

Lysophosphatidic acid activates β -catenin/T cell factor signaling, which contributes to the suppression of apoptosis in H19-7 cells

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Abstract. Lysophosphatidic acid (LPA) is a lipid growth factor that regulates diverse cell functions, including cell proliferation, survival and apoptosis. LPA has been demonstrated to be involved in the regulation of cortical neurogenesis by increasing the survival of neural precursors. Previously, we reported that LPA stimulated the inactivation of glycogen synthase kinase 3 (GSK3) via the G protein-coupled LPA₁ and LPA₂ receptors, by which apoptosis is suppressed in H19-7 cells [an embryonic hippocampal progenitor cell (HPC) line]. Increasing numbers of studies have demonstrated that certain G protein-coupled receptors activate β -catenin/T cell factor (TCF) signaling independently of Wnt, which is involved in cell fate determination, cell proliferation and cell survival. To determine whether LPA activates β -catenin-mediated transcriptional activation pathways and whether β -catenin/TCF signaling is involved in neurogenesis by controlling the survival of neural precursors, β -catenin/TCF signaling cascades induced by LPA were investigated in the HPCs. Activation of β -catenin/TCF signaling was determined by the nuclear translocation of β -catenin and the transcriptional activation of a TCF reporter gene. The activation of β -catenin/TCF signaling was blocked by pertussis toxin (PTX) and a protein kinase C (PKC) inhibitor. The expression of a constitutively active mutated form of GSK3 β activated β -catenin/TCF signaling to comparable levels to those induced by LPA, and protected against apoptosis in differentiating H19-7 cells. These results showed that LPA activates β -catenin/TCF signaling in a PTX- and

PKC-dependent manner, which contributes to LPA-induced cell survival in the HPCs. Activation of β -catenin/TCF signaling by LPA may be involved in neurogenesis by controlling the survival of neural precursors.

Introduction

Lysophosphatidic acid (LPA) affects various cell functions, including cell migration, proliferation, differentiation and survival, through a family of cognate G-protein coupled receptors (GPCRs). The binding of LPA to its cognate receptors elicits a range of LPA downstream signaling through at least three different types of G proteins, including Gi/o, Gq/11 and G12/13 (1). LPA is also involved in diverse neurodevelopmental processes, such as neurogenesis, neuritogenesis and neuronal migration. We have previously demonstrated that LPA protected differentiating hippocampal progenitor cells (HPCs) from apoptosis by inhibiting the phosphorylation of glycogen synthase kinase 3 (GSK3) (2), suggesting that LPA may be involved in hippocampal neurogenesis. GSK3 plays a central role in the canonical Wnt/ β -catenin signaling pathway, which is shown to be important in neurogenesis by supporting the proliferation of neural stem and precursor cells (3). Previous studies have demonstrated novel aspects of Wnt/ β -catenin signaling in neurogenesis. Wnt/ β -catenin signaling is involved in neurogenesis by regulating the differentiation of neural precursors (4-7). Moreover, studies have revealed that β -catenin signaling promotes the survival of neuronal cells, including precursor cells (8-12). This has led to the hypothesis that β -catenin signaling may regulate neurogenesis by modulating the survival of neuronal progenitor cells (NPCs). LPA is also involved in the regulation of cortical neurogenesis by controlling the survival of NPCs in *ex vivo* cortical culture systems, although the molecular mechanism for this is not fully understood (13). Increasing data demonstrate that the activation of G proteins alone, or the activation of GPCRs by agonists such as lipid metabolites and growth factors, may induce the stabilization of β -catenin and the subsequent β -catenin/T cell factor (TCF) transcriptional activation that is independent of Wnt (14,15).

In the present study, it was investigated whether LPA induced β -catenin/TCF transcriptional activation and whether

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β -catenin/TCF signaling by LPA was involved in the control of NPC survival. It was demonstrated that LPA activated the β -catenin/TCF signaling predominantly in Gi/o- and protein kinase C (PKC)-dependent pathways, and that the β -catenin/TCF signaling induced by LPA was involved in the suppression of apoptosis in differentiating H19-7 cells. Therefore, the activation of the β -catenin/TCF signaling pathway by LPA may function in neurogenesis by controlling the survival of neural precursors.

