Visualization of the binding between gintonin, a Panax ginseng-derived LPA receptor ligand, and the LPA receptor subtypes and transactivation of the EGF receptor

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1. Introduction

Ginseng, the root of Korean Panax ginseng Meyer, has been used as a tonic in traditional medicine to maintain vitality and health [1].

Recent reports have shown that gintonin is a novel bioactive ingredient of ginseng and exists in addition to classical ginseng ginsenosides and acidic polysaccharides. Gintonin is a bio-complex of fatty acids, lysophospholipids, phospholipids, and unique ginseng proteins [2]. The functional component of gintonin is lysophosphatidic acid (LPA, 1-acyl-2-hydroxy-sn-glycero-3-phosphate), which is a simple lysophospholipid that plays diverse and important roles in cell migration, proliferation, and survival
through G protein-coupled LPA receptor subtypes. The order of amounts of LPAs found in gintonin is LPA C18:2 > LPA C16:0 > LPA C18:1 [3]. Previous studies from our lab suggest that LPAs bound to gintonin are only partially released after long-term methanol extraction, indicating that LPAs form a remarkably robust complex with gintonin components [4]. Moreover, gintonin exerts various physiologic and pharmacological effects on various organ systems in vitro and in vivo [5]. However, no visual evidence is available on the probable binding between gintonin and the LPA receptor subtypes and it remains uncertain whether gintonin can transactivate any growth factor-related receptor, although gintonin demonstrates functions as an exogenous ginseng-derived G protein-coupled LPA receptor ligand and data suggest that LPA can transactivate the EGF receptor via the LPA1 receptor [6].

In the present study, we constructed gintonin-biotin conjugates, following which we examined whether gintonin could bind to colocalized sites with LPA receptor subtypes and performed experiments to ascertain if biotinylated gintonin could also transactivate the EGF receptor.

2. Materials and methods

2.1. Materials

Gintonin, devoid of ginseng saponins, was isolated from Korean *Panax ginseng* according to previously described methods [4]. Ki16425 was purchased from Cayman Chemicals (Ann Arbor, MI, USA), and AG1478 and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against LPA1, LPA2, and LPA6 (Cat #: ALR-031, 032, 036) were obtained from Alomone labs (Jerusalem, Israel), and those against LPA3 and LPA4 (Cat #: ab23692, 203,290) were purchased from Abcam (Waltham, MA, USA); LPA5 antibody (Cat #: orb157371) was obtained from Biozbyt (Cambridgeshire, UK). The antibodies for phospho-Src and total Src were purchased from Thermo Fisher Scientific, Inc. (Carlsbad, CA, USA) and MyBioSource (San Diego, CA, USA), respectively. The antibodies for the phospho-EGF receptor (Tyr 1173) and total EGF receptor were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Biotinylation of gintonin

To fabricate gintonin-Biotin conjugates, gintonin was biotinylated using the EZ-Link™ Sulfo-NHS–LC-Biotinylation Kit (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. The number of biotin molecules that bound to the gintonin protein region was determined by performing the HABA assay according to the manufacturer’s instructions.

2.3. Western blotting

Cells were harvested and subjected to lysis on ice using a modified RIPA buffer (50 mM Tris-HCl; pH 8.0, 150 mM sodium chloride, 1 % NP-40, 0.25 % sodium deoxycholate, 1 mM EGTA) and protease inhibitor cocktail (1:100; Sigma-Aldrich). Lysates were cleared by centrifugation at 14,000 rpm for 5 min at 4 °C. Cell lysates (50 µg) were loaded on 10 % SDS-PAGE and blotted onto an Immobilon 0.45 mm polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked for 1 h in 5 % BSA using Tris-buffered saline (pH 7.4) containing 0.1 % Tween 20 (TBST), followed by overnight incubation with the primary antibody. Membranes were washed in TBST, and they were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:3000 in TBST) at room temperature. Blots on

the membranes were developed using the enhanced chemiluminescence Western blotting detection system kit (ECL Plus; Amersham, Little Chalfont, UK) and detected using a luminescent image analyzer LAS-4000. Densitometric analyses of the bands were performed using the Multi Gauge software (Fujifilm, Tokyo, Japan).

