Regulation of Brain Glucose Transporters by Glucose and Oxygen Deprivation

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Brain cells are dependent on glucose and oxygen for energy. We investigated the effects of hypoxia, glucose deprivation, and hypoxia plus glucose deprivation on mRNA and protein levels of glucose transporter (GLUT1) and GLUT3 and 2-deoxyglucose (2-DG) uptake in primary cultures of rat neurons and astroglia. Hypoxia for 24 hours did not significantly affect cell viability but increased neuronal GLUT1 and GLUT3 mRNA up to 40-fold and fivefold, respectively, above control levels. Similar changes in GLUT1 mRNA were measured in glia. The effects of hypoxia on GLUT1 and GLUT3 mRNA were reversible. The increase in GLUT1 mRNA could be detected within 20 minutes of hypoxia and was blocked by actinomycin D. Nuclear runoff transcription assays showed that hypoxia did not alter the transcription rate of GLUT1. However, hypoxia enhanced the stability of GLUT1 mRNA in neurons (half-life [$t_{1/2}$]>12 hours) compared with normoxic conditions ($t_{1/2}$ ~10.4 hours), suggesting the existence of a posttranscriptional mechanism for the regulation of GLUT1 transcript levels. Twenty-four hours of normoxia and 1.0 mmol/L glucose increased neuronal GLUT1 mRNA less than threefold above basal, but 24 hours of glucose and oxygen deprivation increased GLUT1 over 111-fold above basal. Induction of neuronal GLUT1 mRNA was temporally associated with increased levels of GLUT1 protein and with stimulation of intracellular 2-DG accumulation. We conclude that hypoxia reversibly increases the transcript levels of GLUT1 and GLUT3 in rat brain cells and stimulates GLUT1 transcript levels by posttranscriptional mechanisms. Although glucose deprivation alone produces minimal effects on GLUT mRNA levels, hypoxia plus glucose deprivation synergize to markedly increase GLUT gene expression.

FROM THE STANDPOINT of intermediary metabolism, the central nervous system (CNS) is a relatively simple tissue, since it relies almost exclusively on glucose as an energy substrate. Thus, the bulk of adenosine triphosphate (ATP) generated in brain cells for normal function depends on the aerobic oxidation of glucose and the integrity of the oxidative phosphorylation pathway. Facilitative glucose uptake into mammalian brain cells occurs by the cell-specific carrier systems designated glucose transport protein 1 (GLUT1) and GLUT3. GLUT1 is most abundant in red blood cells and brain microvessels but is present in almost every tissue, whereas GLUT3 appears to be specific to neurons.[1] In situ mapping of GLUT1 and GLUT3 mRNA in the brain showed that GLUT1 mRNA is distributed in a diffuse pattern throughout the brain, whereas GLUT3 has a more discrete regional localization.[2,3] Neuronal differentiation in the brain of rats[3-5] and probably other species[6,7] is associated with a shift from GLUT1 to GLUT3 expression. GLUT3 determines glucose utilization in neurons and is maximally expressed after neuronal maturation in rats and humans.[8,9]

A deprivation of oxygen, glucose, or both is an important pathologic modifier of CNS function because of the resultant depressed levels of intracellular oxygen and metabolizable substrates. The current study was undertaken to investigate the effect of these perturbants on the expression and activity of GLUT1 and GLUT3 in primary cultures of neurons and glia.

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Submitted April 10, 1997; accepted October 29, 1998.

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Supported by grants from the University of Florida Brain Institute and American Heart Association Research Fellowships (9602008) and (9401206).

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0026-0495/99/4804-0003$10.00/0

MATERIALS AND METHODS

Preparation of Cell Cultures

Pups from timed-pregnant Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were killed 1 or 21 days after birth. The brain was excised, and the overlying pia was removed by dissection. The preparation of primary cultures of neurons and astroglia from whole brain has been described previously.[10-12] To prepare neuronal cultures, dissociated cells were washed in Dulbecco's modified Eagle's medium ([DMEM] GIBCO, Grand Island, NY) with 24 mmol/L D-glucose and supplemented with 10% plasma-derived horse serum ([PDHS] Cocalico Biologicals, Reamstown, PA), 100 μg/mL streptomycin (GIBCO), and 100 U/mL penicillin (Bristol Myers, Princeton, NJ). The cells (1.5×10^6) were plated in 100-mm or 35-mm Falcon tissue culture dishes precoated with poly-L-lysine (Sigma, St Louis, MO). The cultures were grown for 3 days in DMEM at 37°C in a humidified atmosphere containing 6% CO_2. On the third day, the medium was changed and fresh DMEM containing 10 μmol/L cytosine arabinoside (Sigma) was added. This treatment inhibits the growth of rapidly dividing cells, most of which are nonneuronal in origin. After 2 days, the medium was replaced with fresh DMEM, in which cells grew an additional 10 to 14 days before experimentation.

Astroglial cultures were prepared in a similar fashion, except that 10% fetal bovine serum (Hyclone, Logan, UT) was substituted for PDHS and cytosine arabinoside was omitted from the culture medium. A secondary culture, usually 7 to 10 days after plating the primary culture, was performed once the cells were confluent, to achieve a concentration of 1.0×10^5 cells/mL. Cells were allowed to grow an additional 6 days before experimentation.

When primary cultures were initially established, the culture medium contained 24 mmol/L glucose, as previously reported.[10-12] During the 10- to 12-day period of incubation in an atmosphere of 6% CO_2, cells metabolized glucose at the following rates: neurons, 0.047 ± 0.005 mmol/h (n = 5); 1-day-old glia, 0.047 ± 0.002 mmol/h (n = 15); and 21-day-old glia, 0.042 ± 0.002 mmol/h (n = 8). Thus, on the day of experimentation, the medium glucose concentration was 10.4 ± 1.0 mmol/L for neonatal neurons and glia and 12.0 ± 0.7 mmol/L for 21-day-old glia.

Incubation Conditions

On the day of study, culture dishes were placed in airtight incubator chambers (Billups-Rothenberg, Del Mar, CA) with inflow and outflow adapters and a constant 37°C environment. To produce hypoxic conditions, each incubator chamber was continually gassed with 95% N_2/5% CO_2 at a gas flow rate of 5 L/min until the O_2 analyzer (Teledyne Analytical Instruments, City of Industry, CA) measured the outflow gas to contain 0% O_2. The flow rate was maintained at approximately 0.5 L/min for the duration of the experiment. The inflow gas was humidified using a heater humidifier (Therm III; Concha, Arlington Heights, IL), and the chamber humidity was maintained by placing two petri dishes filled with sterile water into each chamber. Oxygen tension in the cell culture medium was measured by an O_2 probe (ABG 100 O2 monitor; Bard, Temsbury, MA). Cells were washed twice on the day of study with glucose-free medium and incubated in DMEM base (glucose-free) supplemented with penicillin, streptomycin, dialyzed serum, 4 mmol/L glutamine, and the amount of glucose (0 to 100 mmol/L) required for a particular experiment.

Chemical Analysis

Glucose and lactate concentrations in the incubation medium were determined in duplicate by standard enzymatic techniques using a YSI glucose/lactate analyzer (Yellow Springs, OH). Cell protein was quantified by a modified Lowry[13] assay using bovine serum albumin as the standard.

