Glycyrrhizic Acid Attenuates the Expression of HMG-CoA Reductase mRNA in High Fructose Diet Induced Dyslipidemic Hamsters

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Abstract: We investigated the hypolipidemic effect of glycyrrhizic acid (GA) focused on the mRNA expression and hepatic HMG-CoA reductase (HMGR) activity in hamsters fed a high-fat diet. Male Syrian Golden hamsters were fed a high fat diet for 8 weeks for induction of dyslipidemia and were treated with GA and fenofibrate. The concentrations of plasma total cholesterol and triglyceride were significantly lower in the GA-treated group than in the control group. The GA treatment significantly decreased Apo B, Lp(a), and cholesterol-ester-transport protein (CETP) concentrations, but increased Apo A-I levels and the Apo A-I/ Apo B ratio. The contents of cholesterol and triglyceride in hepatic tissue were significantly lower in the GA group than in the control group. Real-time PCR analysis revealed that HMGR mRNA expression was significantly lower in the GA group than in the control group. These results indicate that GA treatment reduces plasma cholesterol by down-regulating hepatic HMGR mRNA expression in hamsters fed a high fructose-fat diet.

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Introduction

Dyslipidemia is characterized by increases in plasma cholesterol and triglycerides, accompanied by a reduced high-density lipoprotein (HDL) concentration, are associated with an elevated risk of coronary artery diseases (Miller, 1996). Many drugs are used to reduce the plasma lipid level (Packard, 1998), but although they exert beneficial effects, these drugs have also been shown to cause serious side effects in various clinical settings (Sgro and Escousse, 1991). Many classes of dietary components and natural compounds have been used to regulate plasma lipid concentrations with the aim of reducing the incidence of hyperlipidemia and atherosclerosis (Superko, 1989). It has been reported that glycyrrhizic acid (glycyrrhizin or glygyrrhizinate), the bioactive compound extracted from roots of licorice plants, has anti-diabetic properties (Baltina, 2003). Glycyrrhizic acid (GA), the primary bioactive constituent of the roots of the shrub *Glycyrrhiza glabra* and its pharmacologically active metabolite glycyrrhetic acid act as potent, non-selective inhibitors of both isoforms of 11β-HSD (Alberts et al., 2002; Wamil and Seckl, 2007). Accordingly, the present study aimed to elucidate the mechanism underlying the hypolipidemic action of GA intake by investigating the effect of GA on lipid metabolism and hepatic expression of HMG-CoA mRNA in hamsters with high fat diet-induced hyperlipidemia.

Material and Methods

Material

GA and fenofibrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Casein, cholesterol, cellulose, vitamin and mineral mixtures were purchased from Dyets (Bethlehem, PA, USA). *DL*-methionine and choline chloride were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade or purer.

Table 1 – Composition of high fructose diet (HFD)

Constituents	Amount (g/kg diet)
Fructose[1]	500.0
Casein[1]	190.0
Dalda[3] Vanaspati Ghee	110.0
Wheat+corn+gram flour	150.0
Cholesterol[1]	5.0
Methionine[2]	3.0
Vitamin mix[4]	3.0
Mineral mixture[5]	40

[1]purchased from SRL (Mumbai, India); [2]purchased from HiMedia (Mumbai, India); [3]commercial preparation composed of different vegetable oils; [4]vitamin mix provided the following nutrients (mg/kg of dry diet): retinol 1.8; cholecalciferol 0.019; thiamine 6; riboflavin 4.5; pantothenic acid 21; pyridoxine 3; inositol 45; cyanocobalamin 0.015; ascorbic acid 240; *DL*-tocopherol 51; menadione 12; nicotinic acid 30; paraminobenzoic acid 15; folic acid 1.5; biotin 0.09; [5]mineral mixture: $CaHPO_4$ 430 g; KCl 100 g; NaCl 100 g; MgO 10.5 g; $MgSO_4$ 50 g; Fe_2O_3 3 g; $FeSO_4 \cdot 7H_2O$ 5 g; trace elements (Mn, Cu, Co, Zn, I) 10 g; quantity sufficient to 1,000 g

