Daidzein Modulations of Apolipoprotein B and Fatty Acid Synthase mRNA Expression in Chick Liver Vary Depending on Dietary Protein Levels

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ABSTRACT : This study was designed to determine the effects of daidzein (DE) on hepatic lipid metabolism in chicks fed with low protein (LP) diet based on casein. In experiment 1, the male chicks were fed with one of the three levels of dietary protein containing 10.95%, 21.9% and 43.8% protein content for 2 days. In experiment 2, the chicks were fed one of the three levels of protein with or without DE at 1,000 mg/kg diet for 2 days. Experiment 3 was conducted to compare DE (LP+DE) with estradiol (LP+E2) in chicks fed with LP diet for 7 days. Plasma lipid profiles, hepatic lipid profiles, activities of hepatic malic enzyme and isocitrate dehydrogenase (ICDH) were measured. Transcriptions of hepatic fatty acid synthase, apolipoprotein-B (APO-B), and fructose bisphosphatase mRNA were measured by RT-PCR. Increasing dietary protein levels markedly decreased the concentrations of plasma triglycerides, hepatic total lipids, hepatic TG, and the mRNA transcriptions while the increased dietary protein levels increased hepatic ICDH activities in experiment 1. In experiment 2, the effects of dietary protein levels on blood and hepatic lipid content were more prominent than those of the additional DE. Interestingly, plasma TG levels were affected by DE supplementation ($p<0.05$). In experiment 3, DE inhibited APO-B mRNA expressions and stimulated the accumulation of lipid in the liver through mechanisms different from E2. In this study, we demonstrate that DE has beneficial effects on blood lipid profiles, but that it inhibits APO-B mRNA transcription and aggravates the fatty liver induced by LP diet in chicks. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 2 : 236-244)***

Key Words : Daidzein, Low Protein Diet, Chick, Fatty Liver, Apolipoprotein B mRNA, Fatty Acid Synthase mRNA

INTRODUCTION

Over the past 40 years, numerous studies have been conducted in humans and animals to investigate the lipid-lowering effect of soy protein on blood. Soy protein has been particularly beneficial in the treatment of type IIa hyperlipoproteinemia, which is characterized by increased LDL-cholesterol, and also beneficial in the treatment of type IIb hyperlipoproteinemia, which is characterized by elevated LDL- and VLDL-cholesterol and TG (Sirtori et al., 1995). Because hyperlipoproteinemia is correlated with the developments of cardiovascular disease, atherosclerosis, and type II diabetes in humans, it was believed that soy protein reduced the risk of those chronic diseases. However, the mechanism of hypocholesterolemic effect of soy protein has not been clear. Several studies have proposed that the amino acids composition, peptides, phytoestrogen compounds and saponin of soy protein are responsible for its beneficial effects on blood.

Isoflavones are phytoestrogens found in soy and soy products, and they have been identified as the most common estrogen receptor binding compounds in diets (Bannwart et al., 1984). Isoflavones contained in soy with a relatively high concentration are genistin (GI), daidzin (DI), genistein (GE), and daidzein (DE) (Wang and Muphy, 1994). Recently, soy isoflavones have been shown to have a variety of biological activities, including antioxidative, estrogenic, antiosteoporotic and anticancer activities (Setchell and Cassidy, 1999).

In chicks and pigs, soy bean is the major protein source of diet, and the liver is known as the main tissue for lipid metabolism (Goodridge, 1968; Han et al., 2003; Lu et al., 2003; Yun et al., 2005). Although chicks are administered the isoflavones that are naturally present in soy in large amounts, few studies have been conducted to elucidate the isoflavone effects on hepatic lipid metabolism. Several studies of rodents have reported that GE and DE regulate the transcriptions of several genes in the liver or hepatocytes (Kirk et al., 1998; Borradaile et al., 2002; Ratna, 2002; Xiao et al., 2004). GE and DE stimulate the transcriptions of thyroid hormone receptor β1 and low-density lipoprotein receptor mRNA (Kirk et al., 1998; Borradaile et al., 2002; Xiao et al., 2004), and they inhibit the transcriptions of thyroid receptor α1, apolipoprotein-B (APO-B), microsomal triglyceride transfer protein, 3-hydorxy-3-methylglutaryl Coenzyme A reductase, and estrogen-regulated mRNA stabilizing factor (Kirk et al., 1998; Borradaile et al., 2002; Ratna, 2002). Their results

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Table 1. Composition of diets used in experiment 1

