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Glucose Uptake and Adenoviral Mediated GLUT1 Infection Decrease Hypoxia-induced HIF-1α Levels in Cardiac Myocytes

Ricky Malhotra[1*], David G. W. Tyson[1], Hirohito Sone[1], Kasunori Aoki[1], Arno K. Kumagai[1] and Frank C. Brosius III[1,2]

Departments of [1]Internal Medicine and [2]Physiology, University of Michigan Medical Center, Ann Arbor, Michigan

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R. Malhotra, D. G. W. Tyson, H. Sone, K. Aoki, A. K. Kumagai and F. C. Brosius III. Glucose Uptake and Adenoviral Mediated GLUT1 Infection Decrease Hypoxia-induced HIF-1α Levels in Cardiac Myocytes. *Journal of Molecular and Cellular Cardiology* (2002) **34**, 1063–1073. Hypoxia causes a large array of adaptive and physiological responses in all cells including cardiac myocytes. In order to elucidate the molecular effects of increased glucose flux on hypoxic cardiac myocytes we focused on the basic helix-loop-helix transcription factor, hypoxia inducible factor 1 alpha (HIF-1α), which is rapidly upregulated in hypoxic cells and elicits a number of responses including augmentation of glucose uptake. Primary cultures of neonatal rat cardiac myocytes as well as embryonic rat heart-derived myogenic H9c2 cells demonstrated a significant upregulation of HIF-1α when subjected to hypoxia of 6–8 h in the absence of glucose. Re-addition of extracellular glucose to the medium resulted in a decrease of HIF-1α levels by almost 50%. This glucose effect was blocked by addition of glycolytic inhibitors. In addition, glucose uptake and glycolysis resulted in substantial decreased levels of p53, which is regulated by HIF-1α. Adenoviral infection of cultures of cardiac myocytes with the facilitative glucose transporter, GLUT1 followed by hypoxia of 24 h also resulted in a significant reduction in the protein expression of HIF-1α compared to control vector-infected cultures. GLUT1 infected cultures also demonstrated fewer apoptotic cells and a reduction in the release of cytochrome c after hypoxia. Inhibition of the ubiquitin-proteasomal pathway by a variety of 26S proteasomal inhibitors increased HIF-1α to similar levels under both normoxic and hypoxic conditions and in the presence or absence of glucose. This result suggested that glucose induces HIF-1α degradation via a proteasomal pathway. This conclusion was substantiated by immunoprecipitation experiments of total cell extracts, which demonstrated an increase of ubiquitinated HIF-1α relative to total HIF-1α in the presence of glucose during hypoxia. Thus, glucose as well as GLUT1 overexpression diminishes hypoxia-induced HIF-1α protein via an ubiquitin-proteasomal pathway in hypoxic cardiac myocytes. This represents a novel feedback mechanism that may play an important role in adaptation of cardiac myocytes to hypoxia and ischemia.

Key Words: Glucose; Adenovirus; GLUT1; Cardiac myocytes; Apoptosis; HIF-1α.

*Please address all correspondence to: Ricky Malhotra, PhD, Department of Internal Medicine/Nephrology, University of Michigan Medical Center, 1150 W. Med. Ctr. Dr., 1560 MSRB II, Ann Arbor, MI-48109-0676, USA. E-mail: rmalhotr@umich.edu
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Introduction

Oxygen homeostasis represents a fundamental physiological process that requires the coordinated regulation of an extensive array of genes. Deprivation of oxygen (hypoxia) is a serious extracellular and intracellular stress that compromises cellular survival. In most cells a transcription complex, hypoxia inducible factor-1 (HIF-1), controls gene expression in response to reduction in oxygen tension.[1–3] HIF-1 plays a key role in cellular responses to hypoxia, including the regulation of genes involved in energy metabolism, angiogenesis and apoptosis.[4–6] HIF-1 is an $\alpha\beta$ heterodimer, both subunits of which belong to the larger family of basic helix-loop-helix (bHLH) transcription factors.[7] While the β subunit is constitutively expressed and is identical to the aryl hydrocarbon nuclear translocator, the α subunit's sole but critical function is mediation of the response to hypoxia.[2,7] It is now well documented that HIF-1 helps to restore homeostasis by inducing glycolysis, erythropoiesis, and angiogenesis[4,5] thereby promoting restoration of cellular metabolism and blood flow. In certain circumstances, however, HIF-1α can accelerate cell death by apoptosis,[8–10] perhaps when the cellular response is inadequate to meet metabolic needs. It appears that HIF-1α stimulates mitochondrial apoptotic events, since it is the mitochondrial pathway that leads to cell death in response to a variety of cellular stresses including hypoxia.[11–14]

Glucose uptake and metabolism have been shown to protect cells from hypoxic or ischemic injury. We have reported that glucose uptake and metabolism prevent apoptosis in hypoxic cardiac myocytes primarily by inhibiting the mitochondrial cell death pathway and that glucose transporter overexpression also prevents apoptosis in vascular smooth muscle cells in part by inhibiting JNK activation.[15,16] However, the precise mechanisms by which glucose interferes with apoptosis remain unclear. Recent work has shown that growth factors, known anti-apoptotic factors, control cell survival primarily by maintaining sustained glucose metabolism.[17] Also, the serine/threonine kinase Akt, which is activated by growth factors as well as by hypoxia,[18,19] prevents apoptosis by blocking the mitochondrial pathway.[20] Importantly Akt-mediated survival has been shown to be dependent on promoting glycolysis and maintaining a physiologic mitochondrial potential.[21] Inactivation of Akt also increases HIF-1α expression.[22]

