Anti-obesity effects of ginsenoside Rh2 are associated with the activation of AMPK signaling pathway in 3T3-L1 adipocyte

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Abstract

Metabolic disorders such as obesity are major obstacles in improving the average life span. Therefore, a therapeutic approach using natural compounds has been proposed as a novel strategy for preventing metabolic disorders. Ginsenoside Rh2 is one of the ginsenosides that exert anti-diabetes, anti-inflammatory, and anti-cancer effects. However, the anti-obesity effects of Ginsenoside Rh2 remain unclear. Here, we investigated the anti-obesity ability of ginsenoside Rh2 using cell culture systems. Ginsenoside Rh2 effectively inhibited adipocyte differentiation via PPAR-γ inhibition. Next, to find specific target molecules based on this result, we used cell culture systems to examine whether AMPK activation was involved in the anti-obesity ability of ginsenoside Rh2 since several published papers have indicated that AMPK signaling is involved in the regulation of metabolic disorders. Ginsenoside Rh2 significantly activated AMPK in 3T3-L1 adipocytes. In addition, we also examined the effect of AMPK on lipolysis molecules such as CPT-1 and UCP-2 by using an AMPK inhibitor. Ginsenoside Rh2 effectively induced CPT-1 and UCP-2 and this induction was abolished by AMPK inhibitor treatment. Moreover, we observed that ROS is an important upstream signal for AMPK activation during ginsenoside Rh2 treatment.

Taken together, these results indicate that ginsenoside Rh2 is the most effective candidate for preventing metabolic disorders such as obesity and that it acts via the AMPK signaling pathway. Thus, AMPK signaling might contribute toward improving human health.

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Metabolic disorders are induced by various elements such as uncontrolled food intake, environment, and insufficient exercise. Obesity, one of the metabolic diseases, is a major obstacle in the improvement of human health; it induces various human diseases, including type 2 diabetes, hypertension, cardiac injury, neuronal injury, and cancer.

Therefore, the prevention of metabolic diseases such as obesity by using natural active constituents is proposed as a novel strategy for overcoming metabolic diseases and maintaining a healthy life. Recently, studies have demonstrated that bioactive natural compounds may be potent agents for the prevention of various diseases. Further, naturally occurring products that modulate protein expression have attracted researcher’s attentions for improving human health [1]. One of the best characterized anti-obesity targets, namely, adenosine monophosphate (AMP)-activated protein kinase (AMPK), is a target in the treatment of metabolic disorders using bioactive components that exert their beneficial effects on human health via activation of the AMPK signaling pathway [2]. Previous studies have suggested that AMPK is a key molecule in controlling met-
abolic diseases such as type 2 diabetes, obesity, and cancers and that it is activated by several anti-diabetic drugs such as metformin and rosiglitazone [3]. AMPK is a metabolic sensor that acts as a cellular fuel gauge in eukaryotes. AMPK is activated under ATP-depleting conditions such as hypoxia, ischemia, reactive oxygen species (ROS), heat shock, and glucose deprivation and it subsequently induces ATP-generation pathways for maintaining cellular homeostasis [4]. Therefore, discovering a natural AMPK activator is a novel strategy for overcoming human diseases such as type 2 diabetes, obesity, and cancer.

Ginseng (Panax ginseng) has been widely used for improving human health in oriental countries; it includes certain active components such as ginsenosides [5]. In particular, previous papers have demonstrated that red ginseng, which contains rich ginsenoside Rh2, exerts anti-diabetic, anti-inflammatory, and anti-cancer effects by modulating various signal pathways [6,7]. Although several investigators have proposed that ginsenoside Rh2 exerts preventive effects via the modulation of intracellular signal pathways, its precise mechanisms remain unclear.

Therefore, in this study, we examined whether AMPK signaling is critical for the anti-obesity function of ginsenoside Rh2. In this paper, we suggest that ginsenoside Rh2 is a novel AMPK activator that simultaneously prevents obesity.

Materials and methods

Cell culture and reagent. 3T3-L1 cells were purchased from the ATCC, American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Phospho-AMPK Thr172 and β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, 5-aminoimidazole-carboxamide-riboside (AICAR), 2',7'-dichlorofluorescein diacetate (DCFH), and Hoechst 33342 dye were purchased from Sigma (St. Louis, MO, USA).

