

The Ginsenoside-Rb2 lowers cholesterol and triacylglycerol levels in 3T3-L1 adipocytes cultured under high cholesterol or fatty acids conditions

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The effects of the ginsenoside Rb2 (Rb2) on lipid metabolism were characterized in 3T3-L1 adipocytes to evaluate their utility for treating obesity. While the amounts of total cholesterol and triacylglycerol (TAG) were markedly increased in the adipocytes treated with high amounts of cholesterol and fetal bovine serum (FBS), the test groups treated with Rb2 showed levels that were close to normal. The effect of Rb2 on these cells was comparable to that of lovastatin. Rb2 enhanced the expression of the sterol regulated element binding protein (SREBP) mRNA whereas treatment with cholesterol and FBS led to a reduction in the abundance of this transcript. The activity of fatty acid synthetase (FAS) was lower in the cholesterol group compared to the Rb2 treatment group suggesting that the observed decrease in cholesterol levels and activated SREBP was mediated by Rb2. Treatment with Rb2 also resulted in a decrease in TAG levels in adipocytes cultured under high fatty acid conditions. This effect was mediated by stimulating the expression of SREBP and Leptin mRNA, suggesting that Rb2 might be a valuable component capable of lowering the levels of lipids. [BMB reports 2009; 42(4): 194-199]

INTRODUCTION

Ginsenosides are components of ginseng saponin and they possess various physiological and pharmacological activities including the ability to reduce lipid levels in plasma (1). Yamamoto *et al.* reported that the oral administration of ginseng powder to hyperlipemic patients decreased their plasma levels of triacylglycerol (TAG) and cholesterol (2) and that ginsenosides also lowered plasma lipid levels in rats fed a diet rich in cholesterol (3). Other investigators found that the ginseng saponin fraction was effective in lowering serum cholesterol levels and in preventing the development of atherosclerosis in

animals with hypercholesterolemia (4). In addition, our previous studies showed that the ginsenoside Rb2 decreased the cholesterol level in HepG2 cells by enhancing expression of the low density lipoprotein (LDL) receptor gene through transcriptional induction of sterol regulatory element binding proteins (SREBPs) (5). These SREBPs are transcription factors released from membranes by sterol-regulated proteolysis (6).

SREBPs are global regulators of cholesterol homeostasis and activate specific genes involved in cholesterol synthesis, endocytosis of LDLs, fatty acids synthesis, TAG synthesis and glucose metabolism (7, 8). Under sterol-depletion conditions, SREBPs are cleaved into mature forms capable of binding sterol regulatory elements (SREs) and/or E-box sequences and which then activate the expression of target genes including fatty acid synthetase (FAS), the LDL receptor, acetyl-CoA carboxylase (ACC) and glycerol-3-phosphate acyltransferase (GPAT) (7-11). FAS is the major biosynthetic enzyme for the synthesis of fatty acids from small carbon units and is normally expressed in the liver and in adipose tissue (12). Although adipose tissue plays an important role in the regulation and pathological dysregulation of metabolic homeostasis as well as in the storage of lipid (13, 14), the effect of ginsenosides on lipid metabolism in adipocytes has not well been studied.

Based on our previous finding that the ginsenoside Rb2 could up-regulate the expression of SREBP mRNA in HepG2 cells, we hypothesize that the ginsenoside Rb2 affects the expression of genes relating to lipid metabolism in adipocytes via SREBP-mediated regulation. In this study, we report the effect of the ginsenoside-Rb2 on lipid metabolism in 3T3-L1 adipocytes cultured under high cholesterol conditions, in the presence of fetal bovine serum (FBS) or under fatty acid conditions in terms of the levels of cholesterol and TAG, the expression level of SREBP and leptin, and the activities of FAS and glycerol-aldehyde-3-phosphate dehydrogenase (GPDH).

