Invited Perspective

A_{2B} adenosine receptors stimulate IL-6 production in primary murine microglia through p38 MAPK kinase pathway

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Abstract

The hallmark of neuroinflammation is the activation of microglia, the immunocompetent cells of the CNS, releasing a number of proinflammatory mediators implicated in the pathogenesis of neuronal diseases. Adenosine is an ubiquitous autacoid regulating several microglia functions through four receptor subtypes named A_1, A_{2A}, A_{2B} and A_3 (ARs), that represent good targets to suppress inflammation occurring in CNS. Here we investigated the potential role of ARs in the modulation of IL-6 secretion and cell proliferation in primary microglial cells. The A_{2B}AR agonist 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide (BAY60-6583) stimulated IL-6 increase under normoxia and hypoxia, in a dose- and time-dependent way. In cells incubated with the blockers of phospholipase C (PLC), protein kinase C epsilon (PKC-ε) and PKC delta (PKC-δ) the IL-6 increase due to A_{2B}AR activation was strongly reduced, whilst it was not affected by the inhibitor of adenylyl cyclase (AC). Investigation of cellular signalling involved in the A_{2B}AR effect revealed that only the inhibitor of p38 mitogen activated protein kinase (MAPK) was able to block the agonist's effect on IL-6 secretion, whilst inhibitors of pERK1/2, JNK1/2 MAPKs and Akt were not. Stimulation of p38 by BAY60-6583 was A_{2B}AR-dependent, through a pathway affecting PLC, PKC-ε and PKC-δ but not AC, in both normoxia and hypoxia. Finally, BAY60-6583 increased microglial cell proliferation involving A_{2B}AR, PLC, PKC-ε, PKC-δ and p38 signalling.

In conclusion, A_{2B}ARs activation increased IL-6 secretion and cell proliferation in murine primary microglial cells, through PLC, PKC-ε, PKC-δ and p38 pathways, thus suggesting their involvement in microglial activation and neuroinflammation.

Abbreviations: Ado, Adenosine; AC, adenylyl cyclase; ARs, adenosine receptors; BAY 60-6583, 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide; CHA, N (6)-cyclohexyladenosine; CGS 21680, 2 *p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate; Cl-IB-MECA, 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-β-D-ribofuranuronamide; CNS, central nervous system; DAPI, 4¢,6¢,- diamino-2-phenyl-indole; GFAP, antiglial fibrillary acidic protein; IL-6, interleukin 6; NO, nitric oxide; p44/42 MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinases; PLC, phospholipase C; PSB 603, 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; SB202190, 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; SH5, D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]; siRNA, small interfering RNA; SP600125, 1,9-Pyrazoloanthrone; Rottlerin, 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1- benzopyran-8-yl]-3-phenyl-2-propen-1-one; SQ, 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine; TNF-α, tumor necrosis factor-α; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; U73122, 1[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione

Keywords: Microglial cells; Inflammation; Adenosine receptors; IL-6; Signalling pathway; Proliferation

1 Introduction

Microglial cells are the immunocompetent cells of the central nervous system (CNS) and play a fundamental role in inflammation during CNS pathologies [1]. These cells undergo to change of shape during activation moving from the resting/surveillant ramified cell type in the normal brain to an activated amoeboid form in response to any type of pathologic context causing neuroinflammation. Activated microglia produce diverse substances such as reactive oxygen and nitrogen species like nitric oxide (NO) and cytokines including interleukin (IL)-6, tumor necrosis factor (TNF)-α which affect a variety of neuronal diseases ranging from multiple sclerosis and cerebral ischemia to Parkinson's and Alzheimer's diseases. Therefore, modulating the mediators of inflammation produced by activated microglia may help to attenuate the progression of neuroinflammatory and neurodegenerative diseases [2].

Adenosine (Ado) is an endogenous, ubiquitous molecule that exerts a number of physiological effects by activation of four G-protein-coupled receptors named A_1, A_{2A}, A_{2B} and A_3 (ARs) [3]. A_1 and A_3 receptor subtypes, coupled to Gi/o proteins, inhibit adenylyl cyclase (AC) activity, while A_{2A} and A_{2B} increase it, through activation of Gs proteins. In addition, A_{2B} and A_3 ARs are associated to stimulation of phospholipase C (PLC) activity, through Gq proteins [4,5]. Ado increases in pathophysiological conditions characterized by hypoxia, ischemia and inflammation and exerts important neuroimmunomodulatory effects by regulating both neurons and glial cells [6]. Indeed ado has been found to take part in the principal microglial activation processes spanning from proliferation, process extension, retraction, migration and cytokine production recruiting different AR subtypes [7]. In particular, an inhibitory and stimulatory effect on microglial cell proliferation has been attributed to A_1 and A_{2A} ARs, respectively [8,9]. In addition, A_{2A}ARs upregulation occurs *in vivo* following lipopolysaccharide (LPS) exposure and is responsible for the retraction of microglia processes and assumption of amoeboid morphology, thus contributing to chemotaxis and neuroinflammation [10]. Interestingly, the switch of A_{2A} receptor from an anti- to a pro-inflammatory phenotype has been linked to glutamate levels [11]. Inhibition of cells migration and TNF-α secretion has been observed for A_3ARs activation in LPS injured rats and primary microglial cells [12,13]. However, a role of A_3ARs in the stimulation of cell migration has been demonstrated in ADP-treated microglial cells [14]. A contribution in cells activation has been shown for A_{2A} and A_{2B} ARs through an increase of hypoxia inducible factor 1-α (HIF1-α) and related downstream genes in primary microglial cells, including Glucose transporter-1 (GLUT-1) and NO as well as vascular endothelial growth factor (VEGF) [15]. In addition, as for cytokine production a role for A_{2B} AR has been observed in the increase of the anti-inflammatory IL-10 and reduction of the proinflammatory TNF-α [16,15].

IL-6 has been defined as a major multifunctional cytokine in the CNS exerting both pro- and anti-inflammatory functions by regulating differentiation, proliferation, migration and apoptosis of target cells [17]. Ado has been found to modulate IL-6 through activation of A_{2B}ARs in different cellular types e.g. astrocytes, pituitary folliculostellate cells, bronchial smooth muscle cells, lung fibroblasts, astrocytoma cells, mouse striatum, peritoneal macrophages and cardiac fibroblasts through various signal transduction pathways, including protein kinase C (PKC) and mitogen activated protein kinases (MAPKs) [18–26]. However, no evidence has been reported about the potential regulation of IL-6 in microglial cells by ARs stimulation and the consequent mechanism involved.

Therefore, in this study we examined whether ARs activation could modulate IL-6 secretion and cell proliferation in primary microglial cells. In addition, as inflammation and hypoxic damage may coexist in stroke, ischemia and other CNS disorders the same effects have been investigated also in the presence of 1% oxygen. Our data indicate that stimulation of A_{2B}ARs increases IL-6, via activation of PLC, PKC-ε, PKC-δ and p38 kinase, leading to an increase in cell proliferation.

