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An animal model of PDH deficiency using AAV8-siRNA vector-mediated knockdown of pyruvate dehydrogenase E1α

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ABSTRACT

We evaluated the feasibility of self-complementary adeno-associated virus (scAAV) vector-mediated knockdown of the pyruvate dehydrogenase complex using small interfering RNAs directed against the E1α subunit gene (PDHA1). AAV serotype 8 was used to stereotaxically deliver scAAV8-si3-PDHA1-Enhanced Green Fluorescent Protein (knockdown) or scAAV8-EGFP (control) vectors into the right striatum and substantia nigra of rats. Rotational asymmetry was employed to quantify abnormal rotation following neurodegeneration in the nigrostriatal system. By 20 weeks after surgery, the siRNA-injected rats exhibited higher contralateral rotation during the first 10 min following amphetamine administration and lower 90-min total rotations ($p \leq 0.05$). Expression of PDC E1α, E1β and E2 subunits in striatum was decreased ($p \leq 0.05$) in the siRNA-injected striatum after 14 weeks. By week 25, both PDC activity and expression of E1α were lower ($p \leq 0.05$) in siRNA-injected striata compared to controls. E1α expression was associated with PDC activity ($R^2 = 0.48$; $p = 0.006$) and modestly associated with counterclockwise rotation ($R^2 = 0.51$; $p = 0.07$). The use of tyrosine-mutant scAAV8 vectors resulted in ~17-fold increase in transduction efficiency of rat striatal neurons *in vivo*. We conclude that scAAV8-siRNA vector-mediated knockdown of PDC E1α in brain regions typically affected in humans with PDC deficiency results in a reproducible biochemical and clinical phenotype in rats that may be further enhanced with the use of tyrosine-mutant vectors.

Introduction

The pyruvate dehydrogenase complex (PDC) is a mitochondrial enzyme that plays a central role in aerobic energy metabolism by catalyzing the irreversible oxidation of glucose-derived pyruvate to acetyl-CoA. The complex is entirely nuclear encoded and comprises multiple copies of three structurally distinct, but functionally interdependent, enzymes (E_1 through E_3) [1]. Pyruvate dehydrogenase (E1; EC 1.2.4.1) is a heterotetrameric ($\alpha_2\beta_2$) α-keto acid decarboxylase that oxidizes pyruvate to acetyl CoA and requires thiamine pyrophosphate as cofactor; dihydrolipoamide acetyltransferase (E2; EC 2.3.1.12) forms the structural core of the complex; and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4) is a flavoprotein enzyme that is stably integrated into the complex by an E3-binding protein (E3bp). Rapid regulation of PDC activity occurs by product inhibition and by reversible phosphorylation of serine residues on the E1α subunit that is mediated by a family of PDC kinases (PDK) and phosphatases (PDP) [2,3].

Inborn errors of the PDC are among the more common causes of congenital lactic acidoses [4,5]. Because the PDC catalyzes the first step in the mitochondrial metabolism of carbohydrate, inhibition of pyruvate decarboxylation severely impairs the yield of ATP from glucose and leads to accumulation of lactate and pyruvate. Highly oxidative tissues, such as the central nervous system (CNS), are particularly vulnerable to loss of PDC activity. Thus, the most prevalent clinical manifestations of PDC deficiency reflect progressive neuromuscular and neurobehavioral deterioration, such as mental retardation and developmental delay, hypotonia, exercise intolerance, motor disorders and seizures [5,6]. Indeed, the biochemical and clinical manifestations of PDC deficiency may appear to reflect solely CNS involvement without significant extracranial complications [7]. PDC deficiency is also one of several inborn errors of mitochondrial energetics that may lead to the characteristic neuropathology of Leigh syndrome, with spongy degeneration in the thalamus, basal ganglia and brain stem [7,8]. By far the majority of biochemically proven cases of PDC deficiency are due to defects in the X-linked E1α subunit gene (*PDHA1*) [9].

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There are no proven treatments for PDC deficiency. The recent development of a murine model of the disease demonstrates that complete deficiency of PDC E1α is a lethal defect for the developing male embryo [10,11]. Conditional knockout strategies offer a potential alternative to the more traditional embryonic cell knockout approach. Ribozymes can be used for direct delivery of genes using viral vectors, such as the nonpathogenic human adeno-associated virus 2 (AAV2), and to down-regulate genes *in vivo* in creating animal models of disease [12]. Although hammerhead ribozymes can cause knockdown of E1α activity *in vitro* [13], such an action could not be replicated *in vivo* (unpublished observations). Therefore, we adopted an alternative approach to knockdown the pyruvate dehydrogenase E1α gene (*PDHA1*) by using a combination of self-complementary AAV (scAAV) vector and RNA interference technologies. Delivery of small interfering RNA (siRNA) using scAAV vectors can produce sustained knockdown of *PDHA1* gene in transduced cells *in vitro* [14]. Moreover, scAAV vectors can be used to deliver and express *PDHA1* in cultured mammalian cells and partially restore PDC activity in defective cells [15].

Here we determined the feasibility of recombinant scAAV8-mediated knockdown of PDC E1α using siRNAs directed against the *PDHA1* in the striatum (ST) and substantia nigra (SN) of rats. We also developed capsid-modified tyrosine-mutants that are capable of high-efficiency transduction in the rat striatum *in vivo*.

