Molecular Neurobiology (2019) 56:3280–3294
https://doi.org/10.1007/s12035-018-1308-1

Gintonin, a Ginseng-Derived Exogenous Lysophosphatidic Acid Receptor Ligand, Protects Astrocytes from Hypoxic and Re-oxygenation Stresses Through Stimulation of Astrocytic Glycogenolysis

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Received: 24 October 2017 / Accepted: 8 August 2018 / Published online: 16 August 2018

Abstract

Astrocytes are a unique brain cell-storing glycogen and express lysophosphatidic acid (LPA) receptors. Gintonin is a ginseng-derived exogenous G protein-coupled LPA receptor ligand. Accumulating evidence shows that astrocytes serve as an energy supplier to neurons through astrocytic glycogenolysis under physiological and pathophysiological conditions. However, little is known about the relationships between LPA receptors and astrocytic glycogenolysis or about the roles of LPA receptors in hypoxia and re-oxygenation stresses. In the present study, we examined the functions of gintonin-mediated astrocytic glycogenolysis in adenosine triphosphate (ATP) production, glutamate uptake, and cell viability under normoxic, hypoxic, and re-oxygenation conditions. The application of gintonin or LPA to astrocytes induced glycogenolysis in concentration- and time-dependent manners. The stimulation of gintonin-mediated astrocytic glycogenolysis was achieved through the LPA receptor-G$\alpha_{q/11}$ protein-phospholipase C-inositol 1,4,5-trisphosphate receptor-intracellular calcium ($[Ca^{2+}]_i$) transient pathway. Gintonin treatment to astrocytes increased the phosphorylation of brain phosphorylase kinase, with sensitive manner to K252a, an inhibitor of phosphorylase kinase. Gintonin-mediated astrocytic glycogenolysis was blocked by isofagomine, a glycogen phosphorylase inhibitor. Gintonin additionally increased astrocytic glycogenolysis under hypoxic and re-oxygenation conditions. Moreover, gintonin increased ATP production, glutamate uptake, and cell viability under the hypoxic and re-oxygenation conditions. Collectively, we found that the gintonin-mediated $[Ca^{2+}]_i$ transients regulated by LPA receptors were coupled to astrocytic glycogenolysis and that stimulation of gintonin-mediated astrocytic glycogenolysis was coupled to ATP production and glutamate uptake under hypoxic and re-oxygenation conditions, ultimately protecting astrocytes. Hence, the gintonin-mediated astrocytic energy that is modulated via LPA receptors helps to protect astrocytes under hypoxia and re-oxygenation stresses.

Keywords Gintonin · LPA receptor · Astrocytes · Glycogenolysis · Hypoxia · Cell viability

Introduction

Lysophosphatidic acid (LPA) is a precursor for phosphatidic acid synthesis [1] and found in the mammalian brain [2]. Although LPA is a simple phospholipid, it exhibits diverse cellular effects in the central and peripheral nervous systems [1]. These diverse cellular effects of LPAs are achieved through its six G protein-coupled LPA receptors, which are

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-018-1308-1) contains supplementary material, which is available to authorized users.

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expressed in most cells and organs. The common cellular actions of LPA are to induce intracellular calcium ($[Ca^{2+}]_i$) transients via pertussis toxin-sensitive and toxin-insensitive G proteins. Further, LPA-mediated $[Ca^{2+}]_i$ transients are coupled to diverse in vitro and in vivo effects in early brain development [1]. In the adult brain, LPA receptors also play important roles in hippocampal neurogenesis and in learning and memory functions. Indeed, LPA1 receptor-deficient mice exhibit reductions in hippocampal neurogenesis [3] and impaired performance in hippocampus-mediated spatial memory and cognitive tests [4–6].

Astrocytes are the most abundant cells in the brain and exhibit various functions in normal and abnormal conditions [7]. Compared with other brain cells, one unique property of astrocytes is its ability to store glycogen for brain energy metabolism and for supporting neurons during higher-order brain functions such as cognitive processes [8] and pathophysiological conditions such as hypoxia [9]. According to the astrocyte-neuron lactate shuttle hypothesis, astrocytes metabolize glycogen to lactate, which is then diffused to neurons for oxidative metabolism [10]. Thus, the main function of brain astrocytic glycogen is to serve as an energy reservoir for normal and abnormal conditions, in which the glucose supply to the brain through blood vessels is impaired, such as during hypoxia, ischemia, and hypoglycemia [11].

Recent studies have shown that the LPA concentrations in the brain are relatively high compared with the levels in other organs [2, 12] and that the LPA levels in the brain and cerebral spinal fluid (CSF) are significantly elevated during traumatic injury, including hypoxia [13, 14]. Research has also revealed that traumatic brain injury can induce an increase in LPA receptor gene expression in mouse cortical and spinal cord astrocytes [14, 15]. Hence, the LPA and LPA receptors found in astrocytes may play important roles in brain energy metabolism under physiological and pathophysiological conditions. However, relatively little is known about LPA-mediated astrocytic glycogenolysis in normal and abnormal states.

Ginseng, one type of herbal medicine, is used as a tonic and exhibits anti-neurodegenerative and neuroprotective effects under ischemic or traumatic states [16, 17]. Since ginseng extracts contain various ingredients, it is unknown which ingredient(s) of the ginseng extract are responsible for the neuroprotective effects that are observed during hypoxic ischemia. Furthermore, the molecular mechanisms underlying ginseng's neuroprotective effects against brain ischemia remain unknown [18]. One recent study showed that ginseng extract includes a novel ginseng-derived exogenous G protein-coupled LPA receptor ligand called gintonin [19], which consists of a complex of ginseng major latex-like protein 151 and lipids, and its functional active ingredient is lysophosphatidic acid 18:2 (LPA $C_{18:2}$). The crucial difference between gintonin and other ginseng components is that gintonin uses G protein-coupled LPA receptor signaling pathways to induce in vitro and in vivo effects in nervous systems [20]. Thus, gintonin acts as an exogenous ligand for LPA receptors [20]. Since astrocytes express LPA receptors [21], this raises the possibility that gintonin mediated-$[Ca^{2+}]_i$ transients may also be coupled to the regulation of metabolic activities in astrocytes. Therefore, in the present study, we examined whether gintonin stimulates Ca^{2+}-dependent glycogenolysis in primary cortical astrocytes. In addition, we investigated whether gintonin-mediated astrocytic glycogenolysis plays a role in protecting astrocytes from hypoxia and re-oxygenation stresses.

Our findings show that gintonin stimulates astrocytic glycogenolysis via LPA receptor-mediated Ca^{2+}-signaling pathways by regulating key enzymes involved in glycogenolysis. Gintonin-mediated astrocytic glycogenolysis also stimulates adenosine triphosphate (ATP) production and glutamate uptake and enhances cell viability under hypoxic and re-oxygenation conditions. We further discuss the physiological and pathophysiological roles of gintonin-mediated astrocytic glycogenolysis, as regulated through LPA receptors, in the brain under normoxic, hypoxic, and re-oxygenation conditions.

Methods

Materials

Gintonin, devoid of ginseng saponins, was prepared from *Panax ginseng* according to previously described methods [22]. 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). 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (LPA) was purchased from Avanti Polar Lipids, Inc. (AL, USA). We purchased Ki16425 and isofagomine from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents, including ATP, 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM), glutamate, and K252a were purchased from Sigma-Aldrich (St Louis, MO, USA). The DL-*threo*-β-benzyloxyaspartate (TBOA) was purchased from Tocris Bioscience (Bristol, UK).

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 methods of Kim et al. [22]. 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 to 7 days), cells were harvested with a 0.05% trypsin/ethylenediaminetetraacetic acid solution (Life technologies, Carlsbad, CA, USA), seeded in culture plates previously treated with poly-L-lysine hydrobromide (100 μg/mL; Sigma-Aldrich, St Louis, MO, USA), and grown in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin for further experiments.

Measurement of Astrocytic Glycogenolysis

After the primary culture cells reached confluence, the glycogen assay was performed as described previously [23]. Prior to the glycogen assay, the cell culture medium was replaced by serum-free DMEM containing 5 mM glucose. After incubation in the replaced medium for 4 h, the cells were treated with replaced medium in the presence or absence of gintonin at 37 °C. The intracellular glycogen level was measured using a glycogen assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the procedures provided by the manufacturer. The glycogen content of all samples was normalized to the protein content of each sample.

Induction of Hypoxia or Re-oxygenation

Astrocytes were exposed to hypoxia using a BD GasPak™ EZ anaerobic system [24]. After the cells were exposed to hypoxia for 0, 0.5, 1, 2, or 12 h, they were washed. In the case of re-oxygenation, the culture media was replaced with fresh culture medium, and then the cells were treated with 0–0.3 μg/mL gintonin for 0, 0.5, 2, or 8 h under normal culture condition depending on the experimental protocol.

