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Research Article

Enhancing effect of *Panax ginseng* on Zip4-mediated zinc influx into the cytosol

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ABSTRACT

Background: Zinc homeostasis is essential for human health and is regulated by several zinc transporters including ZIP and ZnT. ZIP4 is expressed in the small intestine and is important for zinc absorption from the diet. We investigated in the present study the effects of *Panax ginseng* (*P. ginseng*) extract on modulating Zip4 expression and cellular zinc levels in mouse Hepa cells.
Methods: Hepa cells were transfected with a luciferase reporter plasmid that contains metal-responsive elements, incubated with *P. ginseng* extract, and luciferase activity was measured. Using $^{65}ZnCl_2$, zinc uptake in *P. ginseng*-treated cells was measured. The expression of Zip4 mRNA and protein in Hepa cells was also investigated. Finally, using a luciferase reporter assay system, the effects of several ginsenosides were monitored.
Results: The luciferase activity in cells incubated with *P. ginseng* extract was significantly higher than that of control cells cultured in normal medium. Hepa cells treated with *P. ginseng* extract exhibited higher zinc uptake. *P. ginseng* extract induced *Zip4* mRNA expression, which resulted in an enhancement of Zip4 protein expression. Furthermore, some ginsenosides, such as ginsenoside Rc and Re, enhanced luciferase activity driven by intracellular zinc levels.
Conclusion: *P. ginseng* extract induced Zip4 expression at the mRNA and protein level and resulted in higher zinc uptake in Hepa cells. Some ginsenosides facilitated zinc influx. On the basis of these results, we suggest a novel effect of *P. ginseng* on Zip4-mediated zinc influx, which may provide a new strategy for preventing zinc deficiency.

1. Introduction

Zinc is an essential nutrient that plays an indispensable role in cell structural components, enzyme activation, and signaling [1,2]. As such, zinc deficiency causes several physical disorders including dysgeusia, anorexia, dermatitis, diarrhea, alopecia, hypogonadism, and immune system dysfunction [3–6]. In mice and humans, nine zinc transporters (ZnT) and 14 Zrt-, Irt-like protein (ZIP) transporters have been determined and are important for zinc homeostasis [2]. These transporters are solute carrier family members that do not require ATP hydrolysis for zinc transfer [2]. The ZnT family reduces intracellular zinc by accelerating zinc efflux from the cytosol into the extracellular space or the lumen of intracellular compartments such as the endoplasmic reticulum or Golgi apparatus. By contrast, ZIP family members increase intracellular zinc by promoting zinc influx from the extracellular space or the lumen of intracellular compartments. Mutations in these transporter genes occur in many inherited diseases that are of significant clinical interest [2].

Mutations in the *ZIP4* (*SLC39A4*) gene is associated with inherited diseases. ZIP4 is present in the small intestine and plays an essential role in zinc absorption from the diet [7]. Therefore, some mutations in this gene result in acrodermatitis enteropathica, which causes severe zinc deficiency within a few days to weeks after birth. Consequently, nutritional supplementation with high doses of zinc is needed to manage the symptoms of this disorder

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[8]. Because of its importance, the regulatory mechanism of ZIP4 expression has been well-studied, and its several post-transcriptional mechanisms have been elucidated. During zinc-deficient conditions, *ZIP4* mRNA is stabilized, and ZIP4 degradation, via endocytosis from the plasma membrane, is attenuated. This results in ZIP4 accumulation on the apical membranes of enterocytes in the small intestine [9–11]. When ZIP4 was overexpressed, zinc uptake and cellular zinc levels were significantly increased. Therefore, a strategy of enhancing the ZIP4 expression would theoretically be beneficial for zinc deficiency prevention [12].

To investigate the regulation of ZIP4 and mouse Zip4, several mouse cell lines were examined whether they regulate Zip4 expression in response to zinc [11–13]. Hepa cells are proven to mimic the regulation of Zip4 expression in the small intestine of mice, in a zinc concentration-dependent manner with multiple post-transcriptional mechanisms. Therefore, ZIP4 target screening of food and food chemicals has been conducted using this cell line [11–13]. From those studies, soybean extracts have been screened, and soyasaponin Bb, a triterpene glycoside, was determined as a compound responsible for increasing Zip4 protein abundance and zinc levels in Hepa cells [12]. However, other daily foods and herbal medicines will also induce Zip4 expression and zinc uptake. Information on such foods and herbal medicines would be useful for improving zinc deficiency by daily intake. Particularly, herbal medicines are familiar to the elderly, who frequently exhibit zinc deficiency [14–16] and are amenable to regular treatment.

