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Glucosamine suppresses platelet-activating factor-induced activation of microglia through inhibition of store-operated calcium influx

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ABSTRACT

Microglia activation and subsequent release of inflammatory mediators are implicated in the pathophysiology of neurodegenerative diseases. Platelet-activating factor (PAF), a potent lipid mediator synthesized by microglia, is known to stimulate microglia functional responses. In this study, we determined that endogenous PAF exert autocrine effects on microglia activation, as well as the underlying mechanism involved. We also investigated the effect of D-glucosamine (GlcN) on PAF-induced cellular activation in human HMO6 microglial cells. PAF induced sustained intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase through store-operated Ca^{2+} channels (SOC) and reactive oxygen species (ROS) generation. PAF also induced pro-inflammatory markers through NFκB/COX-2 signaling. GlcN significantly inhibited PAF-induced Ca^{2+} influx and ROS generation without significant cytotoxicity. GlcN downregulated excessive expression of pro-inflammatory markers and promoted filopodia formation through NFκB/COX-2 inhibition in PAF-stimulated HMO6 cells. Taken together, these data suggest that GlcN may offer substantial therapeutic potential for treating inflammatory and neurodegenerative diseases accompanied by microglial activation.

1. Introduction

In the central nervous system (CNS), microglial cells are the resident macrophages. That have a key part in the regulation of innate immune responses in the healthy or degenerating CNS (Khoo et al., 2001). While actively scanning the microenvironment with their long protrusions (Nimmerjahn et al., 2005), loss of inhibitory signals and the recognition of damage-associated molecular patterns from degenerating neurons lead to the activation of microglia (Cardona et al., 2006; Heneka et al., 2014). When microglial cells are activated, the cells exhibit proliferation and phagocytosis. Moreover, the activated cells can secrete pro- and anti-inflammatory cytokines, chemokines, and neurotrophic factors (Heneka et al., 2014). Therefore, reactive microgliosis is a common hallmark of various neurodegenerative diseases including Alzheimer's disease (Perry et al., 2010), Parkinson's disease (Orr et al., 2002), and multiple sclerosis (Raivich and Banati, 2004).

Platelet-activating factor (PAF) is a potent proinflammatory phospholipid with diverse pathological and physiological effects (Yue and Feuerstein, 1994). PAF is produced by a variety of cells, but especially those involved in host defense, such as platelets, endothelial cells, neutrophils, monocytes, and macrophages (Zimmerman et al., 2002). In the brain, this endogenous phospholipid is produced by microglia in response to ischemic injury or various neurodegenerative diseases (Bate et al., 2006; Prescott et al., 2000; Tuttolomondo et al., 2008). Moreover, PAF stimulates microglia itself (Mori et al., 1996) and induces arachidonic acid metabolism, resulting in the production of neurotoxic factors. It also mediates pathological inflammation in the brain (Mori et al., 1996). It has been reported that PAF immediately induced an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels in human microglial cells (Khoo et al., 2001; Sattayaprasert et al., 2005; Wang et al., 1999). These prolonged increase of $[Ca^{2+}]_i$ by PAF treatment can increase the expression of inflammatory factors, including

Abbreviations: CNS, central nervous system; $[Ca^{2+}]_i$, intracellular Ca^{2+}; COX-2, cyglooxygenase-2; GlcN, D-glucosamine; IκB-α, inhibitor of κB-α; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; PAF, platelet-activating factor; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; SOC, store-operated Ca^{2+} channels; TNF-α, tumor necrosis factor-α.

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cyglooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS) and prostaglandin E_2 (PGE_2) (Choi et al., 2002; Hoffmann et al., 2003).

D-Glucosamine (GlcN) is a dietary supplement widely used for the prevention and/or treatment of rheumatoid arthritis and osteoarthritis (de los Reyes et al., 2000). GlcN can be distributed to the brain at relevant quantities 8 h after oral administration (Setnikar et al., 1984). Our previous study demonstrated that GlcN inhibits exogenous lipopolysaccharide-induced microglia activation (Yi et al., 2005), thus implicating this agent as part of a new inhibitory strategy in targeting activated microglial cells in the CNS. However, the effect of GlcN on endogenous PAF-induced activation of microglia remains largely unknown. Therefore, the aim of the present study was to investigate the effects of GlcN on endogenous PAF-induced increase in $[Ca^{2+}]_i$ and expression of inflammatory factors in HMO6 microglial cells and to evaluate the inhibitory mechanisms involved.

2. Materials and methods

2.1. Materials

FBS and DMEM medium were purchased from Life Technologies (Carlsbad, CA). GlcN was from Sigma–Aldrich (St. Louis, MO). PAF, PK11195 and SKF96365 were from Tocris Bioscience (Bristol, UK). Fura-2 acetoxymethyl (fura-2/AM) ester was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma–Aldrich.

