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Original Article

Short term exposure to ethyl pyruvate has long term anti-inflammatory effects on microglial cells

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ABSTRACT

Ethyl pyruvate (EP) has been increasingly appreciated as an anti-inflammatory and neuroprotective agent with potent pharmacological properties relevant for treatment of various CNS disorders. Microglial cells seem to be particularly sensitive to its effects. In this study, microglial cells were exposed to EP for relatively short periods (10–120 min) and inflammatory properties of the cells were determined after 24 h of cultivation. Application of EP in the short-term periods inhibited production of interleukin-6, tumor necrosis factor and nitric oxide in microglial cells. At the same time, the effects on cell viability, reactive oxygen species generation and expression of F4/80 and CD40 of microglial cells were minor. NFκB activation was not affected by EP in the cells during the short exposures, thus implying that the observed effect of EP on cytokine and nitric oxide generation was performed in NFκB independent way. Importantly, effects of the short term EP treatment on microglial cells were detected by a real time cell analysis, as well. The observed ability of EP to affect microglial cell function after relatively short time of exposure is relevant for its therapeutic potential against inflammatory disorders of the CNS.

1. Introduction

Microglial cells are involved in pathogenesis of a number of inflammation-related disorders of the CNS, including multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease (AD) and stroke [1–4]. While microglia is generally performing protective activity in the CNS, it is shifted toward pro-inflammatory effector phenotype in chronic inflammatory, autoimmune and neurodegenerative CNS diseases [4,5]. Pro-inflammatory microglia produces various inflammatory and neurotoxic mediators, including cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-6 and IL-1β and reactive nitrogen and oxygen species, such as nitric oxide (NO) and it actively participates in CNS tissue destruction [1,3]. Also, inflammatory microglia increases expression of antigen-presentation related molecules, including CD40, which allows it to efficiently present antigens to T cells and thus to further contribute to neuroinflammation [1,5]. Consequently, down-regulation of pro-inflammatory microglial activity has been shown beneficial in the treatment of the CNS disorders [3,5].

Ethyl pyruvate (EP) is a potent redox active compound. It forms Michael-type adducts with thiols and activates Nrf2 in astrocytes, and inhibits NFκB-dependent transcription in different cell types, including LPS-stimulated microglial BV2 cells and macrophage-like RAW 264.7 cells [6–9]. Anti-inflammatory and neuroprotective effects of EP have been observed in animal models of sepsis, uveitis, and asthma [10–12]. EP has been effective also against different neurological insults, such as ischemia/reperfusion [13], intracerebral hemorrhage-induced brain injury [14], traumatic brain injury [15], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [16], LPS [17], and 3-nitropropionic acid [18]. Also, we have recently shown that EP potently ameliorates of experimental autoimmune encephalomyelitis (EAE), an animal model of MS [19].

The aim of our current study was to compare the effects of short-term (10 –120 min) and long time (24 h) exposure of microglial cells to EP. The influence of EP on production of pro-inflammatory cytokines (IL-6, and TNF), generation of NO and expression of CD40 and F4/80 in BV2 cells was determined. Also, effect of short-time exposure to EP on NFκB signaling in these cells was examined. It appears that cytokine and NO production, but not CD40 and F4/80 expression in microglial cells is sensitive to short-term exposure to EP. The observed effects are not paralleled with inhibition of NFκB signaling or with inhibition of reactive oxygen species generation.

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2. Materials and methods

2.1. Cell cultures and reagents

Unless specifically stated chemicals used in the experiments were from Sigma-Aldrich (St. Louis, MO). BV2 cells were grown and cultured in RPMI-1640 culture medium (PAA Laboratories, Pasching, Austria) that was supplemented with 5% heat-inactivated fetal calf serum and at 37 °C in a humidified atmosphere containing 5% CO_2. For the treatments, BV2 cells were seeded at 3×10^5/ml/well in 24-well plates (Sarstedt, Pasching, Austria). BV2 cells were stimulated with 10 ng/ml recombinant mouse interferon-γ (IFN-γ) and 100 ng/ml lipopolyssacharide (LPS) and treated with various concentrations of ethyl pyruvate (EP).