Animals and diets

Four-week-old male Syrian Golden hamsters obtained from Lab Animal Division of our institute were initially fed a chow diet for 7 days. After acclimation, the hamsters (weighing 80–90 g) were randomly divided into three groups and fed a high fructose diet (HF, n=10), for 8 weeks. The composition of the high fructose diet is listed in Table 1. After 8 weeks animals were treated with GA (group II) and fenofibrate (group III) at a dose of 100 mg/kg for 4 weeks, group I acted as control. The animals were maintained at a temperature and humidity of 21–25 °C and 50–60%, respectively, and a 12 h:12 h light:dark cycle (lights on from 06:30 to 18:30 h). All animal procedures were conducted in accordance with the Guidelines for Animal Experimentation of the Institute.

Sample preparation

After treatment for 4 weeks, the hamsters were allowed to fast for 16 h and then sacrificed under anaesthesia. Blood from the orbital plexus was collected in heparinised tubes and centrifuged at 1,500*g* for 15 min to separate the plasma. The livers were excised, weighed, and stored at –70 °C until use. Samples of the respected liver were later used for analysis of the lipid contents and real-time PCR analysis.

Lipid analyses

Plasma total cholesterol, HDL-cholesterol, free cholesterol, free fatty acid, and triglyceride levels were measured using commercial enzyme kits (Roche Diagnostics, Germany) on Roche Cobas Integra fully automated clinical analyzer, as were plasma Apo A-I, Apo B, cholesterol-ester-transport protein (CETP), and Lp(a) concentrations. Hepatic lipids were extracted according to the method of Folch et al. (1957). Hepatic total cholesterol and triglyceride levels were measured using commercial enzyme kits (Eiken, Japan).

Hepatic HMG-CoA reductase activity

Hepatic microsomes were prepared according to the method of Hulcher and Oleson (1973) with slight modifications. Two grams of liver tissue were homogenized in 8 ml of icecold buffer (pH 7.0) containing 0.1 mM triethanolamine, 0.02 mM EDTA, and 2 mM dithiothreitol (DTT; pH 7.0). The homogenates were centrifuged for 10 min at 10,000*g* at 4 °C. The supernatants were then ultracentrifuged at 100,000*g* for 60 min at 4 °C. The resulting microsomal pellets were redissolved in 1 ml of homogenization buffer without DTT, the microsomal protein concentrations were determined using the method of Bradford (1976), and were finally analyzed for their HMG-CoA reductase (HMGR) activities. The microsomal HMGR activities were measured with HMG-CoA as the substrate, based on a modification of the method of Shapiro et al. (1974).

Analysis of mRNA expression

The analysis of mRNA expression (for HMGCR, PPAR-α and LPL genes) were performed by using SYBR green based real-time PCR (polymerase chain reaction). The methodologies followed for this purpose are as follows:

RNA extraction. Total RNA was isolated from livers (five animals per group) using Trizol™ (Gibco, Gaithersburg, MD) according to the manufacturer's instructions. The RNA was then treated with RQ1 RNase-Free DNase (Promega, Madison, WI) for 60 min at 37 °C twice following ethanol precipitation to eliminate the contaminating genomic DNA in the RNA samples. cDNA synthesis was performed using the Bioron Reverse Transcriptase kit (Bioron, Ludwigshafen, Germany).

Real-time quantitative PCR analysis of gene expression (qRT-PCR). Reverse transcription-polymerase chain reaction (RT-PCR) reactions were carried out at 4 °C for 3 min, 40 °C for 30 min, 95 °C for 10 min using Bioron Reverse Transcriptase kit (Bioron, Ludwigshafen, Germany).

