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TrkB agonist, 7,8-dihydroxyflavone, reduces the clinical and pathological severity of a murine model of multiple sclerosis

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ABSTRACT

7,8-Dihydroxyflavone (DHF), is a recently described TrkB agonist that readily crosses the blood brain barrier. We treated C57Bl/6 mice with MOG — induced EAE daily with DHF starting on the day of disease induction. Clinical severity of impairment was reduced throughout the course of disease. Pathological examination of brains and spinal cords on day 28 showed that DHF treatment increased the phosphorylation of TrkB and activated downstream signaling pathways including AKT and STAT3 and reduced inflammation, demyelination and axonal loss compared to EAE controls. DHF treatment duplicated the central nervous system effects of brain derived neurotrophic factor in the EAE.

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1. Background

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) which often produces chronic disability (Compston and Coles, 2008; Koch-Henriksen and Sorensen, 2010). Infiltration of heterogeneous myelin-reactive peripheral immune cells across the blood–brain-barrier (BBB) triggers demyelination, axonal damage and neuronal loss in MS (Rottlaender and Kuerten, 2015). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS in which similar types of auto-reactive cells cause CNS inflammation and neurological impairment (Jager et al., 2009). Immune cell infiltration, demyelination, axonal transection and neurological deficit are common in both MS and EAE (Hendriks et al., 2005; Matthews et al., 1998; Trapp et al., 1998; Wujek et al., 2002). Current approved disease modifying therapies reduce inflammation (Goodin, 2008; Linker et al., 2005; Rudick and Trapp, 2009) but do not prevent long-term axonal damage and neuronal loss (Soulika et al., 2009; Trapp et al., 1998). New therapies are needed that protect neuronal elements and promote myelin repair and reduce the development of long term disability in MS.

Brain derived neurotrophic factor (BDNF) is a pleiotropic cytokine which promotes neuronal survival, axonal growth and neuroprotection that has been identified as a potential therapeutic agent for different neurodegenerative diseases (Gururajan et al., 2015; Numakawa, 2014). BDNF appears to play a protective role in MS and EAE. Glatiramer acetate (GA), an approved drug for MS therapy, stimulates endogenous BDNF production and in animal studies, GA-stimulated cells induced brain derived neurotrophic factor (BDNF) secretion (Chen et al., 2003; Makar et al., 2008). In addition, GA-induced T-cells secrete high levels of BDNF in the brain of EAE mice (Aharoni et al., 2005; Aharoni et al., 2003), and BDNF levels were shown to be increased in the brains of mice after GA injection (Aharoni et al., 2005). However, BDNF treatment has failed in clinical trials in neurodegenerative diseases (Thoenen and Sendtner, 2002) likely due to its short plasma half-life and poor blood brain barrier (BBB) penetration (Poduslo and Curran, 1996). Also, BDNF can induce neuronal cell death through $p75^{NTR}$ receptor binding (Friedman, 2000). To overcome the fact that BDNF does not cross the BBB we engineered hematopoietic stem cells to produce BDNF and

Abbreviations: EAE, Experimental autoimmune encephalomyelitis; MS, Multiple Sclerosis; DHF, 7, 8-dihydroxyflavone; TrkB, Tropomyosin Related Kinase B receptor; MOG, Myelin oligodendrocyte glycoprotein; CNS, Central nervous system; BBB, Blood brain barrier.

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tested the feasibility and effectiveness of a cell-based delivery of BDNF into the CNS in EAE. We demonstrated that BDNF treatment reduces inflammation, axonal damage and neuronal loss in EAE (Makar et al., 2009, 2012, 2014). BDNF treatment is associated with activation of the high affinity receptor Tropomyosin related kinase B (TrkB) (also known as Tyrosine receptor kinase B) and downstream signaling pathways. Activation of TrkB and BDNF-related intracellular signaling may be an alternative approach to treating neurodegenerative diseases including MS.

7, 8-Dihydroxyflavone (DHF) is a small molecule that has recently been identified as a specific TrkB agonist that mimics functions of BDNF. It readily crosses the BBB after peripheral injection (Jang et al., 2010), binds with high affinity to TrkB, activates the downstream PI3K/Akt and other signaling pathways in neurons (Jang et al., 2010; Tsai et al., 2013) and increases cell survival and neurite growth in neurons (Jang et al., 2010). Also, the binding specificity of DHF to TrkB eliminates the adverse effects of $p75^{NTR}$ receptor signaling pathway (Jang et al., 2010). DHF shows neuroprotective activity in different neurodegenerative diseases (Castello et al., 2014; Devi and Ohno, 2012; Jang et al., 2010; Jiang et al., 2013; Makar et al., 2012; Tsai et al., 2013). Taken together, these reports indicate that DHF acts as a TrkB agonist and can be used as a tool to investigate the role of BDNF/TrkB signaling. These findings suggested that DHF might be an orally active agent that could mimic the beneficial effects that we had found with BDNF using engineered hematopoietic stem cells as a delivery device.