2.4. Measurement of [Ca²⁺]i concentrations

Intracellular Ca²⁺ influx was evaluated by performing a dual excitation spectrofluorometric analysis with Fura-2 AM (Ex: 340 nm and 380 nm). Intracellular free calcium levels of cells were assayed using HEPES-buffered saline solution (HBS, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4). Emitted light was reflected through a 515-nm long-pass filter to a cooled charge-coupled device (CCD) camera and ratios of emitted fluorescence were calculated using a digital fluorescence analyzer. All imaging data were collected and analyzed using the MetaFluor software (Universal Imaging Corp. Downing, PA, USA).

2.5. Immunofluorescence labeling, imaging, and quantification of fluorescence signals

Cells were seeded on alcohol-sterilized, flamed coverslips and were rinsed in PBS; thereafter, cells were subjected to treatments with either gintonin or biotin-gintonin for 15 min. Cells were subjected to fixation with 4 % paraformaldehyde for 20 min at room temperature, followed by subjection to washing steps three times with PBS. Subsequently, cells were permeabilized with 1 % Triton X-100 for 30 min. For removing the endogenous biotin, cells were subjected to treatment with endogenous biotin blocking solution (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. Cells were subjected to immunostaining procedures with anti-LPA1 or LPA6 IgG antibodies (1:1000, Alomone, Poway, CA, USA) overnight at 4 °C. Subsequently, the cells were incubated with anti-rabbit IgG serum conjugated (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) with either Alexa 488 (green fluorescence) or streptavidin, Alexa 647 (red fluorescence) for 2 h at 37 °C. Thereafter, cells were subjected to washing steps with PBS, followed by subjection to a single wash in DDW, and were mounted with an antifade mounting medium (Bio-Rad, Hercules, CA, USA). Immunofluorescence images were captured using the Axio200 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany).

2.6. Cell migration

Cell migration was assessed by using modified Boyden chambers (Neuro Probe, Gaithersburg, MD, USA). The underside of membranes were coated with 10 µg/ml rat tail collagen (BD Biosciences, Franklin Lakes, New Jersey, USA) at room temperature for 1 h. Briefly, cells were subjected to washing steps twice with PBS and were re-suspended in serum-free RPMI1640 medium. The lower compartment was filled with gintonin or gintonin-biotin in serum-free RPMI1640. Cells were serum-starved for 2 h 30 min, following which cell suspensions (4 × 10⁶ cells/well) were added to the upper compartment and cells were incubated for 5 h at 37 °C. In other experiments, cells were preincubated with or without an inhibitor for 0.5 h, followed by incubation with gintonin or gintonin-biotin in serum-free RPMI1640 medium. After incubation for 5 h at 37 °C, migrated cells on the membrane were subjected to fixation, staining procedures with Diff Quik (Sysmex, Kobe, Japan), and enumeration via light microscopy (Eclipse 80i; Nikon, Tokyo, Japan).
2.7. Data analysis

All quantitative data are presented as mean ± SD. The differences in averages were statistically analyzed by performing a one-way or two-way analysis of variance (ANOVA), followed by a Bonferroni’s post hoc test; all quantitative data are presented as mean ± SD. A p value of <0.01 has been considered significantly.

