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Rapid communication

Lysophosphatidic acid stimulates astrocyte proliferation through LPA_1

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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

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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.

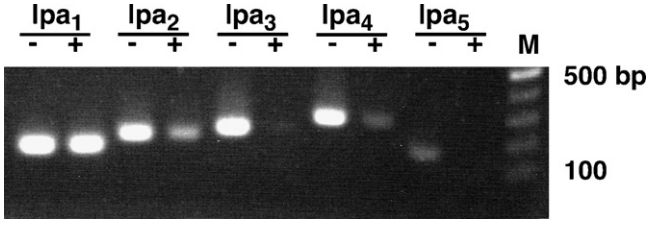

Fig. 1. LPA receptor genes are expressed in cultured astrocytes and altered by DBcAMP treatment. Astrocytes were cultured in the absence (−) or presence (+) of 1 mM DBcAMP for 4 days. Total RNA was extracted and subjected to RT-PCR for LPA receptor subtypes. M, 100 bp DNA ladder.

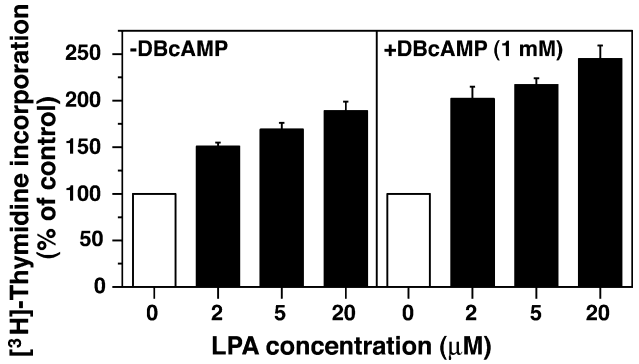

Fig. 2. LPA stimulates DNA synthesis in cultured astrocytes. Astrocytes were cultured in the absence or presence of 1 mM DBcAMP for 3 days. Cells were further cultured with varying concentrations of LPA for 18 h and subjected to [^{3}H]-thymidine incorporation assay. Fatty acid-free bovine serum albumin (FAFBSA, 0.05%) was used as a vehicle. The averages of incorporated radioactivity were 1027 ± 24 and 393 ± 40 cpm/well in undifferentiated and differentiated astrocytes, respectively. Data are means ± S.E.M. from triplicate determinations of the representative experiment.

To investigate this issue directly, we prepared astrocyte cultures from *lpa$_1$* (−/−) mice and examined the effects of LPA on DNA synthesis. As expected, these astrocytes lacked *lpa$_1$* expression and showed gene expression of *lpa$_2$*, *lpa$_3$* and *lpa$_4$* at levels comparable to those in astrocytes derived from *lpa$_1$* (+/−) (Fig. 3A). *lpa$_5$* expression appeared to be relatively increased in *lpa$_1$* (−/−) astrocytes, although the reason for this remains unknown. Enhancement of DNA synthesis by LPA was completely lost in *lpa$_1$* (−/−) astrocytes (Fig. 3B). Thus, these results strongly suggest that LPA-induced stimulation of DNA synthesis in astrocytes is mediated through LPA_1. Because LPA-induced increase in astrocyte proliferation was shown to be pertussis toxin-sensitive (Keller et al., 1997; Tabuchi et al., 2000), the LPA_1-$G_{i/o}$ pathway is a likely mechanism for this response. In addition, because *lpa$_1$* (−/−) astrocytes grew well in serum-containing medium and in a manner indistinguishable from that of wild-type astrocytes (data now shown), it appears that *lpa$_1$* is unnecessary for intrinsic astrocyte growth in culture.

