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Research paper

# Gintonin stimulates gliotransmitter release in cortical primary astrocytes

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## HIGHLIGHTS

- $G\alpha_q$/11 protein-coupled receptor-mediated $[Ca^{2+}]_i$ transients of astrocytes are coupled to release of gliotransmitters.
- Gintonin treatment to astrocytes activates $[Ca^{2+}]_i$ transients pathway via LPA receptor activation.
- Gintonin-mediated $[Ca^{2+}]_i$ transients are coupled to release of ATP and glutamate.
- Gintonin regulates gliotransmitter release via LPA receptor activation in primary astrocytes.

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## ABSTRACT

Lysophosphatidic acid (LPA) is a simple and minor phospholipid, but serves as a lipid-derived neurotransmitter via activation of G protein-coupled LPA receptors. Astrocytes abundantly express LPA receptors and contain gliotransmitters that modulate astrocyte-neuron interactions. Gintonin is a novel ginseng-derived G protein-coupled LPA receptor ligand. Gintonin induces $[Ca^{2+}]_i$ transients in neuronal and non-neuronal cells via activation of LPA receptors, which regulate calcium-dependent ion channels and receptors. A line of evidence shows that neurotransmitter-mediated $[Ca^{2+}]_i$ elevations in astrocytes are coupled with gliotransmitter release. However, little is known about whether gintonin-mediated $[Ca^{2+}]_i$ transients are coupled to gliotransmitter release in astrocytes. In the present study, we examined the effects of gintonin on adenosine triphosphate (ATP) and glutamate release in mouse cortical primary astrocytes. Application of gintonin to astrocytes induced $[Ca^{2+}]_i$ transients in a concentration-dependent and reversible manner. However, ginsenosides, other active ingredients in ginseng, had no effect on $[Ca^{2+}]_i$ transients. The induction of gintonin-mediated $[Ca^{2+}]_i$ transients was attenuated/blocked by the LPA1/3 receptor antagonist Ki16425, a phospholipase *C* inhibitor, an inositol 1,4,5-triphosphate receptor antagonist, and an intracellular $Ca^{2+}$ chelator. Gintonin treatment on astrocytes increased ATP and glutamate release in a concentration- and time-dependent manner. BAPTA and Ki16425 attenuated gintonin-mediated ATP and glutamate release in astrocytes. The present study shows that gintonin-mediated $[Ca^{2+}]_i$ transients are coupled to gliotransmitter release via LPA receptor activation. Finally, gintonin-mediated $[Ca^{2+}]_i$ transients and gliotransmitter release from astrocytes via LPA receptor activation might explain one mechanism of gintonin-mediated neuromodulation in the central nervous system.

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*Abbreviations:* GT, gintonin; LPA, lysophosphatidic acid; [Ca2+]i, intracellular calcium concentration; ATP, adenosine triphosphate; GLP, ginseng major latex-like protein; FBS, fetal bovine serum; HEPES, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid.

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## 1. Introduction

Lysophosphatidic acid (LPA) is a simple phospholipid, but the actions of LPA are diverse with a myriad effects on animal nervous systems [1]. Endogenous LPAs are involved in various events through lipid-derived growth factor-like behaviors. The primary cellular actions of LPA are to elicit transient increases in the intracellular calcium concentration $[Ca^{2+}]_i$ and induce cell proliferation, differentiation, morphological changes, migration, and survival through the activation of LPA receptors. The activations of G protein-coupled LPA receptors are coupled to diverse activities such as brain development, angiogenesis, embryo implantation, spermatogenesis, and wound healing. On the other hand, astrocytes closely modulate neuronal activities by forming a tripartite synapse and releasing gliotransmitters in addition to metabolic support and protection of neurons in the brain [2,3]. Recent studies have shown that astrocytes express LPA receptors abundantly [4,5], although the role of LPA receptors in astrocyte-neuron interactions is not well understood. There are reports that LPA treatment to astrocytes induces neuronal differentiation and axonal outgrowth of neurons, supporting evidence of LPA-mediated release of growth factors via LPA receptor signaling pathways indirectly affecting neuronal activity [6]. However, relatively little is known about LPA effects on gliotransmitter release in astrocytes.

Ginseng is a traditional herbal medicine with diverse biological effects. Recent study shows ginseng contains a novel ginseng-derived LPA receptor ligand, gintonin [7]. Gintonin consists of LPAs such as LPA $C_{18:2}$, LPA $C_{18:1}$, and LPA $C_{16:0}$, as well as ginseng major latex-like protein (GLP) 151 and ginseng ribonuclease-like storage protein [7]. In particular, gintonin induced $[Ca^{2+}]_i$ transients with a low $EC_{50}$ values in cells expressing LPA1, LPA2, LPA3, or LPA5 receptor subtypes, indicating that gintonin is a novel high affinity ligand to these LPA receptors [7]. The primary action of gintonin is to induce $[Ca^{2+}]_i$ transients through activation of G protein-coupled LPA receptor signaling pathways [7]. In addition, gintonin-mediated LPA receptor activation, and the following $[Ca^{2+}]_i$ transients, are linked to the regulation of intracellular $Ca^{2+}$-dependent ion channels and receptors [8]. Further studies showed that gintonin mediated-$[Ca^{2+}]_i$ transients are also coupled to interneuron communication [9]. Although previous reports raise the possibility that gintonin-mediated $[Ca^{2+}]_i$ transients via LPA receptors play a key role in intra- and inter-neuronal communication, it has not been demonstrated that gintonin-mediated activation of G protein-coupled LPA receptors in primary astrocytes is coupled to the regulation of gliotransmitter release.