Ginseng is one of the most-studied herbal medicines, and numerous reports indicated its beneficial effects for various diseases [17]. *Panax ginseng* (*P. ginseng*), which is grown in China and Korea, contains various triterpene glycosides (i.e., ginsenosides), which are the major bioactive compounds in ginseng. Ginsenosides are known as saponins comprising dammarane triterpenoid glycosides. They are divided into either the 20(*S*)-protopanaxadiol (PPD) or 20(*S*)-protopanaxatriol (PPT) group (Supplementary Fig. 1) [18,19]. The PPD group includes ginsenosides Rb1, Rb2, Rc, Rd, Rg3, and Rh2, whereas the PPT group contains ginsenosides Re, Rf, Rg1, Rg2, and Rh1. Each ginsenoside exhibits different pharmacological properties because of their different structure. The pharmacological actions of these ginsenosides in anti-inflammatory [20,21], anti-cardiovascular [22], anti-obesity [23], and anticancer [24] processes have been documented. Other saponins also exhibit pharmacological properties including total plasma cholesterol reduction [25] and antitumor activity [26]. On the basis of these studies, we hypothesized that the *P. ginseng* extract may have the capacity to modulate intracellular zinc levels. However, the effects of *P. ginseng* on Zip4-mediated zinc influx have not been studied.

In the present study, using mouse Hepa cells, we evaluated the effects of *P. ginseng* extract on the Zip4-mediated influx of zinc. *P. ginseng* extract significantly increased cellular zinc levels by inducing *Zip4* mRNA. Some ginsenosides exhibited positive effects on zinc uptake. The regulatory mechanisms of Zip4 expression were also discussed.

2. Materials and methods

2.1. Materials

Dried *P. ginseng* Meyer (China, Lot. 008617007) was purchased from Tochimoto Tenkaido (Osaka, Japan). Ginsenoside Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2 were obtained from Biosynth Carbosynth (Berkshire, United Kingdom). Soyhealth SA (SSA) was purchased from Fuji Oil (Osaka, Japan). All other chemicals were of the highest reagent grade available.

2.2. Preparation of P. ginseng methanol extract

Twenty grams of dried *P. ginseng* powder was extracted three times with 100 mL of 100% methanol while stirring at room temperature for 50 min followed by sonication for 10 min. The extracts were pooled and filtered through filter paper. After evaporation of the methanol using a rotary vacuum evaporator at 40°C, the residue was dissolved in dimethyl sulfoxide at a concentration of 1.0 g/mL and sterilized with a 0.22 μm filter.

2.3. Cell culture

Mouse Hepa cells, kindly provide by Dr. Glen K. Andrews (University of Kansas Medical Center), were grown in a humified 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Heat-inactivated FBS was treated with Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA) to generate a zinc-deficient conditioned medium (CX), as described previously [11–13].

2.4. Dual-luciferase assay

Five copies of a metal-responsive element (MRE) (5′-CTCTGCACACGGCCC-3′), which has a high binding affinity for MRE-binding transcription factor-1 but lacks the ubiquitous Sp1 transcription factor-binding activity [27], was inserted into the multiple cloning region of the pGL4.26[*luc2*/minP/Hygro] vector (Promega, Madison, WI). The *Nhe* I and *Hind* III restriction enzymes were used to generate the plasmid, pGL4.26-MRE. Hepa cells were transfected with the luciferase reporter plasmid pGL4.26-MRE using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The pGL4.74 that carries *renilla luciferase* was also co-transfected as an internal control to normalize transfection efficiency. Luciferase activity was measured using a GloMax 20/20 Luminometer (Promega) and a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.5. Cytotoxicity assay

Hepa cells were subcultured in 96-well plates at a density of 1.0×10^5 cells per well for 24 h and incubated for an additional 24 h with DMEM supplemented with 10% FBS in the absence or presence of various concentrations of *P. ginseng* extract. The cytotoxicity of the extracts was evaluated using a cell counting kit-8 (Dojindo, Kumamoto, Japan), which is based on the conversion of WST-8 to formazan by viable cells. The absorbance at 450 nm (reference at 630 nm) was determined using an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA) to measure viable cells.