3. Results

3.1. Construction of gintonin-biotin conjugates

Although LPA is a lysophospholipid with various biological activities and acts as an LPA receptor ligand, labeling with fluorescent tags for the conduction of LPA binding studies at cytochemical levels is not easy. In the present study, we developed and established a novel biotin labeling method of investigating the gintonin protein region using Sulfo-NHS-LC-biotin, which can easily be used to biotinylate amine groups of proteins. We also isolated pure biotinylated gintonin using a simple procedure [8] (see Materials and methods). The fabrication of gintonin-biotin conjugates was confirmed via western blotting for biotinylated gintonin. As shown in Figure S1, only the biotinylated gintonin was identified through streptavidin-HRP staining. We further confirmed the number of biotin molecules that could bind to the gintonin protein region using HABA assay [8]. The number of biotin molecules per gintonin (MW = 67,000 kD) [4] was estimated to be 2.99 ± 0.13.

3.2. Gintonin-biotin conjugates bind to the plasma membrane sites that are co-localized with LPA1/6 receptors

Next, we studied whether gintonin-biotin conjugates could bind to the plasma membrane of PC3 cells and compared gintonin-biotin conjugates with unbiotinylated gintonin using streptavidin-Alexa fluor 647 (red color). As shown in Fig. 1A and B, treatment of unbiotinylated gintonin following the addition of streptavidin-Alexa fluor 647 to PC3 cells did not present with a strong intensity of red fluorescence; however, treatment involving the use of streptavidin-Alexa fluor 647 after the addition of gintonin-biotin conjugates demonstrated strong intensities of red fluorescence throughout the cells expressing LPA1-LPA6 receptor subtypes. Interestingly, the binding sites of gintonin-biotin conjugates were mainly observed near the oval shaped cell body rather than cell processes in all LPA receptor subtypes. When we compared the fluorescence intensity of streptavidin-Alexa fluor 647 in all LPA receptor subtypes, we found that LPA1 and LPA6 receptor subtypes showed stronger staining intensity than that observed in the remaining LPA receptor subtypes and the order of fluorescence intensity was LPA6 > LPA1 > LPA2 = LPA3 = LPA4 = LPA5 (Fig. 1C and D). Next, we used six different antibodies against each respective LPA receptor subtype. All antibodies used were generated against the extracellular domains of LPA receptors. The binding sites of each LPA receptor subtype was detected by using Alexa fluor 488 (green fluorescence). Interestingly, the LPA1/6 receptor subtypes were detected at the plasma membranes but other LPA receptor subtypes were mainly detected in the cytoplasm, despite the LPA2-LPA5 receptor subtype antibodies being generated at the extracellular domain. When we performed experiments to merge gintonin-biotin conjugates with LPA receptor subtypes, we found that the merged gintonin-biotin conjugates (indicated in red color) and LPA1/6 receptor subtypes (indicated in green color) exhibited white color at the plasma membrane; however, the remaining LPA receptor subtypes were stained separately between the cytosol for the LPA receptor and the membrane for the gintonin-biotin conjugates, indicating that gintonin binding sites were co-localized with the LPA1/6 receptor at the plasma membrane.

![Image](image_url)

**Fig. 1.** Immunofluorescence images depicting the effects of unbiotinylated gintonin (GT), gintonin-biotin conjugates (GT-Biotin), and LPA receptor subtypes in PC3 cells. Cells were subjected to treatment with (A) 1 µg/ml GT or (B) 1 µg/ml GT-Biotin for 15 min. Red fluorescence for detecting against biotins using Streptavidin, Alexa-647 conjugated secondary antibody (detect as red fluorescence). Green fluorescence for detecting against LPA1-6 receptors using LPA receptor subtype specific antibody and Alexa-488 conjugated secondary antibody (detect as green fluorescence). (C) and (D) depict the quantitation results of Alexa-488 and 647 fluorescence intensity determined using the Axio200 inverted fluorescence microscope. The intensity of fluorescence was analyzed by using one-way ANOVA followed by a Bonferroni’s post hoc test (*p < 0.01, GT vs GT-Biotin). Scale bar = 40 μm.
3.3. *siRNA against LPA1/6 receptors abolishes gintonin-biotin conjugate binding at the plasma membrane*

