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Research Paper

Therapeutic innovation: Inflammatory-reactive astrocytes as targets of inflammation

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A B S T R A C T

This study aimed to test pharmaceutical compounds targeting astrocytes showing inflammatory dysregulation. The primary rat brain cultures were treated with different batches of serum with or without microglia added to make the cells inflammatory-reactive. Lipopolysaccharide (LPS) and tryptase were used as inflammatory inducers. Expression levels of Toll-like receptor 4 (TLR4), Na^+/K^+-ATPase, and matrix metalloprotease-13 (MMP-13), as well as actin filament organization, pro-inflammatory cytokines, and intracellular Ca^{2+} release, were evaluated. LPS combined with tryptase upregulated TLR4 expression, whereas Na^+/K^+-ATPase expression was downregulated, ATP-evoked Ca^{2+} transients were increased, actin filaments were reorganized and ring structures instead of stress fibers were observed. Other aims of the study were to prevent astrocytes from becoming inflammatory-reactive and to restore inflammatory dysregulated cellular changes. A combination of the μ-opioid antagonist (−)-naloxone in ultra-low concentrations, the non-addictive μ-opioid agonist (−)-linalool, and the anti-epileptic agent levetiracetam was examined. The results indicated that this drug cocktail prevented the LPS- and tryptase-induced inflammatory dysregulation. The drug cocktail could also restore the LPS- and tryptase-treated cells back to a normal physiological level in terms of the analyzed parameters.

1. Introduction

Inflammatory cells are important contributors to the pathophysiological response to injury. Penetration through the blood–brain barrier (BBB) is achieved by circulating bone-marrow-derived leukocytes or monocytes, which can transform into macrophages/microglia. These cells secrete cytokines that promote tissue damage (Medzhitov, 2008; Waisman et al., 2015). The BBB consists of capillary endothelial cells containing specialized tight junctions and surrounded by a basement membrane, which is composed of extracellular matrix components, pericytes and astrocytic perivascular endfeet (Abbott et al., 2006). The gram-negative bacterial endotoxin lipopolysaccharide (LPS) induces the conversion of endothelial cells into activated fibroblasts that show a myofibroblast-like protein profile. This process is mediated by the Toll-like receptor 4 (TLR4)/NF-κB pathway (Sarmiento et al., 2014).

Astrocytes are now known to exert either potent pro-inflammatory functions or crucial protective anti-inflammatory functions that are regulated by specific signaling inputs (Hansson, 2010; Zeng et al., 2013; Hansson and Skiöldebrand, 2015). Their processes establish contacts with the BBB and the processes of other astrocytes via gap junctions, thereby forming networks of coupled astrocytes (Cornell-Bell et al., 1990; Blomstrand et al., 1999; Guthrie et al., 1999; Nedergaard et al., 2003). At sites of CNS tissue damage, astrocytes become reactive and form scar borders, which serve as functional barriers and have the potential to release diverse molecules that affect nearby cells. Astrocyte dysfunction can be caused by genetic polymorphisms or by exposure to molecular signals derived from infections or trauma, which can alter astrocyte inflammatory regulation and lead to detrimental effects (Sofroniew, 2015), including the alteration of astrocyte signaling mechanisms (Hansson, 2010, 2015).

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LPS as a potent inflammatory activator in astrocytes, has shown to give good results (Forshammar et al., 2011; Block et al., 2012, 2013). However, better inflammatory-induced activity is desired as the mechanisms of actions in the restoration processes are desired to be better expressed.

Mast cells are derived from the bone marrow and play important roles in inflammation, immune responses and tissue repair. They circulate in an immature form until they reach the target tissue site (Skaper et al., 2013). Mast cells are rich in proteases, tryptases and chymases, as well as cytokines such as interleukins, TGFβ and tumor necrosis factor-α (TNF-α). They contact only blood vessels that are ensheathed by astroglial processes, and they can alter the BBB permeability and pass into the CNS (Kahli et al., 2007; Dong et al., 2014). They can upregulate purinergic receptors on astrocytes (Dong et al., 2014) and activate the G protein-coupled protease-activated receptor (PAR-2) in astrocytes by releasing tryptase (Zeng et al., 2013).

Matrix metalloproteinases (MMPs or matrixins) activate signal transduction pathways that control cytokine biosynthesis and barrier immunity. MMP-13, which is produced by both neurons and astrocytes, is upregulated in response to inflammation in the brain, and it remodels the extracellular matrix and degrades substrates as part of the neuroinflammatory response (Cuadrado et al., 2009).

