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# Bradykinin decreases nitric oxide release from microglia via inhibition of cyclic adenosine monophosphate signaling

Sarit Ben-Shmuel, Abraham Danon, Sigal Fleisher-Berkovich*

*Department of Clinical Pharmacology, Ben Gurion University of the Negev, P.O.B. 653, Beer-Sheva 84105, Israel*

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ABSTRACT

Bradykinin (BK) is a major potent inflammatory mediator outside the central nervous system. In Alzheimer's disease, BK release and BK receptor expression in brain tissues are upregulated relatively early during the course of the disease. Hence, BK was believed to promote neuroinflammation. However, BK was recently reported to possess anti-inflammatory and neuroprotective roles. Exposure of BV2 microglial cell line to BK lead to a decrease in NO release from unstimulated cells as well as a dose-dependent attenuation, mediated by both B1 and B2 receptors, in lipopolysaccharide (LPS)-induced NO production. In this study we examined whether cyclic adenosine monophosphate (cAMP) signaling is involved in BK-mediated effect in microglial nitric oxide (NO) production. A protein kinase A (PKA) inhibitor mimicked the effects of BK, while cAMP elevating agents antagonized BK-mediated NO decrease. Moreover, BK inhibited the activation of cAMP responsive element binding protein (CREB). In addition, BK protected microglial cells from death triggered by combinations of LPS and each of the cAMP elevating agents. Finally, the addition of $G\alpha_i$ protein inhibitor abrogated the effects of BK on NO release, and the expression of $G\alpha_i$ protein in the plasma membrane was induced by BK. These results suggest that BK-mediated reduction in microglial NO production depends on coupling to $G_i$ protein and also involves inhibition of cAMP-PKA-CREB signaling.



## 1. Introduction

It is well known that neuroinflammation plays a critical role in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and multiple sclerosis [1]. A major feature of central nervous system (CNS) inflammation is the over-production of pro-inflammatory cytokines, prostaglandins, reactive oxygen species and nitric oxide (NO) by activated microglial cells [2]. NO is often reported as a highly toxic molecule, and is directly associated with neuronal death and CNS tissue destruction [4,31].

Neuropeptides have long been proposed to serve as neurotransmitters and immunomodulators in the CNS [16]. Bradykinin (BK), a member of a group of short-lived peptides called kinins, is a good candidate for the role of an immunomodulator in the brain. BK is formed by the kallikrein-kinin system in response to several pathophysiological events, including inflammation [34,42]. BK is a well known pro-inflammatory mediator outside the CNS, and is capable of inducing pain, vasodilatation and activation of immune cells to produce pro-inflammatory cytokines [42]. BK exerts its effects through two G-protein coupled receptor (GPCR) subtypes designated B1 and B2 receptors (B1R and B2R, respectively) [25]. Both receptors are mainly coupled with $G_q$ proteins, thus leading to increased calcium levels within cells. However, kinin receptors can act through additional signaling pathways, depending on the cell type [30]. BK is frequently regarded as an inflammatory mediator in the brain as well as outside the CNS since it was found to be involved in neuronal cell death associated with various brain injuries [17,35]. In AD cases, amyloid β (Aβ) peptide was shown to activate the release of kinins and induce kinin receptor expression in various brain areas [22,43]. In contrast with these observations, we recently reported that BK attenuated LPS-induced NO and tumor necrosis factor-α (TNF-α) production in microglial cells, an effect probably mediated by inhibition of nuclear factor-κB (NF-κB) activity by BK [36]. We also showed that a B1R-selective agonist decreased prostaglandin $E_2$ ($PGE_2$) production in primary cultured astrocytes, which was accompanied by a decrease in LPS-induced cyclooxygenase (COX)-2 expression [13]. We observed similar effects in a mixed culture of glial cells under both unstimulated and LPS-induced conditions [26]. These data indicate that in the CNS, BK may have anti-inflammatory and neuroprotective effects, the mechanisms of which are not clear yet.

Substantial evidence suggests that the second messenger cyclic adenosine monophsophate (cAMP) is an important regulator of inflammatory and immunological reactions [7]. In the cAMP

* Corresponding author. Tel.: +972 8 647737; fax: +972 8 6479303.
*E-mail addresses:* saritbe@bgu.ac.il (S. Ben-Shmuel), adanon@bgu.ac.il (A. Danon), fleisher@bgu.ac.il, sigalfleisher@gmail.com (S. Fleisher-Berkovich).

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signaling pathway, binding of ligands to GPCRs that are coupled with a stimulatory G protein α subunit (Gαs) triggers the production of cAMP in the cell [45,46]. By contrast, inhibitory Gα subunits ($G\alpha_i$) suppress the production of intracellular cAMP [38]. Increased cAMP levels lead to activation of protein kinase A (PKA) and the subsequent phosphorylation of cAMP response element binding protein (CREB) on its serine 133 residue, resulting in activation of the transcription factor [45].

The aim of the present study was to investigate whether cAMP signaling is a possible mechanism by which BK reduces NO production in the murine microglial cell line, BV2.