RNA Preparation

Cells from two 100-mm culture dishes were pooled to generate RNA for a single time point. RNA was prepared by adding 4 mmol/L guanidinium isothiocyanate solution with 0.1% β-mercaptoethanol and 0.25% sarcosyl to the culture dishes immediately after incubation.[14] The cell lysate was loaded onto a 5.7-mol/L CsCl cushion and centrifuged at 35,000 rpm for 18 hours at 20°C in a Beckman (Fullerton, CA) SW 50.1 rotor. The RNA pellet was retrieved using ethanol precipitation, and the RNA concentration was determined by absorbance at 260 nm.

Northern Blot Analysis

The Northern blot hybridization procedure was adapted from the method of Sambrook et al.[15] Twenty-five micrograms of total RNA was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (Hybond N+; Amersham, Arlington Heights, IL). After cross-linking (Stratagene, La Jolla, CA), the probes were hybridized to blot cDNAs for GLUT1 (2.57 kb) or GLUT3 (3.9 kb). A rat cDNA for copper/zinc superoxide dismutase ([Cu/Zn SOD] 800 bp; provided by Dr H. Nick, University of Florida) was used as a control probe against which GLUT1 and GLUT3 signals were quantitatively normalized. Thus, the results are expressed as the fold change from the control GLUT1 to Cu/Zn SOD ratio. Probes were labeled using a random priming procedure (Stratagene). After hybridization, blots were exposed to Hyperfilm (Amersham) at −70°C and quantified by scanning densitometry (BioRad, Hercules, CA).

RNA Stability Measurement

Neurons were incubated under hypoxic conditions for 3 hours. Then 7.5 μg/mL actinomycin D (Sigma) was added (time 0), and the incubation under hypoxia was continued for 1.5, 3, 6, and 12 hours. Control cultures were maintained at normal atmosphere under similar conditions. RNA from control and hypoxic cultures was isolated, and Northern analyses for GLUT1 were performed as already described. The blots were stripped and reprobed with a rat 18S rRNA oligonucleotide probe (5′-CCG CCC GCC CGC TCC CAA GAT-3′) to normalize for loading. The normalized GLUT1 levels were then expressed as the percent RNA remaining, setting the RNA level at time 0 (time of actinomycin D addition) to 100%.

Nuclear Runoff Transcription

Five petri dishes (100 × 20 mm) of neurons were incubated under hypoxic conditions for 3 or 6 hours. Control cultures were maintained under normoxic conditions. Nuclei were isolated rapidly using the Dounce homogenization–based protocol of Greenberg and Bender[16] and stored in glycerol storage buffer (50 mmol/L Tris chloride, pH 7.5, 40% vol/vol glycerol, and 5 mmol/L $MgCl_2$) at −70°C. Runoff transcription reactions were initiated by addition of an equal volume of 2× reaction buffer (10 mmol/L Tris chloride, pH 8.0, 5 mmol/L $MgCl_2$, 0.3 mol/L KCl, 5 mmol/L dithiothreitol, and 0.5 mmol/L ATP, cytidine triphosphate, or guanosine triphosphate) and 100 μCi [^{32}P]-uridine triphosphate (800 Ci/mmol; ICN, Costa Mesa, CA) to the nuclear suspension and by incubation at 30°C for 30 minutes. The nuclei were then pelleted by centrifugation at 500 × *g* in a JS 4.2 rotor, and RNA was extracted using the RNeasy Mini Kit and QIAshredder columns (Qiagen, Valencia, CA). The radiolabeled runoff transcripts were then used to probe slot blots prepared using 1 μg unlabeled antisense GLUT1 and antisense β-actin (rat) riboprobes. Hybridization was performed at 42°C overnight in 50% formamide, 6× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 2× Denhardt solution, 1% sodium dodecyl sulfate (SDS), and 100 μg/mL tRNA. The blots were washed and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA) for quantitation. GLUT1 was normalized to the β-actin values for each blot, and transcription rates were expressed as a percent of the control, setting the transcription rate under control (normoxic) conditions to 100%.

Western Blot Analysis

Western blot analysis of GLUT1 protein was performed essentially as described previously.[11] A polyclonal antiserum was kindly provided by Dr S. Frost and was generated to a peptide corresponding to the COOH terminus of GLUT1 (CEELFHPLGADSQU) conjugated to keyhole limpet hemocyanin.[12] Cells were washed three times with phosphate-buffered saline (PBS), pH 7.4, and lysed with 20 mmol/L Tris hydrochloride (pH 7.5), 3 mmol/L EDTA, 0.5 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.5% SDS, and 1% mercaptoethanol. After protein determination, samples were subjected to SDS-polyacrylamide gel electrophoresis followed by electroblotting as described previously.[11]

Nitrocellulose membranes were blocked by incubation with 2% gelatin for 30 minutes at 24°C, followed by incubation with a 1:1,000 dilution of anti-GLUT1 antibody. Membranes were also incubated without primary antibody or with normal rabbit serum in place of primary antibody for controls. After rinsing, the membranes were incubated with ^{125}I-protein A (2×10^5 cpm/μL) for 4 hours at 4°C. Excess unbound ^{125}I-protein A was removed, and the membranes were subjected to autoradiography.[17] Bands corresponding to GLUT1 were quantified by scanning densitometric analysis.[18-20]

2-Deoxyglucose Accumulation

^{3}H-deoxyglucose accumulation by cells is a measure of both its uptake and its phosphorylation by hexokinase. 2-Deoxyglucose (2-DG) accumulation in cells cultured in 35-mm dishes was determined after a modification used by Werner et al.[21] After experimentation, the medium was aspirated and the cells were rinsed gently in PBS. Cells were incubated for 5 minutes at 37°C in PBS containing 0.5 mmol/L 2-DG and ^{3}H-deoxyglucose (1 μCi/mL; NEN-Dupont, Boston, MA). The cells were then washed repeatedly with ice-cold PBS and solubilized by incubation in 0.2N NaOH for 1 hour at room temperature. Data for specific activity were used to calculate glucose accumulation in nanomoles per milligram of protein.[22]

Cell Viability

Viability was determined at each time point by quantifying the amount of lactate dehydrogenase retained intracellularly by viable cells compared to that released by damaged or dead cells into the culture medium immediately before and after experimentation, based on established techniques.[23]

Statistical Analysis

Time-specific differences between control values and results obtained under conditions of hypoxia, glucose deprivation, or both were analyzed for significance by the Student *t* test. A two-sample *t* test was used to evaluate the time-dependent trend in steady-state mRNA levels in hypoxic cultures treated with or without actinomycin D. A two-way ANOVA was used to determine the overall effect of hypoxia on mRNA levels from cultured cells incubated in variable glucose concentrations.

RESULTS

Time Course of Induction of Hypoxia

Figure 1 shows a representative experiment in which the time to achieve stable, extreme hypoxic conditions for cultured neurons was determined in the incubation medium and in the atmosphere of the chamber. Atmospheric oxygen became unmeasurable within less than 10 minutes of switching the cells to a 5% CO_2 and 95% N_2 gas mixture, whereas about 25 minutes were required to produce a similar degree of O_2 depletion in the culture medium.

