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# Astrocytes synthesize primary and cyclopentenone prostaglandins that are negative regulators of their proliferation

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ABSTRACT

Recently, the modulation of cellular inflammatory responses via endogenous regulators became a major focus of medically relevant investigations. Prostaglandins (PGs) are attractive regulatory molecules, but their synthesis and mechanisms of action in brain cells are still unclear. Astrocytes are involved in manifestation of neuropathology and their proliferation is an important part of astrogliosis, a cellular neuroinflammatory response. The aims of our study were to measure synthesis of PGs by astrocytes, and evaluate their influence on proliferation in combination with addition of inflammatory pathway inhibitors. With UPLC-MS/MS analysis we detected primary PGs ($1410 \pm 36$ pg/mg $PGE_2$, $344 \pm 24$ $PGD_2$) and cyclopentenone PGs (cyPGs) ($87 \pm 17$ 15d-$PGJ_2$, $308 \pm 23$ $PGA_2$) in the extracellular medium after 24-h lipopolysaccharide (LPS) stimulation of astrocytes. PGs reduced astrocytic proliferation with the following order of potencies (measured as inhibition at 20 μM): most potent 15d-$PGJ_2$ (90%) and $PGA_2$ (80%), > $PGD_2$ (40%) > 15d-$PGA_2$ (20%) > $PGE_2$ (5%), the least potent. However, $PGF_{2\alpha}$ and 2-cyclopenten-1-one, and ciglitazone and rosiglitazone (synthetic agonists of PPARγ) had no effect. Combinations of cyPGs with SC-560 or NS-398 (specific anti-inflammatory inhibitors of cyclooxygenase-1 and -2, respectively) were not effective; while GW9662 (PPARγ antagonist) or MK-741 (inhibitor of multidrug resistance protein-1, MRP1, and CysLT1 receptors) amplified the inhibitory effect of $PGA_2$ and 15d-$PGJ_2$. Although concentrations of individual PGs and cyPGs are low, all of them, as well as primary PGs suppress proliferation. Thus, the effects are potentially additive, and activated PGs synthesis suppresses proliferation in astrocytes.



## 1. Introduction

Recently, the view on cellular inflammatory responses has been dramatically changed, because the identification of endogenous regulators of this process came into the focus of investigations [1,2]. The most attractive regulator molecules are prostaglandins, as they play both pro- and anti-inflammatory roles [1]. The primary prostaglandins (PGs), $PGD_2$, $PGF_{2\alpha}$ and $PGE_2$, are synthesized from arachidonic acid by consecutive action of cyclooxygenase and specific PG synthases. These prostaglandins readily undergo dehydration *in vivo* and *in vitro*, yielding cyPGs of the $J_2$ and $A_2$ series [3–5]. Primary prostaglandins act via G protein-coupled receptors, whereas cyPGs are actively transported into cells and interact with multiple cellular targets, including signaling molecules and specific nuclear receptor transcription factors [3,4]. While primary prostaglandins, especially $PGE_2$, mainly represent pro-inflammatory substances that induce inflammatory responses, cyPGs have anti-inflammatory effects and play important roles in resolution of inflammation [3–5].

*Abbreviations:* BrdU, bromodeoxyuridine; cyPGs, cyclopentenone prostaglandins; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; LPS, lipopolysaccharide; 15d-$PGJ_2$, 15-Deoxy-delta-12,14-prostaglandin $J_2$; $PGA_2$, prostaglandin $A_2$; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

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Inflammatory activation of astrocytes has been implicated in various central nervous system diseases, including multiple sclerosis, human immunodeficiency virus-associated dementia, Alzheimer's and Parkinson's diseases [6]. Reactive astrogliosis in neuroinflammation leads to the formation of glial scars, deregulates astrocyte-neuron networks, and enhances astrocyte proliferation in the damaged areas [6]. Thus, effective control of astrocytes proliferation is important for neuronal survival in post-ischemia and neurodegenerative lesions, but also in astroglial tumors. Although astrocytes have been identified as source of primary prostaglandins in brain upon activation by pro-inflammatory stimuli, including LPS (lipopolysaccharide; toll-like receptor (TLR) 4 agonist) [7–9] and cyPGs possess cellular and brain levels activity [10,11], there are still no data concerning synthesis of cyPG under inflammation. Moreover, no systematic data report the influence of various PGs on the regulation of astrocyte proliferation.

We aimed to 1) measure the amount of primary and cyPGs released by TLR 4-stimulated astrocytes; 2) estimate the influence of primary and cyPGs on astrocyte proliferation; 3) evaluate the effect of inflammatory pathway inhibitors substances as co-stimulators of PGs actions on proliferation.

## 2. Materials and methods

### 2.1. Reagents

Cell culture medium from GIBCO/BRL (Eggenstein, Germany); fetal calf serum (FCS), penicillin, and streptomycin from Biochrom (Berlin, Germany); Cell Proliferation ELISA BrdU kit from Roche (Mannheim, Germany; $PGD_2$, 15d-$PGJ_2$, $PGA_2$, $PGE_2$, $PGF_{2\alpha}$, ciglitazone and rosiglitazone from Cayman Chemical (Steinheim, Germany). Substances were dissolved in dimethylsulfoxide. Chemicals were further diluted in incubation medium for working solutions.

