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Ginsenoside Rb2 Upregulates the Low Density Lipoprotein Receptor Gene Expression through the Activation of the Sterol Regulated Element Binding Protein Maturation in HepG2 Cells

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Abstract : Ginsenosides, a group of *Panax ginseng* saponins, exert the lowering effects of plasma cholesterol levels in animals. We had reported earlier that ginsenoside Rb2 upregulate low-density lipoprotein receptor (LDLR) expression via a mechanism that is dependent of the activation of sterol response element binding protein 2 (SREBP-2) expression. This study was conducted to determine the effects of ginsenoside Rb2 on the expression of the hepatic LDLR expression at cellular levels using HepG2 cells, and to evaluate whether the sterol response element binding protein 1 (SREBP-1) was involved in the regulation of LDLR expression. Incubation of HepG2 cells in serum-free medium supplemented with cholesterol (10 μg/ml) for 8 hours decreased the mRNAs of LDLR mRNA by 12% and SREBP-1 mRNA by 35%. Ginsenoside Rb2 antagonized the repressive effects of cholesterol and increased both LDLR and SREBP-1 mRNA expression to 1.5- and 2-fold, respectively. Furthermore, Western blot and confocal microscopic analyses with SREBP-1 polyclonal antibody revealed that ginsenoside Rb2 enhanced the maturation of the SREBP-1 from the inactive precursor form in ER membrane to the active transcription factor form in nucleus. These results suggest that ginsenoside Rb2 upregulates LDLR expression via a mechanism that is dependent of the activation of not only SREBP-2 expression, but also SREBP-1 expression and maturation, and also indicate that the pharmacological value of ginsenoside Rb2 may be distinguished from that of lovastatin which is reported that it upregulate LDLR through SREBP-2 only, not through SREBP-1.

Key words: Cholesterol, Ginsenoside Rb2, HepG2 cells, LDL receptor, SREBP

INTRODUCTION

The cholesterol content of cells is tightly controlled and the elevated and uncontrolled cholesterol levels in human have been known to develop various disorders including atherosclerosis or coronary heart diseases.[1] Currently, several pharmacological approaches have been conducted for cholesterol reduction in cells and plasma including the inhibition of cholesterol synthesis by 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor like lovastatin, the enhancement of bile acid excretion by bile acid-binding resins, the inhibition of LDL production by nicotinic acid or fibrates, and the stimulation of receptor-independent LDL clearance by probucol.[2] Although all of these approaches have significant effects on reducing elevated cholesterol levels, however, they have also shown potential side effects.[2] Therefore, the development of safe and effective LDL cholesterol-reducing agents for human is substantially important issues for pharmaceutical approach.

When cells meet their sterol requirements because of insufficient cholesterol, rates of both lipoprotein cholesterol uptake and cholesterol biosynthesis are increased. In contrast, under conditions of cholesterol excess, cells prevent accumulation of cholesterol by reduction of both lipoprotein cholesterol uptake and cholesterol synthesis. Of the mechanisms for controlling cholesterol levels in cells and plasma, a control through the low density lipoprotein receptor (LDLR) expression mediated by sterols such as cholesterol plays a critical role for cholesterol clearance in plasma.[3,4,5,6] This sterol-mediated regulation of LDLR gene expression is accomplished by sterol regulatory element binding proteins (SREBPs) that are a member of the basic-helix-loop-helix-leucine zipper family of DNA binding and transcription regulation proteins.[7,8] SREBP is synthesized as a high molecular weight precursor of 125-kDa that is bound to the nuclear

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and endoplasmic reticulum (ER) membranes.[9] When cellular sterol concentrations were reduced, the amino terminal 68-kDa domain of the SREBP precursor is released from the ER membrane, entered the nucleus, and bound to sterol regulatory elements (SREs) in the promoters of SREBP-target genes such as LDLR and HMG-CoA synthase genes, resulting transcriptional activations of these genes.[10]

