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MITOCHONDRIAL CHANGES AND OXIDATIVE STRESS IN A MOUSE MODEL OF ZELLWEGER SYNDROME NEUROPATHOGENESIS

RANI SADIA RAHIM,[a] MO CHEN,[a] C. CATHRIN NOURSE,[a] ADRIAN C. B. MEEDENIYA[b] AND DENIS I. CRANE[a]*

[a] *Eskitis Institute for Drug Discovery, and School of Natural Sciences, Griffith University, Qld, Australia*

[b] *Griffith Health Institute, School of Medical Science, Griffith University, Qld, Australia*

Abstract—Zellweger syndrome (ZS) is a peroxisome biogenesis disorder that involves significant neuropathology, the molecular basis of which is still poorly understood. Using a mouse model of ZS with brain-restricted deficiency of the peroxisome biogenesis protein PEX13, we demonstrated an expanded and morphologically modified brain mitochondrial population. Cultured fibroblasts from PEX13-deficient mouse embryo displayed similar changes, as well as increased levels of mitochondrial superoxide and membrane depolarization; this phenotype was rescued by antioxidant treatment. Significant oxidative damage to neurons in brain was indicated by products of lipid and DNA oxidation. Similar overall changes were observed for glial cells. *In toto*, these findings suggest that mitochondrial oxidative stress and aberrant mitochondrial dynamics are associated with the neuropathology arising from PEX13 deficiency.

Key words: Zellweger syndrome, PEX13 deficiency, mitochondria, oxidative stress, peroxisomes.

INTRODUCTION

Zellweger syndrome (ZS) is a neonatal lethal peroxisome biogenesis disorder that arises from mutations in *PEX* genes and which is characterized by peroxisomal metabolic dysfunction (Distel et al., 1996; Gould and Valle, 2000).

Our understanding of the neuropathogenesis of ZS has been assisted by investigations on mouse models of ZS that have been generated by knockout or conditional inactivation of the *PEX2* (Faust and Hatten, 1997; Faust, 2003), *PEX5* (Baes et al., 1997; Janssen et al., 2000; Kassmann et al., 2007; Krysko et al., 2007; Hulshagen et al., 2008; Bottelbergs et al., 2010, 2012) and *PEX13* genes (Maxwell et al., 2003; Muller et al., 2011). Our investigations have focused on PEX13 mutants. PEX13 is a component of the peroxisome matrix protein import machinery (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) and *PEX13* mutations in humans lead to disease across the Zellweger phenotypic spectrum (Liu et al., 1999; Shimozawa et al., 1999). PEX13 knockout mice model many of the disease phenotype of ZS patients, such as neonatal lethality, abnormal peroxisome metabolism, broad tissue pathology, a neuronal migration defect, and brain dysmorphology (Maxwell et al., 2003). Mice with brain restricted deletion of *PEX13* ("PEX13 brain mutants") have a milder phenotype and have been useful for investigating postnatal brain development; these mutants have impaired granule cell layer migration, abnormal cerebellar Purkinje cell differentiation (Muller et al., 2011) and abnormalities of the serotonergic system (Rahim et al., 2014). Despite the significant findings from these and other mouse models, the cellular mechanisms underlying ZS neuropathology are still unresolved (Crane, 2014).

Although primarily a peroxisome deficiency disorder, a potential role for mitochondria in ZS neuropathogenesis has been evident since early findings of mitochondrial abnormalities in ZS patients (Goldfischer et al., 1973). Mitochondria and peroxisomes carry out a number of similar, non-redundant functions, such as fatty acid β-oxidation and detoxification of reactive oxygen species (ROS), and, in response to metabolic cues, undergo rapid remodeling that involves common components of fission machinery (Schrader, 2006), which together suggest an important inter-dependency. Research on the above-mentioned *PEX* gene mouse models of ZS has also demonstrated significant changes to mitochondrial structure and function in various tissues, such as mitochondrial proliferation and dysmorphology, respiratory chain dysfunction, increased levels of (mitochondrial) SOD2, and increased levels of mitochondria-generated reactive oxygen species (ROS) (Baumgart et al., 2001; Krysko et al., 2007; Muller et al., 2011).

In the context of the neuropathology of ZS, we hypothesize that mitochondrial dysfunction is a plausible consequence of peroxisome deficiency that may be mediated by an oxidative stress-dependent pathway of organelle remodeling. In this regard, there is growing evidence of an important metabolic link between the peroxisome and mitochondrial redox systems (Walton

*Corresponding author. Address: School of Natural Sciences, Griffith University, Nathan, Qld 4111, Australia. Fax: +61-7-37357773.
E-mail address: d.crane@griffith.edu.au (D. I. Crane).
Abbreviations: 8-OHG, 8-hydroxyguanosine; HNE, hydroxy-2-nonenal; IF, immunofluorescence; MEFs, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; TPH2, tryptophan hydroxylase-2; ZS, Zellweger syndrome.

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and Pizzitelli, 2012; Wang et al., 2013). The present study addresses mitochondrial changes arising from PEX13 deficiency, to investigate a possible association between oxidative stress, mitochondrial structure and function, and cellular indicators of tissue pathology.

EXPERIMENTAL PROCEDURES

Animals

PEX13 brain mutants, PEX13 knockout mice, and littermate wild-type mice, were generated and genotyped as reported previously (Maxwell et al., 2003; Muller et al., 2011). Mouse genetic modification was approved by the Griffith University Institutional Biosafety Committee (NLRD/21/07), and animal experimentation was approved by the Griffith University Animal Ethics Committee (BBS/02/09/AEC; ESK/03/13/AEC). The "aged" mouse used in these experiments was a 1-year-old wild-type C57BL/6 J, the background strain of the PEX13 mutants.

