GLUT-1 reduces hypoxia-induced apoptosis and JNK pathway activation

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Lin, Zhiwu, Joel M. Weinberg, Ricky Malhotra, Steven E. Merritt, Lawrence B. Holzman, and Frank C. Brosious III. GLUT-1 reduces hypoxia-induced apoptosis and JNK pathway activation. Am J Physiol Endocrinol Metab 278: E958–E966, 2000.—Many studies have suggested that enhanced glucose uptake protects cells from hypoxic injury. We have previously shown that hypoxia-induced apoptosis can be prevented by glucose uptake and glycolytic metabolism in cardiac myocytes. To test whether increasing the number of glucose transporters on the plasma membrane of cells could elicit a similar protective response, independent of the levels of extracellular glucose, we overexpressed the facilitative glucose transporter GLUT-1 in a vascular smooth muscle cell line. After 4 h of hypoxia, the percentage of cells that showed morphological changes of apoptosis was 30.5 ± 2.6% in control cells and only 6.0 ± 1.1 and 3.9 ± 0.3% in GLUT-1-overexpressing cells. Similar protection against cell death and apoptosis was seen in GLUT-1-overexpressing cells treated for 6 h with the electron transport inhibitor rotenone. In addition, hypoxia and rotenone stimulated c-Jun-NH2-terminal kinase (JNK) activity 10-fold in control cell lines, and this activation was markedly reduced in GLUT-1-overexpressing cells. A catalytically inactive mutant of MEKK1, an upstream kinase in the JNK pathway, reduced hypoxia-induced apoptosis by 39%. These findings show that GLUT-1 overexpression prevents hypoxia-induced apoptosis possibly via inhibition of stress-activated protein kinase pathway activation.

facilitative glucose transport; stress-activated protein kinase; mitogen-activated protein kinase; necrosis

When oxidative metabolism is interrupted by hypoxia, cells markedly augment their rates of glucose uptake and glycolysis. These reproducible increases in glucose uptake and glycolysis may help preserve levels of high energy phosphates and keep cells from undergoing lethal injury during hypoxia. Indeed, many investigators have reported that conditions that enhance cellular glucose uptake and glycolysis during hypoxia appear to prevent cell injury (e.g., 11, 15, 20, 26, 27, 30). However, these studies have not considered the role of glucose transporters in determining cellular glucose uptake and metabolism.

Virtually every mammalian cell expresses one or more facilitative glucose transporter isoforms that transport glucose down its concentration gradient into the cell. In many tissues, such as skeletal, cardiac, and vascular smooth muscle, glucose transport appears to be rate limiting for glucose metabolism (23). Because the transporters present in these cells have high affinities for glucose, with Michaelis-Menten constant, or Km values for glucose generally <5 mM (14), cellular glucose uptake and metabolic flux are relatively insensitive to physiological changes in extracellular glucose levels but are determined by the number and activity of plasma membrane glucose transporters. Accordingly, most cells augment the number of plasma membrane transporters after the onset of hypoxia. This increase in transporter number is mediated by two general mechanisms. In insulin-responsive tissues, hypoxia rapidly stimulates translocation of extant intracellular GLUT-4 glucose transporter molecules to the plasma membrane (7, 34), whereas more prolonged hypoxia, in both insulin-responsive and -unresponsive tissues, induces the expression of the gene encoding another transporter, GLUT-1 (1, 7, 12, 22, 31, 34), which is constitutively expressed on the plasma membrane of most mammalian cells. GLUT-1 expression may be increased by hypoxia or ischemia even in tissues that are not directly hypoxic or ischemic. For example, we have found that regional myocardial ischemia induces GLUT-1 expression in nonischemic as well as ischemic heart regions (5). If increased GLUT-1 expression protects cells from hypoxic or ischemic injury, such an increase in nonischemic regions could be an important adaptive response to minimize injury during subsequent ischemic events.