The expression of HMGCR was determined by qRT-PCR using the primers (Table 2) that are specific for *Mesocricetus auratus*, and normalized to the GDPH gene. The real-time PCR assay was performed with Quantitect SYBR Green PCR kit (Qiagen, USA). In brief, each 20 µl reaction comprises 1X Quantitect SYBR Green PCR kit buffer with pre-optimized Hot start Taq, dNTPs and $MgCl_2$ concentrations, primers (300 nM, each) and 25 ng cDNA. The PCR protocol was followed as per manufacturer's instruction as described: initial denaturation at 95 °C for 10 min and then 30 cycles at 95 °C for 1 min, annealing at 53 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Real-time PCR assays was performed using iCycler real-time PCR system (BIO-RAD, USA) and the standard curve was generated by the iCyclerTM iQ Optical System Software Version 3.0a.

The comparison of expression of HMGCR between control and treated animals were performed using the Comparative Ct (ΔΔCt) Method. Relative quantitation of mRNA expression levels (compounds treated animals/vehicle ratio) was calculated by comparing the target gene/GAPDH of the treated animals to those of the vehicles.

Table 2 – Characteristics of primers used for SYBR green based real-time PCR

Gene	Primer sequence (5'→3')	Primer characteristics			Product size
		length	Tm	GC%	
GAPDH	F: TGGCCTTCCGTGTTCCTACC	20	56	60.0	136
	R: TAGCCCAGGATGCCCTTCAG	20	56	60.0	
HMGCR	F: CAAGGAGCGTGCAAAGACAATC	22	55	50.0	138
	R: GTGAACCATGTGACTTCTGACAAG	24	56	45.8	

Tm – melting temperature

Statistics
All statistical analyses were carried out with ANOVA and Duncan's multiple-range test using SAS (Cary, NC, USA), with a value of $p<0.05$ selected as the cutoff for statistical significance.

Results

Diet consumption, growth, and tissue weight
Treating hamsters fed a high-fat diet for 8 weeks with fenofibrate (FEN group) or glycyrrhizic acid (GA group) led to decreases in the final body weight compared to the HF control group. These reductions in body weight occurred despite the fact that the diet intakes and feed efficiency ratios did not differ significantly between the groups (Table 2). The liver was significantly lighter in the GA treated group than in the HF control group (Table 3), and did not differ between the FEN and HF groups.

Plasma lipids
Table 4 lists the plasma lipid levels of the experimental animals. The total cholesterol level was significantly ($p<0.05$) lower in the FEN and GA groups than in the HF group, and did not differ between the FEN and GA groups. The plasma triglyceride levels were 57% lower in the GA group than in the HF group, and did not differ between the HF and FEN groups. The difference in HDL-cholesterol concentration between the experimental groups was not statically significant. The plasma free-cholesterol concentration was lower in the GA group than in the HF group. The free fatty acid level was not changed in the GA and FEN groups compared with the HF group. The atherogenic index was 24% and 40% lower in the FEN and GA groups, respectively, than in the HF group (Table 3).

Plasma apolipoproteins and CETP
Table 3 also presents the plasma apolipoprotein levels of the experimental animals. The Apo A-I level was higher in the FEN and GA groups than in the HF group. Plasma Apo B and Lp(a) concentrations were both lower in the FEN and GA

Table 3 – Changes in body weight, food intake, feed efficiency ratio and weight of liver

		HF control	FEN	GA
Body weight (g)	Initial	104.4±4.00	103.7±3.95	103.3±3.62
	Final	145.2±1.11	133.2±2.82	129.9±3.59
Food intake (g/day)		6.88±0.15	6.61±0.31	7.19±0.60
Liver weight (g/100 body weight)		6.87±0.12	7.20±0.16	6.03±0.29

Data are mean ± SE values of nine hamsters per group; HF – high-fat diet group; FEN – fenofibrate treated group; GA – glycyrrhizic acid treated group

groups than in the HF group. The CETP level was lower in the GA group than in the HF group, but did not differ between the FEN and HF groups. The Apo A-I/Apo B ratio was higher in the FEN and GA groups than in the HF group.