Cell Viability

Table 1 summarizes the viability of cultured neurons and glia immediately before and after variable periods of hypoxia or glucose deprivation. Neuronal viability remained stable during 6 hours of hypoxia but declined about 12% after 24 hours of hypoxia or 6 hours of combined glucose and oxygen starvation. However, by 24 hours of glucose/oxygen starvation, viability was decreased greater than 50%.

Glucose Transporter mRNA

Twenty-four hours of hypoxia or hypoxia plus glucose deprivation led to a striking elevation in GLUT1 and GLUT3 steady-state mRNA levels in cultures obtained from 1-day-old

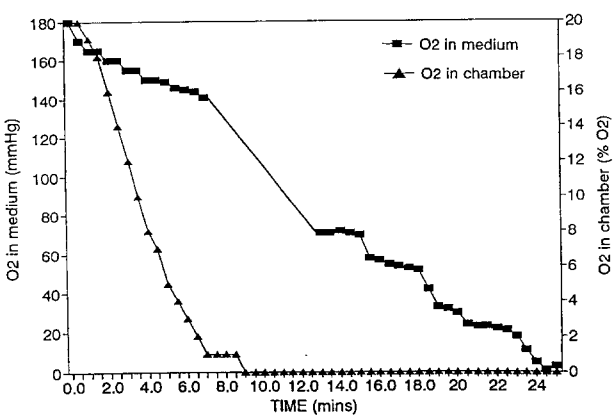

Fig 1. Time course of induction of hypoxia in a primary culture of 1-day-old rat neurons. Shown are the changes in oxygen concentration measured in the culture medium and in the atmosphere of the incubation chamber containing the culture.

Table 1. Cell Viability (%) Before and After 6 or 24 Hours of Oxygen or Oxygen/Glucose Starvation

	Incubation Time and Condition				
Cell Type	0 h $+O_2/+G$	6 h $-O_2/+G$	6 h $-O_2/-G$	24 h $-O_2/+G$	24 h $-O_2/-G$
Neurons	92 ± 3	93 ± 3	82 ± 2	82 ± 3	43 ± 20
1-day-old glia	84 ± 3	80 ± 5	79 ± 4	79, 90	20 ± 5
21-day-old glia	91 ± 1	85 ± 5	90 ± 6	88 ± 3	43 ± 14

NOTE. Neurons and glia were cultured from 1-day-old or 21-day-old rats. Cultures were maintained in DMEM, and the medium was exchanged with fresh DMEM 24 hours before experimentation. Lactate dehydrogenase activity was measured in the cells and medium immediately before experimentation (time 0) and following 6 and 24 hours of incubation in the absence of oxygen (O_2) ± glucose (G). The percentage of viable cells was calculated.[18] Data represent the mean ± SE of ≥4 experiments for each incubation time and condition.

neurons or 21-day-old glia (Figs 2 and 3). The mean fold-increase in neuronal GLUT1 message levels was 41 ± 9 (n = 4, $P < .02$) greater than the control GLUT1 to Cu/Zn SOD ratio (basal level) after 24 hours of hypoxia and 128-fold (n = 2) above basal after 24 hours of combined glucose/oxygen starvation. Similarly, in glia cells exposed to 24 hours of hypoxia with or without glucose deprivation, GLUT1 mRNA levels increased 99-fold and 22-fold, respectively (n = 2). GLUT3 mRNA levels increased approximately sevenfold in neurons after 24 hours of hypoxia. Although no GLUT3 message could be detected in glia cultured from 21-day-old animals, GLUT3 was readily observed after incubating these cells for 24 hours under hypoxic conditions.

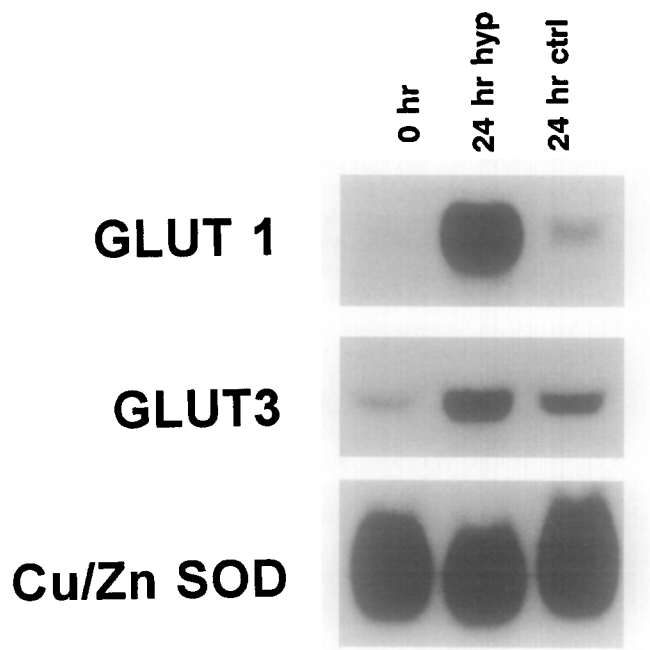

Fig 2. Effect of 24 hours of hypoxia on steady-state mRNA levels for GLUT1 and GLUT3 in neurons cultured from 1-day-old rat brains. In this representative experiment, 24-hour hypoxia led to increased GLUT1 and to a lesser extent GLUT3 mRNA, whereas parallel cultures maintained for 24 hours under air atmosphere showed only modest increments in mRNA GLUT transcript levels. Cu/Zn SOD was used as a constitutively expressed control message.

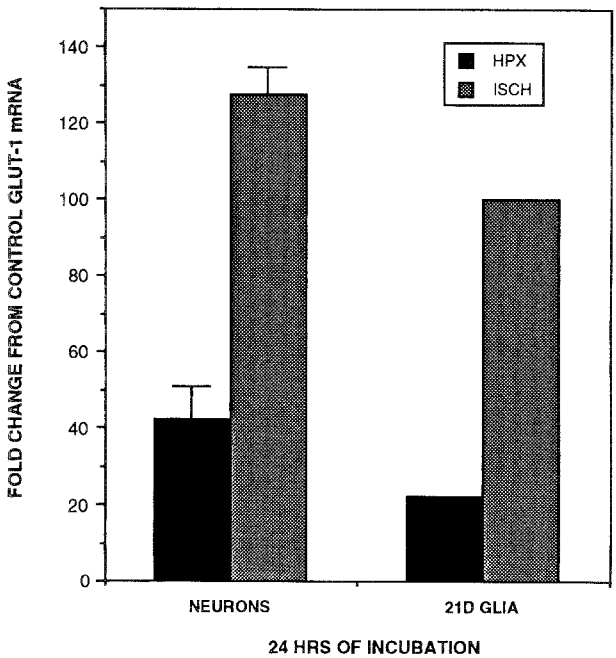

Fig 3. Effect of 24 hours of hypoxia (HPX) or combined oxygen/glucose deprivation (denoted for simplicity as "ischemia," ISCH) on GLUT1 mRNA levels in cultures of 1-day-old neurons or 21-day-old glia. Northern analysis was performed, and message levels were quantified by scanning densitometry. Changes in mRNA levels are expressed as the fold-increase relative to control levels recorded at time 0, before induction of hypoxia.

