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

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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.

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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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

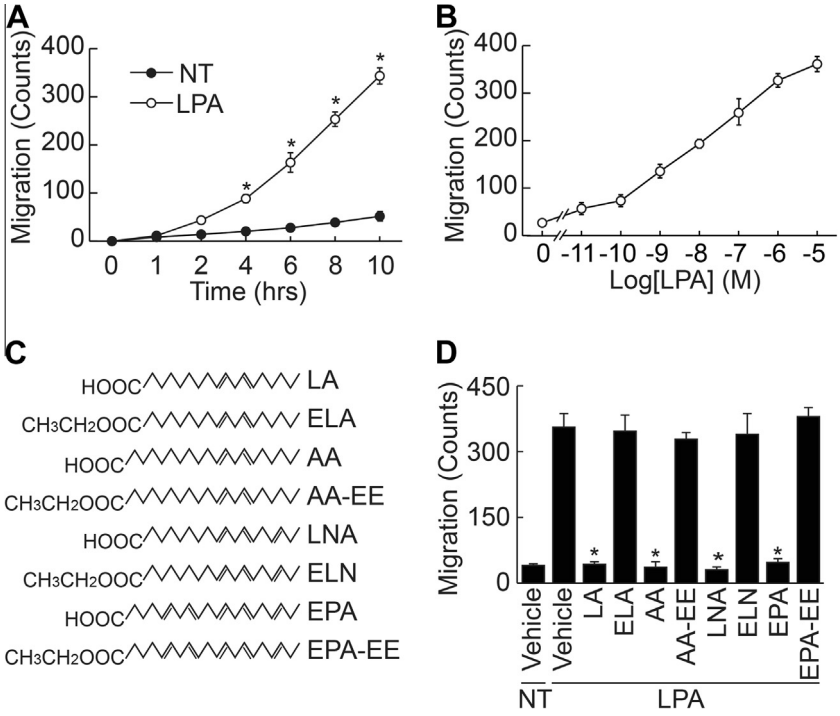

Fig. 1. Carboxylic moiety of PUFAs retains its inhibitory activity on LPA-induced SKOV-3 cell migration. (A and B) LPA-dependent SKOV-3 cell migration was determined in a time- and dose-dependent manner. (C) Chemical structure of PUFAs. LA, linoleic acid; ELA, ethyl linoleate; AA, arachidonic acid; AA-EE, arachidonic acid ethyl ester; LNA, α-linolenic acid; ELN, α-ethyl linolenate; EPA, eicosapentaenoic acid; EPA-EE, eicosapentaenoic acid ethyl ester. (D) LPA-induced migration of SKOV-3 cells was determined in the absence or presence of various PUFAs (10 μM). $^{*}P < 0.05$.

in Fig. 2C, actin stress fiber and focal adhesion formation were strongly induced by treatment with LPA. LNA completely blocked LPA-induced actin stress fiber and focal adhesion formation, whereas ELN did not affect LPA-induced morphological changes. Moreover, pretreatment of SKOV-3 cells with LNA significantly blocked actin stress fiber formation and induced shrinkage. These results suggest that LNA inhibits SKOV-3 cell adhesion and migration through regulation of focal adhesion formation.

3.3. LNA blocks LPA-induced calcium mobilization

It has been reported that LPA substantially evokes calcium mobilization and regulates migration [19]. Since our results showed that LPA-induced SKOV-3 cell migration was inhibited by LNA, we next examined the effect of LNA on LPA-dependent calcium mobilization. As shown in Fig. 3A, LPA-induced calcium mobilization was inhibited by LNA in a dose-dependent manner. In contrast, ELN did not affect LPA-induced calcium mobilization. To assess that LNA-dependent inhibition of calcium mobilization is responsible for cancer cell migration, we determined the effect of L-type calcium channel blocker (nifedipine) on the LPA-induced SKOV-3 cell migration and adhesion. As shown in Fig. 3B, nifedipine significantly but not completely blocked LPA-induced calcium mobilization. Likewise, stimulation of SKOV-3 cells with nifedipine significantly blocked LPA-induced migration and adhesion (Fig. 3C and D). These results suggest that LNA suppresses LPA-induced SKOV-3 cell migration and adhesion through inhibition of calcium mobilization.

