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Cholesterol-Lowering Activity of Soy-Derived Glyceollins in the Golden Syrian Hamster Model

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ABSTRACT: Hypercholesterolemia is one of the major factors contributing to the risk of cardiovascular disease (CVD), which is the leading cause of death in developed countries. Consumption of soy foods has been recognized to lower the risk of CVD, and phytochemicals in soy are believed to contribute to the health benefits. Glyceollin is one of the candidate phytochemicals synthesized in stressed soy that may account for many unique biological activities. In this study, the in vivo cholesterol-lowering effect of glyceollins was investigated. Male golden Syrian hamsters were fed diets including (1) 36 kcal% fat diet, (2) 36 kcal% fat diet containing 250 mg/kg diet glyceollins, or (3) chow for 28 days. Hepatic cholesterol esters and free cholesterol, hepatic total lipid content, plasma lipoproteins, fecal bile acid, fecal total cholesterol, and cholesterol metabolism related gene expressions were measured. Glyceollin supplementation led to significant reduction of plasma VLDL, hepatic cholesterol esters, and total lipid content. Consistent with changes in circulating cholesterol, glyceollin supplementation also altered expression of the genes related to cholesterol metabolism in the liver. In contrast, no change in plasma LDL and HDL, fecal bile acid, or cholesterol content was observed. The cholesterol-lowering effect of glyceollins appeared not to go through the increase of bile excretion. These results supported glyceollins' role as novel soy-derived cholesterol-lowering phytochemicals that may contribute to soy's health effects.

KEYWORDS: *cholesterol, glyceollins, hamster*

INTRODUCTION

Hypercholesterolemia is one of the major factors contributing to the onset and progression of cardiovascular diseases (CVD), which are the leading cause of death in the adult population of industrialized societies.[1] It is estimated that, by 2020, CVD will continue to be the leading cause and account for 37% of all deaths.[2] Therefore, it is imperative to develop preventive strategies against this disease. Besides genetic predisposition, increased consumption of high-fat and high-cholesterol diets adds to the risk of CVD.[3,4] Mechanistically, previous studies have revealed that diet may modulate oxidative status and chronic inflammation, which may play pivotal roles in atherosclerosis.[5,6] Dietary intervention would be an economical and efficient preventive measure.

Soy is one of the primary agricultural commodities in the United States.[7] Soy and soy-derived foods are well-known for their health effects and are a rich source of health-promoting bioactive phytochemicals.[8] Previous research indicated that soy possesses cholesterol-lowering effects,[9,10] but the precise mechanism and the active components remain unclear.[11,12] Glyceollins (Figure 1), which are synthesized in response to environmental stresses, such as infection, are a family of phytoalexins isolated from soy and possess antibiotic activity.[13] Soy phytoalexins also include bioactive isoflavones such as genistein and daidzein (Figure 1).[14] Glyceollins have been reported to exhibit antitumor,[15−17] antiestrogenic,[18] antibacterial,[19] antinematode,[20,21] antifungal,[22,23] antidiabetic,[24−26] and vasodilatory effects in rat.[27] Glyceollins were also reported to possess antioxidant and anti-inflammatory effects.[28−30] The antioxidant and anti-inflammatory properties of glyceollins suggest the potential of glyceollins to prevent, delay, or treat cardiovascular conditions. Hence, accumulating evidence suggests that glyceollins have the potential to be health-promoting phytochemicals and deserve further investigation and characterization. Additionally, the contribution of glyceollins to the cholesterol- and lipid-lowering effects of soy has not been reported.

The golden Syrian hamster (*Mesocricetus auratus*) is widely accepted as a suitable animal model for studying human cholesterol metabolism.[31,32] The lipid profiles and susceptibility to dietary cholesterol of golden Syrian hamster are similar to those of humans.[32−34] The current study takes advantage of this

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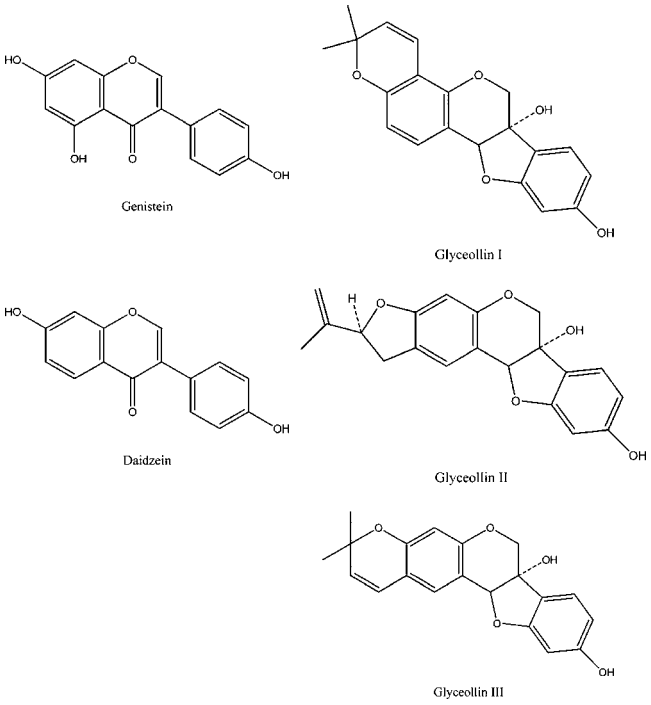

Figure 1. Structures of glyceollins (I, II, and III) and soy phytochemicals genistein and daidzein.

model to test the hypothesis that glyceollins may exert cholesterol- and lipid-lowering effects. Hamsters were fed a high-fat diet or a glyceollin-supplemented high-fat diet. Plasma lipid profiles, liver, fecal lipid contents, expression level of cholesterol and lipid metabolism related genes in the liver were determined to elucidate the cholesterol-lowering activity of glyceollins and the potential mechanisms involved.

