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Ginsenoside Rf, a component of ginseng, regulates lipoprotein metabolism through peroxisome proliferator-activated receptor α

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Abstract

We investigated whether ginseng regulates lipoprotein metabolism by altering peroxisome proliferator-activated receptor α (PPARα)-mediated pathways, using a PPARα-null mouse model. Administration of ginseng extract, ginsenosides, and ginsenoside Rf (Rf) to wild-type mice not only significantly increased basal levels of hepatic apolipoprotein (apo) A-I and C-III mRNA compared with wild-type controls, but also substantially reversed the reductions in mRNA levels of apo A-I and C-III expected following treatment with the potent PPARα ligand Wy14,643. In contrast, no effect was detected in the PPARα-null mice. Testing of eight main ginsenosides on PPARα reporter gene expression indicated that Rf was responsible for the effects of ginseng on lipoprotein metabolism. Furthermore, the inhibition of PPARα-dependent transactivation by Rf seems to occur at the level of DNA binding. These results demonstrate that ginseng component Rf regulates apo A-I and C-III mRNA and the actions of Rf on lipoprotein metabolism are mediated via interactions with PPARα.

Keywords: Ginseng; Ginsenoside Rf; Lipoprotein; PPARα; Wy14,643

Ginseng root extract (GE) is one of the most valuable and widely used herbal medicines in oriental societies. Pharmacological effects of ginseng have been reported in the central nervous system and in endocrine, immune, and cardiovascular systems [1,2], including lipid metabolism [3–6], but the mechanisms of ginseng actions remain unclear. The major metabolites of ginseng are ginsenosides (GS), also referred to as steroidal saponins, which contain most of the pharmacological activity of ginseng [7]. GS belong to a family of steroids and share structural characteristics with steroid hormones [8,9], such as a 4-ring, steroid-like structure with attached sugar moieties [10], suggesting that GS may bind to nuclear receptors and alter gene expression, similar to the actions of steroid hormones [6].

Peroxisome proliferator-activated receptor α (PPARα) is a member of the steroid/thyroid hormone receptor superfamily. Ligand-activated PPARα heterodimerizes with retinoid X receptor (RXR) and binds to PPAR response elements (PPREs) in the promoter region of target genes critical for lipid and lipoprotein metabolism. PPARα has been found to initiate triglyceride-lowering effects through transcriptional activation of apolipoprotein (apo) C-III and lipoprotein lipase [11–13] as well as fatty acid β-oxidizing enzymes [14,15]. PPARα has also been demonstrated to increase the circulating amounts of high density lipoprotein (HDL) levels through induction of apo A-I and A-II gene expression in humans [16,17]. In rat and mice, however, PPARα induces a pronounced decrease in HDL due to a decreased expression of apo A-I, the major HDL apolipoprotein [18,19].

In addition to the well-documented regulation of lipoprotein metabolism by PPARα, we previously reported that in vivo and in vitro treatments of ginseng suppressed

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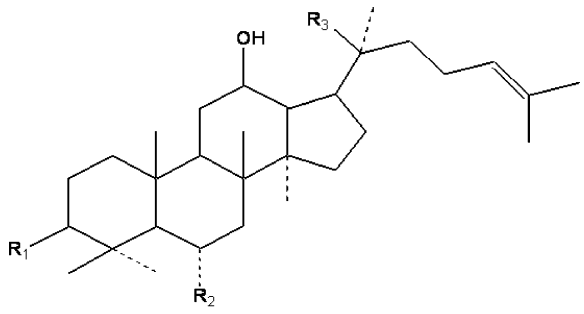
the expression of PPARα target genes encoding peroxisomal enzymes involved in fatty acid metabolism [6], suggesting that ginseng may affect lipoprotein metabolism as a result of interactions with PPARα. We therefore sought to investigate whether ginseng exerts its effects on lipoprotein metabolism via interactions with PPARα, as well as to determine the mechanism by which ginseng suppress PPARα transactivation, and to identify the active ginsenoside. We here report that ginseng regulates lipoprotein metabolism by interfering with the binding of PPARα to DNA. We also found that ginsenoside Rf is the pharmacologically active component in PPARα-mediated lipoprotein metabolism.