Materials and methods

Materials. H19-7 cells were generously provided by Dr. Kwang C. Chung (Yonsei University, Seoul, Republic of Korea). LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), 5-bromodeoxyuridine (BrdU), fatty acid-free bovine serum albumin and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO, USA). Wortmannin and GF109203 were purchased from Tocris Bioscience (Bristol, UK). TOP Flash and FOP Flash luciferase reporters were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Alexa Fluor 568-conjugated Annexin V was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and G418 were obtained from Invitrogen Life Technologies. All other reagents were of analytical grade or the highest purity available.

Cell culture. H19-7 cells, conditionally immortalized, embryonic (day 17) rat hippocampal cells with a temperature-sensitive SV40 large T antigen (16), were maintained at 33°C (the permissive temperature at which SV40 large T is functional) in DMEM supplemented with 10% FBS, 0.2 mg/ml G418, 50 U/ml penicillin and 50 mg/ml streptomycin. For differentiation, H19-7 cells were shifted to 39°C to eliminate the expression of the functional large T antigen, then incubated for 24 h in DMEM supplemented with N2 (N2 medium), and treated with LPA. The differentiation of H19-7 cells into neuronal cells was confirmed by the expression of neuronal markers, such as neurofilament-M and neuron specific enolase, and cell morphology as previously described (17). To determine effect of pharmacological inhibitors on the TCF transcriptional activation and inhibitory phosphorylation of GSK3 β induced by LPA, the H19-7 cells were pretreated with the inhibitor for 1 h before LPA treatment.

Measurement of apoptosis by Annexin V staining. Apoptosis was measured by staining with Annexin V conjugated to Alexa Fluor 568 (Life Technologies, Grand Island, NY, USA) as previously described (2). H19-7 cells were collected, washed with phosphate-buffered saline (PBS) and resuspended in 100 μ l binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM CaCl₂]. The cells were then incubated with 5 μ l Annexin V-Alexa Fluor 568 for 15 min at room temperature in the dark, followed by the addition of 400 μ l binding buffer. The cells were analyzed by flow cytometry using a Guava EasyCyte (GE Healthcare, Piscataway, NJ, USA).

Western blot analysis. H19-7 cells were collected by brief centrifugation at 500 x g and resuspended in lysis buffer

[10 mM HEPES (pH 7.5), 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na₃VO₄, 5 mM NaF and protease inhibitor cocktail]. Following incubation on ice for 10 min, cell lysates were centrifuged at 4,000 x g for 1 min. Supernatants containing cytoplasm were collected and the remaining nuclear pellet was resuspended in lysis buffer containing 0.1% sodium dodecyl sulphate (SDS). The protein concentrations within each lysate were determined by the Bradford assay (Bio-Rad, Richmond, CA, USA). The fractionated extracts were mixed with SDS sample buffer and equal quantities of the samples were loaded and separated by SDS-polyacrylamide gel electrophoresis (8 or 10% reducing gels), transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and blocked with 5% non-fat milk. The membranes were incubated in primary antibody overnight at 4°C. Subsequent to this, the membranes were washed in TBST [10 mM Tris, 140 mM NaCl and 0.1% Tween-20 (pH 7.6)], incubated with the appropriate secondary antibody and washed again in TBST. Bands were visualized by enhanced chemiluminescence and exposed to X-ray film.

Immunocytochemistry. Differentiating H19-7 cells cultivated on chamber slides (Nalgene, Rochester, NY, USA) were treated with LPA. The cells were fixed and permeabilized as described in a previous study (2), processed for immunocytochemistry with mouse anti- β -tubulin III (Sigma) or rabbit anti- β -catenin (Abcam, Cambridge, MA, USA), and subsequently processed with Alexa Fluor 568- or Alexa Fluor 488-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). The stained cells were observed using a fluorescence microscope (Axio Scope A1; Zeiss, Oberkochen, Germany) with a digital camera attachment.

