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Differential regulation of astrocyte prostaglandin response by kinins: Possible role for mitogen activated protein kinases

Nofar Torika [1], Talia Filipovich-Rimon [1], Keren Asraf, Ella Roasso, Abraham Danon, Sigal Fleisher-Berkovich *

Department of Clinical Biochemistry and Pharmacology, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel

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ABSTRACT

The role of kinins, well known as peripheral inflammatory mediators, in the modulation of brain inflammation is not completely understood. The present data show that bradykinin, a B_2 receptor agonist, enhanced both basal and lipopolysaccharide (LPS)-induced cyclooxygenase-2 mRNA and protein levels and prostaglandin E_2 synthesis in primary rat astrocytes. By contrast, Lys-des-Arg^9-bradykinin, which is a bradykinin breakdown product and a selective kinin B_1 receptor agonist, attenuated both basal and LPS-induced astrocyte cyclooxygenase-2 mRNA levels and prostaglandin E_2 production. Pre-treating the cells with p42/p44 MAPK but not with JNK or p38 inhibitors completely abrogated PGE_2 synthesis in cells stimulated with LPS in the presence of bradykinin or bradykinin B_1 receptor agonist. Bradykinin, but not the bradykinin B_1 receptor agonist, augmented p42/p44 MAPK phosphorylation. The phosphorylation of JNK and p38 was not altered upon exposure to Bradykinin or the bradykinin B_1 receptor agonist. These results suggest that the dual delayed effect of kinins on PGE_2 synthesis may be due to differential regulation of COX-2 and signaling molecules such as p42/p44 MAPKs. Thus, kinins may exert opposing actions on brain inflammation and neurodegenerative diseases.

1. Introduction

It is known that glial inflammation plays a major role in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD) (Akiyama et al., 2000). An important feature of glial inflammation is the enhanced production of cytokines, prostaglandins (PGs) and reactive oxygen species by activated astrocytes and microglia (Amor et al., 2010). Elevated levels of PGE_2, a pro-inflammatory molecule, have been found in the cerebrospinal fluid of AD patients following head trauma (Ho et al., 2000).

In the present study, primary rat astrocytes were stimulated in an in vitro model of inflammation by incubation with the bacterial endotoxin lipopolysaccharide (LPS). The LPS receptor CD14 has been shown to interact with fibrils of amyloid β (Aβ) peptide, a major feature of AD plaques. Moreover, antibodies against CD14 as well as genetic CD14 deficiency strongly reduce Aβ-induced microglial activation and neurotoxicity (Fassbender et al., 2004).

Kinins, bradykinin being the most investigated species, are potent regulators of the inflammatory response both in the central and peripheral nervous systems. Although a general role kinins play in glial inflammation has been reported, it remains controversial whether these effects are beneficial or detrimental. Bradykinin exerts its effects through two G-protein coupled receptor (GPCR) subtypes designated bradykinin B_1 and B_2 receptors (Marceau, 1995). Although both receptors are mainly coupled with G_q proteins, thus increasing calcium levels within cells, kinin receptors can act through additional signaling pathways, depending on the cell type (Blaukat, 2003). Bradykinin-B_2 receptor activation participates in various physiological processes such as blood pressure control. The bradykinin metabolite des-Arg^9-bradykinin as well as Lys-des-Arg^9-bradykinin activates the bradykinin-B_1 receptor known to be primarily expressed in inflammatory conditions.

Bradykinin is frequently regarded as an inflammatory mediator in the brain (Thornton, 2010). Viel and Buck (2011) showed a link between kinins and glial inflammation in AD. An increase in bradykinin in the cerebrospinal fluid and in densities of bradykinin B_1 and B_2 receptors in brain areas related to memory, after chronic infusion of Aβ peptide in rats, which was accompanied by memory disruption and neuronal loss, was observed.

On the other hand, anti-inflammatory effects of bradykinin were shown as well. We recently reported that bradykinin attenuated LPS-induced NO and tumor necrosis factor-α (TNF-α) production in

* Corresponding author. Tel.: +972 8 647 7377; fax: +972 8 647 9303.
E-mail address: fleisher@bgu.ac.il (S. Fleisher-Berkovich).
[1] Equal contribution.

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BV2 microglial cells (Sarit et al., 2012). Previously we also reported on a dual role of bradykinin in primary cultured rat glia (Levant et al., 2006).

Substantial evidence suggests that mitogen activated protein kinase (MAPK) family members, including extracellular signal-regulated kinases (ERK) 1 and 2, also named p42/p44 MAPK, c-JunNH2-terminal kinases (JNK) and p38 MAPK, are important regulators of inflammatory and immunological reactions. Bradykinin has been reported to act as an important mediator of activation of p42/p44 MAPK cascades in different cell types (Hsieh et al., 2007). Abnormal p42/p44 MAPK regulation might be implicated in several models of CNS inflammation (Khan and Alkon, 2006).

The aim of the present study was to investigate whether differential COX-2 transcription and MAPK signaling are possible mechanisms by which bradykinin and/or B_1 agonist differentially regulate brain astrocyte inflammation.

2. Materials and methods

2.1. Primary astrocytes

Primary astrocytes were prepared from whole brains of neonatal (24 h) Wistar rats, according to well established protocols. Cells were grown to confluency in 75 cm^2 flasks covered by poly-L-lysine at 37 °C in 8% CO_2 for 19 days. High glucose (4.5 mg glucose/ml) Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 0.2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.2 U/ml insulin, was used as the culture medium. Astrocytes were purified from other glial cells by shaking the flasks with rotary shaker at 1.96 g for 30 min and then again at 3.8 g over night, then the medium was changed to fresh one. One day before each experiment, flasks were shaked again at 3 g for 1 h. Immunocytochemistry studies, using polyclonal rabbit anti-cow antibody against glial fibrillary acidic protein (GFAP), 1:500, DakoCytomation, Denmark), have revealed that these cultures, contained 98% astrocytes (Levant et al., 2006).