Ingredient[1]	Low protein diet	Normal protein diet	High protein diet
	---------- g/kg diet ----------		
Casein	100.0	200.0	400.0
Glucose, monohydrate	762.2	644.7	409.7
Corn oil[2]	30.0	30.0	30.0
α-Cellulose	30.0	30.0	30.0
DL-methionine	2.5	5.0	10.0
L-arginine	5.0	10.0	20.0
Glycine	10.0	20.0	40.0
Choline chloride	2.0	2.0	2.0
Vitamin premix[3]	2.0	2.0	2.0
Mineral premix[4]	56.3	56.3	56.3
Calculated value			
Crude protein (%)	10.95	21.90	43.80
Metabolizable energy (MJ/kg)	14.44	14.44	14.44

[1] Casein was obtained from NZMP Ltd. (Wellington, New Zealand) and contains 92% crude protein. Glucose and corn oil were from Daesang Company (Seoul, Korea). α-Cellulose was from Sigma chemical. DL-methionine were from Degussa Company. L-arginine was from Lancaster (Morecambe, UK). Glycine and choline chloride were form Junsei Chemicals Company (Tokyo, Japan).

[2] Corn oil stabilized by adding butylated hydroxy toluene at a level of 0.0125% of added oil.

[3] Vitamin premix provided the following in mg/kg or IU/kg diet: thiamin HCl, 20; riboflavin, 10; calcium panthothenate, 30; niacin, 50; pyridoxine HCl, 6; folacin, 4; biotin, 0.6; vitamin B12, 0.04; inositol, 100; para-aminobenzoic acid, 2; ascorbic acid, 250; menadione sodium bisulfite, 2; α-tocopherol acetate (source 500 IU/g), 20; retinyl palmitate (source 1,000,000 IU/g), 5,200; cholecalciferol (source 5,000,000 IU/g), 600.

[4] Mineral premix provided the following in g/kg; NaCl, 8.8; $CaCO_3$, 3; $Ca_3(PO_4)_2$, 28; $MgSO_4 \cdot 7H_2O$, 3.5; KH_2PO_4, 9; $MnSO_4 \cdot H_2O$, 0.650; $ZnCO_3$ 0.1; ferric citrate, 0.5; $CuSO_4 \cdot 5H_2O$, 0.02; Na_2SeO_3, 0.0002; KI, 0.04; $CoSO_4 \cdot 7H_2O$, 0.001; H_3BO_3, 0.009; $Na_2MoO_4 \cdot 2H_2O$, 0.009.

supported that isoflavones are responsible for hypolipidemic effect of soy protein at least partially, because the compounds inhibit the transcription of APO-B gene which is the major protein of VLDL and LDL in rats.

The effects of types and levels of protein in diets on the accumulation of hepatic lipids in chicks and rats have been well established (Sato et al., 1996; Adams and Davis, 2001; Ascencio et al., 2004). Casein, a typical animal protein, stimulates the increase in serum insulin. The increase in insulin levels stimulates in turn transcriptions of the sterol-regulated element binding protein-1, the fatty acid synthase (FAS), and the malic enzyme (ME) mRNA. Thus, casein increases the hepatic lipid content in rats (Ascencio et al., 2004). The levels of protein in diets, also, affect the hepatic lipid metabolism in chicks and rats (Sato et al., 1996; Adams and Davis, 2001). Their results demonstrated that feeding LP diet increases the concentration of hepatic lipids, in spite of differences of responses depending on type of protein, for example, casein, whey, and soy protein.

The effects of isoflavones on the fatty liver, which resulted from feeding LP diet, are not well characterized compared to the studies that determined beneficial effects of isoflavones on serum lipid profiles. However, little data are available on the roles of isoflavones in the hepatic lipid metabolism. We were conducted two experiment to determine the effects of dietary protein levels and those of supplemental DE on the hepatic lipid metabolism. In addition, a third experiment was conducted to compare the effects of dietary DE with those of estradiol (E2) administration on the liver lipid metabolism in chicks fed with LP diet.