MATERIALS AND METHODS

Animals and Diets. Male golden Syrian hamsters (approximately 80 g, LVG strain, Charles River, Wilmington, MA, USA) were given free access to water and rodent chow to acclimatize to the environment for 1 week prior to the experiment. For the experiment, hamsters were fed a high-fat diet (36 kcal% fat diet), a high-fat diet with glyceollin supplement (36 kcal% fat diet containing 250 mg/kg diet glyceollins.), or chow (8728C Teklad Certified Rodent Diet, Harlan Laboratories, Inc., Frederick, MD, USA). The experimental diets were formulated and purchased from Research Diets, Inc., New Brunswick, NJ, USA. Animals (10 per group) were fed the respective diets for 4 weeks with water available ad libitum. Food intake was recorded twice a week, and body weights were measured weekly. Diets consisted of 18% protein, 45% carbohydrate, and 36% fat on a caloric basis supplemented with 0.14% cholesterol. Diet compositions are listed in Table 1. Glyceollins were given as a mixture of 68% glyceollin I, 21% glyceollin II, and 11% glyceollin III. Glyceollin mixture was isolated and purified from elicited soy cotyledon as described previously.[35,36] The animal use and care protocol (Protocol 10-014) for this study was reviewed and approved by the USDA, ARS, Beltsville Area Animal Care and Use Committee (BAACUC).

Plasma and Tissue Collection. Hamsters were subjected to 12 h of fasting prior to being anesthetized with CO_2 and sacrificed. Blood was collected by cardiac puncture with syringes previously rinsed with potassium EDTA solution (15% w/v), and plasma was separated after centrifugation at 1500 rpm for 30 min at 4 °C. Livers and retroperitoneal adipose tissues were collected, and one part of the tissue was immediately frozen in liquid nitrogen for analysis; the other part was preserved in RNA Stabilization Solution purchased from Ambion (Austin, TX, USA) and kept at −80 °C.

Plasma Lipoprotein Analysis. Plasma lipoprotein cholesterol concentrations were determined by size exclusion chromatography as previously described.[3] Briefly, an Agilent 1100 chromatograph was employed with a postcolumn derivatization reactor, consisting of a mixing coil (1615-50 Bodman, Aston, PA, USA) in a temperature-controlled water jacket (Aura Industries, New York, NY, USA). A Hewlett-Packard (Agilent, Palo Alto, CA, USA) HPLC pump 79851-A was used to deliver cholesterol reagent (Roche Diagnostics, Indianapolis, IN, USA)

Table 1. Diet Content

	high fat[a]		high fat + glyceollins[a]		chow[b]	
	g%	kcal%	g%	kcal%	g%	kcal%
protein	21.0	18.0	21.0	18.0	24.3	32.0
carbohydrate	52.0	45.0	52.0	45.0	40.2	54.0
fat	19.0	36.0	19.0	36.0	4.7	14.0
others					23.8	
total		100.0		100.0		100.0
kcal/g	4.58		4.58		3.00	
ingredient	g	kcal	g	kcal	g	kcal
casein	222.0	888.0	222.0	888.0		
DL-methionine	3.0	12.0	3.0	12.0		
corn starch	453.0	1812.0	453.0	1812.0		
maltodextrin	100.0	400.0	100.0	400.0		
sucrose						
cellulose	53.0	0.0	53.0	0.0		
corn oil	100.0	900.0	100.0	900.0		
butter	80.0	720.0	80.0	720.0		
menhaden oil	20.0	180.0	20.0	180.0		
mineral mix	45.0	40.0	45.0	40.0		
choline bitartrate	3.0	0.0	3.0	0.0		
cholesterol	1.5	0.0	1.5	0.0	0.05	0.0
glyceollins	0.0	0.0	0.27	0.0	0.0	0.0
total	1080.5	4952.0	1080.5	4952.0	1000.0	3000.0

[a]High-fat and high-fat + glyceollins diets were formulated and purchased from Research Diet (New Brunswick, NJ, USA). [b]Chow diet was purchased from Harlan Laboratories (Frederick, MD, USA).

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at a flow rate of 0.2 mL/min. Bovine cholesterol lipoprotein standards (Sigma-Aldrich, St. Louis, MO, USA) were used to calibrate the signal on the basis of peak areas. Fifteen microliters of plasma was injected via an Agilent 1100 autosampler onto a Superose 6HR HPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). The lipoproteins were eluted with a pH 7.0 buffer solution containing 0.15 M sodium chloride and 0.02% sodium azide at a flow rate of 0.5 mL/min. Plasma lipoprotein concentration was calculated on the basis of the standard curve.

Hepatic Lipid Extraction. Livers were excised and immediately frozen in liquid nitrogen and then stored at −80 °C prior to analysis. The extraction method was modified from the Folch method.[37,38] Approximately 0.15 g of frozen liver was minced and transferred into a test tube. Six milliliters of chloroform/methanol (2:1, v/v) was then added, followed by a 2 min homogenization and 30 s of sonication at 30% power level on ice. Samples were then incubated with shaking for 2 h on a platform shaker at room temperature. After incubation, 2 mL of double-distilled water was added. Samples were then centrifuged for 20 min at 500*g* at room temperature. After centrifugation, the bottom layer was carefully aspirated into a new test tube and incubated overnight. It was then filtered through a 0.22 μm filter and dried by a stream of nitrogen. The dried lipid was weighed and redissolved in isopropanol with 10% Triton X-100 and used for triglyceride and cholesterol analysis as described below.

Triglyceride, Total Cholesterol, and Free Cholesterol in Liver. Hepatic triglyceride, total cholesterol, and free cholesterol were enzymatically determined using commercial kits (Triglyceride-SL, Genzyme Diagnostics PEI Inc., PE, Canada; Cholesterol E and Free Cholesterol E, Wako Chemicals, Richmond, VA, USA) following the manufacturers' protocols.

Fecal Bile Acids and Cholesterol Extraction. Fecal bile acids and cholesterol were extracted using a modified protocol.[39] Fecal samples were collected during 48 h on days 26−28 after initiation of experiment. The samples were lyophilized, pulverized using a pestle and mortar, and weighed. A dried fecal sample (0.10 g) was hydrolyzed in 1.0 mL of 2 M KOH at 50 °C for 5 h. The cooled mixture was then extracted with two 6 mL portions of diethyl ether to remove nonsaponifiable components. Subsequently, 1 mL of 20% sodium chloride followed by 0.2 mL of 12 M hydrochloric acid was added to the remaining mixture. The acidified mixture was extracted with two 6 mL portions of diethyl ether, and the pooled ether extracts were evaporated by nitrogen and redissolved in 0.5 mL of pure ethanol. The samples were used for fecal bile acid and cholesterol determination as follows.