Cell death caused by hypoxia has generally been presumed to occur by necrosis. However, recently it has become clear that much of the cell death associated with hypoxia or ischemia occurs by apoptosis (e.g., 4, 8). Although there has been little active investigation of the effects of glucose uptake and metabolism on apoptosis, we have recently demonstrated that provision of glucose prevents hypoxia-induced apoptosis in cardiac myocytes (24). Other substrates such as lactate, pyruvate, and propionate did not provide protection, and the process was clearly dependent on glycolytic metabolism (24). To determine whether similar protection against hypoxia-induced apoptosis could be obtained by increased...
levels of GLUT-1, independent of changes in extracellular glucose levels, we overexpressed GLUT-1 in a vascular smooth muscle cell line and subjected the cells to hypoxia. We found that increased GLUT-1 expression is accompanied by a major decrease in hypoxia-induced apoptosis. Because stress-activated protein kinase pathways have been implicated in inducing or enhancing apoptosis in many cell types (6, 10, 16, 36, 39, 41), we investigated whether GLUT-1 overexpression affected activity in these pathways. We found that hypoxia appears to induce apoptosis in part via activation of these pathways and that GLUT-1 overexpression is associated with a marked reduction in hypoxia-induced activation of c-j un-NH2-terminal kinase (JNK), suggesting that part of the protective effect of GLUT-1 is due to this reduced JNK pathway activity.

**MATERIALS AND METHODS**

Creation and characterization of GLUT-1-overexpressing cell lines. Subconfluent plates of A7r5 cells [rat embryonal aortic vascular smooth muscle cell (VSMC) line; ATCC no. CRL-1444] were transfected with 2 µg of a recombinant GLUT-1 plasmid cDNA (gift of Dr. David James), comprising the full-length coding sequence of human GLUT-1 cDNA cloned into the Not I and blunted Xba I sites of pRC/CMV (Invitrogen, Carlsbad, CA), by use of Lipofectamine ( Gibco-BRL, Gaithersburg, MD) according to the supplier’s specifications. After establishment of a G418 (Gibco-BRL) killing curve for nonrecombinant A7r5 cells, recombinant GLUT-1-overexpressing colonies and colonies transfected with the pRC/CMV plasmid alone were isolated in 400 µg/ml G418. These cell lines were then maintained in DMEM plus 10% FCS and 200 µg/ml G418. For some experiments, pools of uncloned GLUT-1-overexpressing or control-plasmid cells were selected and maintained in 400 µg/ml G418.

Cellular lysates from 15 out of 70 isolated G418-resistant colonies were used for immunoblotting, as previously described (5), except that an enhanced chemiluminescent system (Amersham, Arlington Heights, IL) was used for detection and quantitation of GLUT-1 polypeptide levels. Cellular lysates (40 µg) were suspended in loading buffer, electrophoresed on 10% (wt/vol) SDS-PAGE gels, and transferred onto nitrocellulose membranes. GLUT-1 was detected with either a 1:5,000 dilution of a specific polyclonal rabbit anti-rat GLUT-1 antiserum directed against the 12 carboxy-terminal amino acids of GLUT-1 (gift of Dr. Bernard Thorens, University of Lausanne, Switzerland) or a 1:10,000 dilution of an antiserum raised against purified rat erythrocyte GLUT-1 [gift of Dr. Christin Carter-Su, University of Michigan (35)]. Specificity for the latter antiserum was determined by preincubation of the antiseraum with 75 µg/ml of partially purified erythrocyte GLUT-1 [gift of Dr. Arno Kumagai, University of Michigan (21)] before incubation of the immunoblot membrane.