2 Materials and methods

2.1 Drugs and materials

A_{2B}ARs siRNAs (sc-29643), and anti-A_{2B}ARs rabbit polyclonal antibodies were from Santa Cruz DBA (Milano, Italy). Rabbit polyclonal anti-HIF-1α was from Cayman, Vinci Biochem (Firenze, Italy). Anti-beta Actin was from Millipore (Milano, Italy). RNAiFect Transfection Kit was purchased from Qiagen (Milano, Italy). 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) was from Promega (Milan, Italy). Anti-beta Actin was from Millipore (Milan, Italy). Antiglial fibrillary acidic protein (GFAP) and anti-CD11b antibodies were from Becton Dickinson (Milano, Italy). The Assays-on- demand™ Gene expression Products for IL–6(Mm00446190_m1), A_{2B}ARs (Mm00839292_m1) and β-actin (VIC-MGB 4352341E-1301018) (were purchased from Life Technologies Italia (Monza, Italy). 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB 603), 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (Cl-IB-MECA) and 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide (BAY 60-6583) were purchased from Tocris, Space Import-Export (Milano, Italy). 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) was purchased by Adipogen (Florence, Italy). D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate] (SH5) and 1,9-Pyrazoloanthrone (SP600125) were from Enzo Life (Florence, Italy). 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) was from Cayman (Florence, Italy). AlphaScreen SureFire phospho(p)ERK1/2(Thr202/Tyr204), pJNK1/3(pThr183/Tyr185), and p-p38αMAPK (pThr180/Tyr182) assay kits were from PerkinElmer (Milan, Italy). PKC-ε translocation inhibitor peptide was purchased by Calbiochem (Milan, Italy). IL-6 ELISA kit was obtained from R&D, Space Import-Export (Milan, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

2.2 Animals

One-day-old Balb/c mice were obtained from Charles River (Calco, Italy). Animal care procedures were in accordance with the guidelines of the European Council directives (86/609/EEC) and were approved by the local Animal Care and Ethics Committee.

2.3 Primary microglia (Please substitute "cultures" with "isolation")cultures

Briefly, after anaesthesia (Zoletil 100, 30 mg kg^{-1}, Virbac Laboratories, France) and decapitation, forebrains from newborn Balb/c mice were excised, meninges were removed and tissue was dissociated mechanically in 0.25% Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Life Technologies, Milan, Italy) for 5 min at 37 °C. An equal volume of culture medium with 10% of fetal bovine serum (FBS) was added to stop trypsinization. Cells were centrifuged, supernatant was discarded and pellet resuspended in 75 cm^2 flasks with Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F-12), supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), at 37 °C in 5% CO_2/95% air. After 21 days *in vitro* (DIV) confluent mixed glial cultures were subjected to mild trypsinization (0.06%) as previously described [27]. In particular mixed glial cells were washed for 1 min in DMEM:F12 to eliminate serum, then treated for 30 min with 0.06% of trypsin in the presence of 0.25 mM of EDTA and 0.5 mM of Ca^{2+}. This results in the detachment of an intact layer of cells containing virtually all the astrocytes and leaves a population of firmly attached cells identified as >98% microglia. Cultures were characterized by immunostaining with Mac-1 anti-CD11b antibody and the absence of astrocytes was confirmed using GFAP antibody.

2.4 Microglial cell cultures

Microglial cells were grown adherently in either 75 cm^2 flasks, 6-well, 24-well or 96-well dishes in the presence of DMEM/F-12, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), at 37 °C in 5% CO_2/95% air. The medium was changed every two days and the cells were observed under light microscope to ensure their were free from any contamination. The cells were sub-cultured and maintained appropriately at 60–70% confluency.

2.5 Cloned A_{2B} adenosine receptor

HEK293 cells transfected with the human A_{2B} have been previously described [15]. The cells were grown adherently and maintained in DMEM/F-12 without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and Geneticin (G418, 0.2 mg/ml) at 37 °C in 5% CO_2/95% air.

2.6 Hypoxic treatment

Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2 (MiniGalaxy, RSBiotech, Irvine, Scotland).

2.7 Cell viability exclusion assay

Microglia (20,000 cells in 24-well dishes) were collected after trypsinization into Eppendorf tubes and centrifuged at 2000 rpm for 15 min at 4 °C. Supernatants were discarded and the pellets were re-suspended in phosphate-buffered saline (PBS) solution mixed with 0.4% trypan blue for 5 min at room temperature, before being examined under the microscope using a Burker chamber. The number of viable cells was evaluated by trypan blue exclusion. In particular, the dead cells that stained blue were scored positive and counted against the total number of cells to determine the percentage of cell death. The results are expressed as the percentage of cells, which excludes the vital trypan blue/total cells.

2.8 Immunofluorescence analysis

Expression of CD11b on primary microglial cells was checked by immunofluorescence analysis as previously described [15]. Briefly, 100–200 cells/mm^2 were seeded on cover slips, and after overnight incubation, fixed with 4% paraformaldehyde for 20 min, permeabilized with cold methanol for 5 min and washed with PBS. Cells were blocked with 3% bovine serum albumin (BSA) in PBS added with Tween-20 0.2% (PBST) for 30 min and incubated for 1 h in PBST with 1% BSA and anti-CD11b and GFAP (1:50). For negative controls, a series of cover slips was also incubated under similar conditions without the primary antibodies. Coverslips were stained with 4′,6-diamidino-2-phenylindole (DAPI), mounted in DABCO glycerol-PBS and observed on Nikon fluorescent microscope (Eclipse 50i) as previously described [15]. Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Milan, Italy).

2.9 Real-time RT-PCR

Microglial cells were plated in 60 mm diameter petri dishes at a density of 700,000 cells/petri and allowed to attach overnight before pharmacological treatments. Total cytoplasmic RNA was extracted from microglial cells by the acid guanidinium thiocyanate phenol method, using the Trizol reagent (Fisher Molecular Biology) according to the manufacturer's instructions. RNA concentration was determined using a spectrophotometer (Beckman DU520) at 260 nm. Residual DNA was removed by treatment with 1 U DNase/1 µg RNA (RQ1 RNase-free DNase, Promega) at 37 °C for 10 min. Identical amounts of RNA (1 µg) were reversely transcribed into cDNA using a commercial RT-PCR

kit Improm II Reverse transcription system (Promega) and random primers in 20 µl at the following conditions 25 °C 5 min, 42 °C 50 min, 70 °C 15 min. Quantitative real-time RT-PCR assays of IL-6 and A_{2B}ARs were carried out in triplicate using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) purchased from Applied Biosystems (IL-6 Mm00446190_m1, A_{2B}ARs Mm00839292_m1 and β-actin VIC-MGB 4352341E-1301018) on a MX3000P Stratagene Real-Time PCR System (M-Medical, Milan, Italy) [28]. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kits was used, and the probe was fluorescent-labelled with VIC™ (Life Technologies Italia, Monza, Italy). Reactions were normalized to β-actin mRNA within the same sample using the ΔΔCT method. A negative control reaction without template DNA yielded no PCR product. The thermal cycling conditions were 30 s at 95 °C, followed by 40 cycles that consisted of a denaturation step at 95 °C for 5 s, annealing and extension step at 60 °C for 34 s.

