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Epilepsy Research (2008) 80(1):18–22.

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Molecular analysis and anticonvulsant therapy in two patients with glucose transporter 1 deficiency syndrome: A successful use of zonisamide for controlling the seizures

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Abstract

Glucose transporter 1 (GLUT1) deficiency syndrome is caused by a deficit in glucose transport to the brain during the pre- and postnatal periods. Here we report two cases of GLUT1 deficiency syndrome diagnosed on the basis of clinical features, reduced GLUT1 activities, and mutations in the *GLUT1* gene. Patient 1 had a novel heterozygous 1-bp insertion in exon 7 that resulted in a shift of the reading frame and the introduction of a premature stop codon at amino acid position 380. His clinical phenotype appeared to be more severe than that of Patient 2 who had a missense mutation in exon 8 resulting in an arginine-to-tryptophan substitution at amino acid position 333. Patient 1 had no meaningful words and could not walk unassisted, while Patient 2 could speak and walk unassisted. Both the patients developed seizures of various types that have been successfully treated with zonisamide. Although several antiepileptic drugs, including barbiturates, diazepam, chloralhydrate, and valproic acid, have been shown to inhibit GLUT1 function, the present study demonstrated no inhibitory effect of zonisamide on GLUT-1-mediated glucose transport. Our data suggested that zonisamide might be preferable if add-on anticonvulsant therapy is required to control the seizures in patients with this disorder.

Key words: Glucose transporter 1; Seizure; Ketogenic diet; Zonisamide; Mutation

1. Introduction

Normal brain development and function are dependent on glucose delivery to the brain during the pre- and postnatal periods. Cell membranes are essentially impermeable to hydrophilic molecules such as glucose. Thus, a membrane-bound glucose transporter is required for glucose transport to the brain. Type-1 glucose transporter (GLUT1) that exclusively mediates glucose transport across the blood-brain barrier (BBB) is expressed on the membranes of brain capillary endothelial cells (Pardridge et al., 1990). GLUT1 is asymmetrically distributed on the brain capillary endothelial luminal and abluminal membranes; it has a 4-fold greater distribution on the abluminal membrane compared to the luminal membrane (Farrell and Pardridge, 1991). This asymmetrical distribution of GLUT1 in the endothelial plasma membranes contributes to maintaining the glucose concentration in the endothelial cytoplasmic compartment lower than that in the blood plasma. Thus, it ensures rapid transport of glucose across the abluminal membrane to the brain parenchyma. A defect in this transporter results in impaired glucose supply to the developing brain, consequently affecting brain development and function. This clinical entity that is characterized by defective glucose transport across the BBB is now recognized as GLUT1 deficiency syndrome (De Vivo et al., 1991). The gene encoding GLUT1 is localized to the short arm of chromosome 1 (1p34.2) (Mueckler et al., 1985; Tao et al., 1995). Several heterozygous mutations have been reported in GLUT1 deficiency syndrome, indicating an autosomal dominant disorder resulting from *GLUT1* haploinsufficiency (Seidner et al., 1998; Wang et al., 2000;

Klepper et al., 2001). Patients with this syndrome present with early-onset seizures and developmental delay. Intractable epilepsy and unresponsiveness to anticonvulsant therapy have been central to the phenotype in the majority of patients.

A ketogenic diet has been suggested as the treatment for patients with GLUT1 deficiency syndrome because ketones enter the brain independent of GLUT1-mediated transport and serve as an alternative fuel for the brain (De Vivo et al., 1991; Nordli and De Vivo, 1997; Klepper and Voit, 2002). Although the ketogenic diet may effectively control the seizures, a subgroup of patients requires add-on anticonvulsant therapy. Notably, GLUT1 function is inhibited by several anticonvulsants, including barbiturates (Honkanen et al., 1995; Klepper et al., 1999a), diazepam (Klepper et al., 2003), chlorhydrate (Klepper et al., 2003), and valproic acid (Wong et al., 2005). Therefore, it is important that patients with GLUT1 deficiency syndrome avoid these drugs in order to preserve their already impaired GLUT1 function. Here we report two cases of GLUT1 deficiency syndrome in which the seizures were successfully controlled with zonisamide prior to being started a ketogenic diet. Studies on glucose uptake by erythrocytes have revealed that zonisamide does not inhibit GLUT1-mediated glucose transport. In keeping with this, our data indicate that zonisamide may be preferable if add-on anticonvulsant therapy is required to control the seizures in patients with GLUT1 deficiency syndrome.

