Biochimica et Biophysica Acta 1851 (2015) 172–183

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Opposing regulation of megakaryopoiesis by LPA receptors 2 and 3 in K562 human erythroleukemia cells

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ARTICLE INFO

Article history:
Received 8 August 2014
Received in revised form 6 November 2014
Accepted 13 November 2014
Available online 21 November 2014

Keywords:
Megakaryopoiesis
Hematopoiesis
Lysophosphatidic acid
Phorbol ester
LPA receptor
CD61

ABSTRACT

Erythrocytes and megakaryocytes (MK) are derived from a common progenitor that undergoes lineage specification. Lysophosphatidic acid (LPA), a lipid growth factor was previously shown to be a regulator for erythropoietic process through activating LPA receptor 3 (LPA_3). However, whether LPA affects megakaryopoiesis remains unclear. In this study, we used K562 leukemia cell line as a model to investigate the roles of LPA in MK differentiation. We demonstrated that K562 cells express both LPA_2 and LPA_3, and the expression levels of LPA_2 are higher than LPA_3. Treatment with phorbol 12-myristate 13-acetate, a commonly used inducer of megakaryopoiesis, reciprocally regulates the expressions of LPA_2 and LPA_3. By pharmacological blockers and knockdown experiments, we showed that activation of LPA_2 suppresses whereas, LPA_3 promotes megakaryocytic differentiation in K562. The LPA_2-mediated inhibition is dependent on β-catenin translocation, whereas reactive oxygen species (ROS) generation is a downstream signal for activation of LPA_3. Furthermore, the hematopoietic transcriptional factors GATA-1 and FLI-1, appear to be involved in these regulatory mechanisms. Taken together, our results suggested that LPA_2 and LPA_3 may function as a molecular switch and play opposing roles during megakaryopoiesis of K562 cells.

1. Introduction

Adult megakaryopoiesis and erythropoiesis take place in the bone marrow where a common stem cell, the megakaryocyte–erythrocyte progenitor (MEP), undergoes lineage specification [1,2]. The control of MEP cell fate is determined by lineage specific transcription factor (TF) networks [3,4], especially the interaction among the GATA-binding factor 1 (GATA-1), the Kruppel-like factor 1 (EKLF), and the Friend leukemia integration 1 (FLI-1) [5,6]. GATA-1 is indispensable for the commitment of both erythroid and megakaryocytic lineages [7]. EKLF guides erythropoiesis at the expense of megakaryocytic differentiation [8], whereas FLI-1 directs megakaryopoiesis by antagonizing EKLF [9]. Molecular switches of these TFs contributing to MEP fate decision are tightly regulated, which can be initiated by micro-RNAs, hematopoietic cytokines, and a variety of micro-environmental factors [10–12].

Lysophosphatidic acid (LPA) is a lipid growth factor found abundantly in blood serum and other biological fluids [13,14]. LPA regulates diverse

Abbreviations: MEP, megakaryocyte–erythrocyte progenitor; TF, transcription factor; GATA-1, GATA-binding factor 1; EKLF, Kruppel-like factor 1; FLI-1, Friend leukemia integration 1; LPA, lysophosphatidic acid; LPA_1, LPA receptor 1; LPA_2, LPA receptor 2; LPA_3, LPA receptor 3; LPA_5, LPA receptor 5; HSC, hematopoietic stem cell; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; MK, megakaryocyte; CD41a, integrin α2b; CD61, integrin β3; ROS, reactive oxygen species; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; OMPT, 1-oleoyl-2-O-methyl-rac-glycerophosphothionate; DMP, dodecyl monophosphate; NAC, N-acetyl-L-cysteine; RT-PCR, reverse-transcription polymerase chain reaction; PBS, phosphate-buffered saline; PI, propidium iodide; DCFDA, 2′,7′-dichlorofluorescin diacetate; BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; ATX, autotaxin; LPC, lysophosphatidylcholine; TCF, T-cell factor; PLC, phospholipase-C; VEGF-C, vascular endothelial growth factor C

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http://dx.doi.org/10.1016/j.bbalip.2014.11.009
1388-1981/

cellular responses through the activation of a family of G-protein coupled LPA receptors [15,16]. To date, six LPA receptors, termed LPA_1 to LPA_6, have been identified, differing in tissue distribution and downstream signaling pathways [17]. LPA has been studied for its role in cell proliferation, migration, tumor progression/invasion, angiogenesis, and cell survival [18]. During hematopoiesis, LPA regulates the invasion of primary hematopoietic stem cells (HSCs) into the stromal layer [19]. Additionally, LPA has been reported to mediate myeloid differentiation in human bone marrow micro-environment [20]. Of note, is our previous study that demonstrated the regulation of erythroid differentiation through activation of the LPA receptor 3 (LPA_3) [21]. More recently, LPA is reported as a developmental cue for the regulation of hemangioblast formation and early hematopoiesis [22]. These results strongly suggest that the commitment of MEP toward either erythroid or megakaryocytic lineage may be affected by LPA in the hematopoietic niche. However, whether LPA signaling plays a role in regulating megakaryopoiesis is currently unclear.

K562 erythroleukemia cell line is a commonly used in vitro model to investigate megakaryopoiesis [23,24]. Treatment with phorbol 12-myristate 13-acetate (PMA), a strong protein kinase C (PKC) activator, induces K562 cells to undergo megakaryocyte (MK) differentiation, characterized by expression of integrin α2b (CD41a) and integrin β3 (CD61) surface markers [25], enhancement of cell adhesion, cell cycle arrest [26], and endomitosis [27]. In this study, we used pharmacological blockers and knockdown strategies to investigate the relationship between LPA receptor signaling and PMA-induced megakaryopoiesis of K562 cells. We demonstrated that LPA_2 and LPA_3 conversely regulate MK differentiation through altering hematopoietic TFs, and identified β-catenin and reactive oxygen species (ROS) as essential mediators in this process. The present data highlight the opposing roles of LPA_2 and LPA_3 that may be critical to control megakaryocytic lineage commitment.

