GLUT1 Deficiency Links Nutrient Availability and Apoptosis during Embryonic Development*

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GLUT1 is essential for human brain development and function, as evidenced by the severe epileptic encephalopathy observed in children with GLUT1 deficiency syndrome resulting from inherited loss-of-function mutations in the gene encoding this facilitative glucose transporter. To further elucidate the pathophysiology of this disorder, the zebrafish orthologue of human GLUT1 was identified, and expression of this gene was abrogated during early embryonic development, resulting in a phenotype of aberrant brain organogenesis consistent with the observed expression of GLUT1 in the embryonic tectum and specifically rescued by human GLUT1 mRNA. Affected embryos displayed impaired glucose uptake concomitant with increased neural cell apoptosis and subsequent ventricle enlargement, trigeminal ganglion cell loss, and abnormal hindbrain architecture. Strikingly, inhibiting expression of the zebrafish orthologue of the proapoptotic protein Bad resulted in complete rescue of this phenotype, and this occurred even in the absence of restoration of apparent glucose uptake. Taken together, these studies describe a tractable system for elucidating the cellular and molecular mechanisms of GLUT1 deficiency and provide compelling in vivo genetic evidence directly linking nutrient availability and activation of mitochondria-dependent apoptotic mechanisms during embryonic brain development.

The cellular uptake of glucose is dependent in part upon the GLUT family of polytopic membrane transporters that facilitate the passive diffusion of this essential nutrient across membranes (1). GLUT1, the prototypic member of this protein family, is highly expressed in erythrocytes and the central nervous system and is believed to play an essential role in the homeostasis of brain glucose in the developing human infant (2). In support of this concept, infants with GLUT1 deficiency syndrome, a rare genetic disorder resulting from inherited heterozygous loss-of-function mutations in the gene encoding GLUT1, develop seizures, acquired microcephaly and profound developmental delay in association with profound hypoglycorrhachia (3). Despite considerable study of these patients, the neurochemical and neuropathologic consequences of GLUT1 deficiency during development are not well understood, and treatment of affected patients remains challenging.

Although abundant in the extracellular milieu, the cellular uptake of glucose is precisely regulated by specific growth factors and signaling pathways (4). Cell culture studies reveal that inhibition of cellular glucose uptake dramatically increases apoptosis under conditions of growth factor restriction and that cell survival under such circumstances is dependent upon the regulation of glucose uptake and metabolism by the proto-oncogene Akt (5–7). A biochemical link between glucose homeostasis and apoptosis was further suggested by studies demonstrating an interaction between the proapoptotic protein, Bad, and the glycolytic enzyme, glucokinase (8). In support of these findings, a recent study now demonstrates a direct link between glucose metabolism and apoptosis in Xenopus laevis oocytes mediated by activation of caspase-2 (9).

In the present study, we have utilized the developing zebrafish embryo to examine the mechanistic role of GLUT1 deficiency in early brain development. We hypothesized that the ease of genetic manipulation, rapid development, and optical transparency of these embryos would provide a unique opportunity to dissect the cellular and biochemical mechanisms following the perturbation of glucose homeostasis associated with loss of function of this transporter (10, 11). Our findings reveal the first in vivo data linking impaired nutrient availability and activation of apoptosis during early vertebrate development and provide new mechanistic insights relevant to the pathogenesis and treatment of affected patients.

MATERIALS AND METHODS

Zebrafish Maintenance and Analysis—Zebrafish were maintained and staged as described previously (12, 13). Wild-type zebrafish stocks were used throughout this study. Embryos were examined with an Olympus SZX12 stereomicroscope. Differential interference contrast (DIC) images were obtained using an Olympus IX71 microscope fitted with a Nomarski objective, and images were acquired with a TH4–100 camera (Olympus) and Olympus microsuite software. Fluorescently labeled embryos were visualized utilizing a laser-scanning confocal microscope (BX61WI FV500; Olympus), equipped with argon 488 and krypton 568 lasers utilizing the ×10 objective. Representative fish were imaged using identical confocal settings, and serial Z stacks were acquired using a pinhole aperture of 150 μm. Images were collected with Fluoview software (Olympus).

