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The ginsenoside Rg3 has a stimulatory effect on insulin signaling in L6 myotubes

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ABSTRACT

The ginsenoside Rg3 is known to have a protective effect against hyperglycemia, obesity and diabetes *in vivo*. In this study, we examined the effect of Rg3 on insulin signaling and glucose uptake in cultured L6 myotubes. Rg3 increased glucose uptake both in the basal and insulin-induced states of L6 myotubes. Consistent with the increase in glucose uptake, Rg3 stimulated the phosphorylation of IRS-1 and Akt. Interestingly, Rg3 dramatically increased IRS-1 protein levels, while the protein level of Akt was not affected. Rg3 regulated IRS-1 expression at the transcriptional level and also increased the level of GLUT4 mRNA. Treatment of ginsam, in which Rg3 is the major compound of ginsenosides, increased the IRS-1 protein levels in OLEFT rats. In addition, we found that this effect of Rg3 on insulin signaling was not mediated by the AMPK pathway. In conclusion, these results suggest that Rg3 improves insulin signaling and glucose uptake primarily by stimulating the expression of IRS-1 and GLUT4.

Introduction

Insulin stimulates glucose uptake by skeletal muscle while repressing glucose production in the liver. A major contributor to the pathogenesis of type 2 diabetes mellitus is an insulin-resistant state in which insulin cannot successfully activate the signaling pathway responsible for the stimulation of glucose uptake [1,2]. Upon binding insulin, the insulin receptor (IR) is activated, and this leads to the phosphorylation of the insulin receptor substrates IRS-1 and/or IRS-2 on tyrosine residues [3]. In skeletal muscle, IRS-1 is primarily responsible for inducing glucose uptake, while insulin signaling in the liver is mediated mainly through IRS-2 [4–6]. IRS-1 activates PI3 kinase (PI3K), which in turn promotes the phosphorylation of a central regulator of glucose uptake, the serine/threonine kinase protein kinase B (PKB/Akt) [7,8].

Various beneficial effects of ginseng, the root of *Panax ginseng*, on cardiovascular disorders, immune responses and metabolic processes have been reported [9,10]. Of interest is that ginseng exhibits anti-hyperglycemic, anti-diabetic and anti-obesity activities *in vivo* [11,12]. However, the mechanisms involved in the activities of ginseng have not been carefully investigated. Ginsenosides are the major active compounds of ginseng, and there are more than 30 ginsenosides that have been isolated from ginseng [13]. Recently, we reported that treatment with ginsam, a vinegar extract from *Panax ginseng*, improves glucose homeostasis and reduces body weight in an obese insulin-resistant rat model [14]. As ginsam is enriched with the ginsenoside Rg3, the above-mentioned effects may be mediated by Rg3.

In this study, we examined whether Rg3 could enhance insulin signaling and glucose uptake in cultured L6 myotubes, and we investigated the mechanism involved in this process.

Materials and methods

Cell culture and differentiation. Rat myoblast L6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were allowed to grow to approximately 80% confluence at which time differentiation into myotubes was induced by culturing the cells in the presence of 2% horse serum for 5–7 days.

Animals. Otsuka Long-Evans Tokushima Fatty (OLETF) rats were handled in compliance with the Guide for Experimental Animal Research of the Laboratory, Clinical Research Institute, Seoul National University Hospital. Rats were grown to 24 weeks of age to acquire obesity and insulin resistance, and then treated with ginsam (500 mg/kg per day) or isotonic NaCl solution (5 mL/day, for the control group) for 8 weeks.

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Glucose uptake. After differentiation, L6 myotubes were treated with the ginsenoside Rg3 (ChromaDex, Inc., Irvine, CA) for 48 h, and then treated with insulin (100 nM) for 30 min. In some experiments, Compound C (Calbiochem, Darmstadt, Germany) was added 1 h before the Rg3 treatment. Cells were then washed in phosphate-buffered saline (PBS) several times and placed in salt/Hepes buffer (130 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.5 mM, NaH_2PO_4, and 10 mM Hepes 0.1% bovine serum albumin) containing 10 μM [^{3}H] 2-deoxyglucose (1.0 μCi/mL) for 15 min at 37 °C. Glucose uptake was terminated by washing five times with cold PBS. The cells were lysed within 0.5 N NaOH and incorporated [^{3}H] were determined by liquid scintillation counting.