Luciferase reporter gene assay. H19-7 cells were transiently transfected with a luciferase reporter and Renilla luciferase (pRL-TK; Promega Corporation, Madison, WI, USA) with the indicated plasmids, using FuGene HD (Roche Molecular Biochemicals, Mannheim Germany). The luciferase reporter constructs TOP Flash and FOP Flash (Upstate Biotechnology) were used to determine the TCF/lymphoid enhancing factor (LEF) transcription activity. Following transfection for 6 h, the cells were cultivated in N2 medium for 18 h and to induce differentiation as described previously. The cells were treated with LPA or vehicle for 4 h and collected. The cell extracts were prepared by rinsing each plate twice with PBS and lysing the cells in 150 μ l Reporter Assay Lysis buffer (Promega Corporation). The cell lysates were collected, normalized for protein content and assayed using a dual luciferase assay system (Promega Corporation). They were analyzed for firefly luciferase (TOP Flash or FOP Flash) activity, and the reactions were quenched and immediately reanalyzed for Renilla luciferase activity using an Autolumat Luminometer (Berthold Technologies, Oak Ridge, TN, USA).

Statistical analysis. Results are presented as the mean \pm SEM. Statistical significance was analyzed by one-way analysis of variance followed by a Dunnett's multiple test or a paired t-test using Prism (GradPad Software, La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

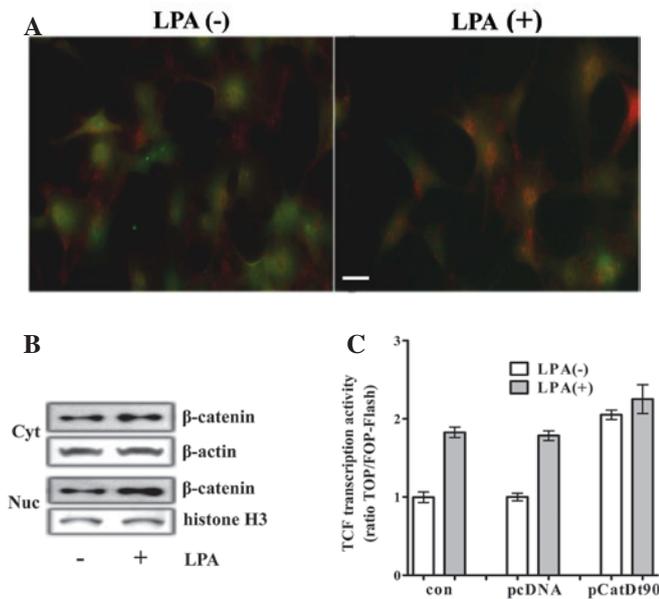


Figure 1. Lysophosphatidic acid (LPA) activated β -catenin/T cell factor (TCF) signaling in differentiating H19-7 cells. H19-7 cells were induced to differentiate and were treated with $5 \mu\text{M}$ LPA or vehicle. (A) The cells were fixed, immunostained with anti- β -catenin or anti-tubulin III and detected with secondary antibody conjugated with Alexa Fluor 568 and Alexa Fluor 488, respectively. Scale bar, $10 \mu\text{m}$. (B) The cells were lysed and fractionated and the levels of β -catenin in the cytoplasm and nucleus were determined by western blot analysis. Representatives of two independent experiments are shown. (C) H19-7 cells were transfected with TOP Flash or POP Flash and pRL-TK, along with additional plasmid encoding CA- β -catenin or empty vector before inducing differentiation. The cells were treated with LPA or vehicle, and TCF reporter transcriptional activity was demonstrated by determining the ratio of TOP-Flash to POP-Flash, as described in the Materials and methods. Each point represents the mean \pm SEM of triplicate samples. Data are representative of three independent experiments.