2. Patients and methods

2.1. Patient 1

A Japanese boy, now aged 8 years, was diagnosed with GLUT1 deficiency syndrome at the age of 6 years. He was born at full term after an uneventful pregnancy. His nonconsanguineous parents were healthy. His neurological symptoms dated back to age 3 months, when his parents noted his first seizures that were described as nodding of the head. He continued to have frequent episodes of head drops, and at age 7 months, he developed generalized tonic-clonic seizures. Over the next few months, he demonstrated inconsolable crying and additional myoclonic seizures. These episodes often appeared upon awakening in the morning. The initial electroencephalogram (EEG) was normal, but subsequent EEGs showed spike discharges over the bilateral frontal areas. Developmental milestones were moderately delayed: head control at 7 months, turning over at 9 months, sitting without assistance at 12 months, and crawling at 24 months. The seizures were unresponsive to anticonvulsants, including phenobarbital, phenytoin, clonazepam, and valproic acid. However, zonisamide administration that was started at the age of 5 years decreased the frequency of the seizures effectively. Physical examination was remarkable for hypotonia, ataxia, and fine motor incoordination. The tendon reflexes at the knees and ankles increased, and Babinski signs were present. A lumbar puncture at the age of 6 years revealed low glucose concentration in the cerebrospinal fluid (CSF) in the setting of normoglycemia

(blood glucose, 86 mg/dl; CSF glucose, 27 mg/dl; and CSF-to-blood glucose ratio, 0.31); this was suggestive of impaired GLUT1-mediated glucose transport to the brain. Magnetic resonance imaging (MRI) performed at that time revealed diffuse cerebral atrophy with the most severe atrophy in the cerebellum. At the age of 6 years when he was diagnosed with GLUT1 deficiency syndrome, a ketogenic diet that comprised a 2:1 ratio of fat : carbohydrate and protein was initiated. After intake of the ketogenic diet, he became more alert and less irritable, particularly in the morning, and returned to school with rare absences. Furthermore, he showed moderate improvement in fine motor coordination. Although he remained on the anticonvulsant zonisamide, only few seizures occurred in a year. However, up to age 8 years, no further developmental gains occurred despite continuance of the dietary treatment; he had no meaningful words and could not walk unsupported.

2.2. Patient 2

A Japanese girl, now aged 6 years, was diagnosed with GLUT1 deficiency syndrome at the age of 4 years. She was born to healthy non-consanguineous parents after an uneventful delivery with normal birth weight and head circumference. Her developmental milestones were mildly delayed; she spoke her first words at 2 years of age and walked unaided with a spastic and ataxic gait at 2.5 years of age. She presented with the first generalized tonic-clonic seizures at the age of 13 months and was treated with valproic acid. The EEG showed spike discharges over the bilateral

frontal areas. At the age of 2 years, she developed additional myoclonic seizures. The seizures were initially infrequent, but subsequently they began to occur in clusters several times a week. The seizures occurred predominantly in the morning before breakfast. She continued to have frequent seizures even after clobazam was included in the treatment. Thus, valproic acid and clobazam proved ineffective for seizure control. At the age of 3 years, after the anticonvulsants administered were substituted with zonisamide, no further seizures occurred. Physical examinations showed mild ataxia and hypotonia. The tendon reflexes at the knees and ankles increased, and Babinski signs were present. A lumbar puncture at the age of 4 years revealed low glucose concentration in the CSF in the setting of normoglycemia (blood glucose, 92 mg/dl; CSF glucose, 31 mg/dl; and CSF-to-blood glucose ratio, 0.34). MRI performed at that time revealed normal brain morphology and myelination. Treatment with ketogenic diet that was initiated at the age of 4 years led to a dramatic improvement in motor and cognitive capacities. Currently, at the age of 6 years, she can speak with minor dysarthria and walk unsupported. The neurological examination is remarkable only for mild hypotonia and ataxia.