Western blot analysis. Protein extracts (15 μg) were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk and hybridized with primary antibodies. After washing, the blots were incubated with horseradish-conjugated secondary antibodies. The blots were developed with enhanced chemiluminescence (Pierce, Rockford, IL). The following antibodies were used for Western blot analysis: antibodies against IRS-1, GLUT4 and GAPDH (Santa Cruz Biotech, Santa Cruz, CA, USA); antibodies against pAkt (Ser473), Akt, p-Jun N-terminal kinase (JNK), JNK, p-p38, p38, p-pErk1/2, Erk1/2 and pAMPK (Cell Signaling Technology, Danvers, MA); an antibody against pIRS-1 (Y612) (Biosource, Camarillo, CA); and an antibody against γ-tubulin (Sigma–Aldrich, Louis, MO).

Northern blot analysis and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany). Aliquots of total RNA (10 μg) were separated by electrophoresis in 1.2% agarose gels and transferred to nylon membranes. The membranes were hybridized with [α-^{32}P] dATP-labeled cDNA fragments. After removal of excess labeled probe, blots were exposed to X-ray films. For RT-PCR, synthesis of cDNA from total RNA was performed using M-MLV reverse transcriptase (Invitrogen). The RT products were amplified by PCR with *Taq* polymerase and specific primers: 5′-CAGTCTTCCC TGCACCCTCC-3′ and 5′-AATGCCTGTCCGCATGTCAG-3′ for IRS-1; 5′-ATCACCATCTTCCAGGAGCGA-3′ and 5′-CATACTTGGCAGGTTTCT CCAGG-3′ for GAPDH; 5′-ACGCCACCATAGGAGCTGGT-3′ and 5′-AGAAGCCAAGCAGGAGGACG-3′ for GLUT4; 5′-CAAGGACGGGCACA TCAAGA-3′ and 5′-TCAGAGGTGACCTGGGGCTT-3′ for Akt.

Statistics. The analysis was performed using GraphPad InStat (Ver 3.05). A *P* values less than 0.05 were considered to be statistically significant.

Results

Rg3 increases glucose uptake in L6 myotubes

To determine whether Rg3 affects glucose uptake in cultured muscle cells, L6 cells were differentiated to myotubes and then treated with Rg3 for 48 h. Insulin modestly increased glucose uptake in the cultured myotubes. Rg3 increased both the basal level and the insulin-induced level of glucose uptake in a dose-dependent manner (Fig. 1).

Rg3 enhances the insulin signaling pathway

We next examined the effect of Rg3 on the insulin signaling pathway that leads to the translocation of glucose transport 4 (GLUT4) to the plasma membrane and increases the uptake of glucose. After L6 myotubes were treated with Rg3 for 48 h, the phosphorylation levels of IRS-1 and Akt were determined. Tyrosine phosphorylation of IRS-1 was induced by insulin, and Rg3 significantly increased the level of phosphorylated IRS-1 (Fig. 2A). Interestingly, in addition to the increase in phosphorylated IRS-1, the

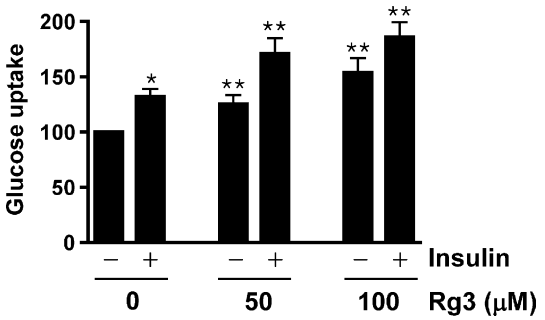

Fig. 1. Effect of Rg3 on glucose uptake by L6 myotubes. Cells were treated with Rg3 (50 or 100 μM) for 48 h and glucose uptake was stimulated by insulin treatment (100 nM) for 30 min. The graphs represent means ± SEM. Basal glucose uptake measured in the cells not treated with Rg3 or insulin was set at 100 and the other values were expressed relative to that. $^*P < 0.05$ compared to the basal value of control cells not treated with Rg3 or insulin; $^{**}P < 0.05$ compared to the corresponding value of control cells not treated with Rg3.