Fecal Bile Acids and Cholesterol Analysis. Fecal bile acid content was enzymatically determined by 3α-hydroxysteroid dehydrogenase (3α-HSD).[40] β-Nicotinamide adenine dinucleotide hydrate (NAD), nitroblue tetrazolium chloride (NBT), diaphorase, 3α-HSD, and cholic acid were obtained from Sigma-Aldrich. NAD, NBT, diaphorase, and 3α-HSD were prepared in 0.01 M phosphate buffer at pH 7.0. The reaction mixture included 40 μL of sample or standard with 4 μL of Triton X-100, 50 μL of NAD (2.5 mM), 50 μL of NBT (0.61 mM), 50 μL of diaphorase (625 U/L), and 50 μL of 3α-HSD (625 U/L). The mixture was incubated for 60 min at ambient temperature, after which 40 μL of phosphoric acid (1.33 M) was added to stop the reaction. The absorbance of each reaction mixture was measured at 530 nm. Cholic acid in ethanol was used to generate a standard curve, and the amount of fecal bile acid obtained was determined using the standard curve. Cholesterol was determined by the same assay as for liver described above.

Total RNA Isolation, cDNA Synthesis, and Gene Expression Analysis. To determine the gene expression changes, liver and adipose preserved in RNALater were cut into 0.1−0.2 g pieces and homogenized using a Precellys 24 (Bertin Technologies, Villeurbanne, France). An RNeasy Mini Kit and an RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) were used for total RNA isolation for liver and adipose, respectively. A StrataScript First Strand complementary DNA synthesis kit from Stratagene (Santa Clara, CA, USA) was used to reverse transcribe complementary DNA. Real-time PCR was performed on an Applied Biosystems 7900HT Sequence detection system using Fast SYBR Green Master Mix by Applied Biosystems (Carlsbad, CA, USA).

Table 2. Sequences of Real-Time PCR Primers

gene	direction	sequence (5′−3′)
		SYBR Green Primers
GAPDH	forward	GAACATCATCCCTGCATCCA
	reverse	CCAGTGAGCTTCCCGTTCA
HMGCoAR	forward	CGAAGGGTTTGCAGTGATAAAGGA
	reverse	GCCATAGTCACATGAAGCTTCTGTA
LDLR	forward	TGAGGAACATCAACAGCATAAAC
	reverse	ATCCTCCAGGCTGACCATCTGT
LXRα	forward	ATTGCCATCAGCATCTTCTCT
	reverse	GCATCCGTGGGAACATCAGT
PPARα	forward	CTCCACCTGCAGAGCAACCA
	reverse	CGTCAGACTCGGTCTTCTTGAT
ABCG5	forward	TGATTGGCAGCTATAATTTTGGG
	reverse	GTTGGGCTGCGATGGAAA
ABCG8	forward	TGCTGGCCATCATAGGGAG
	reverse	TCCTGATTTCATCTTGCCACC
CYP7A1	forward	GGTAGTGTGCTGTTGTATATGGGTTA
	reverse	ACAGCCCAGGTATGGAATCAAC
CYP51	forward	GAGAGAAGTTTGCCTATGTGCC
	reverse	TGTAACGGATTACTGGGTTTTCT
SREBP	forward	GCGGACGCAGTCTGGG
	reverse	ATGAGCTGGAGCATGTCTTCAAA
FA synthase	forward	AGCCCCTCAAGTGCACAGTG
	reverse	TGCCAATGTGTTTTCCCTGA
ACOX	forward	TTACATGCCTTTGTTGTCCCTATC
	reverse	CGGTAATTGTCCATCTTCAGGTA
		TaqMan Primers
GAPDH	forward	GAACATCATCCCTGCATCCA
	reverse	CCAGTGAGCTTCCCGTTCA
	probe	CTTGCCCACAGCCTTGGCAGC
LPL	forward	TTTAACTACCCCCTGGACAATGTC
	reverse	ACCTTCTTGTTGGTCAGACTTCCT
	probe	AGCCTTGGAGCCCACGCTGCT

Primers used in this study are listed in Table 2. Relative mRNA expression levels were calculated using the ΔCt method.[41] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as the housekeeping gene for calculations.

Statistical Analysis. All end-point assays for each sample were conducted in triplicate, and the average was used for group analysis; data for each treatment group were presented as the mean ± standard error. Significance level of differences in means was detected using one-way ANOVA and Tukey's test. Statistics were analyzed using IBM SPSS Statistics 19.0 (2010, IBM Corp., Armonk, NY, USA) or Graphpad Prism 4 (2003, Graphpad Software, La Jolla, CA, USA). Statistical significance was defined at $p \leq 0.05$.

RESULTS

Body Weight and Food Intake. There was no difference in body weight or body weight gain between any of the diet

Table 3. Effect of Different Diets on Body Weight and Food Intake[a]

	high fat	glyceollin	chow
body wt (g)	95.83 ± 5.30a	95.91 ± 4.04a	96.87 ± 3.94a
body wt gain (g)	17.48 ± 4.98a	18.95 ± 6.54a	20.83 ± 3.97a
food intake (g/day)	5.82 ± 0.43a	5.89 ± 0.60a	7.59 ± 0.31b
calorie intake (kcal/day)	26.67 ± 1.97b	26.97 ± 2.76b	22.76 ± 0.93a

[a]Body weight and food intake are presented as the mean ± SEM (n = 10). Significance level of differences in means was detected using one-way ANOVA and Tukey's test. Values marked with different letters are significantly different from each other at $p \leq 0.05$.

treatments during the experimental period (Table 3). Food intake was significantly higher in the group fed chow diet, but caloric intake was higher in the high-fat diet and high-fat diet supplemented with glyceollin groups due to their higher fat content (Table 3).

Plasma Lipoprotein Cholesterol Content. Compared to the chow diet, consumption of the high-fat diet significantly elevated very low-density lipoprotein (VLDL) (366%), low-density lipoprotein (LDL) (482%), and total lipoprotein levels (51%) in hamster plasma (Figure 2). Animals on the high-fat diet supplemented with glyceollins showed significantly lower (30% less) VLDL than animals on the high-fat diet. We also found a trend in reduction of LDL by 19% and total lipoprotein level by 9% in the animals fed glyceollins; however, reductions in LDL and total lipoprotein level did not reach statistical significance (Figure 2). There was no difference in high-density lipoprotein (HDL) levels between the animals on different diets.