### 2.2. Cell cultures of C6 cells and of primary astrocytes

C6, a rat glioma cell line, was maintained on tissue culture plastic in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 20 U/ml penicillin and 20 μg/ml streptomycin, at 37 °C in 10% $CO_2$, humidified. For experiments, C6 cells were seeded into 96-well microplates at a density of $1 \times 10^4$ cells/ml in proliferation medium containing 10% FCS.

Primary astrocytes obtained from 1 or 2 day old Wistar ratpups were obtained according to a protocol approved previously [12]. From animals aseptically decapitated, the brains were isolated, washed in ice-cold Puck's buffer (see Ref. [12]) and minced against meshes (250, 136 μm), then tissue fragments were placed into culture flasks, supplied with DMEM (1 g/l D-glucose, 10% FCS, 50 units/ml streptomycin, 50 μg/ml penicillin) and incubated (37 °C, 5% $CO_2$). Five days later, cultures were shaken to detach microglial cells and given fresh media of same composition. Cells were cultured for additional 6 days with media changed every 2 days. After monolayer formation, cells were treated with trypsin, then the cells were plated into 6-well culture plates at 750.000 cells/well. Cells were used for experiments after two days.

### 2.3. Assay of cell proliferation and cell-viability and cell synchronization

Cells were washed with Hank's solution (see Ref. [12]), detached from plastic with trypsin/EGTA (0.05%/0.02% w/v). Then DMEM containing 10% (v/v) FCS was added to stop trypsin. After that, cells were centrifuged for 5 min at 500x*g*, resuspended in DMEM/FCS, and plated in 96-well plates at a starting density of $1.5 \times 10^5$ cells/ml, 200 μl per well. After 24-h incubation, the cells were subjected to 48-h of serum deprivation with FCS-free DMEM. At time point zero, we returned medium containing 10% FCS, resulting in synchronous re-entry of $G_0$ cells into $G_1$ phase of cell cycle, as described elsewhere [13]. DNA synthesis in proliferating cells was evaluated by measuring bromodeoxyuridine (BrdU) incorporation for 24-h or 3-h using a commercial chemiluminescent Cell Proliferation ELISA kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. The light emission of the samples was measured at 450 nm using a microplate luminometer (Tecan-Spectrafluor Plus). In preliminary studies, all tested substances were estimated for toxicity by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. All tested substances were not toxic (not shown).

### 2.4. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

Cell-free culture media were taken for solid-phase lipid extraction. Oasis® HLB cartridge (60 mg, 3 cc) were obtained from Waters, Eschborn, Germany. For solid-phase-extraction, 1 ml HLB cartridges were washed with 1 ml of methanol and 1 ml of 0.1% formic acid. Half ml of the prepared sample was loaded onto the column and washed with 1 ml 0.1% formic acid and 1 ml 15% ethanol. The cartridges were then eluted with 300 μl of methanol. Lipid mediators were analyzed by 8040 series UPLC-MS/MS (Shimadzu, Japan). UPLC, consisting of a binary pump, an autosampler, and a thermostatted column compartment, was performed with Phenomenex C8 (2.1 mm × 150 mm × 2.6 μm). The flow rate was 0.4 ml/min. The sample cooler and the column temperature were set at 5 °C and 40 °C, respectively. Injection volume was 20 μl. Gradient elution was performed with (A) 0.1% (v/v) formic acid and (B) acetonitrile. The gradient of mobile phase B used was: 10% (0 min)-25% (5 min)-35% (10 min)-75% (20 min)-95% (20.1 min)-95% (25 min)-10% (25.1 min)-10% (30 min). Mass spectrometric detection was achieved with an electrospray ionization source operating in negative mode using nitrogen as nebulizer gas. Quantification and qualification were accomplished in multiple-reaction monitoring mode, and MS was operated at unit mass resolution for both precursor and product ions. Lipid mediator version 2 software was used to operate the mass spectrometer (Shimadzu, Japan). The parameters of mass spectrometry were set: nebulizer gas flow, 3 l/min; drying gas, 10 l/min; heat block temperature, 400 °C; desolvation line temperature, 250 °C; collision induced dissociation pressure, 230 kPa.

Lipid mediators are separated based on chemical properties in UPLC, then we monitored their ion fragments by collision-induced dissociation in conjunction with electrospray ionization-MS/MS. 15d-$PGJ_2$ and $PGA_2$ were identified according to accurate *m*/*z*, retention time, relative retention time of species in the same class, and spectra of MS/MS. For quantitative analysis of $PGE_2$, $PGD_2$, 15d-$PGJ_2$ and $PGA_2$, all samples were examined by LC-MS/MS at both positive and negative ion modes to measure peak areas of detected species. In order to compensate for fluctuations in MS intensities during different runs, peak areas of each individual lipid species were corrected by deuterated internal standards: $PGE_2$-$d_4$, $PGA_2$-d4, 15d-$PGJ_2$-d4 (Cayman Chemical, Ann Arbor, MI). The concentration of prostaglandins was normalized to total protein and expressed as pg/mg. Total protein was determined by Bradford assay.

### 2.5. Data analysis and statistics

Data are expressed as mean ± SEM, All experiments were reproduced three times. Data are presented as mean ± standard error obtained from three independent experiments. Data were

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