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Metformin ameliorates the development of experimental autoimmune encephalomyelitis by regulating T helper 17 and regulatory T cells in mice

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ABSTRACT

Immoderate immunoreaction of antigen-specific Th17 and Treg cell dysfunction play critical roles in the pathogenesis of multiple sclerosis. We examined Th17/Treg immune responses and the underlying mechanisms in response to metformin in C57BL/6 mice with experimental autoimmune encephalomyelitis (EAE). Metformin reduced Th17 and increased Treg cell percentages along with the levels of associated cytokines. Molecules involved in cellular metabolism were altered in mice with EAE. Suppressed activation of mTOR and its downstream target, HIF-1α, likely mediated the protective effects of metformin. Our findings demonstrate that regulation of T cell metabolism represents a new therapeutic target for CNS autoimmune disorders.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease characterized by chronic inflammation and demyelination of the central nervous system (CNS). Although the precise etiology of MS remains unclear, it has been suggested that immoderate immunoreaction of myelin-specific T helper 17 (Th17) cells and dysfunction of regulatory T (Treg) cells, which exert immune tolerance function play important roles in MS pathogenesis (Komiyama et al., 2006; Tzartos et al., 2008; Zozulya and Wiendl, 2008). Therefore, a therapeutic approach that suppresses inappropriate inflammation while promoting Treg responses could benefit patients with MS.

Stimulated T lymphocytes can differentiate into distinct subsets of T cells such as effector T (Teff) or Treg cells, which require different metabolic pathways to support their specific immunological functions. Accumulating evidence suggests that activated effector T cells shift to aerobic glycolysis to meet increased biosynthetic demands and generate sufficient energy. In contrast, the metabolic processes of quiescent and Treg cells acquire energy by oxidative phosphorylation (Pearce, 2010; Wang and Green, 2012).

Helper T cell activation and differentiation are promoted through the integration of various signals that are regulated by the mammalian target of rapamycin (mTOR) (Delgoffe et al., 2011; Esposito et al., 2010). In addition, mTOR plays a central role in regulating cellular size, growth, proliferation, survival, and metabolism (Guertin and Sabatini, 2007). Glucose metabolism in activated lymphocytes is also regulated by mTOR (Chi, 2012), along with the downstream signaling molecule, hypoxia-inducible factor (HIF)-1α, which is an important regulator of Teff cell differentiation and development (Shi et al., 2011). HIF-1α also regulates multiple metabolic genes such as glucose transporter 1 (Glut1) and pyruvate kinase isozyme M2 (PKM2), which are the encoding enzymes involved in glycolysis and differentiation of effector T cells (Macintyre et al., 2014; Shi et al., 2011; Sun et al., 2011).

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a highly conserved cellular energy sensor that is regulated by various metabolic stressors. AMPK and mTOR are opposing pathways that serve as a signaling nexus for regulating cellular metabolism and immunity. AMPK inhibits the mTOR pathway through multiple mechanisms involving phosphorylation of the tuberous sclerosis 2 (TSC2) protein, which is a negative regulator of the mTOR complex 1 (mTORC1) pathway. In addition, AMPK phosphorylates raptor, which is a component

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of mTORC1 (Gwinn et al., 2008; Inoki et al., 2003). AMPK is associated with Th17 cell suppression by inhibiting mTOR and signal transducer and activator of transcription 3 (STAT3) (Nagai et al., 2013). Furthermore, AMPK activation increased the percentage of airway-infiltrating Treg cells in a mouse model of asthma (Michalek et al., 2011).

Metformin (MET) is a member of the biguanide class of drugs widely used for the treatment of type 2 diabetes mellitus. The protective mechanisms of MET are attributed to activation of AMPK (Hawley et al., 2002). In addition to its hypoglycemic effects, MET has anti-inflammatory and antioxidant properties and appears to facilitate the transformation of classically activated macrophages to the alternative-activated form (Alqire et al., 2012; Jin et al., 2014). MET has also shown therapeutic efficacy in the treatment of various other diseases (Kang et al., 2013; Nath et al., 2009; Zhao et al., 2014).

In the present study, we investigated the regulation of Th17/Treg immune responses by MET in C57BL/6 mice immunized with a myelin oligodendrocyte glycoprotein (MOG) peptide to induce experimental autoimmune encephalomyelitis (EAE). This study focuses on the suppression of Th17 differentiation and enhancement of Treg responses induced by MET. Our findings demonstrated that MET attenuated the clinical signs of EAE by impeding Th17 cell proliferation and the associated pro-inflammatory cytokines. Furthermore, MET promoted Treg cell proliferation and enhanced the levels of related anti-inflammatory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10. A decrease in mTOR activity and its downstream target molecules likely mediated the observed alterations in the proportion of Th17/Treg cell populations.

2. Materials and methods

2.1. Animals

Female C57BL/6 wild-type mice, 8–10 weeks of age and weighing 18–20 g, were purchased from Vital River (Beijing, China) and housed under specific pathogen-free conditions with a 12 h light–dark cycle. The mice had free access to food and water, and all experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee Guidelines of Hebei Medical University.