To gain insight into the time course and mechanism of the increase in steady-state GLUT message levels induced by hypoxia and glucose/oxygen deprivation, neurons and glia cultured from 1-day-old rats were exposed to a maximal 6 hours of hypoxia with or without glucose deprivation following a 20-minute pretreatment with actinomycin D, an inhibitor of transcription (Fig 4). In the absence of actinomycin D, an increase in neuronal GLUT1 mRNA was detected by 20 minutes of hypoxia or glucose/oxygen starvation (not shown), and it was increased about threefold after 6 hours of hypoxia and fivefold to 16-fold after 6 hours of glucose/oxygen starvation ($P < .05$). These increments in GLUT1 mRNA were abolished by pretreatment of the cells with actinomycin D and were replicated in two other independent experiments. These results suggest that transcription could be the primary determinant of increased GLUT1 RNA levels. To investigate this, nuclei were rapidly isolated from cultures maintained under normoxic conditions or hypoxic conditions for 3 hours and 6 hours, and nuclear runoff transcription assays were performed. Fig 5A and B shows that hypoxia did not affect the transcriptional rate of GLUT1. Therefore, increased transcription of GLUT1 does not account for the increased abundance of GLUT1 transcript under hypoxia, suggesting the existence of a posttranscriptional mechanism of regulation.

To test whether the stability of GLUT1 mRNA was affected by hypoxic conditions, GLUT1 RNA stability was measured under normoxic and hypoxic conditions (Fig 6). Neurons were exposed to 3 hours of hypoxia or normoxia and 7.5 μg/mL

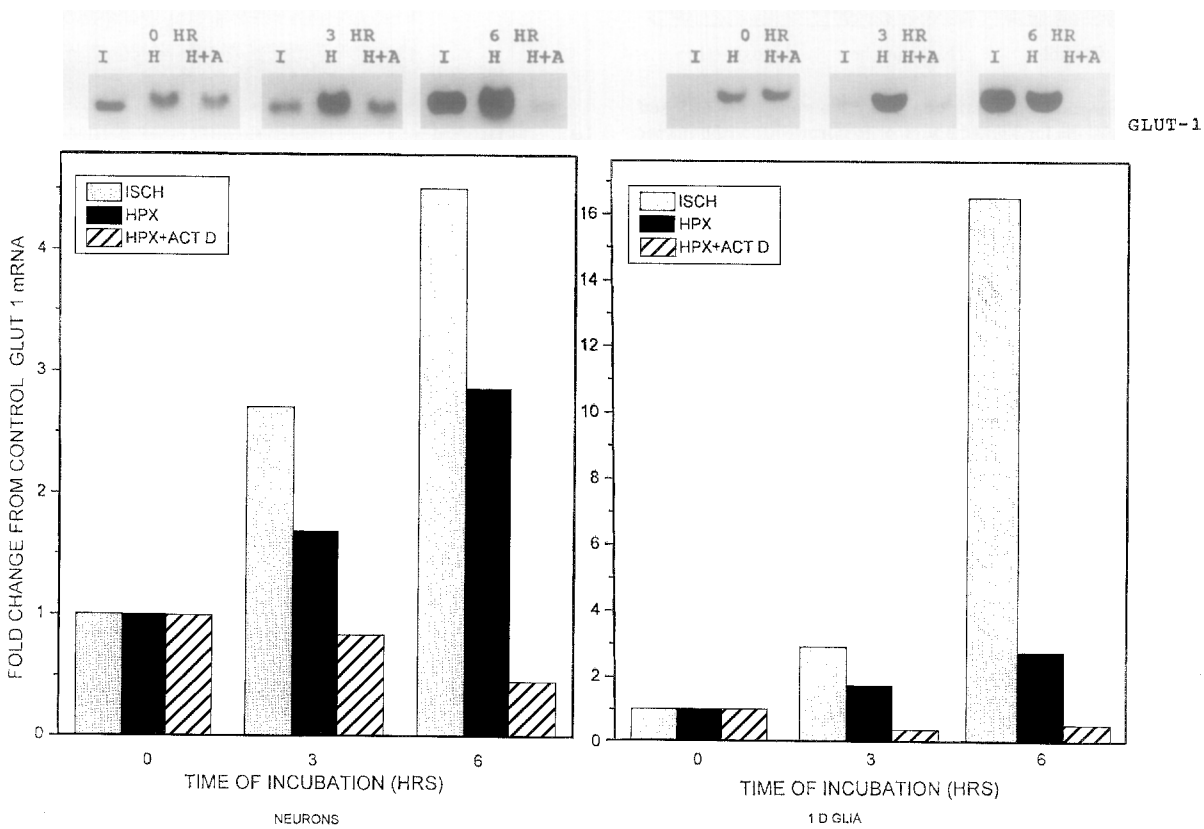

Fig 4. Effect of actinomycin D (A) on GLUT1 mRNA levels derived from cultures of 1-day-old neurons exposed to a maximal 6 hours of hypoxia (H) or combined oxygen/glucose deprivation (denoted for simplicity as "ischemia," I).

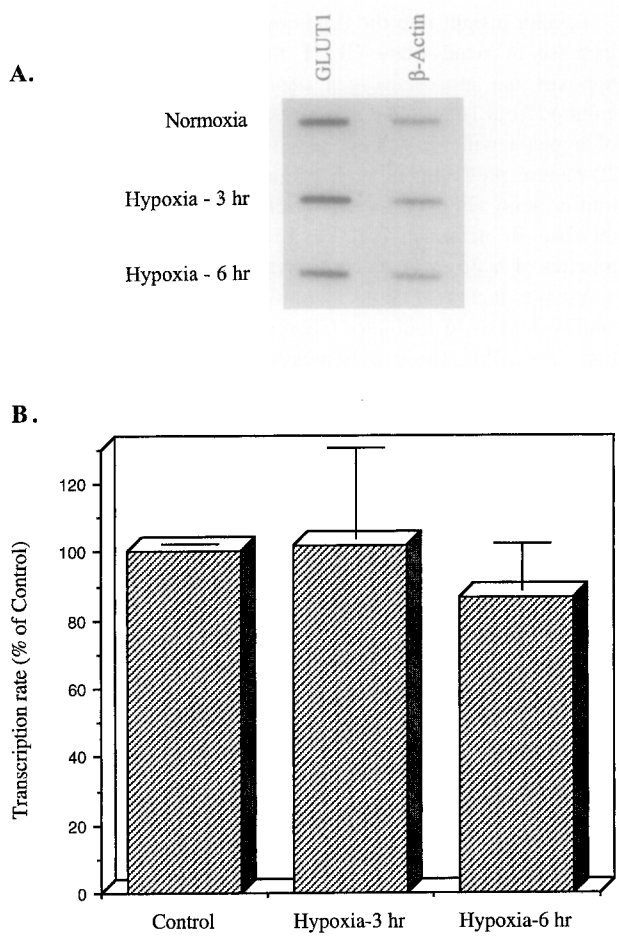

Fig 5. Nuclear runoff transcription assay. Neurons were exposed to hypoxic conditions for 3 and 6 hours, or to normoxic conditions for 6 hours (control). Nuclei were rapidly isolated from each treatment, and nuclear runoff transcription assays were performed. (A) Hybridization patterns for GLUT1 and rat β-actin are shown for the 3 treatments. (B) Quantitations from 3 independent experiments were used to calculate the mean transcription rate (mean ± SD) for GLUT1.

actinomycin D was added. Incubation was then continued under hypoxic or normoxic conditions for 1.5, 3, 6, and 12 hours. The GLUT1 message was extremely stable (half-life [$t_{1/2}$] ~10.4 hours) even under normoxic conditions. Hypoxic conditions further increased GLUT1 stability ($t_{1/2}$ >12 hours). These data suggest that increased GLUT1 transcript levels during hypoxia in neurons are due to increased mRNA stability (Fig 6).

