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Transcriptome profiling of equine vitamin E deficient neuroaxonal dystrophy identifies upregulation of liver X receptor target genes

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ABSTRACT

Specific spontaneous heritable neurodegenerative diseases have been associated with lower serum and cerebrospinal fluid α-tocopherol (α-TOH) concentrations. Equine neuroaxonal dystrophy (eNAD) has similar histologic lesions to human ataxia with vitamin E deficiency caused by mutations in the α-TOH transfer protein gene (*TTPA*). Mutations in *TTPA* are not present with eNAD and the molecular basis remains unknown. Given the neuropathologic phenotypic similarity of the conditions, we assessed the molecular basis of eNAD by global transcriptome sequencing of the cervical spinal cord. Differential gene expression analysis identified 157 significantly (FDR < 0.05) dysregulated transcripts within the spinal cord of eNAD-affected horses. Statistical enrichment analysis identified significant downregulation of the ionotropic and metabotropic group III glutamate receptor, synaptic vesicle trafficking and cholesterol biosynthesis pathways. Gene co-expression analysis identified one module of upregulated genes significantly associated with the eNAD phenotype that included the liver X receptor (LXR) targets *CYP7A1*, *APOE*, *PLTP* and *ABCA1*. Validation of *CYP7A1* and *APOE* dysregulation was performed in an independent biologic group and *CYP7A1* was found to be additionally upregulated in the medulla oblongata of eNAD horses. Evidence of LXR activation supports a role for modulation of oxysterol-dependent LXR transcription factor activity by tocopherols. We hypothesize that the protective role of α-TOH in eNAD may reside in its ability to prevent oxysterol accumulation and subsequent activation of the LXR in order to decrease lipid peroxidation associated neurodegeneration.

1. Introduction

Ataxia with vitamin E deficiency (AVED) is due to genetic mutations in the tocopherol (alpha) transfer protein gene (*TTPA*). Equine neuroaxonal dystrophy (eNAD) is a spontaneous neurodegenerative disease with similar clinicopathologic features to AVED. Clinical signs of eNAD include ataxia (i.e., incoordination) and proprioceptive deficits. Histologic features of eNAD include axonal swellings, or spheroids, localized to the caudal medulla oblongata and spinal cord [1]; similar to histologic lesions observed with AVED [2]. In more severely affected horses, the disease is often termed equine neuroaxonal dystrophy/equine degenerative myeloencephalopathy (eNAD/EDM) and histologic lesions include axonal loss and demyelination of specific neuroanatomic tracts within the cervicothoracic spinal cord [1]. Equine NAD develops in genetically predisposed foals maintained on an α-tocopherol (α-TOH) deficient diet during the first year of life [3]. Similar to AVED, the development of clinical signs can be prevented by early long-term α-TOH supplementation [1,4]. Mutations in *TTPA* are not present with eNAD [5] and the molecular basis remains unknown. While the horse may seem an unlikely model for humans, their lifespan (~30 years), length of their axons and documented similarities in clinical disease may provide unique insights into the molecular mechanism by which α-TOH deficiency impacts neurodegeneration.

Abbreviations: α-TOH, Alpha-tocopherol; ABCA1, ATP-binding cassette transporter; ACTB, Beta-actin; APOE, Apolipoprotein E; AVED, Ataxia with vitamin E deficiency; BCV, biologic coefficient of variation; CDCA, chenodeoxycholic acid; CNS, central nervous system; CSF, cerebrospinal fluid; CYP7A1, Cholesterol 7 alpha-hydroxylase; DET, Differentially expressed transcript; HPRT1, Hypoxanthine Phosphoribosyltransferase 1; LC-MS/MS, liquid chromatography-mass spectroscopy/mass-spectroscopy; LXR, Liver X Receptor; eNAD, Equine neuroaxonal dystrophy; eNAD/EDM, Equine neuroaxonal dystrophy/equine degenerative myeloencephalopathy; PLTP, Phospholipid transfer protein; RXR, Retinoid X Receptor; TTP, Tocopherol (alpha) transfer protein; WGCNA, Weighted correlation network analysis

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To date, candidate gene approaches and genome-wide association studies have not identified a putative functional variant or chromosomal locus for eNAD [5,6]. The overall goal of this study was to further characterize the degree of α-TOH deficiency in eNAD-affected horses and identify differentially expressed genes and pathways in the central nervous system (CNS) by global transcriptome sequencing. Based on comparative phenotypes across species, we hypothesized that the most relevant pathways would include those related to vitamin E transport or metabolism, which is intimately tied to cholesterol homeostasis based on shared biochemical properties [7]. Additionally, vitamin E is the major lipophilic antioxidant to protect against formation of cholesterol oxidation products (oxysterols) that can result in neurodegeneration [8].

2. Materials and methods

2.1. Subjects

All animal procedures were approved by the University of California-Davis and University of Minnesota Institutional Animal Care and Use Committees and owners' consent was obtained for all horses. Over a period of 8 years, samples from 22 post-mortem confirmed eNAD and 21 unaffected horses were collected. All horses were donated by owners for the purpose of this study. Biologic samples (i.e. serum, cerebrospinal fluid [CSF], tissue from liver, spinal cord and medulla oblongata) were available on subsets of eNAD affected and unaffected horses (Table A.1**)**.