Measurement of Intracellular ATP Production and Intracellular Glutamate

The intracellular ATP content was determined with an ATP assay kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. The astrocytes were plated in 6-well plates at 1×10^6 cells/well and allowed to adhere overnight. After incubation in replaced medium for 4 h, the cells were treated with replaced medium in the presence or absence of gintonin under normoxic, hypoxic, and re-oxygenation conditions at the indicated times and concentrations. Then, the cells were washed with cold phosphate-buffered saline (PBS) and collected using a cell scraper in ATP assay buffer. The ATP values were corrected for protein content in the sample and normalized to control ATP values.

For the intracellular glutamate assay, the cells were prepared and treated with or without gintonin, as described above. Then, 100 μM glutamate was added to the medium and terminated after 7 min. Intracellular glutamate was measured colorimetrically using a glutamate assay kit (Abcam) according to the manufacturer's instructions. The glutamate values were corrected for protein content in the samples and normalized to control glutamate values.

2,3-*bis*-(2-Methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide Assays and Cell Morphology

Cell viability was determined using an 2,3-*bis*-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide (XTT) assay kit (Daeillab Service, Seoul, Korea). The cells were seeded in 96-well plates, allowed to adhere overnight, and then treated with gintonin under normoxic, hypoxic, and re-oxygenation conditions. Following 2-h incubation with XTT absorbance was measured at 450 nm using a microplate reader (SpectraMax® Plus 384 Microplate Reader). Cells were planted on cover glasses in 6-well plates (500,000 cells/well). After treatment with gintonin under normoxic, hypoxic, or re-oxygenation conditions, the cells were fixed with 4% paraformaldehyde for 1 h and washed with PBS. The images were acquired on an AE2000 Inverted Phase Contrast Microscope with a × 20 objective.

Immunoblotting of Phosphorylated Glycogen Phosphorylase

Cell lysates were run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin in TTBS (20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20) for 1 h, and then incubated with anti-glycogen phosphorylase isoenzyme BB (GPBB) antibody (Abcam), anti-phosphoglycogen synthase (pGS) or glycogen-synthase (GS) antibody (cell signaling) overnight at 4 °C. Next, the membranes were washed several times with TTBS at 5 min intervals and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Millipore) for 2 h at room temperature. In addition, mouse monoclonal β-actin antibody from Sigma-Aldrich (St Louis, MO, USA) was used. Protein bands were then visualized via chemiluminescence using an ECL kit (Abfrontier, Seoul, Korea).

Immunoprecipitation of Phosphorylated Glycogen Phosphorylase

Cell lysates were prepared by using modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM ethylene glycol tetraacetic acid, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail

(phosStop; Roche, USA)). We incubated 350 μg of the lysates in 3 μg of anti-phosphoserine antibody (Abcam) at 4 °C overnight and precipitated the anti-phosphoserine antibody with protein A-agarose beads (GE Healthcare) at 4 °C overnight. Immunoblotting was performed with anti-GPBB antibody (Abcam). The intensity of each phosphorylation was normalized to that of the untreated control.

Data Analysis

Differences among the groups were determined using unpaired Student's *t* tests, one- or two-way analyses of variance with a Bonferroni post hoc test. Differences were significant at $p < 0.05$. The data are presented as the mean ± the standard error of the mean (SEM).

Results

Effects of Gintonin or LPA on Glycogenolysis and Its Signaling Pathways in Primary Cortical Astrocytes

Given that astrocytes store glycogen in the brain for energy metabolism and that G protein-coupled receptor ligands that are coupled to $[Ca^{2+}]_i$ transients stimulate glycogenolysis in astrocytes [10], we first examined the effects of gintonin on glycogenolysis in cultured mouse cortical primary astrocytes. As shown in Fig. 1a, d, the application of gintonin or LPA $C_{18:1}$ treatment for 30 min to astrocytes stimulated glycogenolysis in a concentration-dependent manner. Gintonin (1 μg/mL) also stimulated astrocytic glycogenolysis in a time-dependent manner (Fig. 1b). Interestingly, gintonin-mediated astrocytic glycogenolysis reached its maximum after 30 min, decreased over time until 2 h, and then increased until 8 h after gintonin treatment.

Next, we evaluated the effects of gintonin (1 μg/mL) on astrocytic glycogenolysis in the absence or presence of the LPA1/3 receptor antagonist Ki16425. As shown in Fig. 1c, d, the presence of Ki16425 significantly attenuated gintonin- and LPA-stimulated glycogenolysis. The phospholipase C inhibitor U73122, inositol 1,4,5-triphosphate (IP_3) receptor antagonist 2-aminoethoxydiphenyl borate (2-APB), and intracellular Ca^{2+} chelator BAPTA-AM also blocked gintonin-stimulated glycogenolysis in astrocytes (Fig. 1c). These results show that gintonin stimulates astrocytic glycogenolysis via LPA1/3 receptor signaling pathway. In addition, we determined the effects of ATP or glutamate on astrocytic glycogenolysis [10, 25]. We found that ATP and glutamate treatment for 30 min also stimulated astrocytic glycogenolysis with concentration-dependent manner (Fig. 1e, f). We examined the changes of glycogenolysis after co-treatment of gintonin with ATP or glutamate. We could observe that co-treatment of gintonin (0.1 μg/mL) with ATP or glutamate (10 μM each) exhibited slight but significant additive effects on astrocytic glycogenolysis (Fig. 1e). However, co-treatment of gintonin (1 μg/mL) with ATP or glutamate (100 μM) did not show additive effects on glycogenolysis, showing saturating effect with high concentration of ligands (Fig. 1f). Interestingly, gintonin had no significant effects on glucose uptake in experiments using a fluorescent glucose, 2-NBDG (Fig. S1).

Effects of Gintonin on Glycogenolysis- and Glycogen Synthase-Related Enzymes in Primary Astrocytes Under Normoxic Conditions

We assessed the effects of the phosphorylase kinase inhibitor, K252a, and glycogen phosphorylase inhibitor, isofagomine, on gintonin- or LPA-stimulated astrocytic glycogenolysis [26]. The results showed that both inhibitors significantly attenuated gintonin- or LPA-stimulated astrocytic glycogenolysis (Fig. 2a, b). In addition, we examined the degree of glycogen phosphorylase phosphorylation, since phosphorylation of glycogen phosphorylase by phosphorylase kinase is a key initiation step in glycogenolysis. As shown in Fig. 2c, d, applying gintonin treatment to astrocytes enhanced the phosphorylation of glycogen phosphorylase in concentration- and time-dependent manners, but the presence of Ki16425 attenuated glycogen phosphorylase phosphorylation (Fig. 2c (b)).

Subsequently, we evaluated the effects of gintonin on glycogen synthase phosphorylation, since increases in glycogen synthase phosphorylation are coupled to decreases in glycogenolysis. As shown in Fig. 2d, treating astrocytes with gintonin reduced glycogen synthase phosphorylation compared with untreated control cells. These results show that gintonin is linked to astrocytic glycogenolysis via activation of the LPA receptor-phospholipase C-intracellular IP_3 receptor-$[Ca^{2+}]_i$ transient pathway and that this pathway, rather than glycogen synthesis, phosphorylates glycogen phosphorylase.

Effects of Gintonin on the Morphologies and Distributions of Astrocytes under Normoxic, Hypoxic, or Re-oxygenation Conditions

Figure 3 shows normal astrocyte cultures under normoxia, hypoxia, and re-oxygenation. Gintonin had no effects on cell morphology under normoxic conditions and slightly elevated the number of cells (Fig. 3a, b). In the absence of gintonin, both exposure to 12 h of hypoxia and 2 h of re-oxygenation after 12 h of hypoxia induced changes in the cell morphologies and cell distributions in culture. Cells under these conditions were not evenly distributed and tended to aggregate; moreover, fewer connections were noted among the cells and more empty spaces were observed between cells when compared with cells under normoxia (Fig. 3c, e). However, the presence of gintonin for 2 h restored the disrupted cell