Ginseng, as one of the major traditional medicines, has been used in Asian countries, especially Korea, Japan, and China, for the treatment of various diseases, including psychiatric and neurologic diseases, as well as cancer.[1,11,12] Ginseng saponins (ginsenosides), the bioactive ingredients of the ginseng root, have been regarded as the principal components responsible for the pharmacological activities of ginseng.[13] Besides anti-tumor effects of ginsenosides, many studies have found that ginsenosides shows the effect of lowering the cholesterol level in rats or rabbits animal models of dietary-induced hypercholesterolemia.[14,15,16] Also, ginsenoside Rb2, one of the ginsenosides prepared from the roots of Panax ginseng C.A. Mayer, has been reported to reduce plasma cholesterol levels.[17,18,19] However, the molecular mechanism of ginsenoside effects on cholesterol reduction in cell has yet to be investigated. We had reported earlier that ginsenoside Rb2 upregulate LDLR expression via a mechanism that is dependent of the activation of sterol response element binding protein 2 (SREBP-2) expression.[20]

In this study, we show that ginsenoside Rb2 enhances the LDLR mRNA transcription in a human hepatoma cell line, HepG2 cells, known to express LDLR amenable to regulation[21] and this transcriptional activation is mediated by the enhancement of SREBP-1 transcription and through the increase of SREBP maturation.

MATERIALS AND METHODS

Ginsenoside

Ginsenoside Rb2 was kindly provided from The Korea Tobacco and Ginseng Central Research Institute. Ginsenoside-Rb2 was resolved in 10% ethanol and the final concentration of ethanol did not exceed to 0.1% (v/v).

Cell and culture conditions

HepG2 cell (KCLB 58065), a human hepatoma cell line, was purchased from Korea Cell Line Bank (KCLB). HepG2 cells were grown in 100 mm dishes containing RPMI 1640 medium (GibcoBRL, USA), supplemented with penicillin/streptomycin (GibcoBRL, USA) and 10% (v/v) fetal bovine serum (FBS; GibcoBRL, USA) at 37°C with 5% CO_2. After cells were grown to 70% confluence, the dishes were washed one time with phosphate buffered saline (PBS). Then, RPMI 1640 media supplemented with penicillin/streptomycin (5%, v/v) and calf lipoprotein-deficient serum (LPDS, GibcoBRL, USA) were added in the dishes and the cells were challenged with cholesterol (10 μg/ml, Sigma, USA) as a control group or cholesterol (10 μg/ml) and ginsenoside Rb2 (10 μg/ml) as a test group. A group with penicillin/streptomycin and LPDS only was similarly set as a normal group. After incubation for 8h, the cells from each group were collected by centrifugation at 1,000 × *g* for 10 min, washed three times with PBS, and used in Northern blot and Western blot analyses.

Northern blot analysis of LDLR and SREBP-1

Total RNAs were extracted from the cells by a protocol using guanidinum thiocyanate.[22] An equal amount (10 μg each) of the extracted total RNAs were separated in a 0.8 % agarose/1.8 M formaldehyde gel and transferred onto Nitran N^+ membrane (Schleicher & Schell, USA) using a Turbo Blotter apparatus (Schleicher & Schell, USA). Northern blotting with chemiluminescence detection for LDLR and SREBP-1a using DIG-labeled 1.9 Kb human LDLR cDNA (pLDLR3, ATCC 57004) and 4.3 Kb human SREBP-1a cDNA (pSREBP-1a, ATCC 79810) probes has been described in DIG application manual (Roche, USA). Signals for LDLR and SREBP-1a were standardized by reference to human β-actin, which was measured with DIG-labeled 1.1 Kb human β-actin cDNA (HHC 189, ATCC 65128) probe, as described above.

Western blot analysis of SREBP-1

Nuclear extract and membrane fractions were prepared from the collected cells, according to a method described in detail with minor modifications.[23] Briefly, the collected cells were suspended in a buffer of 10 mM HEPES at pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.25 M Sucrose, and 0.5% (v/v) Nonidet P-40. The cell suspension was passed through a 22 gauge needle 15 times and centrifuged at 1,000 × *g* at 4°C for 7 min. The resulting 1,000 × *g* pellet was used to prepare a nuclear extract fraction by resuspension in 100 μl of a buffer [20 mM HEPES at pH 7.4, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and 1 mM dithiothreitol], followed by rotation of the suspension at 4 °C for 1 hour and centrifugation at 100,000 × g in a Beckman TLA 100.3 rotor for 30 min at 4°C. The resulting 1,000 × *g* supernatant was used to prepare a membrane