2.2. Crystal violet assay

Viability of BV2 was assessed by crystal-violet (CV) test. At the end of the treatment, cells were washed with PBS to remove non-adherent dead cells, and the remaining cells were fixed with methanol. After staining with 1% CV solution, the plates were thoroughly washed and then the dye was dissolved in 33% acetic acid. The absorbance of dissolved dyes, corresponding to the number of viable cells, was measured in triplicates at 540 nm with a correction at 670 nm, using an automated microplate reader (LKB 5060-006, LKB, Vienna, Austria).

2.3. Detection of NO release

Nitrite accumulation – a measure of NO release, was determined in cell culture supernatants using the Griess reaction. In brief, triplicate aliquots of cell-free supernatants were mixed with an equal volume of Griess reagent (1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4). The absorbance at 540 nm was determined with a microplate reader and compared to a standard curve for $NaNO_2$.

2.4. Detection of reactive oxygen species (ROS) generation

For detection of ROS generation dihydrorhodamine 123 (DHR, Sigma–Aldrich) staining was performed. The cells were incubated in the presence of 1 μM DHR for 30 min and subsequently treated as indicated. The fluorescence was acquired via flow cytometry.

2.5. ELISA test for determination of cytokines

Cytokine concentration in cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Rochild, Denmark) and anti-cytokine paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicates for murine TNF and murine IL-6 (R&D Systems, Minneapolis, MN). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

2.6. Immunoblot

Whole-cell lysates were prepared in a solution containing 62.5 mM Tris–HCl, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% (w/v) bromophenol blue, 1 mM phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride, 1 μg/ml aprotinine, and 2 mM EDTA (pH 6.8). Samples containing 20 μg of proteins (measured by Lowry protein assay) were electrophoresed on a 12% SDS-polyacrylamide gel. The samples were electrotransferred to polyvinylidene difluoride membranes at 5 mA/cm^2, using semi-dry blotting system (Fastblot B43, Biorad, Muenchen, Germany). The blots were blocked with 5% (w/v) non-fat dry milk in PBS with 0.1% Tween-20, and probed with specific antibodies for phosphorylated-IkB and β-actin (Cell Signaling Technology, Boston, MA), followed by incubation with the secondary antibody (ECL donkey anti-rabbit HRP-linked, GE Healthcare, Buckinghamshire, England, UK). Detection was performed by the chemiluminescence (ECL, GE Healthcare) and photographs were made by X-ray films (Kodak, Rochester, NY). Densitometry was performed with Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD) and the results are presented as relative densitometry values (RDV).

2.7. Cytofluorimetry for detection of cell-surface markers

BV2 cells were stained with FITC-conjugated anti-CD40 (BD Biosciences, San Diego, CA) and PE-conjugated anti-F4/80 (eBioscience, San Diego, CA). 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%. BV2 cells were analyzed with a CyFlow Space flow cytometer (Partec, Munster, Germany). Results of cytofluorimetry are presented as proportion of cells bound by an appropriate antibody.

2.8. Real-time cell analysis

Cell activation was characterized in real-time by using an xCELLigence RTCA DP analyzer (Roche Diagnostics, Pleasanton, CA). BV2 cells were seeded at 1×10^4 cells into each well of an E-Plate 16 (Roche Diagnostics). After 60 min of initial adherence, BV2 cells were stimulated and treated as indicated in the results. Impedance measurements were recorded every minute during the first 3 h of cultivation and then every 15 min overnight. Impedance measurements were recorded as a combined cellular index of proliferation, viability, activation and morphology changes.

2.9. Statistical analysis

A Student's *t*-test (two-tailed) was performed for statistical analysis. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Real-time effects on BV2 cells

Impedance measurement in cell cultures is a novel methodology that has been shown informative for proliferation, viability, activity and morphology changes of BV2 cells [20]. BV2 cells were plated overnight and then stimulated with IFN-γ+LPS and simultaneously treated with various concentrations of EP. As presented in Fig. 1, BV2 culture impedance increased in response to IFN-γ+LPS, while EP application inhibited impedance in BV2 cultures immediately after the addition of the agent. EP affected the impedance in a dose dependent manner. Thus, RTCA clearly shows that EP affects BV2 cell biology in relative short time (min).

3.2. Effects on BV2 viability, IL-6, TNF and NO production

BV2 cells were stimulated with IFN-γ+LPS and simultaneously treated with EP for 10, 20, 30, 60 or 120 min. Then, cell culture supernatants were removed from the wells and fresh culturing media without the stimulants and without EP were added in. The additional cultivation of the cells was performed up to total incubation time of 24 h. BV2 cells were also stimulated and treated