Uptake of the glucose analog 2-deoxyglucose (2-DOG) was performed by following the method of Tai et al. (35). Briefly, the medium was removed from 60-mm plates of recombinant cells, which were either confluent or ~40% confluent (nonconfluent). These plates were then washed once with PBS. The PBS was removed and replaced with 0.1 mM unlabeled 2-DOG (Sigma) plus 25 µCi/ml 2-[3H]DOG (Amersham) in Krebs-Ringer phosphate solution (in mmol/l: 128 NaCl, 5.2 KCl, 1.3 CaCl2, 2.6 MgSO4, and 10 Na2HPO4) + 1% (wt/vol) BSA ± 20 nM cytochalasin B, which is an irreversible inhibitor of glucose transport, at 37°C for 5 min. Previous studies had shown that uptake was linear for ~15 min in these cells. The plates were subsequently washed quickly 3 times with ice-cold Krebs-Ringer phosphate solution containing 5.5 mg/dl phlorizin to quench 2-DOG uptake. The samples were then lysed in 0.1% (wt/vol) SDS. A portion of each sample was used for determination of protein concentration by bicinchoninic acid assay (Pierce, Rockford, IL), and the rest was utilized for scintillation counting. Aliquots of the incubation solutions containing 2-[3H]DOG solutions were also counted. 2-DOG uptake was expressed as picomoles cytochalasin B-inhibitable 2-DOG uptake per 5 min per milligram protein. The confluent cell experiments were performed in quadruplicate, whereas the nonconfluent experiment was performed on 10 plates each of the GLUT-1-overexpressing and the vector-control cells. Comparison of cellular protein to cell number was also made for each cell line.

Analysis of cell death. These and all subsequent experiments were performed on the two cell lines with the greatest overexpression of GLUT-1, control transfected cell lines, and on uncloned pools of GLUT-1-overexpressing and control-transfected cells. Before the experiments, all cells were maintained in DMEM (including 25 mM D-glucose and 1 mM sodium pyruvate) plus 10% FCS. All transfected cells were also maintained in 200 µg/ml G418. Cells were then incubated for variable periods in serum-free DMEM inside a heated Plexiglas chamber, which was gassed continuously with 95% N2-5% CO2 at 37°C. In most experiments, 60 µl Oxyrase, a mixture of bacterial membrane monoxygenases and dioxygenases (Oxyrase, Ashland, OH), were added to each milliliter of media to further lower PO2 levels (18). Alternatively, cells in serum-free DMEM were exposed to rotenone (0.5 µg/ml), an inhibitor of electron transport, for variable periods in a conventional tissue culture incubator (95% air-5% CO2). Additionally, in some experiments, cells were incubated in 40 µM benzyloxycarbonylvalinylalanylsapasptfluoromethyl ketone (zVAD.fmk), a nonspecific caspase inhibitor, for 2 h before hypoxia and throughout exposure to hypoxia. Control cells for all experiments were treated identically in serum-free DMEM except for exposure to hypoxia or rotenone. Final time points for analysis were chosen on the basis of the percentage of control cells that showed evidence of cell death (either plasma membrane damage and/or nuclear fragmentation) in initial experiments. Time points in which 25–50% cells showed evidence of cell death were chosen.

At the end of the incubation period, cells in the media were combined with those from the cell layer and stained using the method of Jones and Senft (17) in which cells are incubated in PBS containing a combination of 75 µg/ml fluorescein diacetate and 100 µM propidium iodide (PI). Fluorescein diacetate is cell permeant and metabolized in the cytosol to fluorescein, which is retained by viable cells with normally impermeant plasma membranes. The same cells exclude PI, a nuclear stain. In contrast, cells with necrotic or late apoptotic changes have PI-positive nuclei and do not retain fluorescein. At least three randomly selected, independent fields were counted to determine the percentage of dead cells for each sample.

Identification of apoptosis was based on nuclear morphology in cells permeabilized with 4% methanol (vol/vol). Nuclear condensation and/or fragmentation was determined in three randomly selected, independent fields of >40 cells/field for each set of cells. To confirm apoptosis-associated DNA fragmentation, cells were assessed for free 3'-OH termini using digoxigenin-conjugated dUTP labeling with terminal deoxyribonucleotidyl transferase and anti-digoxigenin-fluorescein following the manufacturer’s protocol (TUNEL assay, Oncor,
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Gaithersburg, MD) except that PI counterstaining was not used.