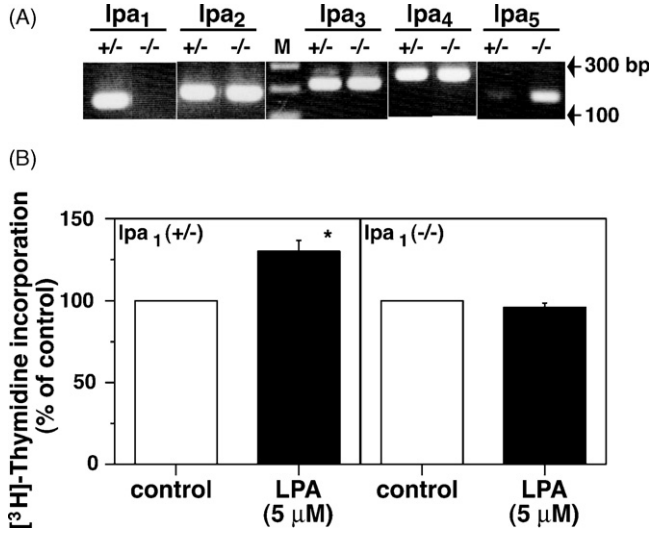

Fig. 3. LPA-stimulated DNA synthesis in astrocytes is mediated through LPA_1. (A) RT-PCR analyses for LPA receptor gene expression in astrocytes derived from *lpa$_1$* heterozygous (+/−) or homozygous (−/−) mice. Astrocytes prepared from *lpa$_1$* (+/−) or *lpa$_1$* (−/−) mice were cultured in serum-free medium for 3 days. Total RNA was extracted and subjected to RT-PCR. M, 100 bp DNA ladder. (B) Effects of LPA on DNA synthesis in astrocytes derived from *lpa$_1$* (+/−) or *lpa$_1$* (−/−) mice. Astrocytes prepared from *lpa$_1$* (+/−) and *lpa$_1$* (−/−) were cultured in serum-free medium for 2 days. Cells were further cultured with or without 5 μM LPA for 18 h and subjected to [^{3}H]-thymidine incorporation assay. FAFBSA (0.05%) was used as a vehicle. The averages of incorporated radioactivity were 988 ± 104 and 808 ± 67 cpm/well in *lpa$_1$* (+/−) and *lpa$_1$* (−/−) astrocytes, respectively. Data are means ± S.E.M. from three to four independent experiments. *$p < 0.05$.

An important role of astrocytes is the regulation of extracellular glutamate concentrations which can be neurotoxic at high concentrations (Gegelashvili and Schousboe, 1997; Schousboe and Waagepetersen, 2005). This regulation is primarily accomplished by uptake of glutamate through glutamate transporters expressed in astrocytes. Undifferentiated astrocytes express GLAST, a subtype of glutamate transporter, while differentiated astrocytes express both GLAST and GLT-1, another subtype of the transporter (Swanson et al., 1997; Schlag et al., 1998). LPA was previously reported to inhibit glutamate uptake by astrocytes (Keller et al., 1996, 1997). Thus, we examined the effects of LPA on