How glucose and its metabolism and HIF-1 interact in regulating responses of cardiac myocytes during hypoxia remains unclear. In the present report we demonstrate in cardiac myocytes that glucose reduces hypoxia-induced HIF-1α levels in cardiac myocytes. We further demonstrate that adenoviral mediated over-expression of the facilitative glucose transporter: GLUT1 in cardiac myocytes reduces hypoxia-induced HIF-1α and p53 levels. This glucose metabolic effect appears to be mediated by increased ubiquitination and enhanced proteasomal degradation of HIF-1α. GLUT1 over-expressing cells also have reduced cytochrome c release and apoptosis in response to hypoxia. The decrease in HIF-1α levels may play a part in glucose-mediated protection of cardiac myocytes from hypoxia-induced apoptosis.

Materials and Methods

Materials

The proteasomal inhibitors MG 262 [Z-Leu-Leu-Leu-B $(OH)_2$] and Clasto-Lactacystin β-Lactone were obtained from BIOMOL Research (Plymouth Meeting, PA, USA) and Epoxomicin was obtained from Affiniti Research (Eexter, UK). The cell culture reagents were obtained from Life Technologies (Gaithersburg, MD, USA) while all other reagents were obtained from Sigma Chemical Company (St Louis, MO, USA).

Myocyte cell culture

Primary cultures of neonatal rat cardiac myocytes were prepared as described[15] with minor changes. Briefly, cardiac myocytes were obtained from ventricular tissue of 1-day old Wistar rats by six to seven 15-min digestions at 37°C in HEPES-buffered solution containing 0.1% collagenase IV, 0.1% trypsin, 15 μg/ml DNase I, and 0.1% chicken serum. The dissociated cells were collected by centrifugation and resuspended in ADS buffer (in g/liter: 6.8 NaCl, 4.76 HEPES, 0.138 NaH_2PO_4, 0.6 glucose, 0.4 KCL, 0.205 $MgSO_4$, 0.0002 phenol red, pH 7.4). The cells were then selectively enriched by differential centrifugation through a discontinuous Percoll (Amersham Pharmacia Biotech.) gradient of densities 1.050, 1.062, and 1.082 g/ml. The enriched cardiac myocytes were washed and suspended in Dulbecco's modified Eagle's medium (DMEM/F12) medium (Life Technologies, Inc.) (1:1, v/v) supplemented with 5% horse serum, 3 mM pyruvic acid, 100 μM ascorbic acid, 1 μg/ml transferrin, 10 ng/ml selenium and 100 μg/ml ampicillin. For most experiments, cardiac myocytes were

plated on precoated 60 mm dishes with gelatin at a density of 2.5×10^6 cells/dish. For adenoviral infection the cells were plated on 30 mm dishes at a density of 2×10^4 cells/dish.

Bromodeoxyuridine at a final concentration of 0.1 mM was added during the first 36 h to prevent proliferation of cardiac fibroblasts.

Hypoxia treatment protocol

For hypoxia experiments, previously published protocols were followed.[15,16] Briefly, after the appropriate treatments, the cells were placed in a Plexiglas chamber, and a constant stream of water-saturated 95% N_2 and 5% CO_2 was maintained over the culture. To lower the PO_2 to <5 mmHg, Oxyrase, (Oxyrase Inc., Ashland, OH, USA) a mixture of bacterial membrane monooxygenases and dioxygenases was added to the culture medium at a final concentration of 6%.

Nuclear extract preparation

After hypoxia treatment the cells were quickly rinsed twice in ice-cold PBS and pelleted. Nuclear extracts were prepared as previously described.[7] Cell pellets were resuspended in 5 packed cell volumes of buffer A (10 mM Tris-HCl [pH 7.6], 1.5 mM $MgCl_2$, 10 mM KCl) supplemented with 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_3VO_4. After incubation on ice for 10 min, cells were pelleted at $2500 \times g$ for 5 min, resuspended in 2 packed cell volumes of buffer A, and lysed by 20 strokes in a glass Dounce homogenizer with type B pestle. Nuclei were pelleted at $10\,000 \times g$ for 10 min and resuspended in 3.5 packed nuclear volumes of buffer C (0.42 M KCl, 20 mM Tris-HCl [pH 7.6], 20% glycerol, 1.5 mM $MgCl_2$) supplemented with 2 mM DTT, 0.4 mM PMSF, and 1 mM Na_3VO_4. Nuclear proteins were extracted by stirring at 4°C for 30 min. After centrifugation at $15\,000 \times g$ for 30 min, the supernatant was dialyzed against buffer Z-100 (25 mM Tris-HCl [pH 7.6], 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 0.4 mM PMSF and 1 mM Na_3VO_4 and 100 mM KCl) at 4°C. The dialysate was clarified by ultracentrifugation at $100\,000 \times g$ for 60 min at 4°C and designated as crude nuclear extract. The nuclear extracts were aliquoted, frozen in liquid N_2, and stored at −80°C. Protein concentration was determined by the method of Bradford with a commercial kit (Pierce) using bovine serum albumin as a standard.