To confirm the binding of gintonin-biotin conjugates with the extracellular co-localized sites of LPA1/6 receptor subtypes, we used siRNAs against both LPA1 and LPA6 receptor subtypes. As shown in Figures S2 and S3, siRNA treatment abolished gintonin-biotin conjugate binding and affected the results of LPA1 and LPA6 receptor subtype staining, compared to the treatment performed with scrambled siRNA. Scrambled siRNA treatment revealed colocalization at the plasma membrane in merged gintonin-biotin conjugate (red color) and LPA1/6 receptor subtype images. These results indicate that gintonin-biotin conjugates bind to specific co-localized sites on LPA1/6 receptor subtypes, respectively.

3.4. Gintonin biotinylation does not affect gintonin-mediated \([\text{Ca}^{2+}]_i\) transient in PC3 cells

We examined whether biotinylation of gintonin affected cellular \([\text{Ca}^{2+}]_i\) transient in PC3 cells that expressed all six LPA receptor subtypes at different levels. As shown in Fig. 2C and S4, it can be inferred that there is no significant difference in the elicitation of \([\text{Ca}^{2+}]_i\) transient between unbiotinylated gintonin and biotinylated gintonin treatment in PC3 cells. Use of the LPA1/3 receptor antagonist, K16425, attenuated gintonin-biotin conjugates-mediated \([\text{Ca}^{2+}]_i\) transient (Fig. S4A), suggesting that gintonin-biotin conjugates also activate the LPA1/3 receptors. These results indicate that biotinylation of the gintonin protein region does not exert effects on physiological aspects of \([\text{Ca}^{2+}]_i\) transient.

3.5. *Repeated treatments of gintonin-biotin conjugates induce changes in cell morphology and desensitization of \([\text{Ca}^{2+}]_i\) transient*

We further examined the binding changes in gintonin-biotin conjugates and LPA1 receptor antibody upon repeated treatment of cells with gintonin-biotin conjugates. Additionally, we also assessed physiological changes in \([\text{Ca}^{2+}]_i\), transient under these conditions. Interestingly, repeated treatment with gintonin-biotin conjugates induced circular morphology instead of the normal bipolar morphology observed with processes. Additionally, the binding patterns of both gintonin-biotin conjugates and antibody against LPA1 receptor subtypes also changed into surrounding whole form of round cell membrane compared to single treated cells (Fig. 2A), in which gintonin-biotin conjugates and antibody against LPA1 receptor mainly bind to around cell bodies but not cell processes (Fig. 2B). Interestingly, repeated treatment of gintonin-biotin conjugates resulted in the generation of diffused cytoplasmic staining patterns of gintonin-biotin conjugates and antibody against the LPA1 receptor complex rather than a limited staining pattern at the plasma membrane observed upon single treatment. Interestingly, repeated treatment with gintonin-biotin conjugates also caused a desensitization of \([\text{Ca}^{2+}]_i\) transient that was observed at first treatment (Fig. 2C). Thus, these results together suggest that gintonin-biotin conjugate-mediated desensitization of \([\text{Ca}^{2+}]_i\) transient may be associated with the
morphological changes occurring in cells and the distribution of the gintonin-biotin conjugate-LPA1 receptor complex.