To determine the reversibility of these transcriptional effects, neuron and glia cultures were exposed to 24 hours of hypoxia and then returned to a normoxic environment (Fig 7). Steady-state GLUT1 mRNA levels increased 25- to 60-fold in neurons ($P < .01$) and 15-fold ($P < .05$) in glia (data not shown) and gradually declined to near-basal levels within 24 hours of reexposure to atmospheric air. GLUT3 mRNA levels increased about sevenfold after 24 hours of hypoxia in neurons, and this effect was also reversible when cells were returned to a normoxic environment.

The effect of glucose concentration on GLUT1 mRNA levels in cells exposed to 6 hours of normoxia or hypoxia was also examined. Neurons were cultured for 24 hours in media containing 1 to 50 mmol/L D-glucose (Fig 8). In neurons incubated in air atmosphere, varying the incubation medium glucose concentration over a wide range had no effect on GLUT1 message (n = 3). In contrast, neuronal GLUT1 mRNA levels were increased threefold by 24 hours of hypoxia (n = 3; $p < .05$ for comparison of grouped data, hypoxia *v* normoxia). This effect occurred regardless of the ambient glucose concentration, but was most evident in cells incubated in the lowest (1 mmol/L) glucose concentration, in which hypoxia stimulated steady-state GLUT1 mRNA levels at least fourfold compared with cells incubated without glucose in an air atmosphere. Similar results were obtained in glia (data not shown). Thus, in both cell types, the steady-state GLUT1 mRNA level could be increased by hypoxia independently of the ambient glucose concentration.

Glucose Transporter Protein

Western blotting was performed to investigate the effect of hypoxia with or without glucose starvation on GLUT1 protein levels in neurons and glia (Fig 9). GLUT1 levels increased steadily in hypoxic neurons, particularly glucose/oxygen-deprived neurons, over 24 hours of incubation. Glia obtained from 1-day-old or 21-day-old rats also showed time-dependent increments in GLUT1 protein levels, and this effect was most

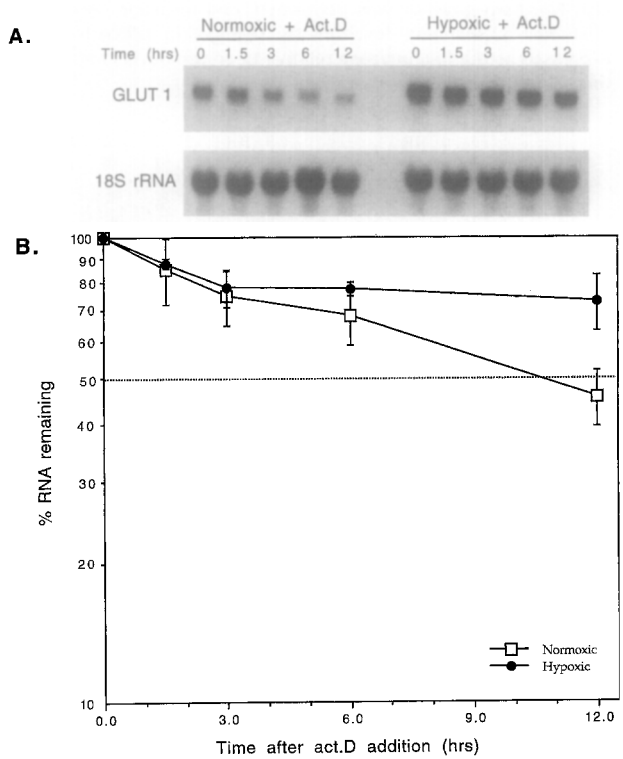

Fig 6. Stability of GLUT1 mRNA under normoxic and hypoxic conditions. (A) Neurons were exposed to normoxic or hypoxic conditions for 3 hours, actinomycin D (7.5 μg/mL) was added, and incubation was continued for 1.5, 3, 6, and 12 hours. Northern blot analyses for GLUT1 and 18S rRNA were performed with 10 μg total RNA per lane. (B) Hybridization patterns of 2-5 independent experiments for each time point (mean ± SD) expressed as the percentage of GLUT1 mRNA remaining, with the mRNA content at time 0 set to 100%.

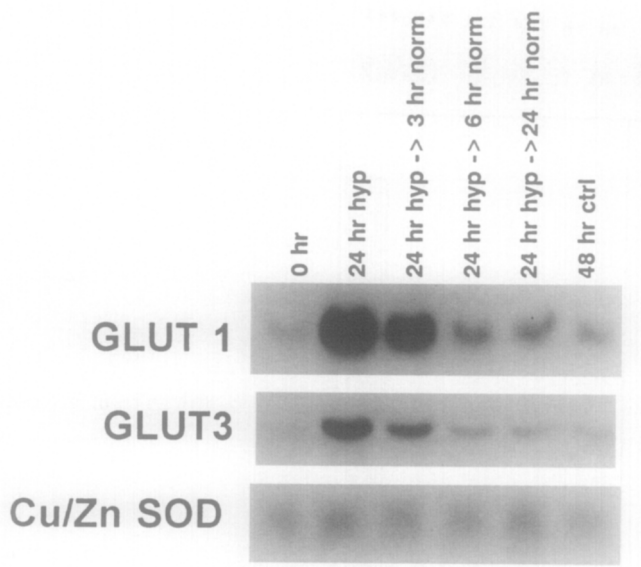

Fig 7. Reversibility of the effect of hypoxia on GLUT1 and GLUT3 mRNA levels in cultures of 1-day-old neurons. Cells were exposed to 24 hours of hypoxia and then returned to an air atmosphere for variable times prior to RNA isolation. Northern blot analysis was performed.

pronounced in 21-day-old cells incubated for 24 hours under conditions of both glucose and oxygen deficiency.

Glucose Transporter Activity

2-DG accumulation by the various cell types, as a measure of 2-DG uptake, phosphorylation, or both, under basal (10 mmol/L glucose, air atmosphere) conditions did not change significantly at any time during 24 hours of incubation. The mean rate of 2-DG accumulation was as follows: neurons, 50.8 ± 6.1 mmol/mg/min (n = 21); 1-day-old glia, 30.7 ± 4.1 (n = 24); and 21-day-old glia, 10.9 ± 1.5 (n = 6).