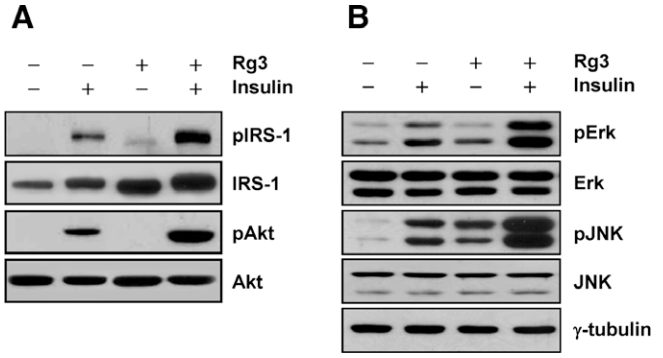

Fig. 2. Effects of Rg3 on the insulin signaling pathway in L6 myotubes. Cells were treated with Rg3 (75 μM) for 48 h and then incubated with or without insulin for 30 min. (A) Western blot analysis of 15 μg of protein with specific antibodies against pIRS-1 (Tyr 612), IRS-1, pAkt (Ser 473) and Akt. (B) Proteins were subjected to Western blot analysis with specific antibodies against pErk, Erk, pJNK, JNK and γ-tubulin, respectively.

total IRS-1 protein level was also enhanced by Rg3. In accord with the activation of IRS-1, phosphorylated Akt also increased significantly, but there was no effect on the level of total Akt. These results indicate that Rg3 strongly enhances insulin signaling, and the increase in total IRS-1 may play an important role in this process.

As insulin is known to activate the MAPK and JNK pathways in addition to the IRS-PI3K-Akt signaling cascade, the effect of Rg3 on these pathways was also monitored. Insulin increased the levels of phospho-Erk and phospho-JNK, and Rg3 further increased this insulin-induced phosphorylation, but no difference in total protein levels was detected after the Rg3 treatment (Fig. 2B).

Rg3 increases the mRNA level of IRS-1

Since the total IRS-1 protein level was dramatically elevated by Rg3 treatment (Fig. 2), we next examined whether the expression of IRS-1 is regulated by Rg3 at the transcriptional level. Total mRNA was prepared from L6 myotubes treated with Rg3, and the IRS-1 transcript level was monitored by RT-PCR. Consistent with the effect on the protein level of IRS-1, Rg3 significantly increased the mRNA level of IRS-1 (Fig. 3A). The effect of Rg3 on the expression of IRS-1 was detected by 12 h after Rg3 treatment and sustained until 48 h. The mRNA level of GLUT4 was also enhanced by Rg3, while Akt's mRNA level was not affected by Rg3. Similarly, IRS-1