Generation of recombinant adenoviral vectors

Adenoviruses expressing the human facilitative glucose transporter, GLUT1 (AdGLUT1) or the empty vector (AdRSV) were generated by Cre-lox recombination *in vitro* according to published procedures.[23] AdGLUT1 was produced by homologous recombination of a sub360 genomic DNA Ad5 derivative with deletion of the E3 region and pAdGT1, a GLUT1 expression plasmid containing the full length cDNA for human GLUT1 under the control of an RSV promoter/enhancer. Recombination was accomplished in a cell-free reaction mixture consisting of only the viral and shuttle plasmid DNAs and Cre recombinase. Viral particles were produced in 293 cells by transfection. After 7–9 days when the cells showed a cytopathic affect the cells were harvested by four cycles of freezing in ethanol/dry ice and rapid thawing at 37°C. The viral lysate was spun and the adenovirus-containing supernatant was collected, purified by centrifugation through cesium chloride gradient and sterilized by passage through 45 μM filters.

Adenoviral infection of primary cultures of cardiac myocytes

Cultures of neonatal rat cardiac myocytes growing in 30 mm dishes were used for adenoviral infections. The cultures were washed twice with serum free DMEM/F-12 medium. Infection of the AdGLUT1 or AdRSV virus at a multiplicity of infection (moi) of 10–1000 was carried out in a final volume of 400 μl/dish. The cultures were transferred back into the CO_2 incubator with rocking of the culture dish every 15 min for 2 h to ensure uniform spread of the virus particles on the cells. At the end of 2 h the virus containing solution was removed and replaced with 2 ml of regular DMEM/F12 medium with 5% horse serum. Protein expression was checked 24 h and 48 h later in total cell lysates using a polyclonal GLUT1 antibody. Since increased GLUT1 protein expression persisted in the cells for at least 48 h after infection (see results section), all hypoxia experiments were finished within 48 h of infection.

Immunoprecipitation and immunoblotting of HIF-1α

For immunoprecipitation, 400 μg of total cell lysates or nuclear extracts from cells exposed to hypoxia for 8–24 h were brought to a total volume of 1 ml with immunoprecipitation (IP) buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCL, 2 mM EDTA, 20% glycerol,

5 mM DTT, and 0.2% Nonidet P-40). Ten μl of preimmune serum were added and incubated for 2 h at 4°C, followed by the addition of 200 μl of a 50% suspension of protein A-Sepharose 4B (Pharmacia) in IP buffer for 1 h at 4°C. The supernatant was collected after centrifugation at $5000 \times g$ for 5 min at 4°C. A 1:1000 dilution of affinity-purified HIF-1α polyclonal antibodies was added to the supernatant which was incubated for 2 h at 4°C, followed by incubation with 100 μl of a 50% suspension of protein A-Sepharose 4B as described above. Pellets were collected by centrifugation for 5 min at $5000 \times g$ at 4°C, washed five times with 900 μl of IP buffer, resuspended in 160 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% [w/v] SDS, 2% mercaptoethanol, and 10 μg/ml bromophenol blue), heated at 100°C for 5 min and fractionated by electrophoresis through a SDS 7%-polyacrylamide gel. Immunoblot assays were performed using a 1:200 dilution of affinity-purified HIF-1αantibodies (Novus Biologicals, Littleton, CO, USA) or a 1:1000 dilution of monoclonal anti-ubiquitin antibodies (Santa Cruz Biotechnology).

Cytochrome c release

Mitochondrial and cytosolic fractions were prepared using a modification of our previously published protocol.[15] Cells (3×10^7) were washed with PBS, suspended in 10 mM HEPES [pH 7.4], 1 mM EDTA, 250 mM sucrose, and homogenized with a type B Dounce (glass/glass) tissue grinder. The homogenates were centrifuged at $10\,000 \times g$ for 30 min to obtain a pellet highly enriched in mitochondria. This pellet was resuspended in the same buffer and reserved while the supernatant was further spun at $160\,000 \times g$ for 1 h. The supernatant from this final ultracentrifugation represented the cytosolic fraction. The resulting protein samples were electrophoresed on 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed as previously reported[15] using the cytochrome c antibody (Pharmingen, San Diego, CA, USA) at a final concentration of 1 μg/ml in 5% non-fat dry milk. To ensure that cytochrome c release was not due to physical disruption of mitochondria during isolation, both the mitochondrial and cytosolic fractions were probed with monoclonal antibodies to cytochrome oxidase (subunit IV) (mAb 20E8-C12, Molecular Probes, Eugene, OR, USA) at a dilution of 0.1 μg/ml. Signals were detected by enhanced chemiluminescence (ECL, Amersham).

Morphological analysis

For morphological studies the cardiac myocytes were grown in eight-well gelatin-coated Falcon glass culture slides (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The cells were rinsed in PBS, pH 7.4, and fixed for 30 min in 4% paraformaldehyde in PBS at room temperature. After a rinse in PBS, the cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. The cells were rinsed in PBS and then stained with the karyophilic dye Hoechst 33258 (5 μg/ml) for 10 min at room temperature. After a final rinse in PBS the cells were mounted in moiwol, an antifade agent, and visualized under ultraviolet light with a Leitz Orthoplan microscope. The percentage of cells displaying chromatin condensation and nuclear fragmentation were counted in five different fields by a blinded observer.

Densitometric analysis

Quantitation of HIF-1α and p53 protein levels in various experiments was performed either by phosphorimager analysis or densitometric analysis and NIH Image software.

Statistical analysis

Quantitative data are represented as means $\pm$ SEs. The data were subjected to ANOVA using Scheffe's method for post-hoc analysis. Significance was accepted at $P < 0.05$.