fraction by centrifugation at 100,000 × g for 30 min at 4 °C, followed by resuspension of the $10^5 \times g$ pellet in 100 µl of SDS lysis buffer (10 mM Tris-HCl at pH 6.8, 1% SDS, 1 mM EDTA, and 1 mM EGTA). Protein concentration was measured with a protein assay kit (Bio-Rad, USA). An equal amount of samples (50 µg each) was mixed with 3 × SDS loading buffer prior to SDS-PAGE on 7.5% gel. After electrophoresis, the proteins were transferred to Immobilon-P membrane (Millipore, USA). Western blot analysis was carried out with 5-bromo-4-chloro-3-indolylphosphate / nitroblue tetrazolium (BCIP/NBT) detection kit (GibcoBRL, USA) according to the manufacturer's protocols except that the membrane was blocked in PBS containing 0.05% (v/v) Tween 20 and 5% nonfat dry milk. Immunodetection of SREBP-1 was carried out with SREBP-1 polyclonal antibody (K-10, 1:1,000 dilution; Santa Cruz Biotechnology, USA), followed by a alkaline phosphatase conjugated secondary antibody (1:2,000 dilution. Sigma, USA).

Immunohistochemistry of SREBP-1

HepG2 cells were grown on coverslips coated with 1% (w/v) gelatin for 2 days and treated as described below. The cultured cells were fixed in 3.7 % formaldehyde-PBS solution, rinsed with PBS for 10 min, and permeabilized in 2 % formaldehyde-PBS with 0.2 % (v/v) Triton X-100 for 10 min. The treated cells on coverslips were washed three times for 5 min with PBS. The cells were incubated with a SREBP-1 polyclonal antibody (1:100 dilution, Santa Cruz Biotechnology, USA) for 16 h at 4°C, and washed three times with PBS for 10 min intervals. After then, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibody (1:200 dilution. Chemicon International. USA) and 5 µg/ml of propidium iodide for 1 h at room temperature. The stained cells were washed with PBS several times, mounted on microscope slides, and visualized with MRC 1024 confocal microscope system (Bio-Rad, USA) at the indicated magnification.

Statistics

The results were expressed as mean ± SEM. Statistical evaluation was done using with the Student t test, comparing treatment groups with the control group. A value of $P < 0.05$ was taken as the criterion of significance.

RESULTS

Effect of ginsenoside Rb2 on LDLR transcription

Our preliminary observations revealed that ginsenoside Rb2 at the 10 µg/ml of concentration maximally reduced the cholesterol level in HepG2 cells cultured in an enriched cholesterol condition (10 µg/ml) up to 40%. To know whether this cholesterol reduction in HepG2 cells by ginsenoside Rb2 is related to LDLR expression, HepG2 cells were challenged with serum-free media only (normal group), cholesterol only (10 µg/ml; control group), or cholesterol plus ginseonside Rb2 (10 µg/ml each, test group). After 8 hours, total RNAs from the treated cells were extracted and Northern blot analysis was performed with human LDLR cDNA probe as described in Materials and Methods. The results showed that when the level of LDLR mRNA in the cholesterol-untreated cells (normal group) was set as 100%, the level of LDLR mRNA in the cholesterol-treated cells (control group) was 12% less than the normal group (Fig. 1A), indicating that LDLR mRNA expression in HepG2 cells was slightly repressed by cholesterol. When HepG2 cells were cultured in the cholesterol-enriched control media supplemented with ginsenoside Rb2 at the 10 µg/ml of concentration for 8 hours and the levels of LDLR mRNA were evaluated, the results unexpectedly showed that ginsenoside Rb2 antagonized the depressive effects of cholesterol and increased LDLR mRNA abundance up to 1.5-fold (Fig. 1A), indicating that ginsenoside-Rb2 has an enhancing effect on LDLR expression at the transcription level, but the cellular cholesterol level was decreased by ginsenoside Rb2.

