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Research paper

Gintonin attenuates depressive-like behaviors associated with alcohol withdrawal in mice

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ABSTRACT

Background: *Panax ginseng* Meyer extracts have been used to improve mood and alleviate symptoms of depression. However, little is known about the extracts' active ingredients and the molecular mechanisms underlying their reported anti-depressive effects.
Methods: Gintonin is an exogenous lysophosphatidic acid (LPA) receptor ligand isolated from *P. ginseng*. BON cells, an enterochromaffin cell line, and C57BL/6 mice were used to investigate whether gintonin stimulates serotonin release. Furthermore, the effects of gintonin on depressive-like behaviors following alcohol withdrawal were evaluated using the forced swim and tail suspension tests.
Results: Treatment of BON cells with gintonin induced a transient increase in the intracellular calcium concentration and serotonin release in a concentration- and time-dependent manner via the LPA receptor signaling pathway. Oral administration of the gintonin-enriched fraction (GEF) induced an increase in the plasma serotonin concentration in the mice. Oral administration of the GEF in mice with alcohol withdrawal decreased the immobility time in two depression-like behavioral tests and restored the alcohol withdrawal-induced serotonin decrease in plasma levels.
Limitations: We cannot exclude the possibility that the gintonin-mediated regulation of adrenal catecholamine release in the peripheral system, and acetylcholine and glutamate release in the central nervous system, could also contribute to the alleviation of depressive-like behaviors.
Conclusion: The GEF-mediated attenuation of depressive-like behavior induced by alcohol withdrawal may be mediated by serotonin release from intestinal enterochromaffin cells. Therefore, the GEF might be responsible for the ginseng extract-induced alleviation of depression-related symptoms.

1. Introduction

The root of *Panax ginseng* C.A. Meyer, is a popular natural medicine consumed worldwide. The *P. ginseng* extract is reported to alleviate anxiety, depression, general fatigue, and chronic fatigue syndrome by improving health and vitality (Lee et al., 2016). Extracts of *P. ginseng* contain several active ingredients including acidic polysaccharide, a polymer of carbohydrates, and ginsenoside, one of the plant's saponins. However, these ingredients cannot fully explain the molecular mechanism(s) responsible for the *P. ginseng* extract-mediated mood elevation. Recently, our laboratory isolated a novel component from *P. ginseng* called gintonin, a non-carbohydrate polymer and a non-saponin. Gintonin acts as a ginseng-derived novel G protein-coupled lysophosphatidic acid (LPA) receptor ligand, which induces an intracellular calcium concentration $[Ca^{2+}]_i$ transient through LPA receptor signaling pathways (Choi et al., 2015). This gintonin-mediated $[Ca^{2+}]_i$ transient is further linked to calcium-dependent intra- and inter-cellular communication through the regulation of ion channels and receptors, and neurotransmitter release in the peripheral and central nervous systems (Choi et al., 2015).

It is well known that nearly 95% of the body's serotonin, or 5-hydroxytryptamine (5-HT), originates in the gut. Majority of the 5-HT is stored in gastrointestinal enterochromaffin (EC) cells (Bertrand and Bertrand, 2010; McLean et al., 2007). Stimulation of the intestinal EC cells in the gut induces 5-HT release in a Ca^{2+}-dependent manner (Racké and Schwörer, 1993). Moreover, 5-HT released from the

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intestinal EC cells affects intestinal motility and secretion of digestive enzymes and juices (Gershon, 2013; McLean et al., 2007). The intestinal EC cells are also a primary source of plasma 5-HT (McLean et al., 2007). However, despite being the main source of 5-HT, large amounts of EC cells are difficult to derive from the intestines of experimental animals. Instead, BON cells, which originate from human intestinal carcinoids and contain enterochromaffin granules with 5-HT, are well characterized in terms of their 5-HT release properties (Townsend et al., 1993), and have been used as an alternative EC cell model for 5-HT-related studies of the intestinal enterochromaffin system (Townsend et al., 1993; Vandamme et al., 2015). Although the effects of 5-HT derived from intestinal systems on the central nervous system are not clearly defined, recent studies have shown that intestinal 5-HT influences the vomiting center in the brain stem through intestinal afferent systems to the brain (Andrews and Horn, 2006; Endo et al., 2000). In addition, it has been recently shown that LPA1 (Lysophosphatidic acid 1) receptor-null mice consume more alcohol during alcohol withdrawal and exhibit anxiety-like behaviors (Castilla-Ortega et al., 2016). Moreover, the plasma concentration of 5-HT decreases in patients with alcohol withdrawal with depression (Almeida-Montes et al., 2000; Patkar et al., 2013). However, a link between 5-HT released from intestinal EC cells and alcohol withdrawal-induced depressive-like behavior is still not established.