Results

Glucose reduces the protein expression of HIF-1α in cardiac myocytes and H9c2c cells

In order to investigate the interaction of glucose with HIF-1α, we cultured Percoll-enriched neonatal rat cardiac myocytes as well as rat embryonic heart-derived H9c2 cells and subjected them to 8 h of hypoxia as described in the Methods Section. At the end of the hypoxia incubation the cells were immediately harvested and nuclear extracts prepared to avoid degradation of HIF-1α upon exposure to normal oxygen tension. Under normoxic conditions there were no detectable levels of HIF-1α protein. However, 8 h of hypoxia dramatically increased the protein expression of HIF-1α, in cells cultured in glucose-deficient medium. The presence of 17.5 mM glucose in the extracellular medium (the standard

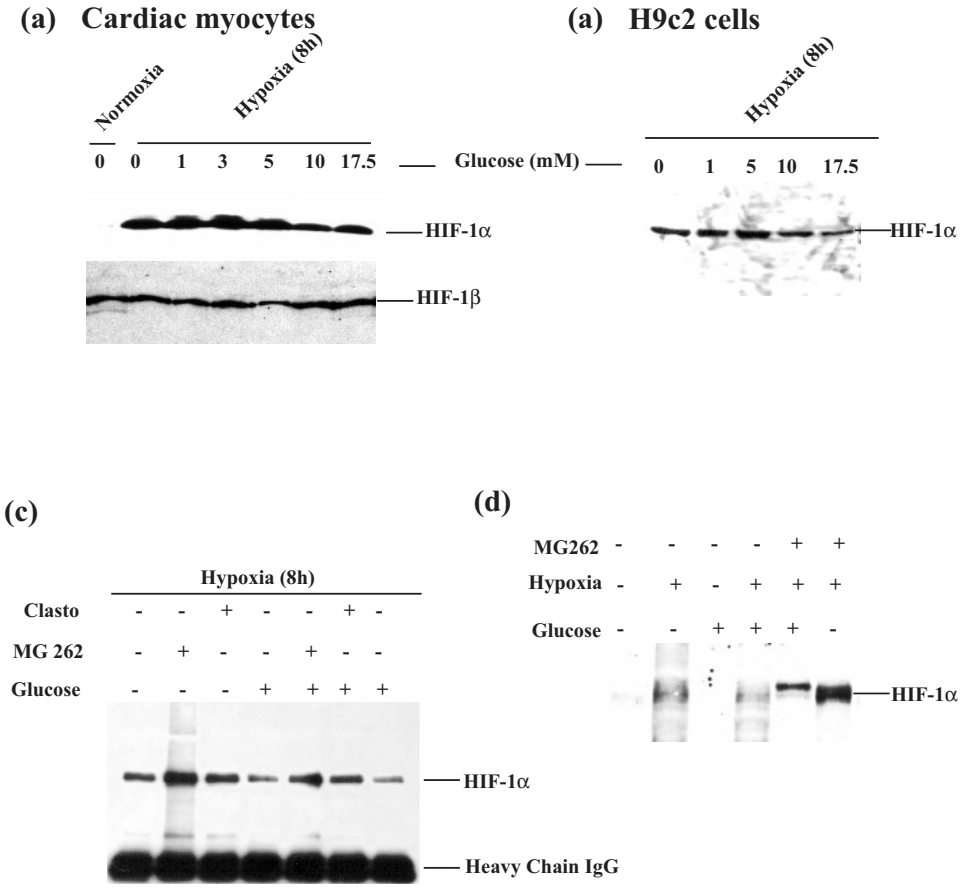

Figure 1 HIF-1α levels in cardiac myocytes and embryonic rat heart derived H9c2 cells. (a) Cardiac myocytes were cultured and exposed to 8 h of hypoxia either in the absence of glucose or in various concentrations of glucose. After the end of hypoxic incubation the cells were immediately harvested and nuclear extracts prepared as described in methods. Equal amounts (50 μg) were run on a 6% SDS-PAGE gel and immunoblotted with HIF-1α and HIF-1β specific antibodies. (b) Nuclear extracts from H9c2 cells were also immunoblotted with HIF-1α antibody. (c) Total cell lysates were prepared from cardiac myocytes exposed to 8 h of hypoxia. Equal amounts (500 μg) of the lysates were immunoprecipitated with HIF-1α antibody and the immune complexes after extensive washing were fractionated on a 6% SDS-PAGE gel and blotted with HIF-1α antibody. (d) Cardiac myocytes were exposed to 8 h of hypoxia in the presence and absence of glucose. Equal amounts (500 μg) of nuclear proteins were immunoprecipitated overnight with HIF-1α specific antibody and the resulting immune complexes were probed with HIF-1α antibody. For C and D the proteasomal inhibitors MG 262 and Clasto-Lactacystin β-Lactone (Clasto) were added to the cells at a final concentration of 100 μM, 4 h prior to hypoxia.

glucose concentration in the medium of cultured cardiac myocytes) resulted in a $50.5 \pm 6.4\%$ decrease of HIF-1α protein expression, compared to levels in cells incubated in 0 mM glucose ($P < 0.01$, $n = 4$) (Fig. 1a and b). Equal protein loading was determined by Ponceau S staining of the blots as well as by immunoblotting equal amounts of nuclear extract proteins with an antibody specific for HIF-1β, which is constitutively expressed and heterodimerizes with HIF-1α in the nucleus (Fig. 1a). A reduction in HIF-1α protein expression in response to glucose was also seen when Jurkat cells were cultured under identical conditions and subjected to hypoxia (data not shown). Immunoprecipitation experiments involving total cellular HIF-1α from whole cell lysates instead of nuclear HIF-1α also demonstrated a similar reduction in HIF-1α protein expression in cardiac myocytes cultured in the presence of glucose and subjected to hypoxia (Fig. 1c). Glucose deprivation alone for up to 8 h had no effect on HIF-1α levels (data not shown). Neither hypoxia nor glucose deprivation, alone or together, caused a significant change in the protein expression of HIF-1β, the dimerization partner of HIF-1α (Fig. 1a).