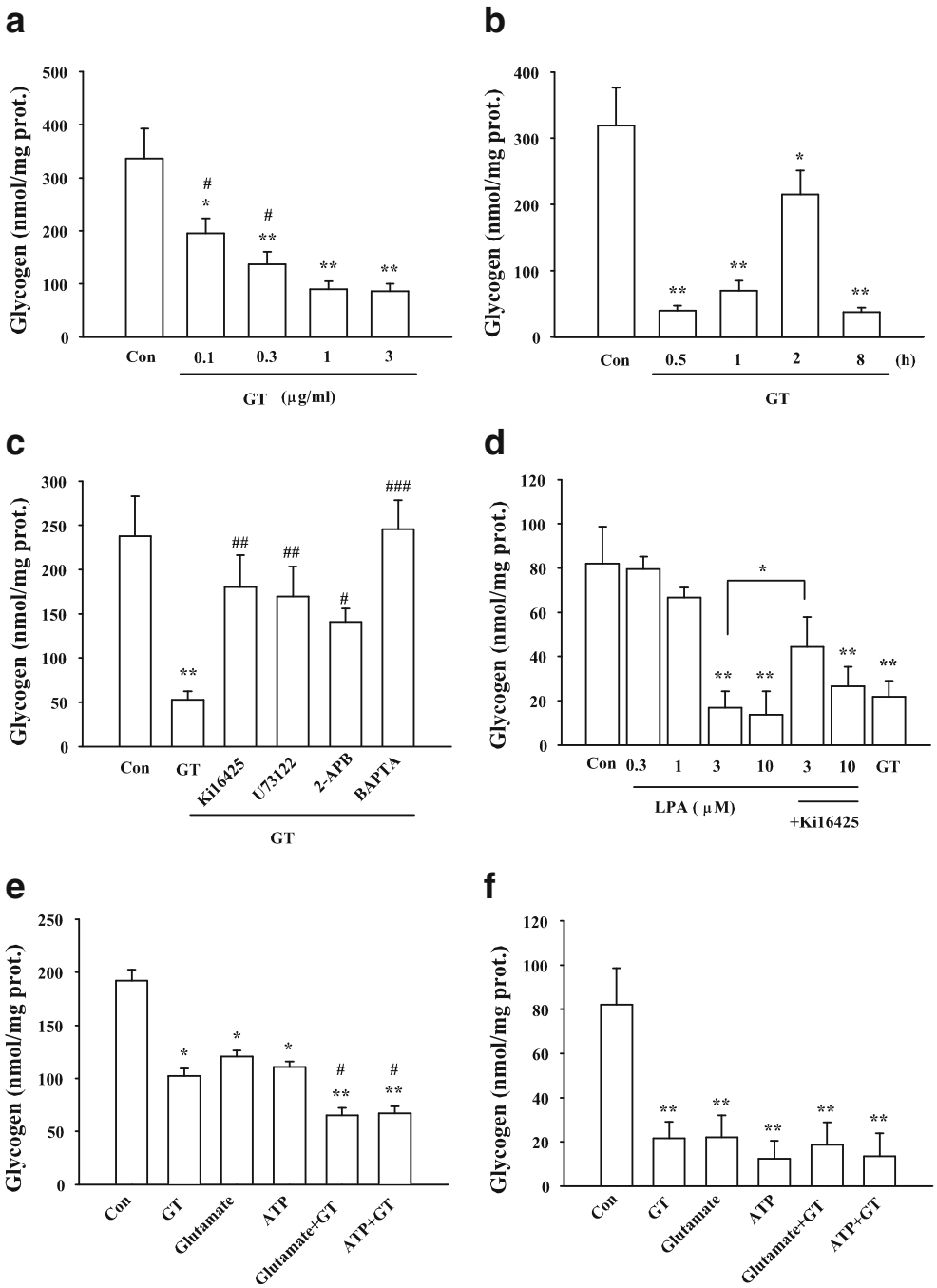

Fig. 1 Effects of gintonin (GT) and LPA on astrocytic glycogenolysis for 0.5 h and its signaling pathway in primary astrocytes. **a**, **b** Concentration- and time-dependent effects of GT-mediated astrocytic glycogenolysis in normoxia. $^{*}p < 0.01$ and $^{**}p < 0.001$ compared with control (Con). $^{\#}p < 0.05$ compared with GT (1 and 3 μg/mL). **c**, **d** Effects of signaling regulators on GT-mediated glycogenolysis; a LPA1/3 receptor antagonist (Ki16425, 10 μM), phospholipase C inhibitor (U73122, 5 μM), IP_3 receptor antagonist (2-aminoethoxydiphenyl borate, 100 μM), or intracellular Ca^{2+} chelator (BAPTA-AM, 50 μM) were added before GT application. $^{*}p < 0.01$ compared with the absence of Ki16425; $^{**}p < 0.001$ compared with control (Con). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with GT (1 μg/mL) alone. **e**, **f** Effects of ATP (10 or 100 μM) or glutamate (10 or 100 μM) alone or together with GT (0.1 or 1 μg/mL) on astrocytic glycogenolysis. $^{*}p < 0.01$ and $^{**}p < 0.001$ compared with control (Con). $^{\#}p < 0.05$ compared with GT, ATP, or glutamate alone. Each point represents the mean ± SEM ($n = 4$)

distributions and morphologies that were observed under the hypoxic and re-oxygenation conditions to levels that were similar to those observed during normoxia (Fig. 3d, f). These results indicate that gintonin protects astrocytes from hypoxia and re-oxygenation-induced injuries.

Effects of Gintonin on Astrocytic Glycogenolysis under Hypoxic and Re-oxygenation Conditions

Since gintonin treatment protected astrocytes from the cell morphology and distribution changes that are induced by hypoxia and re-oxygenation stresses (Fig. 4) and given that astrocytic glycogenolysis tended to increase under hypoxic conditions in previous reports [24], we investigated how gintonin treatment affects astrocytic glycogenolysis under hypoxic or re-oxygenation conditions. As shown in Fig. 4a, b, gintonin-mediated astrocytic glycogenolysis exhibited biphasic effects under hypoxic and re-oxygenation conditions; specifically, gintonin-mediated astrocytic glycogenolysis increased 30 min and 1 h after hypoxia, slightly increased over time until 2 h, but rather increased the glycogen content 12 h after gintonin treatment. These biphasic effects may reflect