Materials and methods

Materials. The PPARα ligand (4-chloro-6-(2,3-xylidine)-pyrimidinylthio)acetic acid (Wy14,643) was purchased from ChemSyn Science Laboratories (Lenexa, KS). Korean red GE powder was commercially prepared from ginsengs cultivated with care in well-fertilized fields for 6 years (Korea Ginseng). GS were obtained from extractions of the GE powder as described previously [6]. Each ginsenoside was isolated from GS according to the method of Nah et al. [20]. Fig. 1 shows the structures of the eight main ginsenosides that were tested.

Animal treatments. Eight-week-old male PPARα (+/+) and PPARα (−/−) mice on a C57BL/6 background were used, and bred under specific pathogen-free conditions and a standard 12-h light/dark cycle. Mice received intraperitoneal injections of either saline (control), GE (5 mg per mouse), GS (0.5 mg per mouse), each of the eight main ginsenosides (0.05 mg per mouse) for 10 days, or a diet containing 0.1% Wy14,643 (Wy14,643) for 1 day. For co-treatment studies, mice were treated for 10 days with GE, GS, or each of the eight main ginsenosides, followed by Wy14,643 for 1 day. At the end of each study, animals were sacrificed by cervical dislocation, tissues were harvested, weighed, and snap frozen in liquid nitrogen and stored at −80 °C until use.



Ginsenosides	R_1	R_2	R_3
Rb_1	-O-Glc^2-Glc	-H	-O-Glc^6-Glc
Rb_2	-O-Glc^2-Glc	-H	-O-Glc^6-Ara (Pyr)
Rc	-O-Glc^2-Glc	-H	-O-Glc^6-Ara (Pyr)
Rd	-O-Glc^2-Glc	-H	-O-Glc
Re	-OH	-O-Glc^2-Rha	-O-Glc
Rf	-OH	-O-Glc^2-Rha	-OH
Rg_1	-OH	-O-Glc	-O-Glc
Rg_2	-OH	-O-Glc^2-Rha	-OH

Fig. 1. Chemical structures of the eight main ginsenosides tested. Based on chemical structure, they differ at three side chains attached to the common steroid ring. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara (pyr), arabinopyranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links the two carbohydrates.

Northern blot analysis. Total RNA was prepared using Trizol reagent (Gibco-BRL, Grand Island, NY) and analyzed by electrophoresis on 1.2% agarose gels containing 0.22 M formaldehyde. The separated RNA was transferred to Nytran membranes (Schleicher & Schuell, Dassel, Germany) by downward capillary transfer in the presence of 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0), UV-crosslinked, and baked for 2 h at 80 °C. Probe hybridization and washing were performed using standard techniques. Blots were exposed to PhosphorImager screen cassettes and visualized using a Molecular Dynamics Storm 860 PhosphorImager system (Sunnyvale, CA). The probes used in this study were ^{32}P-labeled by the random-primer method using a Ready-to-Go DNA Labeling kit (Amersham–Pharmacia Biotech, Piscataway, NJ), as described previously [21]. Densitometric analysis of the mRNA signals was performed using ImageQuant image analysis software (Molecular Dynamics).

Western blot analysis. Five microliters of serum aliquots from wild-type and PPARα(−/−) mice was mixed with an equal volume of 2× sodium dodecyl sulfate (SDS)-loading buffer and boiled for 10 min. After separation on 10% SDS–polyacrylamide gels, serum proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with a rabbit anti-mouse apo A-I antibody (BioDesign International, Saco, ME) and rabbit anti-mouse apo C antibody that reacted with mouse apo C-III (BioDesign International) as primary antibodies and a horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) as a secondary antibody. Antibody bindings were visualized by chemiluminescence with the ECL kit (Amersham–Pharmacia Biotech, Piscataway, NJ).

Transient transfection assay. The expression vectors, pSG5-mPPARα and $PPRE_3$-tk-luc reporter gene were generously provided by Dr. Frank J. Gonzalez (National Cancer Institute, NIH, Bethesda, MD). Murine liver cell line NMu2Li cells were routinely cultured in DMEM containing 10% fetal bovine serum (Gibco-BRL, Grand Island, NY), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (0.25 μg/ml), and 2-mercaptoethanol (50 μM). Cells were seeded in six-well tissue culture plates (2×10^4 cells/well) 24 h prior to transfection. For all transfections, 200 ng/well of each of the appropriate plasmids were used. Transfections were performed using GeneSHUTTLE-40 (Q-Biogene, Carlsbad, CA) according to the manufacturer's instructions. After 6 h, the culture medium was changed and the test compounds, Wy14,643, GE, GS, or each of the eight ginsenosides were added. After incubation for 24 h in the presence of the aforementioned chemicals, cells were washed twice with PBS and assayed for luciferase and β-galactosidase activity using commercial kits according to the manufacturer's instructions (Promega, Madison, WI).

