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Inhibition of 5-lipoxygenase activity in mice during cuprizone-induced demyelination attenuates neuroinflammation, motor dysfunction and axonal damage ☆

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Increased expression of 5-lipoxygenase (5-LO), a key enzyme in the biosynthesis of leukotrienes (LTs), has been reported in MS lesions and LT levels are elevated in the cerebrospinal fluid of MS patients. To determine whether pharmacological inhibition of 5-LO attenuates demyelination, MK886, a 5-LO inhibitor, was given to mice fed with cuprizone. Gene and protein expression of 5-LO were increased at the peak of cuprizone-induced demyelination. Although MK886 did not attenuate cuprizone-induced demyelination in the corpus callosum or in the cortex, it attenuated cuprizone-induced axonal damage and motor deficits and reduced microglial activation and IL-6 production. These data suggest that during cuprizone-induced demyelination, the 5-LO pathway contributes to microglial activation and neuroinflammation and to axonal damage resulting in motor dysfunction. Thus, 5-LO inhibition may be a useful therapeutic treatment in demyelinating diseases of the CNS.

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1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterized by recurrent and progressive demyelination/remyelination cycles, resulting in development of scleroses in both the white and gray matter of the CNS [1], axonal damage, neuroinflammation and neuronal loss [2,3]. Demyelination is accompanied by depletion of oligodendrocyte precursor cells, loss of mature oligodendrocytes, astrogliosis, and infiltration of macrophages/microglia and T lymphocytes [4].

The cuprizone model of demyelination [5] is characterized by apoptotic death of mature oligodendrocytes [6,7], and is accompanied by neuroinflammation [8] and motor dysfunction [9]. Four patterns have been described in MS lesions, with patterns I and II showing similarities to T-cell-mediated or T-cell plus antibody-mediated autoimmune encephalomyelitis, respectively, and patterns III and IV lesions suggesting a primary oligodendrocyte damage and degeneration, reminiscent of virus- or toxin-induced demyelination rather than autoimmunity [10]. Thus, the cuprizone model of demyelination is closer to patterns III and IV lesions in reproducing a primary demyelination that is independent from the immune system, and axonal damage. Mice show progressive demyelination when they are kept on a 0.2% cuprizone diet, with a peak in demyelination observed after 5 weeks of cuprizone [6,11]. Cuprizone withdrawal from the diet results in a remyelination after several weeks [12].

Omega-6 fatty acids, such as linoleic acid, γ-linoleic acid and arachidonic acid, have been implicated in demyelinating disease. Linoleic acid and γ-linoleic acid have shown protective effects in MS and experimental autoimmune encephalomyelitis (EAE) [13–16]. On the other hand, arachidonic acid cascade is suggested to become activated during demyelination[17,18]. Increased expression of 5-lipoxygenase (5-LO) in lesions [19,20] and of 5-LO-derived leukotriene (LT) products in the cerebrospinal fluid [21] has been reported in patients with MS. 5-LO, a key enzyme in the biosynthesis of LTs [22] is activated by 5-LO-activating protein (FLAP) and converts arachidonic acid to LTA_4, which is then converted to LTB_4 by LTA_4 hydrolase [23,24], or to a cysteinyl-LT (such as LTC_4, LTD_4 or LTE_4) by LTC_4 synthase [25,26].

MK886 is a 5-LO inhibitor that binds to FLAP and thereby prevents 5-LO activation [27]. *In vitro*, MK886 inhibits LTs

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biosynthesis in leukocytes [28]. *In vivo*, systemic administration of MK886 has been shown to inhibit LPS-induced hypothalamic LT production [29] and cortical cysteinyl-LT production induced by permanent occlusion of the middle cerebral artery [30].

In this study, to determine whether 5-LO activity contributes to the pathological events associated with demyelination and associated neuroinflammation, we administered MK886 to cuprizone-exposed mice. We demonstrated that, although MK886 did not attenuate cuprizone-induced demyelination in the corpus callosum and cortex, it reduced microglial activation, IL-6 production, axonal damage and motor dysfunction. These data suggest that the 5-LO pathway is involved in microglial activation and neuroinflammation and contributes to axonal damage and motor dysfunction.