2.10 Elisa

The levels of IL-6 protein secreted by the cells in the medium were determined by mouse Quantikine ELISA kits (R&D Systems, Milan, Italy). In brief, subconfluent microglia, 20,000 cells in 24-well dishes, were plated and allowed to attach overnight, then changed into fresh medium in the presence of solvent or various concentrations of drugs. The medium was collected, and IL-6 protein concentrations were measured by ELISA according to the manufacturer's instructions. The results were normalized to the number of cells per well. The data are presented as mean ± SE from four independent experiments performed in triplicate.

2.11 Western blotting

Microglia were plated in six-well plates at a density of 250,000 cells/well and allowed to attach overnight before pharmacological treatments. Whole cell lysates from murine primary microglia were prepared as described previously [28]. In brief, cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA) containing protease inhibitors (aprotinin, leupeptin, pepstatin: 1 µg/ml each) and 10 µM PMSF. The protein concentration was determined using a BCA protein assay kit (Pierce, Euroclone, Milan, Italy). Proteins were denaturated at 100 °C for 10 min then equivalent amounts of protein (40 µg) were subjected to electrophoresis on sodium dodecyl sulfate-acrylamide gel (7.5% for HIF-1α and 10% for A_{2B}AR and β-actin). The gel was then electroblotted onto a nitrocellulose membrane and blocked with 5% defatted milk powder in TBST (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature to reduce aspecific binding. Subsequently the membranes were probed with anti-HIF-1α or anti-A_{2B}AR antibody (1:1000 dilution) overnight at 4 °C. Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against rabbit IgG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Perkin Elmer, Milan, Italy). Actin (1:1000) was used to ensure equal protein loading.

2.12 Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Mean densitometry data from independent experiments were normalized to the results in control cells.

2.13 Small interfering RNA (siRNA) treatment

Microglia were plated in 60 mm diameter petri dishes at a density of 500,000 cells/petri or in six-well plates containing 200,000 cells/well, for RNA and western blotting analysis, respectively, before transfection. Transfection of siRNA-A_{2B} was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit. Cells were cultured in complete media, and RNA and total proteins were isolated at 24, 48 and 72 h for mRNA and western blot analysis of A_{2B}AR proteins. A randomly chosen non-specific siRNA was used under identical conditions as control [28]. A time of 72 h of transfection was chosen for the experiments.

2.14 AlphaScreen SureFire pMAPK assays

AlphaScreen SureFire phospho(p)ERK1/2(Thr202/Tyr204), pJNK1/3(pThr183/Tyr185), and *p*-p38 MAPK (pThr180/Tyr182) assay kits (Perkin Elmer, Milan, Italy) were utilized. Upon MAPK phosphorylation and excitation at 680 nm, fluorescent signals at 615 nm are emitted. Microglial cells were seeded in 100 µl culture medium into 96-well plates (4000/well), and incubated at 37 °C, in both normoxic and hypoxic conditions, for 24 h. Cells were pretreated with various inhibitors for 30 min. Then, receptors were maximally stimulated using 100 nM BAY60-6583 and incubated for 5–15 min (ERK1/2, p38MAPK) or 5–30 min (JNK) at 37 °C. After agonist removal, lysis buffer was added, then donor and acceptor beads linked to specific anti-*p*-MAPK- and anti-MAPK-antibodies were dispensed, according to manufacturer instructions. Finally, fluorescent signals were detected through an Ensight Perkin Elmer-multimode plate reader (Perkin Elmer, Milan, Italy). Data were normalized to fold activation above basal pMAPK levels (=100). For pp38 inhibitor graphs, raw data were transformed into percentages relative to controls (basal level = 100%) in order to merge data from several experiments.

2.15 MTS assay

The MTS assay was performed to determine microglial cell proliferation according to the manufacturer's protocol from the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Milan, Italy). Cells were plated in 96-multiwell plates (4000 cells/well), allowed to attach overnight, then 100 µl of complete medium was added to each well in the absence and in the presence of the A_{2B}AR agonist/antagonist and inhibitors of cell signalling. The cells

were then incubated for 0, 12, 24, 48 and 72 h in normoxia and hypoxia. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 570 nm.

2.16 Statistical analysis

All values in the figures and text are expressed as mean ± standard error (SEM) of independent experiments. Each experiment was performed by using the microglial cells derived from one single mouse, and was performed in triplicate. The experiments were repeated at least four times as indicated from n values that represent the number of mice used [15]. Data sets were examined by one-way analysis of variance (ANOVA) and Dunnett's test (when required). Two-way ANOVA and Bonferroni's post hoc test were used when time and treatment were compared (Fig. 6B). A P-value less than 0.05 was considered statistically significant.

3 Results

3.1 Microglia cells morphology

Primary microglial cells showed a morphology characterized by bipolar or unipolar processes and elongated cell bodies, as shown by the myeloid cell surface antigen CD11b staining. The purity of the cells was verified through the lack of astrocyte-specific protein GFAP staining (Fig. 1).

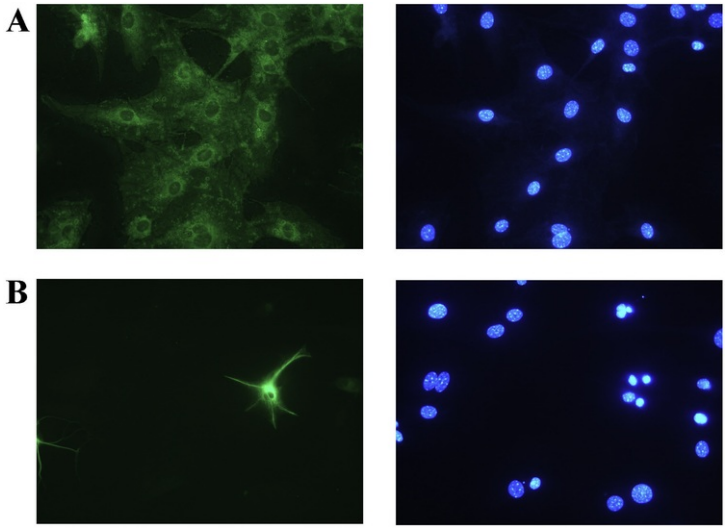

Fig. 1 Microglial cells morphology by immunofluorescence analysis. Staining of microglia with the myeloid cell surface antigen CD11b (A) and astrocyte-specific protein GFAP (B). The blue-fluorescent DAPI nucleic acid stain is shown.