2.6. Zinc uptake

Hepa cells were seeded in 24-well plates and incubated for 24 h with DMEM supplemented with 10% FBS in the absence or presence of 1000 μg/mL of *P. ginseng* extract. The Hepa cells were washed with wash buffer (10 mM HEPES pH 7.4, 142 mM NaCl, 10 mM glucose, 5 mM KCl) and then incubated with 0.5 μM $ZnCl_2$ containing 1.87 nM $^{65}ZnCl_2$ (18.5 kBq/well) in a zinc-deficient conditioned medium at 37°C for 20 min. Zinc uptake was stopped by adding an equal volume of ice-cold wash buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA). After removing the buffer, the cells were washed two times with the same buffer and dissolved in 200 mM NaOH. Cell-associated radioactivity was measured with an automatic gamma counter (2480 Wizard^2, Perkin Elmer, Waltham, MA), and protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific,

Waltham, MA). Zinc uptake rates were calculated and normalized to protein content.

2.7. RNA isolation and quantitative RT-PCR

Total RNA was extracted from Hepa cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription for real-time PCR was conducted using the ReverTra Ace qPCR RT Master Mix kit with gDNA Remover (Toyobo, Osaka, Japan). The mRNA levels were quantified by real-time PCR using the Thunderbird SYBR qPCR Mix (Toyobo) and a LightCycler 96 (Roche, Basel, Switzerland). The primer sequences and reaction conditions were described in previous studies [12,28]. *β-actin* was used as an internal control to normalize expression in each sample. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8. Immunoblot analysis

Cells were subcultured in six-well plates at a density of 1.0×10^6 cells per well in DMEM containing 10% FBS. After incubating for 24 h, the cells were washed with PBS and incubated with a normal medium in the absence or presence of various concentrations of *P. ginseng* extract or zinc-deficient conditioned medium for 24 h at 37°C. Cells were resuspended in 700 μL of cold homogenization buffer (250 mM sucrose, 20 mM HEPES, and 1 mM EDTA) and homogenized with 40 strokes of a tight-fitting 1 mL Dounce homogenizer. The homogenate was centrifuged for 5 min at 1,000 × *g* at 4°C to remove the nucleus. The supernatant was centrifuged for 30 min at 20,600 × *g* at 4°C. The pellet was dissolved in 1% Triton X-100 and sonicated. The expression of Zip4, Na,K-ATPase, and GRP78 were examined by immunoblot analysis using a monoclonal anti-Zip4 antibody (1:1000 dilution), which detects the extracellular amino-terminal domain [12,29], the monoclonal anti-Na,K-ATPase antibody (Cell Signaling Technology, Danvers, MA) (1:1000), and the polyclonal anti-GRP78 BiP antibody (Abcam plc, Cambridge, UK) (1:1000), respectively.

2.9. Statistical analysis

The statistical significance of the differences between mean values was analyzed using an unpaired Student's *t*-test. Multiple comparisons were conducted using Dunnett's test following analysis of variance. Differences were considered significant at $P < 0.05$ (two-tailed).

3. Results

3.1. Effects of P. ginseng extract on intracellular zinc levels

The reporter plasmid pGL4.26-MRE, which contains five MRE copies, was constructed to monitor changes in intracellular zinc levels (Supplementary Fig. 2A). We co-transfected pGL4.26-MRE and pGL4.74, an internal control plasmid for normalizing transfection efficiency, into Hepa cells, and evaluated the response to cellular zinc levels (Supplementary Fig. 2B). Firefly luciferase activity was significantly lower in zinc-deficient medium (CX), and its activity was returned to a normal level by adding $ZnSO_4$. When soybean extract SSA, which contains soyasaponin Bb, was added to the normal medium, the luciferase activity was significantly enhanced as reported previously [12]. This probably occurred by the inhibition of Zip4 protein degradation and an increase of zinc uptake. The result indicates that the assay system functioned properly. Next, we examined whether *P. ginseng* extracts affect intracellular zinc levels. The luciferase activity was significantly increased with the addition of *P. ginseng* extract at 1000 μg/mL, the same concentration used for SSA treatment (Fig. 1A). Since the *P. ginseng* extract did not exhibit cytotoxicity in Hepa cells below a concentration of 1000 μg/mL (Supplementary Fig. 3), we treated the Hepa cells with different concentrations of *P. ginseng* extract. The luciferase activity was increased by the addition of extract in a concentration-dependent manner (Fig. 1B). At 1000 μg/mL, the enhancing effects of *P. ginseng* on luciferase activity were significantly higher than that of control cells cultured in a normal medium. These results suggest that by the addition of *P. ginseng* extract, intracellular zinc levels in Hepa cells were increased, resulting in elevated luciferase activity. Indeed, *P. ginseng* extract enhanced zinc uptake in Hepa cells (21.69 ± 0.41 vs. 24.10 ± 0.65 % dose/mg cell protein) (Fig. 2).