MATERIALS AND METHODS

Chemicals and reagents

Casein (92% crude protein) was purchased from NZMP Ltd. (Wellington, New Zealand), DE and Glycine from TCI organic chemicals (Tokyo, Japan), and cellulose, Tris HCl, Trizma Base, isocitric acid, L-malic acid, EDTA sodium salt, triolein, ammonium acetate, 2,4-pentandione, 17β-estradiol (E2) and dithiothreitol from Sigma chemical. L-arginine was purchased from Lancaster (Morecambe, UK), DL-methionine from Degussa Company, and isopropanol, methanol and acetic acid from J. T. Baker Inc. (Phillipsburg, NJ). All vitamins for the vitamin premix were purchased from Roche. Choline chloride, potassium hydroxide, glycine, aluminium oxide and all of the reagents for mineral premix were purchased from Junsei Chemicals Company (Tokyo, Japan); total cholesterol, ALT and AST analysis kits from Wako Pure Chemicals Company (Tokyo, Japan) and cryo-tubes (2 ml) were from TRP® (Swiss); Agarose (Seakem® LE type) from FMC bioproducts (Maine, USA). TRIzol reagent, random primers and SuperScript™ II RNase H^- Reverse Transcriptase were purchased from Invitrogen Life Technologies (Carlsbad, CA); TaKaRa Ex Taq™ from TaKaRaKoreaBiomedical Company (Seoul, Korea).

Animals and diets

Hy-line® Variety Brown Single Comb Leghorn (layer type) male chicks (1 day old, n = 100) were obtained from Hanyang Hatcheries (Kyunggi-do, Korea) for each experiment. The chicks were housed in thermostatically controlled and electrically heated wire floor cages (15 birds/cage) at 31°C and were lighted for 24 h/d. The chicks had free access to water and to purified type of normal protein level diet (Normal protein diet; Table 1) during the adaptation periods. Four (experiment 1) or seven days (experiment 2, 3) after hatching, the chicks were sorted and were assigned to each dietary treatment. One (experiment 3) or two (experiment 1, 2) birds were housed in stainless steel

Table 2. Composition of diets used in experiment 2[1]

Ingredient	Low protein diet	Normal protein diet	High protein diet
	---------- g/kg diet ----------		
Casein	100.0	200.0	300.0
Glucose, monohydrate	762.2	644.7	409.7
Corn oil	30.0	30.0	30.0
α-Cellulose	30.0	30.0	30.0
DL-methionine	2.5	5.0	7.5
L-arginine	5.0	10.0	15.0
Glycine	10.0	20.0	30.0
Choline chloride	2.0	2.0	2.0
Vitamin premix	2.0	2.0	2.0
Mineral mix	56.3	56.3	56.3
Calculated value			
Crude protein (%)	10.95	21.90	32.85
Metabolizable energy (MJ/kg)	14.44	14.44	14.44

[1] All of the ingredients and the compositions of vitamin premix and mineral premix were same as using in experiment 1 (Table 1). Daidzein was added to each protein level diet at 1,000 mg/kg diet.

wire cages, and body weight distributions were similar among the cages. Animal care in this study conformed to the Guide for the Care and Use of Laboratory Animals published by U.S. National Institute of Health.

Blood samples were obtained using EDTA treated test tubes after the chicks were anesthetized with diethyl ether at the end of each experiment, and the plasma was separated with centrifuge (1,100×g, J6-MC, Beckman Co., USA) at 4°C and stored at -70°C until analysis. The chicks were killed by cervical dislocation to obtain liver samples. About 0.2 g of the each liver was put into 2 ml cryo tube to be used for RNA analysis and measurement of the enzyme activities. Then the tubes were frozen immediately in liquid nitrogen and stored at -70°C.

Experiment 1

After adaptation period of 4 days, the 36 male chicks were selected and allocated into one of the three dietary treatments (Table 1), and two birds were housed in each stainless steel wire cage for 2 days. To certify isoflavones-free, casein was used as a protein source of diets, and the NP diet was based on standard reference diet for chicks, chemically casein diet, by NRC (1994), except for cellulose added at level of 3% as a fiber source. Protein content of the experimental diets was 10.95%, 21.9% and 43.8% for the LP, NP and HP diet, respectively. Body weight and food consumption were recorded at the end of this experiment.

Experiment 2

In experiment 2, protein content of the HP diet was reduced from 43.80% of experiment 1 to 32.85% (Table 2). DE was added to each diet at 1000 mg/kg. Therefore, the dietary treatments were the LP, LP+DE, NP, NP+DE, HP and HP+DE. All of the other protocols were the same as in experiment 1.

Experiment 3

This experiment was designed to compare the effects of DE with E2 on lipid metabolism of the fatty liver that was induced by the LP diet in chicks. The treatments to achieve the objectives of this experiment were the LP diet as a control, DE added to the LP diet at 1,000 mg/kg diet (LP+DE) and E2 administration (LP+E2). E2 was resolved in corn oil at 2 mg/ml level and administrated to the chick though intra-muscular injection three times at 2 mg/kg body weight with 2 d interval. The other chicks, in the LP and LP+DE dietary treatments, received the same volume of the vehicle. The experimental periods were 7 days to sufficiently show different effects between DE and E2.