Cholesterol and Lipid Content of Liver. In animals fed the high-fat diet, hepatic cholesterol esters and free cholesterol increased 759 and 27%, respectively, compared to the animals on the chow diet and cumulatively resulted in a 266% increase in total cholesterol (Figure 3). Glyceollin supplementation in the high-fat diet reduced hepatic cholesterol esters and free cholesterol by 20 and 14% (Figure 3B,C). Overall, the total lipid content in liver of high-fat diet animals was 82% higher than that of chow diet animals (Figure 3E). Total lipid in the livers of glyceollin-supplemented animals was 18% lower than in high-fat diet animals. There was no difference in hepatic triglyceride level between the animals from different diet groups (Figure 3D). Consistent with the lower lipid level in glyceollin-treated animals, we also observed that the livers of the glyceollin-fed animals appeared dark red, smooth, and more similar to that of chow-fed animals,

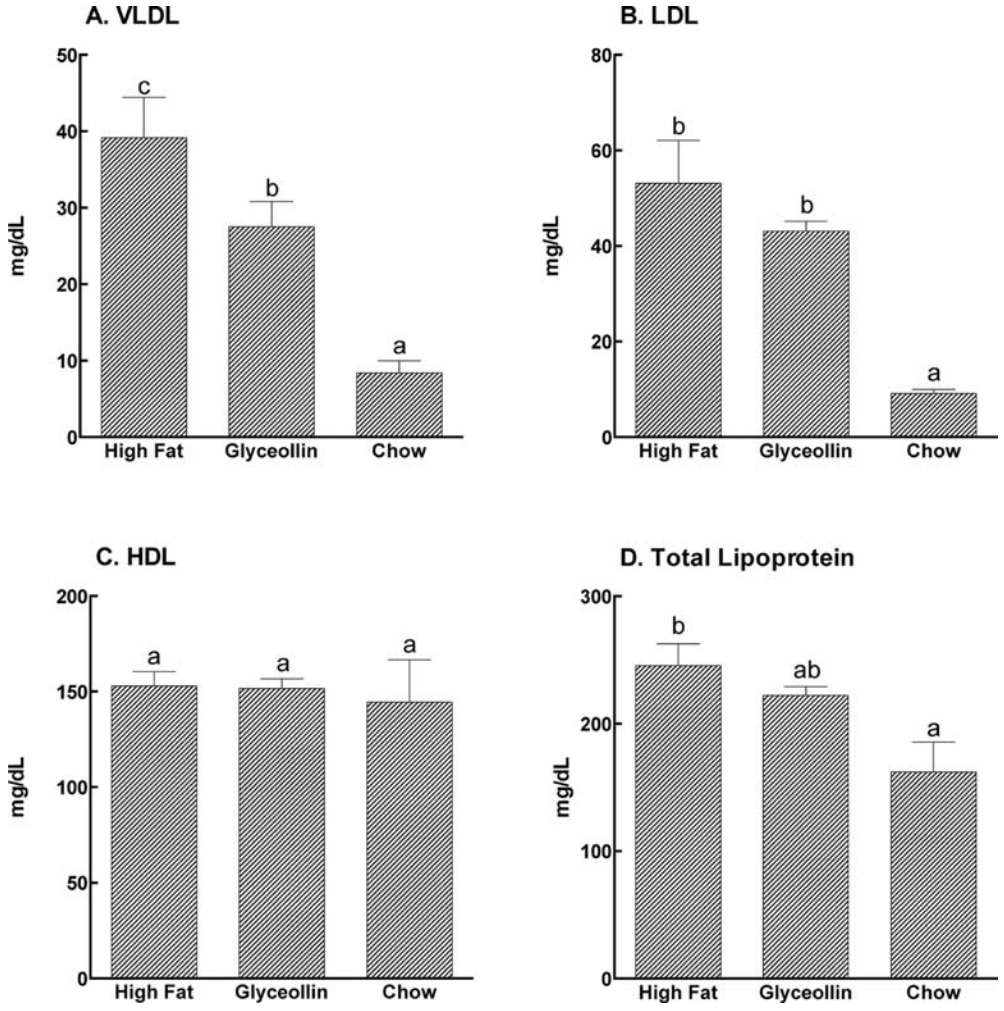

Figure 2. Effects of glyceollin supplement on plasma lipoprotein cholesterol levels: (A) very low-density lipoprotein (VLDL): (B) low-density lipoprotein (LDL); (C) high-density lipoprotein (HDL); (D) total lipoprotein. Hamster plasma from different diet groups was harvested, and plasma lipoprotein cholesterol concentrations were determined by size exclusion chromatography as described under Materials and Methods. Each column represents the mean ± SEM (n = 10). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

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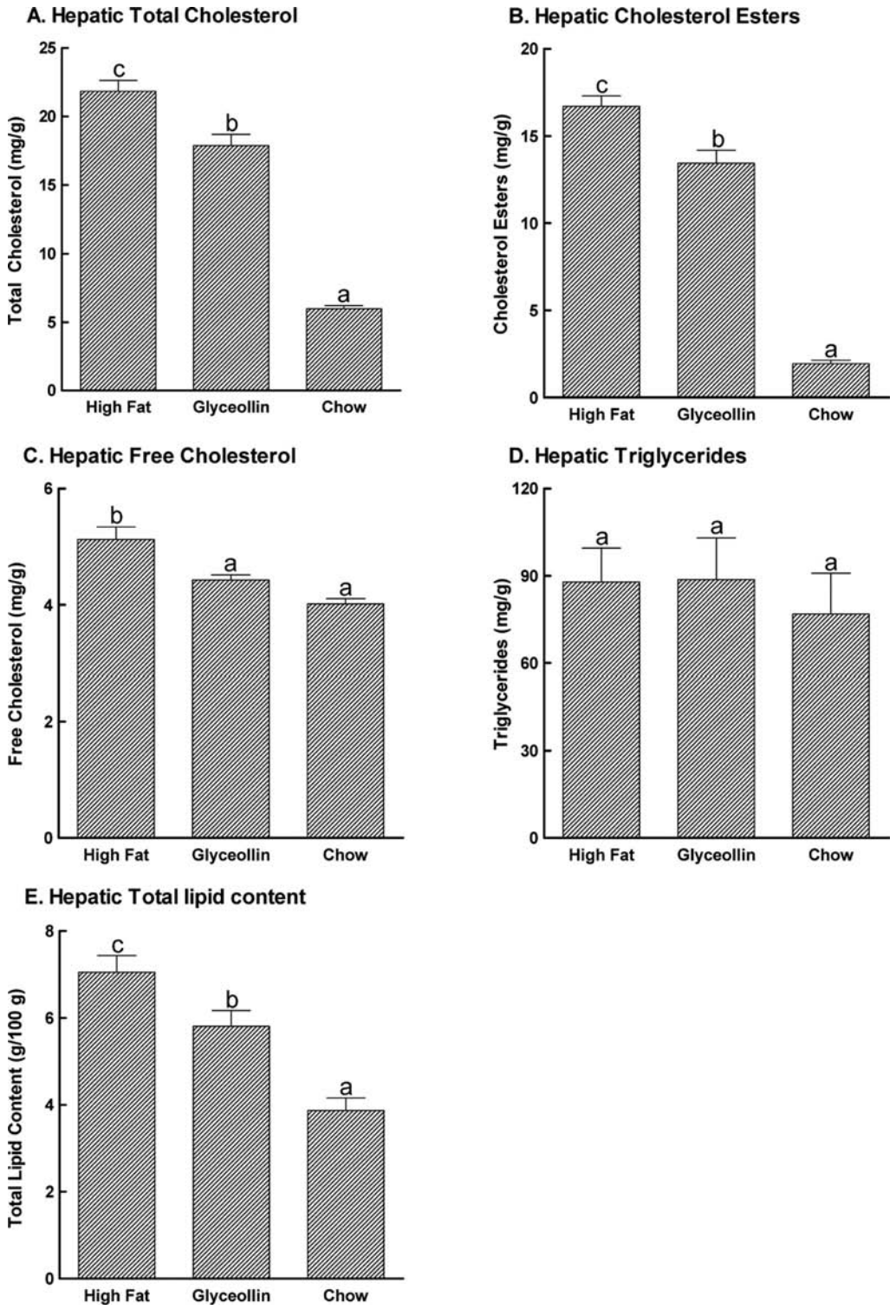