alt-text: Fig. 1

3.2 BAY60-6583 stimulates IL-6 secretion in primary murine microglial cells

We evaluated the effect of treatment with 100 nM ARs agonists for 24 h on IL-6 secretion in normoxia and hypoxia in murine primary microglial cells. As shown in Fig. 2A the A_{2B} agonist BAY60-6583 but not the A_1 agonist N(6)-cyclohexyladenosine (CHA), the A_{2A} agonist 2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate (CGS 21680), and the A_3 agonist 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (Cl-IB-MECA) produced an increase of IL-6 secretion in both normoxic and hypoxic conditions. The effect of BAY60-6583 was time- and dose-dependent, with maximal stimulation obtained after 24 h of treatment with BAY60-6583 1 μM (Figs. 2B, C, respectively). To confirm the involvement of A_{2B} receptors in this effect, microglia were treated with 300 nM 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB 603) for 30 min, before addition of 100 nM BAY60-6583 for 24 h in normoxia and hypoxia. As shown in Fig. 2D, the stimulatory effect on IL-6 production induced by BAY60-6583 was blocked by PSB 603 in both conditions. Antagonist alone did not modify IL-6 basal levels in microglial cells. No significant difference between normoxia and hypoxia was observed (Fig. 2).

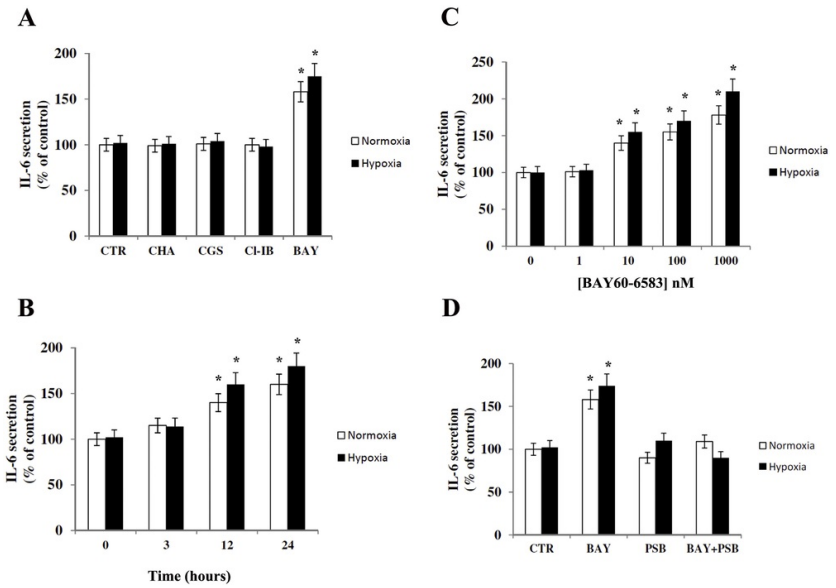

Fig. 2 BAY60-6583 stimulates IL-6 secretion in primary murine microglial cells. (A) Cells were incubated in normoxia and hypoxia for 24 h in the absence (CTR, 100%) and in the presence of 100 nM CHA, CGS21680 (CGS), Cl-IB-MECA (Cl-IB) and BAY60-6583 (BAY) and IL-6 protein secretion was evaluated by ELISA. (B) Cells were incubated in the presence of 100 nM BAY60-6583 under normoxia and hypoxia for 0, 3, 12 and 24 h and IL-6 protein secretion was evaluated by ELISA. (C) Cells were incubated with increasing concentrations of BAY60-6583 (0–1000 nM) for 24 h under normoxia and hypoxia and IL-6 protein secretion was evaluated by ELISA. (D) Cells in the absence or in the presence of 300 nM PSB603 (PSB) were exposed to 100 nM BAY60-6583 for 24 h in normoxia and hypoxia and IL-6 protein secretion was evaluated by ELISA. *P < 0.01 compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA, followed by Dunnett's test.

alt-text: Fig. 2

3.3 BAY60-6583 increases IL-6 transcription in primary murine microglial cells

Activation of microglial cells with 100 nM BAY60-6583 produced a stimulation of IL-6 mRNA transcription after 4 and 8 h of treatment. The effect was investigated in both normoxic and hypoxic conditions with a fold increase of 1.7 ± 0.1 and 2.3 ± 0.2 in normoxic and 1.8 ± 0.1 and 2.6 ± 0.2 in hypoxic conditions, respectively (Fig. 3).

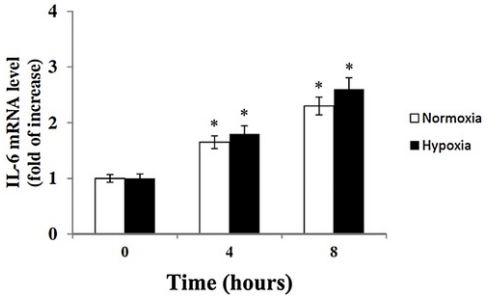

Fig. 3 BAY60-6583 increases IL-6 transcription in primary murine microglial cells. Cells were incubated in normoxia and hypoxia for 0, 4, 8 h with 100 nM BAY60-6583 and IL-6 mRNA levels were evaluated by real-time RT-PCR. Data were expressed as fold of increase *vs* cells in the absence of BAY60-6583, arbitrarily fixed as 1. *P < 0.01 compared with 0. Each point is the mean ± SEM of four independent experiments (N = 4). Analysis was by one way ANOVA, followed by Dunnett's test.

alt-text: Fig. 3

3.4 Hypoxia stimulates hypoxia-inducible factor HIF-1α and A_{2B}ARs in a time-dependent way

Microglial cells were exposed to 1% O_2 for 4, 8 and 24 h and HIF-1α and A_{2B} receptor modulation were assessed through western blot analysis. As shown in Fig. 4 both proteins HIF-1α (Fig. 4A) and A_{2B} AR (Fig. 4B) were upregulated in a time-dependent way by hypoxia thus confirming the effect of a hypoxic environment in our experimental conditions. The additional band in A_{2B} western blot may be due to homo- or hetero-oligomers as previously observed [15].