2.3. Mutation analysis of the GLUT1 gene

After informed consent had been granted, genomic DNA was extracted from the patients' white blood cells and used as the template for polymerase chain reaction (PCR). Appropriate primers were used to yield DNA fragments spanning the entire

GLUT1 coding region and intron-exon boundaries (Wang et al., 2000). The PCR fragments were analyzed by automated sequencing. To further confirm the mutations identified by the direct DNA sequencing, a separate set of PCR fragments of exon 7 or 8 were digested with *Nci I* or *NgoM IV*, respectively. The reaction products were then visualized by ethidium bromide staining following electrophoresis on a 12% acrylamide gel. To amplify the DNA fragments encompassing the mutation sites, the following primers were used: for exon 7, 5'-cccacatccactgctacagag-3' and 5'-cctagtgcccttctgaacc-3'; and for exon 8, 5'-aggccccaacagtttctctt-3' and 5'-tatgaagcccaggcaaactc-3'.

2.4. Erythrocyte 3-O-methyl-D-glucose uptake studies

Studies on glucose uptake into erythrocytes by using non-metabolisable 3-O-methyl-D-glucose (3-OMG) that have been shown to be representative of the transendothelial glucose transport across the BBB were performed as described previously (Klepper et al., 1999b). Briefly, blood samples were obtained simultaneously from the patients and their mothers; the samples were collected in a citrate-dextrose-phosphate solution. The samples were immediately placed on wet ice and processed within 24 h. All procedures were performed at 4°C. The blood samples were washed 3 times in phosphate-buffered saline, and 50- μ l aliquots were incubated with increasing concentrations of 3-OMG (0.25 - 8 mmol/L). Uptake was terminated after 20 s with ice-cold phosphate-buffered saline containing 50 μ M

phloretin. The aliquots were washed 3 times, lysed, bleached, and counted using a scintillation counter. The following reagents were used: 3-OMG and phloretin from Sigma Chemical Co. (St. Louis, MO, USA), and [¹⁴C]-3-OMG (56.4 mCi/mmol) from PerkinElmer (Waltham, MA, USA).

2.5. Effects of anticonvulsants on GLUT1-mediated glucose transport

This assay was performed as described earlier (Klepper et al., 2003). Briefly, the 50- μ l aliquots of washed erythrocytes were incubated in 50 μ l phosphate-buffered saline containing increasing concentrations of pentobarbital or zonisamide for 1 h at 4°C prior to analyzing the 3-OMG uptake at a concentration of 5 mmol/L. The anticonvulsants, pentobarbital and zonisamide, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3. Results

3.1. Identification of the GLUT1 gene mutations

The diagnosis of GLUT1 deficiency syndrome was confirmed by mutations in the *GLUT1* gene in both patients. Patient 1 had a novel heterozygous 1-bp insertion mutation (1081-1082 insC) in exon 7 (Fig. 1A) that resulted in a premature stop codon

at amino acid position 380 (p.A301fsX380, amino acid numbering commenced at the first methionine). Patient 2 had a heterozygous C-to-T transition at nucleotide 1176 in exon 8 (Fig. 1B), resulting in an arginine-to-tryptophan substitution at amino acid position 333 (p.R333W). This R333W mutation has been reported in unrelated patients, indicating that it is a hot-spot mutation (Wang et al., 2005; Fujii et al., 2007). The insertion mutation in Patient 1 created a new *Nci I* restriction site, and the missense mutation in Patient 2 eliminated an *NgoM IV* restriction site. Consequently, PCR restriction-digestion analysis of DNA from Patients 1 and 2 showed novel fragments in addition to the estimated wild-type fragments, further confirming the heterozygous mutations in these patients (Fig. 1C and D).