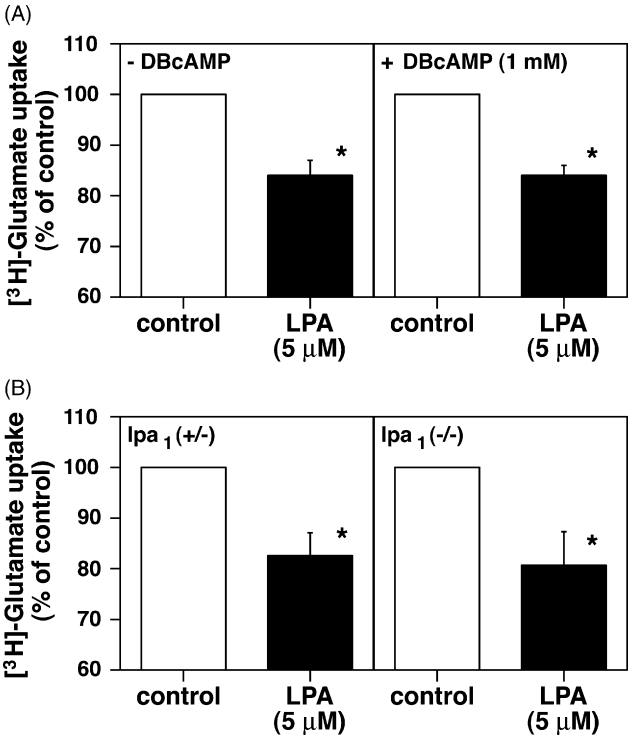

Fig. 4. LPA inhibits glutamate uptake independently of LPA_1. (A) Effects of LPA on [^{3}H]-glutamate uptake in cultured astrocytes. Astrocytes were cultured in the absence or presence of 1 mM DBcAMP under serum-free conditions for 3 days, and subjected to [^{3}H]-glutamate incorporation assay. The levels of incorporated glutamate in undifferentiated and differentiated cells were 22,321 ± 485 and 22,926 ± 1182 cpm/well, respectively. (B) Effect of LPA on [^{3}H]-glutamate uptake in cultured astrocytes prepared from *lpa$_1$* (+/−) or *lpa$_1$* (−/−) mice. Astrocytes cultures were prepared from *lpa$_1$* (+/−) or *lpa$_1$* (−/−) mice, and subjected to [^{3}H]-glutamate incorporation assay. The levels of incorporated glutamate in undifferentiated and differentiated cells were 29,558 ± 1327 and 22,502 ± 1286 cpm/well, respectively. Data are means ± S.E.M. from three to four independent experiments. *$p < 0.05$.

glutamate uptake in both undifferentiated and differentiated astrocytes. Pretreatment of cells with LPA resulted in an approximately 16% reduction in glutamate uptake (Fig. 4A). Inhibition of glutamate uptake by LPA treatment was also observed to a degree similar to that in undifferentiated astrocytes (Fig. 4A).

To examine the involvement of LPA_1 in glutamate uptake, *lpa1* (−/−) astrocytes were used for the same assay. LPA exposure resulted in 19% inhibition of glutamate uptake in *lpa1* (−/−) astrocytes, which was equivalent to that (17% inhibition) observed in *lpa1* (+/−) astrocytes (Fig. 4B). Taken together, these results indicated that LPA-induced inhibition of glutamate uptake is not mediated through LPA_1. *lpa3* is undetectable in differentiated astrocytes; therefore, *lpa2*, *lpa4*, *lpa5* or other unidentified LPA receptor might be involved in glutamate uptake inhibition by LPA.

We further explored the intracellular signaling pathways through which LPA inhibits glutamate uptake. LPA_2, LPA_4 and LPA_5 activate one or more of $G_{i/o}$, G_q and $G_{12/13}$-mediated pathways (Ishii et al., 2000; Noguchi et al., 2003; Lee et al., 2006), leading to stimulation of divergent signaling molecules, such as MAP kinase, phosphoinositide 3-kinase (PI3K), or Rho kinase. We tested the effects of pertussis toxin (a $G_{i/o}$ inhibitor), PD98059 (a MAP kinase inhibitor), wortmannin, (a PI3K inhibitor), and Y27632 (a Rho kinase inhibitor). Although these reagents blocked their corresponding kinase-dependent responses in other cell types employed in our laboratory, each of them failed to inhibit the effects of LPA on glutamate uptake in both undifferentiated and differentiated astrocytes (data not shown). Thus, the signaling pathways are yet to be determined, and identification of the LPA receptor and signaling pathways as well as involvement of non-receptor mechanisms are currently underway.

To date, *lpa1* expression has been observed in oligodendrocytes but not astrocytes in adult brains (Weiner et al., 1998; Stankoff et al., 2002). However, astrocytes respond to LPA with astrogliosis, reflecting astrocyte proliferation leading to glial scar formation, when LPA is directly injected into mouse brains (Sorensen et al., 2003). This finding suggests that astrocyte expression of LPA receptor genes in normal brains is undetected using typical assay conditions for *in situ* hybridization. Alternatively, LPA receptor gene expression may be induced upon physical or LPA stimulation in astrocytes *in vivo*. Although neural cells are one source of LPA production in the brain (Fukushima et al., 2000), platelets are known to produce high levels of LPA in serum (Eichholtz et al., 1993; Tigyi et al., 1995). If platelet-derived LPA enters the brain due to impairment of the blood–brain barrier following brain injury or ischemia (Eichholtz et al., 1993; Tigyi et al., 1995), LPA-induced astrocyte responses, such as glutamate uptake inhibition and cell proliferation, may induce neuronal cell death or accelerate neurodegeneration triggered by other stimuli, such as glutamate. Further investigation of the roles of LPA receptor in astrocyte responses would benefit our understanding of neuropathology and help develop medicinal treatments for neurodegenerative diseases.

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