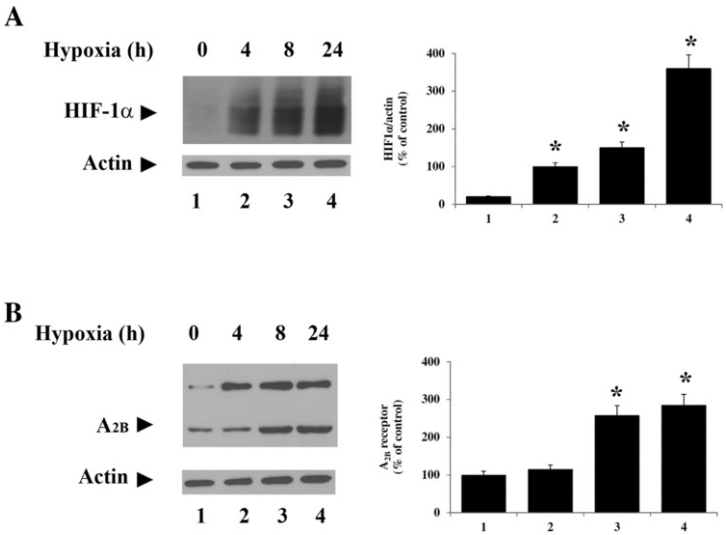

Fig. 4 Hypoxia induces HIF-1α accumulation and A_{2B}AR upregulation in primary microglial cells. Cells were incubated for up to 24 h in the absence or presence of hypoxia (1%) and the protein levels of HIF-1α (A) and A_{2B}AR (B) were determined by western blotting in whole cell lysates and actin served as control for equal protein loading. Densitometric quantification is the mean ± SEM of four independent experiments (N = 4); control (cells in the presence of 4 h hypoxia (A), cells in normoxia (B)) was set to 100%. Values are the mean ± S.E. of four independent experiments (N = 4); *P < 0.05 compared with Control; analysis was by one way ANOVA, followed by Dunnett's test.

alt-text: Fig. 4

3.5 Signalling pathways involved in the IL-6 production induced by BAY60-6583 in primary murine microglial cells

The involvement of AC, PLC, PKC-ε and PKC-δ in the IL-6 increase due to A_{2B}AR activation was investigated. Microglial cells were incubated with 50 μM SQ22,536, 10 μM U73122, PKC-ε translocation inhibitor peptide and rottlerin as inhibitors of AC, PLC, PKC-ε and PKC-δ, respectively. As shown in Fig. 5A, all inhibitors alone did not significantly affect IL-6 levels, whilst blockers of PLC, PKC-ε and PKC-δ were able to antagonize the stimulatory effect of BAY60-6583 on IL-6 secretion, suggesting the involvement of these molecules in the A_{2B} agonist effect. Furthermore, to examine whether MAPK and Akt pathways were involved in the stimulatory effect mediated by BAY60-6583 on IL-6 secretion, primary microglia were pretreated with 1 μM SB202190, U0126, SP600125 and SH5, inhibitors of p38, ERK1/2, JNK1/2 MAPK kinases and Akt respectively, before exposure to 100 nM BAY60-6583, in normoxia and hypoxia. As shown in Fig. 5B, only SB202190 strongly reduced the effect of BAY60-6583 on IL-6 secretion, whilst U0126, SP600125 and SH5 did not. All inhibitors alone did not significantly regulate IL-6 levels in microglia.

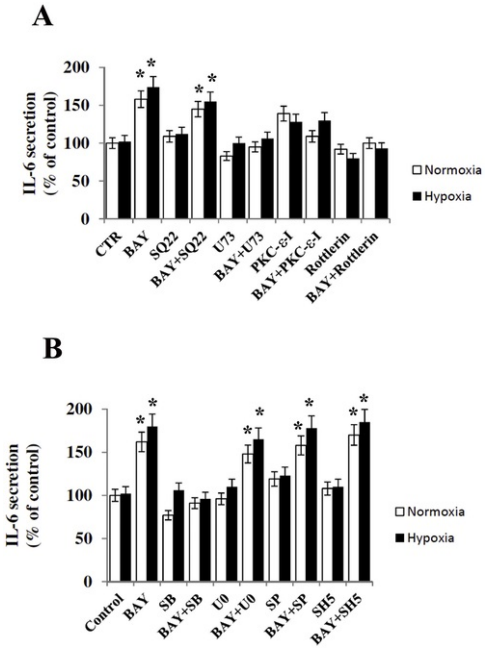

Fig. 5 Signalling pathways involved in the IL-6 production induced by BAY60-6583 in primary murine microglial cells. Effect of 100 nM BAY60-6583 on IL-6 protein secretion under normoxia and hypoxia (24 h) and antagonism by 50 μM SQ22,536, 10 μM U73122, PKC-ε blocker and rottlerin (A), 1 μM SB202190, U0126, SP600125 and SH5 (B) evaluated by ELISA. *P < 0.01 compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

alt-text: Fig. 5

3.6 Effect of BAY60-6583 on p38 MAPK kinase

To confirm our data, phosphorylation of p38 was assessed at 0–15 min in the absence and in the presence of 100 nM BAY60-6583, as stimulator of A_{2B}ARs, following normoxia and hypoxia (Fig. 6A,B, respectively). The extent of p38 phosphorylation was strictly similar in normoxic and hypoxic conditions with a maximal effect after 5 min of incubation that decreased at 15 min in both conditions. Phosphorylation of p38 was maximal after treatment with 100 nM BAY60-6583 for 5 min both in normoxia and hypoxia (Fig. 6C,D respectively). The effect of 100 nM BAY60-6583 was potently antagonized by pretreatment of cells with 300 nM PSB603 for 30 min, in both normoxic and hypoxic conditions (Fig. 6E,F, respectively). BAY60-6583 did not alter significantly ERK1/2, JNK1/2 and Akt phosphorylation (data not shown). p38 phosphorylation induced by exposure to 100 nM BAY60-6583 for 5 min in microglia was reduced in the presence of PLC, PKC-ε and PKC-δ inhibitors in normoxia and hypoxia, but not after incubation with the AC blocker (Fig. 6G,H, respectively).