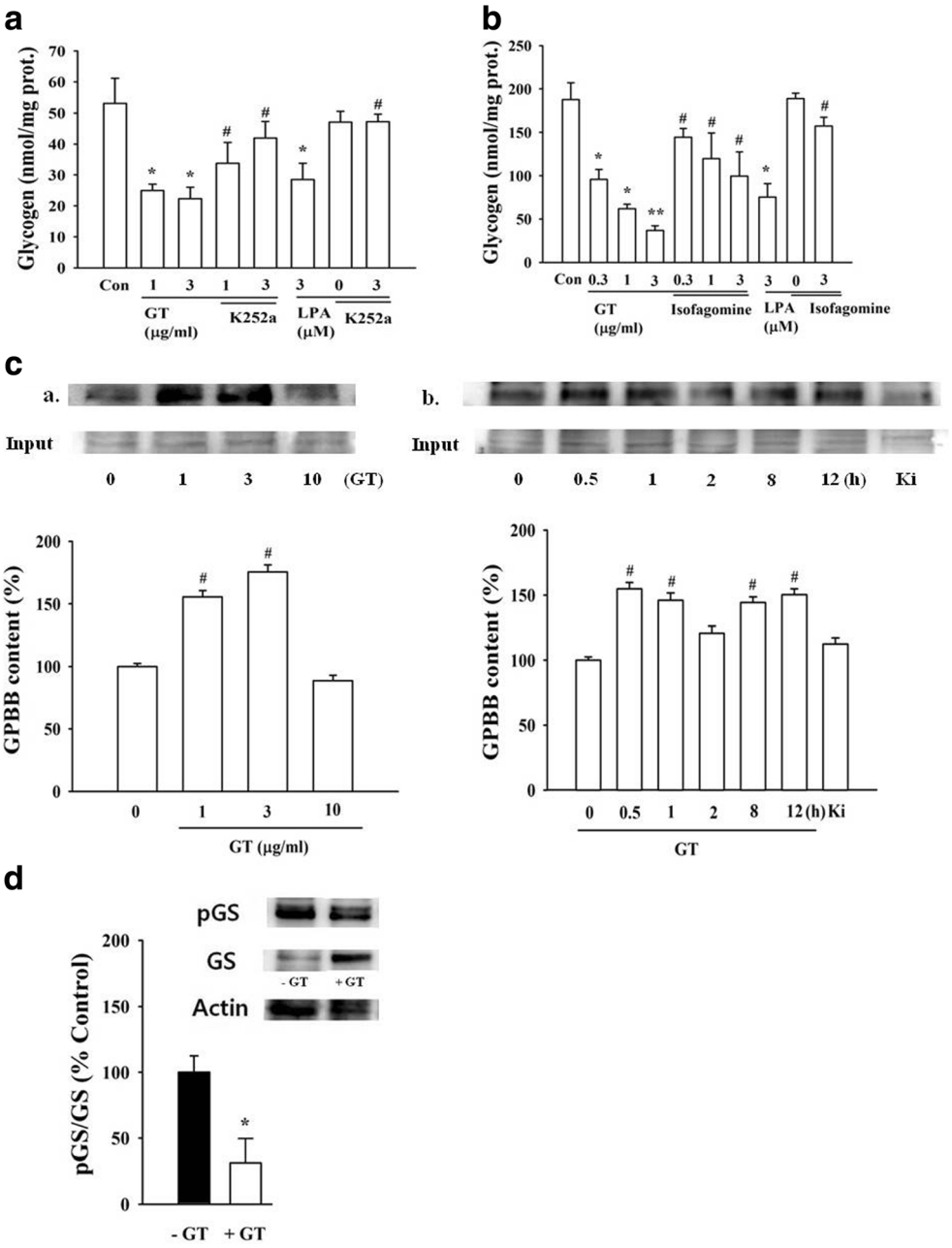

Fig. 2 Effects of phosphorylase kinase or glycogen phosphorylase inhibitor on gintonin (GT) or LPA-mediated astrocytic glycogenolysis and effects of gintonin on glycogen synthase in normoxia for 0.5 h. **a**, **b** K252a (0.5 μM), an inhibitor of phosphorylase kinase, and isofagomine (300 μM), an inhibitor of glycogen phosphorylase, attenuated GT (1 μg/mL) and LPA (1 μM)-mediated astrocytic glycogenolysis. $^{*}p < 0.01$ and $^{**}p < 0.001$ compared with GT-untreated or control cells (Con); $^{\#}p < 0.05$ compared with GT or LPA alone treatment. **c** Concentration- and time-dependent effects of GT on the phosphorylation of glycogen phosphorylase. *Inset*, the representative immunoblotting bands after immunoprecipitation show GT-mediated elevations in glycogen phosphorylase phosphorylation, brain form (GPBB). *Inset* also shows 10% input lysate (Input). $^{\#}p < 0.05$, compared with GT-untreated cells. **d** Immunoblotting on effect of GT on the phosphorylation of glycogen synthase. GS, glycogen synthase, pGS, phosphorylated glycogen synthase. $^{*}p < 0.01$ compared with the absence of GT. Data are presented as the mean ± SEM ($n = 4$–5)

that when applied to astrocytes, gintonin augments astrocytic glycogenolysis in the initial stages after hypoxia but rather increases glycogen accumulation in the later stages. Similarly, treating astrocytes with gintonin and exposing them to 12 h of hypoxia and 30 min of re-oxygenation stimulated astrocytic glycogenolysis in a concentration-dependent manner, while 8 h of re-oxygenation after 12 h of hypoxia elevated glycogen accumulation compared with astrocytes that were not treated with gintonin (Fig. 4c, d). These results indicate that under hypoxic and re-oxygenation conditions, gintonin-mediated astrocytic glycogenolysis exhibits biphasic effects depending on the duration of hypoxia or re-oxygenation (Fig. 4).

Effects of Gintonin on ATP Production Under Normoxic, Hypoxic, and Re-oxygenation Conditions

We further investigated whether gintonin-mediated astrocytic glycogenolysis is coupled to ATP production under normoxic, hypoxic, and re-oxygenation conditions. As shown in Fig. 5a, gintonin treatment to astrocytes amplified ATP production in a concentration-dependent manner in normoxia. In the time-dependent experiment, gintonin enhanced ATP production maximally after 30 min, with slight elevations in ATP occurring thereafter (Fig. 5b). However, this gintonin-mediated enhancement of ATP production in astrocytes was blocked by

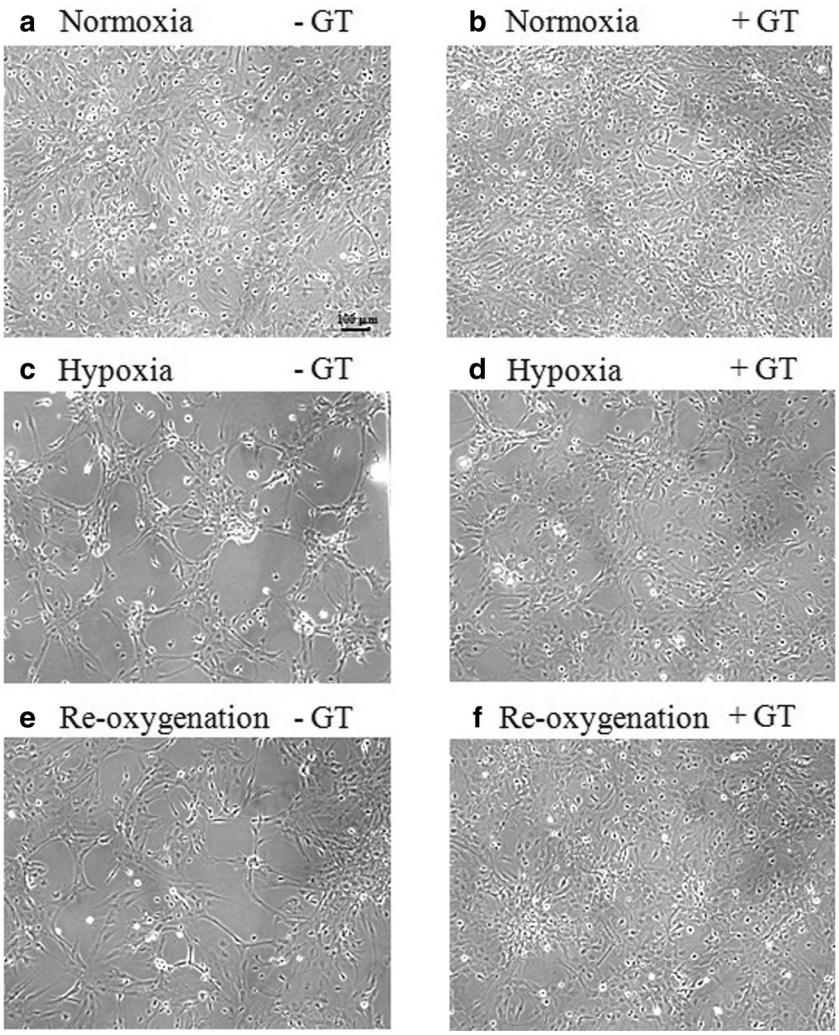

Fig. 3 Effects of gintonin (GT) on astrocytic cell distribution under normoxia, hypoxia, or re-oxygenation. Cortical astrocytes were cultured in the absence (**a**, **c**, and **e**) or presence of GT (1 μg/mL) (**b**, **d**, and **f**) under normoxia for 12 h (**a** and **b**), hypoxia for 12 h (**c**, **d**), and 2 h of re-oxygenation after hypoxia for 12 h (**e**, **f**). Hypoxia and re-oxygenation stresses induced changes in the cell distributions, but GT treatment restored the cell distributions to patterns that resembled those under normoxia. Scale bars represent 100 μm

isofagomine, a glycogen phosphorylase inhibitor (Fig. 5a). Hypoxia significantly reduced ATP production, indicating that the astrocytes were suffering from metabolic stress under hypoxia. Gintonin treatment applied under hypoxia significantly elevated the ATP level, while isofagomine inhibited this gintonin-mediated ATP production (Fig. 5c). As shown in Fig. 5d, increasing the hypoxic duration further reduced ATP production. However, the presence of gintonin during the hypoxic state prevented this ATP depletion and maintained ATP production at the control level (Fig. 5d).

We also determined the effects of gintonin on the ATP level following 2 h of re-oxygenation after 12 h of hypoxia. We found that re-oxygenation lowered the ATP level when compared with the control level. However, gintonin raised the ATP level, with the maximum effect occurring at 1 μg/mL, although the effect was not concentration dependent, as was the case during hypoxia. In the re-oxygenation experiments, gintonin stimulated ATP production in a time-dependent manner, with saturation occurring at 2 h (Fig. 5e, f). But isofagomine also blocked gintonin-mediated ATP production in normoxia, hypoxia and re-oxygenation conditions (Fig. 5a, c, and e). These results indicated that the gintonin-mediated amplification of ATP production might be closely associated with astrocytic glycogenolysis and that treating astrocytes with gintonin attenuates the ATP depletion that is observed following exposure to hypoxia and re-oxygenation stresses.

