Available online at www.sciencedirect.com
ScienceDirect
Neurochemistry International 52 (2008) 216–220
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Rapid communication

Lysophosphatidic acid stimulates astrocyte proliferation through LPA_1

Shinya Shano [a], Ryutaro Moriyama [a], Jerold Chun [b], Nobuyuki Fukushima [a,c,*]

[a] *Division of Molecular Neurobiology, Department of Life Sciences, School of Science and Engineering, Kinki University, Kowakae 3-4-1, Higashiosaka 577-8502, Japan*

[b] *Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA*

[c] *Research Institute of Science and Technology, Kinki University, Kowakae 3-4-1, Higashiosaka 577-8502, Japan*

Received 19 June 2007; received in revised form 3 July 2007; accepted 3 July 2007
Available online 10 July 2007

Abstract

Lysophosphatidic acid (LPA) is an extracellular lipid mediator that regulates nervous system development and functions through multiple types of LPA receptors. Here we explore the role of LPA receptor subtypes in cortical astrocyte functions. Astrocytes cultured under serum-free conditions were found to express the genes of five LPA receptor subtypes, *lpa1* to *lpa5*. When astrocytes were treated with dibutyryl cyclic adenosine monophosphate, a reagent inducing astrocyte differentiation or activation, *lpa1* expression levels remained unchanged, but those of other LPA receptor subtypes were relatively reduced. LPA stimulated DNA synthesis in both undifferentiated and differentiated astrocytes, but failed to do so in astrocytes prepared from mice lacking *lpa1* gene. LPA also inhibited [^{3}H]-glutamate uptake in both undifferentiated and differentiated astrocytes; and LPA-induced inhibition of glutamate uptake was still observed in *lpa1*-deficient astrocytes. Taken together, these observations demonstrate that LPA_1 mediates LPA-induced stimulation of cell proliferation but not inhibition of glutamate uptake in astrocytes.

Keywords: Lysophosphatidic acid; LPA_1: astrocyte; DNA synthesis; Glutamate uptake

1. Introduction

Lysophosphatidic acid (LPA) is an extracellular lipid mediator produced by platelets and present in serum (Moolenaar, 1999). LPA-induced cellular responses include enhanced cell proliferation, cytoskeletal rearrangement, and reduced cell death, depending on cell type. These effects are mediated through G protein-coupled LPA receptors. To date, five types of LPA receptor genes (*lpa1*–*lpa5*) have been identified, and *lpa1*, *lpa2*, and *lpa3* are known to be structurally related with over 50% amino acid identity (Fukushima et al., 2001; Ishii et al., 2004). Recently identified LPA receptor subtypes *lpa4* (GPR23) and *lpa5* (GPR92) share ~35% amino acid identity and are phylogenically distant from *lpa1*, *lpa2* and *lpa3* (Noguchi et al., 2003; Lee et al., 2006). All five LPA receptor genes are expressed in developing and adult brains, suggesting involvement in cellular functions of neural cells. Indeed, *lpa1* has been shown to be involved in neuronal differentiation in developing cerebral cortex (Kingsbury et al., 2003; Fukushima et al., 2007).

Recently, LPA was shown to induce diverse cellular responses in cultured astrocytes, which express *lpa1*, *lpa2* and *lpa3* genes (Rao et al., 2003; Sorensen et al., 2003). These responses include cell proliferation, inhibition of glutamate uptake and increased production of radicals, all which relate to neurodegeneration (Keller et al., 1996, 1997; Rao et al., 2003; Sorensen et al., 2003), although there are some conflicting reports (Fuentes et al., 1999; Pebay et al., 1999). However, it is unclear which LPA receptor subtype is involved in these LPA-induced astrocyte responses. Moreover, whether recently identified *lpa4* and *lpa5* are expressed in astrocytes remains unknown. Here we examine the expression profiles of LPA receptor subtypes in cultured astrocytes and explore the role of LPA receptors in astrocyte proliferation and glutamate uptake in culture.