ATP determinations. Floating and attached cells were placed into 6% (wt/vol) TCA, vortexed, and centrifuged at 2,000 rpm to remove the precipitated protein. The supernatant was neutralized and extracted with equal volumes of 0.5 M trioctylamine (Aldrich, Milwaukee, WI) in Freon-113 (Matheson, East Rutherford, NJ) and then centrifuged at 13,000 g for 5 min at room temperature. The aqueous layer was removed, filtered through a 0.45-µm nitrocellulose filter, and stored at −20°C. ATP was quantified as previously reported (37) on 20-µl aliquots of sample by HPLC at an absorbance of 254 nm with a Beckman (Fullerton, CA) C18 reversed-phase ion pairing column by use of an isocratic mobile phase consisting of 5% acetonitrile, 20 mM KH3PO4, 20 mM tetrabutylammonium dihydrogen phosphate (pH 3.5) at 24°C and a flow rate of 1 ml/min.

J NK activity analysis. GLUT-1-overexpressing and control cells were exposed to hypoxia or rotenone as described above. Cell lysates were prepared in 1 ml of a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 20 mM β-glycerophosphate, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml pepstatin, 20 µg/ml leupeptin, and 20 µg/ml aprotinin). Equal protein amounts of each cell lysate (usually 100 µg) were incubated for 3 h at 4°C in the presence of purified GST-c-Jun un(1—79), a gift of Dr. Alan Saltiel, Parke-Davis (Ann Arbor, MI), bound to glutathione-agarose beads (5 mg GST-c-jun/5 ml beads), as previously described (13). Beads were washed 3 times with PBS containing 1% (vol/vol) Nonidet P-40 and 2 mM vanadate, once with 100 mM Tris (pH 7.5) and 0.5 M LiCl, and once in kinase reaction buffer (in mM: 12.5 MOPS, pH 7.5, 75.5 β-glycerophosphate, 7.5 MgCl2, 0.5 EGTA, 0.5 NaF, and 0.5 vanadate). Samples were then resuspended in 30 µl kinase reaction buffer containing 1 µCi [γ-32P]ATP and 20 µM unlabeled ATP. After 20 min at 30°C, reactions were terminated by the addition of SDS loading buffer, boiled, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and used for autoradiography or counted in a phosphorimager. The same, or identically processed, membranes were immunoblotted with a J NK1 (C-17) antibody (rabbit anti-carboxy-terminal peptide antiserum, Santa Cruz Biotechnology, Santa Cruz, CA) as an internal control of J NK1 capture and to determine levels of J NK1 expression in the different cell lines. Quantitation of J NK activity was performed either by phosphorimager analysis or densitometric analysis of autoradiographs by use of a videoimaging system and NIH Image software as previously reported (5).

p38MAPK and ERK1/2 immunoblotting. Cell lysates were prepared from GLUT-1-overexpressing and control cells, and equal amounts of protein from each sample (50 µg) were separated using 10% SDS-PAGE, as described above. Immunoblots were performed as noted above with the following antibodies, which detected either phosphorylated forms of p38MAPK and ERK1/2 or both phosphorylated and nonphosphorylated forms of the kinases: phosphospecific p38MAPK (Tyr 182) antibody (rabbit anti-phosphopeptide antiserum, New England Biolabs, Beverly, MA), phosphospecific MAPK (Tyr 204) antibody (rabbit anti-phosphopeptide antiserum, New England Biolabs), p38 (C-20) antibody (rabbit anti-carboxy-terminal peptide antiserum, Santa Cruz Biotechnology), and ERK2 (C-14) antibody (rabbit anti-carboxy-terminal peptide antiserum, Santa Cruz Biotechnology).

Effects of transient overexpression of MEKK1 on hypoxia-mediated apoptosis. A7r5 cells were transiently cotransfected with a vector encoding Escherichia coli β-galactosidase (pCMVβ, Invitrogen, Carlsbad, CA) and a fivefold greater amount of an expression vector encoding MEKK1(K432M), a catalytically inactive form of MEKK1, which competitively inhibits signaling through J NK (41, 5), or a control vector (pcDNA-3, Invitrogen). After 48 h, cells were exposed to hypoxia for 4 h and then fixed in 0.5% glutaraldehyde for 5 min, washed with PBS/1 mM MgCl2, and incubated in a PBS solution containing 1 mM MgCl2, 1.64 g/l K3Fe(CN)6, 2.1 g/l K4Fe(CN)6, 3H2O, and 1 mg/ml X-gal to identify transfected cells. Transfected cells, identified by blue staining, were then examined under Nomarski optics for plasma membrane blebbing and nuclear changes characteristic of apoptosis (40) by an observer blinded to the MEKK1(K432M) transfection status of the cells. The percentage of apoptotic cells per plate was determined on the basis of counts of ≥3 fields.