RESULTS

The effects of the ginsenoside Rb2 on total cholesterol and TAG levels in 3T3-L1 adipocytes cultured under high cholesterol conditions or in the presence of FBS

When 3T3-L1 adipocytes were cultured under high cholesterol

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conditions (10 µg/ml) or in the presence of 10% FBS for 8 hours, the amount of total cholesterol in the differentiated adipocytes was increased up to 30% ($P < 0.01$) or 86% ($P < 0.01$) compared to the normal group without cholesterol and FBS, respectively (Fig. 1A). Also, the amount of TAG in the differentiated adipocytes cultured under high cholesterol (10 µg/ml) conditions or in the presence of 10% FBS were similarly increased when compared to the normal group (Fig. 1B). However, the experimental groups supplemented with 10 µg/ml of the ginsenoside Rb2 showed reduced cholesterol and TAG levels in the presence of 10 µg/ml cholesterol (decreased up to 19% ($P < 0.01$) and 44% ($P < 0.01$), respectively) or 10% FBS in culture medium (decreased up to 36% ($P < 0.001$) and 26% ($P < 0.01$), respectively). The experimental groups supplemented with 10 µg/ml lovastatin also showed similar effects when cultured in the presence of 10 µg/ml cholesterol (decreased up to 13% ($P < 0.01$) and 50% ($P < 0.001$), respectively) (Fig. 1A and B).

The ginsenoside Rb2 enhances the expression of the SREBP mRNA in 3T3-L1 adipocytes cultured under high cholesterol conditions or in the presence of FBS

SREBP mRNA expression levels were determined in 3T3-L1 adipocytes cultured under various conditions. In adipocytes cultured under high cholesterol conditions, the abundance of the SREBP mRNA was decreased by up to one half when compared to the normal group. When the ginsenoside Rb2 (10 µg/ml) was added to cells cultured under high cholesterol conditions, however, the expression of the SREBP mRNA was greatly enhanced (Fig. 1C), revealing that the ginsenoside Rb2 can counter the effects of cholesterol on SREBP mRNA expression.

The ginsenoside Rb2 increases the activity of FAS and GPDH in 3T3-L1 adipocytes cultured under high cholesterol conditions or in the presence of FBS

To determine the effects of the ginsenoside Rb2 on fatty acid biosynthesis in adipocytes, FAS and GPDH activities were measured in differentiated adipocytes cultured under various conditions. A treatment of 10% FBS in the differentiated adipocytes led to a 24% increase in FAS activity when compared to the normal group. However, cells grown under high cholesterol conditions (a control group) showed a 21% decrease in the activity of FAS when compared to the normal group (Fig. 2A). When the ginsenoside Rb2 was added to the differentiated adipocytes cultured under high cholesterol conditions or in the presence of FBS, FAS activity increased by 74% ($P < 0.05$) or 39%, respectively, compared to the control group. Similarly, GPDH activity also increased by 16% ($P < 0.05$) or 34% ($P < 0.01$), respectively, compared to the control group (Fig. 2B).

The ginsenoside Rb2 lowers TAG levels in 3T3-L1 adipocytes cultured under high fatty acid conditions

3T3-L1 adipocytes were cultured under high fatty acid conditions containing 2% albumin and a 2 mM mixture of fatty