Effects of Gintonin on the Intracellular Glutamate Level Under Normoxia, Hypoxia, and Re-oxygenation

Glutamate is a major neurotransmitter released from neurons during normal synaptic activities in the brain. In addition, brain ischemia induces the release of a large amount of glutamate from neurons [27, 28]. One of the main roles of astrocytes is to reduce the extracellular glutamate concentration through glutamate uptake, as the accumulation of extra-neuronal glutamate induces excitatory neurotoxicity in the nervous system [29]. Astrocyte-mediated glutamate uptake requires a constant ATP supply to maintain a normal balance of glutamate. As such, we explored whether the gintonin-mediated augmentation of ATP production in astrocytes is coupled to elevations in the intracellular glutamate level under

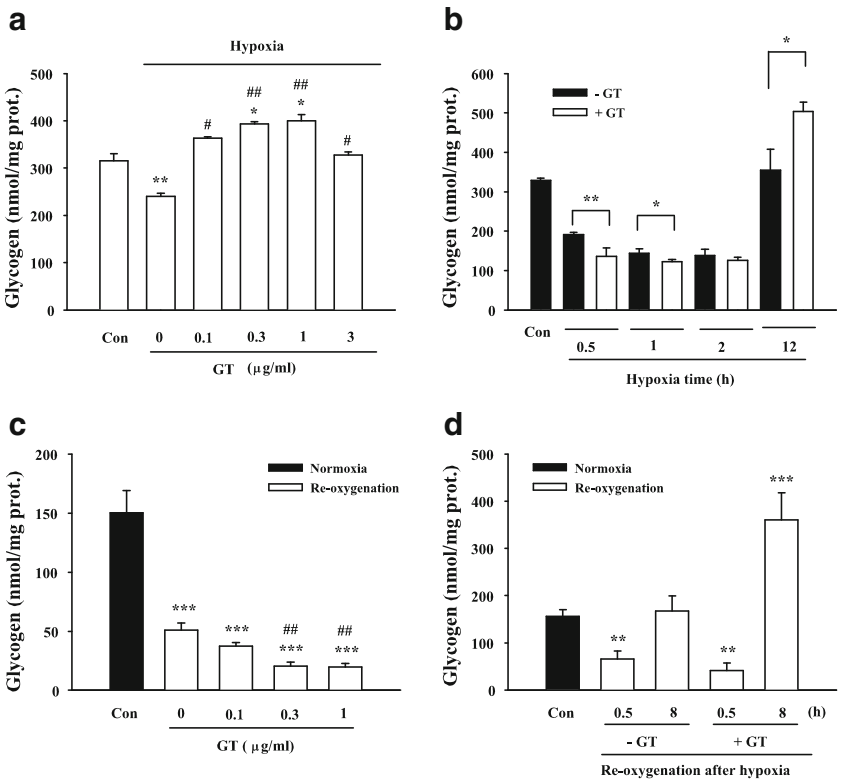

Fig. 4 Effects of gintonin (GT) on astrocytic glycogenolysis after exposure to hypoxia or re-oxygenation stresses. **a**, **b** Concentration- and time-dependent effects of GT on astrocytic glycogenolysis under the hypoxic condition. Hypoxia was induced for 12 h (**a**) or for the indicated time (**b**). $*p < 0.05$ and $**p < 0.01$ compared with control (Con) (**a**) or the presence of GT (**b**). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the absence of GT. **c**, **d** Concentration- and time-dependent effects of GT on astrocytic glycogenolysis under the re-oxygenation condition. Hypoxia was induced for 12 h, and then the cells were re-oxygenated for 0.5 h. $***p < 0.001$ compared with normoxia (Con); $^{\#\#}p < 0.01$ compared with the absence of GT (C). $**p < 0.01$ compared with normoxia (Con) (**d**); $***p < 0.001$ compared with normoxia (Con) (**d**). Data are presented as the mean ± SEM ($n = 4$–5)

normoxic, hypoxic, and re-oxygenation conditions (Fig. 6). During normoxia, gintonin treatment elevated the intracellular glutamate level in astrocytes in time-dependent manner (Fig. 6b). Interestingly, the gintonin-mediated intracellular glutamate level reached its maximum at a gintonin concentration of 0.3 μg/mL, while slight reductions in the glutamate levels were observed at higher gintonin concentrations (Fig. 6a). In terms of time, the glutamate level nearly reached saturation after 30 min when gintonin (0.3 μg/mL) was applied to astrocytes (Fig. 6b). However, co-treatment of astrocytes with the glutamate transporter inhibitor, TBOA and gintonin (0.3 μg/mL) blocked the gintonin-induced increase in intracellular glutamate (Fig. 6a). These results support that the gintonin-mediated elevation in the intracellular glutamate concentration involves the glutamate transporter.

Astrocytes exposed to hypoxia exhibited lower intracellular glutamate levels than did cells under normoxic conditions, whereas gintonin (0.3 μg/mL) treatment maximally augmented the intracellular glutamate level compared with cells that were not treated with gintonin (Fig. 6c). Gintonin treatment increased the intracellular glutamate level from 1 to 12 h compared with gintonin-untreated control cells (Fig. 6d). The intracellular glutamate level was also lower in astrocytes under the re-oxygenation condition than it was in control cells under normoxia. However, gintonin treatment further increased the astrocytic intracellular glutamate level in concentration- and time-dependent manners compared with cells that were not treated with gintonin (Fig. 6e, f). These results show that the augmented intracellular ATP that was observed with gintonin-mediated astrocytic glycogenolysis is coupled to glutamate uptake through the glutamate transporter.

Effects of Gintonin on Astrocytic Viability under Normoxic, Hypoxic, and Re-oxygenation Conditions

To examine the effects of gintonin-mediated astrocytic glycogenolysis on astrocytic viability during normoxia, hypoxia, and re-oxygenation, we used the XTT and bromodeoxyuridine (BrdU) assays, which assesses cell viability and proliferation as a function of cell number based on metabolic activity [30]. As shown in Fig. 7a and Fig. S2, under normoxia, treatment of gintonin (0.3 μg/

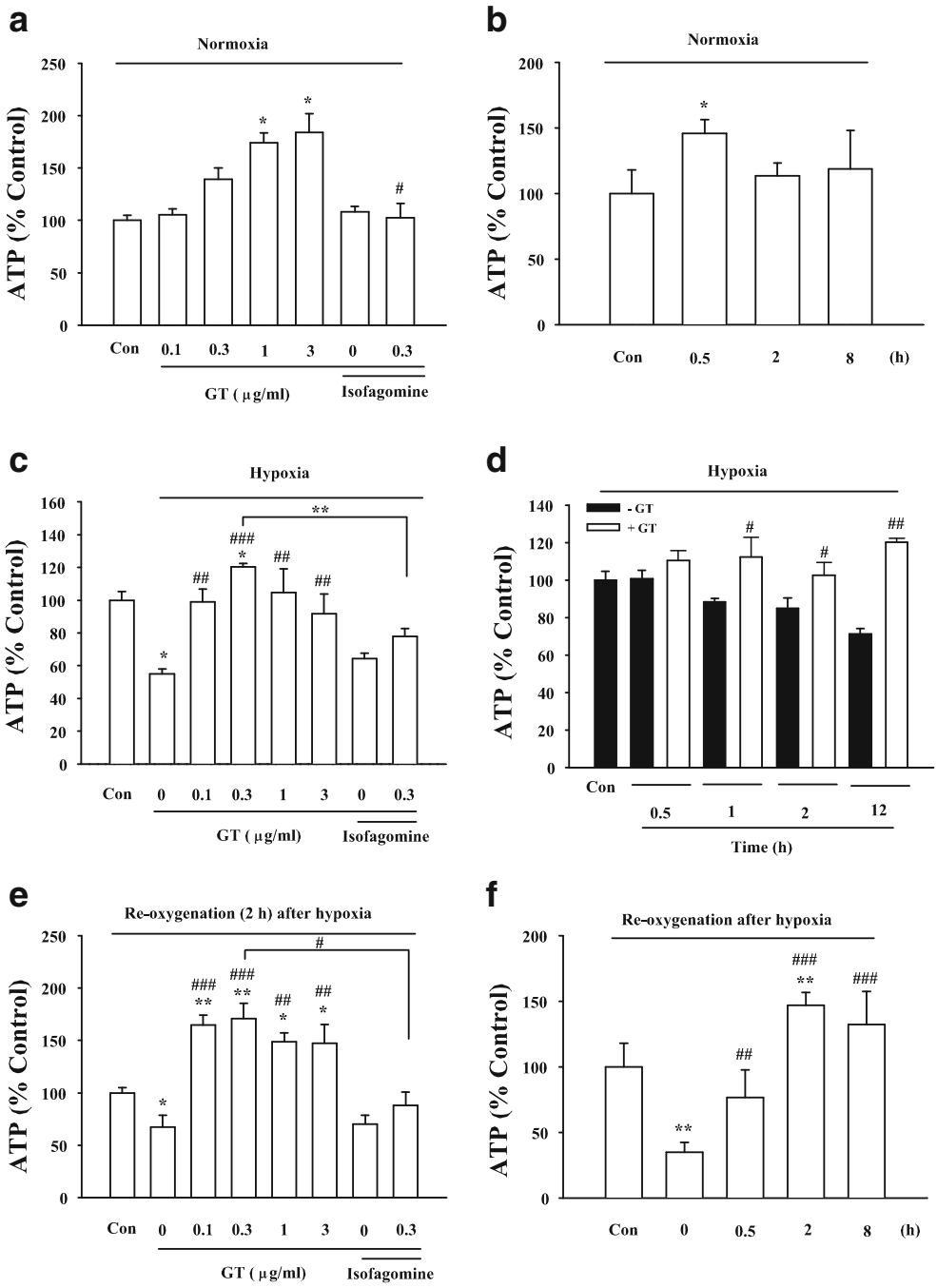

Fig. 5 Effects of gintonin (GT) on ATP production in astrocytes under normoxia, hypoxia, or re-oxygenation. **a**, **b** GT increased ATP production in concentration- and time-dependent manners under normoxia. Treatment with isofagomine (300 μM), an inhibitor of glycogen phosphorylase, for 30 min inhibited GT-mediated ATP production in normoxia. $^{*}p < 0.01$ compared with control; $^{\#}p < 0.05$ compared with GT (0.3 μg/mL). **c**, **d** GT treatment increased ATP production following 12 h of hypoxia in concentration- and time-dependent manners. $^{*}p < 0.01$ compared with control; $^{**}p < 0.001$ compared with GT (0.3 μg/mL). In the time-course experiments, GT (0.3 μg/mL) was applied for 2 h re-oxygenation after 12 h hypoxia. GT treatment elevated ATP production, whereas cells that were not treated with GT showed reduced ATP production over time under hypoxia. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.005$ compared with the absence of GT (C); $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the absence of GT (**d**). **e**, **f** GT increased ATP production in time-dependent manners in the re-oxygenation condition (2 h of re-oxygenation after 12 h of hypoxia). In the concentration-dependent experiments using isofagomine (300 μM), GT (0.3 μg/mL) was used. $^{*}p < 0.01$ and $^{**}p < 0.001$ compared with control; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.005$ compared with the absence of GT; $^{\#}p < 0.005$ compared with the presence of isofagomine. Data are presented as the mean ± SEM ($n = 4$–5)