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MTT assay. Cells were cultured in 24-well plates with each stimulus, and then incubated with 30 μl of MTT solution (5 mg/ml in PBS) for 2 h at 37 °C. After discarding the medium, cells were exposed to DMSO and then relative cell viability was determined by an ELISA reader.

Oil-red O staining. After differentiation, cells were washed with phosphate-buffered saline (PBS) and then incubated with Oil-red O dye. The stained lipid droplets were dissolved in isopropanol and quantified by either ELISA (510 nm) or fluorescence microscopy.

Reverse transcription PCR. After treatment of each stimulus, RNA was extracted with Tri-zol reagent (Life Technologies, Glasgow, UK) and then cDNA synthesis was performed with 1 g of total RNA. Synthesized cDNA was used for amplification of specific target. PCR products were separated on 1% agarose gels and stained with ethidium bromide. GAPDH was used as the RNA loading control.

Western blotting. Cells were lysed with lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 0.2% protease inhibitor cocktail, and pH 7.2). Western blot analysis was performed with specific antibodies.

DNA fragmentation detection with Hoechst 33342 dye. Cells were fixed with 3.5% formaldehyde in PBS for 15 min, then washed twice with PBS, and stained with 10 μM Hoechst 33342 for 30 min at room temperature. The cleaved nuclei were observed by a fluorescence microscope (Olympus Optical, Tokyo, Japan).

PPAR-γ transactivation assay. HEK293 cells were transiently transfected with expression plasmid, PPAR-γ, RXRα, luciferase reporter vector, containing a PPAR-responsive element (PPRE) and β-galactosidase. After eighteen hours, the cells were treated with rosiglitazone, PPAR-γ agonist, in the absence or presence of ginsenoside Rh2 for 24 h. The cells were then used for luciferase reporter gene assay. Luciferase activity was assayed by the Luciferase Assay System (Promega, WI).

ROS measurement. 3T3-L1 cells were fixed with 3.5% formaldehyde in PBS for 15 min, washed twice with PBS, and stained with 10 μM 2',7'-dichlorofluorescein diacetate (DCFH) dye at 10 μM for 30 min at room temperature. Released ROS was detected by green fluorescence in fluorescent microscopy (Olympus Optical, Tokyo, Japan).

Statistical analysis. Data are presented as means ± SD. Significance was P < 0.05. Statistical analyses were conducted using SPSS 9.0 (SPSS Inc., Chicago, IL).

Results

Ginsenoside Rh2 inhibits 3T3-L1 adipocyte differentiation

Ginsenoside Rh2, an active component of Panax ginseng, has been reported to prevent cancer and cellular injury [7,8]. However, the anti-obesity effect of ginsenoside Rh2 remains unclear. In this study, we first examined the safety of ginsenoside Rh2. 3T3-L1 cells were exposed to ginsenoside Rh2 in a dose-dependent manner, and subsequently, intracellular toxicity was measured by MTT or Hoechst staining. Ginsenoside Rh2 exerted cellular toxicity at doses of 80–160 μM; moreover, at these concentrations, DNA fragmentation was significantly increased (Fig. 1B and C). We next examined the ability of ginsenoside Rh2 to prevent obesity by using adipocyte differentiation processing condition. As shown in Fig. 1D and E, ginsenoside Rh2 effectively blocked adipocyte differentiation processing as assessed by Oil-red O staining in 3T3-L1 adipocytes. These results suggest that ginsenoside Rh2 can be used for obesity prevention and concentrations <80 μM might be appropriate for the treatment of 3T3-L1 adipose cells.