Plasma triglyceride and total cholesterol concentrations

Plasma TG concentrations were measured using a spectrophotometer following the method of Neri and Frings (1973) slightly modified. Briefly, 80 μl of plasma was added to the test tube allocated with 4,920 μl isopropanol and about 1 g of aluminum oxide, preliminarily, and the tube was shaken with the shaker (VRX IKA-Vibrax®, IKA) for 15 min. And then, the test tube was centrifuged (1,000×g, 4°C, J6-MC, Beckman) for 10 min and 2,000 μl aqueous phases were collected to a new test tube. The test tube was added 200 μl saponification reagent [10% potassium hydroxide in 25 isopropanol/75 water (= v/v) solution] and was incubated at 70°C for 10 min. After

Table 3. HPLC analytical conditions for plasma daidzein concentration

Item	Analytical conditions
Pump	60 F, Waters Co. (Milford, MA)
Controller	600 series, Waters Co.
Detector	L-ECD 6A, Shimadzu Co. (Japan)
Injection module	717 plus autosampler, Waters Co., equipped with 200 μl sample loop
Data processing module	Peak simple 202, SRI Instrument (USA)
Column	Mightysil C18, 250×4.5 (L×OD, mm), Cica®, Kanto Chemical Co. (Tokyo, Japan)
Column temperature	30°C
Mobile phase	Methanol/0.1 M ammonium acetate (pH 6.0)/25 mM EDTA = 40/50/1
Flow rate	1 ml/min
Electrode potential	0.75 V

Table 4. Primers employed for RT-PCR

Gene	Primers (5'-3')	Predicted size (bp)
FAS		282
Left	gagctgcatcttcgtggata	
Right	gctatcaagacagcggtcaa	
APO-B		320
Left	agctgtacaaggacgcaatg	
Right	tgtgaggcgtgtaaccaagt	
Fructose diphosphatase		284
Left	gcacatgcgttattgtgtca	
Right	acagttgacacctccagcag	
β-Actin		209
Left	gcaagcaggagtacgatgaa	
Right	actgctgctgacaccttcac	

cooling, 1,000 μl acetylacetone reagent (6,000 μl aetylacetone in 1 L of 2 mol/L ammonium acetate) was added to the test tube and incubated at 70°C for 10 min. Absorbance of the sample was measured using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech) at 405 nm. Triolein was used as a standard.

The concentrations of total cholesterol, ALT and AST in plasma were measured using commercial enzymatic kits (Wako Pure Chemicals Co., Japan) according to the manufacturer's instruction.

Plasma daidzein concentrations

The concentrations of DE in plasma were analyzed using HPLC (King et al., 1996). 200 μl of plasma was sampled for DE analysis. HPLC Detector was the electron chemical detector (Table 3).

Liver total lipid and triglyceride content

The content of hepatic total lipids was determined according to the method of Folch et al. (1957) and the concentration of TG in total lipid extracts was determined as described above, after all the organic solvent of the extract was volatilized under N_2 stream.

Malic enzyme and isocitrate dehydrogenase assay

Hepatic malic enzyme [L-malate-$NADP^+$ oxidoreductase (decarboxylating), E.C. 1.1.1.40, ME] and isocitrate dehydrogenase [L-isocitrate-$NADP^+$ oxidoreductase (decarboxylating), E.C. 1.1.1.42, ICDH] activities were estimated in continuous spectrophotometer assays in which the reduction of NADP was monitored at 340 nm at room temperature (MacDonald, 2002). The liver was homogenized with 9 parts ice cold 250 mmol/L sucrose containing 1 mmol/L reduced glutathione and centrifuged at 700×g for 10 min using J6-MC centrifuge (Beckman, USA) at 4°C. The supernatant fraction was collected and recentrifuged at 15,900×g for 10 min (Mega-17R, Hanil Co., Korea) at 4°C. The supernatant was recovered and ultracentrifuged at 100,000×g for 1 hour (Optima XL-100K, Beckman, USA) at 4°C, and it was used to estimate enzyme assays.

The enzyme reaction mixture for activity of ME contained NADP (0.5 mmol/L), $MgCl_2$ (4 mmol/L), dithiothreitol (0.1 mmol/L) and Tris buffer (50 mmol/L, pH 7.8) and the mixture for activity of ICDH contained NADP (0.5 mmol/L), $MnCl_2$ (4 mmol/L), dithiothreitol (0.1 mmol/L) and Tris buffer (50 mmol/L, pH 7.8). The background rate in the complete enzyme reaction mixture containing cytosol excluding the substrate was measured during 30 seconds (from 15 to 45 sec) at room temperature. The enzyme reaction started with the addition of substrates (L-malic acid for ME and isocitric acid for ICDH). Concentrations of malic and isocitric acid, in the final enzyme reaction mixture, were 1 mmol/L and 0.5 mmol/L, respectively. The total rate was measured during next 30 seconds (form 60 to 90 sec). The background rate that was considered as natural conversion of NADP was subtracted from the total rate to evaluate the net rate that was attributed to the enzymes. Protein concentration of the cytosol was measured by protein assay kits (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The unit of enzyme activity was nmol/min/mg protein.