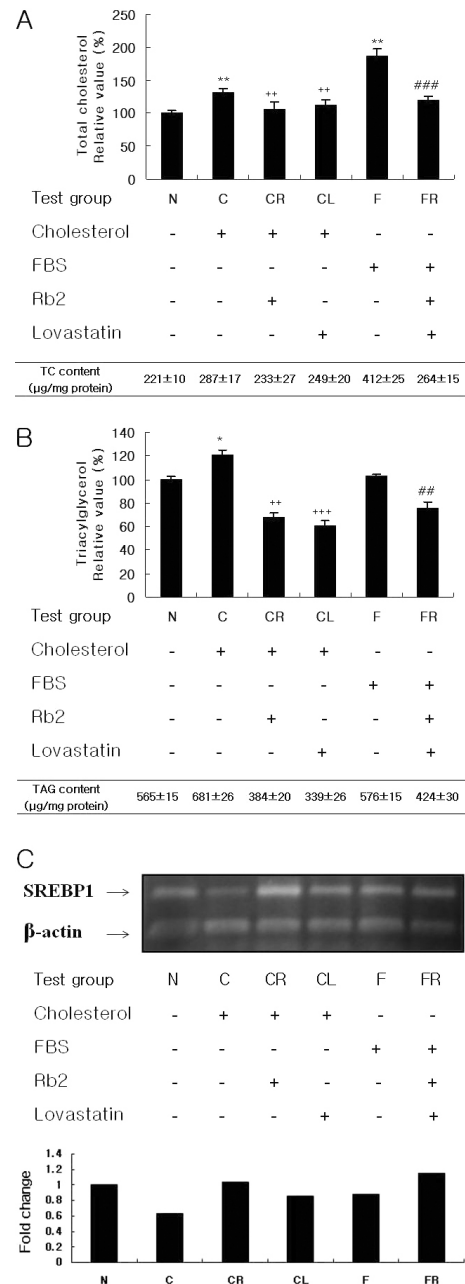


Fig. 1. Levels of total cholesterol (A) and triacylglycerol (B), and the abundance of SREBP-1 mRNA (as determined by RT-PCR) (C) in 3T3-L1 adipocytes cultured under various conditions. Normal 3T3-L1 adipocytes (N) were cultured in serum free-DMEM for 8 hours after complete differentiation. Control groups were cultured in serum free-DMEM containing cholesterol (C, 10 µg/ml) or FBS (F, 10%). Test groups were cultured under the same conditions as the control group except for the addition of the ginsenosides Rb2 (R, 10 µg/ml) or lovastatin (L, 10 µg/ml), respectively. Data are given as the mean ± SD. n=3. Bars represent the standard deviation of each mean. * $P < 0.05$ vs. the N group; ** $P < 0.01$ vs. the N group; + $P < 0.01$ vs. the C group; +++ $P < 0.001$ vs. the C group; ## $P < 0.01$ vs. the F group; ### $P < 0.001$ vs. the F group.

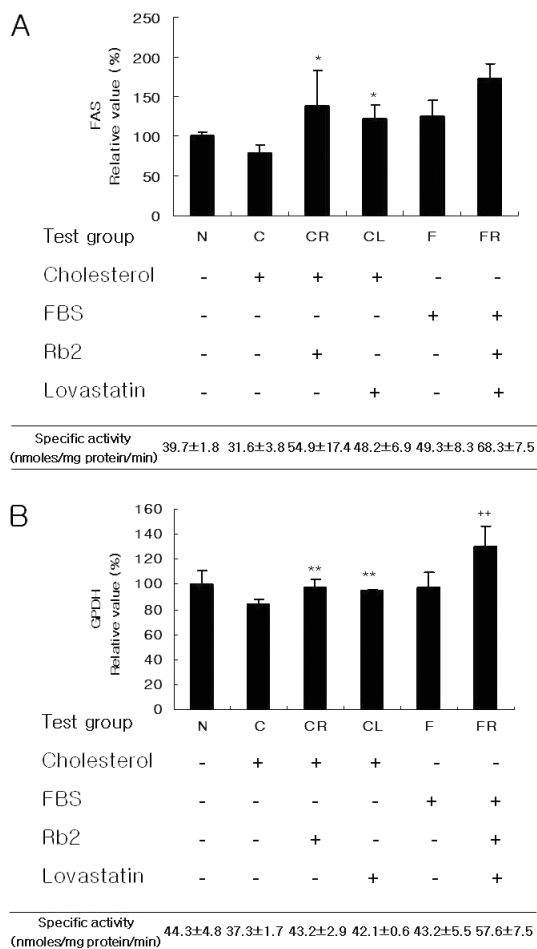


Fig. 2. The activities of FAS (A) and GPDH (B) in 3T3-L1 adipocytes cultured under various conditions were determined. Normal 3T3-L1 adipocytes (N) were cultured in serum free-DMEM for 8 hours after complete differentiation. Control groups were cultured in serum free-DMEM containing cholesterol (C, 10 µg/ml) or FBS (F, 10%). Test groups were cultured under the same conditions as those of the control group except for the addition of the ginsenosides Rb2 (R, 10 µg/ml) or lovastatin (L, 10 µg/ml), respectively. Data are given as the mean ± SD n=3. Bars represent the standard deviation of each mean. *P < 0.05 vs. the C group; **P < 0.01 vs. the C group; ***P < 0.01 vs. the F group.