Identification and Cloning of Zebrafish glut1 Orthologue—The Ensembl zebrafish genome browser data base (available on the World Wide Web at www.ensembl.org; zebrafish assembly version 4 (Zv4)) was searched using the human GLUT1 protein sequence and the BLASTP program. A genomic clone, AL772391.17, was identified on chromosome 23 that contained an annotated region with the highest level of sequence identity (maximum BLAST probability, e−26) predicted to encode for a member of the facilitative glucose transporter family. The predicted amino acid sequence was reciprocally compared against the National Center of Biotechnology Information human protein sequence data

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probe, Inc.) and incubated at room temperature. At 5 and 60 min after injection, uptake of 2-deoxy-\(^{3}H\)glucose (Sigma) was measured as previously described (14). For inhibition experiments, oocytes were incubated with 20 \(\mu\)M cytochalasin B (Sigma) for 5 min prior to uptake. Experiments were repeated three times with at least 10 oocytes/experimental group.

**In situ Hybridization and Immunohistochemistry**—To generate an in situ probe, an 894-bp PCR product was amplified from zebrafish glut1 (forward, 5\(^\prime\)-GGCCTACATCACTAAGGAGGATCCAC-3\(^\prime\); reverse, 5\(^\prime\)-GGGCAGATTCAACTCTGTA-3\(^\prime\)) and used as template to prepare riboprobes using the DIG-labeling kit (Roche Applied Science). Various stage embryos were collected and fixed in fresh 4% paraformaldehyde overnight at 4 °C. Fixed embryos were dechorionated, dehydrated with an ascending methanol series (30, 50, and 100%), and stored in methanol at −20 °C overnight. In situ hybridization was performed as previously described (15). Antibody staining of whole-mounted embryos with anti-acetylated tubulin (Sigma), ZN-12 (antineuronal specific antigen) and 4D9 (antiengrailed) (Developmental Studies Hybridoma Bank, University of Iowa) was performed as described previously, and staining was detected using the ABC method following the manufacturer’s instructions (Vectastain).

**Morpholino Designs and Injections**—To inhibit the expression of glut1, antisense morpholinos targeting the translational start site (5\(^\prime\)-GGCCATCATCAGCTAAGGAGGATCCAC-3\(^\prime\)) and the exon–splice acceptor site of exon 3 (5\(^\prime\)-TTGAGTTCTCTGTTGGAAGATCACA-3\(^\prime\)) were synthesized (Gene Tools LLC, Philomath, OR). The inverse sequence of the glut1 translational start site (5\(^\prime\)-GGTGACTCCTGCTGAGGAGTCACC-3\(^\prime\)) and a nonspecific morpholino (5\(^\prime\)-CAGTTACAATTTATA-3\(^\prime\)) were designed. 2.5 ng of morpholinos were microinjected into embryos—48-hpf MO-treated embryos were incubated with 50 ng of mRNA prepared from zebrafish Glut1 cDNA, human GLUT1 cDNA, and zebrafish Glut1 inverse cDNA (negative control) (14). At 48 h after injection, uptake of 2-deoxy-\(^{3}H\)glucose (Sigma) was measured as previously described (14). For inhibition experiments, oocytes were incubated with 20 \(\mu\)M cytochalasin B (Sigma) for 5 min prior to uptake. Experiments were repeated three times with at least 10 oocytes/experimental group.

**Glucose Uptake**—The yolk sac of 24-hpf MO-treated embryos was injected with 5 mg/ml 2-(\(N\)-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminol)-2-deoxyglucose (2-NBDG, Molecular Probes) at 0.1 mM for 1 h followed by three washes with egg water. Embryos were mounted in a thin layer of 0.4% low melt agarose, and 1000 pl of fluorescein dextran (M, 70,000, Molecular Probes) was injected into the midbrain. Embryos were immediately imaged using confocal microscopy utilizing the argon 488 laser.

**RESULTS**

Identification and Characterization of Zebrafish glut1 Orthologue—To identify a zebrafish glut1 orthologue, a bioinformatics search was performed using the BLASTX program (available on the World Wide Web at www.ncbi.nlm.nih.gov). The identification of human GLUT1 as the closest match in this reverse search confirmed this gene as the zebrafish glut1 orthologue.

