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Anti-encephalitogenic effects of ethyl pyruvate are reflected in the central nervous system and the gut

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ABSTRACT

Ethyl pyruvate is a redox analogue of dimethyl fumarate (Tecfidera), a drug for multiple sclerosis treatment. We have recently shown that ethyl pyruvate ameliorates experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. It affects encephalitogenic T cells and macrophages *in vitro*, as well as in lymph nodes draining the site of encephalitogenic immunization and within the central nervous system (CNS). Here, in vivo effects of ethyl pyruvate on EAE are thoroughly investigated in the CNS and within the gut associated lymphoid tissue. Ethyl pyruvate reduced infiltrates within the CNS and number of activated macrophages/microglia ($ED1^+$/$Iba1^+$) and proliferating astrocytes ($GFAP^+$). Furthermore, it reduced expression of HMGB1 in activated macrophages/microglia. It also reduced number of activated T cells and antigen-presenting cells and expression of Th1/Th17-related molecules in mesenteric lymph nodes and Peyer's patches. These results contribute to our understanding of anti-encephalitogenic effects of ethyl pyruvate as they provide evidence of its effects within the CNS and imply that these effects are related to reduction of inflammatory immune response in gut associated lymphoid tissue.

1. Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Although there are numerous drugs available for the treatment of multiple sclerosis, none of the drugs is a cure for the disease. Furthermore, high price of the drugs makes it unavailable for a large proportion of the patients. Therefore, novel therapies are needed. Ethyl pyruvate (EP) is a redox analogue of dimethyl fumarate (Tecfidera), one of the drugs currently used for multiple sclerosis treatment [1]. We have recently demonstrated that EP potently reduces experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis [2]. Its effects on EAE were mediated through inhibition of chief pathogenic T helper (Th) cells, i.e. interferon (IFN)-γ-producing Th1 cells and interleukin (IL)-17-producing Th17 cells [3]. Also, it suppressed macrophage release/production of IL-6, tumor necrosis factor (TNF) and reactive nitrogen and oxygen species which promoted neuroinflammatory and neurodegenerative events in multiple sclerosis. For instance, IL-6 is known to potentiate the resistance of effector T cells to regulatory T cells in multiple sclerosis [4], while TNF actively contributes to demyelination and axonal degeneration in neuroinflammation [5]. Likewise, reactive species contribute to loss of oligodendrocytes, blood-brain barrier dysfunction, T cell infiltration, and neurodegeneration [6].

Our previous study was focused on *in vitro* and *ex vivo* effects of EP, while *in vivo* effects were not explored in details. Therefore, here we were focused on the effects of EP on the CNS and gut-associated lymphoid tissue (GALT) in EAE. We were interested in the CNS, as a potential drug for multiple sclerosis should have anti-inflammatory and neuroprotective effects within the target tissue. Also, GALT has recently become appreciated in pathogenesis of multiple sclerosis, especially as a possible playground for the initiation and regulation of CNS-directed autoimmune response under the influence of gut microbes [7].

Our results indicate that EP reduces inflammation within the CNS and that it has immunomodulatory effects in the GALT. These properties of EP are important for its anti-encephalitogenic activity.

Abbreviations: CFA, complete Freund's adjuvant; CNS, central nervous system; c.s., clinical signs; DA, dark agouti; EAE, Eexperimental autoimmune encephalomyelitis; EP, ethyl pyruvate; GALT, gut-associated lymphoid tissue; IFN, interferon; IL, nterleukin; MLN, mesenteric lymph node; MLNC, MLN cells; MMLV, moloney murine leukemia virus; p.i., post immunization; PBS, phosphate buffer saline; PP, Peyer's paches; PPC, PP cells; RT, reverse transcription; SCH, spinal cord homogenate; Th, T helper; TNF, tumor necrosis factor

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2. Material and methods

2.1. Experimental animals and EAE induction

Dark Agouti (DA) rats (female, 2-4 months old) were used in this study. The rats were maintained in the animal facility of the Institute for Biological Research "Siniša Stanković". Animal manipulation and experimental procedures were approved by the local Ethics Committee (Institute for Biological Research "Siniša Stanković", N° 01-09/16). EAE was induced with rat spinal cord homogenate (SCH) in phosphate buffer saline (PBS, 50% w/v) mixed with equal volume of complete Freund's adjuvant (CFA, Difco, Detroit, MI) and supplemented with *M. tuberculosis* (to 5 mg/ml). Each rat was injected with 100 µl of the emulsion into one hind hock. Animals were monitored daily for EAE clinical signs (c.s.), and scored according to the following scale: 0 − no clinical signs; 1 − flaccid tail; 2 − hind limb paresis; 3 − hind limb paralysis; 4 − moribund state or death. Cumulative c.s. was calculated as the sum of daily clinical scores, while average c.s. was calculated as cumulative c.s. divided by the number of days with clinically manifested EAE. Rats were treated intraperitoneally with EP (300 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) or vehicle (PBS), once per day, starting from day 8 post immunization.

2.2. Tissue preparation

Rats were sacrificed on day 12 post immunization (p.i.). Lumbar regions of spinal cord were rapidly dissected and immersed in cold 4% paraformaldehyde overnight for fixation followed by dehydration in series of alcohol and xylene. Afterward, sections were embedded in paraffin and 5 micrometers of sections were cut on a microtome RM2265 (Leica, Wetzlar, Germany) and placed on a Superfrost glass slides (Thermo Scientific, Waltham, MA) in a row.