3.4. LNA suppresses calcium-dependent RhoA activation

In order to explore the role of calcium mobilization in LPA-dependent cancer cell migration, we observed whether calmodulin (CaM), which is a key regulator of the calcium mobilization signaling pathway, is involved in the LPA-induced SKOV-3 cell migration. LPA-induced SKOV-3 cell migration and adhesion were markedly attenuated by CaM antagonist W7 (Fig. 4A and B). Moreover, W7 also abolished the focal adhesion formation (Fig. 4C). To ascertain the molecular mechanism by which CaM regulates the LPA-induced migration, we next examined the participation of CaM-dependent protein kinase II (CaMKII) on the LPA-induced migration of SKOV-3 cells. As shown in Fig. 4A and B, CaMKII-specific inhibitor (KN93) completely blocked LPA-induced SKOV-3 cell migration and adhesion, whereas KN92, an inactive analog of KN93, had no effect. In addition, LPA-dependent focal adhesion formation was abrogated by KN93 but not by KN92 (Fig. 4C). Since RhoA regulates stress fiber formation and thereby enhances cell migration, we performed a pull-down assay to assess how LNA affects LPA-induced RhoA activity. As shown in Fig. 4D, LPA-induced RhoA activity was abrogated by LNA, nifedipine, W7, and KN93. However, ELN and KN92 did not affect LPA-induced RhoA activation. These results suggest that carboxyl group-containing omega-3 controls CaMKII as well as the RhoA signaling pathway, thereby regulating LPA-induced SKOV-3 cell migration and adhesion.

4. Discussion

In the present study, we explored the ability of omega-3 and omega-6 structures differentially regulate LPA-induced cancer cell migration and adhesion. Many reports suggest that LPA is a major component of ascites from ovarian cancer patients and an important predictor of cancer diagnosis [18,20]. Indeed, LPA drastically stimulated migration of SKOV-3 ovarian cancer cells (Fig. 1A and B). Thus, modulation of LPA-induced cancer cell migration and adhesion seems to be an important issue in cancer biology.

Around eighty percent of cancer patients die with metastasis. Metastasis begins with cell migration and adhesion during intravasation and extravasation, understanding migration and adhesion is thus crucial for development of cancer therapeutics. Appropriate ingestion of PUFAs such as omega-6/omega-3 has