3.6. Gintonin-biotin conjugates transactivate the EGF receptor

Previous reports have shown that activation of LPA receptors is coupled to the transactivation of the EGF receptor [6,9]. However, it was not demonstrated whether gintonin-biotin conjugates could also transactivate the EGF receptor. In the present study, we examined whether gintonin-biotin conjugate treatment was associated with the transactivation of the EGF receptor. Therefore, we first determined whether gintonin-biotin treatment increased phosphorylation levels of the EGF receptor. As shown in Figure S5A (green color), the binding of gintonin-biotin conjugates (white arrows in Figure S5A) as well as the conduction of unconjugated gintonin treatment significantly increased the intensity of fluorescence staining for phospho-EGF receptors in immunocytochemical assay compared to the control. Additionally, western blotting showed that gintonin-biotin conjugate treatment increased the levels of phospho-EGF receptor and use of the LPA1/3 receptor antagonist, K1i6425, attenuated gintonin-biotin conjugate-mediated increases in phospho-EGF receptor expression (Fig. 3A, B and C), suggesting that gintonin-biotin conjugates transactivated the EGF receptor via the LPA1/3 receptor. Also application of these cells with gintonin-biotin conjugates was phosphorylated c-Src at 3 min but not 30 min (Fig. 4A–C and Fig. 4D–F). Since the EGF receptor activation is coupled to c-Src and ERK activation, we also examined whether gintonin-biotin conjugate treatment increased phospho-Src and phospho-ERK levels. Treatment of these cells with gintonin-biotin conjugates phosphorylated c-Src and ERK, as shown in Fig. 3D, E, and F, and Fig. 4.

Since it is known that the EGF receptor-cSRC-ERK pathway is physiologically coupled to cell migration, gintonin-biotin conjugates may be used to stimulate cell migration. When we examined cell migration upon treatment with gintonin-biotin conjugates along with an EGF receptor antagonist, AG1478, cell migration was inhibited compared to that observed in the gintonin-biotin conjugate-control (Fig. 5F). Thus, activation of the gintonin-biotin conjugate-mediated LPA receptor transactivates the EGF receptor via c-Src phosphorylation and activated EGF receptor is also coupled to ERK activation, resulting in cell migration that is blocked by the EGF receptor inhibitor (Fig. 6).
4. Discussion

Although LPAs function via a G protein-coupled LPA receptor ligand for their diverse physiological and pharmacological activities [10], LPAs cannot be easily labeled with isotopes or fluorescent tags for ligand binding studies without creating changes in its innate chemical properties, since they are small molecules [11]. Additionally, LPAs are vulnerable to hydrolysis with a short half-life and cannot perform their role as a lipid-derived growth factor [12]. In previous reports, we have demonstrated that gintonin, a ginseng-derived LPA receptor ligand, exhibits diverse biological effects ranging from in vitro neurotransmitter release and synaptic transmission to in vivo neuroprotective functions [13–16]. One of the merits of gintonin is that gintonin LPAs tightly bind to ginseng protein components and are poorly hydrolyzed in aqueous solutions, and this prolongs its cellular effects [17,18]. We utilized these properties of gintonin for biotin labeling of the protein component of gintonin, since amine groups of gintonin protein components can be easily biotinylated to produce biotin conjugates. Therefore, we developed a gintonin-biotinylation and streptavidin-fluorescent tag method to characterize gintonin binding to endogenous LPA receptors expressed on the plasma membrane.

In the present study, we constructed, for the first time, gintonin-biotin conjugates with three purposes. The first purpose was to visualize ginseng gintonin binding to LPA receptor subtypes, since there is no report available to prove that ginseng component(s) act as a ligand and directly bind at the plasma membrane target proteins, thereby eliciting biological effects. The second purpose was to ascertain whether repeated treatment of gintonin could change binding patterns of biotinylated gintonin and antibody against LPA receptors. Additionally, the aim was to determine if the biotinylation of amine residues of the protein region affected the biological effects of gintonin. The final purpose was to visualize if the binding of gintonin-biotin conjugates could result in the transactivation of EGF receptors.