Gel electrophoretic mobility shift assay. The binding of PPARα to the PPAR-specific oligonucleotide probe was assayed by adding 8 μg of liver nuclear extract to each gel shift reaction mixture. An oligonucleotide containing rat acyl-CoA oxidase PPRE (5′-CTAGCGATATCATGACC TTTGTCCTAGGCCTC-3′) was used along with an oligonucleotide of complementary sequence [22]. The oligonucleotides were mixed (50 ng/μl final concentration) and denatured by heating to 95 °C for 10 min in 0.1 M Tris–Cl, 50 mM $MgCl_2$ (pH 7.9) and allowed to anneal by slowly cooling to room temperature. The annealed oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase according to the supplier's instructions (Promega, Madison, WI). In a total volume of 20 μl of binding buffer (25 mM Tris–Cl, pH 7.5, 40 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol), the following components were combined: 1 μg of poly(dI–dC), 2 μl of nuclear extract, and the test compounds Wy14,643 (10 μM), GE (10 μg/ml), GS (10 μg/ml), or Rf (10 μM) dissolved in Me_2SO. After a 20-min incubation at room temperature, 20,000 cpm of the labeled oligonucleotide was added, and the incubation was continued for a further 20 min. For a supershift experiment, 2 μg of goat anti-human PPARα antibody (Santa Cruz Technology, Santa Cruz, CA) was added to the reaction mixture prior to addition of the oligonucleotide probe. Samples were analyzed on a 5% non-denaturing polyacrylamide gel, containing

2.5% glycerol, in 0.4× TBE (1× = 89 mM Tris–Cl, 89 mN boric acid, 2 mM EDTA). After drying, the gels were exposed to PhosphorImager screen cassettes and were visualized using a Molecular Dynamics Storm 860 PhosphorImager system.

Statistics. Unless otherwise noted, all values are expressed as mean ± standard deviation (SD). All data were analyzed by the unpaired, Student's *t* test for significant differences between the mean values of each group using SigmaPlot 2001 (SPSS, Chicago, IL).

Results

Hepatic apo A-I and C-III mRNA levels

To study the effects of ginseng on lipoprotein metabolism, the levels of apo A-I and C-III mRNA were measured in wild-type (+/+) and PPARα (−/−) mice treated once daily with GE, GS or each of the eight ginsenosides for 10 days. Compared with chow-fed control (+/+) mice, administration of GE, GS, and ginsenoside Rf to PPARα (+/+) mice exhibited significant increases in mRNA levels of apo A-I and C-III, although Rf showed a trend toward increasing mRNA levels of apo A-I (Fig. 2). However, treatment of PPARα (−/−) mice with GE, GS or Rf resulted in no significant changes in apo A-I and C-III mRNA levels.

To determine the effects of ginseng on PPARα activator Wy14,643-suppressed apo A-I and C-III gene expression, mice were treated for 10 days with GE, GS, or each of the eight main ginsenosides, followed by Wy14,643 for 1 day. Rodent hepatic apo A-I and C-III mRNAs have been shown to be downregulated by PPARα activators such as Wy14,643 [12,18]. As expected, hepatic apo A-I and C-III mRNA levels were substantially lowered in wild-type mice fed a diet containing 0.1% Wy14,643 (Wy14,643) for 1 day than in chow-fed control mice (Fig. 3). However, compared to Wy14,643-only treated wild-type mice, concomitant treatment with Wy14,643/GE, Wy14,643/GS or Wy14,643/Rf resulted in significantly increased hepatic expression of both apo A-I and C-III genes. In contrast to the results in wild-type mice, ginseng treatment had no effects on apolipoprotein mRNA levels in PPARα-null mice. These results suggest that in vivo treatment with ginseng may significantly increase hepatic mRNA levels of PPARα targets apo A- I and C-III and that