Perfusion-fixation of mouse brain

For immunofluorescence analysis, brains were collected as follows: P20 animals were first anesthetized with 80 mg/kg ketamine and 10 mg/kg xylazine, then perfused transcardially with 10 mM phosphate-buffered saline, pH 7.4 (PBS) containing 0.5% sodium nitrite at a rate of 0.5 mL/min to remove blood, followed by perfusion with modified Zambonie's fixative reagent (2% paraformaldehyde, 0.2% picric acid, 0.1 M PBS, pH 7.2). Brains were then removed and retained in fixative overnight in vacuo. The following day, fixed brains were rinsed with PBS three times for 30 min to remove fixative and stored in PBS/0.5% azide (PBS/azide) at 4 °C for later processing.

Tissue cryo-processing and immunofluorescence analysis

Brains were washed twice with PBS for 30 min then placed in 30% sucrose containing PBS/azide overnight at 4 °C. Brains were then passed through a series of graded OCT solutions (20%, 30%, 50%, 70% OCT, prepared in 30% sucrose in PBS/azide, and finally 100% OCT) for 60 min each and then molded in cryomolds (25 cm × 22 cm) with 100% OCT solution and stored at −80 °C until sectioned (Nguyen et al., 2010). Whole brain was sectioned using a Leica CM 3050 s cryostat and sections were collected in a 24-well plate containing PBS/azide as free-floating sections or on supra-frost slides as adherent sections, and kept at 4 °C and −80 °C respectively, until further analysis. 40-μm-thick coronal brain sections from three wild-type and three PEX13 brain mutants (six sections for each) were carefully matched through reference to the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001). Brain sections were rinsed with PBS containing 0.1% Triton X-100 (PBS/Triton) and permeabilized using 100% DMSO (Sigma) for 20 min. To block non-specific binding sites on tissue, sections were incubated in blocking buffer (PBS/Triton X-100 containing 10% donkey serum (v/v) for 1 h at room temperature. Various combinations of primary antibodies were used: mouse anti-GFAP, at a dilution of 1:800 (Sapphire Biosciences, Waterloo, NSW, Australia); rabbit anti-IBA-1, 1:2000 (Wako Chemicals, Richmond, VA, USA); goat anti-tryptophan hydroxylase-2 (TPH2) (Everest Biotech Ltd, Oxfordshire, UK), rabbit anti-TPH2 (ThermoFisher Scientific, Inc, Rockford, IL, USA), rabbit anti-5HT (Sigma Aldrich Ltd, NSW, Australia); rabbit anti-SOD2, 1:500 (Abcam plc, Cambridge, UK), sheep anti-SOD2, 1:1000 (EMD Millipore, Billerica, MA, USA), rabbit anti-NOS, 1:500 (Merck Millipore, Billerica, Massachusetts, USA), mouse anti-PMP70, 1:600 (Maxwell et al., 2003), rabbit anti-PEX14 (Maxwell et al., 2003), rabbit anti-PGC-1α (Santa Cruz Biotech Inc., Dallas, TX, USA). Antibodies were diluted in blocking buffer and incubated with sections overnight at 4 °C. Sections were then washed thoroughly in PBS/Triton and incubated with the relevant Alexa Fluor-conjugated secondary antibodies diluted in PBS/Triton, for 3 h at room temperature – donkey anti-rabbit 488 or 594 (1:400; Life Technologies, Carlsbad, CA, USA); donkey anti-goat 594 or 488 (1:400, Invitrogen); donkey-anti-goat 650, 1:50 (Abcam plc, Cambridge, UK), donkey-anti-sheep 594 (1:400; Life Technologies Carlsbad, CA, USA), donkey-anti-mouse 594 (1:400; Life Technologies, Carlsbad, CA, USA). To reduce non-specific background staining from secondary antibodies, multiple washing steps (up to 10 times depending on the antibody) using PBS/TritonX-100 were employed.

Preparation and analysis of cultured mouse embryonic fibroblasts (MEFs)

MEFs were prepared from stage 14 embryos and cultured as described previously (Nguyen et al., 2006), except that culture flasks were pre-coated with 2% gelatin to enhance cell adhesion. Immunofluorescence analysis of cultured MEFs was also as described (Nguyen et al., 2006). Other assays used fluorescence microscopy of MEFS following incubation with specific probes: mitochondrial morphology and distribution were assessed following incubation with 200 nM MitoTracker Red CMXRos (Invitrogen) for 30 min at 37 °C, and rabbit SOD2 antibody (1:200) (Abcam plc, Cambridge, UK); levels of mitochondrial superoxide were assessed following incubation with 5 μM MitoSOX (Invitrogen) for 15 min at 37 °C; mitochondrial membrane potential was measured following incubation with 1 μM JC-1 dye (Invitrogen) for 30 min at 37 °C. As positive controls for superoxide generation and membrane potential, MEFs were incubated with 2 μM rotenone for 30 min at 37 °C. Partial inhibition of catalase enzymatic activity was achieved by incubating wild-type MEFS with 2 mM 3-amino-1,2,4,-triazole (K&K Laboratories) for 4 h at 37 °C (Walton and Pizzitelli, 2012). Catalase activity in MEF homogenates was determined using a spectrophotometric analysis as previously described (Crane et al., 1985). To assess the direct effect of oxidants on mitochondria, 250 μM hydrogen peroxide was added to culture medium of wild-type MEFs for 2 h (Stamer et al., 2002). Antioxidant effect was tested following addition of 1 mM N-acetylcysteine to culture medium for 24 h (Kageyama et al., 2012).