mL) to astrocytes started to increase cell viability in a concentration-dependent manner. Gintonin treatment also increased cell viability under 12 h of hypoxia with concentration-dependent manner (Fig. 7b; Fig. S2). However, the presence of isofagomine, a glycogen phosphorylase inhibitor, attenuated the gintonin-mediated improvements in cell viability (Fig. 7c), indicating that gintonin-mediated astrocytic glycogenolysis plays a key role in cell viability. The gintonin-mediated astrocytic viability showed a slightly different pattern under the re-oxygenation condition compared with those observed during normoxia and hypoxia (Fig. 7d, f). Thus, the maximum cell viability occurred when gintonin (0.3 μg/mL) was applied for 30 min and 2 h after re-oxygenation. In addition, gintonin exhibited concentration-dependent improvements in cell viability, with the maximum cell viability occurring when gintonin was applied for 8 h after hypoxia and re-oxygenation. Isofagomine also attenuated gintonin-mediated increase of cell viability under re-oxygenation (data not shown). Interestingly, the cell viability under hypoxia was lower than was that during re-oxygenation. These results indicate that gintonin exhibits

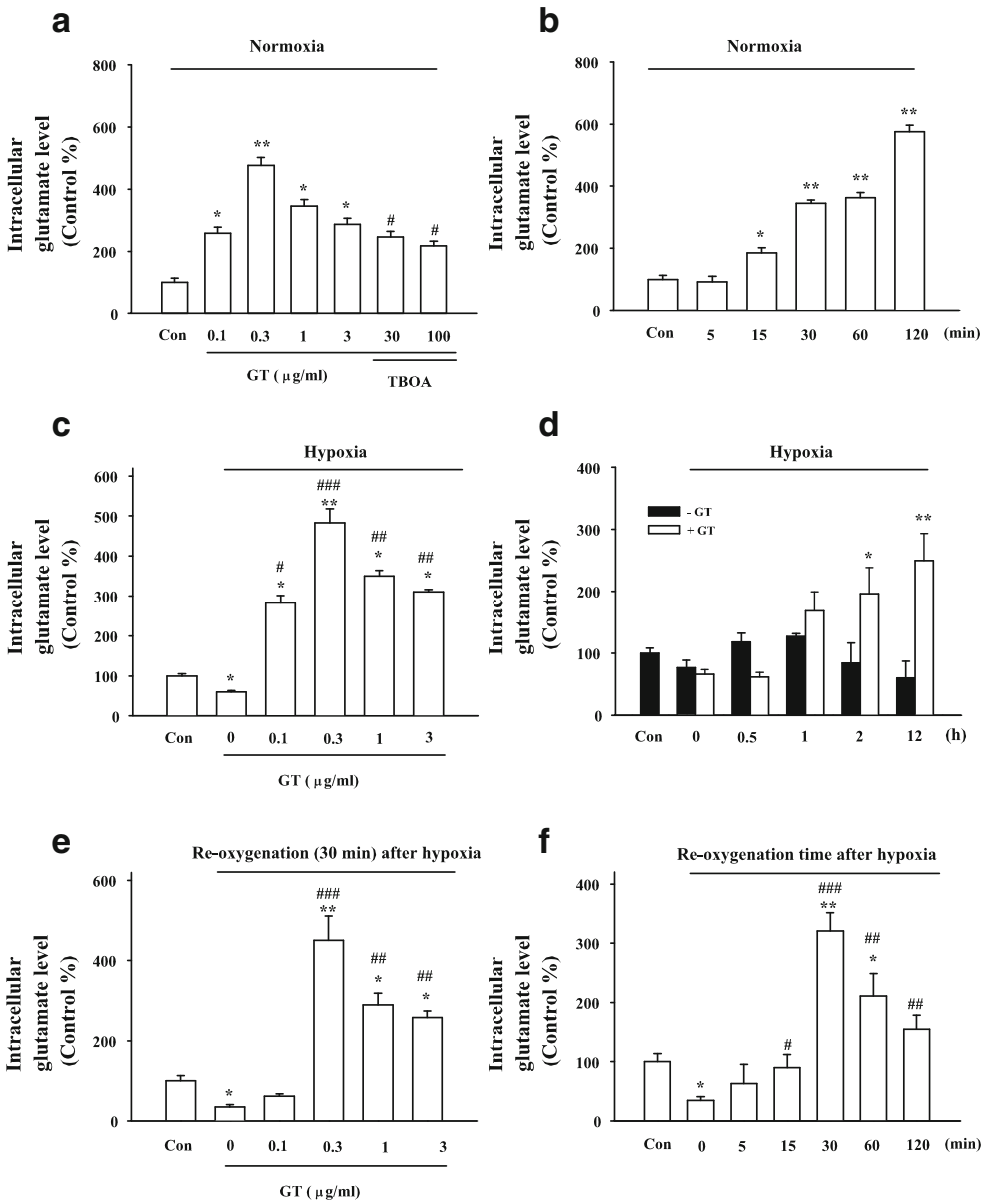

Fig. 6 Effects of gintonin (GT) on intracellular glutamate uptake in astrocytes under normoxia, hypoxia, or re-oxygenation. **a**, **b** GT enhanced glutamate uptake in concentration- and time-dependent manners under normoxia. Treatment with TBOA (30 or 100 μM), a glutamate transporter inhibitor, blocked the GT-mediated (0.3 μg/mL) elevations in glutamate uptake. $*p < 0.01$ and $**p < 0.005$ compared with control (Con); $^{\#}p < 0.01$ compared with GT (0.3 μg/mL). **c**, **d** GT increased glutamate uptake in concentration- and time-dependent manners after 12 h of hypoxia. In the concentration- and time-course experiments, GT treatment (0.3 μg/mL) induced elevations in glutamate uptake after 1 h. $*p < 0.01$ and $**p < 0.005$ compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with the absence of GT. **e**, **f** GT increased glutamate uptake in concentration- and time-dependent manners during the re-oxygenation condition (0.5 h of re-oxygenation after 12 h of hypoxia). In the concentration-dependent experiments, GT treatment more than 0.3 μg/mL induced elevations in glutamate uptake after 0.5 h under re-oxygenation. In the time-course experiments, GT treatment (0.3 μg/mL) induced elevations in glutamate uptake after 0.5 h under re-oxygenation conditions. $*p < 0.01$ and $**p < 0.005$ compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with the absence of GT. Data are presented as the mean ± SEM ($n = 4$–5)

better protective effects when applied during the re-oxygenation process after hypoxia. Altogether, our data suggest that gintonin restores the hypoxia-induced reductions in cell viability and that gintonin-mediated astrocytic glycogenolysis contributes to astrocytic survival after exposure to hypoxia and re-oxygenation stresses.

Discussion

Brain energy metabolism is one of the main themes in brain physiology, since the brain consumes about 20% of the body's energy and requires a constant glucose supply [31]. To produce this energy, i.e., ATP, neurons use glucose or lactate [32].