Figure 3. Effects of glyceollins on hepatic triglyceride and cholesterol levels: (A) hepatic total cholesterol; (B) hepatic cholesterol esters; (C) hepatic free cholesterol; (D) hepatic triglycerides; (E) hepatic total lipid content. Livers were harvested from animals on different diets, and hepatic lipid was extracted and enzymatically determined as described under Materials and Methods. Each column represents the mean ± SEM (n = 10). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

whereas livers in high-fat diet animals were pale and spotted (data not shown).

Bile Acid and Cholesterol Content of Feces. There was no significant difference between the diet groups for fecal bile acid content (Figure 4). Compared to animals on the chow diet, higher fecal total cholesterol (170%) levels were detected in high-fat diet fed animals, but there was no difference between animals fed with or without glyceollins.

Relative Expression of Genes Related to Cholesterol and Bile Acid Metabolism. Expression of hepatic genes related to cholesterol, bile acid, and fatty acid metabolism was determined to elucidate potential mechanisms of action.

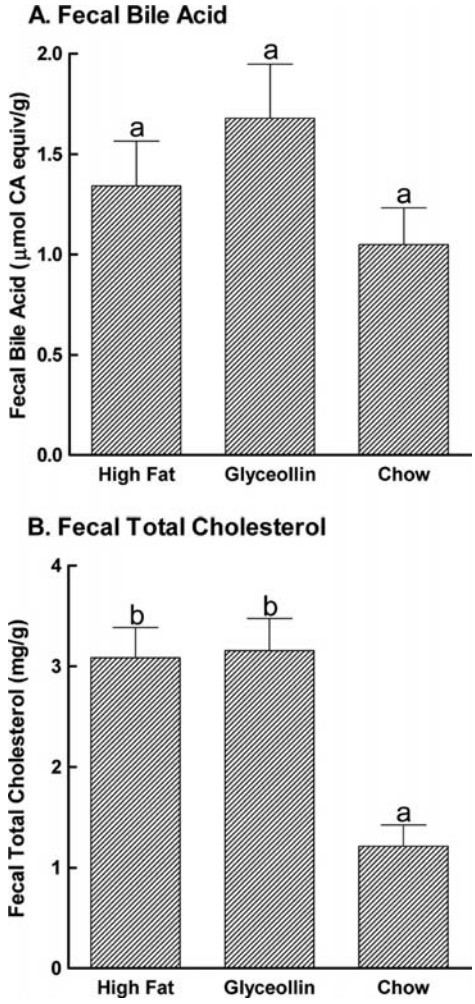

Figure 4. Effects of glyceollins on fecal bile acid and cholesterol levels: (A) fecal bile acid; (B) fecal total cholesterol. Two-day fecal samples were collected for animals on different diets, and fecal bile acid and total cholesterol were extracted and determined as described under Materials and Methods. CA equiv stands for cholic acid equivalents. Each column represents the mean ± SEM (n = 10). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

There was no difference in the expression of the mRNA levels of LDL receptor, which binds to LDL particles in circulation, between animals fed different diets (Figure 5A). Compared to the chow diet, the high-fat diet significantly decreased the hepatic mRNA level of the HMG-CoA reductase (32%) (Figure 5B), which is the rate-limiting enzyme of the mevalonate pathway involved in cholesterol synthesis.[42] Animals fed the glyceollin-supplemented diet showed a similar decrease in hepatic HMG-CoA reductase as animals fed the high-fat diet. Another rate-limiting enzyme in cholesterol synthesis, CYP51, which converts lanosterol to cholesterol,[43] was down-regulated ~80% in high-fat diets with or without glyceollins supplemented as compared to the chow diet (Figure 5C). Liver X receptor (LXR) α and peroxisome proliferator activated receptor (PPAR) α, transcription factors regulating cholesterol synthesis and metabolism,[44,45] did not exhibit difference between the diets (Figure 5D,E).

ABCG5 and ABCG8 function as half-transporters to limit intestinal absorption and promote biliary excretion of sterols.[46] The high-fat diet significantly elevated both ABCG5 and ABCG8 mRNA expression by 309 and 128%, respectively (Figure 6A,B). Animals fed glyceollin-supplemented diet expressed significantly lower levels of ABCG5 (29%) and ABCG8 (25%) mRNA as compared to the high-fat diet (Figure 6A,B). CYP7A1 encoding the enzyme for the initial rate-limiting step of bile acid synthesis[47] was increased by 63% in the glyceollin-supplemented diet, although it did not reach statistical significance (Figure 6C), whereas the high-fat diet and chow diet exhibited similar expression levels of CYP7A1.