Since HIF-1α translocates to the nucleus by hypoxia to bind with its dimerization partner HIF-1β, we determined whether glucose reduced HIF-1α levels in the nucleus as well as in total cell lysates. A reduction in nuclear HIF-1α was seen in cells subjected to hypoxia in the presence of glucose compared to hypoxic cells without glucose (Fig. 1d).

Glycolytic inhibitors prevent the glucose-induced decrease of HIF-1α levels

In order to confirm that the glucose effect on HIF-1α levels required glycolytic metabolism, we examined the effect of 2 glycolytic inhibitors, 2-deoxyglucose (2-DOG) and iodoacetate on hypoxia-induced HIF-1α levels. 2-DOG is phosphorylated by hexokinase but is not further metabolized and blocks glycolysis by depleting cellular ATP. Iodoacetate blocks glycolysis by inhibiting the enzyme glyceraldehyde-3-phosphate-dehydrogenase. Cardiac myocytes were pre-incubated with 2-DOG (3 mM) or iodoacetate (4 mM) and then exposed to hypoxia for 8 h in the presence and absence of glucose. Nuclear extracts were prepared and subjected to immunoblotting with HIF-1α specific antibodies. Both 2-DOG and iodoacetate prevented the glucose-mediated reduction effect in HIF-1α levels (Fig. 2).

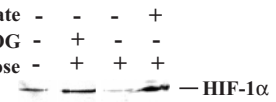

Figure 2 Primary cultures of cardiac myocytes were exposed to hypoxia of 8 h in the presence and absence of glucose and also after a 30 min pre-incubation with the glycolytic inhibitors 2-deoxyglucose (2-DOG) (3 mM) and iodoacetate (4 mM) respectively. Equal amounts (50 μg) of nuclear extracts were subjected to immunoblot analysis with HIF-1α specific antibodies.

Adenoviral infection of cardiac myocytes with GLUT1 leads to decreased hypoxia-induced HIF-1α levels

We tested whether overexpression of glucose transporters also reduced hypoxia-induced HIF-1α levels, by infecting cultures of cardiac myocytes with adenoviruses containing the human GLUT1 cDNA. Cultures of cardiac myocytes infected with either AdGLUT1 or AdRSV at a multiplicity of infection (moi) of 10—1000 were subjected to hypoxia of 8 h and 24 h in the presence of glucose. Figure 3a demonstrates an almost ten-fold increase in the expression of GLUT1 in cultures of cardiac myocytes infected with AdGLUT1 compared to those infected with AdRSV, both after 24 and 48 h of infection. Nuclear extracts prepared from the GLUT1 infected cells and exposed to 8 h hypoxia (Fig. 3b) as well as 24 h of hypoxia (Fig. 3c) showed a significant reduction in HIF-1α levels compared to control-infected as well as wild-type cells. There was no detectable HIF-1α in cardiac myocytes infected with either AdGLUT1 or AdRSV under normoxic conditions (data not shown).

Proteasomal inhibitors increase the protein expression of HIF-1α under normoxic conditions

Under normoxic conditions HIF-1α is rapidly ubiquitinated and degraded in the proteasome, a central non-lysosomal protein degradation complex present in all cells.[24–26] To confirm that proteasomal inhibitors increased protein expression of HIF-1α under

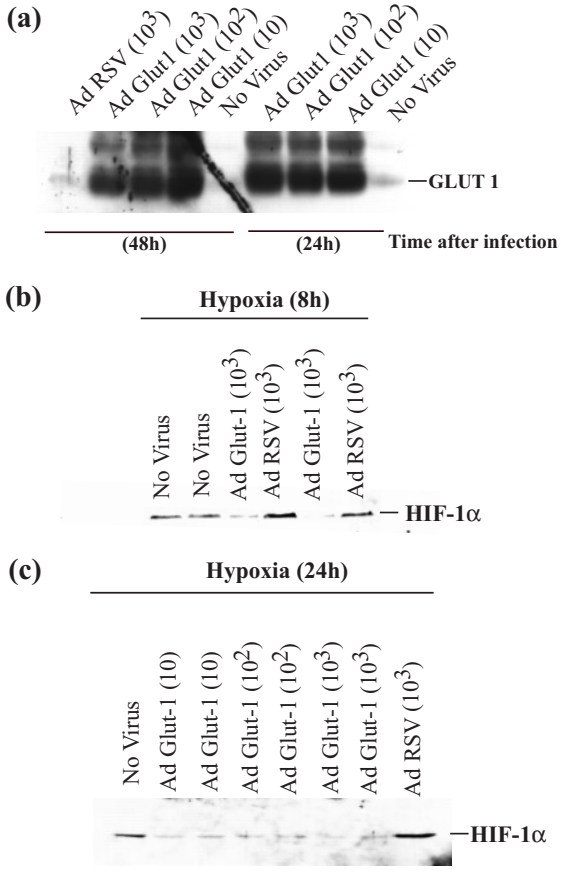

Figure 3 Primary cultures of cardiac myocytes were infected with various multiplicities of infection (indicated in parentheses) of adenoviruses containing the full-length human facilitative glucose transporter gene GLUT1 (Ad GLUT1) and also the empty vector (Ad RSV). The cells were then exposed to hypoxia of 8 and 24 h in the presence of glucose. (a) Immunoblot analysis of total cell lysates immunoblotted with the GLUT1 antibody 24 and 48 h after infection with the adenovirus. (b) Nuclear extracts were prepared from cells exposed to 8 h hypoxia and equal amounts of protein were fractionated on a 6% SDS-PAGE gel and immunoblotted with HIF-1α specific antibody. (c) Cardiac myocytes were infected with AdGLUT1 at a moi of either 10, 10^2 or 10^3 and then exposed to hypoxia of 24 h. Equal amounts of nuclear extract protein were fractionated on a 6% SDS-PAGE gel and immunoblotted with HIF-1α specific antibody.

