Research Article

Gintonin regulates inflammation in human IL-1β-stimulated fibroblast-like synoviocytes and carrageenan/kaolin-induced arthritis in rats through LPAR2

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1. Introduction

Ginseng has a higher concentration of lysophosphatidic acids (LPAs) compared to other edible plants and Chinese herbs [1,2]. In ginseng, there exists a glycolipoprotein complex with a special form of lipid LPAs called Gintonin [3]. Lysophosphatidic acid (LPA) is a metabolic intermediate that is produced by phospholipases in both plants and animals as well as by autotaxins (ATX) in animals. In the plant system, LPA is considered to be a small phospholipid. However, in animals, LPA activates the G-protein coupled LPA receptor and is also considered to act as a growth factor derived from lipids [4–6]. The subtypes of LPA that can be found in Gintonin include LPA C18:2, LPA C18:3, and LPA C16:0. Besides, Gintonin contains ginseng protein and other bioactive lipids such as phosphatic acid and lysophospholipids. Gintonin is characterized by the fact that lysophosphatic acid (LPA) C18:2 is found in a relatively higher concentration than other LPA types, and it was observed that LPA C18:2 most strongly suppressed the activity of ATX [7,8].

Rheumatoid arthritis (RA) is one of the autoimmune diseases characterized by chronic inflammation of the synovial membrane (synovitis), which leads to the destruction and deformation of cartilage [9]. Common symptoms of arthritis include pain, edema, stiffness of joints, and fatigue [10]. The cause of rheumatoid arthritis has not been determined, but the onset of RA begins with the induction of an inflammatory immune response to the synovial membrane. Important events in RA are regulated by the complex
interaction of pro-inflammatory cytokines, chemokines, and MMPs in synovial fluid and synovial tissue. These suggest that blocking the pro-inflammatory cytokines, chemokines, and MMPs in synovial fluid and synovial tissue would be a measure to treat arthritis [11–15]. LPA receptors are expressed in a wide array of cells in the body which include the cells of the synovial membrane sublining [16]. There have also been reports that the mRNAs of LPA1, LPA2, and LPA3 receptors are expressed in human Fibroblast-like synoviocytes (FLS) [17]. Lysophospholipase D or ATX mRNA has also been observed to be expressed in the FLS of RA patients [18], ATX is known to be an enzyme that produces the majority of the extra- cellular LPA and also acts as an autocrine tumor cell motility stimulator [19]. Emerging evidence that may be related to the pathogenesis of RA is the Ca\(^{2+}\) flux. Compared to normal T cells, significant differences in endoplasmic reticulum (ER) Ca\(^{2+}\) concentration in synovial fluid T cells from RA were found [20,21].

Gintonin has higher affinity and selectivity for LPA receptors than other lipid-associated receptors, and also activates these LPA receptors [1,22]. The LPA receptor activated by Gintonin activates the cell membrane signaling system linked to the G protein, releasing free Ca\(^{2+}\) from the calcium storage ER. This increased calcium contributes to various effects on calcium-dependent intracellular, intercellular, and living organism functions [23–25]. Gintonin has also been reported to inhibit ATX [8,26]. Since Gintonin acts as an exogenous LPA receptor-ligand, studies are underway regarding the function of LPA receptors in the nervous system [27–29]. In addition, reports on the relationship between rheumatoid arthritis and LPA continue to be reported [17,30,31]. However, there are no reports of whether Gintonin/LPA2 mediation can also involve in an anti-arthritic effect. Therefore, the purpose of this study is to confirm that Gintonin has a therapeutic effect on rheumatoid arthritis, a type of autoimmune disease, through LPA receptors.

2. Materials and methods

2.1. Reagents

Gintonin was supplied from the Ginsengology Research Laboratory of Konkuk University (Seoul, Korea). All reagents used in cell culture were supplied by WELGENE Inc. (Gyeongsan, Korea). Carrageenan and kaolin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Human IL-1β was supplied by Bio Vision Inc. (Milpitas, CA, USA). H2LS186303 was obtained from R&D systems (Minneapolis, MN, USA). Antibodies for β-actin, phosphorylated- and total-forms of p38, JNK/SAPK, ERK1/2, IKKα, IKKβ, IκBα, and NF-κB/p65 were purchased from Cell Signaling Technology (Danvers, MA, USA) and iNOS, TNF-α, IL-6, and COX-2 were supplied by Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell cultures

Fibroblast-like synoviocytes (FLS), which are primary fibroblast-like cells derived from the synovial tissue of a RA patient, were obtained from Cell Applications, Inc. (San Diego, CA, USA). FLS cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin under a humidified 5% CO\(_2\) atmosphere at 37 °C. FLS cells between 3rd and 5th passages were used and media was changed every day until confluence reached 80%.