To generate a full-length cDNA, total RNA was extracted from 24-h postfertilization (hpf) embryos and reverse-transcribed to single-stranded cDNA using Superscript III (Invitrogen) according to the manufacturer’s instructions. PCR was performed using glut1-specific primers (forward, 5\(^\prime\)-GAGTCTTAATTTAAGGAGGTGACT-3\(^\prime\); reverse, 5\(^\prime\)-GAATCTGCCCC-3\(^\prime\)) and five independent clones were sequenced.

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formed using the amino acid sequence of human GLUT1. This search identified a 488-amino acid protein with a predicted molecular mass of 54 kDa that shares 74% sequence identity with human GLUT1 and contains the signature sequence motifs implicated in conferring glucose transport activity including a QLS motif in helix 7, glutamine 161 in helix 5, and tryptophan 388 in helix 10 (Fig. S1 (16)). Phylogenetic analysis of this sequence revealed that this protein was more closely related to the GLUT1 homologs of other species, including humans and pufffish (fugu) than with any of the other GLUT family members, including GLUT3, GLUT14, or GLUT4 (Fig. S2). Functional conservation of zebrafish Glut1 was confirmed by in vitro uptake of 2-deoxyglucose (2-DOG) into Xenopus oocytes (Fig. 1). Uptake into oocytes injected with zebrafish Glut1 mRNA and human GLUT1 mRNA was much greater than control oocytes injected with inverse zebrafish Glut1 mRNA (56.7 ± 12.2 versus 32.6 ± 14.6 versus 0.5 ± 0.1 pmol of 2-DOG/oocyte/30 min, respectively), and uptake was inhibited by cytochalasin B (Fig. 1). In situ hybridization of 6-hpf embryos showed ubiquitous expression of glut1 within the developing blastoderm, whereas at 18 hpf, expression of glut1 was more restricted to cells of the pronephric ducts and the central nervous system (Fig. 2, A and B). At 24 hpf, expression was observed in the central nervous system surrounding the tectal and hindbrain ventricles with continued expression in the developing kidney (pronephric ducts) (Fig. 2, C–E).

Knockdown of glut1 Resulted in Alteration in CNS Morphology—To abrogate the expression of glut1, two MOs, one to inhibit translation at the start methionine (ATG MO) and a second designed to interfere with splicing between exon 2 and exon 3 (Ex/In MO) were utilized. As shown in Fig. 3, inhibiting the expression of glut1 with either MO resulted in a similar and dramatic phenotype. At 24 hpf, glut1 morphant embryos were characterized by increased tissue opacity in the head, resulting in the loss of key morphological markers in the CNS (Fig. 3, A, C, and E). In addition, we consistently observed a thinner/smaller yolk sac extension (ye) and the fin fold (ff). By 48 hpf, glut1 morphant embryos (D and F) are characterized by enlarged tectal (tctv) and hindbrain (hbv) ventricles compared with control (B). G and H, dorsal view of 48-hpf embryos imaged using the membrane-specific dye BODIPY TR (red channel) and ventricle staining with fluorescein isothiocyanate-dextran (green channel) reveals the significant hindbrain enlargement of the morphant embryos and loss of the midbrain/hindbrain boundary (mhb).