The mRNA levels of fatty acid synthase (FAS) and acyl-CoA oxidase (ACOX), genes encoding the rate-limiting enzymes in fatty acid synthesis and peroxisomal β-oxidation,[48,49] were not affected by the different diets (Figure 7A,B). Sterol regulatory element-binding protein (SREBP)-1c is a transcription factor that binds to the sterol regulatory element DNA sequence upstream of genes for fatty acid biosynthesis. Animals on the high-fat diet, with or without glyceollin, had higher hepatic SREBP-1c expression (~170%) than animals on the chow diet (Figure 7C).

The LPL gene encodes lipoprotein lipase, which has the dual functions of triglyceride hydrolase and ligand bridging factor for receptor-mediated lipoprotein uptake.[50] In this study, the LPL expression level in the liver was elevated in the high-fat diet by 158%, whereas glyceollin supplementation reduced liver LPL by 27% (Figure 7D). On the other hand, adipose LPL mRNA levels were significantly lower (35%) in animals that consumed glyceollins as compared to animals on the high-fat or chow diets (Figure 7E).

DISCUSSION

Glyceollins are a family of major phytoalexins and phytoestrogens in stressed soy[51,52] (Figure 1). We have previously shown that a soy protein diet enriched with isoflavones and glyceollins reduced cholesterol in postmenopausal female Cynomolgus monkeys, with isoflavone- and glyceollin-supplemented diet showing greater change in cholesterol-metabolizing genes and lower serum cholesterol than in diet supplemented with isoflavone alone,[53] which suggested that glyceollin might be responsible for the effects in cholesterol reduction. This study assessed the short-term (4 weeks) effect of glyceollin supplementation on lowering cholesterol levels in animals fed a high-fat Western style diet. As shown in Figure 2A, glyceollins significantly reduced the plasma concentration of VLDL by 30% in hamsters fed a high-fat diet. VLDL is responsible for transporting endogenous triglycerides, phospholipids, cholesterol, and cholesterol esters. Elevated VLDL is a critical factor in the onset and progression of cardiovascular conditions.[54,55] The higher circulating level of VLDL in animals consuming a high-fat diet is reflective of increased lipid and cholesterol intake. Our results suggested glyceollin supplementation may be useful in the prevention of potential detrimental effects of high fat intake through reduction high fat diet induced increase of VLDL. The plasma concentrations of LDL and total lipoprotein were also slightly lowered by 19 and 9% upon glyceollin supplementation (Figure 2B,D), whereas the HDL level was unaffected (Figure 2C).

The consumption of the high-fat diet induced an increase of hepatic cholesterol esters and free cholesterol compared to the chow diet (Figure 3). However, glyceollin supplementation significantly reduced hepatic cholesterol esters, free cholesterol, and total lipid content. These effects of glyceollins suggest less cholesterol resided in the liver and less is available for packaging into VLDL, therefore leading to lower circulating VLDL level. Additionally, livers from glyceollin-fed animals were dark red and smooth, similar to those of chow-fed animals. The high-fat diet animals' livers, on the other hand, were pale and mottled, which

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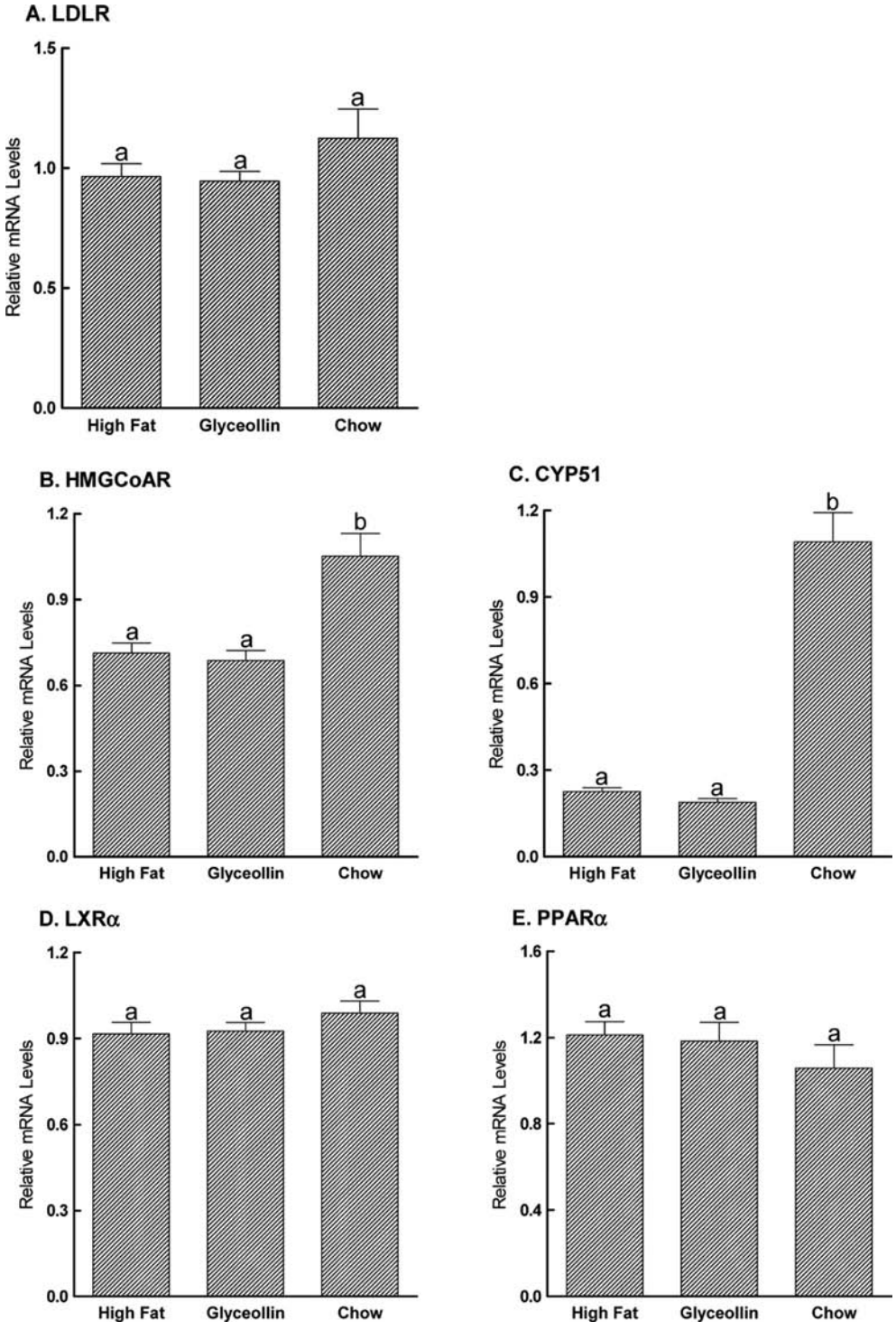