In the present study, we examined the effects of gintonin on $[Ca^{2+}]_i$ transients and the release of adenosine triphosphate (ATP) and glutamate in cultured mouse cortical astrocytes. We report that gintonin induces $[Ca^{2+}]_i$ transients via membrane signaling transduction pathways of LPA receptors, and that gintonin-mediated $[Ca^{2+}]_i$ transients are coupled to the stimulation of ATP and glutamate release. Additionally, we discuss the pharmacological roles of gintonin-mediated neuromodulation via the release of gliotransmitters from astrocytes.

## 2. Materials and methods

### 2.1. Materials

Gintonin, devoid of ginseng saponins, was prepared from *Panax ginseng* according to previously described methods [10]. Gintonin was dissolved in deionized water and diluted with medium before use. Dulbecco's Minimum Essential Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Camarillo, CA, USA). LPA (1-oleoyl-2-hydroxy-*sn*-Glycero-3-phosphate, 857130P) was purchased from Avanti Polar Lipids, Inc. (Alabama, USA). All other reagents, including ATP and *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (HEPES), were purchased from Sigma–Aldrich (St Louis, MO, USA).

### 2.2. Mouse cortical primary astrocytes culture

Primary astrocyte cultures were prepared from the cerebral cortices of postnatal day 1 ICR (CD-1®) mice according to the method of Shano et al. [5]. Briefly, primary astrocyte cultures were prepared from the cerebral cortices of 1-day-old neonatal ICR mice. Cells were seeded in culture plates coated with poly-L-lysine hydrobromide (100 μg/mL; Sigma–Aldrich) and grown in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% $CO_2$ at 37 °C. At the time of primary cell confluence (5–7 days), cells were harvested with a 0.05% trypsin/EDTA solution (Life technologies, Carlsbad, CA), seeded in culture plates previously treated with poly-L-lysine hydrobromide (100 μg/mL; Sigma–Aldrich), and grown in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin for further experiments.

### 2.3. Measurement of intracellular calcium concentration

The free $[Ca^{2+}]_i$ was measured by dual excitation spectrofluorometric analysis of cell suspensions loaded with Fura-2 AM, as previously described [7]. Briefly, astrocytes were harvested with a trypsin/EDTA solution and re-suspended in a HBS. The cells were incubated with Fura-2 AM (final concentration 2.5 μM) in HBS at 37 °C for 40 min. Extracellular Fura-2 AM was removed by centrifugation. Each aliquot of $3 \times 10^6$ cells was loaded into a cuvette and free calcium mobilization was measured using a RF-5301PC spectrofluorophotometer and Supercap software (Ex: 340 nm and 380 nm; Em: 520 nm) (Shimadzu, Tokyo, Japan).

### 2.4. Measurement of ATP and glutamate release

For ATP assay experiments, cells were seeded on 24-well plates at a density of $4 \times 10^4$ cells per well. For measurement of ATP release, astrocytes were treated with DMEM in the presence or absence of gintonin at 37 °C. For glutamate assay experiments, cells were seeded on 6-well plates at a density of $2 \times 10^5$ cells per well. To determine glutamate release, astrocytes were treated with HEPES-buffered saline solution (HBS: 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, 25 mM HEPES, pH 7.4) in the presence or absence of gintonin at 37 °C. The supernatants from control and treated astrocytes were collected, centrifuged at 12,000 rpm for 5 min, and the concentrations of ATP and glutamate in the supernatant were determined according to the manufacturer's instruction—ATP Bioluminescent Assay Kit (Sigma–Aldrich) and Glutamate Assay Kit, fluorometric (Abcam, Cambridge, MA), respectively.

### 2.5. Data analysis

To obtain concentration-response curves for the effects of gintonin on $[Ca^{2+}]_i$ transients, the peak increase of $[Ca^{2+}]_i$ transient amplitudes at different concentrations of gintonin were plotted. Origin software (OriginLab, Northampton, MA, USA) was used to fit the data to the Hill equation: $y/y_{max} = [A]^{nH}/([A]^{nH} + [EC_{50}]^{nH})$, where $y$ is the peak at a given concentration of gintonin, $y_{max}$ is the maximal peak in the absence of gintonin, $EC_{50}$ is the concentration of gintonin producing a half-maximal effect, $[A]$ is the concentration of gintonin, and nH is the Hill coefficient. All values are presented as the mean ± the standard error of the mean (S.E.M).

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