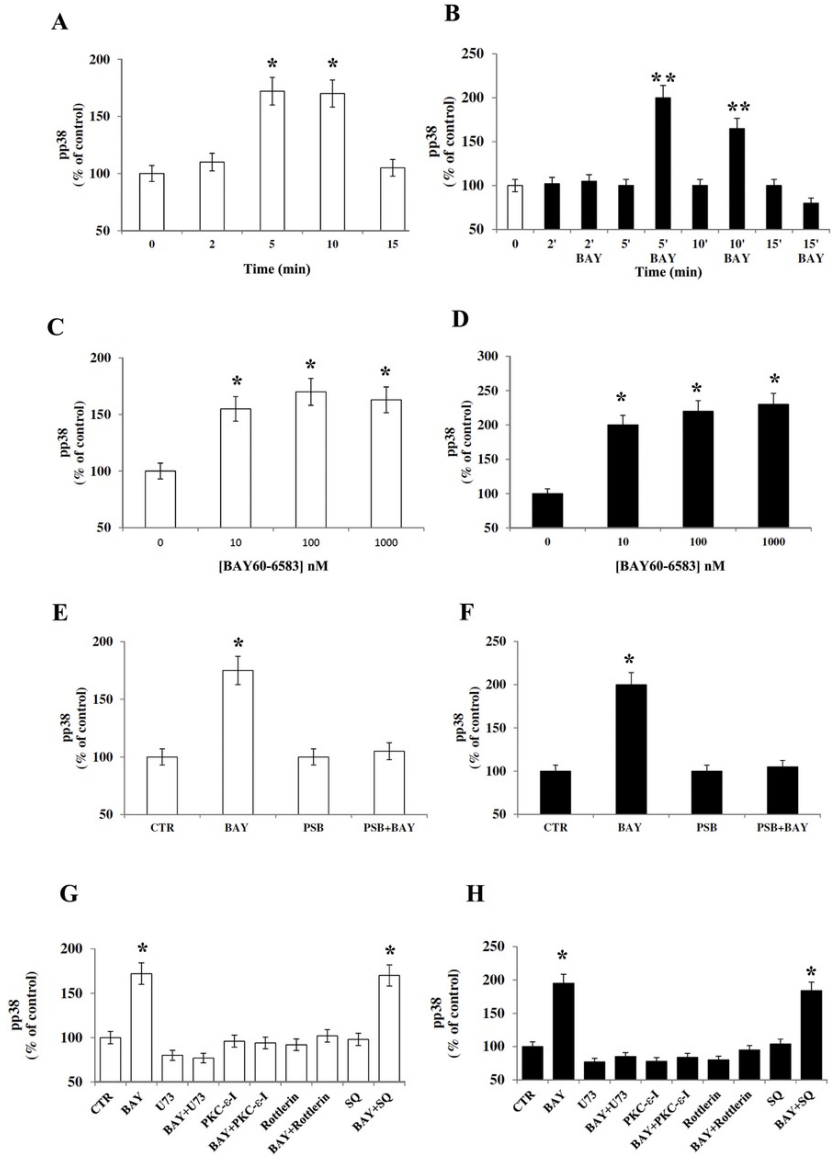

Fig. 6 Effect of BAY60-6583 on p38 MAPK activation. Cells were incubated in the presence of 100 nM BAY60-6583 for 0, 2, 5, 10 and 15 min and pp38 protein levels were evaluated by AlphaScreen SureFire pMAPK assays under normoxia (A) and hypoxia (B). Cells were incubated with increasing concentrations of BAY60-6583 (0–1000 nM) for 5 min and pp38 protein levels were evaluated by AlphaScreen SureFire pMAPK assays under normoxia (C) and hypoxia (D). Cells in the absence or in the presence of 300 nM PSB603 (PSB) were exposed to 100 nM BAY60-6583 for 5 min and pp38 protein levels were evaluated by AlphaScreen SureFire pMAPK assays in normoxia (E) and hypoxia (F). Effect of 100 nM BAY60-6583 on pp38 protein levels under normoxia (G) and hypoxia (H) (5 min) and antagonism by 10 μM U73122, PKC-ε blocker, rottlerin and 50 μM SQ22,536 evaluated by AlphaScreen SureFire pMAPK assays. *P < 0.01 compared with CTR. Analysis was by one-way ANOVA followed by Dunnett's test; **P < 0.001 compared with cells without BAY60-6583 in normoxia. Analysis was by two-way ANOVA followed by Bonferroni's test. Interaction F(4,20) = 40.82, P < 0.0001; Time F(4.20)=40.02, P < 0.0001; BAY60-6583 F(1,20)=69.76, P < 0.0001. Means ± S.E. values from four experiments are shown.

alt-text: Fig. 6

3.7 Effect of BAY60-6583 on microglial cell proliferation

The effect of 100 nM BAY60-6583 on microglial cell proliferation was assessed after 12, 24, 48 and 72 h of treatment. Cell proliferation started to increase after 12 h and reached a maximum effect after 48 h of incubation (Fig. 7A). This effect was antagonized by addition of 300 nM PSB603, 1 μM SB202190, U73122, inhibitor of PKC-ε and rottlerin, thus suggesting the involvement of A_{2B}ARs, PLC, PKC-ε, PKC-δ and p38 signalling in cell proliferation (Fig. 7B).

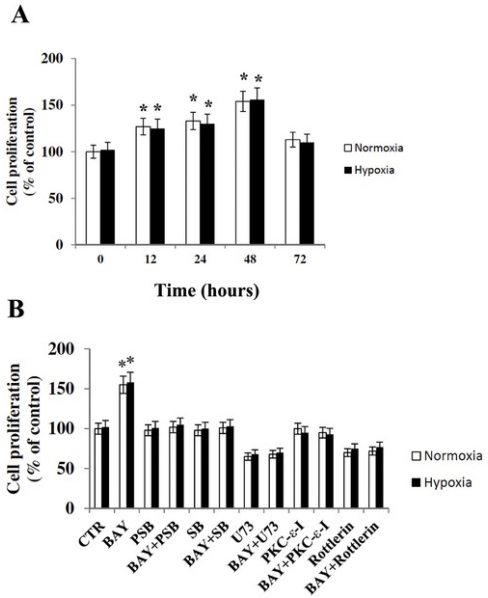

Fig. 7 Effect of BAY60-6583 on microglial cells proliferation. Cells were incubated in the presence of 100 nM BAY60-6583 for 12, 24, 48 and 72 h and cell proliferation was evaluated by MTS assay under normoxia and hypoxia (A). Cells in the absence or in the presence of 300 nM PSB603, 1 μM SB202190, 10 μM U73122, PKC-ε blocker and rottlerin were exposed to 100 nM BAY60-6583 for 48 h and cell proliferation was evaluated by MTS assay under normoxia and hypoxia (B). *P < 0.01 compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA, followed by Dunnett's test.

alt-text: Fig. 7

3.8 Effect of A_{2B} AR silencing on p38 MAPK kinase, IL-6 and cell proliferation

In order to further verify the role of A_{2B}AR in p38 phosphorylation, IL-6 protein secretion and cell proliferation we knocked-down it. Microglial cells were transfected with siRNA targeting A_{2B} ARs. After 48 and 72 h post-transfection, A_{2B}ARs mRNA and protein levels were significantly reduced in comparison to control cells transfected with non-specific random control ribonucleotides (siRNA scrambled, siRNActr) (Fig. 8A and B, respectively). A_{2B}ARs siRNA treatment for 72 h inhibited the increase of p38 phosphorylation induced by 100 nM BAY60-6583 applied for 5 min (Fig. 9A). Furthermore, A_{2B}ARs stimulation for 24 h did not increase IL-6 secretion in microglial cells preincubated with A_{2B}ARs siRNA for 48 h, suggesting the involvement of A_{2B}AR subtype in this effect (Fig. 9B). Similarly, cell proliferation was not more affected by 100 nM BAY60-6583 added for 48 h to the cells transfected with A_{2B}ARs siRNA (Fig. 9C). No significant difference between normoxia and hypoxia was observed.