Liver lipids contents

Table 5 lists the effects of GA on liver total cholesterol, triglyceride, and total lipid levels in the hamsters. The liver total cholesterol level was lower in the GA group than in the HF group, and the liver triglyceride level was 36% lower ($p<0.05$) in the GA group than in the HF group. The liver total lipid contents were 16% and 20% lower in the FEN and GA groups, respectively, than in the HF group, but there was no difference between the GA and FEN groups.

Hepatic HMG-CoA reductase activity and expression of HMGR mRNA

The activities of the hepatic cholesterol biosynthesis-regulating enzyme HMGR after eight weeks of GA feeding on animals are shown in Figure 1. Liver of GA

Table 4 – Plasma lipids and lipoproteins level

Group	HF control	FEN	GA
Dose (mg/kg/day)	–	100	100
Plasma lipid profile			
T-Chol (mg/dl)	266.2±11.8	168.1±18.4***	110.5±6.1***
TG (mg/dl)	545.5±80.9	425.6±93.9	287.1±33.6*
HDL-C (mg/dl)	70.8±8.2	94.4±6.6*	82.7±4.0
LDL-C (mg/dl)	71.5±6.4	47.6±4.2***	55.1±5.0
Free fatty acid (μmol/l)	871.6±34.8	765.0±29.3	644.1±41.9*
Glycerol (μmol/l)	1,076.9±87.7	797.0±152.7	564.1±86.9*
Oxidised LDL (U/l)	82.6±2.97	58.1±2.32***	56.2±3.56***
Plasma apolipoproteins			
Apo A-I (mg/dl)	24.8±1.6	33.8±2.0***	27.9±1.6
Apo B(mg/dl)	39.8±1.9	28.8±1.2***	33.9±2.3
Lp(a) (mg/dl)	23.2±1.2	17.1±0.8***	19.6±0.9
Apo A-I/Apo B	0.64±0.06	1.18±0.08***	0.85±0.09
CETP (pmol/μl/h)	24.67±0.61	22.84±0.54	19.63±0.46
LCAT (470/390 emission intensity)	3.53±0.16	3.85±0.33	4.49±0.23*

Data are mean ± SE values of nine hamsters per group; *p<0.05; ***p<0.001 as assessed using Dunnet's test; HF – high-fat diet group; FEN – fenofibrate treated group; GA – glycyrrhizic acid treated group

Table 5 – Effects on hepatic lipid contents

	HF control	FEN	GA
Total lipid (mg/g liver)	211.5±11.2	177.5±4.4**	168.4±3.3**
Cholesterol (mg/g liver)	85.4±1.9	80.1±2.5	72.3±1.7**
Triglycerides (mg/g liver)	57.9±1.4	44.5±2.4***	37.0±1.4**

Data are mean ± SE values of nine hamsters per group; **p<0.01; ***p<0.001 as assessed using Dunnet's test; HF – high-fat diet group; FEN – fenofibrate treated group; GA – glycyrrhizic acid treated group

treated groups hamsters showed significant reduction (55.7%, $p<0.001$) in HMGCR activity whereas FEN group showed no significant change in hepatic HMGCR activity. The effects of GA treatment on HMGCR mRNA levels in the liver were examined by real-time PCR. The levels of HMGR mRNA were the same in the HF and FEN groups. However, treatment with GA resulted in a significant decrease in liver HMGR mRNA levels (Figure 2), which correlated with reduced enzyme activities.

Discussion

In this study, we investigated the hypolipidemic effect of glycyrrhizic acid (GA) in hamsters with diet-induced hyperlipidemia. Lipoprotein metabolism in hamsters is comparable to that in humans because of similarities in the metabolic components and mechanisms for metabolism of both lipoproteins and bile acids (Guo et al., 2001). The hamster therefore represents an ideal small-animal species for studying lipid and lipoprotein metabolism. A cholesterol-rich diet appears to induce free-radical production followed by hypercholesterolemia, which is a major risk factor for atherosclerosis and is related to occlusive vascular diseases (Wissler, 1992). In the present study we found that the plasma cholesterol concentration was significantly lowered by the treatment with GA. Concentrations of plasma triglycerides, free cholesterol and total cholesterol, and the atherogenic index were lower in the GA group than in the HF group, and both GA and fenofibrate increased the ratio of Apo A-I to Apo B. Stein et al. (1990) reported that a diet supplemented with 2% cholesterol resulted in a rapid development of