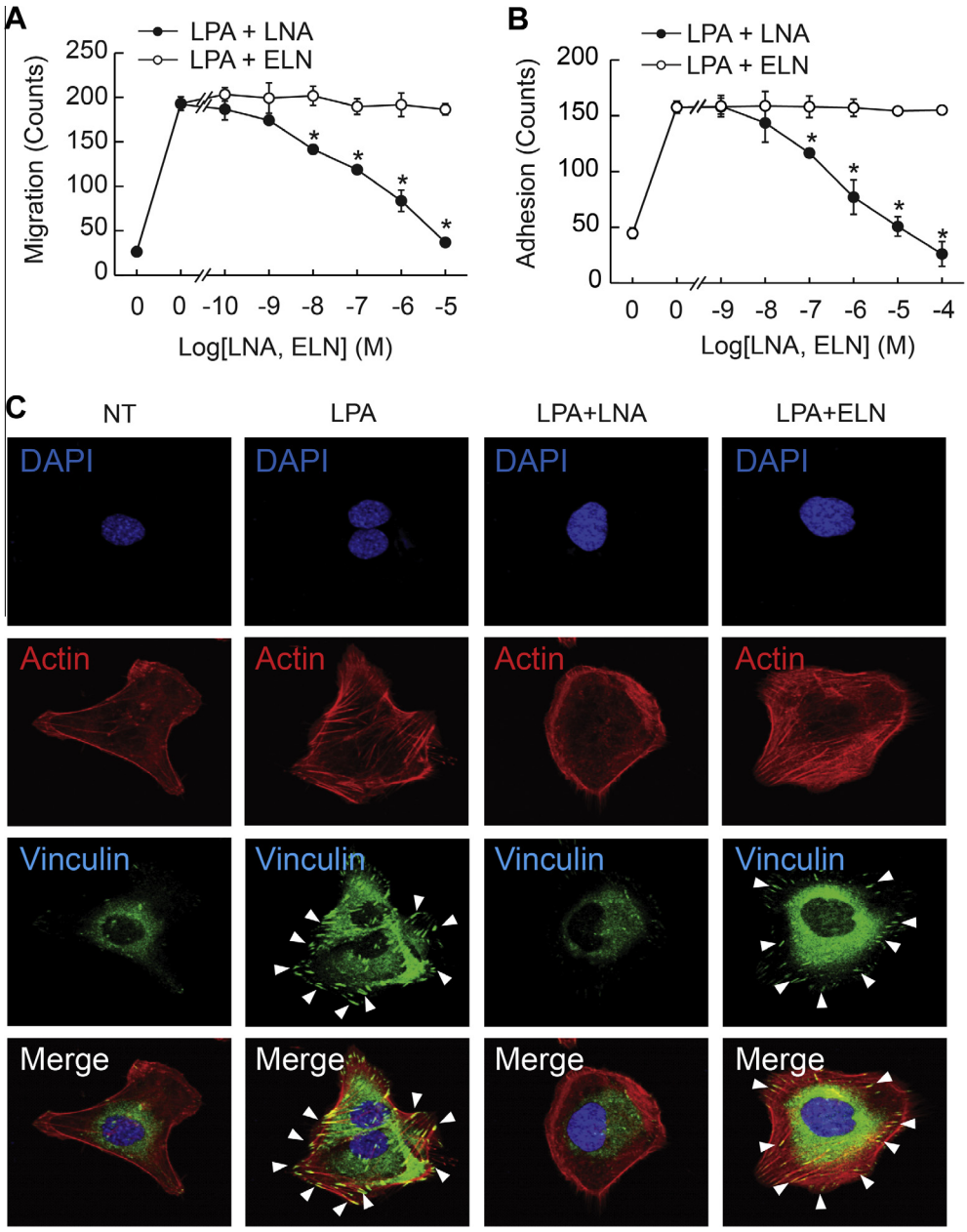

Fig. 2. LNA suppresses LPA-dependent SKOV-3 cell migration and adhesion in a dose-dependent manner. (A and B) LPA-dependent migration and adhesion of SKOV-3 cells were determined in the absence or presence of the indicated concentration of LNA or ELN. (C) SKOV-3 cells were pretreated with LNA (10 μM) or ELN (10 μM) followed by stimulation with LPA (10 μM) for 3 min. Cells were stained with DAPI, rhodamine-phalloidin, and vinculin antibody. Images were captured with confocal laser microscopy at 40× magnification and enlarged 2X in silico. Focal adhesions were indicated by arrow heads. $^{*}P < 0.05$.

recently been suggested to have some beneficial effect on colon and prostate cancer patients [11–13]. However, the underlying mechanism for this beneficial effect on cancer patients is still unclear. Therefore, we investigated the role of various PUFAs on LPA-induced cancer cell migration and adhesion. As a result, we identified LA, AA, LNA, and EPA as negative regulators of LPA-induced cancer cell migration and adhesion (Figs. 1D, 2A and B). Thus, it seems that PUFAs may have beneficial effects for cancer patients through modulation of cancer cell migration and adhesion.

One important issue arising from this study is the structural properties of PUFAs. It has been reported that different PUFAs have unique cognate receptors. For example, short chain fatty acids (FAs) are specific agonists for GPR41 and GPR43 [21] and medium-chain FAs for GPR84 [22]. Long-chain FAs can activate GPR40 [23] and GPR120 [24]. In particular, it has been reported that GPR120 is an omega-3 receptor mediating potent anti-inflammatory and insulin-sensitizing effects [25]. In addition to these structural properties of chain length, our results demonstrated that the carboxyl group is a crucial moiety for the inhibitory effect of PUFAs. For instance, LNA potently suppressed LPA-induced migration, adhesion, focal adhesion formation, and calcium mobilization whereas ELA which contains an ethyl ester group at the carboxyl terminus, had no effect (Figs. 1D, 2, and 3). Likewise, LA, AA, and EPA suppressed LPA-induced migration of SKOV-3. However, their ethyl ester form did not have this suppressive effect on LPA-induced SKOV-3 cell migration (Fig. 1D). Therefore, it is likely that the carboxyl terminal group of PUFAs has functional activity in the inhibition of LPA-induced cancer cell migration. In this regard, it is worthwhile to evaluate