PPAR-γ signaling was involved in the inhibition of adipocyte differentiation

To uncover the molecular mechanisms underlying ginsenoside Rh2-induced inhibition of adipogenesis, we next examined whether the peroxisome proliferator-activated receptor (PPAR)-γ signaling pathway was influenced by ginsenoside Rh2 treatment. PPAR-γ is a well-known adipogenic marker that leads to the inhibition of adipocyte differentiation [9]. Therefore, we next examined whether PPAR-γ inhibition was involved in the ginsenoside Rh2-mediated inhibition of adipocyte differentiation. 3T3-L1 cells were treated with ginsenoside Rh2 at the indicated concentrations and the cells were then lysed with Trizol reagent. After cDNA synthesis, PPAR-γ expression was detected by the reverse transcription polymerase chain reaction (RT-PCR) method. As shown in Fig. 2A,
PPAR-\(\gamma\) expression was significantly blocked by ginsenoside Rh2 treatment. We next examined the PPAR-\(\gamma\) transcriptional activity after ginsenoside Rh2 treatment by using a reporter gene-based assay. HEK293 cells were transfected with the plasmid indicated in Materials and methods; subsequently, transcriptional activity was assessed using luciferase activity assay. Further, as shown in Fig. 2B, ginsenoside Rh2 significantly inhibited the PPAR-\(\gamma\) agonist rosiglitazone-induced PPAR-\(\gamma\) transcriptional activation. These results indicate that the anti-obesity ability of ginsenoside Rh2 is exerted due to its antagonist action against PPAR-\(\gamma\).

**AMPK activity also involved in the inhibition of adipocyte differentiation**

It has also been reported that AMPK signaling is critical for controlling metabolic disorders such as diabetes, obesity, and cancer [10]. More recent studies have suggested that certain naturally derived active components prevent metabolic diseases [11]. Therefore, in this study, we next determined whether AMPK activation is involved in ginsenoside Rh2-induced adipocyte differentiation blockage. To this end, 3T3-L1 cells were treated with ginsenoside Rh2 in a dose-dependent manner. As shown in Fig. 3A, AMPK was significantly activated by ginsenoside Rh2 treatment (20–40 \(\mu\)M). To confirm this AMPK activation, 3T3-L1 cells were pretreated with the AMPK inhibitor compound C and then exposed to ginsenoside Rh2 for 3 h. AMPK activation was determined by western blot analysis. As shown in Fig. 3B, AMPK activation induced by ginsenoside Rh2 was abrogated by pretreatment with AMPK inhibitor. We next examined the effect of AMPK on fatty acid oxidation molecules such as carnitine palmitoyltransferase (CPT)-1 and uncoupling protein (UCP)-2 using an AMPK inhibitor. As shown in Fig. 3C, ginsenoside Rh2 effectively induced CPT-1 and UCP-2 and this induction was abolished by AMPK inhibitor treatment.

These results suggest that the AMPK signaling pathway is required for the anti-adipogenic function of ginsenoside Rh2.

**ROS is upstream signal for Rh2-stimulated AMPK activation**

According to previous papers, ROS is involved in the inhibition of fat accumulation by natural compounds; moreover, they are required for the activation of the
AMPK signaling pathway [12]. Hence, we evaluated whether ROS release is involved in AMPK signaling pathway activation. As shown in Fig. 4A, ROS was rapidly increased by ginsenoside Rh2 treatment, whereas its release was blocked by treatment with the ROS scavenger N-acetyl cysteine (NAC) (B). Under the same conditions, in order to evaluate ROS participation in AMPK activation, 3T3-L1 cells were pretreated with 5 mM NAC and then exposed to 40 μM ginsenoside Rh2 for 3 h. As shown in Fig. 4B, ginsenoside Rh2-mediated AMPK activation was surprisingly blocked by NAC treatment. Taken together, these results strongly suggest that ginsenoside Rh2 is a candidate for the treatment of obesity and its effects were induced via AMPK signaling pathway activation.

Discussion

Obesity is associated with the development of metabolic diseases such as type 2 diabetes, hypertension, dyslipidemia, and myocardial infarction. Therefore, many investigators have attempted to discover natural active compounds for the treatment of obesity. Ginsenoside Rh2 is an active component derived from Panax ginseng; it exerts beneficial effects in various diseases such as cancer, inflammatory diseases, and ischemia [13,14]. However, the anti-obesity effect of ginsenoside Rh2 was poorly understood.

In this study, we evaluated the anti-obesity effects of ginsenoside Rh2 using 3T3-L1 adipocytes. In our experiments, we first examined the effect of ginsenoside Rh2 on adipogenic processing. Ginsenoside Rh2 effectively decreased

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fat accumulation in a dose-dependent manner; at a dose of 40 μM, ginsenoside Rh2 treatment nearly blocked adipocyte differentiation. Further, it appeared to be safe.