2.1.1. α-TOH concentrations

Alpha-tocopherol (α-TOH) concentrations were assessed in the serum (n=21 eNAD-affected, n=12 unaffected), CSF (n=17 eNAD-affected, n=8 unaffected), liver (n=18 eNAD-affected, n=11 unaffected) and spinal cord (n=5 eNAD-affected, n=7 unaffected) of phenotyped horses (Table A.1). These horses were selected to be age matched (≤3 years of age) with samples that had been collected prior to (serum) or immediately following (CSF and liver) euthanasia. Spinal cord tissue was prioritized for RNA-sequencing and assessment of cholesterol concentrations and therefore spinal cord samples from the same location (i.e. cervical vertebra 1) for assessment of α-TOH concentrations were only available from a limited subset of horses. Remaining spinal cord tissue was used for a complete histologic evaluation with eNAD horses confirmed as previously described [1]. Unaffected horses were euthanized for reasons other than neurologic disease and a full neuropathologic evaluation was performed. Alpha-TOH concentrations were assessed as previously described [1,3] and concentrations compared using a Mann-Whitney test with significance set at $P < 0.05$.

2.1.2. RNA-sequencing

Horses selected for RNA-sequencing were between 1 and 2 years of age and matched for sex. Transcriptome sequencing of the caudal medulla oblongata was performed on 4 eNAD-affected Quarter Horses (n=2 male, n=2 female; n=1 classified as eNAD and n=3 classified as eNAD/EDM at post-mortem [1]) and 4 unaffected Quarter Horses (n=2 male, n=2 female). Subsequently, transcriptome sequencing of the cervical spinal cord was performed on 5 severely affected horses (i.e. classified as eNAD/EDM on post-mortem examination; n=3 Quarter Horses, n=1 Hanoverian/Thoroughbred, n=1 Shire; n=3 males, n=2 female; 1–2 y of age, median 1.5 y) and 5 unaffected horses (n=3 Quarter Horses, n=1 Percheron, n=1 Pony of the Americas; n=2 males, n=3 females; 0.5–2 y of age, median=1.5 y). Based on phenotypic criteria (i.e. more severely affected horses were available at the time of spinal cord sequencing) and tissue availability, 5 horses (2 eNAD/EDM-affected and 3 unaffected) had transcriptome sequencing performed on both tissues (Table A.1). None of the affected horses were related within 3 generations. Two of the unaffected Quarter horses used for both medulla oblongata and spinal cord sampling (Unaffected 1 and Unaffected 2) were half-siblings.

2.1.3. Nervous tissue sampling

All clinically suspect eNAD-affected horses were euthanized with an overdose of pentobarbital (> 100 mg/kg IV) and a full post-mortem examination was performed. Samples of caudal medulla oblongata were collected immediately caudal to the level of the obex and flash-frozen in liquid nitrogen. Samples of the spinal cord were collected at the level of cervical vertebra 1, cross-sectioned and flash-frozen in liquid nitrogen. Dorsal root ganglia (DRG) were collected and frozen separately on only a subset of horses. All tissues for RNA preparation were collected within 3 h of euthanasia. The remaining brain and spinal cord were formalin-fixed and a complete histologic evaluation was performed. A moderate phenotype (eNAD) or a severe phenotype (eNAD/EDM) was diagnosed with histologic lesions as previously described [1] (Table A.1). Unaffected horses were euthanized for reasons other than neurologic disease (pseudohermaphroditism, type I polysaccharide storage myopathy, lameness) and samples were collected in an identical manner. As expected for a primary myopathy associated with a glycogenosis [9], the unaffected horse with type 1 polysaccharide storage myopathy did not have any evidence of neuropathic muscle atrophy or neurodegeneration.

2.2. RNA isolation and quality control

RNA samples isolated from the region of the lateral accessory cuneate nucleus of the caudal medulla oblongata were utilized for RNA-seq analysis based on histologic evidence of spheroids, or axonal swellings in this region of eNAD-affected horses [1]. Based on our additional work determining that the nuclei for the affected neurons in eNAD originate in the dorsal root ganglia of the cervical spinal cord and terminate in the caudal medulla oblongata, [10] transcriptome sequencing was subsequently performed in the spinal cord, at the level of cervical vertebrae 1, in 5 eNAD-affected and 5-unaffected horses. For all samples, total RNA was extracted using TRIzol reagent (Thermofisher, Wilmington, DE, USA). The resulting amount of RNA and integrity scores are included in Table A.2. For RT-qPCR, RNA was washed and eluted on columns (Direct-zol™ RNA MiniPrep Plus, Zymo, Irvine, CA) and treated with TURBO DNase (Thermofisher, Wilmington, DE, USA) according to manufacturer's instructions. For RNA-seq, in an effort to include non-polyadenlyated long non-coding RNAs in the sequencing, ribosomal RNA was depleted (Ribo-Zero, Illumina, San Diego, US). Quantification and quality of RNA, along with degree of rRNA contamination, was assessed using the Pico chip on the Agilent Bioanalyzer 2100 (Santa Clara, CA, USA), with a RNA integrity number (RIN) ≥7. Ribosomal RNA depletion was successful in all samples (amount of remaining rRNA contamination 0–2.6%; mean 2%).

2.3. RNA-sequencing and exploratory data analysis

Strand-specific libraries (TruSeq Stranded Total RNA Library pre kit, San Diego, US) underwent next-generation sequencing (100-bp pair-end sequences with an Illumina HiSeq 2000, San Diego, US) at a targeted 20 million reads/sample across one lane. After quality control trimming with trimmomatic using a sliding window of 3 and quality score minimum of 28 [11], these sequences were aligned and reads quantified using two pseudoalignment methods for transcript quantification using both salmon [12] and kallisto [13] with 30 bootstraps. Gene annotation files included Ensembl (http://www.ensembl.org/info/data/ftp/index.html) and a custom annotation file (https://github.com/drtamermansour/horse_trans). RNA-seq was subsequently performed as described above on the spinal cord at the level of cervical vertebra 1 in 5 eNAD-affected and 5 unaffected horses (Table A.2), with library preparation and sequencing similar to medulla oblongata samples.