The first aim of the present study was to test different substances of relevance to get astrocytes more inflammatory reactive compared to LPS-induced inflammatory-reactivity. The astrocyte cultures were treated with different batches of serum with or without microglia. Furthermore, LPS and tryptase were applied in combination. Cellular changes were analyzed according to several parameters, such as expression of TLR4, Na^+/K^+-ATPase, and MMP-13, as well as actin filament organization, pro-inflammatory cytokine levels, and intracellular Ca^{2+} release. The second aims were to 1) prevent astrocytes from becoming inflammatory-reactive and 2) restore cellular changes and disturbances, which already were inflammatory-reactive back to physiological levels. We have earlier shown that a combination of a μ-opioid receptor antagonist in ultra-low concentrations, naloxone, a μ-opioid agonist, endomorphin-1, and an agent attenuating IL-1β release, levetiracetam, can restore cellular parameters induced by inflammation. The responses have been tested in astrocytes (Block et al., 2013) and in post-surgical neuropathic pain patients in vivo (Block et al., 2015). We wanted now to replace endomorphin-1/morphine with the non-addictive μ-opioid agonist (−)-linalool and evaluate if this substance had similar restorative effects as endomorhin-1.

2. Materials and methods

The studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.1. Primary astrocyte cultures and treatments

Primary cortical astrocytes, from Sprague-Dawley rats at embryonic day 19, were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) and prepared according to the manufacturer's instructions with some modifications. Briefly, one vial containing 1×10^6 viable cells were plated at a seeding density of 1×10^4 cells per cm^2 on uncoated glass coverslips (no. 1, 20 mm in diameter) (Bergman Labora, Stockholm, Sweden) and placed in 12-well plates. The medium was replaced twice per week. The astrocytes were used after 16–17 days in culture.

2.2. Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.3. Inflammatory-reactive substances added to astrocyte cultures

Cultures were treated with two different batches of serum: 15% fetal bovine serum from Invitrogen, called astroglia serum, or 15% fetal bovine serum (Biochem AG, Berlin, Germany) called microglia serum, as it was used to increase the amount of microglia (Persson et al., 2005). Some cultures were shaken to promote growth of microglia. Exogenous microglia were added to the astrocyte cultures.

LPS (10 ng/ml) (Forshammar et al., 2011), which was used to promote inflammatory reactivity, was added 24 h before the experiments. To further increase the inflammatory reactivity, tryptase (10 ng/ml) (Zeng et al., 2013) was added together with LPS 24 h before the experiments.

2.4. Immunocytochemistry

Immunocytochemistry was done according to Block et al. (2013). Glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark) and a mouse monoclonal antibody against OX42 (Serotec, Oxford, UK) were used. The cells were viewed using a Nikon Eclipse 80*i* microscope. Pictures were taken with a Hamamatsu C5810 color-intensified 3CCD camera.

2.5. Viability assay

A LIVE/DEAD viability assay kit (Invitrogen Molecular Probes) for mammalian cells was used according to Forshammar et al. (2011). viewed using a Nikon Eclipse 80*i* microscope. Pictures were taken with a Hamamatsu C5810 color-intensified 3CCD.

2.6. Calcium imaging

Calcium imaging was done as earlier described (Block et al., 2013). The total areas under the curve (AUC), which reflects the amount of Ca^{2+} released (Berridge, 2007), were analyzed. The amplitude was expressed as the maximum increase of the 340/380 ratio. The area under the Ca^{2+} peaks (AUC) was calculated in Origin (Microcal Software Inc., Northampton, MA, USA). Forty cells were used for each experimental set-up and were taken from four different coverslips and from two different seeding times.

2.7. SDS-PAGE and western blot

SDS-page and western blot were done as earlier described (Block et al., 2013).

2.8. Actin visualization

The astrocyte cytoskeleton was stained using Alexa™488-conjugated phalloidin (Invitrogen) (Block et al., 2013).

2.9. Actin assay

Actin quantitation was performed as recommended by the F-actin/G-actin In Vivo Assay Biochem Kit (Cytoskeleton, Inc., Denver, CO, USA) and SDS-page performed as described above, with the exception of the sample preparation. The primary antibody, rabbit polyclonal anti-actin (Cytoskeleton, 1:500), and the secondary antibody, HRP-conjugated donkey anti-rabbit IgG $F(ab')_2$ fragments