In the present study, we investigated whether the *in vitro* gintonin-mediated $[Ca^{2+}]_i$ transient in BON cells is coupled to 5-HT release, and examined *in vivo* changes in plasma 5-HT levels after oral administration of the gintonin-enriched fraction (GEF). We further investigated whether the GEF-mediated increase in plasma 5-HT levels can relieve the alcohol withdrawal-induced depressive-like behavior in tests such as the forced swimming test (FST) and tail suspension test (TST) in mice. We found that the treatment of BON cells with gintonin induced $[Ca^{2+}]_i$ transients and 5-HT release through LPA receptor signaling pathways, while oral GEF administration increased the plasma 5-HT levels. Furthermore, oral administration of gintonin attenuated alcohol withdrawal-induced depressive-like behavior in mice. We also discuss the molecular mechanisms of 5-HT release from intestinal EC cells, and how the elevation of plasma 5-HT levels after oral administration of gintonin is linked to anti-depressive effects in mice with alcohol withdrawal.

2. Materials and methods

2.1. Animals and drug treatments

Male wild-type C57BL/6 mice (12-week-old) were purchased from Koatech Co., Ltd. (Pyongtaek, Korea). Gintonin was prepared according to previous method (Choi et al., 2011). Given the scarcity of gintonin in animal experiments, we used the gintonin-enriched fraction (GEF). The GEF was prepared as follows. One kilogram of 4-year-old ginseng was ground into small pieces (> 3 mm) and refluxed with 70% fermented ethanol eight times for 8 h each at 80 °C. The extracts (150*g*) were concentrated, dissolved in distilled, cold water at a ratio of 1:10, and stored at 4 °C for 24–96 h. The supernatant and precipitate of water fractionation after ethanol extraction of ginseng were separated by centrifugation (3000 rpm, 20 min). The precipitate after centrifugation was lyophilized. This fraction was referred to as the GEF since this fraction contains most of the gintonin (Choi et al., 2015). Mice were treated with the GEF (50 or 100 mg/kg) through oral administration daily for 7 days. Mice were housed in groups of 5 per cage, and were allowed access to water and food ad libitum. Animals were kept under a constant temperature (23 ± 1 °C and humidity (55 ± 5%) on a 12:12 h light/dark cycle (lights on from 07:00 until 19:00 h). All the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Konkuk University (Permit Number: 16–206). All the surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

2.2. BON cell culture and measurement of intracellular calcium concentration

The human carcinoid cell line, BON, is a mixed population of large round cells and small cells with dendrite-like extensions. The cells were subcloned by serial dilution, and clone 5, which displayed the highest gintonin-induced 5-HT release, was selected. The free $[Ca^{2+}]_i$ was measured by dual excitation spectrofluorometric analysis of the cell suspension loaded with Fura-2 AM, as previously described (Shin et al., 2012). Briefly, BON cells were harvested with a trypsin/ethylenediaminetetraacetic acid (EDTA) solution and re-suspended in a HEPES-buffered solution (HBS). The cells were incubated with Fura-2 AM (final concentration, 2.5 μM) in HBS at 37 °C for 40 min. The extracellular Fura-2 AM was removed by centrifugation. Each aliquot containing 3×10^6 cells was loaded into a cuvette and the free calcium mobilization was measured using an RF-5301PC spectrofluorophotometer and Supercap software (excitation, 340 nm and 380 nm; emission, 520 nm) (Shimadzu, Tokyo, Japan).

2.3. Induction of alcohol withdrawal

Male C57BL/6 mice were provided with a Lieber/DeCarli Regular Liquid Diet for Rodents-Ethanol (Dyets, Bethlehem, PA, USA) and water *ad libitum* for 1 month. Subsequently, mice were given continuous access to 2 bottles, 1 with water and the other with a 6% ethanol solution for 2 weeks. Water and ethanol consumption were recorded every day. The experiment involved four different groups: 1) saline control, 2) alcohol withdrawal alone, 3) GEF (100 mg/kg) alone, and 4) GEF (100 mg/kg)+alcohol withdrawal. After 14 days of drinking ethanol, mice were withdrawn of ethanol for 5 consecutive days. At the end of the fifth withdrawal day, behavioral tests were performed in these mice in order to assess depression-like behavior following ethanol withdrawal.

2.4. Forced swim test (FST) and tail suspension test (TST)

During the FST, mice were placed in an open cylinder-shaped flask (diameter, 10 cm; height, 25 cm) filled with 19 cm water at 24 ± 1 °C. The FST, a selective standard animal test for testing the effect of antidepressant treatment, was used to assess behavioral immobility in mice (Haj-Mirzaian et al., 2015; Porsolt et al., 1977). Mice were allowed to swim for a total of 6 min. A mouse was assumed immobile when it stopped to struggle and floated motionlessly in the water, with only limited movements to keep its head above the water surface. The immobility time was recorded during the last 4 min of the test. In the TST, each mouse was suspended on the edge of a rod, 50 cm above a table top, using adhesive Scotch tape placed approximately 1 cm away from the tip of the tail. Tail climbing was prevented by passing the mouse's tail through a small plastic cylinder prior to suspension. The duration of immobility was manually measured for a 6-min observation period. Mice were considered immobile when they hung down passively and were completely motionless (Cryan et al., 2005; Steru et al., 1985). Compounds that decrease immobility and increase active behavior in the FST and TST are considered to suppress the indices of depression.

2.5. Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) or one-way repeated measures ANOVA followed by *post-hoc* Fisher's least significant difference (LSD) test. Unpaired 2-tailed *t*-test and Pearson's correlation were used for *in vitro* and *in vivo* statistical comparisons using IBM SPSS ver. 21.0 (IBM, Chicago, IL,