Effect of ginsenoside Rb2 on SREBP transcription

Because the sterol-mediated regulation of LDLR gene expression is accomplished by SREBPs, the membrane-bound transcription factors[7,8], we tested the effect of ginsenoside Rb2 on SREBP transcription in HepG2 cells cultured in enriched cholesterol conditions. When the level of SREBP mRNA in the cholesterol-untreated cells (normal group) was set as 100%, this in the cholesterol-treated cells (control group) was decreased to 35% (Fig. 1B), indicating that SREBP mRNA expression in HepG2 cells was also repressed by cholesterol. Consistent with the effect of ginsenoside Rb2 on LDLR mRNA expression shown above, however, ginsenoside Rb2 also antagonized the depressive effects of cholesterol on SREBP expression and increased SREBP mRNA abundance up to 2-fold (Fig. 1B). We next examined the effect of actinomycin-D as a transcription inhibitor on SREBP-1 mRNA expression. The result showed that actinomycin-D (5 µg/ml) could inhibit the SREBP-1 mRNA transcription to 50% (data not shown), indicating that the ginsenoside Rb2

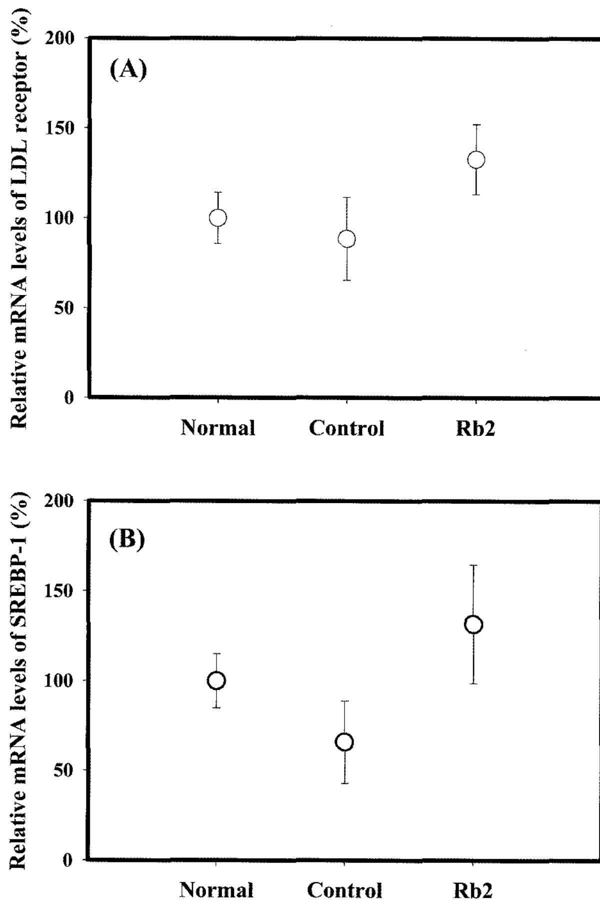


Fig. 1. Effect of ginsenoside-Rb2 on transcription of LDL receptor and SREBP-1 mRNA in HepG2 cells cultured in cholesterol enriched condition. Normal HepG2 cells were cultured in serum free-RPMI 1640 medium. Control group was cultured in serum free-RPMI 1640 medium containing 0.1% alcohol and 10 μg/ml cholesterol. Ginsenoside-Rb2 treated test group(Rb2) was cultured under the same conditions as those of the control group with ginsenoside-Rb2 (10 μg/ml) for 8 hours. Total RNA was extracted from the collected cells of each group, and each mRNA was separated and identified by northern blotting as described in detail in the Materials and Methods. (A): relative mRNA level of LDL receptor, (B): relative mRNA level of SREBP-1.

enhanced de novo synthesis and transcription of SREBP mRNA. Taken together, the results showed that the transcriptional activation of LDLR in HepG2 cells by ginsenoside Rb2 is truly mediated through de novo synthesis and transcriptional activation of SREBP mRNA.