3.2. Effects of P. ginseng extract on Zip4 expression

To understand the mechanism underlying the enhancement of intracellular zinc levels, we investigated the effects of *P. ginseng* extract on Zip4 mRNA and protein expression. Consistent with previous studies [9–11], zinc-deficient conditions upregulated the expression of Zip4 by stabilizing the mRNA and inhibiting protein degradation (~75 kDa) (Fig. 3A and B). The expression of Zip4 mRNA and protein was significantly enhanced by the addition of *P. ginseng* extract in a concentration-dependent manner (Fig. 3A and B). Thus, *P. ginseng* extract increased Zip4 expression at the mRNA and protein level, which resulted in higher zinc uptake in Hepa cells.

Next, we investigated the time-dependent effects of *P. ginseng* extract on *metallothionein 1* (*Mt1*) mRNA expression. As the *Mt1* promoter contains MREs, it can act as a substitute for monitoring the changes of intracellular zinc levels [12]. *Mt1* mRNA decreased after a 6 h incubation under zinc-deficient conditions (CX) and these decreased levels were maintained for 24 h (Fig. 4A). SSA also affected *Mt1* mRNA expression as early as 6 h and the differences in *Mt1* mRNA expression were significant at all-time points following treatment. By contrast, *P. ginseng* extract increased *Mt1* mRNA expression at 12 and 24 h but not at 6 h. Interestingly, *Zip4* mRNA expression was significantly increased at all-time points by *P. ginseng* extract, and at 12 and 24 h, the expression exhibited greater than a two-fold increase (Fig. 4B). In contrast to a higher induction of mRNA expression, Zip4 protein increased slightly at 12 and 24 h when compared with the control (Fig. 4B and C). These results suggest that *P. ginseng* extracts induced Zip4 expression at the mRNA and protein level, which are responsible for higher zinc uptake in Hepa cells.

3.3. Effects of ginsenosides on intracellular zinc levels

We examined whether ginsenosides are responsible for facilitating the transport of zinc into the cytosol. Hepa cells co-transfected with the pGL4.26-MRE and the pGL4.74 plasmids were incubated with various concentrations of the representative ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2) for 24 h to investigate the effect of the major ginsenosides on intracellular zinc levels (Fig. 5). To examine and compare the effects of ginsenosides on intracellular zinc levels, we have tested the response of luciferase activity by various concentrations of ginsenosides (1, 2.5, 5, 10, 50, 100, 250, 500 μM). Treatment with higher concentration (>10 μM) of ginsenosides did not exhibit significant cytotoxicity and also did not influence luciferase activity in Hepa cells (data not shown). As shown in Fig. 5C and E, luciferase activity was significantly increased by the addition of 1 μM ginsenoside Rc and 2.5 μM ginsenoside Re. Treatment with 1 μM ginsenoside Rb1 and 2.5 μM ginsenoside Rb2 also tended to increase luciferase activity (Fig. 5A and B). The effective concentrations of these ginsenosides were