Prior to an actual experiment, the cells were incubated in 1 ml serum-free medium for 4 h. The test agents were added to serum-free medium containing 0.1% bovine serum albumin and 10 mM HEPES which was used for incubation of the cells for a period of 15 h. At the end of each experiment, cells were harvested with a mixture of trypsin and serum-free medium (1:1), and counted using Z1 Coulter counter (Coulter Electronics, Miami, Florida, USA).

The following agents: bradykinin acetate, [Lys-des-Arg^9]-Bradykinin (B1 receptor agonist), lipopolysaccharide from *Escherichia coli* 055:B5, were purchased from Sigma-Aldrich, St. Louis, MO.

2.2. Prostaglandin E_2 assay

Assessment of prostaglandin E_2 that had accumulated in the unextracted samples of the medium was performed using a single antibody radioimmunoassay with dextran-coated charcoal precipitation. The assay was performed in duplicate for each sample. The assay is based on competitive binding of labeled and unlabeled prostaglandins to specific antiserum. Samples were incubated with rabbit antibody to prostaglandin E_2 for 30 min. Precipitation of unbound prostaglandins was achieved by incubation of the samples with dextran-coated charcoal for 10 min and centrifugation at 2000*g*, at 4 °C for 15 min.

Radioactivity was measured in the resulting supernatant on β-scintillation counter (TriCarb 2100, Packard, Meriden, CT, USA). Medium did not interfere with the assay. The sensitivity of the assay was 0.07 ng/ml. Rabbit antibody to prostaglandin E_2 was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA), and tritium-labeled prostaglandin E_2 (160 Ci/mmol) was supplied by the Radiochemical Center (Amersham, UK).

2.3. Western blot analysis

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), cells were harvested, collected and lysated. Protein concentrations ware determined by using a well established Bradford method. Lysated samples containing Laemmli buffer and β-mercaptoethanol were boiled for 5 min at 95 °C and electrophoresed on a 10% SDS-tris gel. Proteins were transferred to nitrocellulose membranes and probed with specific anti COX-2, anti total-ERK (t-ERK) 1/2 or phospho ERK (P-ERK) 1/2, anti t-JNK or P-JNK, anti t-p38 or anti P-p38. Non-specific binding sites were blocked by incubating the membranes with 5% non-fat dry milk powder in TBS buffer containing Tween 20 (TTBS). The washing of the membranes was performed using TTBS. Thereafter, the membranes were incubated with the appropriate secondary antibody (IgG-HRP conjugates for 90 min at room temperature). Immunoreactivity was detected using enhanced chemiluminescence (ECL) solution followed by exposure to X-ray film (Fuji medical X-ray film, FujiFilm). Semi-quantitative analysis was performed using a computerized image analysis system (EZ Quant-Gel 2.2, EZQuant Biology Software Solutions Ltd., Israel). To ensure that blots were loaded with equal amounts of protein, β-actin protein levels were measured using the proper primary and secondary antibodies. The following antibodies were used: horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). HRP-conjugated goat anti-mouse and bovine anti-goat antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Rabbit polyclonal anti-p38 antibody, mouse monoclonal anti-phospho p44/42 MAP kinase antibody, mouse monoclonal anti-phospho JNK antibody were from Cell Signaling Technology® (Danvers, MA). Mouse monoclonal anti-phosphor-p38 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Mouse monoclonal anti-COX-2 antibody was from BD Biosciences (San Jose, CA), Rabbit monoclonal anti p44/42 antibody and mouse monoclonal anti-β-actin antibody was from Sigma-Aldrich (St. Louis, MO).

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Astrocytes were seeded onto 10 cm diameter petri dishes, 25×10^6 cells per dish. Test agents (LPS 0.1 μg/ml, the B_1 receptor agonist 100 nM and bradykinin 100 nM) were added for 3 h. Isolation and purification of total RNA was performed using EZ-RNA II kit (Biological industries, Beit Haemek, Israel) according to the manufacturer's instructions. Cells were washed in ice-cold PBS and scraped with a rubber policeman in reagent A (denaturing solution). Lysates were transferred into microcentrifuge tubes and incubated for 5 min at room temperature. Lysates were added with reagent B (water-saturated phenol) followed by the addition of reagent C (1-bromo-3-chloropropane) and incubation for 10 min at room temperature. Samples were centrifuged at 12,000*g* for 15 min at 4 °C. The topmost phase (containing RNA) was collected into a new tube, added with isopropanol and incubated for 10 min at room temperature. Samples were centrifuged at 12,000*g* for 8 min at 4 °C, stored at −20 °C for 30 min and centrifuged again. Pellets were washed with ethanol 75% in DEPC-treated water and centrifuged at 7500*g* for 5 min at 4 °C. Dried pellets were re-suspended in DEPC-treated water and incubated at 55 °C for 10 min. Total RNA concentrations were determined by a spectrophotometer (Thermo Scientific NanoDrop™ 1000 Spectrophotometer, Pittsburgh, PA).

Reverse transcription was carried out using Verso™ cDNA kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions. RNA (1 μg/μl) was denatured by incubation at 70 °C for 5 min. Then RNA was added with RT buffer, first strand primer, dNTPs, RT enhancer and RTase in a total reaction volume of 20 μl and incubated at 43 °C for 30 min and then at 95 °C for 2 min.