acids (oleic acid:palmitic acid = 2:1), or under normal conditions without albumin or the mixture of fatty acids for up to 24 hours. TAG levels were determined at various time points and were increased up to 1.7, 3.4 (P < 0.001), 4.0 (P < 0.01) or 4.4 (P < 0.01) fold after 4, 8, 12 and 24 hours, respectively, in the cells grown under high fatty acid conditions, compared to the normal group (Fig. 3A). However, the experimental groups (high fatty acid conditions) supplemented with 10 µg/ml of the ginsenoside Rb2 showed significantly reduced TAG levels (4-47% (P < 0.01)) at each time point (Fig. 3A).

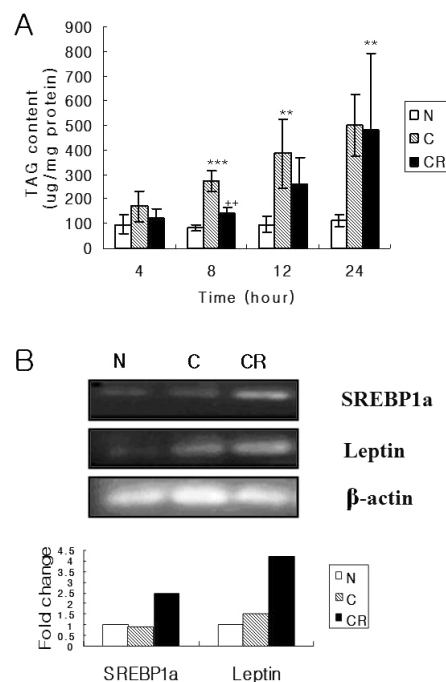


Fig. 3. Time-dependent accumulations of TG (A) and RT-PCR/Southern blot analysis of various mRNA (B) in 3T3-L1 adipocytes cultured under various conditions. Normal 3T3-L1 adipocytes (N) were cultured in serum free-DMEM medium. The control group (C) was cultured in serum free-DMEM medium containing 2% albumin and a 2 mM mixture of fatty acids (oleic acid:palmitic acid = 2:1) for various amounts of time. Experimental groups were cultured under the same conditions as those of the control group except for the addition of the ginsenoside-Rb2 (R, 10 µg/ml). Data are given as the mean ± SD. n=3. Bars represent the standard deviation of each mean. **P < 0.01 vs. the N group; ***P < 0.001 vs. the N group; **P < 0.01 vs. the C group.

The ginsenoside Rb2 enhances the expression of SREBP and leptin mRNAs in 3T3-L1 adipocytes cultured under high fatty acid conditions

The mRNA levels in adipocytes cultured under high fatty acid conditions showed no change in (SREBP) and only a slight increase (leptin), compared to the normal group. When the ginsenoside Rb2 (10 µg/ml) was added to cells grown under high fatty acid conditions, however, the abundance of SREBP and leptin mRNA were greatly enhanced (Fig. 3B), revealing that the ginsenoside Rb2 can counter the effects of high fatty acid growth conditions on the levels of TAG in cultured adipocytes by enhancing the expression of these mRNA.

DISCUSSION

Ginseng is widely used in oriental societies and is considered a highly valuable medicine. Although the putative therapeutic effects of ginseng have prompted tremendous efforts to reveal the cellular mechanism of its action (15, 16), it remains poorly

understood. In this study, we examined the effect of the ginsenoside Rb2 on lipid metabolism in 3T3-L1 adipocytes cultured under high cholesterol conditions, in the presence of FBS or under high fatty acid conditions to investigate the mechanism of its action. Our data showed that the ginsenoside-Rb2 has the ability to lower cholesterol and TAG levels in the differentiated 3T3-L1 adipocytes. When these effects of the ginsenoside Rb2 were compared to those of lovastatin, we found that the ginsenoside Rb2 has comparable effects. Lovastatin is used to reduce the levels of serum cholesterol and for the prevention of atherosclerosis because of its inhibitory activity on 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase.