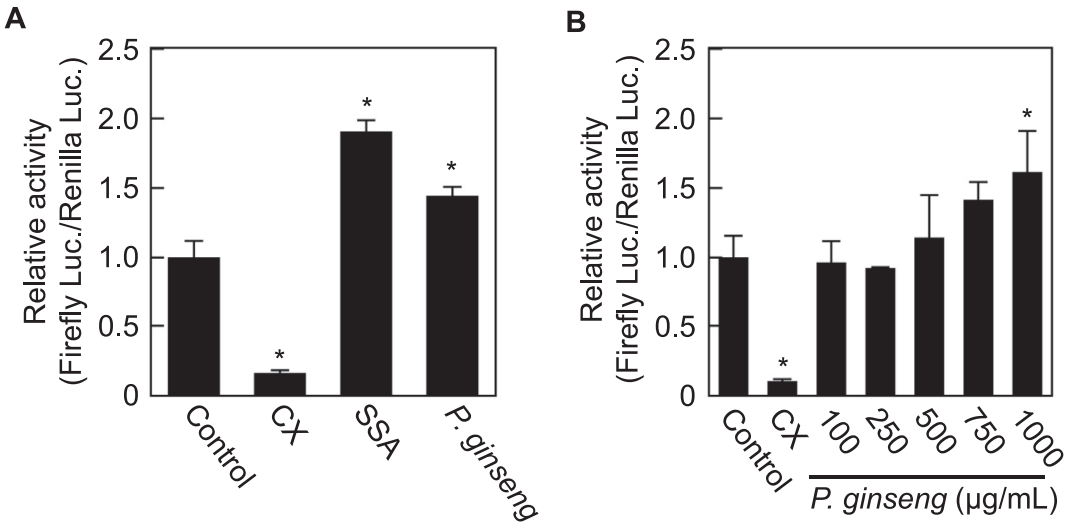

Fig. 1. Effects of *P. ginseng* extract on luciferase activity driven by intracellular zinc levels. Hepa cells co-transfected with pGL4.26-MRE and pGL4.74 were incubated for 24 h in normal medium (control), zinc-deficient medium (CX), and in the presence of 1000 μg/mL Soyhealth SA (SSA) or 1000 μg/mL *P. ginseng* extract in normal medium (A), or in the presence of the indicated concentrations of *P. ginseng* extract in normal medium (B). *$P < 0.05$, significantly different from control. Each bar represents the mean ± S.D. (n = 3).

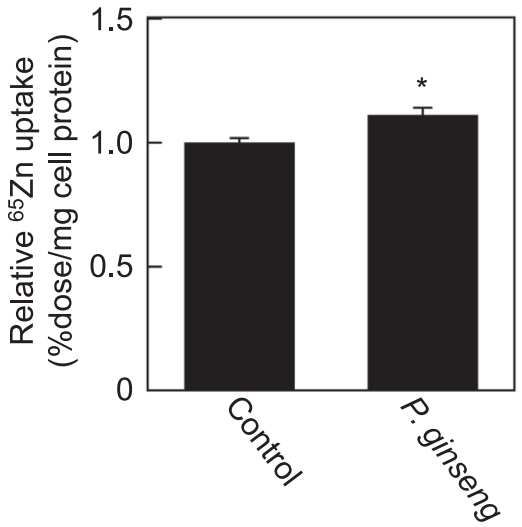

Fig. 2. *P. ginseng* extract induces zinc uptake in Hepa cells. Hepa cells were incubated for 24 h in normal medium (control) in the absence or presence of 1000 μg/mL *P. ginseng* extract. Zinc uptake rates for 20 min were calculated and normalized to protein concentrations. *$P < 0.05$, significantly different from control. Each bar represents the mean ± S.D. (n = 3).

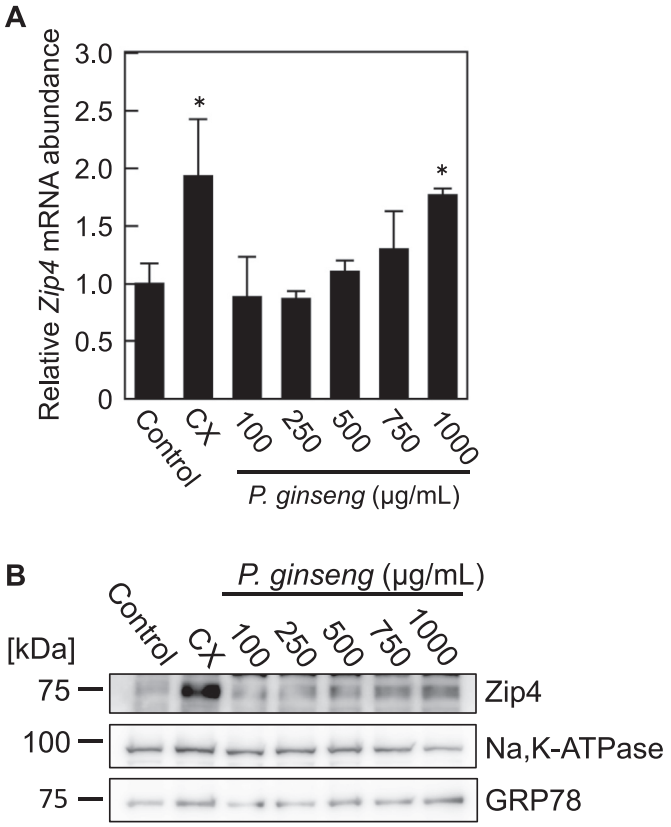

Fig. 3. Induction of Zip4 expression by *P. ginseng* extract in a concentration-dependent manner. (A) Total RNA isolated from Hepa cells cultured under the indicated conditions for 24 h was subjected to RT-qPCR. *$P < 0.05$, significantly different from control. Each bar represents the mean ± S.D. (n = 3). (B) Membrane proteins (18 μg) from Hepa cells treated with the indicated concentrations of *P. ginseng* extract for 24 h were separated by 10% SDS-PAGE. The expression of Zip4, Na,K-ATPase, or GRP78 was detected with specific antibodies.

comparable to those of the ginsenosides in *P. ginseng* extract (1000 μg/mL) (Table 1; Supplementary Fig. 4). Although ginsenoside Rc enhanced luciferase activity at 1 μM, it tended to decrease at 5 μM ($P = 0.088$). Treatment with 5 μM ginsenoside Rf decreased luciferase activity (Fig. 5F). These results indicate that each ginsenoside may have different effects on intracellular zinc levels, and their concentration is important for efficacy.