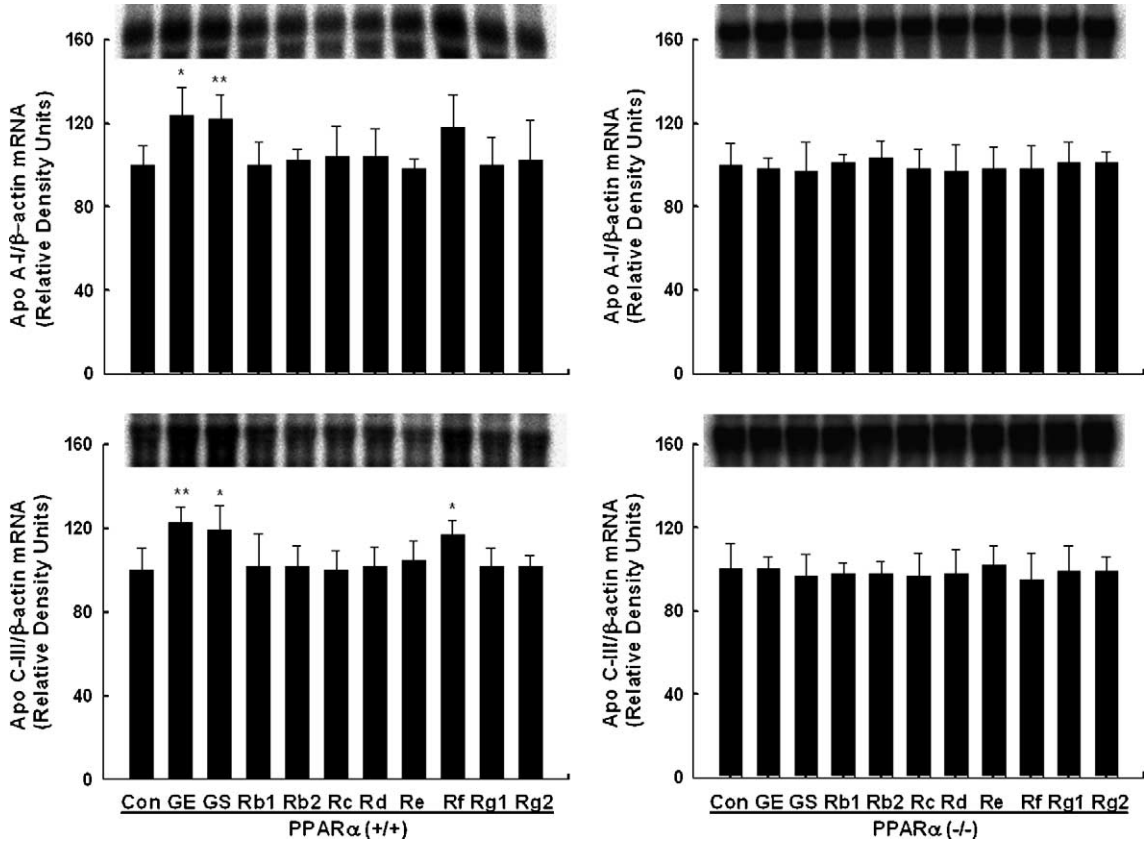


Fig. 2. Regulation of hepatic apo A-I and C-III mRNA levels by ginseng in PPARα (+/+) and (−/−) mice. PPARα (+/+) and (−/−) male C57BL/6 mice were treated with saline (Con), 0.1% Wy14,643 (Wy), GE (5 mg per mouse), GS (0.5 mg per mouse), or each of the eight main ginsenosides (0.05 mg per mouse) as described, and samples were collected and analyzed as described in Materials and methods. All values are expressed as means ± SD of five animals and are expressed taking the apolipoprotein/β-actin mRNA values of control as 100%. * Significantly different versus control, $p < 0.05$. ** Significantly different versus control, $p < 0.01$.