Cholesterol homeostasis in mammalian cells is controlled by a family of membrane-bound transcription factors called SREBPs. We found that the ginsenoside Rb2 up-regulates the expression of the SREBP mRNA in HepG2 (5) and hypothesized that the ginsenoside Rb2 may also affect the expression of genes that can modify lipid metabolism in adipocytes via SREBP target genes such as FAS. The effects of the ginsenoside Rb2 on the expression of SREBP-1 mRNA level were characterized and we demonstrated that it enhanced SREBP transcription, whereas the cholesterol treatment group showed a reduction in SREBP transcription.

We measured FAS activity because it is known to be regulated by sterol and SREBPs (17). FAS is a key lipogenic enzyme that catalyzes all steps in the biosynthesis of long-chain fatty acids from acetyl-CoA precursors. During active lipogenesis it is expressed at high levels in lactating mammary glands, the liver, and in adipose tissue and is subjected to tight hormonal and nutritional controls. FAS control is exerted primarily at the level of transcription (17-20). Key physiological regulators of FAS expression such as glucose (18), insulin, cAMP (19), thyroid hormone (20) and cholesterol have been identified. Down-regulation of FAS and ACC gene expression were demonstrated by exogenous cholesterol, a major signal inhibiting SREBPs cleavage, and it suggested that fatty acids and cholesterol biosynthetic pathways might be controlled coordinately (21). Our data showed that cholesterol decreased FAS activity in adipocytes as predicted, and that the ginsenoside Rb2 can counter this reduction in 3T3-L1 adipocytes. This effect mediated by the ginsenoside Rb2 can be obtained by a combination of a decrease in cholesterol levels and an enhancement in the expression of SREBP. Moreover, ginsenoside Rb2 treatment slightly increased the enzyme activity of GPDH, an enzyme that is highly expressed in mature adipocytes and which catalyzes the conversion of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (22). Interestingly, although FAS and GPDH activity increased, total cholesterol and TAG levels were reduced in the ginsenoside-Rb2 treatment group. We thought that there may be other mechanisms controlling total cholesterol levels and TAG degradation or turn over. There were reports that SREBPs induced the expression of lipoprotein lipase (LPL) and leptin. Leptin is reported to lower tissue TAG content by increasing intracellular oxidation of free fatty acid

(23, 24). Our data also showed that the ginsenoside Rb2 significantly enhanced the expression of leptin mRNA and that the levels of TAG were decreased in 3T3-L1 adipocytes cultured under high fatty acids conditions.

Taken together, it can be suggested that the ginsenoside Rb2 might be a valuable component for the development of drugs for lowering lipid levels because it is capable of lowering TAG levels in 3T3-L1 adipocytes cultured under high energy conditions by stimulating the expression of SREBP and Leptin mRNA.

MATERIALS AND METHODS

Ginsenoside

The ginsenoside Rb2 was kindly provided by The Korea Tobacco and Ginseng Central Research Institute by the official request of The Korean Society of Ginseng for this study. The ginsenoside Rb2 was dissolved in 10% ethanol and the final concentration of ethanol did not exceed 0.1% (v/v).

Cell culture

3T3-L1 preadipocytes (Korean Cell Line Bank, Korea) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). When cells were confluent, differentiation was induced by adding 0.5 mM isobutylmethylxanthine (IBMX) and 1 µM dexamethasone to the cultures. After 2 days cells were allowed to differentiate further by adding 10% FBS and 10 µg/ml insulin and the medium was changed every 2 days. For these studies, normal groups (N) were cultured in serum free-DMEM for 8 hours after the cells have completely differentiated (at day 10). Control groups were cultured in serum free-DMEM containing 10 µg/ml cholesterol (C) or 10% FBS (F). For high fatty acids conditions, cells were cultured in serum free-DMEM containing 2% albumin and a 2 mM mixture of fatty acids (oleic acid:palmitic acid = 2:1). Experimental groups were cultured under the same conditions as those of the control group except for the addition of the ginsenosides Rb2 (R) or lovastatin (Sigma, USA) (L) as a comparative agent, respectively.