Fat accumulation is associated with the activation of specific molecules such as PPAR-γ, a well-known adipogenic marker, and with increase in adipocyte proliferation or differentiation processing [15]. In this study, we also found that ginsenoside Rh2 downregulated both rosiglitazone-stimulated PPAR-γ transcriptional activity and expression itself. However, we did not detect any upstream or downstream signal molecule of PPAR-γ, which was inhibited by ginsenoside Rh2.

To detect specific target molecules via which ginsenoside Rh2 exerts its anti-adipogenic effects, we next evaluated the role of AMPK in anti-adipogenesis due to ginsenoside Rh2 treatment. AMPK belongs to the metabolic sensor protein kinase family and is activated by intracellular stress conditions such as hypoxia, ischemia, and ROS; it inhibits cellular ATP-consuming pathways by inhibiting cell proliferation [16]. AMPK has also been proposed to play a beneficial role in the prevention of metabolic diseases such as type 2 diabetes, obesity, and cancer [17]. Our present study suggested that certain naturally derived sources activated the AMPK signaling pathway and exerted anti-obesity or anti-cancer effects both in vitro and in vivo. It has also been reported that berberine inhibits adipocyte differentiation by modulating the AMPK and PPAR-γ pathways and that genistein also inhibits adipocyte differentiation via AMPK activation [18,19]. On the other hand, ginsenoside Rh2 is an active component of Panax ginseng that is a type of natural product exerting preventive effects in various diseases. A previous paper indicated that ginsenoside Rh2 inhibited cancer cell proliferation by inducing apoptotic proteins in either ovarian cancer cell [20]. Ginsenoside Rh2 also inhibits MCF-7 cell proliferation by modulating cyclin-dependent kinase and p21 [21].

For this reason, we speculated that ginsenoside Rh2 possessed the ability to inhibit cell proliferation because AMPK is a cell proliferation inhibitor and ginsenoside Rh2 exerted effects on AMPK activation as well as on adipocyte differentiation. In this study, ginsenoside Rh2 significantly activated AMPK in a dose-dependent manner.
and subsequently, ginsenoside Rh2-mediated AMPK activation in 3T3-L1 adipocytes was inhibited by compound C, an AMPK-specific inhibitor. Progression of fatty acid oxidation is also regulated by several molecular markers such as mitochondrial UCP and CPT that have also been reported to be associated with the AMPK signaling pathway [22]. In this study, we also evaluated the role of AMPK in the expression of fatty acid oxidation markers such as UCP and CPT. Ginsenoside Rh2 significantly induced the expression of UCP-2 and CPT-1, whereas simultaneous treatment with AMPK inhibitor abolished the UCP-2 and CPT-1 expression induced by ginsenoside Rh2. Moreover, we observed that ROS transmitted upstream signals that activated AMPK, thereby mediating the anti-obesity effect of ginsenoside Rh2. A previous paper suggested that ROS is involved in the process of c-Jun N-terminal kinase 1 (JNK1)-induced apoptosis mediated by ginsenoside Rh2 in HeLa, MCF10A-ras, and MCF-7 cells [23]. Furthermore, ginsenoside Rh2-induced cell death is associated with the generation of ROS in brain tumors [24]. A more recent paper indicated that epigallocatechin gallate (EGCG) released ROS that could be used for anti-adipogenesis via AMPK signal activation [25]. Theaflavins, the major component of black tea, also activated AMPK via the LKB1 and ROS pathways, indicating that AMPK is critical for decreasing lipid accumulation [26]. These results show that ROS is critical for activating AMPK signaling during treatment with active components such as ginsenoside Rh2.

Taken together, these data strongly suggest that AMPK is a precise target of anti-adipogenesis and is induced by ginsenoside Rh2; further, this anti-adipogenic ability may be exerted due to its anti-proliferative abilities.

In conclusion, this study is the first to demonstrate that ginsenoside Rh2, a Panax ginseng-derived active compound, inhibits adipocyte differentiation via the AMPK signaling pathway, and that AMPK might be used to prevent obesity and obesity-induced diseases.

Acknowledgments

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References


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