2.2. Cell culture

Human microglial cells (HMO6) were a gift from S.U. Kim (University of British Columbia, Vancouver, Canada). HMO6 cells were established as immortalized clonal cells of human microglia from human embryonic telencephalon tissue by using a retroviral vector encoding v-myc and were investigated by immunochemistry and fluorescence-activated cell sorting analyses (Nagai et al., 2005). HMO6 cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in DMEM medium containing 5% heat-inactivated FBS, 5% heat-inactivated horse serum and 20 μg/mL gentamycin.

2.3. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Microfluorescent imaging of $[Ca^{2+}]_i$ was performed on HMO6 cells loaded with the calcium indicator dye fura-2/AM. Some of the procedures used in calcium imaging in this experiment have been described (Yi et al., 2005). Fura-2/AM (3 μM) was added to HMO6 cells bathed in 1.8 mM Ca^{2+} containing physiological saline solution (126 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES [pH 7.4], 0.2% BSA and 10 mM glucose) at room temperature for 30 min followed by a 30-min wash in dye-free saline solution to allow esterase conversion to the free form of fura-2. Cover slips were placed on the stage of an inverted microscope and imaging was performed with a dual-wavelength system (Intracellular Imaging, Cincinnati, OH). $[Ca^{2+}]_i$ was calculated as the relationship between the ratio of emissions at 510 nm from excitation at 340 and 380 nm, respectively. Ratio images were processed every 5 s and converted to $[Ca^{2+}]_i$ as compared to a range of such ratios obtained by measurement of fura-2 in the presence of known concentrations of calcium (Calcium Calibration Buffer Kit, Molecular Probe, Eugene, OR). When Ca^{2+}-free solution was used, Ca^{2+} was omitted and 2 mM EGTA was added. After the establishment of a stable baseline $[Ca^{2+}]_i$ level, the cells were pre-treated with or without 1 mM GlcN for 2 min prior to stimulation with 300 nM PAF for 1 min. Each experimental data point represents the mean $[Ca^{2+}]_i$ calculated from at least 12 individually measured cells from three separate cultures. All imaging experiments were done at room temperature (20–22 °C).

2.4. Western blot analysis

Cells were treated with 300 nM PAF in the presence or absence of 1 mM GlcN for 4 h. Crude cell extracts were subjected to SDS-PAGE and immunoblotted with anti-COX-2 (Abcam, Cambridge, UK), anti-Iκb-α (Cell Signaling, Danvers, MA) and anti-β actin (Sigma–Aldrich) antibodies. The immunoreactive bands were visualized using a horseradish peroxidase-conjugated secondary antibody (Abcam) and enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK). The experiments were repeated at least three times.

2.5. Quantitative real-time PCR analysis

After exposure of cells to 300 nM PAF in the presence or absence of 1 mM GlcN for 4 h, total cellular RNA was extracted from HMO6 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA was eluted with Rnase-free water. Reverse transcription was performed using the first strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) according to the manufacturer's protocols. Real-time PCR amplification was performed using the SYBR Green master mix (Applied Biosystems, Foster City, CA) and the Prism 7500 real-time PCR detection system. Relative amounts of mRNA were normalized by use of the gene encoding glyceraldehyde 3-phosphate dehydrogenase and calculated using the ΔΔCT (cycle threshold) method. The specific primers for iNOS were 50-GTT CTC AAG GCA CAG GTC TC-30 (forward) and 50-GCA GGT CAC TTA TGT CAC TTA TC-30 (reverse). The primers for IL-1β were 50-TTA CAG TGG CAA TGA GGA TGA-30 (forward) and 50-TGT AGT GGT GGT CGG AGA TT-30 (reverse). The primers for TNF-α were 50-GGA GAA GGG TGA CCG ACT CA-30 (forward) and 50-CTG CCC AGA CTC GGC AA-30 (reverse).

2.6. Measurement of reactive oxygen species (ROS) by confocal microscopy

Intracellular ROS were measured using the cell-permeable fluorescent dye H_2DCFDA. First, HMO6 cells were treated with 300 nM PAF in the presence or absence of 1 mM GlcN for 1 h. After incubation, wells were loaded with 5 μM H_2DCFDA and incubated for 30 min at 37 °C. The cells were then washed twice with Krebs Ringer Bicarbonate buffer to ensure the removal of unbound dye. After washing, the cells were incubated for an additional 10 min. Images were obtained by subjecting the cells to confocal laser microscopy (LSM 5 EXCITER; Carl Zeiss, Jena, Germany) using excitation and emission wavelengths of 488 and 525 nm, respectively.

2.7. Measurement of PGE_2

The levels of PGE_2 were measured using ELISA kits (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions. Briefly, HMO6 cells were loaded in 24-well plates and treated with 300 nM PAF in the presence or absence of 1 mM GlcN for 1 h. A total of 100 μL of culture medium supernatant was collected to determine PGE_2 concentration by ELISA.

2.8. Statistical analyses

Results were expressed as the mean ± SEM. SPSS version 20.0 (SPSS, Chicago, IL) was used for statistical analyses. The AUC was calculated using Microcal Origin software version 9.1 (Northampton, MA). Comparisons between the two groups were performed using a Student's two-tailed *t* test. For comparisons involving more