Effect of ginsenoside Rb2 on SREBP maturation

To test the effect of ginsenoside Rb2 on SREBP expression at protein levels, nuclear extract and membrane fractions were obtained from the cells described above and a Western blot analysis was performed with SREBP-1 polyclonal antibody. The result showed that in cholesterol enriched condition, the 125-kDa sized SREBP-1 precursor was detected only in membrane fraction, but not in nuclear extract (Fig. 2B), indicating that cholesterol inhibited the proteolytic maturation of SREBP-1 precursor form. In sterol-deprived condition (normal), the mature nuclear form of 68-kDa size was detected in nuclear extract (Fig. 2A), showing that the SREBP precursor was proteolytically cleaved from membrane and moved to nucleus. Interestingly, when ginsenoside Rb2 was added in the cholesterol enriched condition, the mature nuclear form of SREBP was predominantly detected in nuclear extract (Fig. 2C), proposing that ginsenoside Rb2 relieves the SREBP maturation step blocked by cholesterol. To further convince and visualize the effect of ginsenoside Rb2 on SREBP-1 maturation and nuclear translocation, immuno-fluorescence localization studies were performed with FITC-conjugated SREBP-1 polyclonal antibody using a laser confocal microscope. The results showed that FITC-conjugated SREBP-1 antibody staining recognized SREBP proteins (green color) as reticular staining patterns throughout HepG2 cell bodies in all the tested cells (Fig. 3), indicating the localization of the SREBP-1

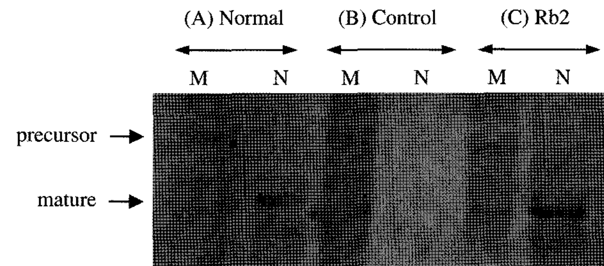
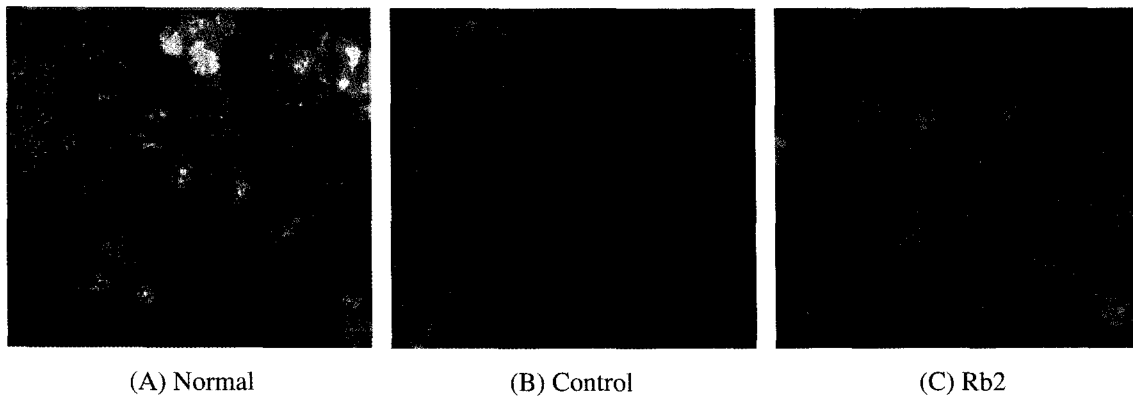


Fig. 2. Effect of ginsenoside-Rb2 on maturation of SREBP-1 in HepG2 cells cultured in cholesterol enriched condition. Normal HepG2 cells were cultured in serum free-RPMI 1640 medium (A). Control group was cultured in serum free-RPMI 1640 medium containing 0.1% alcohol and 10 μg/ml cholesterol (B). Ginsenoside-Rb2 treated test group was cultured under the same conditions as those of the control group with ginsenoside-Rb2 (10 μg/ml) for 8 hours (C). Nuclear extract (N) and membrane fractions (M) were prepared from the collected cells of each group as described in detail in the Materials and Methods. Cellular proteins were separated and identified by using SDS-PAGE and western blotting with polyclonal SREBP-1 antibody. The Precursor and the mature form of SREBP-1 were located at band position of 125 Kd and 68 Kd, respectively.