normoxic conditions in cardiac myocytes, as has been previously shown in other cell types,[24] cardiac myocytes were incubated with 10—100 μM concentrations of various proteasomal inhibitors for 4 h. At the end of the incubation period, nuclear extracts were prepared and analyzed for HIF-1α protein expression after inmmunoblotting with HIF-1α specific antibodies. Figure 4 shows that HIF-1α expression was increased by all inhibitors including epoxomicin, a potent and highly selective compound that inhibits the proteasome by covalently modifying the α and β catalytic subunits of the 26S proteasome core.[27]

Proteasomal inhibitors prevent glucose-induced reduction in HIF-1α levels

Since the presence of glucose decreased the levels of HIF-1α during hypoxia we tested the participation of the proteasome in glucose-mediated HIF-1α degradation. Figure 5 demonstrates that hypoxia

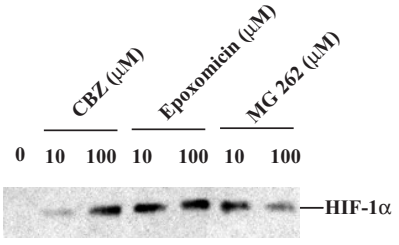

Figure 4 Cardiac myocytes were cultured under normoxic conditions in the absence and presence of proteasomal inhibitors CBz-LLL (CBZ) (10 and 100 μM). Epoxomicin (10 and 100 μM) and MG 262 (10 and 100 μM). Equal amounts (50 μg) of nuclear extracts were subjected to SDS-PAGE and immunoblotted with HIF-1α specific antibodies.

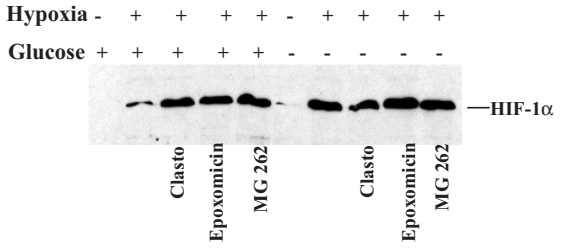

Figure 5 Cardiac myocytes were cultured in the presence and absence of glucose and exposed to hypoxia for 8 h. Nuclear extracts were prepared and equal amounts of protein (50 μg) was subjected to gel electrophoresis followed by immunoblotting with HIF-1α specific antibodies. The cells were pre-incubated for 4 h with 100 μM each of the proteasomal inhibitors, Clasto-Lactacystin β-Lactone (Clasto), MG-262 and Epoxomicin respectively.

increased levels of HIF-1α in cells pre-incubated with the proteasomal inhibitors, to equal levels in the presence and absence of glucose, suggesting that glucose induced HIF-1α degradation via a proteasomal pathway.

Glucose increases the ubiquitination of HIF-1α under hypoxic conditions

In order to explore mechanisms responsible for the reduction of HIF-1α levels during hypoxia in the presence of glucose, we examined the ubiquitination of HIF-1α both in the presence and absence of glucose. Under identical conditions of proteasomal inhibition and hypoxia, there was greater ubiquitination of HIF-1α when glucose was present in the medium (Fig. 6). Densitometric quantitation of the immunoblots revealed a 3.2- to 9.25-fold increase of ubiquitinated HIF-1α relative to total HIF-1α in the presence of glucose during hypoxia, compared to those in glucose-free medium.

Hypoxia leads to increased expression of p53

HIF-1α can induce transcription of p53 and increase p53 expression.[6,28,29] We determined whether glucose metabolism also had effects on this downstream mediator of HIF-1α effects.[6,10,29] Cardiac myocytes were cultured both in the presence and absence of glucose and subjected to hypoxia of 1—8 h. At the end of hypoxia total cell lysates were prepared and immunoblotted with a p53 specific antibody. p53 protein expression after 8 h of hypoxia

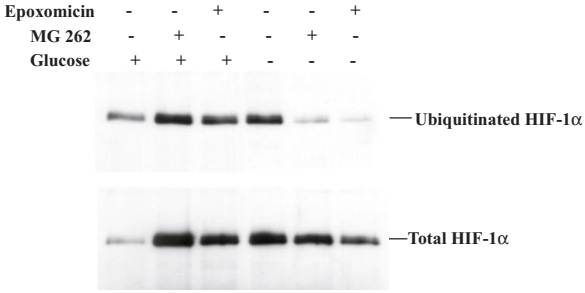

Figure 6 Cardiac myocytes were exposed to hypoxia of 8 h both in the presence and absence of glucose and also with and without a 4 h pre-incubation with 100 μM either of the proteasomal inhibitors, Epoxomicin and MG 262. Total cell lysates were immunoprecipitated with HIF-1α antibody and the resulting immune complexes were subjected to Western blot analysis followed by immunoblotting with anti-ubiquitin antibody or HIF-1α specific antibody.