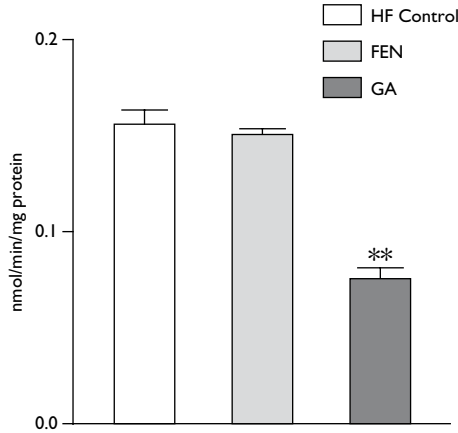

*Figure 1 – Activity of hepatic HMG-CoA reductase. (**p<0.01 as compared to HF control)*

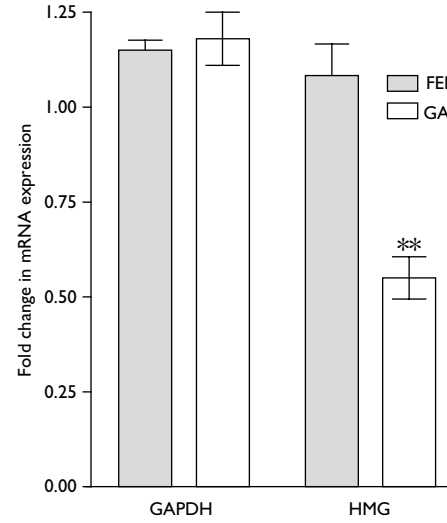

*Figure 2 – Fold change in hepatic HMG-CoA reductase mRNA expression. (**p<0.01 as compared to GAPDH)*

hypercholesterolemia and increased CETP activity in hamsters. A reduction in CETP activity was observed after GA treatment in this study, suggesting that GA indirectly affected the intravascular processing of lipoproteins by reducing the transfer of cholesteryl ester from HDL to VLDL. Although there is controversy as to whether the role of CETP is pro- or anti-atherogenic, studies in humans suggest that if CETP deficiency is associated with lower HDL-cholesterol concentrations, then the role of CETP appears to be proatherogenic (Sonia et al., 2001). In the present study, GA lowered plasma CETP activity with causing increase in HDL-cholesterol concentrations. Thus, a beneficial effect of GA in reducing proatherogenic lipoproteins can be postulated. However, the hypolipidemic action of GA is complex and warrants further investigation in a clinical setting.

A high-fat and cholesterol-containing diet induces hepatic cholesterol and triglyceride accumulation and an overweight liver (Seifalian et al., 1999). However, in this study GA treatment decreased the liver weight and lowered hepatic cholesterol and triglyceride contents, GA and fenofibrate treatment also caused decrease in plasma triglyceride concentration.

HMG-CoA reductase is an enzyme that has received extensive study. It catalyzes the reduction of HMG-CoA to CoA and mevalonate, which is the rate-limiting reaction in the *de novo* synthesis of cholesterol. HMG-CoA reductase is the target of statins, a class of drugs that are highly effective in controlling hypercholesterolemia; statins inhibit cholesterol biosynthesis by blocking the substrate's access to the active site of the enzyme (Istvan, 2002). The present study found that the activity of hepatic HMGR was lower in the GA group than in the HF group. Endo (1992) reported that, similar to atorvastatin, red wine polyphenolics inhibited HMGR. Moreover, we found that treatment with GA decreased HMGR mRNA expression. This indicates that GA inhibits cholesterol synthesis by altering hepatic HMGR. The present results also suggest that GA is very effective as a lipid-lowering agent in hamsters with diet induced hyperlipidemia. These effects of GA may be mediated via decreased HMGR activity and down-regulation of HMGR mRNA expression.

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