3.2. Functional deficit in the mutated GLUT1

The glucose uptake studies revealed a functional defect in the mutated GLUT1. 3-OMG uptake by erythrocytes was reduced in both the patients as compared to their mothers. The V_{max} value for glucose influx in Patients 1 and 2 was 284 and 315 fmol/s per 10^6 erythrocytes, which was 38% and 76% of the corresponding values for the mothers', respectively. The V_{max} values for the glucose influx in the mothers of Patients 1 and 2 were 748 and 414 fmol/s per 10^6 erythrocytes, respectively. The large variation in V_{max} values has been reported in control individuals and may reflect environmental factors such as caffeine, which inhibits GLUT1 (Ho et al., 2001). Alternatively, the variation may reflect genetic factors such as polymorphisms in the

GLUT1 or other genes that modify the expression of GLUT1 (Wang et al. 2005). . . .

3.3. No inhibitory effect of zonisamide on GLUT1-mediated glucose transport

Treatment with zonisamide effectively reduced seizure frequency in Patient 1 and ceased the seizures in Patient 2. GLUT1 function has been shown to be inhibited by several anticonvulsants such as barbiturates (Honkanen et al., 1995; Klepper et al., 1999a), valproic acid (Wong et al., 2005) and diazepam (Klepper et al., 2003). However, it remains to be elucidated whether zonisamide inhibits GLUT1 function. To address this question, we assayed the effect of zonisamide on the 3-OMG influx into erythrocytes. The erythrocytes of these 2 patients and 3 controls were preincubated with pentobarbital or zonisamide at various concentrations (therapeutic to toxic ranges), following which 3-OMG influx was assayed. Pentobarbital inhibited the 3-OMG influx into the erythrocytes in a dose-dependent manner, as reported previously (Fig. 2A) (Honkanen et al., 1995; Klepper et al., 2003). However, zonisamide showed no inhibitory effect on the 3-OMG influx at therapeutic (50-150 μ M) and toxic (> 250 μ M) concentrations (Fig. 2B).

4. Discussion

We report the cases of 2 Japanese patients with GLUT1 deficiency syndrome who were

diagnosed on the basis of the clinical and laboratory features, and mutations in the *GLUT1* gene. These 2 patients showed early-onset seizures of various types, decreased muscle tone, ataxia and, significant delays in neurological development, all of which have been demonstrated as invariable clinical features of GLUT1 deficiency syndrome (Klepper et al., 1999c). Furthermore, a lumbar puncture revealed hypoglycorrachia in these patients, and the other causes of low glucose concentration in the CSF, such as hypoglycemia and meningitis, were excluded. The diagnosis of GLUT1 deficiency syndrome was then confirmed by reduced erythrocyte uptake of glucose and mutations in the *GLUT1* gene.

Patient 1 had a 1-bp insertion in exon 7 that resulted in a shift of the reading frame and the introduction of a premature stop codon. His clinical phenotype appeared to be more severe than that of Patient 2 who had a missense mutation in exon 8. Patient 1 had no meaningful words and could not walk unassisted, while Patient 2 could speak and walk unassisted. Furthermore, brain MRI revealed severe atrophic changes in the cerebellum of Patient 1 but not of Patient 2. Patient 1 also had lower residual GLUT1 activity than Patient 2 whether expressed as a percentage of his mother's GLUT1 or the controls' activity (Fig. 2). However, there appears to be no correlation between 3-OMG uptake values in erythrocytes and phenotypic severity (Klepper et al., 2002; Wang et al., 2005). Patient 1 may be more severely affected because Patient 1 started on the ketogenic diet at an older age than Patient 2. Thus, Patient 1 may have had a longer period of brain energy deficiency.