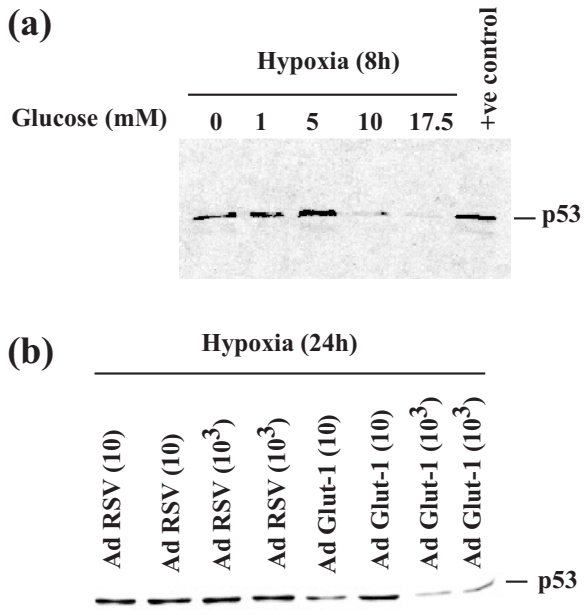

Figure 7 (a) Cardiac myocytes were cultured in the absence of glucose and also in the presence of 0, 1, 5, 10 and 17.5 mM of glucose and then exposed to hypoxia for 8 h. Equal amounts (100 μg) of total cell lysates were fractionated on a 10% SDS-PAGE gel and immunoblotted with a monoclonal antibody against p53 (Oncogene Research Products, Cambridge, MA). p53 fusion protein was used as a positive control. (b) Cardiac myocytes were infected with either AdRSV or AdGLUT1 at a moi of 10 or 10^3 respectively and then exposed to hypoxia of 24 h. Equal amounts (100 μg) of total cell lysates were fractionated on a 10% SDS-PAGE gel and immunoblotted with a monoclonal antibody against p53.

was decreased by $82.6 \pm 7.4\%$ in cells incubated with 10 or 17.5 mM glucose compared to p53 levels in cells incubated in 0 mM glucose ($P < 0.001$, $n = 3$) (Fig. 7a). Further, we also examined p53 protein expression in cultures of cardiac myocytes infected with AdGLUT1 or AdRSV and exposed to hypoxia of 24 h. p53 protein expression after 24 h of hypoxia was decreased by $75.4 \pm 8.3\%$ in cells infected with AdGLUT1 compared to p53 levels in cells infected with AdRSV or wild type cells ($P < 0.001$) (Fig. 7b).

Adenoviral infection with GLUT1 prevents hypoxia-induced cytochrome c release and apoptosis in cardiac myocytes

Finally, we examined the effect of adenoviral mediated GLUT1 infection in cardiac myocytes on hypoxia-induced apoptosis. After exposure to 24 h of hypoxia, AdGLUT1 infected cells demonstrated a significant reduction in cytochrome c release compared to cells infected with AdRSV or wild-type uninfected cells (Fig. 8a). In addition an examination of nuclear morphology also revealed a greater number of condensed and fragmented nuclei in AdRSV infected cells after exposure to 24 h of hypoxia compared to cells infected with AdGLUT1 and wild type cells (Fig. 8b). In these studies, the number of apoptotic AdGLUT1 infected cells at the highest multiplicity of GLUT1 infection was almost half that of the AdRSV cells (21.2% apoptotic cells in AdGLUT1 infected cells compared to 43.4% in AdRSV infected cells) (Fig. 8c).

Discussion

HIF-1 serves as a master switch that directs the cellular response to hypoxia and ischemia. As noted above, HIF-1 activity is regulated by the cellular levels of the HIF-1α subunit, which then heterodimerizes with HIF-1β to form the active transcription factor.[7] Much of the programmatic response directed by HIF-1 is involved in protecting cells from hypoxic and ischemic damage. The induction of erythropoietin, VEGF and other pro-angiogenic factors, as well as enhanced GLUT1 gene expression and glycolytic metabolism are examples of this protective program.[30] However, when these protective mechanisms fail and HIF-1 levels remain elevated, HIF-1 can also participate in the programmed death of hypoxic and ischemic cells.[8,9] Similarly, HIF-2α, which is related to HIF-1α in structure and function, has been demonstrated to play a major role in inducing apoptosis in response to hypoglycemia.[31]

In the present study, we have explored the interaction of glucose and its metabolism with the expression of HIF-1α. We have demonstrated that glucose and GLUT1 gene expression result in decreased hypoxia-induced HIF-1α levels, simulating the regulation of HIF-1α levels under normoxic conditions. Recent reports have elucidated the specific mechanisms by which oxygen tension regulates HIF-1α levels that allowed us to investigate some of the glucose-mediated effects on HIF-1α.[32] Under normoxic conditions, HIF-1α of molecules are rapidly ubiquitinated and proteosomally degraded, thereby inhibiting formation of the HIF-1 complex. This process is initiated by the hydroxylation of a proline residue in the oxygen-dependent degradation domain of HIF-1α by one or more members of a family of dioxygenases, PHD1-3.[32,33] The prolyl hydroxylation allows recognition of HIF-1α by the von Hippel—Lindau tumor suppressor protein (VHL) which then targets HIF-1α for ubiquitination and proteosomal degradation.[34] The activity of PHDs1-3 is predictably oxygen dependent and is quite low under hypoxic conditions. This leads to reduced VHL