(A) Normal (B) Control (C) Rb2

Fig. 3. Effect of ginsenoside-Rb2 on translocation of truncated SREBP-1 in HepG2 cells cultured in cholesterol enriched condition. Normal HepG2 cells were cultured in serum free-RPMI 1640 medium (A). Control group was cultured in serum free-RPMI 1640 medium containing 0.1% alcohol and 10 μg/ml cholesterol (B). Ginsenoside-Rb2 treated test group was cultured under the same conditions as those of the control group with ginsenoside-Rb2 (10 μg/ml), for 8 hours (C). After 8 hours, cells were fixed and permeabilized, then immunofluorescene detection was done by Confocal microscope; magnifications: 200X with zoom 2.4. The first antibody was a polyclonal SREBP-1 antibody and secondary antibody was FITC-conjugated goat anti-rabbit IgG. (See details in the Materials and Methods.)

precursor form to intracellular membranes. However, yellow color in nucleus resulting from overlapping images between green-colored SREBP protein by FITC fluorescence and red-colored nucleus stained by propidium iodide was observed only in cells cultured in cholesterol-deprived condition (Fig. 3A) or cholesterol enriched condition with ginsenoside Rb2 challenge (Fig. 3C), but not in cholesterol enriched condition only (control) (Fig. 3B). The specific FITC staining in nucleus was not due to non-specific binding of the FITC-conjugated SREBP-1 antibody to nucleus because omitting the first antibody in staining procedures emitted fluorescence in the FITC band only at background levels (data not shown), suggesting that the ginsenoside Rb2 up-regulate the LDLR gene expression in HepG2 cells through the activation of SREBP-1 maturation and translocation of the truncated SREBP-1.

DISCUSSION

Ginsenosides, a group of Ginseng saponins, are known to inhibit absorption of both dietary cholesterol and endogenous cholesterol excreted into the intestinal lumen via the bile[24,25] and reduce plasma cholesterol levels through the regulation of LDLR expression in experimental animals.[14] Therefore, it can be used in the treatment of hypercholesterolemia as a potential herbal medicine without any side effects. Because hepatic cholesterol reduction is highly correlated with cholesterol absorption inhibition and induced compensatory increases in both hepatic HMG-CoA reductase activity for cholesterol synthesis and hepatic LDLR levels for cholesterol uptake, the present study was undertaken to examine the effects of ginsenoside Rb2 on cholesterol regulation in a human hepatoma cell line, HepG2 cells that known to express LDLR amenable to regulation.[21] In this study, HepG2 cells in an enriched cholesterol condition (10 μg/ml) reduced cholesterol uptake rate through down-regulation of LDLR gene transcription as previously reported[26,27], but ginsenoside Rb2 addition in an enriched cholesterol condition (10 μg/ml) enhanced LDLR gene transcription to 1.5 fold. Ginsenoside Rb2 also up-regulated SREBP-1 gene transcription repressed by cholesterol up to 2-fold, suggesting that ginsenoside Rb2 relieves SREBP-1 gene transcription repressed by cholesterol and the gene activation consequently enhances LDLR gene transcription in HepG2 cells. In Western blot and confocal microscopic analyses, our results consistently demonstrated that ginsenoside Rb2 enhanced the conversion of SREBP-1 from the inactive precursor form in ER membrane to the active transcription factor form in nucleus. Therefore, this activation of SREBP-1 maturation by ginsenoside Rb2 consequently accompanied the activation of the LDLR gene expression in HepG2 cells because LDLR gene is one of the target genes activated by SREBPs.[28] Despite this apparent increase in LDL receptor gene transcription by ginsenoside Rb2, however, the fact that cholesterol did not accumulate within the HepG2 cells as the cellular

cholesterol remained decreased by 40% is contradictory. A possible explanation could be that ginsenoside Rb2 appears to increase the export of cholesterol from the cells into media to such an extent that the increase in the LDLR does not fully compensate for the loss of cell cholesterol. Indeed, ginsenoside Rb2 addition in an enriched cholesterol condition increased the activity of acyl CoA:cholesterol acyl-transferase (ACAT) which is the enzyme responsible for the acylation of the intracellular free cholesterol to form cholesterol esters in HepG2 cells up to 2-folds (unpublished observation), suggesting that the increased conversion of cholesterol to cholesterol esters lowers intracellular cholesterol concentration in HepG2 cells.