In both neurons and glia, hypoxia and particularly glucose/oxygen starvation stimulated 2-DG accumulation by the cells (Fig 10). 2-DG uptake increased significantly 1.6-fold after 24 hours of exposure to hypoxia and 1.9-fold under conditions of combined glucose/oxygen starvation. More striking changes in 2-DG accumulation were observed in glia cells cultured from 1-day-old or 21-day-old rats. By 24 hours of incubation, 2-DG accumulation by neonatal cells increased 2.5-fold after 24 hours of hypoxia and ninefold after 24 hours of glucose/oxygen deficiency. In 21-day-old glia, 6 hours of hypoxia with or without glucose deprivation stimulated intracellular 2-DG accumulation over twofold above control rates. By 24 hours of incubation, 2-DG accumulation by hypoxic cells was 3.6-fold above the control, while 2-DG uptake by glucose/oxygen-starved cells was 15-fold above the level measured in cells incubated for 24 hours in an air atmosphere and with glucose.

To determine the effect of the ambient glucose concentration on glucose transporter activity in cultured neurons and glia, 2-DG accumulation was determined after incubating cells for 24 hours under normoxic or hypoxic conditions and at variable glucose levels (Fig 11). In cells exposed to air atmosphere, the glucose concentration had little effect on 2-DG accumulation, except in 21-day-old glia cells cultured in 1.0 mmol/L glucose. 2-DG accumulation either was not significantly changed or was inhibited when neurons or glia from 1-day-old animals were incubated for 24 hours in air atmosphere and media glucose concentrations of 1.0 to 100 mmol/L. In contrast, intracellular 2-DG accumulation increased significantly when 1-day-old glia were incubated in 0 mmol/L glucose, particularly under hypoxic conditions. Cells from 21-day-old rats demonstrated a marked stimulation of 2-DG accumulation that was reciprocally related to the media glucose level over the 0- to 50-mmol/L concentration range.

DISCUSSION

At least three genetically distinct glucose transporter proteins (GLUT1, GLUT3, and GLUT5) coexist in the CNS of mammals.[5,24] Although the functional significance of the GLUT5 protein for brain cells is uncertain, GLUT1 and GLUT3 are considered responsible for determining the rate of glucose translocation in neurons and glia, and can be regulated in part by hormones, nutrients, ontogeny, and disease.[1-9,25,26]

Our data indicate that oxygen deprivation, particularly glucose/oxygen deprivation, represents an additional perturbant of glucose transporter expression in mammalian brain. Steady-state message levels of GLUT1 and GLUT3 increase within 1 to 3 hours of exposure of cultured neurons or glia to oxygen starvation, and this effect is magnified when cells are deprived of both oxygen and glucose. The changes in GLUT1 and

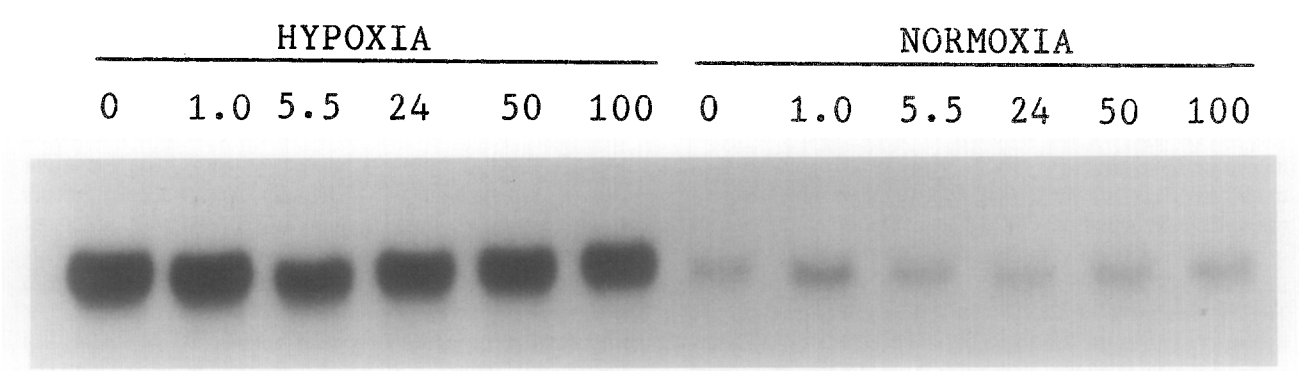

Fig 8. Effect of glucose concentration (0-100 mmol/L) on GLUT1 mRNA levels in cultures of 1-day-old neurons. On the day of experimentation, cultures were washed with glucose-free medium and incubated in culture medium containing the desired concentration of glucose. Northern blot analysis was performed.