Materials and methods

Construction of the recombinant AAV vector encoding siRNA and virus preparation

The design and construction of scAAV-based plasmid encoding siRNA to target rat PDHA1 sequences was described previously [14]. The plasmid construct includes the EGFP gene driven by a chicken β-actin (CBA) promoter [16]. Vectors were packaged at the University of Florida Powell Gene Therapy Center. Vector titer (viral particles/mL) was determined by dot–blot hybridization analysis. The titers of scAAV8-EGFP and scAAV8-si3-PDHA1-EGFP were 3.3×10^{11} and 4.89×10^{12} viral genomes/mL, respectively.

Construction of surface-exposed tyrosine residue mutant AAV8 capsid plasmid

We utilized a procedure based on the QuikChangeII site-directed mutagenesis (Stratagene, La Jolla, CA) according to the manufacturer's protocol and as we previously described [17]. The nucleotide sequences of primers used for site-directed mutagenesis of surface-exposed tyrosine residues are shown in Table 1. The introduction of tyrosine to phenylalanine (Y-F) mutations was confirmed by DNA sequencing. The titers of scAAV8-Y447F-EGFP and scAAV8-Y733F-EGFP were 1.6×10^{12} and 2.7×10^{12} viral genomes/mL, respectively.

Table 1
Nucleotide sequences of primers used for site-directed mutagenesis of surface-exposed tyrosine residues.

Mutants	Primer sequences (5′–3′)
Y447F	Tyr→Phe XbaI
AAV8Y447-F	CCAGTACCTGTAC**TTC**TTGTCTAGAACTCAAACAACAGGAGG
AAV8Y447-R	CCTCCTGTTGTTTGAGTTCTAGACAAGAAGTACAGGTACTGG
Y733F	MluI Tyr→Phe
AAV8Y733-F	CCCCATTGGCACGCGT**TTC**CTCACCCGTAATC
AAV8Y733-R	GATTACGGGTGAGGAAACGCGTGCCAATGGGG

The codon triplets are shown in bold; red nucleotide denotes the mutation from phenylalanine to tyrosine (Y–F) residues. Green indicates the silent mutations to create the restriction enzyme sites shown (underlined), which were used to obtain the desired clone.

Animal surgeries

All surgical procedures and care were approved by the UF Institutional Animal Care and Use Committee. The brain coordinates (calculated relative to the bregma) for injections for the ST were: anterior–posterior (AP) −0.5 mm, medial-lateral (ML) −3.5 mm, and dorso-ventral (DV) −5.0 mm from dura. For the SN, the coordinates were: AP +5.6 mm, ML −2.4 mm, and DV −7.2 mm from dura. Briefly, after the rats were anesthetized, they were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and received a subcutaneous injection of marcaine at the incision site. Rats were continuously under isoflurane anesthesia during the injection procedure. Injections were performed with a 5-μl Hamilton syringe fitted with a glass micropipette with an opening of approximately 60–80 μm. Vectors were injected at a rate of 0.5 μl/min. The titer of vector stocks was adjusted to attain the same titer for each vector. For SN injections, the needle was left in place for 5 min before being slowly removed from the brain. For ST injections, the needle was retracted an additional 1 mm/min after the cessation of the injection and was then left in place for an additional 4 min before slowly withdrawing it from the brain.

Recombinant AAV-mediated siRNA vector-mediated knockdown of PDC E1α in the striatal and nigral regions of the rat brain

Fifteen female Sprague–Dawley rats (225–250 g, Charles River Laboratories) underwent stereotaxic surgery (described above) into the right striatum (1.5×10^{10} viral particles) and substantia nigra (7.3×10^{9} viral particles) to deliver scAAV8-si3-PDHA1-EGFP ($n=10$, PDHA1 knockdown) or scAAV8-EGFP ($n=5$, positive control) vectors. Five rats served as negative controls (no surgery). The left (uninjected) hemisphere was used as control tissue from each rat for the biochemical and immunohistochemical assays. Animals were monitored weekly and showed no difference in body weight over a 6-month period of observation.

Rotational behavior

Rotational activity was monitored starting 4 weeks after surgery by intraperitoneally injecting animals with 2.5 mg/kg amphetamine sulfate (Sigma-Aldrich, St. Louis, MO). Amphetamine causes neuronal release of dopamine that leads to measurable changes in rotational behavior in rats. This technique has become a standard and robust method for quantifying abnormal behavior following chemical or genetically induced neurodegeneration in the rat nigrostriatal system [18–21]. Animals were fitted with small, non-restricting harnesses and placed in automated rotometer bowls [22]. Full 360 clockwise and counterclockwise rotations were measured during a 90-min period after amphetamine injection.

Tissue processing for biochemical and immunohistochemical studies

After 14 weeks, 5 PDHA1-siRNA-injected rats and 3 rats from each control group were sacrificed for tissue analyses. The remaining rats, 5 siRNA-injected and 2 rats from each control, were followed until week 24 and were sacrificed for tissue analyses on week 25.

Rats were deeply anesthetized with pentobarbital and decapitated. The brains were rapidly removed and sectioned coronally at the level of the cerebral peduncles. The ST was dissected from the region anterior to the cut while the SN were dissected from the region posterior to the cut. The left and right ST were separated and isolated from the surrounding tissue. The tissue from each ST hemisphere was homogenized and separated into two tubes, sample weights were recorded and specimens were quickly frozen in liquid nitrogen. Tissues were stored in −80 °C until further biochemical analyses. The brain section containing the SN were post-fixed for 24 h in 4%