The ginsenoside Rb2 effect on LDLR expression shown in this study is somewhat similar to the effect of green tea in that an ethyl acetate extract of green tea, containing 70% (w/w) catechins, increased the LDLR at protein and mRNA levels and the maturation of the SREBP-1 protein, but it decreased the cell cholesterol concentration up to 30%.[29] However, there were some differences between ginsenoside Rb2 and green tea effects in Hep G2 cells that ginsenoside Rb2 decreased the expression of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis (unpublished observation), but green tea increased the HMG-CoA reductase mRNA in HepG2 cells[29], proposing that the upregulation mechanisms of LDLR expression by ginsenoside Rb2 and green tea extracts are similar, but their cholesterol lowering mechanisms in HepG2 cells may differ. Another report showed that conjugated linoleic acid (CLA) antagonized the depressive effects of 25-hydroxycholesterol and increased both LDLR protein and mRNA abundance twofold, but both abundances had no effects on an acyl CoA:cholesterol acyltransferase (ACAT) inhibitor (58-035) treatment and CLA had no effect on SREBP-abundance, suggesting that CLA upregulates LDLR expression via a mechanism that is independent of ACAT and SREBP-1.[30]

LDLR mediates the endocytic uptake of cholesterol-carrying lipoproteins, thereby controlling cholesterol levels in cells and plasma.[8] SREBPs are transcription factors that were first isolated as a result of their properties for binding to the sterol regulatory element and conferring sterol regulation to several genes including LDLR gene.[9] The SREBPs are synthesized as 125-kDa precursor forms that contain two transmembrane domains for insertion into the ER membrane and the N-terminal domain, which is a 68-kDa, (i.e. mature SREBP) is released for nuclear translocation by a sterol-dependent proteolytic cascade.[9] The cleavage of SREBPs for activation is a complicated process having multiple steps. The SREBP cleavage activating protein (SCAP), known as a primary candidate molecule for sensing the cellular cholesterol levels[31], is prerequisite for SREBPs cleavage and the SREBP precursor and SCAP form a complex on the ER membrane.[32,33] When sterol is depleted, the SREBP-SCAP complex moves to Golgi and targets to two consecutive proteolytic cleavages by site-1 and site-2 proteases for liberating the active N-terminal domain of SREBP.[33,34] Also, importin β is involved in this nuclear transport of mature SREBP.[35] In this study using Western bolt and confocal microscopic analyses, we clearly demonstrated that cholesterol blocked the SREBP maturation in HepG2 cells and ginsenoside Rb2 could retrieve the SREBP maturation step repressed by cholesterol. We do not currently know how ginsenoside Rb2 activates SREBP maturation. Ginsenoside Rb2 may play a role either in enhancement of the SREBP-SCAP complex targeting to Golgi or in activation of site-1 and site-2 proteases for SREBP cleavage. Both possibilities remain to be solved.

In conclusion, ginsenoside Rb2 as an active ingredients of ginseng saponins up-regulate the LDLR gene expression in HepG2 cells through the activation of not only SREBP-2, but also SREBP-1 expression and maturation, and this enhancement of the LDLR can effectively reduce the cholesterol concentration in plasma. Moreover, the pharmacological value of ginsenoside Rb2 may be distinguished from that of lovastatin which is reported that it upregulate LDLR through SREBP-2 only, not through SREBP-1. The clearance effect of ginsenoside Rb2 on intracellular excess cholesterol in response to a facilitated turn over of cholesterol to cholesterol ester via a possible activation of ACAT and to the bile via a possible activation of cholesterol 7α-hydroxylase[36] may play a role in the hypocholesterolemic effect to the subject in hypercholesterolemia *in vivo* and may ultimately expand the options for the pharmacologic treatment of lipid disorders by natural substance without side-effects.

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