2.3. Cell viability

Cell viability was measured using Quanti-Max™ WST-8 cell viability assay kit (BIOMAX Co., Seoul, Korea). Cells were seeded to 2 × 10^4 cells/well in a 96-well plate. Various concentrations of Gintonin were then added and after 1 hour, treated with IL-1β (10 ng/ml) for 24 hours. To measure cell viability, 10 μl WST-8 was added for 1 h and then read using a microplate reader at 450 nm (Molecular Devices, San Jose, CA, USA).

2.4. Measurement of ROS levels

FLS cells (2.5 × 10^5 cells/well in a 24-well plate) were prepared by treating the cells under the same conditions as the cell viability assay. The intracellular ROS levels were detected using 7-diethylrhodamine-2-carboxylic acid (DHICA, Sigma-Aldrich). In brief, the cells were stained with H2DCFDA solution dissolved at 50 μM in pre-warmed PBS for 30 minutes at 37 °C and measured at an excitation of 485 nm and emission of 530 nm using a fluorescence reader (BioTek Instruments, Winooski, VT, USA). Mitochondrial ROS production was measured with MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen, Waltham, MA, USA) using the same method in accordance with a previous report [32]. Briefly, MitoSOX working solution (5 μM) dissolved in HBSS/ Ca/Mg was added to each well and incubated for 10 minutes at 37 °C. The fluorescence was monitored with a microplate reader set to 510 nm excitation and 595 nm emission wavelengths.

2.5. Western blot

All cells were lysed using RIPA buffer (ELPIS Biotech Inc., Daejeon, Korea). The proteins were separated using 12% SDS-PAGE, transferred to a PVDF membrane, and were incubated in a chamber at 4 °C with the primary antibodies overnight, and with the secondary antibodies for 1 hour. The membranes were developed using the enhanced chemiluminescence (ECL) detection kits (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6. Carrageenan/kaolin-induced arthritic rat model

Six-week-old male Sprague-Dawley (SD) rats (200–230 g) were randomly divided into five groups (n = 6); a non-treated normal group (NOR), an arthritis control group by carrageenan/kaolin injection (ART), an arthritis group with Gintonin treatment at 25 mg/kg (ART + GIN25), an arthritis group with Gintonin treatment at 50 mg/kg (ART + GIN50), and an arthritis group with Gintonin treatment at 100 mg/kg (ART + GIN100). The carrageenan/kaolin-induced model is well established as a rheumatoid arthritis model [33]. Referring to a previous study, the rats were injected with 5% carrageenan/kaolin (150 μl) into the left knee joint, inducing arthritis on day 0 [34]. Gintonin (25, 50, and 100 mg/kg) dissolved in saline was administered (P.O.) to treatment groups while normal and arthritis groups received only saline (P.O.) once a day until day 6. The experimental procedures were carried out according to the animal care guidelines of the NIH and the Ewha Womans University Institutional Animal Care and Use Committee.

2.7. Assessment of arthritis symptoms

To evaluate the progress of carrageenan/kaolin-induced arthritis in rats, three different parameters, knee joint thickness, squeaking score, and weight distribution ratio (WDR), were measured 1 hour after treatment every day starting from day 0 until day 6. The thickness of the hind leg knee (left side) was assessed using an electronic digital caliper. The squeaking score was recorded as the average number of vocalizations when each hind leg was bent. The WDR is a measure of the force of both hind legs bearing its weight and this was recorded using an incapacitation meter (UGO-BASIL Biological Research Apparatus). The WDR percentage was
calculated as percentage \( WDR = \frac{\text{weight borne by ipsilateral limb}}{\text{total weight borne by both limbs}} \times 100 \). After Gintonin administration and behavioral tests, rats were sacrificed on day 6. In order to evaluate the effectiveness of Gintonin in the histopathology of the carrageenan/kaolin model, the knee joint tissue obtained from the rats of each group was paraffinized, embedded in paraffin, and sectioned. Subsequently, the formation of pannus and synovitis were evaluated through H&E staining, and scored as described [35]. All parameters were measured by investigators blinded to the experimental groups.