2. Experimental procedures

2.1. Astrocyte cultures

Astrocytes were prepared from cerebral cortices of postnatal day 1 ICR mice (SLC, Japan), or *lpa1* heterozygous (*lpa1* (+/−)) or *lpa1* homozygous (*lpa1* (−/−)) mice (Contos et al., 2000). Pups from *lpa1* (+/−) females crossed with *lpa1* (+/−) males were genotyped by PCR using genomic DNA prepared from

* Corresponding author. Tel.: +81 6 6730 5880x4140; fax: +81 6 6723 2721.
E-mail address: nfukushima@life.kindai.ac.jp (N. Fukushima).

0197-0186/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2007.07.004

tail tissue. These *lpa*$_1$ (+/−) mice were of C57BL/6N background (backcrossed 5 generations). Cerebral cortices were dissected and dissociated with 0.25% trypsin/0.1 mM EDTA. Cortical cells were plated in T75 flasks or T25 flasks (cells from 1 mouse/25 cm^2) and cultured to confluence in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemicals, Osaka, Japan) containing 10% fetal calf serum (FCS). Cells were then harvested with trypsin/EDTA, replated in 24-well plates, and cultured in 10% FCS-containing DMEM for 1 day. Cells were further cultured in serum-free Opti-MEM (Invitrogen, Tokyo, Japan) and subjected to assay. Between 80 and 90% of the cells were glial fibrillary acidic protein (GFAP)-positive by immunocytochemical staining using anti-GFAP antibody (Dr. Watanabe, Hokkaido University). The ratios of microglia, oligodendrocytes, and neurons were less than 5, 1, and 1%, respectively.

2.2. RT-PCR

Total RNA was prepared from cultured astrocytes using Tri Reagent (Sigma, St. Louis, MO) and treated with RNase-free DNase; cDNAs were synthesized using an oligo(dT) primer (all reagents from Invitrogen). The resultant cDNAs (0.025 μg) were amplified by PCR using GoTaq green master mix (Promega, Tokyo, Japan) for LPA receptor family members. The primers (Nippon EGT, Toyama, Japan) used were: for *lpa*$_1$, forward lpa1-s3 (5′-AGTTCTGGACCCAGGAGGAATCGG-3′) and reverse lpa1-as3 (5′-ACTTCTCATAGGCCAGGACATCGCA-3′), producing a 157-bp product; for *lpa*$_2$, lpa2-s2 (5′-CACTCAGCCTAGTCAAGACGGTT-3′) and lpa2-as2 (5′-GCATCTCGGCAGGAATATACCACT-3′), producing a 193-bp product; for *lpa*$_3$, lpa3-s1 (5′-GGTGGCGGTATACGTACGCATCT-3′) and lpa3-as1 (5′-ACGTGTTGCACGTTACACTGCTTG-3′), producing a 216-bp product; for *lpa*$_4$, lpa4-s1 (5′-TGGTGACACCCTCTGTAAGATCTC-3′) and lpa4-as1 (5′-GGAGAAGCCTTCAAAGCAAGTGG-3′), producing a 262-bp product; for *lpa*$_5$, lpa5-s1 (5′-GGTGAGCGTGTACATGTGCA-3′) and lpa5-as1 (5′-GCTGCCGTACATGTTCATCTGG-3′), producing a 157-bp product. The cycling protocol was performed as follows: 60 s at 95 °C; 33 cycles of 20 s at 95 °C, 20 s at 56 °C, and 40 s at 72 °C; and a final extension period of 7 min at 72 °C. These conditions were sufficient for semi-quantitative detection of all LPA receptor subtype genes. The absence of genomic DNA contamination was confirmed by using other primers for *lpa*$_1$ that span the intron.

2.3. [^{3}H]-Thymidine incorporation assay

Astrocytes were treated with [^{3}H]-thymidine (0.5 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO) in Opti-MEM for 4 h, washed with ice-cold PBS, and solubilized with 200 μl of 0.2 M NaOH. Lysates were transferred to a 1.5-ml tube and mixed with 40 μl of 1 M HCl and 60 μl of 100% trichloroacetic acid (TCA). DNA was precipitated by centrifugation at 15,000 × *g* and washed twice with 5% TCA, solubilized with 0.1 M NaOH, and measured for radioactivity using a scintillation counter.