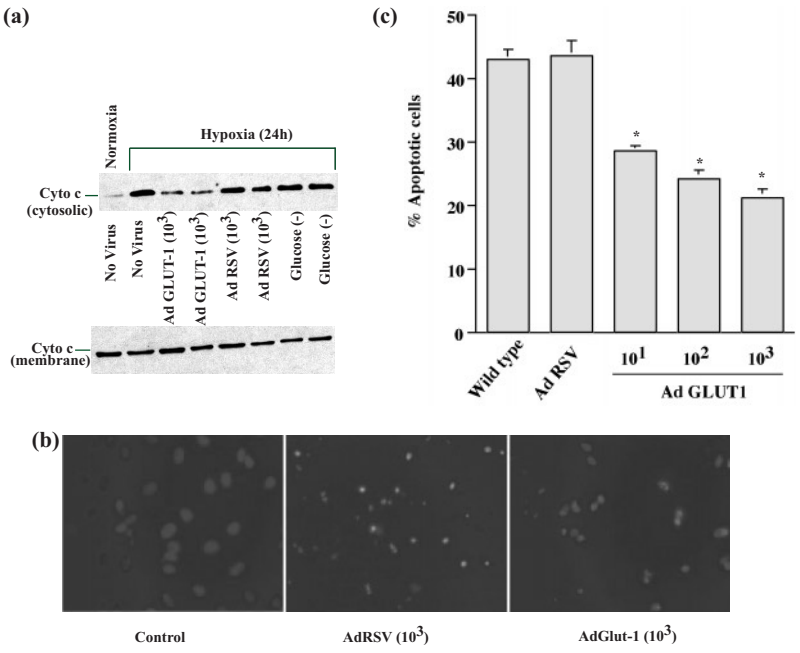

Figure 8 Cardiac myocytes were infected with adenoviruses containing the full-length human facilitative glucose transporter gene GLUT1 (AdGLUT1) and also the empty vector (AdRSV) at various multiplicities of infection. Twenty-four hours after infection the cells were exposed to hypoxia of 24 h both in the presence and absence of glucose. (a) After subcellular fractionation both the membrane and high speed cytosolic supernatants were subjected to western blot analysis followed by immunoblotting with monoclonal antibodies specific for cytochrome c. (b) Representative Hoechst labelled images of control cardiac myocytes as well as cardiac myocytes infected with either AdRSV or AdGLUT1 and exposed to 24 h hypoxia. (c) Quantitative analysis of apoptotic cells after staining with karyophilic dye Hoechst 33258. * Denotes $P < 0.05$ ($n = 5$).

binding, decreased ubiquitination and proteosomal degradation of HIF-1α, and activation of HIF-1.[34]

Our observations demonstrate that glucose promotes ubiquitination of HIF-1α in cardiac myocytes and thereby augment targeting and degradation of this polypeptide by the ubiquitin proteasomal pathway, even in the presence of hypoxia. These effects were prevented by glycolytic inhibitors suggesting that glycolysis was required for HIF-1α induction. This glucose-mediated reduction in HIF-1α levels apparently serves as a feedback mechanism, whereby HIF-1α induces glucose uptake and glycolysis which in turn promote HIF-1α degradation. This is the first demonstration of this feedback response in any cell type. At this point, we have not determined whether the glucose and GLUT1 effects on HIF-1α are mediated by increased prolyly hydroxylation and/or HIF-1α association with VHL, or through some other mechanism. In addition to the effects on HIF-1α, GLUT1 and enhanced glycolysis result in reduction of p53 levels in hypoxic cardiac myocytes. This reduction in p53 levels may well be in response to decreased HIF-1 activity.

We have also demonstrated that cardiac myocytes infected with adenoviruses expressing GLUT1 display decreased apoptosis in response to hypoxia. These findings corroborate our earlier studies in cardiac myocytes[15] and vascular smooth muscle cells[16] in which glucose uptake, glycolysis and GLUT1 overexpression (in vascular smooth muscle cells) prevented hypoxia-induced apoptosis. It seems likely that the glucose-mediated reduction in HIF-1α and p53 levels accounts for at least some of the anti-apoptotic effects of glucose uptake and metabolism. However, corroboration of such awaits experiments that specifically regulate HIF1 and p53 activity in this system. Other potential mediators of the glucose effects on apoptosis include Akt/protein kinase B and its substrate, glycogen synthase kinase-3 (GSK-3). Recent reports have demonstrated that activated Akt helps to maintain mitochondrial integrity and prevent cytochrome c release, and that this effect requires glucose uptake and glycolysis.[17,20,21] Studies from our laboratory have demonstrated that hypoxia reduces Akt and GSK3β phosphorylation whereas glucose or GLUT1 overexpression prevent these effects. We have also demonstrated that increased phosphorylation of GSK-3β and Akt are accompanied by a decrease in apoptosis (R. Loberg, F.C. Brosius III, unpublished data).

In summary, we have found that glucose and glycolysis partially prevent hypoxia-induced

increases in HIF-1α levels in and do so by augmenting the ubiquitin-proteosome pathway of HIF-1α degradation. These observations introduce a novel regulatory pathway that may play an important feedback role by signaling to the cell that metabolism is adequate. By abrogating prolonged HIF-1 activity, which can lead to cardiac myocyte apoptosis, glucose metabolism can insure cardiac myocyte survival during hypoxia and ischemia. Understanding how glucose metabolism modulates HIF-1α levels may help identify important new mechanisms by which cellular responses can be modulated during cardiac myocyte ischemia.

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