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Research Paper

Dimethyl fumarate modulates antioxidant and lipid metabolism in oligodendrocytes

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ABSTRACT

Oxidative stress contributes to pathology associated with inflammatory brain disorders and therapies that upregulate antioxidant pathways may be neuroprotective in diseases such as multiple sclerosis. Dimethyl fumarate, a small molecule therapeutic for multiple sclerosis, activates cellular antioxidant signaling pathways and may promote myelin preservation. However, it is still unclear what mechanisms may underlie this neuroprotection and whether dimethyl fumarate affects oligodendrocyte responses to oxidative stress. Here, we examine metabolic alterations in oligodendrocytes treated with dimethyl fumarate by using a global metabolomic platform that employs both hydrophilic interaction liquid chromatography–mass spectrometry and shotgun lipidomics. Prolonged treatment of oligodendrocytes with dimethyl fumarate induces changes in citric acid cycle intermediates, glutathione, and lipids, indicating that this compound can directly impact oligodendrocyte metabolism. These metabolic alterations are also associated with protection from oxidant challenge. This study provides insight into the mechanisms by which dimethyl fumarate could preserve myelin integrity in patients with multiple sclerosis.

Introduction

Metabolic reactions drive fundamental cellular processes, including energy production, signaling, and the generation of precursors for macromolecules. Perturbation of these pathways by disease results in cellular pathology; therefore, the profiling of metabolites has the potential to uncover new disease biomarkers, identify novel therapeutic targets, and provide information on drug mechanism [1,2]. The comprehensive analysis of global metabolism allows the identification of alterations in endogenous small molecules without bias; however, a significant hurdle for these studies is the chemical variability contained within the metabolome. This factor makes a single separation and detection method unlikely to provide complete coverage of all metabolite species [3]. Here we introduce a global metabolomic platform that utilizes both direct-infusion mass spectrometric analysis of lipids (shotgun lipidomics) and a hydrophilic interaction liquid chromatography–mass spectrometric method (HILIC–MS) that measures polar metabolites associated with the tricarboxylic acid cycle (TCA), amino acid, and nucleotide metabolism [4]. Direct infusion of the lipid samples significantly reduces processing time while still detecting high abundance lipids that include biologically important classes such as glycerophospholipids, sphingolipids, and triacylglycerols [5]. The combination of these two methods provides coverage of diverse metabolic pathways critical for cellular function with reduced analysis times.

We sought to utilize our platform to identify metabolic pathways important for neuroprotection, specifically the maintenance of myelin. The myelin sheath is produced by oligodendrocytes and wraps around neuronal axons, facilitating rapid nerve conduction and providing trophic support to neurons [6]. Damage to the myelin or oligodendrocytes leads to the development of lesions within the central nervous system and this is a defining pathological feature of the autoimmune disease, multiple sclerosis [7]. Several mechanisms have been postulated to result in demyelination during multiple sclerosis, including the release of toxic mediators such as reactive oxidative species as well as direct killing of oligodendrocytes by inflammatory cells [8,9]. The activation of cellular pathways that interrupt these processes may not only prevent demyelination, but also inhibit the axonal degeneration that accompanies the loss of the oligodendroglia-neuronal connection [10].

A number of agents have been tested for their ability to act as neuroprotective agents during central nervous system inflammation. The small molecule drug, dimethyl fumarate (DMF,

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Tecfidera®) is currently approved for the treatment of multiple sclerosis [11]. Data from human and animal studies suggests that this compound has both anti-inflammatory and antioxidant properties [12–14]. Clinically, a recent study showed that patients treated with DMF had increases in brain magnetization transfer ratio (MTR) and this was thought to correlate with the preservation of myelin density [15]. However, it is unclear whether the myelin protection seen in multiple sclerosis patients treated with DMF is due to the direct modulation of oligodendrocyte functions or if this result is due to its effect on astrocytes, neurons, or inflammatory cells. Analysis of DMF treatment during cuprizone-induced demyelination showed no impact on demyelination and short-term treatment of an oligodendroglial cell line in vitro did not prevent hydrogen peroxide-mediated death [16]. These results suggest that the myelin protection induced by DMF may be secondary to its anti-inflammatory actions. In this study, we sought to clarify whether DMF directly impacts oligodendrocyte physiology by using mass spectrometry-based metabolomics to identify changes in metabolism induced by drug treatment as well as determine the impact of these alterations on oligodendrocyte responses to oxidative stress.

Materials and methods

Chemicals

Ammonium acetate (HPLC, $\geq$99.0%), ammonium hydroxide solution (LC/MS, $\geq$25% in H_2O), and DMF were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC/MS-grade water, acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol was purchased from DeconLaboratories, Inc. (King of Prussia, PA, USA). Chloroform (HPLC Grade, $\geq$99.5%) was obtained from Alfa Aesar (Ward Hill, MA, USA). For cell culture experiments, Dulbecco's Modification of Eagle's Medium (DMEM), Penicillin/streptomycin, Phosphate-Buffered Saline (PBS) and Fetal Bovine Serum (FBS) were purchased from Corning (Manassas, VA, USA).