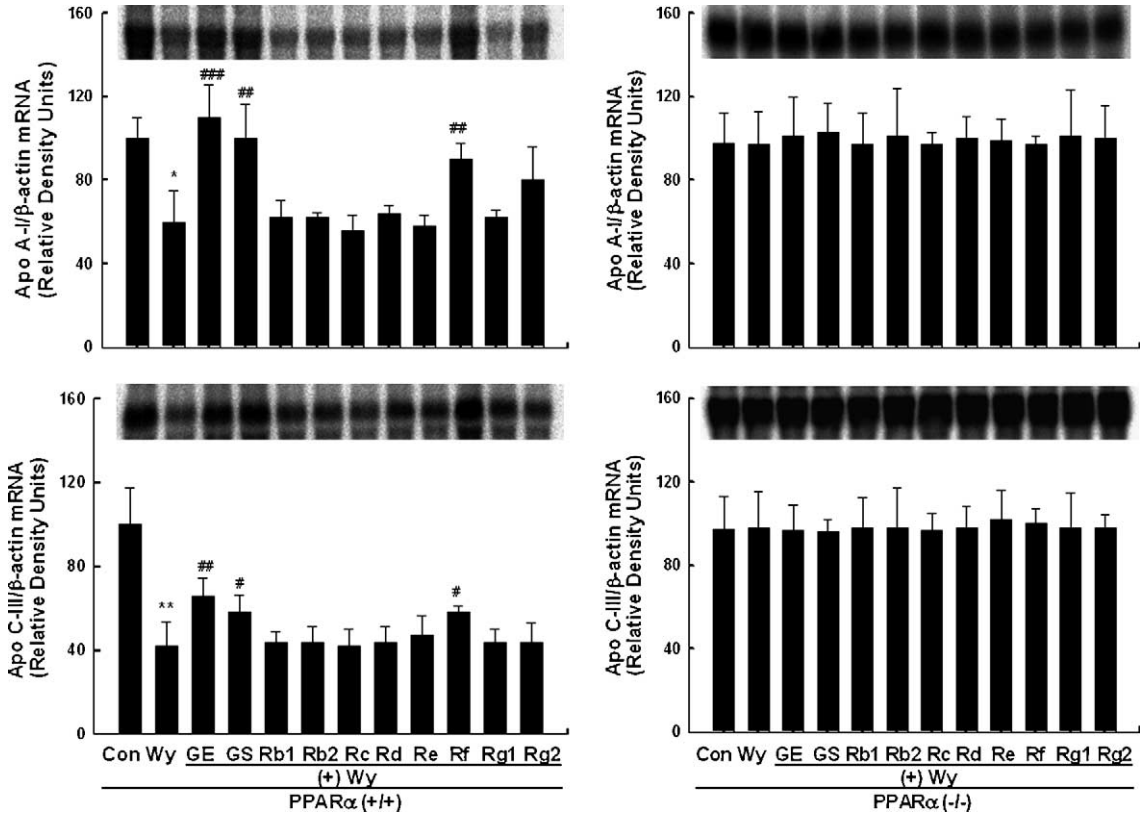


Fig. 3. Induction of Wy-14,643-inhibited apo A-I and C-III gene expression by ginseng in PPARα (+/+) and (−/−) mice. PPARα (+/+) and (−/−) male C57BL/6 mice were treated with either saline (Con) for 10 days or a 0.1% Wy14,643 diet (Wy) for 1 day. For co-treatment studies, mice were treated for 10 days with GE (5 mg per mouse), GS (0.5 mg per mouse), or each of the eight main ginsenosides (0.05 mg per mouse), followed by Wy for 1 day, as described, and samples were collected and analyzed as described in Materials and methods. All values are expressed as means ± SD of five animals and are expressed taking the apolipoprotein/β-actin mRNA values of control as 100%. *Significantly different versus control, $p < 0.01$. **Significantly different versus control, $p < 0.001$. #Significantly different versus Wy diet-only, $p < 0.05$. ###Significantly different versus Wy diet-only, $p < 0.01$. ####Significantly different versus Wy diet-only, $p < 0.001$.

ginsenoside Rf seems to exert ginseng's effects on hepatic apo A-I and C-III mRNA.

Serum apo A-I and C-III levels

We next studied the effects of ginseng on serum apo A-I and C-III levels. Compared with control (+/+) mice, both GS and Rf decreased serum apo A-I and C-III levels in wild-type mice. We also found that treatment with Wy14,643 decreased serum apo A-I and C-III levels in PPARα (+/+) mice and that these effects were almost completely prevented by treatment with GS and Rf (Fig. 4). However, administration of Wy14,643, Wy14,643/GS or Wy14,643/Rf to PPARα (−/−) mice had no effect on serum

