Bar graph showing Akt_{Ser473} levels under hypoxic conditions with Vector Control and GLUT1 expression.](image)
Enhanced glycogen synthase kinase-3β activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism

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