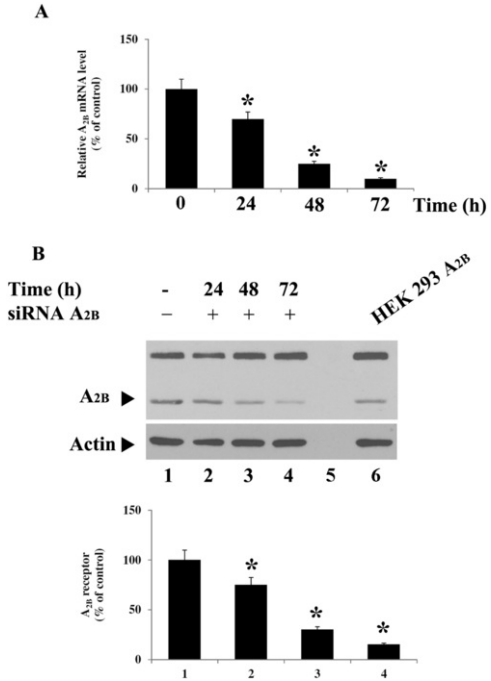

Fig. 8 A_{2B}AR silencing by siRNA transfection. (A) A_{2B}AR mRNA quantification, related to β-actin mRNA, by real-time RT-PCR. Microglial cells were transfected with siRNAA_{2B} by RNAiFectTM Transfection reagent and cultured for 24, 48 and 72 h. Plots are mean ± S.E. (n = 4); *P< 0.01 compared with the control (time = 0). (B) Western blot analysis using anti A_{2B}AR polyclonal antibody of protein extracts from microglial cells treated with siRNA-A_{2B} and cultured for 24, 48 and 72 h; cells transfected with siRNActr for 72 h and HEK293 cells transfected with A_{2B}AR as positive control (line 1 and 6, respectively). Actin shows equal loading protein. *P < 0.05 vs cells transfected with siRNActr for 72 h. Analysis was by one way ANOVA, followed by Dunnett's test.

alt-text: Fig. 8

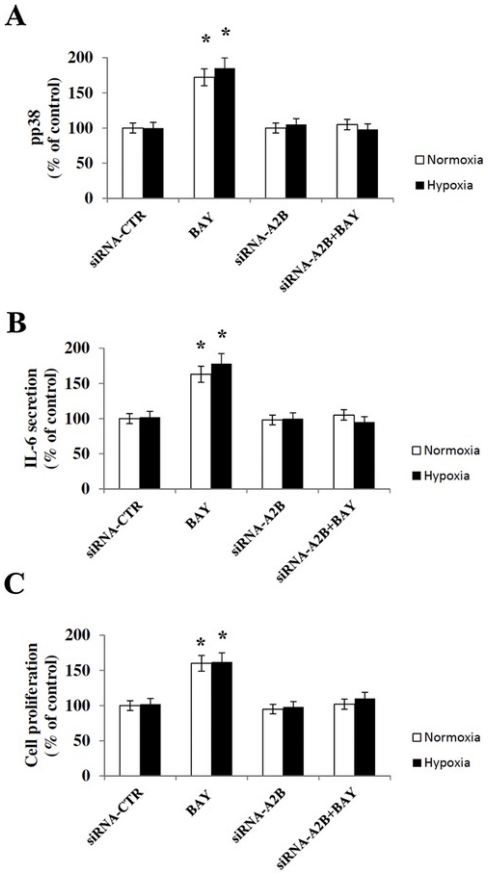

Fig. 9 pp38, IL-6 protein levels and proliferation in microglia, A_{2B}ARs silenced, stimulated by BAY60-6583. (A) Microglial cells were incubated with both siRNA scrambled (siRNA-ctr) and siRNA of A_{2B}ARs (siRNA-A_{2B}) and cultured for 72 h before addition of 100 nM BAY60-6583 for 5 min and pp38 levels were evaluated by AlphaScreen SureFire pMAPK assays under normoxia and hypoxia. (B) Microglial cells were incubated with both siRNA-ctr and siRNA-A_{2B} and cultured for 48 h before addition of 100 nM BAY60-6583 for 24 h and IL-6 levels were evaluated by ELISA under normoxia and hypoxia. (C) Microglial cells were incubated with both siRNA-ctr and siRNA-A_{2B} and cultured for 24 h before addition of 100 nM BAY60-6583 for 48 h and cell proliferation was evaluated by MTS under normoxia and hypoxia. *$P < 0.01$ compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA, followed by Dunnett's test.

alt-text: Fig. 9

3.9 Viability of microglial cells following pharmacological treatments

To evaluate the status of the cells following the different pharmacological treatments used during the study we have checked their viability through the trypan blue exclusion assay. In particular, microglia were treated with 100 nM CHA, CGS21680, Cl-IB-MECA, 50 μM SQ22,536, 1 μM SB202190, U0126, SP600125 and SH5 for 24 h or with 100 nM BAY60-6583, 300 nM PSB 603, 10 μM U73122, 10 μM PKC-ε translocation inhibitor peptide, 10 μM rottlerin for 48 h or with siRNActr and A_{2B}ARs siRNA for 72 h, in both normoxia and hypoxia. As shown in Fig. 10 hypoxia by itself and the various pharmacological treatments in normoxia and hypoxia did not significantly affect cell viability of these cells.

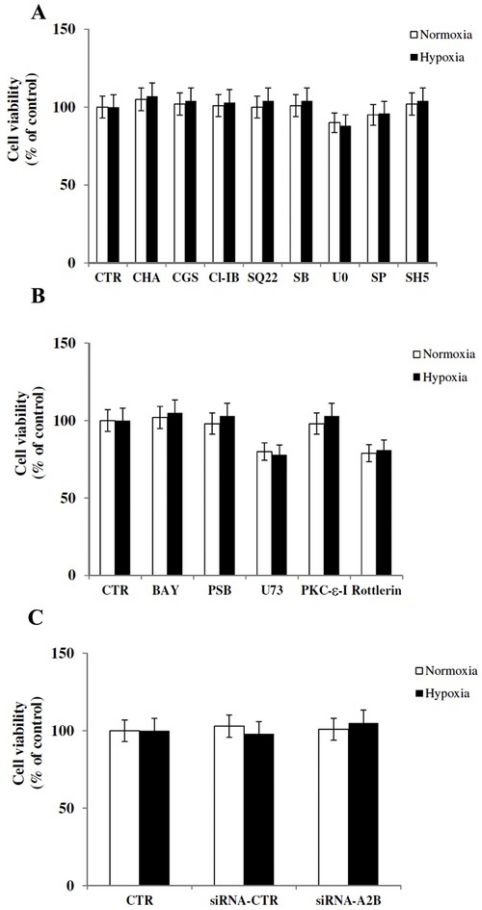

Fig. 10 Microglia viability assessed by Trypan Blue Exclusion assay. Cells were treated with 100 nM CHA, CGS21680 (CGS), Cl-IB-MECA (Cl-IB), 50 μM SQ22,536 (SQ22), 1 μM SB202190 (SB), U0126 (U0), SP600125 (SP) and SH5 for 24 h (A) or with 100 nM BAY60-6583 (BAY), 300 nM PSB 603 (PSB), 10 μM U73122 (U73), 10 μM PKC-ε translocation inhibitor peptide (PKC-ε-I), 10 μM rottlerin for 48 h (B) or with siRNActr and or A_{2B}ARs siRNA for 72 h (C) in both normoxia and hypoxia (A-C). The results are expressed as the percentage of cell viability observed in different treatment conditions compared with untreated cells (considered as 100%). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA.

alt-text: Fig. 10

4 Discussion

Microglia represent a significant fraction of the CNS cells, including 5% to 20% of the total glial population, and are as numerous as neurons [29]. Microglia are crucial in maintaining tissue homeostasis, very sensitive to environmental changes in the brain and become activated in case of tissue damage or invading microorganisms e.g in infection, traumatic injury, ischemia, neurodegenerative disease [30]. This implies a change of morphology and functions, cell proliferation, increase in the production of a variety of cytotoxic mediators contributing to the damage of surrounding healthy neurons [31,32]. For these reasons microglial responses must be tightly regulated since their sustained activation results in chronic neuroinflammation. Therefore, the reduction of inflammation induced by activated microglia may slow the progression of several CNS pathologies [33].