2.8. Paw-pressure test

The nociceptive removal threshold was evaluated using a paw-pressure analgesy meter (Ugo Basile Biological Research Apparatus Co., Comerio-Varese, Italy) in accordance with the Randall-Selitto test protocol provided by previous studies [36,37]. An hour after oral administration of Gintonin (100 mg/kg), 100 \( \mu l \) of 1% carrageenan was injected in the footpad of the rat, causing hyperalgesia. Then, the assessment was conducted three hours later in a blind manner. A gradually increasing force was added to the plantar and dorsal part of the paw of the rat until a withdrawal response resulted. In order to maintain its position over repeated trials, the action points were marked with ink. Six rats were used per group.

2.9. Measurement of cytokine levels in serum

Serum used for cytokine measurement was obtained from the heart of rats. The rats that received Gintonin for each concentration were sacrificed on day 6. The measurement of the levels of TNF-\( \alpha \) (Abnova Corp., Taipei, Taiwan), IL-6 (Elabscience biotechnology Inc., Houston, TX, USA), and PGE\(_2\) (Abcam Inc., Cambridge, MA, USA) levels were conducted using ELISA kits according to the protocol provided by each supplier.

2.10. Statistical analysis

All experiments were performed at least three times with duplicate samples. Data analyses were carried out using Prism 5.0 (GraphPad Software, San Diego, CA, USA). All data are presented as means ± S.E.M., and statistical comparisons were identified using one-way ANOVA with Tukey’s multiple comparison test and two-way ANOVA with Bonferroni’s post-hoc testing. \( p < 0.05 \) were considered to indicate statistical significance.

3. Results

3.1. Gintonin reduced the production of ROS.

Before starting in vitro experiments in Fibroblast-like synoviocytes (FLS), it was confirmed that there was no cytotoxicity in the presence or absence of Gintonin and IL-1\( \beta \) (10 ng/ml) (data not shown). To determine whether Gintonin affected ROS production in FLS cells, ROS products were measured 24 hours after stimulating the cells with IL-1\( \beta \) (10 ng/ml) following pretreatment with Gintonin (5, 10, 15, and 20 \( \mu g/ml \)) for 1 hour according to each concentration. Both intracellular accumulation of ROS products through \( \text{H}_2\text{DCFDA} \) (Fig. 1A) and superoxide production in the mitochondrial matrix through MitoSOX red (Fig. 1B) were significantly reduced by Gintonin.

**Fig. 1. Gintonin inhibited the production of ROS and protein expression of pro-inflammatory mediators in IL-1\( \beta \)-treated FLS cells.** FLS cells were pretreated with Gintonin (5, 10, 15, 20 \( \mu g/ml \)) for 1 h, followed by IL-1\( \beta \) (10 ng/ml) for 24 h. (A) \( \text{H}_2\text{DCFDA} \) for the measurement of intracellular ROS and (B) MitoSOX red for the detection of mitochondrial ROS production were stained in cells. (C) Western blot analysis using FLS cells pretreated with Gintonin for 1 h and stimulated later with IL-1\( \beta \) (10 ng/ml) for 6 h. The representative results from three independent experiments are shown. Quantification data are presented at the right panel. The data are expressed as means ± S.E.M. (n = 3). * \( p < 0.05 \), and *** \( p < 0.001 \) vs. untreated group (None), ** \( p < 0.01 \) and *** \( p < 0.001 \) vs. IL-1\( \beta \)-treated group without Gintonin treatment.
3.2. Gintonin inhibited inflammatory mediator expression.

To verify anti-inflammatory effects in IL-1β-stimulated FLS cells, the protein expressions were measured through western blot. Representative inflammatory mediators, iNOS, TNF-α, IL-6, and COX-2, were evaluated. IL-1β stimulation resulted in protein level increases of each inflammatory mediator, and cells with Gintonin pretreatment decreased the levels of those inflammatory mediators dose-dependently (Fig. 1C). Therefore, these results suggested that Gintonin has an anti-inflammatory effect in FLS cells.

3.3. Gintonin regulated the MAPK signaling pathway in IL-1β-stimulated FLS cells.

To determine the molecular mechanism behind the suppression of the aforementioned inflammatory mediators induced by Gintonin in FLS cells, the MAPK pathways, which are involved in the upstream signaling inflammatory processes, were investigated using western blot. Western blot analysis was conducted with antibodies against the phosphorylated- and total- forms of extracellular signal regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 (Fig. 2). Gintonin significantly reduced the phosphorylation of JNK and ERK MAPKs in IL-1β-stimulated FLS cells. On the other hand, Gintonin was not able to reduce the phosphorylation of p38 MAPK.