Figure 5. Effects of glyceollins on liver LDL receptors, cholesterol-synthesizing enzymes, and cholesterol-metabolizing transcription factors mRNA levels: (A) liver LDL receptor; (B) HMGCoAR; (C) CYP51; (D) LXRα; (E) PPARα gene. Livers were harvested from animals on different diets, total mRNA was extracted, and the mRNA level was determined using real-time PCR as described under Materials and Methods. Results are expressed as relative expression levels (mean $\pm$ SEM, $n = 10$) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

was suggestive of lipid accumulation. These results supported our previous conclusion suggesting that glyceollin supplementation may contribute to the reduction of cholesterol level in plasma.[53]

Typical dietary cholesterol-reducing agents, such as cholestyramine, increase fecal bile acid excretion.[56] However, fecal excretion of bile acid and cholesterol appeared not to be responsible for glyceollins' effect on VLDL, and no change in these parameters was observed in this study (Figure 4).

To further elucidate the mechanism of glyceollin action, we also examined expression of genes involved in lipid metabolism. Our results indicated the changes in gene expression due to feeding glyceollins were limited but appeared also reflective of

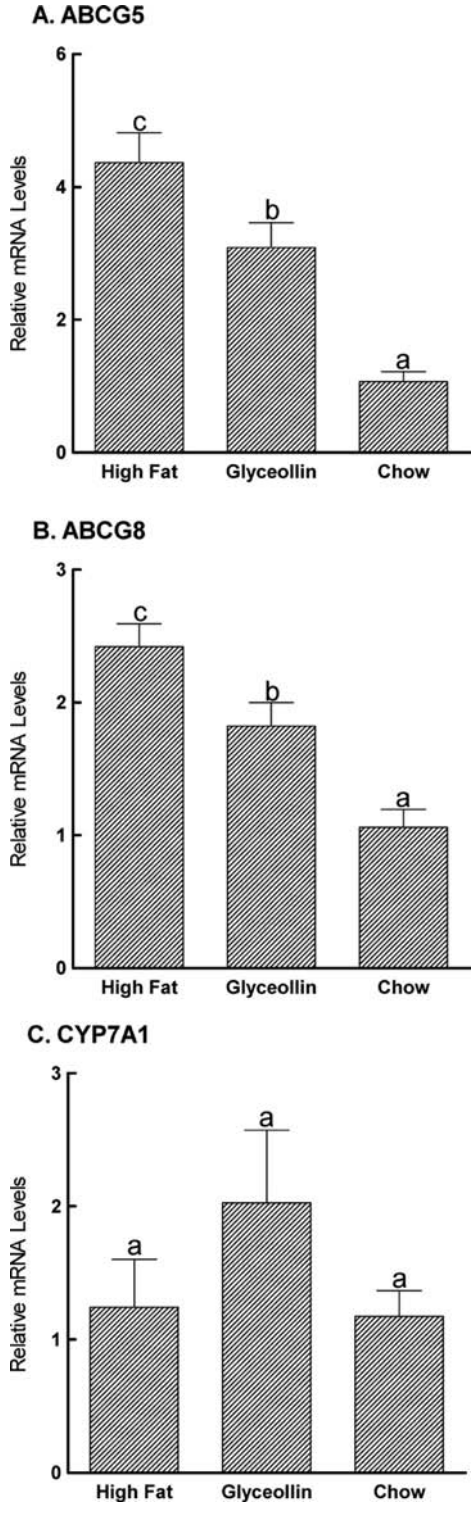

Figure 6. Effects of glyceollins on liver cholesterol transporting and catabolizing enzymes mRNA levels: (A) ABCG5; (B) ABCG8; (C) CYP7A1. Livers were harvested from animals on different diets, total mRNA was extracted, and mRNA level was determined using real-time PCR as described under Materials and Methods. Results are expressed as relative expression levels (mean ± SEM, $n = 10$) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

glyceollins' effect on liver lipid and cholesterol contents. Because the LDL receptor functions to bind and internalize circulating LDL-cholesterol and liver removes ~70% LDL from circulation through LDL receptors,[57,58] it can be a source of increased hepatic cholesterol. In this study, liver LDL receptor gene expression did not appear to change significantly regardless of the type of diet (Figure 5A). ABCG5 and ABCG8, two transporters for cholesterol excretion, were significantly up-regulated by the high-fat diet as compared to chow, but expressions of both genes in animals fed glyceollins were significantly lower than in animals consuming the high-fat only diet (Figure 6A,B). LPL, a dual function enzyme involved in cholesterol/lipid transport, was significantly up-regulated in high-fat diet fed animals compared to chow-fed animals. The glyceollin-supplemented diet significantly lowered hepatic LPL expression compared to the animals fed the high-fat only diet. The changes in ABCG5, 8, and LPL appeared to be reflective of hepatic lipid/cholesterol status in glyceollin-fed animals. We did observe a trend of bile synthetic enzyme CYP7A1 up-regulation and increased bile acid excretion in glyceollin-fed animals compared to high-fat only animals. However, there was a large difference between animals in these parameters and was not statistically significant. It is possible that a longer feeding period or an increase in animal number might confirm this observation and warrant further study. One unique observation in our study was that glyceollin-fed animals appeared to have a significantly lower adipose LPL mRNA level than high-fat or chow-fed animals. This result suggested that glyceollins might have an effect on adipose tissues, such as preventing accumulation of lipid in adipose tissues. Because our study was designed to investigate short-term effects and no body weight or adipose weight changes were observed, further long-term studies would be necessary to elucidate the biological significance and mechanisms of glyceollins' effect on adipose LPL.