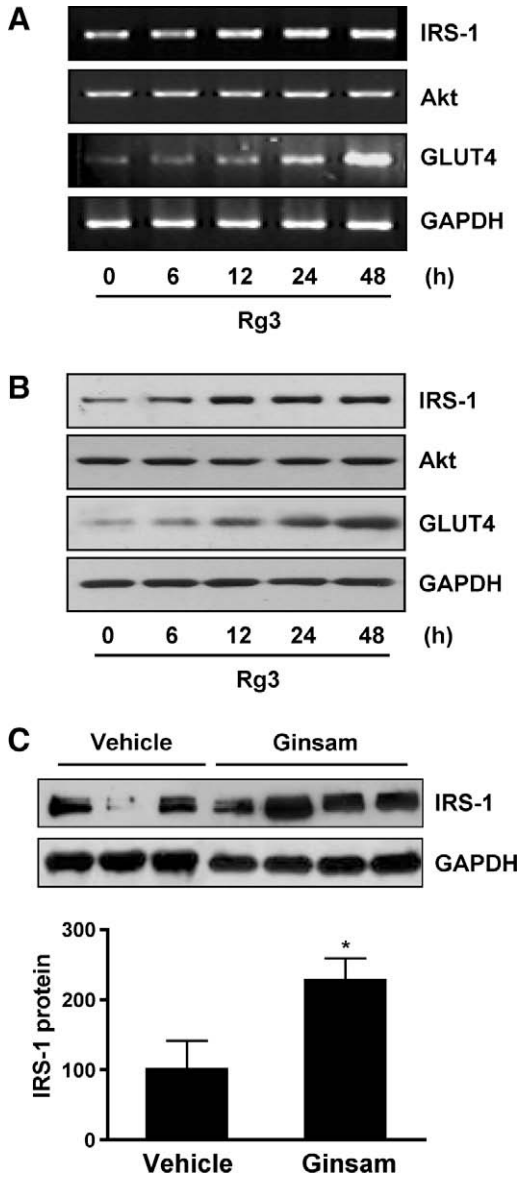

Fig. 3. Rg3 increases the mRNA level of IRS-1. (A) Total RNA was prepared from cells treated with Rg3 (75 μM) for the indicated periods and then subjected to RT-PCR with specific primers for IRS-1, Akt, GLUT4 or GAPDH. Similar results were obtained in Northern blot analyses (data not shown). (B) Cell lysates were prepared from cells treated with Rg3 for the indicated periods and then subjected to immunoblot analysis. (C) Protein extracts were prepared from the skeletal muscle of vehicle- ($n = 3$) or ginsam-treated ($n = 6$) OLETF rats, and immunoblot analysis was performed with specific antibodies against IRS-1 and GAPDH (upper panel). The band intensity of IRS-1 was normalized to that of GAPDH, and the mean value obtained from the vehicle-treated rats was designated as 100, and the other values were expressed relative to that (lower panel). Data represent means ± SEM. $^{*}P < 0.05$.

and GLUT4 protein levels increased within 12 h after Rg3 treatment (Fig. 3B).

Ginsam increases the expression of IRS-1 in OLETF rats

To determine the effect of Rg3 on the expression of IRS-1 *in vivo*, IRS-1 protein levels were compared in obese rats (OLETF) treated or untreated with ginsam. As mentioned above, Rg3 is the major ginsenoside in ginsam. In the rats treated with ginsam (500 mg/kg per day), blood glucose levels were significantly lower than in the untreated rats starting 7 days after the onset of treatment [14]. Consistent with the in vitro result obtained with Rg3, ginsam treatment increased the expression of IRS-1 in the muscles of OLETF rats (Fig. 3C). These results indicate that Rg3 in the ginsam may play a role in the increase of IRS-1 expression and affect insulin signaling *in vivo*.

The effect of Rg3 on insulin signaling is not mediated by the AMPK pathway

It has been demonstrated that Rg3 increases the phosphorylation of AMP-activated protein kinase (AMPK) [14,15], and activation of AMPK is known to induce the expression of GLUT4 in muscle [16,17]. Therefore, we tested whether AMPK activation mediates the effect of Rg3 on insulin signaling. In the presence of Compound C, an inhibitor of AMPK, Rg3 still increased glucose uptake and increased total IRS-1 protein levels in L6 myotubes (Fig. 4A and B). Therefore, the AMPK pathway seems not to mediate the effect of Rg3 on insulin signaling.

Discussions

In this study, we showed that Rg3 treatment for 48 h increases glucose uptake by L6 myotubes both in their basal and insulin-stimulated states; similarly, the tyrosine phosphorylation of IRS-1 was enhanced by Rg3. Interestingly, total IRS-1 protein levels were also enhanced by Rg3, and the expression of IRS-1 was regulated at the transcriptional level, as the mRNA level of IRS-1 was elevated 12 h after Rg3 treatment (Fig. 3A). While Rg3 induced the expression of IRS-1, the expression levels of Akt, Erk and JNK were not affected (Fig. 2). These results indicate that the enhancement of glucose uptake and insulin signaling by Rg3 is at least in part caused by the increase in IRS-1 transcripts.