## 2. Materials and methods

### 2.1. Cell culture

BV2 immortalized murine microglial cell line was a kind gift from Prof. Rosario Donato (Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Italy). BV2 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine, and were maintained in a humified incubator at 37 °C with 5% $CO_2$. For experiments, cells were seeded on a 24-well plate at a concentration of $3 \times 10^5$ cells per well, or a 6-well plate at a concentration of $1 \times 10^6$ cells per well, and allowed to settle for 24 h.

Prior to each experiment, the cells were incubated with 0.5 ml serum-free medium (SFM) for 4 h. Then, SFM was removed and test agents in SFM containing 0.1% BSA and 10 mM HEPES buffer, pH 7.4 were added for the indicated times.

The following agents were used: bradykinin, Lys-(des-Arg9, Leu8)-Bradykinin and HOE-140, lipopolysaccharide from *Escherichia coli* Serotype 055:B5, $N^6$-2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP) and H-89 were from Sigma–Aldrich (St. Louis, MO). Forskolin (*Coleus forskohlii*) and pertussis toxin (*Bordetella pertussis*, PTX) were from Calbiochem (Darmstadt, Germany).

### 2.2. Cell count

At the end of each experiment, cells were harvested after incubation with 1 ml SFM for 1 h at 4 °C, and counted using Z1 Coulter counter (Coulter Electronics, Miami, FL).

### 2.3. Determination of NO levels (Griess reaction)

NO levels in cultured media were determined by measuring the amount of nitrite in the culture supernatants. 100 μl of each culture supernatant were mixed with the same volume of the Griess reagent (Sigma–Aldrich) in a 96-well plate and incubated for 15 min in the dark, at room temperature. Absorbance was subsequently read at 540 nm using a microplate reader (model 680, Bio-Rad). Nitrite concentrations were calculated with reference to a standard curve of sodium nitrite.

### 2.4. Cell fractionation

Cell nuclear fraction was obtained for the study of CREB phosphorylation, cells were collected by scraping in PBS and centrifuged at $1000 \times g$ for 5 min at 4 °C. The cell pellets were resuspended in hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM NaF and 1 mM $Na_3VO_4$) supplemented with a protease inhibitors cocktail. After the addition of 0.5% (v/v) Nonidet P-40 (Sigma–Aldrich) and vigorous vortexing for 10 s, the nuclei were pelleted by centrifugation ($14{,}000 \times g$ for 30 s at 4 °C). Supernatants were collected for further analysis of the cytosolic fraction. The nuclei were washed with PBS, resuspended in extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF and 1 mM $Na_3VO_4$) supplemented with a protease inhibitors cocktail and vortexed for 10 s. After incubation on ice on a rocking platform (150 rpm) for 30 min, vigorous vortexing for 30 s and centrifugation ($14{,}000 \times g$ for 10 min at 4 °C), the supernatants were collected for further analysis of the nuclear fraction.

Cell membrane fraction was obtained for the study of Gαi expression. Cells were collected by scraping in lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM $MgCl_2$) supplemented with a protease inhibitors cocktail and homogenized at 25,000 rpm on a Polytron (PT 1200, Kinematica AG, Switzerland) for 1 min. Homogenates were centrifuged at $1000 \times g$ for 10 min at 4 °C. Supernatants were collected and further centrifuged at $30{,}000 \times g$ for 30 min at 4 °C. Supernatants were discarded, while pellets were washed with lysis buffer (as described above) and centrifuged again ($30{,}000 \times g$ for 30 min at 4 °C). Supernatants were discarded and pellets (the membrane fraction) were resuspended in lysis buffer (as described above with the addition of 1% triton 100× and 1% deoxycholic acid). Lysates were briefly sonicated for 2–3 s (Sonicator ultrasonic processor, Misonix Incorporated, USA).

Protein concentration of cell lysates was determined by the Bio-Rad protein assay (Bradford assay).

### 2.5. SDS polyacrylamide gel electrophoresis and Western blot analysis

5–15 μg protein of nuclear fractions, or 25 μg protein of the membrane fractin were separated on 10% polyacrylamide-SDS gels and transferred to nitrocellulose membranes. The membranes were blocked with 4% BSA for 90 min at room temperature and incubated overnight at 4 °C with specific anti-phospho-CREB (Ser133), anti-CREB, or anti-$G\alpha_i$ antibodies. 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 (for membrane fraction) and lamin B (for nuclear fraction) 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 monoclonal anti-phospho-CREB (Ser133) antibody and rabbit polyclonal anti-$G\alpha_i$ antibody were from Cell Signaling Technology® (Danvers, MA). Rabbit polyclonal anti-CREB antibody was from Millipore (Temecula, CA). Goat polyclonal anti-lamin B antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-β-actin antibody was from Sigma–Aldrich (St. Louis, MO).

### 2.6. Statistical analysis

Results are presented as the mean ± SEM for each experiment. To assess the statistical significance of differences between treatment groups, one-way analysis of variance (ANOVA) was performed, followed by a post hoc multiple comparison test (Tukey–Kramer Multiple Comparison Test). Unpaired, two-tailed