4. Discussion

Zinc is an essential nutrient, and its absorption from the small intestine via ZIP4 is vital to human health. In the present study, we examined the effect of *P. ginseng* extract on Hepa cells as a screen for herbal medicines that enhance zinc absorption. *P. ginseng* extract significantly increased zinc uptake in Hepa cells (Figs. 1 and 2). This higher uptake was likely caused by enhanced Zip4 expression at the mRNA and protein levels (Figs. 3 and 4). Some

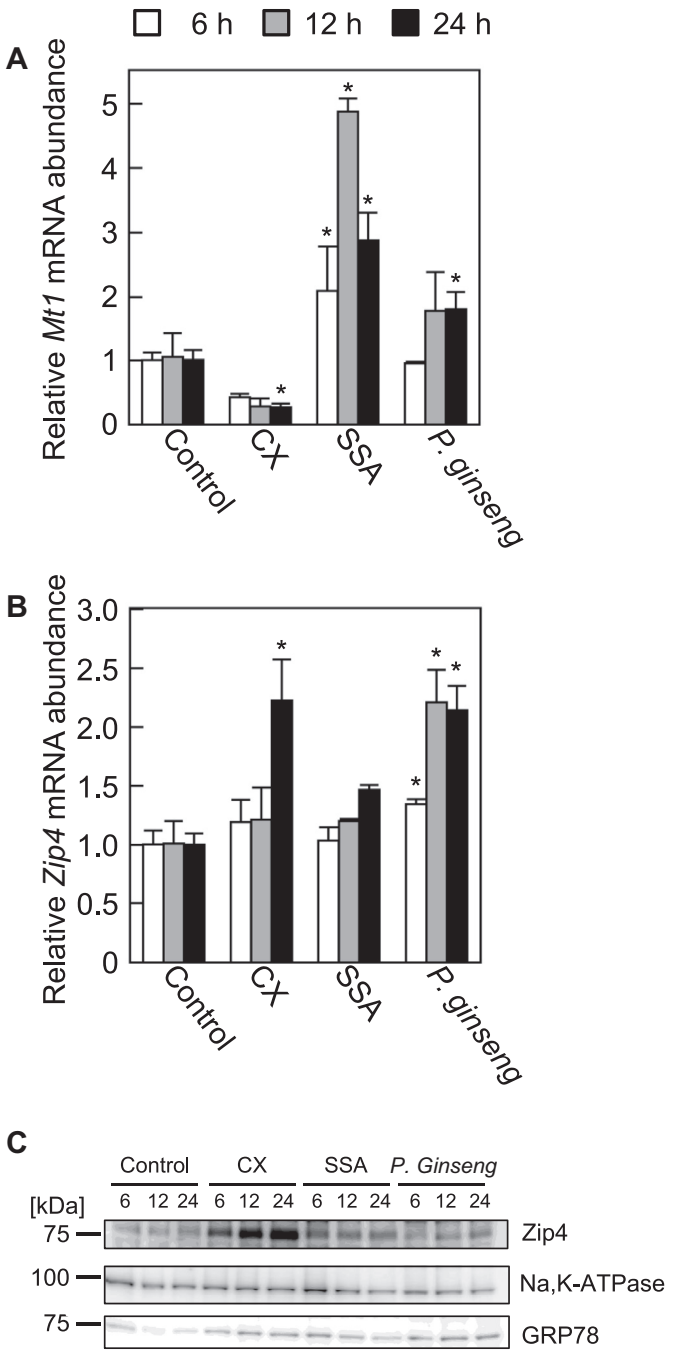

Fig. 4. Time-dependent expression of Mt1 and Zip4 after treatment with *P. ginseng* extract. (A) *Mt1* mRNA and (B) *Zip4* mRNA expression levels were measured via qRT-PCR in Hepa cells cultured in normal medium (control), zinc-deficient medium (CX), and in the presence of 1000 μg/mL SSA or1000 μg/mL *P. ginseng* extract in normal medium. *$P < 0.05$, significantly different from control. Each bar represents the mean ± S.D. (n = 3). (C) Membrane proteins (10 μg) from Hepa cells treated under the indicated conditions were separated by 10% SDS-PAGE.

ginsenosides at least partially contributed to the enhanced zinc uptake in Hepa cells (Fig. 5).

