FEBS Letters xxx (2014) xxx–xxx

FEBS Letters
journal homepage: www.FEBSLetters.org

Inhibitory role of polyunsaturated fatty acids on lysophosphatidic acid-induced cancer cell migration and adhesion

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ARTICLE INFO

Article history:
Received 18 April 2014
Revised 20 May 2014
Accepted 21 May 2014
Available online xxxx

Edited by Lukas Huber

Keywords:
α-Linolenic acid
Lysophosphatidic acid
Migration
Adhesion
Vinculin
Calcium mobilization

ABSTRACT

Polyunsaturated fatty acids (PUFAs) have important pharmacological effects on mammalian cells. Here, we show that carboxyl group-containing PUFAs inhibit lysophosphatidic acid (LPA)-induced focal adhesion formation, thereby inhibiting migration and adhesion. Carboxyl group-containing PUFAs inhibit LPA-induced calcium mobilization, whereas ethyl ester-group containing PUFAs have no effect. In addition, carboxyl group-containing PUFAs functionally inhibit LPA-dependent RhoA activation. Given these results, we suggest that PUFAs may inhibit LPA-induced calcium/RhoA signaling pathways leading to focal adhesion formation. Carboxyl group-containing PUFAs may have a functional role in this regulatory mechanism.

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1. Introduction

Cancer is a disease of complex etiology, defined as uncontrolled growth of cells. The transformation of normal cells to cancerous involves three distinct phase: initiation, promotion, and progression [1]. During the initiation and promotion steps, cancer cells attain several cancerous features caused by genetic changes. At the end of tumorigenesis, cancer cells acquire the ability to spread to distant organs through so-called metastasis. The major leading cause of the high mortality rates associated with cancer is metastasis. Indeed, metastases are the cause of 90% of cancer patients' deaths [2]. Therefore, cancer therapies should be focused on not only tumor development but also metastasis.

Migration is a key process for normal physiologies such as embryonic development, immune function, and angiogenesis. It is also associated with inflammatory diseases, vascular impairment, tumor cell invasion and metastasis [3,4]. To attain motility, a cell must coordinate a number of different extracellular stimuli into appropriate cellular responses. The cell is polarized in the direction of migration by extending lamellipodial and/or filopodial protrusions. Nascent adhesions are acquired by assembly of the branched actin network of the lamellipodium. This process allows the maturation of adhesions to anchor the protrusion. These adhesions also provide the traction forces necessary to pull the cell body forward and break adhesions at the rear of the cells. Perturbation of any of these events affects the migratory ability of the cells [5].

Cell adhesion is regulated by a complex of proteins that localizes to sites of focal adhesions (FAs) [6]. Vinculin is a key regulator of FAs [7], and targeted disruption of vinculin reduces adhesion to a variety of extracellular matrix (ECM) proteins, increases migration rates, and results in fewer and smaller adhesions compared with wild-type cells [8]. Despite the profound role of vinculin in cell adhesion and motility, the molecular mechanisms by which vinculin exerts these distinct effects are poorly understood.

Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) are essential fatty acids for mammals, indicating that mammals can neither synthesize nor interconvert omega-3 and omega-6. Therefore, they have to be consumed in the daily diet as vegetable oils and fish oils. Appropriate ingestion of omega-6/omega-3 is recommended for human health [9,10]. Some evidence suggests that omega-3 is beneficial in prevention of colon [11] and prostate

Abbreviations: AA, arachidonic acid; CaM, calmodulin; CaMKII, CaM-dependent kinase II; DHA, docosahexaenoic acid; ECM, extracellular matrix; ELA, ethyl linoleate; ELN, ethyl linolenate; EPA, eicosapentaenoic acid; FAs, focal adhesions; LA, linoleic acid; LNA, α-linolenic acid; LPA, lysophosphatidic acid; PUFAs, polyunsaturated fatty acids

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http://dx.doi.org/10.1016/j.febslet.2014.05.052
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Please cite this article in press as: Kim, E.K., et al. Inhibitory role of polyunsaturated fatty acids on lysophosphatidic acid-induced cancer cell migration and adhesion. FEBS Lett. (2014), http://dx.doi.org/10.1016/j.febslet.2014.05.052

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cancer [12,13]. Omega-6 and omega-3 have a carbon–carbon double bond at the sixth carbon and third carbon from the methyl end of the carbon chain, respectively [14]. However, little is known about structure- and chain length-relationship in the regulation of cancer cell migration. In the present study, we explored the effect of omega-3 and omega-6 on cancer cell migration and adhesion, and suggest that LPA-induced cancer cell migration and adhesion is regulated by the carboxylic acid group of omega-3 and omega-6 through the calcium/CaM/CaMKII signaling pathway.

2. Materials and methods

2.1. Reagents and antibodies

Reagents and antibodies used in this study were described in Supplementary file.

2.2. Cell culture and Western blotting

SKOV-3 cell culture and Western blotting were performed as described in a previous report [15].

