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Dimethyl fumarate alters microglia phenotype and protects neurons against proinflammatory toxic microenvironments

Haiyan Peng *[,1], Huo Li, Adam Sheehy, Patrick Cullen, Norm Allaire, Robert H. Scannevin [2]

Biogen, Inc., 225 Binney St, Cambridge, MA 02142, United States

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ABSTRACT

Delayed-release dimethyl fumarate (DMF) is an approved treatment for multiple sclerosis (MS). Microglia are considered central to MS pathophysiology, however the effects of DMF and the primary metabolite monomethyl fumarate (MMF) on microglia are not well characterized. We demonstrated that DMF and MMF altered transcriptional responses in primary microglia related to the nuclear factor (erythroid-derived 2)-like 2 pathway. Additionally, through an NRF2 independent manner, DMF, but not MMF significantly reduced production of proinflammatory mediators in classically activated microglia, and further rescued mitochondrial respiratory deficits in primary cortical neurons that were induced by activated microglia. These data suggest the mechanism of action of DMF may involve modulation of microglia inflammatory responses and attenuation of neurotoxicity.

1. Introduction

Microglia are the resident macrophages of the central nervous system (CNS). Occupying approximately 5–12% of the CNS cell population, microglia have critical functions in both health and disease. In vivo imaging studies revealed that microglia in the healthy brain have highly dynamic processes and consistently survey the local microenvironment (Nimmerjahn et al., 2005). Further studies have indicated that microglia play a role in the elimination and refinement of synaptic connections in healthy brain tissue (Schafer et al., 2012; Wu et al., 2015; Hong et al., 2016). Disruption of microglia may result in learning deficits in adult animals, which is correlated with a reduction of synaptic formation in neurons (Schafer and Stevens, 2015). Currently available evidence suggests that microglia are critical in shaping the neural network and maintaining synaptic homeostasis. However, during diseases such as multiple sclerosis (MS), microglia may sustain and propagate neuroinflammation and drive disease progression (Cunningham, 2013, Goldmann and Prinz, 2013, Huang and Feng, 2013, Ransohoff and Prinz, 2013, Perry and Holmes, 2014, Maphis et al., 2015). Positron emission tomography imaging studies have demonstrated that activated microglia persist in lesions of relapsing remitting MS (RRMS), and are further dispersed in the secondary progressive forms of MS where infiltrating immune cells are largely reduced (Politis et al., 2012; Giannetti et al., 2014). This may indicate a shift from adaptive immunity mediated inflammation to compartmentalized inflammation maintained by microglial activation, which is likely to drive continuous progression of the disease. In addition to the clinical evidence, preclinical studies also suggest a detrimental role of microglia. Activation of microglia has been observed in the spinal cord of experimental autoimmune encephalomyelitis (EAE) mice prior to disease onset and continuing into the chronic phase of the disease. To address the function of activated microglia, Goldmann and colleagues generated conditional knockout mice that specifically targeted microglia associated inflammation during autoimmune disease (Goldmann et al., 2013). Interestingly, their data suggested that depletion of (TGF)-β-activated kinase 1 in microglia only suppressed EAE, reduced CNS inflammation and diminished axonal and myelin damage by cell autonomous inhibition of nuclear factor κB (NF-κB), c-Jun amino-terminal kinase and extracellular signal-regulated kinases 1 and 2 pathways (Goldmann et al., 2013). Although the exact mechanisms through which microglia activation leads to neuronal damage and neurodegeneration is not fully understood, accumulating evidence, at least in vitro, suggests that upon activation, microglia releases toxic factors such as proinflammatory cytokines and reactive oxygen specifies (ROS) that subsequently result in neuronal damage (Frakes et al., 2014; Brown and Vilalta, 2015, Peng et al., 2015; Papageorgiou et al., 2016). This evidence supports the notion that reducing microglia pathological activation could be a

Abbreviations: DMF, dimethyl fumarate; MS, multiple sclerosis; MMF, monomethyl fumarate; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; RRMS, relapsing remitting MS; ROS, reactive oxygen species; NO, nitric oxide; 1,2-Dimethoxycarbonylethy, *N*-acetyl-*S*; DMF-GSH, DMF glutathione conjugate.

* Corresponding author at: Biogen, Inc., 225 Binney St, Cambridge, MA 02142, United States.
E-mail address: Haiyan_Peng@Eisai.com (H. Peng).
[1] Current address: Eisai Inc. 4 Corporate Drive, Andover, MA 01810, United States.
[2] Current address: Yumanity Therapeutics, 790 Memorial Drive, Cambridge, MA 02139, United States.

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viable therapeutic approach for treating patients with neurological disorders. Ideal therapies will be required to cross the blood brain barrier (BBB) in order to exert their direct effects on microglia.

Delayed-release of dimethyl fumarate (DMF) was approved as an effective treatment of RRMS in 2013 (Fox et al., 2012). DMF has a short-half life and is converted to its primary metabolite, monomethyl fumarate (MMF), quickly. Although MMF was shown to penetrate brain parenchyma (Brennan et al., 2016), DMF itself has been undetectable in vivo. Subsequently, the biological relevance of DMF in the central nervous system (CNS) remains to be seen. In this study, for the first time, we provide evidence demonstrating that some DMF may survive absorption after oral dosing, and thus has the potential to exert pharmacological activity throughout the body. Furthermore, the effects of both DMF and MMF on pathological activity of microglia were also characterized in vitro. The results of these studies indicate that DMF, but not MMF, significantly reduces production of inflammatory cytokines and nitric oxide (NO) by primary microglia after inflammatory challenge, which subsequently reduces the toxicity of these cells towards primary neurons.