2. Materials and methods

2.1. Animal procedures

All animal experiments were performed under a NIH approved animal protocol (NICHD #08-026) approved by the NIH, NICHD Animal Care and Use Committee, in accordance with the NIH guidelines on the care and use of laboratory animals. C57BL/6 male mice (Taconic Farms, Germantown, NY) were received at our facility at 8–10 weeks of age and were fed *ad libitum* a powdered diet (Purina #5002; formulated by Research Diets, New Brunswick, NJ) containing 0.2% cuprizone (bis-cyclohexanone oxaldihydrazone; Sigma, St. Louis, MO). In a preliminary experiment, mice were fed with cuprizone diet for 6 weeks to investigate time-dependent changes of 5-LO gene and protein expression during the demyelination process (Fig. 1A). In the following experiments, mice were fed with cuprizone diet up to 5 weeks, which represents the peak of demyelination, to investigate the effects of 5-LO inhibition on demyelination, neuroinflammation and motor function (Fig. 1D). Mice were maintained on a 12/12 h light/dark cycle. Mice (n=7–8 per group) were euthanized with Nembutal and forebrain containing frontal cortex and corpus callosum was dissected on ice. Cerebellum, thalamus, hippocampus, striatum and olfactory bulb were excluded from the dissected samples. Forebrain was rapidly frozen in 2-methylbutane at −50 °C, and stored at −80 °C until use for molecular analysis. For histology, mice (n=5 per group) were intracardially perfused with 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde, subsequently cryoprotected in a 30% sucrose solution, snap frozen and stored at −80 °C until use [31].

2.2. Treatment with MK886

MK886 (Cayman Chemical, Ann Arbor, MI) was dissolved in saline with 5% DMSO, 25% polyethylene glycol-15-hydroxystearate (Solutol, BASF, Ludwigshafen, Germany). MK886 was administered at a dose of 3 mg/kg by intraperitoneal (i.p.) injection once-daily for the last 7 days (weeks 4–5) of cuprizone exposure. Control mice on a normal cuprizone-free diet were treated in parallel with MK886 at the same dose once-daily for 7 days.

2.3. Western blotting

The cytosolic fraction was prepared from forebrain as described [32]. Forebrains (n=6 per group) were homogenized in a homogenizing buffer containing 20 mM Tris–HCl (pH 7.5), using a Polytron® homogenizer. The supernatant was centrifuged at 100,000g for 60 min at 4 °C. The supernatant was collected and protein concentration was measured using a Dc Protein Assay kit (Bio-Rad, Richmond, CA). Western blotting was performed as previously described [32]. Briefly, proteins (50 μg) were loaded on Criterion gels (Bio-Rad), transferred onto a polyvinylidene difluoride membrane (Bio-Rad) and immunoblotted with antibodies against 5-LO (1:500; Cayman Chemical) and β-actin (1:3000; Sigma) as loading control. An Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NB,) was used to detect and quantify protein levels.

2.4. Measurement of IL-6 levels

Forebrains (n=5 per group) were homogenized in a lysis buffer containing 25 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA with a complete protease inhibitor cocktail (Roche, Indianapolis, IN). The homogenates were centrifuged at 14,000g for 20 min, and the supernatant was immediately assayed using a mouse IL-6 ELISA kit (Invitrogen, Carlsbad, CA).

2.5. Histology

Thirty μm coronal brain sections were cut on a cryostat (Bright Instrument Company, Ltd.; Huntingdon, England) and mounted on gelatin-coated glass slides. Sections were stained with Black Gold II (Histo-Chem, Jefferson, AR) as previously described [33]. Briefly, sections were incubated in a 0.2% Black Gold II solution for 12 min, rinsed in distilled water, fixed in 2% sodium thiosulfate, rinsed in tap water and air-dried. Sections were then cleared in Histo-Clear (National Diagnostics, Atlanta, GA) and coverslipped using DPX (Sigma) mounting medium. Immunohistochemistry was performed using rat anti-mouse CD11b (1:200; Serotec, Oxford, UK) as primary antibody at room temperature overnight and visualized using VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) and counterstained them with VECTOR hematoxylin QS (Vector). Double immunofluorescence was performed using anti-mouse amyloid precursor protein (APP) (1:200; Chemicon, Temecula, CA) and anti-rabbit neurofilament 200 (NF200) (1:80, Sigma), as follows. Sections were incubated with a mixture of two primary antibodies at room temperature overnight, followed by incubation at room temperature for 1 h with a mixture of the two secondary antibodies (Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG; 5 μg/ml, Invitrogen). Stained sections were imaged with a Leica TCS SP5confocal microscope system (Leica Microsystems, Wetzlar, Germany). All images were imported into Image J, CD11b-positive cells (corpus callosum and cortex) and APP-NF200 double positive axons in the corpus callosum were counted, and densities (counts/mm^2) calculated.

2.6. Quantification of demyelination in the corpus callosum and cortex

Black Gold stained sections were selected between Bregma −0.22 and −0.58 mm. Section were photographed (Olympus U-CMAD3 camera) at 10× magnification, the images were opened with Spot Advanced 4.1 software and imported into Image J, which was used to measure the mean optical density within the middle of the corpus callosum, at the level of the fimbria, and of the cortex (primary somatosensory cortex and motor cortex). Optical density in no tissue area was used as blank (background) and blank was subtracted using Spot Advanced 4.1 software. Myelin densities for each mouse were normalized against optical density values in unchallenged mice using the following formula: myelin score (%)=(density reading/unchallenged density average) × 100.