2.3. RhoA activation assay

The level of active GTP-bound RhoA was determined by pulling-down GTP-bound RhoA with GST-Rhotekin-RBD coupled to glutathione-agarose beads. Cells were stimulated with LPA for 5 min and then lysed with lysis buffer containing 50 mM Tris (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged, and supernatants were incubated with beads coupled to GST-Rhotekin-RBD for 2 h at 4 °C. Beads were washed with lysis buffer and GTP-loaded RhoA was eluted with sample buffer. The amount of active RhoA was determined by Western blot analysis.

2.4. Migration assay

SKOV-3 cells were grown and serum-starved for 10 h before plating on a ChemoTx chamber (Neuro Probe Inc.). Cells were detached with trypsin-EDTA and washed with serum-free RPMI. For the migration assay, the bottom side of the ChemoTx membrane (8-μm pore size) was coated with type I collagen for 30 min, and 1×10^4 serum-starved cells in 50 μl volume were placed on the top side of ChemoTx membrane per each well. Migration was induced by placing the cells on an overlaid ChemoTx membrane on top of serum-free medium for 10 h. The ChemoTx membrane was fixed with 4% paraformaldehyde, and non-migratory cells on the top side of the membrane were removed by gently wiping with a cotton swab. The membrane was stained with DAPI, and migrating cells were counted under the fluorescence microscope at 10× magnification (Carl Zeiss).

2.5. Adhesion assay

To explore the effect of omega-3 on adhesion ability of SKOV-3 cells, 96-well plates (Falcon, Becton-Dickinson, Mountain View, CA) were incubated with collagen type I for 12 h, then blocked with PBS containing 0.2% BSA for 50 min at 37 °C. SKOV-3 cells were trypsinized and suspended in the presence or absence of omega-3 in LPA at a density of 1×10^5 cells/ml, and 0.1 ml of the cell suspension was then added to each well of the plates. After 2 h, unattached cells were removed by rinsing twice with PBS. Attached cells were counted under the microscope at ×100 magnification after staining with DAPI.

2.6. Immunocytochemistry

SKOV-3 cells were grown in 6-well plates on coverslips, serum-starved for 12 h, and then stimulated with LPA (10 μM). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with anti-vinculin and rhodamine-phalloidin for an hour followed by DAPI and Alexa Fluor 488-conjugated secondary antibody for 30 min. Samples were mounted with anti-fading reagent (2% N-propylgalate in 80% glycerol/phosphate-buffered saline solution), and images were obtained with a confocal microscope at 40× magnification and enlarged 2X in silico (OLYMPUS FV-1000).

2.7. Measurement of intracellular calcium concentration

Intracellular calcium concentration was measured using fura-2/AM, a calcium-sensitive fluorescent dye, as described previously [16]. Briefly, a total of 1×10^6 SKOV-3 cells were incubated with 3 mM fura-2/AM at 37 °C in fresh serum-free RPMI medium with stirring for 50 min. Cells (1×10^5) were aliquotted into Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, 2.2 mM $CaCl_2$, and 0.2 mM EGTA) for each assay. Fluorescent emission at 500 nm was measured at excitation wavelength of 340/380 nm.

2.8. Statistical analysis

Results are expressed as the means ± S.D. of two independent experiments ($n = 3$ for each experiment). When comparing two groups, an unpaired Student's t-test was used to address differences. P-values < 0.05 were considered significant and indicated by *.

3. Results

3.1. LPA-induced cancer cell migration is regulated by carboxyl group-containing omega-3 and omega-6

LPA was originally identified as a tumor-stimulating factor that promotes cancer cell migration [17,18]. Likewise, our results also showed that LPA strongly induced the migration of SKOV-3 cells (Fig. 1A and B). To elucidate the potential role of omega-3 and omega-6 during LPA-induced cancer cell migration, we examined the effect of various omega-3 and omega-6 analogs on the LPA-induced SKOV-3 cell migration (Fig. 1C). LPA-induced SKOV-3 cell migration was attenuated by pre-treatment with carboxyl group-containing omega-3 and omega-6 such as linoleic acid (LA), arachidonic acid (AA), α-linolenic acid (LNA) and EPA. However, ethyl linoleate (ELA), AA ethyl ester, α-ethyl linolenate (ELN) and EPA ethyl ester which have the ethyl ester structure were not effective (Fig. 1D). These results suggest that carboxyl group-containing omega-3 and omega-6 play crucial roles in LPA-induced cancer cell migration.

3.2. Carboxyl group-containing omega-3 regulates cancer cell migration and adhesion

Adhesion of cells to a substrate is necessary for cell spreading and migration. Therefore, we validated the effect of LNA on LPA-dependent cancer cell migration and adhesion. As shown in Fig. 2A and B, LPA-induced cancer cell migration and adhesion were abolished by LNA, whereas ELN was not effective. Since cell adhesion is regulated by adhesion-associated proteins such as FAK, integrin, talin, paxillin, and vinculin, we examined morphological changes by staining cells with actin and vinculin. As shown

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