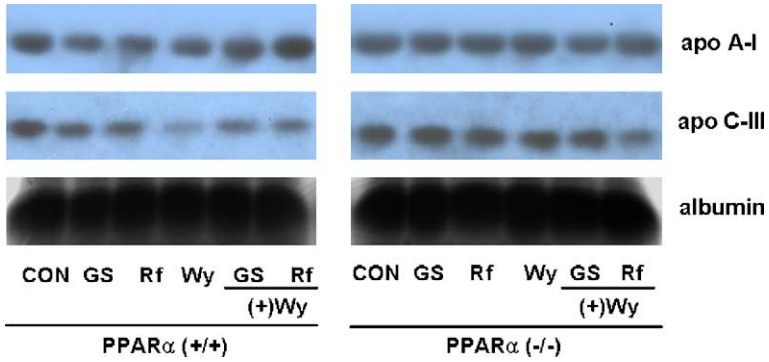


Fig. 4. Changes in circulating apo A-I and C-III levels by ginseng in PPARα (+/+) and (−/−) mice. PPARα (+/+) and PPARα (−/−) male C57BL/6 mice were treated with saline (Con), 0.1% Wy14,643 (Wy), GS (0.5 mg per mouse), or ginsenoside Rf (0.05 mg per mouse). For co-treatment studies, mice were treated for 10 days with either GS (0.5 mg per mouse) followed by Wy for 1 day, or ginsenoside Rf (0.05 mg per mouse) followed by Wy for 1 day, as described, and samples were collected and analyzed as described in Materials and methods. Representative Western blots showing the differences in serum apo A-I and C-III levels in PPARα (+/+) and (−/−) mice.

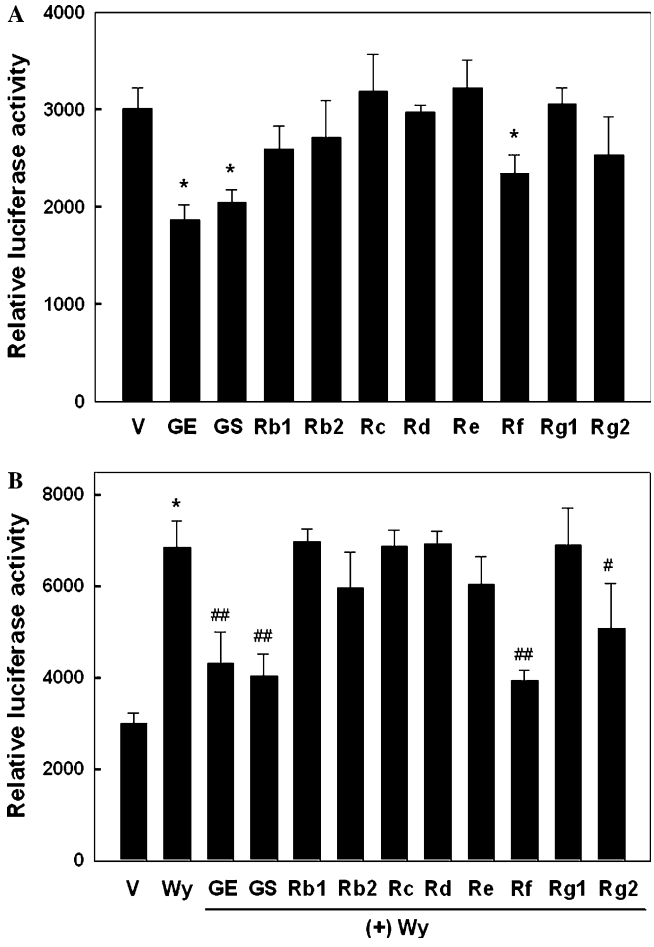


Fig. 5. Inhibition of PPARα reporter gene expression by ginsenosides. NMu2Li cells were transiently transfected with expression plasmids for PPARα, a luciferase reporter gene construct containing three copies of the PPRE from the rat acyl-CoA oxidase gene and β-galactosidase gene. (A) Inhibition of endogenous PPARα reporter gene expression by ginseng. Cells were treated with GE (10 μg/ml), GS (10 μg/ml) or each of the eight ginsenosides (10 μM) at the initial time of culture. Following incubation for 24 h, cells were harvested, lysed, and subsequently assayed for luciferase and β-galactosidase activities. All values are expressed as the mean ± SD of relative luciferase units/β-galactosidase activity. Experiments were performed at least three times. *Significantly different versus vehicle, $p < 0.01$. (B) Inhibition of Wy14,643-induced PPARα reporter gene expression by ginseng. Cells were treated with Wy14,643 (Wy, 10 μM) alone or with GE (10 μg/ml), GS (10 μg/ml) or the eight ginsenosides (10 μM). *Significantly different versus vehicle, $p < 0.01$. #Significantly different versus Wy, $p < 0.05$. ##Significantly different versus Wy, $p < 0.01$.

apo A-I and C-III levels. These results suggest that ginseng regulates serum apo A-I and C-III through PPARα.