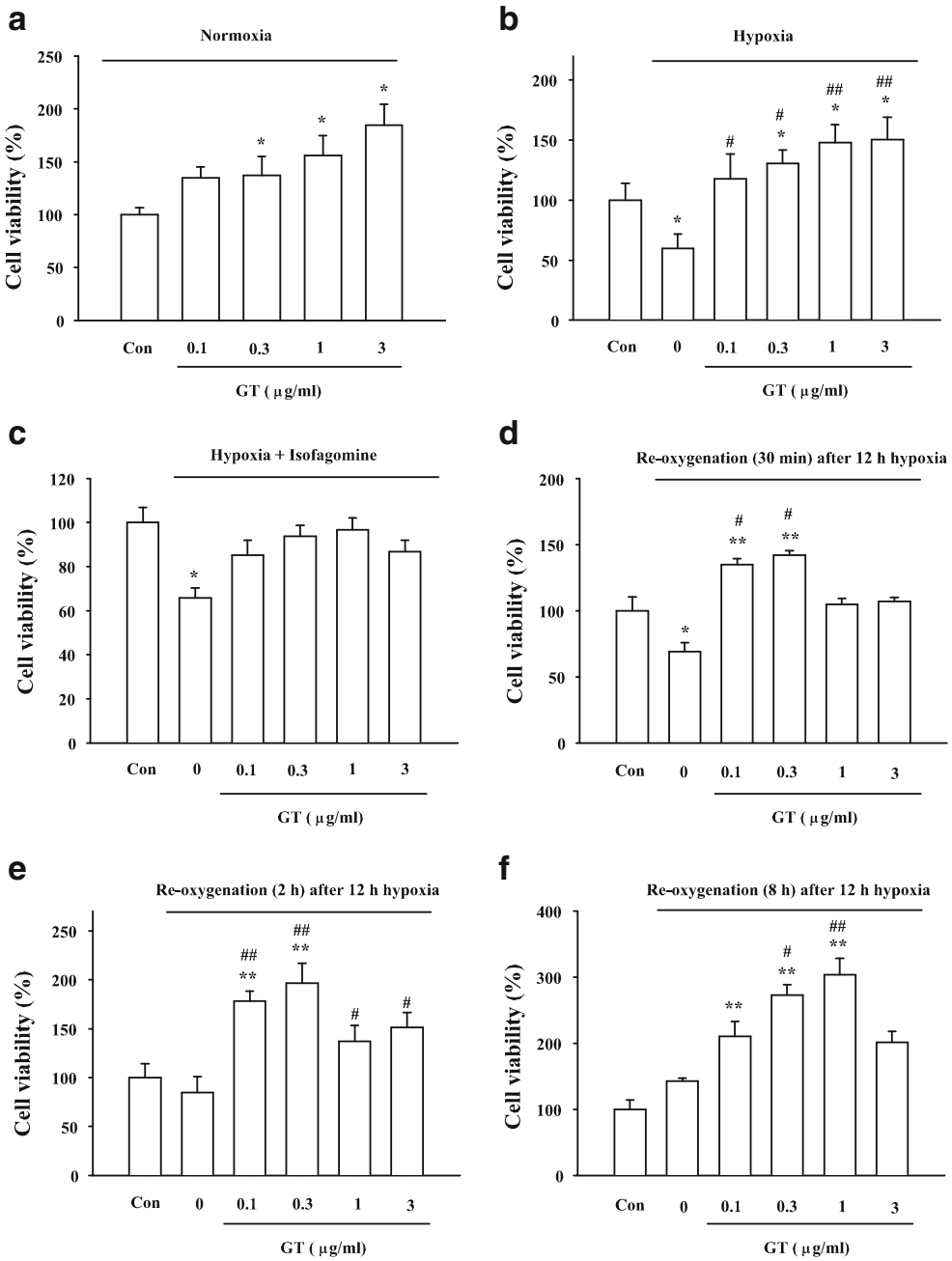

Fig. 7 Effects of gintonin on cell viability under the normoxic, hypoxic, or re-oxygenation conditions. **a** Cells were treated with the indicated concentration of gintonin for 0.5 h under normoxia. $^{*}p < 0.05$ compared with control. **b**, **c** Cells were treated with the indicated concentration of gintonin for 0.5 h after 12 h of hypoxia. $^{*}p < 0.05$ compared with control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the absence of GT. The attenuating effect of isofagomine on gintonin-mediated cell viability under hypoxia. **d**, **f** Cells were treated with the indicated concentration of gintonin for the indicated time of re-oxygenation after 12 h of hypoxia. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the absence of GT. Astrocyte viability was estimated with an XTT assay. Data are presented as the means ± SEM ($n = 4$–5)

When glucose is used, it is obtained directly from brain-blood vessels through the glucose transporter system [31]. Alternatively, neurons can use lactate to produce energy, and obtain lactate through astrocytic glycogenolysis, since astrocytes store glycogen in the brain [31]. It is well characterized that LPA receptors are highly expressed in the developing brain and that these receptors are critical during the prenatal stages of brain development [1]. However, in the adult brain, LPA receptors are mainly expressed in glia rather than in neurons. Unfortunately, the glial functions of LPA receptors in the adult brain remain unclear [1, 14]. In addition, although it is known that LPA exists in the brain and that brain astrocytes store glycogen and express LPA receptors, relatively little is known about the relationship between LPA and cortical astrocytic LPA receptor-mediated energy metabolism under normoxic, hypoxic, and re-oxygenation conditions.

In the present study, we revealed that gintonin and LPA stimulated glycogenolysis in astrocytes via the LPA receptor-mediated-$[Ca^{2+}]_i$ transient-glycogenolytic enzyme pathway and demonstrated the effects of gintonin during hypoxia and re-oxygenation (Fig. 8). Our data imply that gintonin actively participates in brain energy metabolism via the LPA receptor-signaling pathway. Supporting this notion, we found that gintonin-mediated astrocytic glycogenolysis was blocked

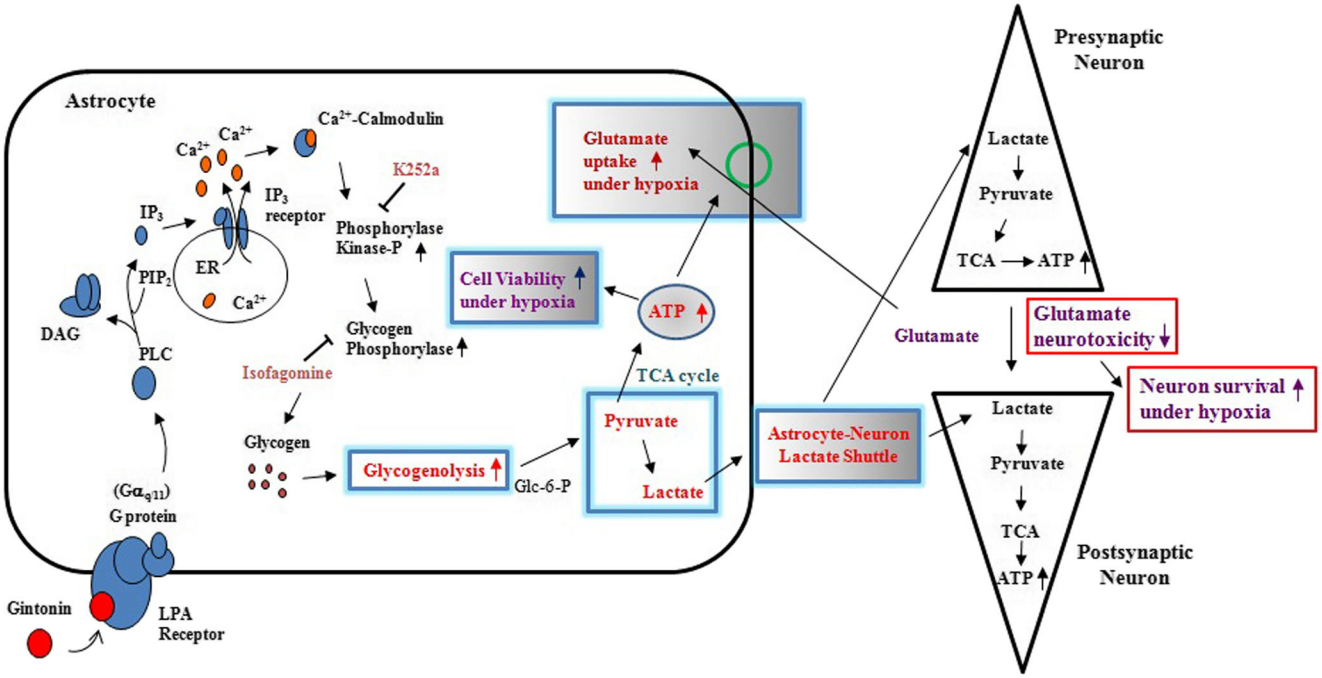

Fig. 8 Schematic diagram illustrating the intra- and intercellular effects of gintonin-mediated astrocytic glycogenolysis in primary cortical astrocytes via LPA receptor membrane signaling pathways. Gintonin-mediated astrocytic glycogenolysis might be coupled to dual intracellular and intercellular effects. The intracellular effects of gintonin-mediated astrocytic glycogenolysis are to produce ATP, increase glutamate uptake, and improve cell viability under normoxia and hypoxia- and re-oxygenation-induced stress. The intercellular effects of gintonin-mediated astrocytic glycogenolysis are also coupled to the astrocyte-neuron lactate shuttle, allowing neurons use lactate under hypoxia or re-oxygenation stress and to attenuate glutamate neurotoxicity by removing excessive extracellular glutamate after synaptic transmission or under hypoxia

by an LPA1/3 receptor antagonist and attenuated by a phosphorylase kinase inhibitor. Gintonin also stimulated glycogen phosphorylase phosphorylation, and gintonin-mediated glycogenolysis was inhibited by a blocker of glycogen phosphorylase, a key enzyme involved in glycogenolysis. Regarding gintonin's effects during hypoxia and re-oxygenation, we showed that gintonin stimulated astrocytic glycogenolysis under these stresses (Fig. 4), indicating that gintonin-mediated astrocytic glycogenolysis may also play crucial roles in abnormal conditions. Collectively, our data support that gintonin regulates glycogen metabolism in primary cortical astrocytes under normoxic, hypoxic, and re-oxygenation conditions. However, gintonin had no significant effects on glucose uptake, indicating that gintonin's effects on $[Ca^{2+}]_i$ transients via LPA receptor signaling pathways may not be linked to glucose transporter systems in primary astrocytes (Fig. S1).