FIGURE 3. Abrogation of glut1 causes severe morphant phenotype. A–F, phenotype of embryos injected with a MO targeting the glut1 translational start site (ATG MO) or the exon/intron boundary between exons 2 and 3 (Ex/In MO) compared with embryos injected with a control MO (Con MO). At 24 hpf (A, C, and E), the following brain structures are distinct in the control embryos but are not apparent in the glut1 morphants: diencephalon (dl), telencephalon (tel), tectal ventricle (tctv), midbrain/hindbrain boundary (mhb), tegmentum (tgm), and hindbrain ventricle (hbv). Instead, the neuronal compartment appears dense and opaque. Also illustrated are the yolk sac extension (ye) and the fin fold (ff). By 48 hpf, glut1 morphant embryos (D and F) are characterized by enlarged tectal (tctv) and hindbrain (hbv) ventricles compared with control (B). G and H, dorsal view of 48-hpf embryos imaged using the membrane-specific dye BODIPY TR (red channel) and ventricle staining with fluorescein isothiocyanate-dextran (green channel) reveals the significant hindbrain enlargement of the morphant embryos and loss of the midbrain/hindbrain boundary (mhb).
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To characterize the central nervous system in glut1 morphants, immunohistochemical analysis utilizing antibodies directed against neuronal specific antigens was performed. By 24 hpf, a simple axon scaffold has formed in the embryonic zebrafish brain, consisting of two bilaterally symmetrical longitudinal tracts connected by commissures, providing a template for subsequent development (Fig. 5, A and C) (17, 18). In contrast, the neuronal architecture of glut1 morphants was clearly altered, revealing thinner, poorly fasciculated longitudinal tracts and an apparent reduction in the trigeminal ganglion neurons as illustrated by staining with the neuron-specific antibody, antiacetylated tubulin (Fig. 5B). The disorganized neuronal architecture and hypothesized reduction in total neurons were further demonstrated by panaxonal staining with ZN-12, highlighting a reduction in the lateral and medial longitudinal fascicles (Fig. 5D). Additionally, a reduction in cells within the midbrain/hindbrain region was observed by staining for Engrailed, a transcription factor specifically expressed by cells within this region (Fig. 5, E and F) (19).

Glucose Uptake Was Inhibited and Apoptosis was Enhanced When glut1 Expression Was Abrogated—To assess the functional impact of inhibiting Glut1 expression, glucose uptake experiments utilizing a fluorescently labeled nonmetabolizable glucose analog, 2-NBDG, were performed. Within 5 min after injection, fluorescently labeled 2-deoxyglucose was observed in embryos treated with control MO at a level 7-fold higher than that observed in glut1 morphant embryos (Fig. 6, A and B, and Fig. S4A). Whereas a minimal level of fluorescence was visible in the morphant embryos at the 60-min time point, there was still a qualitative increase in 2-NBDG uptake observed in control embryos compared with the glut1 morphants (Fig. 6, C and D). With increased tissue opacity in the head and the apparent reduction of neurons observed by immunohistochemistry, we hypothesized that inhibiting Glut1 expression may be affecting cell survival in these regions. Therefore, to assess the presence of increased programmed cell death in the CNS of glut1 morphants, apoptosis was assayed by staining with the vital dye acridine orange. At both 19 and 24 hpf, an overall increase in apoptosis was observed in morphant embryos compared with controls (Fig. 6, E–H). At 19 hpf, there was a generalized increase in apoptosis throughout the embryo, whereas at 24 hpf, apoptosis appeared to be localized primarily to the CNS, with concentrated areas observed in the head and tail. There was an average 2-fold increase in apoptosis in glut1 morphant embryos compared with control embryos (Fig. S4B). A similar pattern of apoptosis was observed using terminal dUTP nick end labeling, a method that specifically labels fragmented DNA, an established marker of programmed cell death (Fig. S5).
An aberrant splice product, confirmed by nucleotide sequence analysis, in embryos injected with the splice site MO (Fig. S6).

Whereas the inhibition of bad expression reversed neuronal apoptosis and subsequent degeneration, the attenuation of bad expression did not rescue the impaired glucose uptake function conferred by abrogating glut1 expression alone (Fig. 7, E-G, and Fig. S6). Decreased expression of bad alone had no effect on glucose transport function of treated embryos (data not shown).

**DISCUSSION**

The data in this study reveal a critical role for Glut1 in the development of the embryonic vertebrate brain. Bioinformatic and 2-deoxyglucose uptake analyses confirmed the identification of a functional zebrafish glut1 orthologue (Figs. S1, S2, and 1). Expression analysis by in situ hybridization revealed that this transporter is expressed throughout the brain tectum at 24 hpf (Fig. 2), and abrogation of this expression resulted in a phenotype of impaired brain organogenesis specifically rescued by human GLUT1 mRNA (Figs. 3–5). These findings are probably a direct consequence of impaired glucose homeostasis, as evidenced by the marked decrease in 2-deoxyglucose uptake from the yolk sac of affected embryos (Fig. 6). Whereas the predominant phenotype observed in this study is brain neurodegeneration, a role for Glut1 in other aspects of early development is suggested by the complex phenotype (Fig. 3) and excess apoptosis observed throughout the embryo (Fig. 6).