The expression of Zip4 is regulated at the post-transcriptional level by multiple mechanisms. A previous study revealed that SSA decreased Zip4 endocytosis in response to zinc and increased the abundance of apically localized Zip4 [12]. SSA showed little effect on *Zip4* mRNA levels [12], which is consistent with the results of the present study (Fig. 4). By contrast, *P. ginseng* extract increased *Zip4* mRNA levels more than two-fold at 12 and 24 h, which would contribute to the enhanced Zip4 protein levels (Fig. 4). Luciferase activity and *Mt1* mRNA expression in cells treated with *P. ginseng* extract are lower than those in cells treated with SSA. These data suggested that the abundance of Zip4 localized to the plasma membrane in the cells treated with *P. ginseng* extract might be lower than that in the cells treated with SSA. Therefore, the induction mechanism of Zip4 expression by *P. ginseng* extract is different compared with that of SSA.

The stabilization of *Zip4* mRNA is induced by zinc deficiency; however, the mechanism by which zinc stabilizes *Zip4* mRNA remains to be elucidated [11]. The transcription of *Zip4* mRNA is controlled by Krüppel-like factor 4 (KLF4), which is highly expressed in the intestinal epithelium [30,31]. KLF4 belongs to the Krüppel-like transcription factor family, and its expression and activity are regulated by phosphorylation, acetylation, ubiquitination, and methylation [32–35]. For example, the activity of KLF4 is negatively regulated by ERK1 and ERK2 phosphorylation and by p300/CBP acetylation [32,33]. Some traditional Chinese medicines, such as Sijunzi decoction and Tongxinluo, which both contain *P. ginseng* as a component, modulate KLF4 expression and activity [36,37]. Tongxinluo enhances endothelial cell proliferation by increasing the phosphorylation of KLF4 [37]. Therefore, as one possible mechanism of Zip4 induction, *P. ginseng* extract may regulate the abundance of *Zip4* mRNA via the modulation of KLF4-related signalling.

P. ginseng contains various active constituents, particularly, the ginsenosides. A previous study has reported that PPD inhibits the activation of MAP kinase family members, including ERK1, ERK2, JNK, and p38 MAPK [38]. Additionally, ginsenoside Rc is known to decrease forkhead box O1 acetylation by CBP, which leads to the upregulation of various genes involved in cellular metabolism [39]. In the present study, some ginsenosides enhanced luciferase activity driven by intracellular zinc levels (Fig. 5), although the effects of each ginsenoside were lower than that of the whole *P. ginseng* extract. Multiple ginsenosides of *P. ginseng* may exhibit combinatorial and synergistic effects on Zip4 expression and may produce greater effects than that of any single ginsenoside, as is the case for antioxidant activity and activation of Nrf2 by Rb1, Rg1 and 20S [40]. Additionally, *P. ginseng* contains several other active compounds, including polysaccharides, alkaloids, and phenolic acids [19]. As the non-saponin fraction of *P. ginseng* decreases glucose uptake and transport [41], other compounds in the extract may modulate Zip4 expression. Further studies will provide insight into these active compounds and their physiological roles.

While zinc is an essential nutrient, a high concentration of zinc is toxic to the cells. Zinc availability is also involved in tumor growth and progression. Moreover, ZIP4 is a potential marker in pancreatic cancer, in which ZIP4 is ectopically overexpressed [42]. Thus, the use of *P. ginseng* extract might need to be careful if used in large quantities. Since some ginsenosides, i.e., Rc and Rf, showed the tendency to decrease luciferase activity in this study, further analysis of such compounds might lead to the identification of effective compounds against cancer cells in the future.

In conclusion, we demonstrated that *P. ginseng* extract enhanced the Zip4-mediated zinc influx into the cytosol. *P. ginseng* is an important herbal medicine used globally, especially in Asian countries. It is also familiar to elderly people who likely experience decreased zinc levels in serum or plasma with age [15,16]. Therefore, the daily intake of *P. ginseng* extract may contribute to efficient zinc absorption from the diet. Our study will help in developing new therapeutic agents to enhance the ZIP4-mediated zinc influx and to provide new strategies for preventing zinc deficiency.