PPARα reporter gene expression

Most pharmacological actions of ginseng are attributed to ginsenosides. More than 30 ginsenosides have been isolated and novel structures continue to be reported [1,2]. To identify the ginsenoside that gives its biological activity on PPARα-mediated transactivation, NMu2Li cells were cotransfected with PPARα and RXRα expression constructs as well as a luciferase reporter construct ($PPRE_3$-tk-luc) containing three copies of the PPRE from the rat ACOX gene. Transfected cells were treated with GE, GS, and eight ginsenosides at doses that did not show any cytotoxic effects as measured by trypan blue exclusion. We found that GE, GS and the ginsenoside Rf caused significant decreases in endogenous expression of a luciferase reporter gene (Fig. 5A). Moreover, GE, GS, and the ginsenosides Rf and Rg2 significantly decreased reporter gene activation in the presence of Wy14,643, with Rf having a greater effect than Rg2 (Fig. 5B). Thus, of the eight ginsenosides examined, Rf was most effective for inhibiting PPARα reporter gene expression.

DNA binding of PPARα

To determine whether ginseng interfered with the binding of PPARα to a oligonucleotide containing rat acyl-CoA oxidase PPRE (5′-CTAGCGATATCATGACCTTTGTC CTAGGCCTC-3′), we performed a gel electrophoretic mobility shift assay. Treatment of a nuclear extract containing PPARα with Wy14,643 increased DNA binding (Fig. 6). The binding of PPARα to DNA was decreased, however, by treatment with GE, GS, or Rf. Compared with Wy14,643, the combination of Wy14,643 with GE, GS or Rf decreased the DNA binding to PPARα, and these changes in the DNA binding activity of PPARα were confirmed by a supershift experiment using antibody to PPARα. These findings indicate that the inhibition of PPARα-dependent action by ginseng occurs at the level of DNA binding.

Discussion

Ginseng is a herbal medicine widely used in the Far East to maintain health, and it is also gaining popularity in the West. Many studies have attempted to determine the pharmacological activities of ginseng. Based on the suggestion in oriental medicine that ginseng modulates lipid metabolism, the structural similarities of ginsenosides to steroidal hormones, and the role of PPARα in the expression of genes encoding apolipoproteins, we investigated the role of PPARα in the regulation of lipoprotein metabolism by ginseng, as well as the mechanism of action of ginseng on lipoprotein metabolism at the molecular level. Here, we demonstrate that ginseng regulates lipoprotein metabolism via interactions with PPARα and that a ginsenoside in ginseng, Rf, is an active component of ginseng modulating lipoprotein metabolism.

As reported previously, the potent PPARα ligand Wy14,643 decreased hepatic apo A-I mRNA and serum apo A-I levels in wild-type mice [17,23]. These effects were almost completely prevented by 10 days of treatment with GE or GS prior to 1-day exposure to Wy14,643. GE and GS substantially increased Wy14,643-downregulated serum apo A-I and hepatic apo A-I mRNA levels. None of these effects was observed in PPARα-null mice.

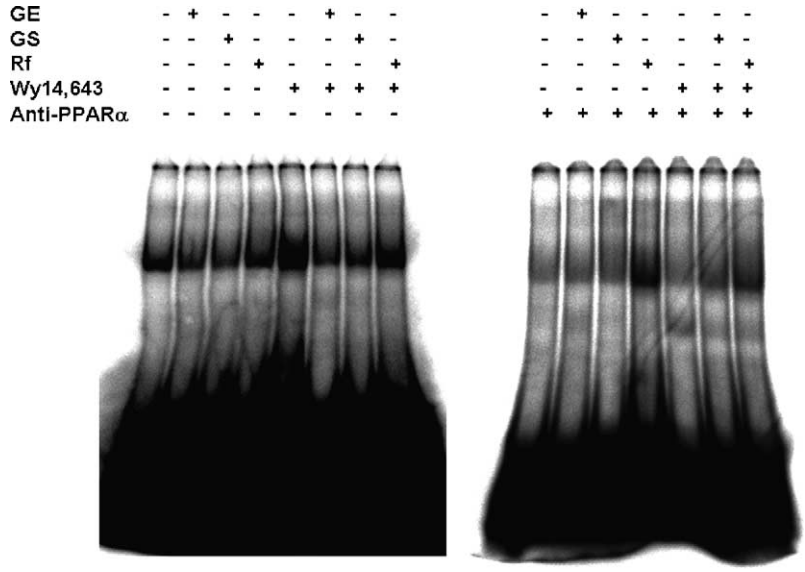


Fig. 6. DNA Binding of PPARα by gel electrophoretic mobility shift assay. Gel electrophoretic mobility shift assays were performed using nuclear extracts from liver tissues of PPARα (+/+) and (−/−) mice. GE, GS, or Rf decreased binding of PPARα to an oligonucleotide containing rat acyl-CoA oxidase PPRE, which was increased by Wy14,643.