Most importantly, our findings provide a direct in vivo link between glucose homeostasis and the mitochondria-dependent apoptotic pathway during development as evidenced by the attenuation of glut1-dependent apoptosis and rescue of the neurodegenerative phenotype in the absence of bad expression (Fig. 7). Strikingly, this inhibition of apo-
ptosis occurs without apparent restoration of glucose availability, suggesting that cell death in the absence of Glut1 is driven not by the gradual decline of metabolism due to insufficient cellular fuel per se but rather by induction of the mitochondrial cell death pathway due to the sensing of inadequate nutrient availability. The concept that activation of glucose-dependent apoptosis is not the result of impaired metabolism but rather that the sensing of intracellular energy levels is acting to directly regulate the mitochondrial cell death pathway is strongly supported by recent elegant studies of *X. laevis* oocyte survival directly linking glucose metabolism and apoptosis via activation of caspase-2 (9).

A role for glucose in cell survival is consistent with prior cell culture studies demonstrating that inhibition of cellular glucose uptake dramatically increases apoptosis under conditions of growth factor restriction and that cell survival under such circumstances is dependent upon the regulation of glucose uptake and metabolism by the proto-oncogene Akt (5–7). The involvement of Bad in this process is supported by findings that Akt promotes cell survival by phosphorylating and thus inhibiting the proapoptotic protein Bad (20) as well as biochemical studies revealing a late the mitochondrial cell death pathway is strongly supported by that the sensing of intracellular energy levels is acting to directly regulate glucose-dependent apoptosis is not the result of impaired metabolism but rather that the sensing of intracellular energy levels is acting to directly regulate the mitochondrial cell death pathway is strongly supported by recent elegant studies of *X. laevis* oocyte survival directly linking glucose metabolism and apoptosis via activation of caspase-2 (9).

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The presented studies demonstrate that zebrafish provide a tractable system for elucidating the cellular and molecular mechanisms of GLUT1 deficiency. This vertebrate model may allow further elucidation of the signaling events linking glucose homeostasis and apoptosis by analysis of a group of zebrafish mutants termed *spacehead* (22, 23). These mutants, consisting of at least 30 independent complementation groups, have a phenotype that is identical to that observed in the *glut1* morphants (Fig. 3), suggesting that at least some of these loci may encode genes in the signaling pathways critical for coupling glucose homeostasis and cell survival during development. Such findings will have important fundamental implications for understanding the role of metabolic intermediates as mediators of cell signaling and would extend the recently developed paradigm of metabolic intermediates as ligands in signal transduction pathways (24).

Our finding that vertebrate brain development is critically dependent upon Glut1 expression may also permit new approaches to elucidating the pathogenesis of human GLUT1 deficiency syndrome. Importantly, our current data are not yet a precise model for this disease, since affected patients retain one functional GLUT1 allele and present in the postnatal period with signs and symptoms reflecting the acute sensitivity of the central nervous system to impaired glucose homeostasis. However, further elucidation of the role of glucose homeostasis in regulating the mitochondrial death pathway during vertebrate brain development should provide insights into the cellular and molecular basis of the complex neurological phenotypes observed in these patients including mental retardation and impaired cognition. Furthermore, manipulation of Glut1 levels in the zebrafish embryo by adjusting morpholino dose or through the identification of a *glut1* mutant as noted above are both feasible and may permit novel therapeutic approaches in affected patients, including a more thorough understanding of the efficacy of treatment with ketogenic substrates. Such an approach has been successfully employed utilizing a zebrafish model for pyruvate dehydrogenase deficiency, where neurological dysfunction was rescued using a ketogenic diet (25). Taken together, these data provide the foundation for future studies that should provide new insights into the role of glucose homeostasis in the development of the mature nervous system.

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