In addition to pro-inflammatory functions through M1 polarization, myeloid cells exert protective effects by M2 phenotype resulting in the neuroprotection after brain injury [34–36]. Extracellular nucleotides/nucleosides and the related purinergic receptors are important actors into the transformation process of microglia in pathological conditions, where the autacoid ado increases its levels in a significant way [7]. Ado affects almost all functions of microglia from process extension and retraction, migration, proliferation and cytokine production, recruiting different ARs subtypes, that may represent important targets

for drug development [37].

This work was aimed to evaluate whether ARs contribute to neuroinflammation occurring following microglia activation, in both normoxic and hypoxic conditions. We have demonstrated that A_{2B}ARs activation increased both mRNA and protein levels of IL-6, as well as cell proliferation through PLC, PKC-ε, PKC-δ and p38 signalling. Inhibition of A_{2B}ARs either pharmacologically by the A_{2B}ARs antagonist PSB603 or by siRNA-induced A_{2B}ARs knockdown was associated with a reduction in p38 phosphorylation, IL-6 secretion and microglial cells proliferation. Microglial cells express all ARs but only the A_{2B}AR subtype was involved in this effect [38,16,7,15]. Surprisingly, IL-6 was not differentially modulated in normoxia and hypoxia even though both HIF-1α and the A_{2B}AR were increased by 1% O_2. Therefore as microglia are less sensitive to hypoxia than any other brain parenchymal cell population it might be speculated that i) more severe hypoxic stimuli should be applied to reveal possible differences between normoxia and hypoxia; ii) the IL-6 increase observed following A_{2B}AR stimulation has already reached its plateau. As IL-6 is pivotal in inflammation by regulating a number of cell functions, including cell proliferation, it is possible to hypothesize that the increased number of microglia observed in our study, dependent on A_{2B} ARs, PLC, PKC-ε, PKC-δ, p38, involves also IL-6 signalling. Our results are in agreement with literature data reporting the modulation of IL-6 through A_{2B}ARs activation, in a variety of cells such as astrocytes, pituitary folliculostellate cells, bronchial smooth muscle cells, lung fibroblasts, astrocytoma cells, mouse striatum, peritoneal macrophages and cardiac fibroblasts, indicating that A_{2B}ARs represent an important target in neuroinflammation [18–26]. Accordingly, the signal transduction pathway linked to A_{2B}AR modulation of IL-6 was attributed to PLC, PKC and p38 in murine pituitary folliculostellate cells, U373 human astroglioma cells and murine cardiac fibroblasts but not in rat astrocytes where it was associated to PKA and cAMP signalling [20,23,26,19]. Indeed the involvement of PKC in the regulation of IL-6 formation has been widely reported and in particular the IL-6 secretion induced by A_{2B}ARs activation has been earlier attributed to the enrollment of PKC-ε and PKC-δ isoforms [23,26]. Therefore, in our study we took in consideration these particular isoforms by using specific inhibitors, thus confirming their role in the pathway triggered by A_{2B}ARs to increase IL-6 production and microglial cell proliferation. Anyway, we cannot exclude the involvement of other PKC isoforms in this effect modulated by A_{2B} ARs in microglia.

It is well known that three independent subgroups of MAPK and Akt are expressed in microglia affecting several cell functions and that ARs are all coupled to them [39–41]. Therefore by using specific and selective inhibitors we investigated the roles of MAPK and Akt in the pathway triggered by A_{2B}AR activation. We found that only p38 was responsible for the stimulatory effect of A_{2B}AR on IL-6 secretion and cell proliferation. Accordingly, an association between p38 and A_{2B}AR activation has been already found in the increase of IL-10, HIF-1α, iNOS, VEGF and GLUT-1 in activated microglial cells [16,15].

Interestingly, this is the first time that an increase in cell proliferation was observed in microglial cells related to A_{2B}ARs stimulation, suggesting a role for this ado subtype in one of the early step in microglial activation, with possible implication in microglia protective functions.

Previous data confirmed a stimulatory effect of AR agonists on microglial cell proliferation, attributing this role to A_1 and A_2ARs [42]. Then a contribution of A_{2A}AR was observed in cell proliferation induced by LPS stimulation in both N9 and primary microglial cells, through a pathway dependent on brain derived neurotrophic factor [9]. In contrast, it has been reported a role for A_1 and possibly A_{2B}ARs in the reduction of proliferation in microglial cells [8]. It would be possible that the high and not more selective doses of AR agonists used to investigate thymidine incorporation in the BV2 microglia cell line may explain the different effect observed in that study.

However, it has to be reported that the *in vitro* approach in the study of microglial functions retains some limitations linked to the microglia limited motility, difficulty to acquire a ramified morphology and the lack of exposure to signals from other cell types e.g. neurons, suggesting that *in vivo* studies are necessary to corroborate our findings. In summary, our results show for the first time that ado by activating A_{2B}ARs increases IL-6 protein levels and cell proliferation through a pathway dependent on PLC, PKC-ε, PKC-δ, and p38 signalling. These findings add a new important piece of knowledge on the role of ARs in microglia activation and neuroinflammation.

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not-for-profit sectors.

Conflict of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.11.024.

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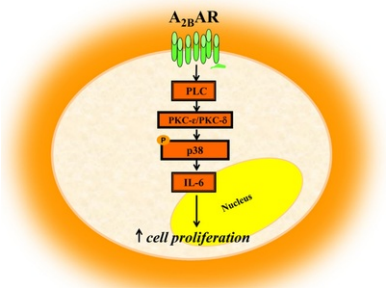
Appendix A. Supplementary data

The following is Supplementary data to this article:

Multimedia Component 1

Graphical abstract



Queries and Answers

Query: Please check the dochead, and correct if necessary.
Answer: Please correct with original article

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Answer: Regular issue is ok.

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Query: This closing parenthesis does not have a corresponding opening parenthesis. Please insert the parenthesis in the appropriate position.
Answer: 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl]phenyl]-1-propylxanthine