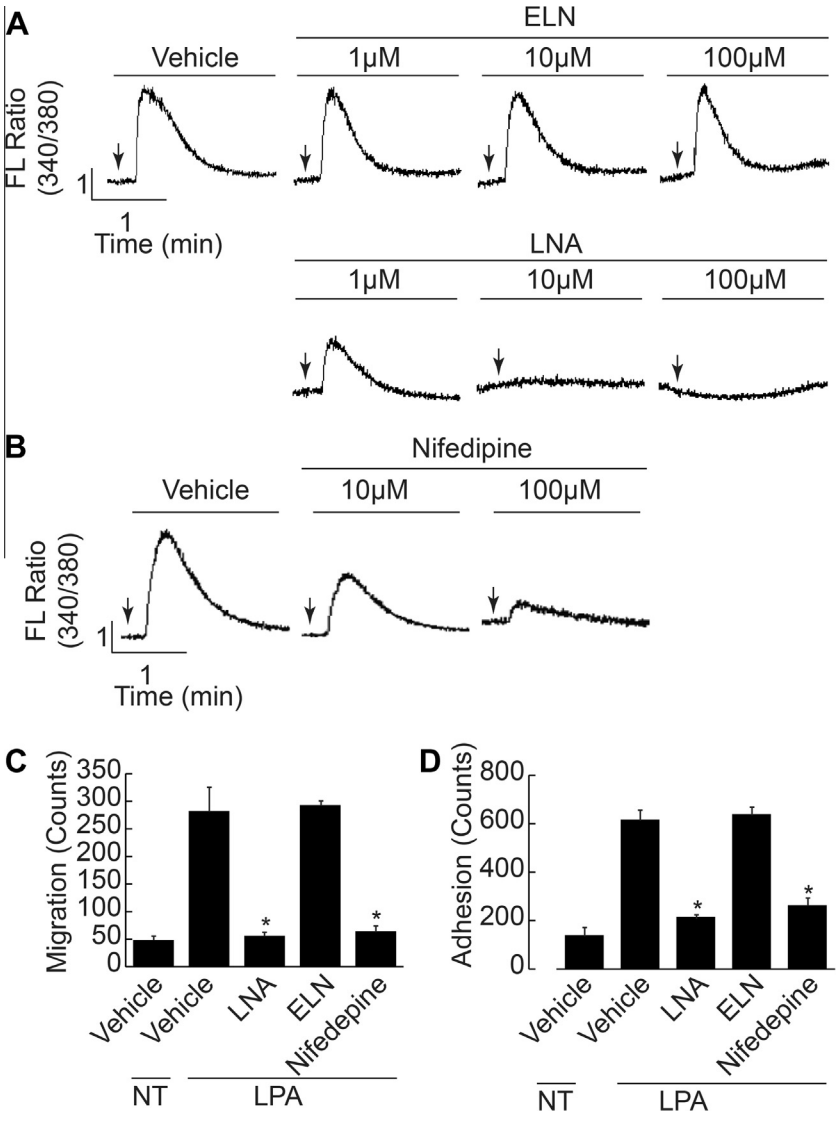

Fig. 3. LNA blocks LPA-induced calcium mobilization. (A) SKOV-3 cells were pre-incubated with the indicated concentration of LNA or ELN for 20 min and then stimulated with LPA (10 μM). Calcium mobilization was measured as described in 'Section 2'. (B) SKOV-3 cells were pre-treated with indicated concentration of nifedipine prior to stimulation with LPA (10 μM), and calcium mobilization was determined. (C and D) LPA-induced migration and adhesion of SKOV-3 cells were measured in the absence or presence of LNA (10 μM), ELN (10 μM), and L-type calcium channel blocker (nifedipine, 100 μM). $^{*}P < 0.05$.

the effect of the carboxyl group on the biological activity of fatty acid binding receptors.