Results

LPA induces activation of β -catenin/TCF signaling in H19-7 cells. In a previous study, it was demonstrated that LPA stimulated the inactivation of GSK-3 in H19-7 cells through LPA₁ and LPA₂ (2). As β -catenin is a substrate for GSK3 and is degraded following phosphorylation by GSK3, and as LPA activates G proteins, such as Gq, Gi/o and G12/13 (via coupling to LPA₁ and LPA₂), LPA may activate β -catenin/TCF signaling in H19-7 cells. To determine whether LPA activated β -catenin/TCF signaling, cytoplasmic and nuclear levels of β -catenin in the H19-7 cells treated with LPA were investigated by immunocytochemistry and western blot analysis. An increase in the level and the nuclear translocation of β -catenin was detected by immunocytochemistry in the H19-7 cells (Fig. 1A). In addition, an increase in the cytoplasmic levels of β -catenin and the subsequent increase in the levels of nuclear β -catenin was demonstrated by western blot analysis (Fig. 1B). An increase in the level of nuclear β -catenin was also detected within 30 min (data not shown). To determine whether LPA activated β -catenin-mediated transcriptional activation, the TCF reporter activity was analyzed in differentiating H19-7 cells transfected with TCF luciferase reporter. TCF with LPA reporter activity was ~ 2 -fold that without LPA, and was similar to that induced by transfection with a plasmid encoding β -catenin truncated N-terminal 90 amino acids

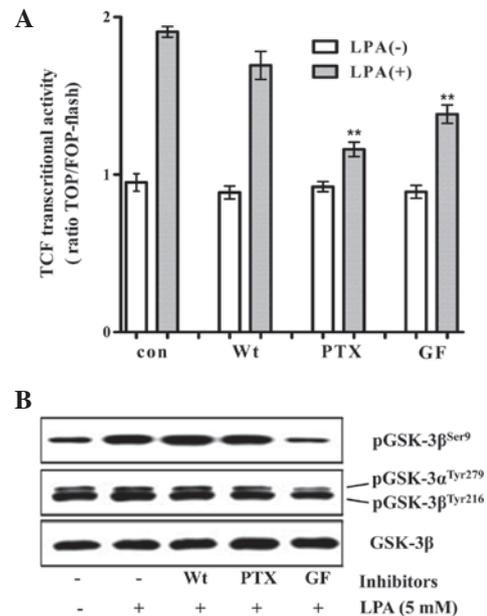


Figure 2. Effect of pharmacological inhibitors on the T cell factor (TCF) transcriptional activity and inhibitory phosphorylation of glycogen synthase kinase (GSK)3 β . (A) H19-7 cells were transiently transfected with TOP Flash or FOP Flash, and differentiation was induced. The differentiating cells were pretreated with $10 \mu\text{M}$ GF109203, $2 \mu\text{M}$ wortmannin and 100 ng/ml pertussis toxin (PTX) for 1 h, and treated with LPA ($5 \mu\text{M}$) or vehicle for 4 h. The cells were collected and TCF reporter activity was determined. Results are presented as the mean of triplicate samples from three independent experiments. The value in the absence of inhibitor (control) versus value in the presence of inhibitor are significantly different ($n=9$, $**P<0.001$). (B) Differentiating H19-7 cells were treated with $10 \mu\text{M}$ GF109203, $2 \mu\text{M}$ wortmannin and 100 ng/ml PTX for 1 h, and treated with LPA ($5 \mu\text{M}$) or vehicle for 4 h. Inhibitory phosphorylation of GSK3 β was detected using anti-GSK-3 β Ser9 (Cell Signaling Technology Inc., Beverly, MA, USA) by western blot analysis. Data are representative of three independent experiments.

(b-Cat $\Delta 90$) acting as a constitutively active mutant of β -catenin (CA- β -catenin) (Fig. 1C).