An invariant clinical feature of GLUT1 deficiency syndrome is early-onset

seizures within the first year of life (Leary et al., 2003). Both Patients 1 and 2 also presented with seizures of various types that included generalized tonic-clonic, myoclonic and atonic seizures. The seizures did not respond to phenobarbital, phenytoin, clonazepam, clobazam and valproic acid. However, zonisamide effectively decreased seizure frequency in Patient 1 and ceased the seizures in Patient 2.

Zonisamide is a broad-spectrum antiepileptic drug that has been used to treat patients with partial or generalized seizures (Ohtahara, 2006). Although several antiepileptic drugs have been shown to inhibit GLUT1 function, the effects of zonisamide on GLUT1 function remained to be elucidated. Our study demonstrated that zonisamide did not inhibit GLUT1-mediated glucose transport; this was consistent with our clinical observation. Therefore, zonisamide might be preferable if add-on anticonvulsant therapy is required to control the seizures in patients with GLUT1 deficiency syndrome.

Acknowledgements

We sincerely thank the members of the families described here, whose help and participation made this work possible.

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valproate inhibits glucose transport and exacerbates Glut1-deficiency in vitro. J. Cell Biochem. 96, 775-785.

Figure legends:

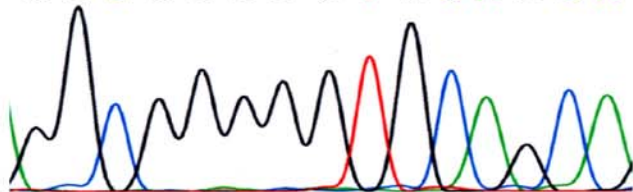
Fig. 1. Mutations in the *GLUT1* gene in Patients 1 and 2. Automated DNA sequencing using the PCR product from Patient 1 showed a 1-bp insertion at nucleotide 1081-1082 (numbering according to GenBank accession no. NM006516) in exon 7 of the *GLUT1* gene, as indicated by the arrow (A), which resulted in a shift of the reading frame and the introduction of a premature stop codon. In Patient 2, a C-to-T transition was identified at nucleotide 1176 in exon 8, as indicated by the arrow (B). These mutations were not observed in the normal controls (A and B). *Nci I*-digestion of the exon 7 PCR product showed additional fragments (212-bp and 113-bp) in Patient 1, which resulted from a 1-bp insertion mutation creating a new *Nci I* restriction site (C). These additional fragments were observed with the 325-bp wild-fragment, confirming the heterozygous mutation in Patient 1. *NgoM IV*-digestion of the exon 8 PCR product showed an additional fragment (469-bp) in Patient 2, which resulted from a C-to-T transition eliminating a *NgoM IV* restriction site (D). This additional fragment was observed with the wild-type fragments (333-bp and 136-bp), confirming the heterozygous mutation in Patient 2.

Fig. 2. Effects of anticonvulsants on GLUT1 - mediated glucose transport. Isolated erythrocytes from 2 patients and 3 controls were preincubated for 60 min at 4°C in the indicated concentrations of pentobarbital (A) or zonisamide (B) prior to analyzing 3-OMG uptake. Pentobarbital inhibited the 3-OMG uptake into erythrocytes in a dose-dependent manner, while no inhibitory effects of zonisamide were observed even with concentrations increasing into the toxic range. The results indicate the 3-OMG uptake as a percentage of the values of the controls in the absence of drugs and are shown as mean \pm SEM. The controls included 3 healthy volunteers, but not the patients' parents. Closed circles represent the data of controls (n=3); and squares represent the data of Patient 1 (open squares) and 2 (closed squares).