A complex signaling mechanism regulates cell adhesion and spreading [26], and integration of these signals leads to assembly of focal adhesion proteins such as talin and vinculin to attachment and spreading of cells [27–31]. In line with this, our results also showed that LPA drastically regulated focal adhesion formation (Figs. 2C and 4C). Calcium mobilization is a key regulatory mechanism for LPA-induced focal adhesion formation. For example, LPA stimulates calcium mobilization in a variety of cells [32] and activates focal adhesion kinase and paxillin [33]. The inhibitory mechanism of LNA seems to be related with calcium mobilization. In fact, LPA-induced calcium mobilization was completely blocked by LNA, whereas the ethyl ester form of LNA (ELN) was not effective (Fig. 3A). Furthermore, blocking of calcium mobilization by L-type calcium channel blocker (nifedipine) also significantly blocked LPA-induced SKOV-3 cell migration and adhesion (Fig. 3C and D). Thus, the carboxyl group-containing omega-3 may exert its effect on LPA-mediated cancer cell migration and adhesion *via* calcium mobilization.

One possible mediator for calcium mobilization is the CaM/CaMKII signaling pathway. Indeed, it has been reported that activation of the CaM/CaMKII signaling pathway plays a key role in LPA-induced migration hBMDS cells [34]. Likewise, several lines of evidence support that LPA-dependent SKOV-3 cell migration is mediated by the CaM/CaMKII signaling pathway. First, blocking calcium mobilization with L-type calcium channel blocker or LNA significantly attenuated LPA-induced SKOV-3 cell migration and adhesion (Fig. 3C and D). Second, direct inhibition of CaM (W7) or CaMKII (KN93) significantly blocked LPA-induced SKOV-3 cells migration and adhesion whereas the inactive form of KN93 (KN92) was not effective (Fig. 4A and B). Therefore, the CaMKII signaling pathway may be important for LPA-induced SKOV-3 cell migration as well as adhesion, and carboxyl group-containing omega-3 may exert its inhibitory effect on calcium mobilization and thus on CaMKII activation.

The activation of Rho GTPase is the most important determinant for many types of cell migration [35,36]. Activated RhoA eventually controls stress fibers and assembly of focal adhesion complexes such as vinculin [37–39]. It has been reported that intracellular