LPA activates β -catenin/TCF signaling in a PTX- and PKC-dependent manner. Our previous study demonstrated that LPA induced cell survival in a PTX-dependent manner by the post-translational upregulation of Mcl-1 through the inhibitory phosphorylation of GSK3 in differentiating H19-7 cells. The inhibitory phosphorylation of GSK3 is mediated predominantly by PKC and partly by mitogen activated protein kinases, PKA and phosphatidylinositol 3-kinase (PI3K) in the H19-7 cells (2). To determine the mechanism by which LPA stimulates β -catenin/TCF signaling, the effect of pharmacological inhibitors of PKC, PI3K and Gi/o on the LPA-induced activation of the TCF reporter activity and on the inhibitory phosphorylation of GSK3 β at Ser9 by LPA was analyzed. GF109203 (a PKC inhibitor) and PTX (a Gi/o specific inhibitor) significantly decreased the LPA-induced activation of TCF reporter activity, whereas wortmannin (a PI3K inhibitor) did not significantly decrease this activation. By contrast, the LPA-induced inhibitory phosphorylation of GSK3 β was marginally decreased by PTX and not by wortmannin, while GF109203 completely inhibited the phosphorylation (Fig. 2). The observation that PTX significantly inhibited the LPA-induced β -catenin/TCF signaling, with a marginal decrease in the inhibitory phosphorylation of GSK3 β ,

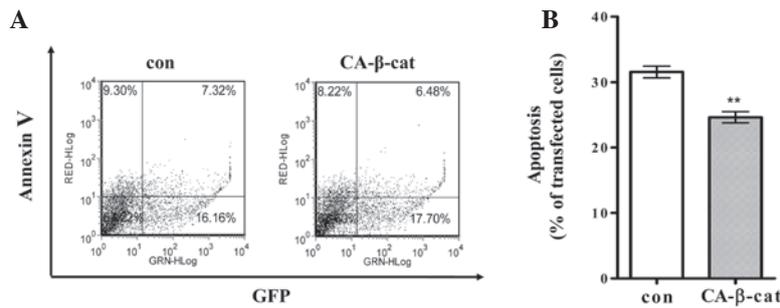


Figure 3. β -catenin/T cell factor signaling by lysophosphatidic acid (LPA) suppresses apoptosis in differentiating H19-7 cells. (A) Cells were transfected with pCatDt90, a plasmid expressing CA- β -catenin, or empty vector (control) with phosphorylated enhanced green fluorescent protein (pEGFP). Differentiation was induced and cells were treated with LPA at 39°C for 4 h. The cells were harvested, stained with Annexin V conjugated with Alexa Fluor 568 and analyzed by flow cytometry. The intensity of the red fluorescence of Annexin V-Alexa Fluor 568 is shown on the y-axis, the intensity of GFP is shown on the x-axis and the populations of apoptotic cells among cells expressing b-Cat Δ 90 or control cells in the quadrant are shown. Data are representative of two independent experiments. (B) Quantitation of apoptotic cells among the transfected cells with pCatDt90 or empty vector (pcDNA3). Apoptosis was significantly reduced in H19-7 cells transfected with pCatDt90 compared with the control. Results are the mean of triplicate samples from two independent experiments (n=6; **P<0.01, vs. control).

suggested that LPA may stimulate β -catenin/TCF transactivation via signaling pathways that are independent of the direct inhibition of GSK3.

Activation of β -catenin/TCF transcription by LPA suppresses the apoptosis of H19-7 cells. LPA induced cell survival in a dose-responsive manner in the differentiating H19-7 cells; however, the H19-7 cells treated with LPA were not stained with BrdU, suggesting that LPA induced cell survival by protecting H19-7 cells from cell death, not by inducing cell proliferation (data not shown). To determine whether LPA-mediated β -catenin/TCF signaling was involved in protecting H19-7 cells from cell death, the cells were transfected with a plasmid expressing either CA- β -catenin or empty vector and a plasmid expressing GFP. Transfected cells differentiated and apoptosis was detected by flow cytometry as described in the Materials and methods. Apoptosis was significantly suppressed in the H19-7 cells expressing CA- β -catenin compared with that of the control cells (Fig. 3).