Astrocytic glycogenolysis can be activated by neurotransmitters in two ways [33, 34]. First, norepinephrine and vasoactive intestinal peptide can induce astrocytic glycogenolysis via their respective receptor-mediated cyclic adenosine 3′,5′-monophosphate (cAMP) signaling pathway [34, 35]. The increase in cytosolic cAMP in astrocytes is associated with the activation of enzymes that are involved in glycogenolysis [23, 36]. Interestingly, the gintonin-mediated astrocytic glycogenolysis exhibited similar effects, as shown by norepinephrine [23], although gintonin utilizes Ca^{2+} rather than cAMP signaling pathway. Second, ATP and glutamate can use the Ca^{2+} signaling pathway to induce astrocytic glycogenolysis via their respective receptors [37]. Here, when we examined whether co-treating astrocytes with gintonin and ATP or glutamate would induce additive effects on astrocytic glycogenolysis, we could observe additive effects at non-saturating concentration but could not observe further significant elevations in astrocytic glycogenolysis at saturating concentration (Fig. 1e, f). These results raise a possibility that gintonin may not share common signaling pathways with ATP or glutamate for activating astrocytic glycogenolysis, since they interact with different membrane receptors. To our knowledge, this is the first study to show that gintonin and LPA, in addition to ATP and glutamate, regulate astrocytic glycogenolysis through LPA receptor signaling pathways.

It is important to consider the roles of gintonin-stimulated astrocytic glycogenolysis through LPA receptors in the brain. Astrocytes are closely associated with neurons in the brain. The main roles of astrocytes are to supply energy to neurons and to reduce excessive extracellular glutamate [9]. Astrocytes can provide energy to neurons in the form of lactate through astrocytic glycogenolysis in two ways [32]. The first is when a continuous and large source of energy is required by neurons during normal higher-order brain activities such as extensive

synaptic transmissions or long-term potentiation for the consolidation of long-term memory [10]. In previous reports, Trimbuch et al. [38] and Vogt et al. [39] demonstrated that LPA acts as an excitatory neurotransmitter by stimulating the release of glutamate from hippocampal presynaptic sites through LPA receptors. A previous study by our laboratory also showed that treating mouse hippocampal slices with gintonin induces glutamate release, enhanced synaptic transmission, and long-term potentiation via LPA receptors [40]. We further showed in previous reports that administering gintonin orally to mice enhanced hippocampus-dependent memory [40], while administration of gintonin to animal model of Alzheimer's disease and older patients with early dementia or mild cognitive impairment improved cognitive function [41, 42]. Thus, in addition to the gintonin-mediated modulations of synaptic transmission and cognitive function, gintonin-mediated astrocytic glycogenolysis can be speculative to have two functions in the normal state. One is to produce energy to maintain homeostasis within the astrocyte. The other function is to supply energy to neurons and lower the amount of glutamate that is released during synaptic transmission [38, 39], since excess extracellular glutamate can induce excitotoxicity in neighboring neurons (Fig. 8). The present study expands on this knowledge by showing that LPA receptor activation by LPA or gintonin may be important for brain energy metabolism and synaptic transmission.

Gintonin also has effects under pathophysiological states such as during exposure to hypoxia and re-oxygenation stresses. Ischemia is one major cause of hypoxia, which results in decreased synaptic transmission and neural death owing to ATP depletion and excessive glutamate-induced excitotoxicity [13, 43]. Moreover, it is thought that astrocytic glycogenolysis is activated under hypoxia to provide lactate to neurons and impede ATP loss [44]. Our data revealed that gintonin stimulated astrocytic glycogenolysis and boosted ATP production under hypoxic and re-oxygenation conditions. These results imply that gintonin, via LPA receptors, provides an additional energy supply to neurons under abnormal brain conditions, helping to meet the additional energy needs that are required during hypoxia and re-oxygenation. Furthermore, the gintonin-induced elevations in astrocytic glycogenolysis that we observed during hypoxia and re-oxygenation were linked to neuronal energy supplies through the intercellular astrocyte-neuron lactate shuttle (Fig. 8). Since astrocytic glycogen also serves as an energy store of fuel for glutamate uptake during hypoxia [45–47], our results indicate that the elevated ATP production and glutamate uptake that occurred through gintonin-mediated astrocytic glycogenolysis may be closely associated with improved astrocyte survival under hypoxic and re-oxygenation stresses (Fig. 8). However, we cannot exclude a possibility besides astrocytic glycogenolysis that the gintonin-mediated ATP synthesis also involves metabolic use of glutamate, which can fuel tricarboxylic acid cycle via anaplerotic reactions. Further study will be required to elucidate an exact molecular mechanism on gintonin-mediated ATP production.

Previous reports have shown that the endogenous levels of autotoxin, which produces LPA, in the human brain, as well as the astrocytic LPA1 receptor expression levels, increase following trauma or hypoxia [16, 17]. However, the functions of the elevated LPA and LPA receptors during these pathophysiological conditions have not been clearly defined. Based on the present results, it can be proposed that the gintonin- and LPA-mediated stimulation of astrocytic glycogenolysis through LPA receptors is beneficial because it provides ATP to astrocytes for glutamate uptake, improves cell survival, and maintains the neuronal energy balance by supplying lactate to neurons under pathophysiological conditions (Fig. 8).

Conversely, we observed interesting gintonin-mediated astrocytic glycogen metabolism patterns. That is, gintonin induced biphasic effects on glycogenolysis depending on the treatment duration in normoxic, hypoxic, and re-oxygenation conditions. In the normoxic condition, short-term treatment with gintonin for 30 min to 1 h stimulated glycogenolysis, but treatment with gintonin for 2 h elevated the glycogen content (Fig. 2b). Under hypoxic conditions, short-term treatment with gintonin for 30 min to 1 h stimulated glycogenolysis, while long-term treatment with gintonin for 12 h increased the glycogen content (Fig. 4a, b). As for the re-oxygenation condition after hypoxia, gintonin treatment for 30 min to 1 h stimulated astrocytic glycogenolysis, but gintonin treatment for 8 h elevated the glycogen content. These findings are consistent with previous reports; for instance, Sorg and Magistretti [23] first reported that vasoactive intestinal peptide and norepinephrine induced biphasic effects on astrocytic glycogenolysis under normoxic conditions. In an in vivo stroke model, Hossain et al. [48] observed that the glycogen content of a damaged region decreased at 3 h after stroke induction but significantly increased 6 to 24 h after stroke induction. In addition, an in vitro hypoxia study using astrocytes demonstrated patterns of astrocytic glycogenolysis and glycogen re-synthesis that were similar to those observed in the present study. The increased glycogen accumulation that was observed following the induction of astrocytic glycogenolysis may be regarded as an astrocytic compensation phenomenon that is similar to the supercompensation of glycogen re-synthesis that is observed in the skeletal muscle following exercise [49]. However, the exact mechanisms underlying the gintonin-mediated elevation in glycogen content following astrocytic glycogenolysis are currently unknown. Further studies are required to elucidate the underlying molecular mechanisms.

In summary, although brain astrocytes express LPA receptors, the roles of these receptors in astrocytes are not well understood. In the present study, we demonstrated that gintonin, a ginseng-derived exogenous LPA receptor ligand, activates the astrocytic glycogenolysis pathway and that

gintonin-mediated astrocytic glycogenolysis is linked to increases in ATP production, glutamate uptake, and cell viability under normal and abnormal conditions. Finally, the present study proposes that the gintonin-induced alterations in brain energy that is mediated through astrocytic glycogenolysis may provide a molecular basis for the beneficial effects of ginseng that are observed under physiological and pathophysiological conditions.

Acknowledgments This study was supported by a grant from the Basic Science Research Program from the Brain Research Program (NRF-2017R1D1A1A09000520) through the NRF of Korea funded by the Ministry of Science, ICT and Future Planning (NRF-2016M3C7A1913845) to S-Y. Nah.

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