In the present study we evaluated the effects of DHF in EAE to determine whether DHF activates TrkB and downstream signaling pathways in vivo and produces reductions in clinical severity, immunological changes, neuronal loss and demyelination similar to those seen with cell-based delivery of BDNF in EAE (Makar et al., 2009, 2012, 2008). Other flavonoids have been found to show anti-inflammatory activity in EAE mice (Abd-Alla et al., 2015; Zeng et al., 2007), but the effect of DHF in EAE has not been investigated. We report here that DHF increased the phosphorylation of TrkB in vivo, activated pathways downstream from TrkB and reduced the clinical and pathological severity of MOG induced EAE in C57Bl/6 mice. We showed that DHF reduced axonal loss and demyelination and increased remyelination.

2. Methods

2.1. Animals

Female C57Bl/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under pathogen-free conditions at the animal facility of the University of Maryland School Of Medicine, Baltimore. All experimental procedures were conducted following NIH guidelines under an Institutional Animal Care and Use Committee-approved protocol from the University Of Maryland School Of Medicine, Baltimore.

2.2. EAE induction

All animals were used at 8 weeks of age. EAE induction, evaluation and scoring the clinical severity of the disease was carried out as described (Makar et al., 2009, 2008). EAE was induced with 200 μg myelin oligodendrocyte glycoprotein 35–55 (MOG) peptide (Biomer Technology, Pleasanton, CA, USA) (Nimmagadda et al., 2013). Mice were evaluated clinically daily.

2.3. Clinical evaluation

EAE evaluation and scoring the clinical severity of the disease was carried out as described (Makar et al., 2009, 2008). Mice were given a clinical score of increasing severity: 1- limp tail; 2-hind limb paresis or partial paralysis; 3-total hind limb paralysis; 4-hind limb paralysis and body/front limb paresis/paralysis; and 5-moribund. Briefly, from day 9 onwards mice were assessed daily for signs of paralysis by two independent observers in a blinded fashion. End point evaluation included mean severity of disease over time and mean day of disease onset (first day of score >0).

2.4. Drug

We administered DHF (Tokyo Chemical Industry) intraperitoneally at a dose of 5 mg/kg in 200 μl vehicle of 17% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS). EAE mice were divided in to vehicle-treated EAE (EAE) and DHF-treated EAE (EAE + DHF). EAE + DHF mice received a single dose once daily from the day of EAE induction (day 0). Vehicle-treated mice received 200 μl of 17% DMSO in PBS.

2.5. Tissue pathology

Mice were euthanized on day 28. Spinal cords and brains were removed for analysis. Paraffin sections of spinal cord were prepared as previously described (Makar et al., 2009). 7 μm thick sections were stained with hematoxylin and eosin (H&E) (to detect inflammatory infiltrates) and Luxol Fast Blue (for demyelination) following standard protocols for conventional light microscopy. Axonal loss was determined by Silver Nitrate staining using Hito Bielschowsky Optim Stain Kit (Hitobiotec Inc., Wilmington, DE, USA). Slides were examined using standard bright-field microscopy.

2.6. Immunohistochemistry

Immunohistochemistry of was performed as previously described (Makar et al., 2012) using VECTASTAIN ABC kits (Vector Laboratories, Burlingame, CA, USA). 7 μm thick sections were used. Primary antibodies used are listed in Table 1. Nuclei were counterstained with hematoxylin. Slides were examined using standard bright field microscopy.

2.7. Immunofluorescence

Immunofluorescence was performed on spinal cord sections, as previously described (Nimmagadda et al., 2013). 7 μm thick paraffin sections were used. Primary Abs used are listed in Table 1. Slides were examined using standard fluorescence microscopy.

2.8. TUNEL assay for apoptotic cell death

Paraffin embedded spinal cord sections (7 μm) were examined by in situ terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL). Apoptosis was detected on paraffin sections by an ApopTag peroxidase in situ apoptosis detection kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin.

2.9. Preparation of brain extracts for Western blots

Cytosol and membrane fractions were prepared from the mouse brains using Thermo Scientific MEM-PER plus kit (Thermo Scientific, Rockford, IL, USA) following the protocol provided with the kit. Protein content of the extracts was determined by Bradford method (Sigma-Aldrich, St Louis, MO, USA).

2.10. Western blot analysis

Protein samples were boiled for 5 min with a gel-loading buffer (Thermo Fisher Scientific, Waltham, MA, USA). Equal amount of protein for each sample were separated by SDS–polyacrylamide gel