A

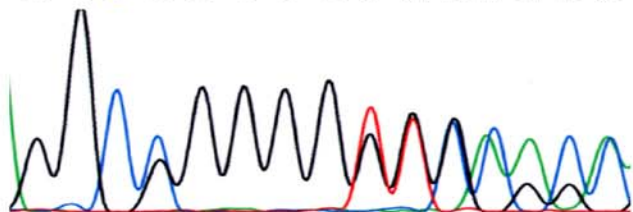
Control

G G C G G G G G T G C A G C A



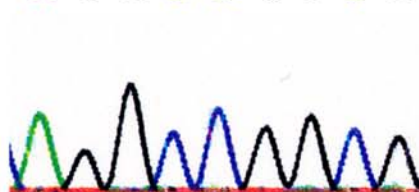
Patient 1 ↓

G G C G G G G G N N N N N N

**B**

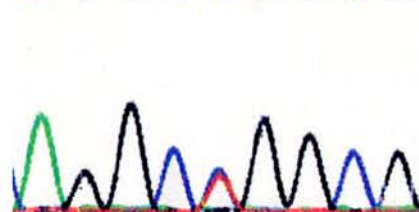
Control

A G G C C G G C G

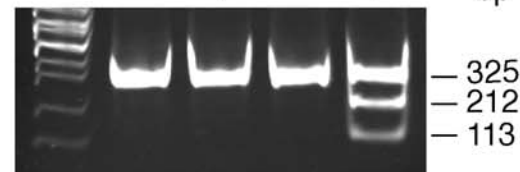


Patient 2 ↓

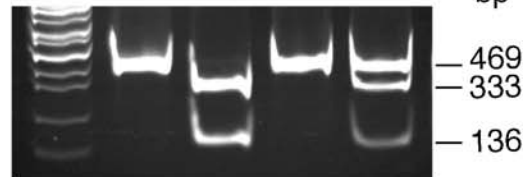
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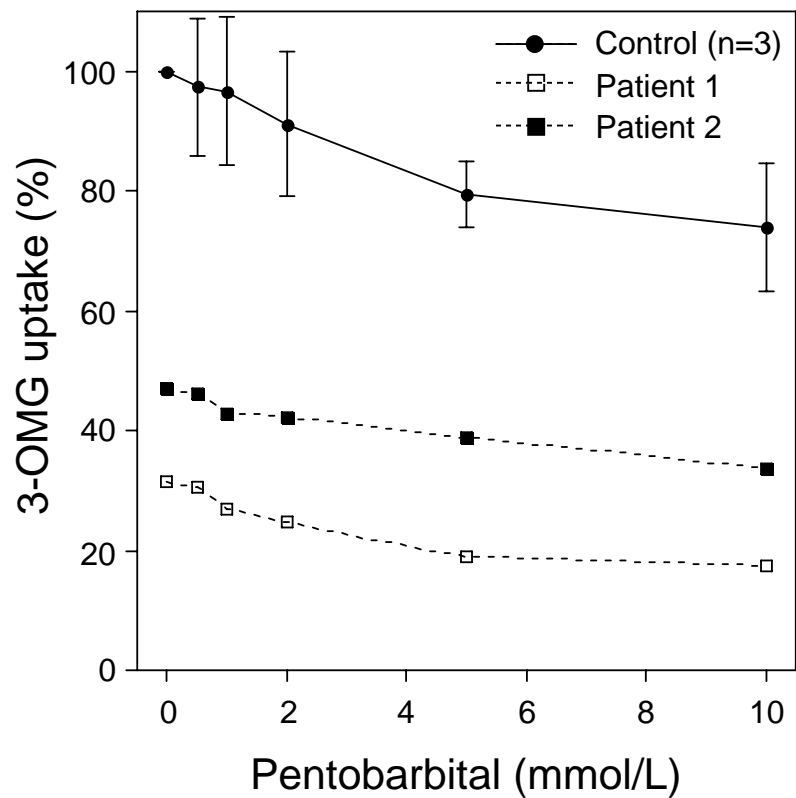
**C**

Nci I	M	Control		Patient 1		bp
		-	+	-	+	
						325
						212
						113

**D**

NgoM IV	M	Control		Patient 2		bp
		-	+	-	+	
						469
						333
						136



A**B**