Statistical evaluation. Data were expressed as means ± SE unless fewer than four experiments were reported, in which case data were expressed as means ± SD, and so indicated in RESULTS. An unpaired two-tailed Student's t-test was used to compare results from two populations, except for MEKK1(K432M) transfection experiments in which a paired Student's t-test was utilized because those experiments were performed on a series of paired cotransfection populations. ANOVA with a Scheffé intervariable test was performed when more than two populations were analyzed. Differences were deemed to be statistically significant when P < 0.05.

RESULTS

Characterization of GLUT-1-overexpressing cells. Two cell lines were identified that had substantial increases in total cellular GLUT-1 content (Fig. 1A). Uptake of the glucose analog 2-DOG (Fig. 1B) was increased in both GLUT-1-overexpressing cell lines. When measured in confluent cultures, the increase in 2-DOG uptake in the GLUT-1-overexpressing lines was relatively modest (2.8- and 1.8-fold, respectively). However, when these experiments were performed with culture conditions identical to those used for the hypoxia experiments (nonconfluent cultures), the relative increase in 2-DOG uptake in the GLUT-1-overexpressing cell lines was significantly (2.8- and 1.8-fold, respectively). However, when these experiments were performed with culture conditions identical to those used for the hypoxia experiments (nonconfluent cultures), the relative increase in 2-DOG uptake in the GLUT-1-overexpressing cell lines was significantly higher (6.8-fold greater than in the control-transfected cells).
Hypoxia was used to compare control to GLUT-1-overexpressing cells. After 4 h of hypoxia, the number of PI-positive GLUT-1-overexpressing cells was approximately one-half the number seen in the nontransfected and control-transfected cells (Fig. 2A). In these experiments, PI-positive cells likely comprised both necrotic and late apoptotic cells, although the proportions of each were not determined by this experimental approach. For cells kept in a normoxic environment without exposure to Oxyrase, incubation in serum-free medium caused no morphological changes or increase in PI-positive cells. Fewer than 1% of normoxic cells were PI positive.

A striking change in the control-transfected and nontransfected cells exposed to hypoxia was in the degree of apoptotic nuclear changes. These morphological features were virtually absent in the two GLUT-1-overexpressing lines (Figs. 2B and 3). The nuclear apoptotic changes were also prevented by preincubation of either vector control or GLUT-1-overexpressing cells in 40 µM zVAD.fmk, a nonspecific caspase inhibitor, in two separate experiments (not shown).
To rule out clonal variation as the cause of the differences between GLUT-1-overexpressing and control cell lines, pools of stably transfected GLUT-1-overexpressing and control-transfected cells were examined. The GLUT-1-overexpressing cell pools demonstrated about twofold greater GLUT-1 expression and, after exposure to hypoxia, showed a more than twofold reduction in the number of apoptotic cells compared with control cell pools (not shown). Changes of apoptosis were noted in <1% of control cells, whether incubated in serum-containing or serum-free media.

To confirm the results of the hypoxia experiments, cells were exposed to 0.5 µg/ml rotenone, an inhibitor of mitochondrial electron transport. In initial experiments on control cells, there were <10% either PI-positive or apoptotic cells after 4 h of exposure (not shown). However, after exposure to rotenone for 6 h, the numbers of PI-permeable and apoptotic cells increased dramatically. In these experiments, 57.9 ± 10.2% (n = 5) of control cells vs. 9.04 ± 2.1% (n = 5) and 11.4 ± 1.2% (n = 2) of the GLUT-1-overexpressing cells were PI permeable, and 58% of control cells vs. 1.2 and 1.1% of the GLUT-1-overexpressing cells (n = 2 for each line) showed apoptotic changes.