We found that gintonin biotinylation did not affect cellular effects. The levels of $[\text{Ca}^{2+}]_i$ transient were not affected as compared to those observed with the treatment involving the use of unbiotinylated gintonin (Fig. 2 and S4), suggesting that the protein component of gintonin, which is directly biotinylated biotin, not directly related to gintonin functions (as an LPA receptor ligand). This finding is consistent with that reported in a previous study, indicating that trypsin digestion of gintonin does not affect gintonin-mediated $\text{Ca}^{2+}$-activated $\text{Cl}^-$ channel activation in...
Xenopus oocytes [19]. Additionally, using gintonin-biotin conjugates, we could visualize gintonin binding on the cell membrane with two different streptavidin-fluorescent labeling systems (Fig. 1). Interestingly, we found that in PC3 cells, gintonin was highly co-localized at sites of LPA1/6 receptors rather than the sites with other four LPA receptor subtypes. PC3 cells express all 6 types of LPA receptor subtypes; however, the LPA1/6 receptor subtypes are predominant compared to other LPA receptor subtypes [20,21].
Thus, the reason that gintonin-biotin conjugates bind to co-localized sites with LPA1/LPA6 receptors may be associated with and proportional to the expression levels of endogenous LPA receptor subtypes [22]. Additionally, the binding pattern of both gintonin-biotin conjugates and LPA1/6 receptors is mainly observed at cell membranes rather than at long cellular processes, indicating that LPA1/6 receptors may be more localized on cellular bodies rather than at cell processes (Figs. 1 and 2). Moreover, the immunocytochemical analyses using antibodies against LPA2, LPA3, LPA4, and LPA5 receptor subtypes revealed an increased intensity of cytosolic staining compared to that observed at the plasma membrane, although the antibodies were generated for binding with the plasma membrane LPA receptors.

Next, when we examined whether repeated treatment of gintonin-biotin conjugates affected the distribution of both gintonin-biotin conjugates and LPA1 receptors. We found that both the LPA1 receptor and the gintonin-biotin conjugates were localized in the cytoplasm rather than at the plasma membrane (Fig. 2A and B). This suggested that the binding of gintonin-biotin conjugate to LPA1 receptor might induce endocytosis of the gintonin-biotin/LPA1 receptor complex via LPA1 receptor desensitization [23,24]. Supporting this assumption is the observation that repeated treatments with the gintonin-biotin conjugates do not further elicit $[\text{Ca}^{2+}]_i$ transient in PC3 cells (Fig. 2C). Consequently, repeated treatments with the gintonin-biotin conjugates rendered morphological changes from an oval shape to a round shape without any processes; however, both gintonin-biotin conjugates and an antibody against the LPA1 receptor binds at the periphery of the cells (Fig. 2B). These results also provide an evidence that repeated gintonin binding to the LPA1 receptor induces desensitization, accompanying morphological changes in the distributions of gintonin-biotin conjugates.

Activation of the EGF receptor leads to the regulation of diverse physiological and pathophysiological phenomena in multiple organ systems including cell proliferation and migration [25,26]. In the present study, we found that gintonin-biotin conjugates trans-activated the c-Src-EGF receptor-ERK pathway via LPA1 receptor regulation in PC3 cells. The use of an EGF receptor inhibitor blocked gintonin-biotin conjugate-mediated cell migration, thereby indicating that the binding of gintonin-biotin conjugates to the LPA1 receptor could transactivate the EGF receptor even in the absence of EGF. On the other hand, ginsenosides such as ginsenoside Rd and Rg3 exhibited anti-tumor activity through inhibition of EGF receptor-ERK pathway activation or by increasing EGF regulation to mediate EGF-dependent cell migration [27,28]. Thus, the previous studies based on the use of ginsenosides, and the present study based on the use of gintonin-biotin conjugates, show a possibility that although both are ginseng components, their biological activities are different from each other. These results also show a possibility that gintonin and ginsenoside may exhibit differential effects in biological systems [13].

In summary, for the first time, we visualized gintonin binding sites in cell membranes using biotinylated gintonin and fluorescent tag. Moreover, we also proved that LPA receptor subtypes co-localized with gintonin-biotin conjugates at the plasma membrane. We further demonstrated that gintonin could exhibit its biological effects through transactivation of the EGF receptor-Src-ERK pathway via LPA receptor regulation. Finally, we could provide a visual evidence that ginseng gintonin bound directly to the LPA receptor subtypes to mediate its physiological and pharmacological effects via transactivation of the EGF receptor.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.10.004.

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