Discussion

Activation of the cognate GPCRs by lipid metabolites, growth factors or a P2Y receptor agonist was shown to activate β -catenin/TCF signaling, although the mechanism of action is unclear. The GPCR-mediated β -catenin/TCF transcriptional activation (except for the P2Y receptor-mediated TCF activation) was demonstrated to be implicated in oncogenicity, including cell proliferation and epithelial-mesenchymal transition in cancer cells, but not in normal physiological processes (18-23). In the present study, it was demonstrated that LPA activated β -catenin/TCF signaling predominantly via the Gi/o- and PKC-dependent pathways in differentiating HPCs, and that the β -catenin/TCF signaling induced by LPA was involved in suppressing apoptosis in the HPCs. Activation of β -catenin/TCF signaling was determined by an increase in the nuclear levels of β -catenin and the stimulation of TCF reporter activity (Fig. 1). The observation that β -catenin/TCF transactivation induced the expression of Mcl-1, an anti-apoptotic Bcl2 family member (24), was consistent with a previous study demonstrating that LPA-induced cell

survival was mediated by post-translational and transcriptional upregulation of Mcl-1 (2). Whether the direct inhibition of GSK3 β is involved in β -catenin stabilization and subsequent β -catenin/TCF transactivation remains controversial. LPA was previously shown to induce the inhibitory phosphorylation of GSK3 β by PKC, mitogen-activated protein kinase and protein kinase A, but not PI3K, in H19-7 cells (2). Therefore, in the present study, it was investigated whether inhibitory phosphorylation of GSK3 β by upstream kinases was involved in the β -catenin/TCF transactivation. LPA-induced activation of β -catenin/TCF signaling was significantly inhibited by PTX, although PTX marginally decreased the inhibitory phosphorylation of GSK3 β . Moreover, the PKC inhibitor decreased the β -catenin/TCF signaling less potently than that by PTX, although the PKC inhibitor completely abolished the inhibitory phosphorylation of GSK3 β . Thus, these results suggested that LPA may activate β -catenin/TCF transactivation via signaling pathways that are independent of the direct inhibition of GSK3. However, β -catenin/TCF transactivation may have occurred through direct inhibition of GSK3 β , as the PKC inhibitor significantly decreased the activation of β -catenin/TCF signaling and the inhibitory phosphorylation of GSK3 β . Previous studies have suggested that GSK3 β only modifies β -catenin when GSK3 forms the Axin/APC/ β -catenin destructive complex (25,26), and that only a small pool of GSK3 β exists in the Axin scaffolding complex within which GSK3 β is not readily accessible to upstream kinases (27). Thus, GPCR-mediated activation of β -catenin/TCF signaling may occur without direct inhibitory phosphorylation of GSK3 β in the Axin scaffolding complex, although upstream kinases are involved in this signaling pathway and inhibitory phosphorylation of GSK3 β in the other pool is increased. Alternatively to the phosphorylation of GSK3 within the Axin complex, the upstream kinases may activate β -catenin/TCF signaling by acting on other targets involved in the activation of β -catenin/TCF signaling, such as 14-3-3-mediated nuclear efflux of β -catenin by Akt or PKC and KSRP-mediated stabilization of β -catenin mRNA by PI3K/Akt (28-30). Thus, PKC may activate β -catenin/TCF signaling without inhibitory phosphorylation of GSK3 β in the Axin complex, although the total inhibitory phosphorylation

of GSK3 β is increased.

Expression of CA- β -catenin suppressed apoptosis in differentiating H19-7 cells, suggesting that the activation of the TCF reporter activity by LPA was involved in cell survival of HPCs. Apoptosis of the H19-7 cells was suppressed by 6% following expression of CA- β -catenin (Fig. 3). Suppressing effects of β -catenin/TCF activation on the survival of the H19-7 cells were marginal, but may be physiologically relevant, as small changes in the survival of progenitor pools may produce a marked effect on neurodevelopment. It was suggested that only a 3.5% increase in survival and a 2.5% increase in terminal mitosis of progenitor cells induced an ~30% increase in the thickness of the cerebral cortical wall (13). In concordance with the present results, previous studies demonstrated that β -catenin/TCF signaling was involved in the survival of neural precursors, although the molecular mechanisms remain unknown (31-33). In conclusion, the results suggested that LPA may regulate β -catenin/TCF signaling in NPCs, as LPA is involved in neurogenesis by controlling the survival of neural precursors.

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