3.4. Gintonin regulated nuclear translocation of NF-κB/p65 in IL-1β-stimulated FLS cells.

NF-κB signaling pathway is known to be an important upstream modulator for the expression of cytokines and NF-κB pathway activity suppression leads to anti-inflammatory responses of cytokines and chemicals. Thus, the NF-κB signaling pathway was analyzed using western blot (Fig. 3). As expected, the results showed that the expression of phosphorylated IKKβ, IkBz, and NF-κB/p65 were enhanced in IL-1β-stimulated FLS cells without Gintonin. On the other hand, the pretreatment of Gintonin down-regulated these activated protein expressions and therefore suggesting that Gintonin blocked NF-κB/p65 from translocating to the nucleus of IL-1β-stimulated FLS cells.

3.5. Gintonin regulated inflammatory mediator expression in IL-1β-stimulated FLS cells via LPA receptor.

Next, to investigate whether LPA receptors were involved in the anti-inflammatory function of Gintonin, FLS cells were pretreated with H2L5186303 (H2L5), an LPAR2 antagonist, for 1 hour prior to adding Gintonin and then after 1 hour of Gintonin pretreatment, the cells were stimulated with IL-1β for 6 hours. Western blot analysis was then performed. As shown in Fig. 4A, H2L5 at 3 μM significantly reversed the results of Gintonin-mediated suppression of TNF-α, IL-6, and COX-2. The LPAR2 antagonist reversed the
downregulation of Gintonin against not only inflammatory factors but also NF-κB signaling pathway. Gintonin-mediated suppression of phosphorylated- IKKαβ, IκBα, and NF-κB/p65 was inhibited by H2L5 at 3μM. These results indicated that the LPAR2 plays an important role in the anti-inflammatory mechanisms of Gintonin in FLS cells.

3.6. Gintonin ameliorated arthritis behavioral parameters in the carrageenan/kaolin-induced arthritis model.

Based on the previous results, the anti-inflammatory effects of Gintonin were identified in FLS cells. This was then followed with experiments related to the effects of Gintonin on arthritis, an inflammatory disease, through in vivo carrageenan/kaolin-induced arthritis model in rats. The measured knee joint thickness, squeaking score, and WDR are indicators of arthritis symptoms in rats. Through the measurement of the knee joint thickness, all groups were indicated to have a severe case of arthritis starting on day 1 (Fig. 5A). Both the ART + GIN50 and ART + GIN100 groups showed a decrease in knee joint thickness from day 3 and the ART + GIN25 group knee joint thickness declined significantly starting from day 4. In the number of vocalizations indicated by the squeaking score (Fig. 5B), all groups had the highest score on day 1, the day after the arthritis was induced, indicating that the arthritis model was well induced. All groups showed a significant drop in the number of vocalizations from day 4. Among them, ART + GIN100 group showed the most significant mitigation. Next, the pain behavior against inflammation in rats was evaluated through WDR (Fig. 5C), a comparison of the distributed weight between the paws of rats after inducing arthritis. The NOR group WDR was normal at 50%. Prior to the injection of carrageenan/kaolin, the values in all groups were not significantly different from the NOR group. However, on the first day after the carrageenan/kaolin injection, there was a significant change in the WDR ratios. Arthritis was markedly reduced, with ART + GIN25 and ART + GIN100 groups having a recovery effect from Day 3, and the ART + GIN50 group from Day 4. All three indicators decreased considerably on the sixth day of treatment in comparison to day 1 when the arthritis symptoms were severe. The extent of this decrease was also statistically significant.

3.7. Gintonin showed analgesic tendencies in carrageenan-induced paw edema in rats.

Paw-pressure test was performed to assess whether Gintonin has an anti-nociceptive effect (Fig. 5D). The value of the pain withdrawal pressure for three groups was obtained by applying the Randall-Selitto probe. The rats squeaked at the threshold of
mechanically added pressure. CON group had a distinctly low pressure tolerance compared to NOR group. The average value of GIN100 group was slightly higher than that of the CON group. The value of GIN100 group was statistically significant, suggesting that Gintonin has a potential analgesic effect.

3.8. Gintonin alleviated histological outcome on the knee joints and decreased pro-inflammatory cytokines expression in serum.