Because hypoxia induces GLUT-1 expression in many cells (1, 12, 22, 31, 33), we investigated whether the hypoxia led to increased GLUT-1 expression in the control-transfected cells. After 4 h of hypoxia, there was a substantial increase in native GLUT-1 mRNA levels in both control-transfected and GLUT-1-overexpressing cells (Fig. 4A). In the control-transfected cells, the increase in GLUT-1 polypeptide levels was less than twofold (Fig. 4B) and therefore did not approach those found in the GLUT-1-overexpressing cells (Fig. 4C).

Reoxygenation may also induce apoptosis and cell membrane damage in certain cell types (9, 32). Therefore, we determined the extent to which these events occurred in cells that survived the initial period of hypoxia.

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Fig. 3. Photomicrograph of digoxigenin-conjugated dUTP labeling with terminal deoxynucleotidyl transferase (TUNEL assay) of control-transfected cell line (A) and one of the GLUT-1-overexpressing cell lines, AG14 (B). Many control cells demonstrate bright TUNEL staining and morphological changes (nuclear fragmentation and condensation) of apoptosis.

Fig. 4. Effect of hypoxia on native GLUT-1 expression in control-transfected and GLUT-1-overexpressing cells. A: GLUT-1 Northern analysis of 20 µg of total RNA harvested from vector-control cells (vect.) and GLUT-1-overexpressing cells (AG14) grown under normal oxygen conditions (Control) or 4 h of hypoxia. Transfected GLUT-1 mRNA (trans. GLUT1) has a mobility of ~2 kb, whereas native GLUT-1 mRNA (n. GLUT1) has a mobility of ~2.8 kb. B: GLUT-1 immunoblot of lysates (40 µg) from control-transfected cells exposed to the same conditions used for Northern analysis. C: GLUT-1 Immunoblot with antiserum directed against rat erythrocye GLUT-1 on GLUT-1-overexpressing cells. Immunoblot on right (+ competition) was incubated with GLUT-1 antiserum after preincubation of antiserum with 75 µg/ml of partially purified GLUT-1 that eliminated specific GLUT-1 detection on immunoblot. Several nonspecific bands induced by hypoxia were not competed away by this maneuver.
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DISCUSSION

The increase in glucose transport and metabolism that reproducibly accompanies hypoxia and ischemia has been studied for over a century, and a number of reports have suggested that augmented glucose uptake and metabolism can reduce cell death due to hypoxia or ischemia. To our knowledge, only two previous reports have directly examined the effect of glucose on hypoxia-induced apoptosis. Schmaltz et al. (29) demonstrated that hypoxia promoted apoptosis in oncogenically transformed mouse embryonic fibroblasts. Their data suggested that it was the degree of acidosis and not the presence or absence of glucose that determined the extent of apoptosis (29). In contrast, in a recent study from our laboratory, hypoxia-induced apoptosis in rat neonatal cardiac myocytes was prevented by the presence of as little as 1 mM glucose in the medium (24). Glucose inhibited translocation of cytochrome c from the mitochondria to the cytosol and cleavage of the death substrate poly[ADP-ribose] polymerase (PARP). Cleavage of PARP and DNA laddering were prevented by preincubation with the caspase inhibitors, z-VAD.fmk and z-DEVD.fmk, indicating participation of activated caspases in the apoptotic process. The alternate substrates lactate, pyruvate, and propionate provided no protection from apoptosis, and the effect of extracellular glucose on apoptotic biochemical and morphological changes was eliminated when glycolysis was blocked, indicating that glycolysis was necessary for glucose-induced protection (24).