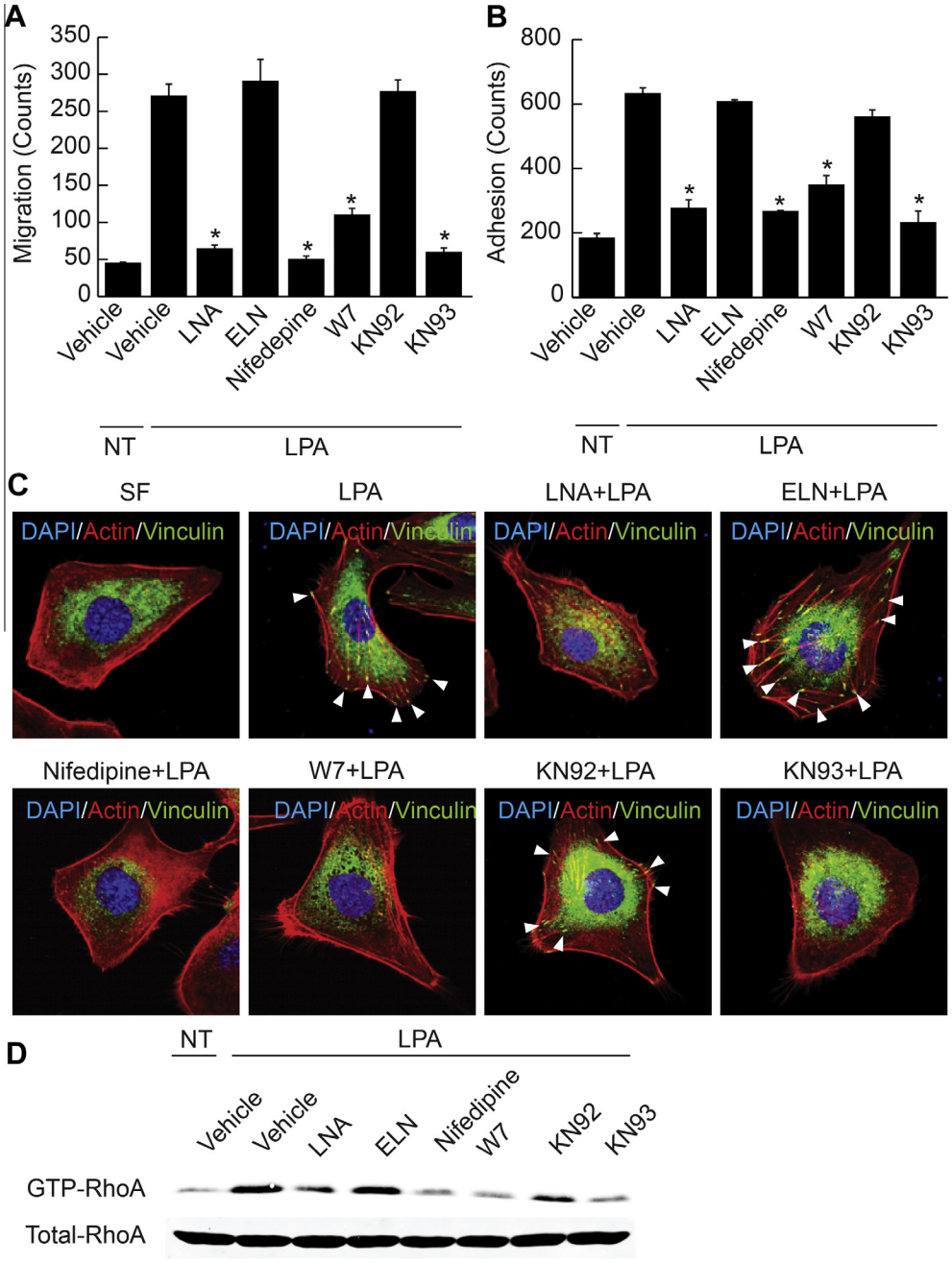

Fig. 4. LNA suppresses LPA-induced focal adhesion formation and RhoA activation. (A and B) SKOV-3 cells were pre-treated with LNA (10 μM), ELN (10 μM) nifedipine (100 μM), W7 (CaM inhibitor, 5 μM), KN93 (CaMKII inhibitor, 2.5 μM), or KN92 (inactive analog of KN93, 2.5 μM), and LPA-induced migration and adhesion were determined. (C) Under the same conditions as A and B, cells were stained with DAPI, rhodamine-phalloidin, and vinculin antibody. Images were captured with confocal laser microscopy at 40× magnification and enlarged 2X in silico. Focal adhesions were indicated by arrow heads. (D) SKOV-3 cells were pretreated with various inhibitors for 20 min then stimulated with LPA (10 μM) for 3 min. Activation of RhoA was determined by measuring the amount of GTP-bound RhoA as described in 'Section 2'. *$P < 0.05$.

calcium redistributes Rho GTPase localization and regulates axonal guidance in neuronal cells [40]. In correlation with this, inhibition of calcium mobilization or CaMKII drastically regulated LPA-induced focal adhesion formation in SKOV-3 cells (Fig. 4C). In particular, LNA significantly abolished LPA-induced focal adhesion formation whereas the ethyl ester form of LNA (ELN) was not effective. These results might account for the involvement of Rho GTPase in the regulation of LPA-induced focal adhesion formation in SKOV-3 cells. Indeed, LPA-dependent activation RhoA was significantly abolished by calcium channel blocker or inhibitors of CaMKII signaling pathways (Fig. 4D). Notably, LNA also blocked LPA-dependent RhoA activation whereas ethyl ester form of LNA (ELN) had no effect. Therefore, these results suggest that omega-3 regulates cancer cell migration through inactivation of RhoA signaling pathways. Currently, downstream signaling pathway of RhoA is still ambiguous. However, recent evidences suggest that phospholipase D (PLD) is overexpressed in the cancer tissues and its PLD-dependent cancer cell migration is regulated by small G proteins such as Arf, Rac, and Rho [41]. Therefore, it is possible that PUFAs regulates PLD activity through modulation of upstream small G proteins such as RhoA.

In conclusion, cancer cell migration is suppressed by PUFAs such as omega-3 and omega-6 and the carboxylic moiety is important for functional activity. Inhibition of calcium mobiliza-

tion and subsequent activation of CaMKII/RhoA signaling pathway might be the mechanistic pathway for inhibition of focal adhesion formation as well as migration. These results provide mechanistic insight into the inhibition of cancer cell migration based on structural properties.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.05.052.

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