2. Materials and methods

2.1. Cell culture and pharmacological treatment

K562 human erythroleukemia cells obtained from ATCC (Manassas, VA, USA) were cultured in RPMI-1640 (HyClone, South Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Cell density was maintained between 10^5 and 10^6 cells/mL. PMA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). For MK differentiation, K562 cells were induced by PMA treatment at a final concentration of 25 nM unless otherwise specified. 293T cells were cultured in DMEM (HyClone, South Logan, Utah, USA) containing 10% FBS for lentivirus preparation.

1-Oleoyl-2-O-methyl-rac-glycerophosphothionate (OMPT) (Cayman Chemicals, Ann Arbor, MI, USA), Ki16425 (Ki) (Cayman Chemicals), quercetin (Sigma-Aldrich), and GRI compound 977143 (Genome Research Institute, University of Cincinnati Drug Discovery Center, Cincinnati, OH, USA), were dissolved in DMSO. Dodecyl monophosphate (DMP) (Sigma-Aldrich) was dissolved in ethanol. N-acetyl-L-cysteine (NAC) (Sigma-Aldrich) was diluted in double distilled H_2O. For all the pharmacological treatments, K562 cells were starved in serum-free RPMI medium for 12 h. After starvation, 2×10^5 cells were seeded on 6-well plates and cultured in RPMI medium containing 0.5% FBS, PMA and above chemicals for 48 h.

2.2. LPA_3 siRNA transfection

LPA_3 siRNA (siRNA (h): sc-37088S, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted in 100 μL serum-free medium at a final concentration of 100 nM, and reacted with 6 μL HiPerfect Transfection Reagent (QIAGEN, Valencia, CA, USA) for 10 min to form the transfection complex. Subsequently, 2×10^5 cells/mL K562 cells in 100 μL culture medium containing 10% FBS were incubated with the transfection complex for 18 h and treated with PMA to induce megakaryopoiesis.

2.3. Lentivirus packaging and LPA_2 shRNA transfection

LPA_2 shRNA template inserted in pLKO.1 lentivector was purchased from National RNAi Core Facility Platform, Academia Sinica (Taipei, Taiwan). The shRNA target sequence was: 5′-CCTGGTCAAGACTGTTGT CAT-3′ (shLPAR2, TRCN0000221131). Lentiviral stock contained 7.5 μg of lentivector, 7.5 μg of viral packaging plasmids and 2.5 M calcium chloride. Co-transfection of 293T cells was performed by adding the lentiviral stock to the cell culture followed by incubation for 16 h. The culture medium was then replaced with DMEM containing 10 mM of sodium butyrate and incubated for an additional 24 h. The conditioned medium was collected and added to K562 cell cultures for infection. Colonies of infected K562 cells were selected using 1 μg/mL puromycin (InvivoGen, San Diego, CA, USA), and then expanded under normal culture condition.

2.4. RNA extraction and quantitative real-time PCR reaction

Total RNA was isolated using the Trizol Reagent (Invitrogen), and was converted to cDNA with reverse-transcription polymerase chain reaction (RT-PCR). Real-time PCR with reagent SYBR® Green Master Mix (Bio-Rad, Hercules, CA, USA) was carried out using iCycler iQ Realtime detection system (Bio-Rad) to quantify the expression levels of target mRNA. Each target mRNA level was evaluated from the real-time threshold cycle and normalized by the amount of GAPDH. Specific primer sequences (human) were:

ATX
forward (5′ → 3′): ACA ACG AGG AGA GCT GCA AT
reverse (5′ → 3′): AGA AGT CCA GGC TGG TGA GA

LPA_1
forward (5′ → 3′): TTC AAC TCT GCC ATG AAC CCC
reverse (5′ → 3′): CTA AAC CAC AGA GTG GTC ATT

LPA_2
forward (5′ → 3′): ACA CTT CTG GCA CTG CCT CT
reverse (5′ → 3′): AGG CTG AGT GTG GTC TCT CG

LPA_3
forward (5′ → 3′): TCA GCA GGA GTG ACA CAG GCA G
reverse (5′ → 3′): GGA AGT GCT TTT ATT GCA GAC T

LPA_5
forward (5′ → 3′): CTC GGT GGT GAG CGT GTA CAT G
reverse (5′ → 3′): GCG TAG CGG TCC ACG TTG AT

CD61
forward (5′ → 3′): GTG ACC TGA AGG AGA ATC TGC
reverse (5′ → 3′): TTC TTC GAA TCA TCT GGC C

GATA-1
forward (5′ → 3′): CAA GAA GCG CCT GAT TGT CAG
reverse (5′ → 3′): AGT GTC GTG GTG GTC GTC TG

EKLF
forward (5′ → 3′): CGG CAA GAG CTA CAC CAA G
reverse (5′ → 3′): CCG TGT GTT TCC GGT AGT G

FLI-1
forward (5′ → 3′): ATG ACC ACC AAC GAG AGG G
reverse (5′ → 3′): TGT GTC GAT CTC CAT CAA GC

GAPDH
forward (5′ → 3′): AAG GTG AAG GTC GGA GTC
reverse (5′ → 3′): TGT AGT TGA GGT CAA TGA AGG

2.5. Flow cytometry

To monitor the expression of CD61, K562 cells were suspended in phosphate-buffered saline (PBS), and stained with anti-CD61-FITC (BD Bioscience Pharmingen, San Diego, CA, USA) for 30 min on ice. For DNA content analysis, cells were collected in PBST (0.1% Triton X-