2.2. Induction, assessment, and treatment of EAE mice

Mice were randomly placed into three groups including non-EAE healthy controls (healthy control group), EAE mice receiving MET (MET-treatment group), and EAE mice receiving saline as a vehicle control (EAE group). EAE was induced by subcutaneous injection of 250 μg of MOG35-55 peptide (Lysine Bio-System, Xian, China) emulsified in complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA) containing 4 mg/mL of heat-inactivated *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, USA). Then, at 0 h and 48 h post-immunization, EAE mice were intraperitoneally (i.p.) injected with 500 ng of pertussis toxin (Alexis, San Diego, CA, USA) in phosphate-buffered saline (PBS). Subsequently, the EAE mice were administered 100 mg/kg (body weight) MET dissolved in saline solution (Sigma-Aldrich, St. Louis, MO, USA) or saline only by i.p. injection from day 0 until the end of the study. The non-EAE healthy mice were also administered i.p. saline as a vehicle control. The mice were weighed daily and scored for clinical signs of EAE until they were euthanized. Clinical signs were scored as follows: 0, no paralysis; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; and 5, moribund and death.

2.3. Histological evaluation

On day 20 post-immunization, a specified number of mice from each group were perfused with 4% (*w*/*v*) paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), and the lumbosacral enlargements were carefully removed then embedded in paraffin. Then, 5-μm-thick transverse sections were stained with hematoxylin and eosin (H&E) to evaluate CNS immune cell infiltration using light microscopy. The degree of inflammation in three non-serial sections from each mouse was blindly and semi-quantitatively assessed as previously described (Zhang et al., 2003).

2.4. Western blot analysis

Lumbosacral enlargements of the spinal cords and splenocytes were isolated from mice on day 20 post-immunization. Total proteins were prepared using a total protein extraction kit (Applygen, Beijing, China) according to the manufacturer's instructions and a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay reagent kit (Novagen). Equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes (PVDF, Millipore). The membranes were blocked in 5% (*w*/*v*) non-fat dry milk or 5% (*w*/*v*) bovine serum albumin (BSA) in Tris-buffered saline plus Tween (TBST) for the phosphoproteins at room temperature for 1 h and then incubated overnight at 4 °C with primary antibodies against IL-17 (1:500, Bioworld), HIF-1α (1:500, Novus), AMPKα (1:1000, Cell Signaling Technology), phospho-AMPKα (1:1000, Cell Signaling Technology), S6K1(1:1000 Abcam), phospho-S6K1 (1:200 Abcam), and β-actin (1:3000, Bioworld). After washing with TBST thrice, the bound antibodies were detected using corresponding secondary antibodies. Relative levels of the target protein were quantified by densitometry with an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA).

2.5. Total RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

On day 20 post-immunization, a specified number of mice were euthanized and their spinal cords dissected. Then, RORγt, Foxp3, IL-17A, IL-10, and TGF-β mRNA transcripts were evaluated relative to the β-actin control using qRT-PCR. Additionally, splenic mononuclear cells were isolated and RORγt, Foxp3, IL-17A, IL-10, TGF-β, HIF-1α, Glut1, and PKM2 mRNA transcripts were analyzed relative to the β-actin control using qRT-PCR. Total RNA was extracted from the mouse spinal cord and splenic mononuclear cells using TRIzol reagent (Life Technologies) according to the manufacturer's protocol; cDNA synthesis was performed using a HiFiScript first-strand cDNA synthesis kit (CWBio, Beijing, China) according to the manufacturers' instructions. PCR amplification was accomplished using UltraSYBR mixture (CWBio, Beijing, China) with the Roche LightCycler 480II system (Roche Diagnostics GmbH, Mannheim, Germany), and the following specific primers: Foxp3 sense 5′-CTCTAGCAGTCCACTTCACCAA-3′ and anti-sense 5′-CACCCACCCTCAATACCTCTCT-3′; RORγt sense 5′-GGTCCAGACAGCCA CTGCATTC-3′ and anti-sense 5′-GGTGCGCTGCCGTAGAAGGT-3′; IL-17 sense 5′-CAACCGTTCCACGTCACCCT-3′ and anti-sense 5′-CCAGCTTTCC CTCCGCATT-3′; IL-10 sense 5′-CTGGACAACATACTGCTAACCGACTC-3′ and anti-sense 5′-AACTGGATCATTTCCGATAAGGC-3′; TGF-β sense 5′-GCCCTGGATACCAACTATTGCTTCA-3′ and anti-sense 5′-TAGGGGCA GGGTCCCAGACAGAAGT-3′; HIF-1α sense 5′-TGAACATCAAGTCAGCAA CG-3′ and anti-sense 5′-CACAAATCAGCACCAAGCAC-3′; Glut1 sense 5′-CGCTTCCTGCTCATCAATCGTAA-3′ and anti-sense 5′-CCGACCCTCTTC TTTCATCTCC-3′; and PKM2 sense 5′-CCATCAAGAATGTCCGTGAAGCC-3′ and anti-sense 5′-ATGTAAGCGTTGTCCAGGGTGA-3′. The qRT-PCR parameters included an initial incubation for 10 min at 95 °C, followed by 40 cycles of the following: denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. β-Actin was amplified in all samples as a housekeeping gene to which target gene expression was normalized. The data were analyzed using the Sequence Detection Systems software, and the levels of each target gene mRNA transcripts were calculated relative to the control using the $2 - \Delta\Delta CT$ method.