Cell culture

The MO3.13 human oligodendrocyte cell line was purchased from CELLutions Biosystems Inc. (Burlington, Ontario, CA). MO3.13 cells were cultured at 37 °C in 5% CO_2 in DMEM containing 10% FBS and 1% penicillin/streptomycin for all experiments. Cells were seeded in 6-well plates at a density of 3.0×10^5 cells/mL for the 24 h experiments or 1.0×10^5 cells/mL for experiments lasting 72 h.

DMF treatment and metabolite extraction

MO3.13 cells were allowed to adhere and subsequently treated with 10 μM DMF diluted in 0.2% ethanol/PBS or vehicle alone as a control. Treatments were carried out for 24 or 72 h prior to the metabolite extraction [16,17]. Following treatment, a modified form of the Bligh and Dyer Extraction was used to obtain both hydrophilic and lipophilic metabolites for metabolomic analysis [18]. MO3.13 cells were lysed by the addition of 180 μL HPLC grade water with 20 μL methanol and removed from the tissue culture dishes by gentle scraping. The cell suspensions were then subjected to three cycles of freezing in liquid nitrogen, thawing, and sonication. 750 μL of 1:2 (v:v) $CHCl_3$:MeOH and 125 μL $CHCl_3$ were added to each sample, the samples were vortexed, and an additional 250 μL of water was added. After incubation at −20 °C for 1 h, samples were centrifuged at 1000 × *g* for 10 min at 4 °C to give a two-phase system: an aqueous layer on top, an organic layer below, and a protein disk interphase. The aqueous and organic phases were collected into 1.5 mL tubes separately. All the extracted samples were dried in a CentriVap Concentrator (LABCONCO, Kansas, MO, USA) and then preserved at −80 °C until resuspension and analysis. Protein pellets were used to normalize extracted metabolites quantities based on protein concentration with a Bicinchoninic Acid (BCA) protein assay (G-Biosciences, St. Louis. MO, USA) [19].

Hydrogen peroxide treatment and MTT assay

MO3.13 cells were seeded in a 96-well plate at a density of 7.5×10^3 cells/mL for the 24 h treatment, or 2.5×10^3 cells/mL for the 72 h treatment (12 replicates per treatment group). Cells were treated with 1 or 10 μM DMF, PBS, or 0.2% ethanol (vehicle). PBS-treated cells were used for viability normalization based on absorbance. After drug treatment, 400 μM hydrogen peroxide was added to the cells for 2 h. 20 μL of a 5 mg/mL Thiazolyl Blue Tetrazolium Bromide (MTT) PBS solution was subsequently added to all the wells and cultures were incubated at 37 °C for 3.5 h. The media was then aspirated and 150 μM of lysis solvent (4 mM HCl, 0.1% Nondet P-40 (NP_40) in isopropanol) was added to dissolve the insoluble purple formazan product into a colored solution. The plates were then covered with aluminum foil and agitated on an orbital shaker for 15 min. The absorbance was read at 590 nm with a reference filter of 620 nm by using a SpectraMax™ M2 plate reader [20].

HILIC–MS profiling of metabolites

Chromatographic separation was carried out on a Micro200 LC (Eksigent, Redwood, CA, USA) equipped with a hydrophilic interaction liquid chromatography (HILIC) column (Luna 3 μ NH_2 100 Å, 150 mm × 1.0 mm, Phenomenex, Torrance, CA, USA). The polar metabolites were re-suspended in 200 μL of a 35:65 (v:v) acetonitrile: water solution, and 5 μL of the sample was injected into the column. The liquid chromatographic method consisted of a mobile phase A of water and a mobile phase B of acetonitrile, each with the addition of 5 mM ammonium acetate and 5 mM ammonium hydroxide. The flow rate was 30 μL/min. The gradient consisted of the following linear changes in mobile phase B over time: 0 min 98%, 0.5 min 98%, 1 min 95%, 5 min 80%, 6 min 46%, 13 min 14.7%, 17 min 0%, 17.1 min 100%, 23 min 100%.

Samples were analyzed on the 5600+ TripleTOF Mass Spectrometer (AB SCIEX, Framingham, MA, USA) in both positive and negative mode and were processed with Information Dependent Acquisition (IDA). The ion source nebulizer gas (GS1) used was set at 15 psi, heater gas (GS2) was 20 psi, and the curtain gas (CUR) was 25 psi. In the IDA experiment, a TOF MS scan was selected to perform a survey scan for the mass range of 60–1000 Da. This survey scan utilized a 250 ms accumulation time for precursor ion acquisition. In the positive mode, +5000 V ionspray voltage was used and a +100 V declustering potential (DP) was selected to increase precursor ions detection. The background threshold for candidate ion selection was set to 10 counts/s to eliminate peaks with low abundance as well as a low signal to noise (*S*/*N*) ratio. Fragmentation data were subsequently collected by using a collision energy spread (CES) of +(25−40) V. Samples analyzed in negative mode used the same GS1, GS2 and CUR in IDA criteria. A −4500 V ionspray voltage was used and a −100 V declustering potential (DP) was selected for better precursor ion detection. Fragmentation data were subsequently collected by using a collision energy spread (CES) of −(40–25) V.