In studies in which cells were exposed to other types of apoptotic stimuli, glucose uptake and metabolism were also associated with improved survival in cells. For example, Binder et al. (3) showed that increased glucose transport reduced tumor necrosis factor-α-associated apoptosis in a human breast cancer cell line, and Kan et al. (19) showed that apoptosis due to growth factor withdrawal was reduced in mast cells that overexpressed the v-Abl tyrosine kinase. These authors showed that this enhanced survival was due to increased glucose uptake and increased plasma membrane GLUT-1 expression. When glucose uptake was blocked by incubation with cytochalasin B (19), apopto-
sis proceeded without hindrance. In this latter study, alternate substrates, such as glutamine and pyruvate, did confer resistance to apoptosis, in contrast to their lack of efficacy in hypoxia-induced apoptosis (24). In a study of cultured cardiomyocytes by Bialik et al. (2), serum and glucose withdrawal were found to induce biochemical and morphological signs of apoptosis, suggesting that glucose uptake was critical for preventing such events, although the specific effects of glucose withdrawal alone vs. those of serum withdrawal were not reported. Moley et al. (25) found that elevated extracellular glucose levels led to decreased GLUT-1 expression, which in turn resulted in diminished glucose uptake and intraembryonic free glucose levels. These embryos underwent apoptosis at a fourfold greater rate, increasing Bax expression and apoptosis in blastocysts and embryos from diabetic mouse mothers (25). More recently, in studies that are most relevant to our current findings, Moley et al. found that antisense-GLUT-1 expression in murine blastocysts increased apoptosis in murine blastocysts incubated in normal glucose concentrations. These authors also noted that elevated glucose levels had no effect on apoptosis in embryos deficient in either Bax or p53, showing that activation of these proapoptotic factors is necessary to transduce the effects of glucose and glucose transport (Moley et al., unpublished data).

In the present report, we demonstrate that changes in glucose transporter expression, independent of extracellular glucose concentrations, can affect the cellular response to hypoxia. Augmented GLUT-1 expression probably protects cells via enhanced glycolytic metabolism, as demonstrated in the previous reports, although
this was not formally tested. Surprisingly, there was no significant difference in the ATP levels between control and GLUT-1-overexpressing cells. This contrasts with other reports suggesting that augmented glucose metabolism during hypoxia results in stabilization of ATP levels (e.g., 24, 28). One potential explanation for this unexpected finding is that GLUT-1 overexpression enhanced ATP production only in a subsarcolemmal compartment and that this difference was not detected by our total cellular ATP measurements. Because carbohydrate metabolism in VSMCs is highly compartmentalized (23), this possibility is a reasonable one.

Our results also suggest that GLUT-1 effects on the activation of the stress-activated protein kinase system comprise a part of that mechanism by which augmented GLUT-1 expression and glucose uptake reduce hypoxia-induced apoptosis. We found that GLUT-1 overexpression reduced JNK activation due to hypoxia. Although total JNK levels and basal JNK activities were similar in control and GLUT-1-overexpressing cells, hypoxia and rotenone caused much greater JNK activation in control cells than in GLUT-1-overexpressing cells. Although stress-activated protein kinase activation has been associated with apoptosis and is a consistent marker of cellular stress, the reduction in JNK activation and in apoptosis in our system may not be causally related. However, the expression of a catalytically inactive mutant of MEKK1 in A7r5 cells did reduce hypoxia-induced apoptosis in our system. Because this mutant inhibits activation of MEKK1 in most settings (e.g., 13), it is possible that reduced stress-activated protein kinase pathway activation may be one mechanism by which the GLUT-1-overexpressing cells were protected from apoptosis. The interaction of the stress-activated protein kinase and apoptotic pathways is complex and varies on the basis of cell type and apoptotic stimulus. In apoptosis due to cellular stress (e.g., hypoxia, serum withdrawal, ultraviolet irradiation), it appears that activated caspase 3-related caspases can directly cleave and thereby activate MEKK1 (6, 38). Activated MEKK1, perhaps through downstream activation of the JNK pathway, can then promote further caspase cleavage and activation (6), thereby amplifying the apoptotic response. Thus GLUT-1 reduction of JNK activation may be indirect, via reduced caspase activation and reduced caspase-mediated MEKK1 and JNK activation.

In this study, we have demonstrated a novel effect of glucose transporter expression: prevention of hypoxia-induced apoptosis in VSMCs. Should such a phenomenon be reproducible in vivo and in other cell types, maneuvers that increase or induce GLUT-1 expression may provide important protection of tissues at risk of hypoxic or ischemic damage.

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