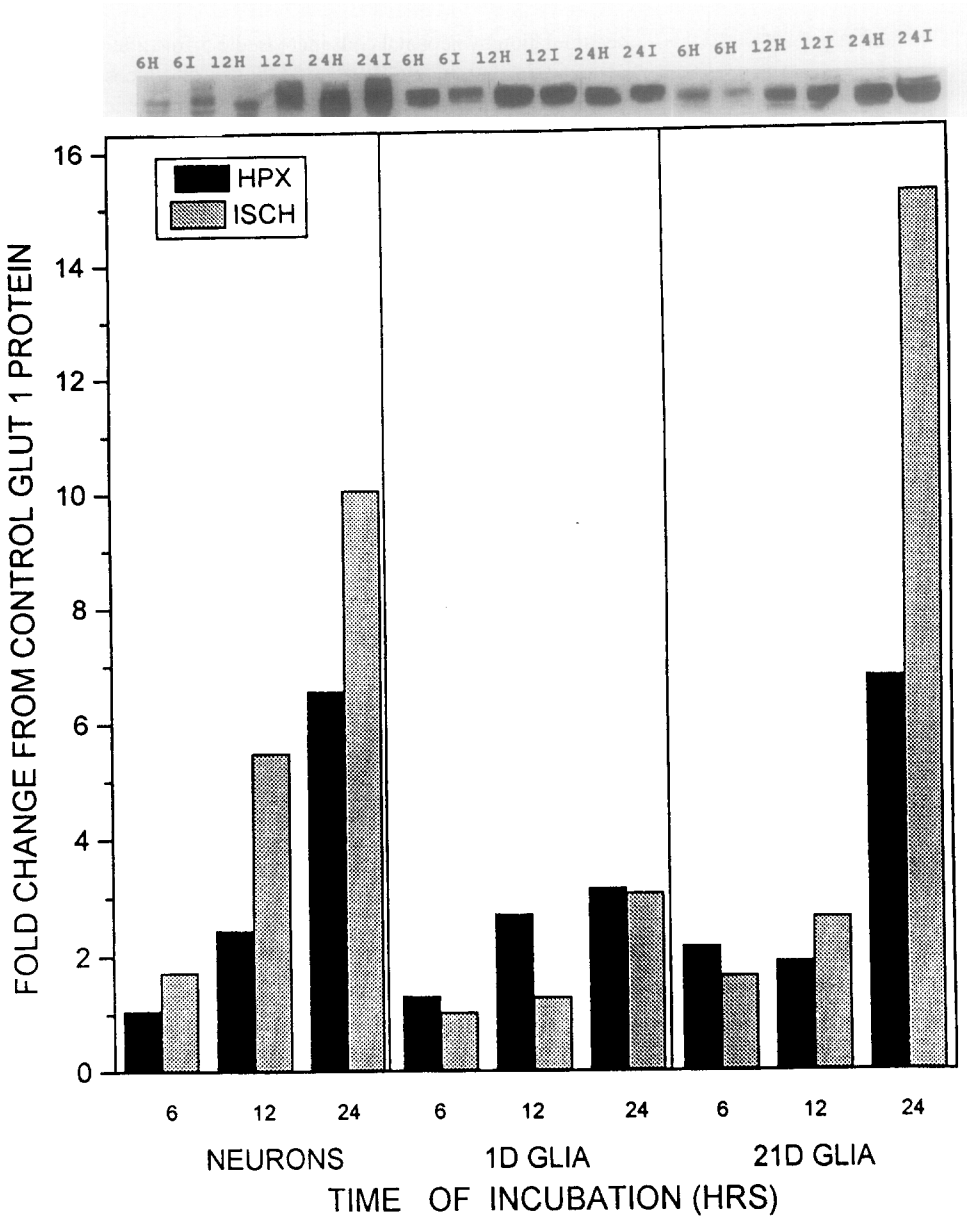

Fig 9. Effect of hypoxia (HPX) or oxygen/glucose deprivation (denoted for simplicity as "ischemia," ISCH) on GLUT1 protein levels in cultures of 1-day-old neurons or glia or 21-day-old glia. Western blot analysis was performed, and protein levels were quantified by scanning densitometry.

GLUT3 expression are similar to those observed by in situ hybridization in rats following transient occlusion of the middle cerebral artery.[27] The increase we measured in GLUT1 (and presumably GLUT3) mRNA induced by hypoxia is time-dependent and reversible, and the induction of transcript levels can be blocked by the addition of actinomycin D before incubation under hypoxic conditions. However, nuclear runoff transcription assays show that hypoxia does not affect the transcriptional rate of the GLUT1 gene. Therefore, the addition of actinomycin D must inhibit the expression of other genes important for increasing GLUT1 mRNA steady-state levels. The measurement of GLUT1 mRNA stability showed that hypoxia increases the RNA stability of GLUT1 ($t_{1/2}$ >12 hours) compared with normoxic conditions ($t_{1/2}$ ~10.4 hours). Therefore, the induction of GLUT1 transcript levels during hypoxia in neurons appears to be primarily a consequence of enhanced mRNA stability. A similar mechanism of regulation of GLUT1 has been reported in brain capillary endothelial cells, where glucose deprivation enhances RNA stability without affecting transcription of the gene.[28]

It should be noted that the extremely long $t_{1/2}$ of GLUT1 in neurons measured under normoxic conditions is not typical of other cell systems that have been studied. For example, the GLUT1 $t_{1/2}$ in C6 (rat glial tumor cell line) cells is about 0.52 hours,[29] about 3 hours in mouse primary juxtaglomerular cells,[30] about 4.8 hours in 3T3-L1 adipocytes,[31] about 1.5 hours in clone 9 cells (a nontransformed rat liver cell line),[32] and about 5 hours in rat skeletal muscle L5E9 myoblast/myotubes.[33] This wide disparity in the GLUT1 $t_{1/2}$ in different cell types points to cell/tissue-specific regulation of GLUT1 mRNA stability. UV cross-linking has identified protein factors (120, 44, and 41 kd) that interact with the 3′-untranslated region of the GLUT1 message and influence its stability.[34,35] Selective downregulation of the 44-kd complex (rapid decay factor) in cerebral hemangioblastoma compared with glioblastoma multiforme has been linked with overexpression of GLUT1 protein in the

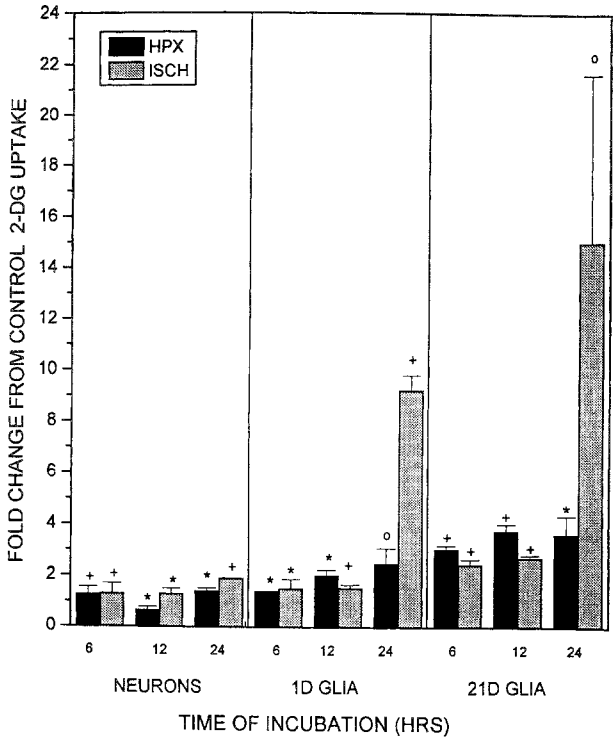

Fig 10. Stimulation of 2-DG uptake by hypoxia (HPX) or oxygen/glucose deprivation (denoted for simplicity as "ischemia," ISCH) in cultures of 1-day-old neurons or glia or 21-day-old glia. Following the indicated periods of hypoxia or ischemia, cells were washed and incubated in PBS containing 0.5 mmol/L 2-DG and 1 μCi/mL ^{3}H-deoxyglucose. Data are the mean ± SE. $^+P < .05$, $^*P < .01$, and $^\circ P < .001$ *v* control values. The number of individual experiments was 6-24, depending on the experimental condition.

former and underexpression in the latter.[35] Therefore, primary rat neurons seem to possess elaborate mechanisms for GLUT1 mRNA stabilization ($t_{1/2}$ ~10.4 hours) even under normoxic conditions, with the capability to further increase RNA stability under hypoxia. It is reasonable to speculate that neurons have evolved such elaborate posttranscriptional mechanisms to preserve constitutive high-level expression of molecules like the GLUT1 protein critical to substrate utilization and energy metabolism.

It is noteworthy that a low concentration of glucose in the incubation medium was itself insufficient to provoke changes in GLUT1 or GLUT3 mRNA as long as the atmospheric O_2 concentration was normal. Instead, a lack of glucose appeared to exert a permissive effect to increase glucose transporter expression in response to oxygen deficiency beyond that achieved by low O_2 alone.