2.3. Histochemistry

In order to stain inflammatory infiltrates, hematoxylin staining was performed. The stained nuclei of mononuclear cells were counted from 4 animals. Five micrometers of transversal lumbosacral sections (L1–L5) were cut and 8 successive slices were placed on the same glass slide. Number of infiltrates and cells per infiltrate were counted from histological data obtained from 8 sections per rats.

2.4. Immunohistochemistry

In order to block unspecific labeling, normal donkey serum (10% solution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Single peroxidase immunohistochemistry was obtained using rabbit anti-HMGB-1 antibody (1:500, Abcam, MA, USA) and anti-GFAP antibody (1:500, clone: 73-240, NIH NeuroMab, Davis, CA, USA) followed by donkey anti rabbit IgG (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-Iba1antibody (1:200, Abcam) followed by donkey anti-goat (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-ED1 antibody (1:200, Abcam) and anti-SMI-32 (1:1000, Sigma-Aldrich) followed by donkey anti-mouse IgG (1:200, Santa Cruz Biotechnology). The immunoreaction products were visualized with 3′3-diaminobenzidine (DAB, Dako, Glostrup, Denmark) according to manufacturer instructions. For double immunofluorescence labeling, sections were incubated with rabbit anti-HMBG-1 antibody (1:500), followed by incubation with the antibodies against specific cell markers: mouse anti-GFAP (1:500, clone: 73-240, NIH NeuroMab, Davis, CA, USA), mouse anti-ED1 (1:200, Abcam, MA, USA), mouse anti-SMI-32 (1:200, Sigma-Aldrich, St. Louis, MO, USA), goat-anti Iba1 (1:200, Abcam, MA, USA) antibodies. Immune complexes were visualized with donkey anti-rabbit Alexa Fluor 555 (1:250), donkey anti-mouse IgG Alexa Fluor 488 (1:250) and donkey anti-goat Alexa Fluor 488 (1:250) all purchased from Invitrogen (Carlsbad, CA, USA). Nuclei were visualized with DAPI nuclear staining. The sections were mounted in Mowiol (Calbiochem, Millipore, Germany) and captured on Zeiss Axiovert fluorescent microscope equipped with camera and EC Plan-Apochromat, using the Apotome system for obtaining optical sections.

2.5. Isolation of cells

Mesenteric lymph nodes (MLN) and Peyer's patches (PP) were isolated from non-immunized rats and from rats on day 12 post immunization. Four MLN were isolated from each rat and MLN cells (MLNC) were prepared by mechanical disruption. PP were obtained from the small intestine and PP cells (PPC) were obtained by mechanical disruption.

2.6. Cytofluorimetry

Cells were stained with the following antibodies: PE-conjugated anti-CD4 (mouse monoclonal OX35, eBioscience) and anti-MHC class II (mouse monoclonal His19, eBioscience), FITC-conjugated anti-CD8 (mouse monoclonal OX8, AbD Serotec, Oxford, UK), anti-CD25 (mouse monoclonal NDS601, AbD Serotec), anti-CD86 (mouse monoclonal 24F, eBioscience) and anti-CD134 (mouse monoclonal OX40, BD Pharmingen, San Jose, CA). FoxP3 staining with rat monoclonal antibody (FJK-16s) was performed according to the procedure suggested by the manufacturer (eBioscience). Appropriate isotype control antibodies were used where necessary to set gates for cell marker positivity. Typically, proportion of isotype control antibody-stained cells was < 1%. Analyses were performed on a Partec CyFlow Space cytometer (Partec, Munster, Germany). Results of cytofluorimetry are presented as proportion of cells bound by an appropriate antibody.

2.7. Reverse transcription − real time polymerase chain reaction

mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) was used for isolation of total RNA. Reverse transcription (RT) was performed by using random hexamer primers and MMLV (Moloney Murine Leukemia Virus), according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified by using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the recommendations of the manufacturer in a total volume of 20 µl in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermocycler conditions comprised an initial step at 50 °C for 5 min, followed by a step at 95 °C for 10 min and a subsequent 2-step PCR program at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. The PCR primers (Metabion) were as follows GAPDH: 5'-ACA GCC TTG GCA GCA CCA GT-3'; 5'-TCA CCA TCT TCC AGG AGC GAG-3'; β-actin: 5'-GCT TCT TTG CAG CTC CTT CGT-3'; 5'-CCA GCG CAG CGA TAT CG-3'; HMGB1: 5'-TAA GAA GCC GAG AGG CAA AA-3'; 5'-GCA GAC ATG GTC TTC CAC CT-3'; IFN-γ: 5′-TGG CAT AGA TGT GGA AGA AAA GAG-3′; 5′-TGC AGG ATT TTC ATG TCA CCA T-3′; IL-17: 5′-ATC AGG ACG CGC AAA CAT G-3′; 5′- TGA TCG CTG CTG CCT TCA C-3′; T-Bet:5′-CCA ACA ATG TGA CCC AGA TGA T-3′; 5′-CTG GCT CAC CGT CAT TCA-3′; RorγT: GAC AGG GCC CCA CAG AGA-3'; 5'-TTT GTG AGG TGT GGG TCT TCT TT-3'; IL-6: 5'-GCC CTT CAG GAA CAG CTA TGA-3'; 5′-TGT CAA CAA CAT CAG TCC CAA GA-3′. For the analysis of the results 7500 System Software was used. Relative RNA expression is determined as 2^{-dCt}, where dCt is Ct value of a gene of interest − Ct value of the endogenous control (GAPDH for spinal cord homogenates, β-actin for MLNC).

2.8. Statistical analysis

A Student's t test (two-tailed) and Mann–Whitney U test were performed for statistical analysis. A p value less than 0.05 was considered statistically significant.