Consumption of a high-fat, high-cholesterol diet can lead to gene changes that would compensate for changes in dietary intake.[59] In our current hamster study, as expected, significant down-regulation of the cholesterol synthesis related gene HMG-CoA reductase and CYP51 (Figure5B,C) was observed in hamsters on the high-fat diets compared to control group, with CYP51 being more sensitive to the influence of dietary cholesterol. This is consistent with the fact that abundant cholesterol is available to cells upon consumption of a high-fat diet; de novo synthesis of cholesterol appeared to be unnecessary. As important as HMG-CoA reductase is in the process of cholesterol synthesis, no effect was observed in this study, which suggested a lack of glyceollin influence on this cholesterol synthesis pathway. Similar results were also observed for SREBP-1c; however, in this case the high-fat diet led to significant up-regulation of this gene as compared to the chow-fed animals. Glyceollins did not affect SREBP-1c expression when compared to high-fat diet animals.

Our results indicated that several pathways appeared not to be affected by dietary perturbation at the transcriptional level. We did not observe significant differences in LXRα, PPARα, FAS, or ACOX mRNA expression between the different diets. None of these pathways were affected by glyceollin supplementation. These data would support the notion that, under our experimental condition, cholesterol synthesis and excretion were the main pathways animals used to adapt to the high-fat diet.

For the first time, our study gathered direct evidence that glyceollin supplementation in a high-fat diet significantly reduced VLDL, hepatic cholesterol esters, hepatic free cholesterol, and hepatic total lipids in animals. The effects of glyceollins on hepatic lipid and cholesterol levels appeared to contribute to molecular changes in hepatic ABCG5, 8, and LPLs mRNA levels.

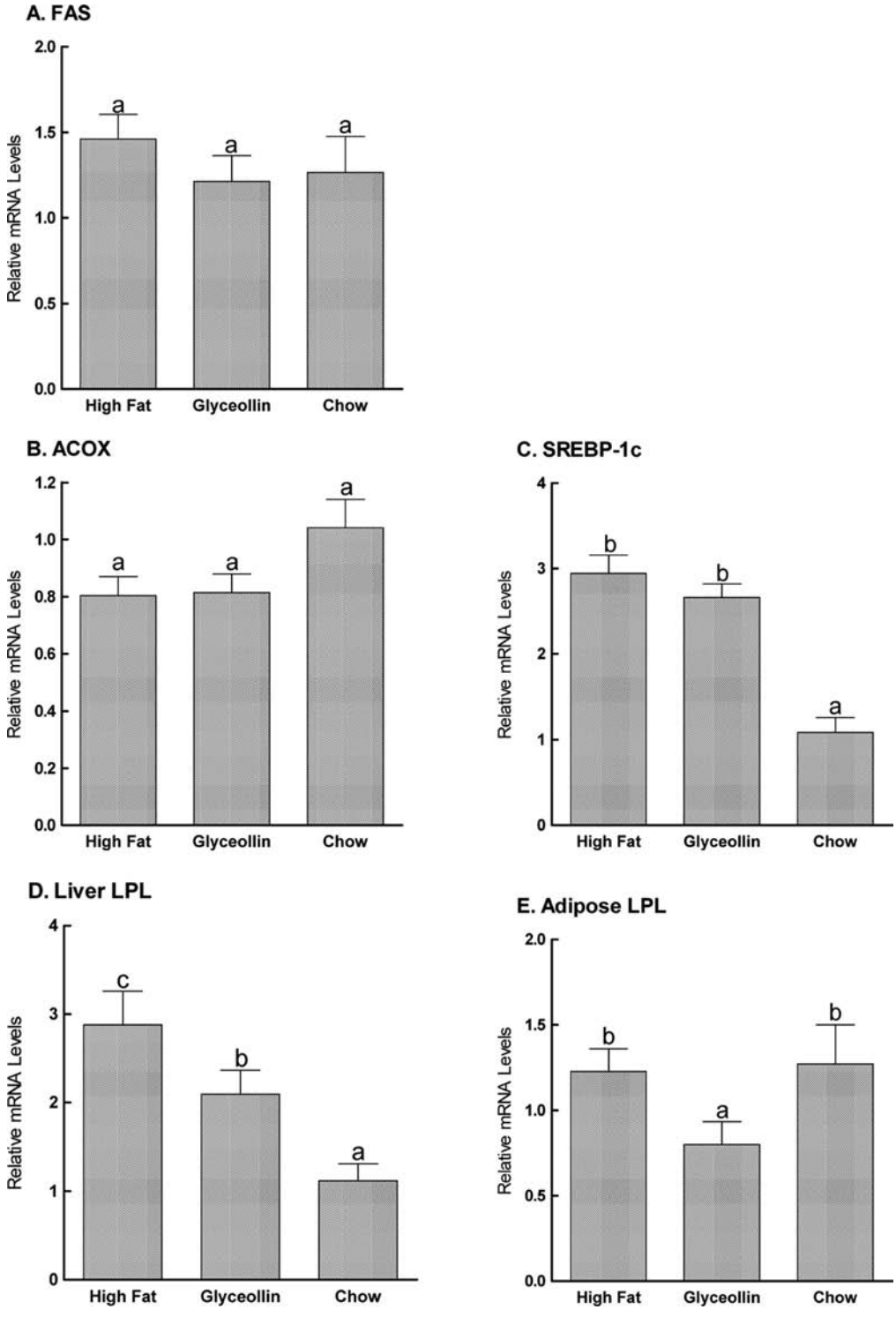

Figure 7. Effects of glyceollins on lipid-metabolizing genes in liver and adipose tissue: (A) FA synthase; (B) ACOX; (C) SREBP-1c; (D) liver LPL; (E) adipose LPL. Livers and adipose tissues were harvested from animals on different diets, total mRNA was extracted, and mRNA level was determined using real-time PCR as described under Materials and Methods. Results are expressed as relative expression levels (mean ± SEM, $n = 10$) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

However, the actual molecular mechanism of the cholesterol-lowering effect of glyceollins in vivo remained unclear and needs further investigation. Given that glyceollins are naturally derived phytochemicals in stressed soy, our results suggested that the inclusion of glyceollins in diet may benefit the population by lowering VLDL cholesterol and decreasing the risk for cardiovascular disease.

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■ ABBREVIATIONS USED

ABCG5/8, ATP-binding cassette subfamily G member 5/8; ACOX, acyl-CoA oxidase; CVD, cardiovascular disease; CYP51, lanosterol 14 α-demethylase; CYP7A1, cholesterol 7 alpha-hydroxylase; FA synthase, fatty acid synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDL, high-density lipoprotein; HMGCoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; LXRα, liver X receptor alpha; PPARα, peroxisome proliferator-activated receptor alpha; SREBP, sterol regulatory element-binding protein; VLDL, very low-density lipoprotein

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