2. Materials and methods

Unless noted otherwise, reagents were obtained from Sigma (St. Louis, MO).

2.1. Animal procedures and exposure analysis

All procedures involving animals were performed in accordance with standards established in the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. All animal protocols were approved by the Biogen, Inc. Institutional Animal Care and Use Committee. Biogen, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Male Sprague-Dawley rats weighing 300-350 g were used (Charles River, Wilmington, MA). Standard water and chow ad libitum were given throughout the duration of the experiment. All rats were acclimated at least one week before the study. On the day of the study, rats were dosed orally (PO) at 10 ml/kg with a single dose of DMF (100 mg/kg) or the glutathione conjugate of DMF (DMF-GSH; 100 mg/kg) as a suspension in 0.8% hydroxypropyl methylcellulose, and the dosing schedule was counterbalanced within groups to mitigate time of day effects. Separate cohorts of animals were sacrificed at 2, 5, 10, 30 or 60 min after dosing and blood and brain tissue were collected. Brain and blood samples were processed as previously described (Brennan et al., 2016). Briefly, brain tissue was snap frozen on dry ice, and blood was processed to plasma in the presence of sodium fluoride as a stabilizing agent. A liquid chromatography–mass spectrometry/mass spectrometry-based bioanalytical assay was used to determine MMF and DMF-GSH concentrations in plasma and brain tissue.

2.2. Primary microglia culture

Primary microglia enriched culture was prepared as described previously with modifications (Butovsky et al., 2014). In brief, microglia cultures were obtained from primary mixed glial cultures from 1- to 3-day-old C57BL/6 mice. To obtain mixed glial cultures, cerebral cortices were dissected, and digested with 0.25% trypsin-EDTA solution for 25 min at 37 °C. Trypsinization was stopped by adding an equal volume of culture media, to which 0.02% deoxyribonuclease-I was added. The culture media consisted of Dulbecco's modified Eagle media (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 0.1% penicillin-streptomycin. Cells were pelleted (10 min, 1300 rpm), resuspended in culture media, and brought to a single cell suspension by repeated pipetting followed by passage through a 70 μm-pore mesh. Cells were seeded at a density of 3.5×10^5 cells/ml (1.2×10^5 cells/cm^2) and cultured at 37 °C in a 5% CO_2 humidified atmosphere. Media was replaced every 5–7 days. Microglial cultures were prepared by shaking the mixed glia culture at 37 °C for 1 h at 200 rpm. Detached microglia cells were then collected and seeded in Poly-D-lysine (PDL) coated plates. The microglial cultures were rested in 2% FBS DMEM media for 48 h prior to any treatment. Due to their lipophilic properties, DMF and MMF were dissolved in Dimethyl sulfoxide (DMSO) as stock solutions and further diluted (1:2000) into PBS upon use. Microglia were treated with DMF, MMF (30 μM each) or DMSO control before stimulation with lipopolysaccharide (LPS; 100 ng/ml) + interferon γ (IFN-γ; 20 ng/ml; R&D Systems, Minneapolis, MN).

2.3. Microarray analysis

Total RNA from primary microglia was isolated using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. RNA integrity was assessed using the Agilent 2100 Bioanalyzer system and Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA). Total RNA from DMF treated primary microglia in the presence or absence of LPS (10 ng/ml) and IFN-γ (20 ng/ml) were amplified using the Ovation Pico WTA System (NuGEN, San Carlos, CA). The Encore Biotin Module Kit (NuGEN) was used for fragmentation and labeling of cDNA for further analysis. Samples were hybridized using Affymetrix GeneChip Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara CA). Gene expression data were analyzed using Affymetrix Expression Console software (Affymetrix), R/Bioconductor AFFY package (Gentleman et al., 2004), TIBCO Spotfire (TIBCO, Somerville, MA). Data were normalized by gcRMA method (Irizarry et al., 2003) and filtered for transcripts, which were differentially expressed between treated conditions and controls. Significance ($p \le 0.05$) was calculated using an analysis of variance (one way ANOVA), adjusted for multiple comparison using Benjamini-Hochberg procedure. Only transcripts with an absolute change in expression level of at least 1.5-fold were considered. Gene ontology and pathway enrichment was calculated using Ingenuity pathway analysis (Qiagen) database and software.

2.4. Luminex assays

Levels of eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage (GM)-CSF, IFN-γ, interleukin (IL)-1α, IL-1β, IL-2, IL-3 IL-4, IL-5, IL-6, IL-7, IL-9 IL-10, IL-12p70, IL-12p40, IL-13, IL-15, IL-17, IFN-γ-inducible protein (IP)-10, keratinocyte chemoattractant (KC), leukemia inhibitory factor (LIF), monokine induced by gamma interferon (MIG), LPS-induced CXC chemokine (LIX), monocyte chemotactic protein (MCP)-1, macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein (MIP)-1α, macrophage inflammatory protein (MIP)-1β, MIP-2, regulated upon activation normal T cell expressed and presumably secreted (RANTES), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF) were simultaneously determined using Luminex xMAP technology (EMD Millipore, Billerica, MA). Procedures were conducted according to manufacturer protocols, and a standard curve for each of the target proteins was prepared by serial dilution in assay buffer. Supernatants from microglia cultures were collected at 24 h after LPS addition. Undiluted supernatant solutions (25 μL/well) were assayed in duplicate. Experimental samples were analyzed in duplicate and concentrations were calculated by comparison to the appropriate reference standard curve. Quality-control samples (provided by the manufacturer) were analyzed simultaneously and used to validate the accuracy of the assay.

2.5. Quantification of nitrite and nitrate

Conditioned media (CM) from primary microglia cell cultures were collected at 48 h after LPS addition. Production of NO was determined