Determining the levels of cellular cholesterol and TAG

Cellular lipids were extracted with hexane/isopropanol (3/2, v/v), dried by centrifugation and dissolved in isopropanol. Total cholesterol and TAG (amounts per mg protein) were determined using an assay kit employing a fluorescent enzymatic method according to the manufacturer's instructions. Protein concentrations were determined using the Bradford method using dissolved cells in 0.1 N NaOH and bovine serum albumin as a standard (25).

FAS assay

For the FAS activity assay, cells were washed twice with PBS and homogenized with ice-cold buffer (pH 8.0) containing 20

mM Tris, 2 mM EDTA and 5 mM GSH. Differentiated adipose cell homogenates were centrifuged to generate cytosolic fractions and the activity of FAS in these fractions was determined. The assay was performed in 100 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM NADPH and 25 μ M acetyl-CoA. After preincubation at 30°C for 5 min, the reaction was started by the addition of 60 μ M malonyl-CoA and the rate of decrease in extinction at 340 nm was recorded. A decrease of 1.0 in the optical density at 340 nm corresponds to the oxidation of 80.5 nmoles of NADPH. One unit (U) of FAS activity was defined as the quantity of enzyme catalyzing the malonyl-CoA-dependent oxidation of 1 nmole NADPH per minute at 30°C (26).

GPDH assay

For the GPDH activity assay, cells were washed twice with PBS and homogenized with ice-cold buffer (pH 7.3) containing 20 mM Tris, 1 mM EDTA and 1 mM β -mercaptoethanol. Mechanical disruption of cells was carried out by six consecutive passages through a 21-gauge needle. A reaction mixture (0.1 M triethanolamine, 2.5 mM EDTA, 0.1 mM β -mercaptoethanol, 125 μ M NADH, pH 7.7) was added to the homogenate and preincubated for 10 min at 37°C. Dihydroxyacetone phosphate (DHAP) was added to start the reaction (200 μ M) and changes in the absorbance at 340 nm were measured using a spectrophotometer. The extinction coefficient for NADH ($\epsilon = 0.002724 \mu\text{M}^{-1}$) was used and one unit of enzyme is defined as the amount of enzyme catalyzing the oxidation of 1 nmole NADH per minute at 37°C (22).

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA were isolated from 3T3-L1 cells at day 10 of differentiation using TRIzol reagent (SIGMA) according to the manufacturer's instructions. First-strand cDNA was generated from 1 μ g of RNA using primers specific for mouse mRNA. The resulting RT reaction mixture was used for PCR with primers specific for mouse SREBP1 cDNA (sense: 5'-CTCAGGTCATGTTGGAA ACC-3', antisense: 5'-AGACAGGGAGTTCTCAGATG-3', GeneBank accession No. AF374266), and leptin cDNA (sense: 5'-G GAATTCAGGAAAATGTGCTGGAGA-3', antisense: 5'-GGAA TTCTCAGCATTAGGGCTAAC-3', GeneBank accession No. NM008493). Thirty cycles of PCR were performed using the following parameters: 92°C for 40 sec, 55°C for 40 sec, 72°C for 40 sec. β -actin primers were used as an endogenous control (sense: 5'-TCGTGCGTGACATTAAGGAGGTG-3', antisense: 5'-TTGCGCTCAGGAGGCAAT-3'). The PCR products were electrophoresed on a 1% agarose gel and the intensity of the bands were compared.

Statistical analysis

Data are presented as means \pm SD. Significance was analyzed using the Student's t-test. Differences were considered significant when $P < 0.05$.

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