2.4. [^{3}H]-Glutamate uptake assay

Astrocytes were treated with 100 μM [^{3}H]-glutamate (0.2 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO) in Opti-MEM for 30 min, washed twice with ice-cold Opti-MEM, and lysed with 100 μl of lysis buffer. Radioactivity of the lysates was measured using a scintillation counter.

2.5. Materials

Oleoyl-LPA was purchased from Avanti Polar Lipid (Alabaster, AL), dissolved in sterile H_2O at 10 mM and stored at −30 °C until use. Fatty acid-free bovine serum albumin (FAFBSA) was purchased from Sigma, and stored at 10% (w/v) in sterile phosphate-buffered saline at −30 °C. Dibutyryl cyclic adenosine monophosphate (DBcAMP) was purchased from Nacalai Tesque Chemicals (Kyoto, Japan), dissolved in sterile H_2O at 100 mM, and stored at −30 °C until use. Pertussis toxin, PD98059, wortmannin, and Y27632 were from Calbiochem (La Jolla, CA).

2.6. Statistics

Analysis of variance (ANOVA) followed by a post hoc test was applied to data to determine statistical significance by using the statistical software, StatView 4.5 (Abacus Concepts, Berkeley, CA).

3. Results and discussion

Astrocytes have long been known to respond to cAMP-elevating reagents with stellate morphology and increased expression of GFAP, indicating astrocyte differentiation. The stellate morphology also resembles that of reactive astrocytes *in vivo* (Sensenbrenner et al., 1980). We, therefore, examined the gene expression of LPA receptor family members in both undifferentiated and differentiated (DBcAMP-treated) astrocytes. Astrocytes were cultured under serum-free conditions, because serum contains LPA at μM levels that may affect LPA receptor gene expression. RT-PCR analysis demonstrated that genes of each LPA receptor subtype (*lpa*$_1$–*lpa*$_5$) were expressed in undifferentiated astrocytes, although the *lpa*$_5$ signal was barely visible under these conditions (Fig. 1). Treatment of astrocytes with DBcAMP induced a stellate morphology in most cells and raised GFAP expression (data not shown), indicating these astrocytes were differentiated, as previously reported (Sensenbrenner et al., 1980). In these differentiated astrocytes, the *lpa*$_1$ expression level remained unchanged (Fig. 1). In contrast, the expression of *lpa*$_2$ and *lpa*$_4$ were reduced, and those of *lpa*$_3$ and *lpa*$_5$ became undetectable (Fig. 1). Thus, astrocyte differentiation may result in alterations in LPA receptor gene expression. Alternatively, it is also possible that DBcAMP-mediated intracellular signaling influences the promoter activities of these genes with the exception of *lpa*$_1$.

We next examined the effects of LPA on DNA synthesis in astrocytes. Undifferentiated astrocytes responded to LPA in a concentration-dependent manner with an increase in [^{3}H]-thymidine incorporation into DNA (Fig. 2), in accordance with previous reports (Tabuchi et al., 2000; Rao et al., 2003; Sorensen et al., 2003). In differentiated astrocytes, LPA also exerted similar concentration-dependent effects on DNA synthesis (Fig. 2). Considering the constant level of *lpa*$_1$ expression observed in undifferentiated and differentiated astrocytes, this result suggests that LPA_1 primarily mediates the LPA-induced stimulation of DNA synthesis.

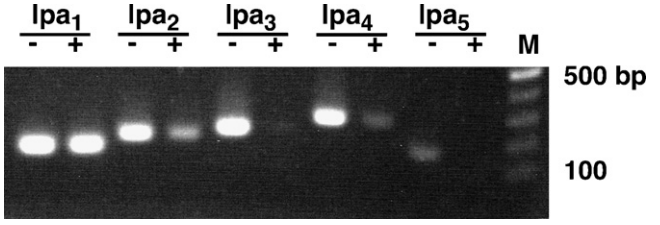

Fig. 1. LPA receptor genes are expressed in cultured astrocytes and altered by DBcAMP treatment. Astrocytes were cultured in the absence (−) or presence (+) of 1 mM DBcAMP for 4 days. Total RNA was extracted and subjected to RT-PCR for LPA receptor subtypes. M, 100 bp DNA ladder.