In parallel, our previous results showed that serum HDL cholesterol and total cholesterol levels were significantly decreased by Wy14,643 and that both GE and GS reversed these effects of Wy14,643 [6]. In contrast, serum lipid levels in PPARα-null mice were unaffected by ginseng treatment (not shown). These results indicate that not only PPARα may be involved in the regulation of apo A-I gene expression by ginseng, but the changes in hepatic apo A-I mRNA and serum levels are also correlated with serum total cholesterol and HDL cholesterol following ginseng treatment.

Consistent with the report that PPARα has a major role in regulating apo C-III levels accompanying major changes in triglyceride metabolism [24–27], we found that Wy14,643 significantly decreased hepatic apo C-III mRNA and serum apo C-III levels in wild-type animals, and these effects of Wy14,643 were almost completely prevented by GE and GS. These results suggest that ginseng-regulated apo C-III metabolism requires the presence of PPARα, in accordance with our previous data showing increases in circulating triglycerides by ginseng [6]. Together, these observations show that ginseng may act as an antagonist of PPARα and its downstream effects [6].

Ginsenosides are responsible for most of the pharmacological effects of ginseng [2,7–9]. To identify the active compound of ginseng that regulates PPARα-mediated lipoprotein metabolism, we tested the effects of the eight main ginsenosides on the mRNA and serum levels of apo A-I and C-III. Our results showed that in vivo administration of ginsenoside Rf to wild-type mice significantly increased basal levels of apo C-III mRNA and tended to increase apo A-I mRNA levels compared with a chow diet. Rf also substantially elevated the Wy14,643-reduced apo A-I and C-III gene expression in wild-type mice. Similarly, serum levels of apo A-I and C-III were reduced by treatment of wild-type mice with Wy14,643, but these effects were inhibited by Rf. These results suggest that Rf is an active component of ginseng to regulate apo A-I and C-III gene expression.

In accordance with our in vivo data, we found that both GE and GS significantly decreased PPARα reporter gene activation. Of the eight main ginsenosides, Rf was most effective in inhibiting PPARα-dependent reporter gene expression and Rg2 significantly decreased PPARα reporter gene activity caused by Wy14,643, whereas the other ginsenosides had relatively little effects on luciferase activity. Unlike the other ginsenosides, Rf and Rg2 are present in very small amounts in some species of ginseng [1,20], suggesting that these active ingredients present in ginseng in only trace amounts regulate PPARα transactivation.

The molecular mechanism by which ginseng inhibits PPARα transactivation was also examined by EMSA. These experiments showed that ginseng and a particular component of ginseng, Rf, inhibited DNA binding by PPARα. Treatment of a nuclear extract containing the PPARα/RXRα complex with GE, GS or Rf decreased DNA binding and interfered with the DNA binding of PPARα caused by Wy14,643. These results indicate that ginseng may regulate PPARα-dependent lipoprotein metabolism by inhibiting PPARα binding to DNA. These results are supported by the findings showing that ginsenosides Rb1, Rg1, Rh1, and Rh2 bind to nuclear hormone receptors, such as glucocorticoid receptor and estrogen receptor [28–32]. In these reports, ginsenosides competitively inhibited the binding of the ligands to steroid hormone receptors, indicating that they are functional ligands of the nuclear hormone receptors. Subsequently, ginsenoside-occupied receptors regulated the transcription of target genes after binding to specific response elements.

Similar to these findings, our results also suggest that Rf binds to PPARα through competition with PPARα ligands, acting as a negative regulator of PPARα function and thus contributing to its inhibitory effect although, to date, no binding studies have been performed [21,33–35].

In conclusion, our results provide evidence that ginseng regulates lipoprotein metabolism by interacting with PPARα. In addition, these data suggest that Rf is the most potent of major ginsenosides in its interaction with PPARα, and acts as a PPARα antagonist. This ginsenoside may have therapeutic applications since PPARα is an important molecular target for the development of lipid disorder, cardiovascular disease, obesity, and diabetes.

Acknowledgment

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