Ginsam treatment increased the expression of IRS-1 in the muscles of an obese rat model (Fig. 3C). Although Rg3 is the major ginsenoside in ginsam, we cannot exclude the possibility that other ginsenosides contribute the change in the IRS-1 expression. How-

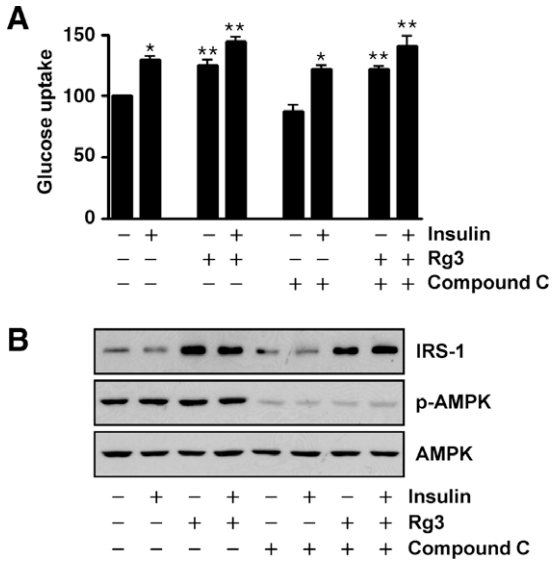

Fig. 4. The AMPK pathway is not involved in the effect of Rg3 on insulin signaling. L6 myotubes were treated with Rg3 (75 μM) for 48 h in the absence or presence of Compound C (10 μM), and then treated with insulin for 30 min. (A) Glucose uptake was measured as described in Materials and methods. Basal glucose uptake measured in the cells not treated with Rg3, insulin or Compound C was set as 100 and the other values were expressed relative to that. The graphs represent means ± SEM. $^{*}P < 0.05$ compared with the basal value of control cells not treated with Rg3, insulin or Compound C; $^{**}P < 0.05$ compared with the corresponding value of control cells not treated with Rg3. (B) Immunoblot analysis was performed with antibodies against pAMPK, AMPK and IRS-1.

ever, this study indicates that IRS-1 expression is induced at least in part by Rg3 *in vivo*. It has been reported that overexpression of TFE3 improves insulin signaling through transcriptional activation of hepatic IRS-2 [18]. It has also been reported that IRS-1 expression is reduced in diabetic subjects, and a low level of IRS-1 expression is associated with age and impaired insulin-stimulated glucose uptake [19,20]. Therefore, it seems likely that total IRS protein level is closely related to insulin sensitivity. In addition to IRS-1, Rg3 also increased the expression of GLUT4 in L6 myotubes (Fig. 3), consistent with the observation in the previous study that GLUT4 protein levels in rats treated with ginsam were elevated [14]. These results indicate that Rg3 improves glucose uptake through the induction of IRS-1 and GLUT4 expression.

As shown in Fig. 3, transcriptional activation of IRS-1 and GLUT4 was detected 12 h after Rg3 treatment. We showed that AMPK activation may not mediate the effect of Rg3 on this process (Fig. 4). Therefore, further study will be required to determine what mediates the effect of Rg3 on the expression of these genes. As shown in Fig. 2, insulin moderately increases the phosphorylation of Erk and JNK, and Rg3 further increases the phosphorylation of these proteins in the presence of insulin. Therefore, it is possible that Rg3 has an insulin-mimicking activity. Furthermore, as phosphorylated Erk and JNK inhibit the tyrosine phosphorylation of IRS-1 [21], the effects of Rg3 on insulin signaling seem not to be so simple. In conclusion, these results suggest that Rg3 increases insulin signaling and that this effect is primarily due to the enhancement of IRS-1 expression.

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