The stimulation of GLUT1 mRNA levels in neurons and glia by 24 hours of hypoxia was associated with an increase in both GLUT1 protein, determined by Western blotting, and glucose transporter activity, measured by cellular accumulation of 2-DG. Both of these effects were accentuated by the absence of oxygen plus glucose. Compared with neurons, glial cells obtained from neonatal or 21-day-old rats had lower basal 2-DG accumulation but a higher response of 2-DG accumulation to glucose/oxygen deprivation. The reason for the differential response in 2-DG accumulation by neurons and glia is unknown. However, glia are reported to have a greater capacity than neurons to store glycogen[36,37] and thus may act as a potential reservoir of metabolizable substrate for neurons under conditions of glucose deprivation. It is noteworthy that the magnitude of change in GLUT protein and activity induced by oxygen starvation or combined glucose/oxygen deprivation was considerably less than the observed increase in GLUT1 and GLUT3 mRNA levels. If these apparent discrepancies also

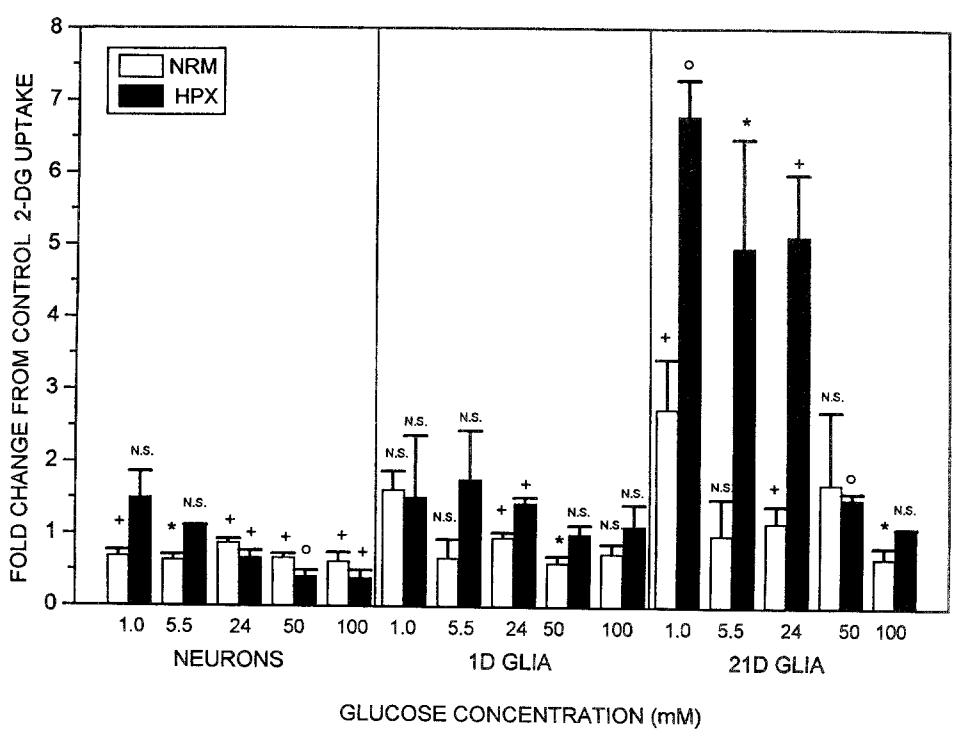

Fig 11. Effect of variable glucose concentration on 2-DG uptake by cultures of 1-day-old neurons or glia or 21-day-old glia incubated under hypoxic (HPX) or normoxic (NRM) conditions. See Fig 10 for experimental details. Data are the mean ± SEM. $^+P < .05$, $^*P < .01$, and $^\circ P < .001$ *v* control values.

occur in vivo, then cell damage may be due in part to the inability of brain cells to translate GLUT messages sufficiently during conditions of limited availability of oxygen and glucose. Such a marked quantitative discrepancy in the magnitude of change in mRNA levels and protein induced by hypoxia is consistent with evidence demonstrating a much greater vulnerability of translational versus transcriptional events in response to ischemia or other cellular injuries.[38,39] At present, it is unknown whether important differences exist in the energy threshold for disruption of GLUT1 protein synthesis and transport activity. Nevertheless, the fact that GLUT1 protein levels and glucose transporter activity are increased by a lack of oxygen with or without glucose suggests that at least some of the glucose transporter message induced by these perturbations is translated into functional protein, a conclusion supported by similar results in studies of cultured endothelia[40] and intact rat hearts.[41] However, a caveat to this interpretation is the possibility that glucose and oxygen deficiency also stimulate glucose uptake, phosphorylation, or both by facilitating the translocation of preformed GLUT proteins from an intracellular site to the plasma membrane. Such an effect on GLUT4 has been reported to occur in rat hearts perfused with O_2-depleted medium[42] and in rat skeletal muscle incubated under hypoxic conditions.[43]

Regardless of the underlying mechanism(s) for the changes we observed, an increase in the rate of glucose transport in response to glucose deprivation[44,45] or hypoxia[46-48] has been reported for a number of different cell types, and probably reflects a common mechanism to facilitate substrate availability for energy production, particularly when glucose uptake is rate-limiting for carbohydrate metabolism. Indeed, GLUT1, and perhaps other glucose transporters, is considered to belong to a glucose-regulated family of stress-induced proteins expressed to varying degrees in many cell types.[49,50] To date, glucose deprivation has been the principal stimulus used to induce these proteins. However, it is reasonable to assume that hypoxia or glucose/oxygen starvation could also regulate the expression of this family of molecules, the function of which may have survival value for the cell by optimizing substrate fuel availability. In this regard, it is noteworthy that the striking elevations of GLUT1 and GLUT3 mRNA we measured in hypoxic neurons and glia are severalfold greater than the increments in GLUT messages observed in non-CNS cells subjected to similar degrees and durations of hypoxia, glucose deprivation, or both.[40-43]

The fact that hypoxia stimulates glucose uptake and induces glucose transporter transcription in the brain (this report) and other cell types[40,41] in the presence of variable glucose concentrations implies that depletion of ambient glucose alone may not always be sufficient for enhanced GLUT expression, although the combination of hypoxia and glucose deprivation appears necessary to elicit the maximal effect of hypoxia on GLUT transcriptional and posttranscriptional regulation. Likewise, the ambient O_2 concentration itself is unlikely directly responsible for the altered GLUT expression, since inhibitors or uncouplers of oxidative phosphorylation provoke increases in glucose transporter activity and GLUT1 mRNA levels in cultured endothelial cells similar to the increases achieved by hypoxia alone.[51-53] If so, the absence of a direct O_2-sensing mechanism by eukaryotic cells may distinguish them from bacteria, in which oxidation of an OxyR protein activates transcription of stress-inducible genes that protect against oxidative damage.[54]

In conclusion, the major findings of this study are as follows: (1) hypoxia stimulates a reversible increase in GLUT1 (and probably GLUT3) steady-state mRNA levels in primary cultures of rat neurons and glia; (2) this effect appears independent of the specific type or age of the cells; (3) enhanced RNA stability, not increased transcription, may be primarily responsible for the hypoxia-induced steady-state levels of GLUT1; (4) although the ambient glucose concentration per se has little effect on mRNA induction, glucose deprivation plus hypoxia synergize to further increase steady-state message levels; (5) the increase in GLUT mRNA is temporally associated with increased GLUT1 protein and transporter activity; and (6) these findings are consistent with the hypothesis that the induction of GLUT1 and GLUT3 gene expression by hypoxia or combined glucose/oxygen deprivation is translated into biologically active glucose transporters that facilitate substrate entry into brain cells in an effort to maintain glycolytic energy supply under conditions of limited oxidative phosphorylating capacity.

ACKNOWLEDGMENT

We thank M. Riedy for manuscript preparation, M. Rupp for technical assistance, C.S. Sumners, University of Florida, for providing neuronal cultures for some experiments, W.A. Dunn, University of Florida, for providing autoantibodies for immunofluorescent studies, and H.S. Nick, University of Florida, for providing cDNA probes.

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