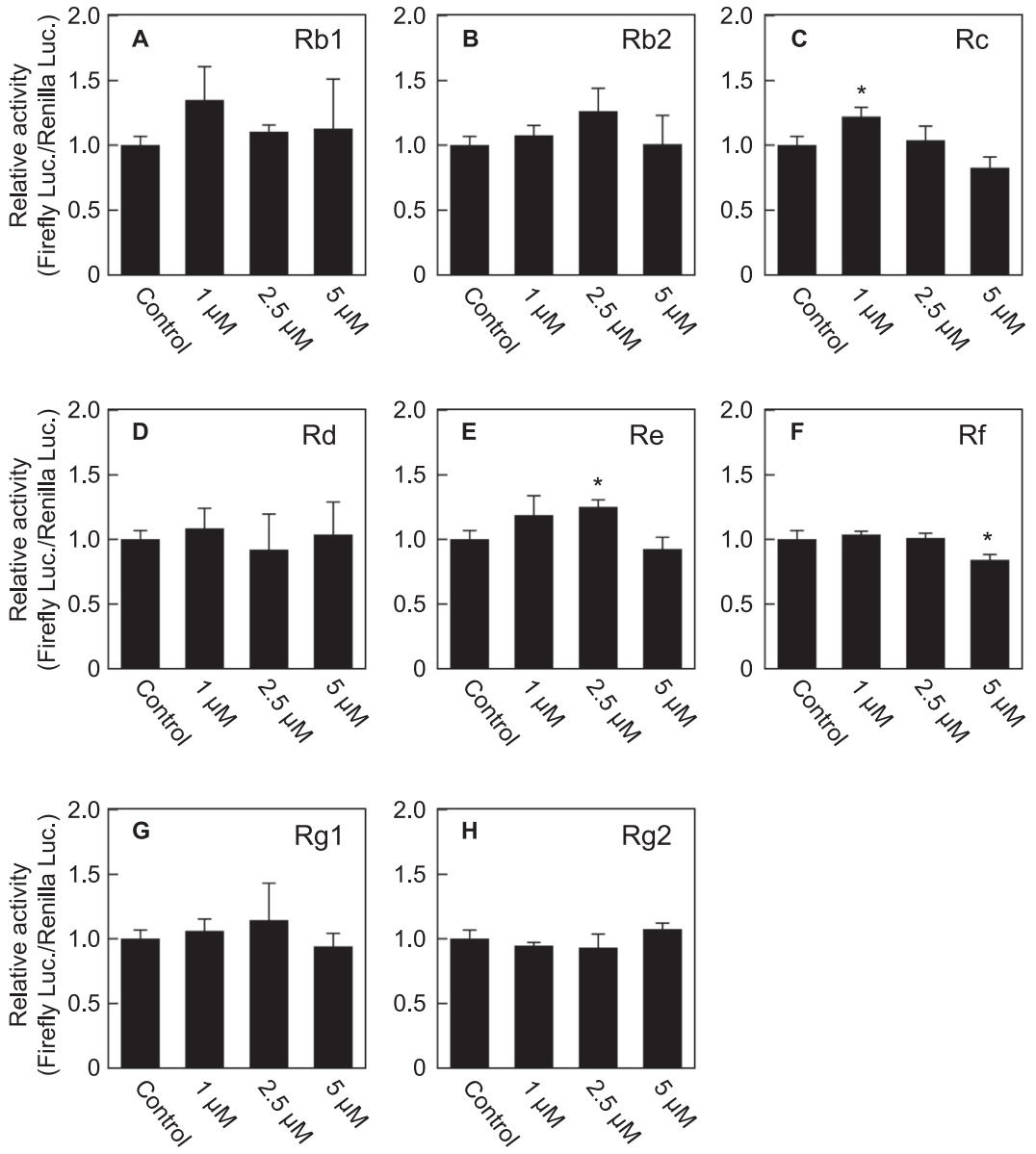

Fig. 5. Effects of ginsenosides on luciferase activity driven by intracellular zinc levels. Hepa cells, co-transfected with the pGL4.26-MRE and the pGL4.74 plasmids, were incubated in normal medium (control) and the indicated concentrations of ginsenoside Rb1 (A), Rb2 (B), Rc (C), Rd (D), Re (E), Rf (F), Rg1 (G), and Rg2 (H) for 24 h. *$P < 0.05$, significantly different from control. Each bar represents the mean ± S.D. (n = 3).

Table 1
Content of Ginsenosides in 1000 μg/mL of *P. ginseng* Extract

Ginsenoside	Content (μM)
Rb1	1.35
Rb2	1.52
Rc	1.47
Rd	0.88
Re	4.04
Rf	0.92
Rg1	5.59
Rg2	2.11

Declaration of competing interest

The authors declare that no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.06.006.

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