The scores were evaluated mainly with a focus on the growth of the pannus and cartilage–pannus junction, number of infiltrated immune cells, and the thickness of the synovial membrane. Gintonin generally showed a dose-dependent anti-arthritic effect compared to the ART group. In the GIN100 treatment group, the development of the pannus decreased significantly enough to be visually identifiable. Thus, the anti-arthritic effects of Gintonin was identified in the in vivo carrageenan/kaolin arthritis rat model.

To explore the levels of inflammatory factors involved in the effects of Gintonin in vivo, TNF-α, IL-6, and PGE2 were determined in rat serum (Fig. 6B). ART group, which did not have any treatment with Gintonin and only had induced arthritis by carrageenan/kaolin, had significantly increased levels of TNF-α, IL-6, and PGE2. As the concentration of Gintonin increased, there was greater inhibition in the levels in each inflammatory factor. Overall, these data showed that Gintonin recovered carrageenan/kaolin-induced arthritis in rats.

4. Discussion

LPA is an important phospholipid signaling molecule that binds to seven-transmembrane domain receptors acting through specific G protein-coupled receptors (GPCRs), leading to the regulation of various biological activities such as cell migration, proliferation, apoptosis, differentiation, inflammatory cytokine secretion, and much more [38,39].

In a previous study, we demonstrated that Gintonin contributes to anti-inflammatory and anti-arthritic effects through the poly-arthritis mouse model and in vitro experiments. However, it did not provide how the mechanism of these effects by Gintonin works. Gintonin with an exogenous LPA ligand is expected to relieve RA by binding to the LPA receptor present in animal cell membranes. There is a report that among LPA receptors Gintonin has highest affinity with LPA2 followed by LPA5, LPA1, LPA3, and LPA4, consecutively [1]. Thus, we hypothesized that activating LPA2 receptor by Gintonin would have a suppressive effect on the arthritis.

In the first part of this study, the antioxidant and anti-inflammatory effects of Gintonin were verified using IL-1β-stimulated Fibroblast-like synoviocytes (FLS). The treatment of IL-1β-induced pro-inflammatory signaling through the MAPK cascade. In the study, Gintonin displayed an anti-arthritic effect by decreasing the nuclear translocation of NF-κB/p65 through the ERK and JNK MAPK signaling pathways. As Gintonin has the highest affinity with LPAR2 out of the LPA receptors, we used the H2L5186303, an LPAR2 antagonist, to confirm whether LPAR2 is involved with Gintonin and its anti-arthritic effect in FLS cell lines. As a result, inhibiting LPAR2 signaling resulted in the reversal of the action of Gintonin. Although distinct sub mechanisms of Gintonin/LPAR2 have not yet been identified, we have fully demonstrated that the LPAR2 pathway plays an important role in the anti-arthritis mechanism of Gintonin in IL-1β-stimulated FLS cells.

We have also showed, not only in in vitro but also in in vivo experiments, the anti-arthritic effect of Gintonin. In the second part of this study, we then carried out the in vivo experiment, using the carrageenan/kaolin-induced arthritis rat model and conducted three behavioral experiments to measure the development of arthritis. In the same way as the results of previous study about the
anti-arthritic effect of Gintonin in a polyarthritis mouse model. Gintonin relieved the symptoms of arthritis in the carrageenan/kaolin-induced arthritis rat model, a local arthritis model. The knee thickness was measured to assess the degree of edema caused by arthritis and the squeaking score was performed to evaluate cartilage damage and pain. Weight distribution ratio (WDR) was also used to measure the symptom mitigation of arthritis. In a previous study, the WDR was reported to be a useful and objective indicator for measuring the relief of arthritis. All three concentrations (25, 50, and 100 mg/kg) significantly alleviated the symptoms of arthritis development over six days. We then histologically analyzed the joints of the arthritis model after treatment with Gintonin. The histological analysis of the joints in Gintonin groups showed that the formation of the pannus had been reduced. Through further hyperalgesia experiments, it was found that Gintonin tended to have an anti-nociceptive effect. Gintonin also significantly reduced the production of TNF-α, IL-6, and PGE2, which are pro-inflammatory intermediaries, in serum and inflammatory synovial membrane tissues in RA patients. PGE2 also induces and amplifies edema and inflammatory diseases, which can eventually lead to cartilage and bone erosion.

In conclusion, Gintonin alleviates inflammation in synoviocytes and attenuates arthritis in an animal model through the LPAR2. It strongly suggests that Gintonin can be applied as an agent for arthritis therapeutic development.

**Declaration of competing interest**

The authors report no conflicts of interest.

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