The extraction of total RNA and RT-PCR assay

Total RNA was isolated from the liver using TRIzol reagent (Invitrogen, Carlsbad, CA) based on Chomczynski and Sacchi (1987). The RNA quantity was measured using the spectrophotometer (Ultrospec 2000, Pharmacia Biotech) and cDNA was prepared using Random Primers (Invitrogen, Carlsbad, CA) and SuperScript™ IIRNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Each Gene sequence was obtained from GenBank, and primers were selected by Primer3 software (Table 4). The cDNA product was amplified by PCR with TaKaRa Ex Taq™ (TaKaRaKoreaBiomedical Inc., Seoul, Korea). The PCR condition was 94°C for 2 min, 94°C for 30 s, annealing temperature for 30 s, and 72°C for 1 min for 25-28 cycles, 72°C 5 min. Samples were resolved on agarose gel and visualized with ethidium bromide. ImageQuant™ TL (Amersham Biosciences) was used for band analysis.

Statistical analysis

All values were represented as means±SEM. Data were analyzed by ANOVA according to General Linear Model procedure. The means were compared by Tukey's Studentized Range (HSD) test to detect significant differences at $p<0.05$. In experiment 2, the effects of the levels of dietary protein and addition of DE were evaluated by the two-way ANOVA. All statistical procedures were done with the SAS® software package (Release 8.02, 2001).

Table 5. Growth performance and total cholesterol, triglyceride, ALT and AST of plasma, total lipid, triglyceride, enzyme activities of liver in chicks fed various levels of protein in experiment 1[1]

	Low protein	Normal protein	High protein
Body weight (g/bird, n = 6)			
Initial	61.6±0.3	61.2±0.2	60.9±0.3
Final	74.0±0.7^{ab}	77.3±0.9^{a}	73.1±1.2^{b}
Gain	12.5±0.7^{b}	16.2±0.8^{a}	12.2±1.0^{b}
Food intake (g/bird/day, n = 6)	13.12±0.83^{a}	12.88±0.85^{a}	7.72±0.75^{b}
Food/gain ratio (n = 6)	2.11±0.04^{a}	1.59±0.04^{b}	1.27±0.05^{c}
Plasma (n = 10)[2]			
Triglyceride (mmol/L)	7.28±0.35^{a}	6.59±0.43^{a}	4.48±0.22^{b}
Cholesterol (mmol/L)	4.57±0.17^{a}	3.87±0.15^{b}	3.80±0.22^{b}
ALT (IU/ml)	3.59±0.40^{a}	1.66±0.38^{b}	1.30±0.26^{b}
AST (IU/ml)	28.97±1.94	22.82±2.01	26.73±0.93
Liver (n = 10[3])			
Total lipid (%)	20.76±1.48^{a}	9.07±0.50^{b}	4.69±0.21^{c}
Triglyceride (μmol/g)	238.74±14.81^{a}	106.46±4.78^{b}	36.50±4.17^{c}
ME (nmol/min/mg protein)	42.05±4.25	48.76±5.86	48.77±7.11
ICDH (nmol/min/mg protein)	313.49±10.04^{b}	376.59±24.44^{b}	510.34±42.19^{a}

[1] Values are means±SEM. Means in a row without a common superscript differ, $p<0.05$.
[2] ALT: Alanine transaminase; AST: Aspartate transaminase.
[3] ME: Malic enzyme; ICDH: Isocitrate dehydrogenase.

RESULTS AND DISCUSSION

Experiment 1: Effects of dietary protein level on liver lipid metabolism of chicks

Our study confirmed that feeding LP diet elevated the concentrations of hepatic total lipid and TG in chicks (Table 5). The final body weight and F/G ratio were lower in chicks fed with LP diet than in those fed with NP diet ($p<0.05$). Food intake was lower and F/G ratio was higher in the HP treatment than other dietary treatments ($p<0.05$). An insufficient protein intake in the chicks fed with LP diet caused a decrease in body weight gain and F/G ratio, whereas the NP dietary treatment showed an increase ($p<0.05$).

The chicks fed with LP or NP diets exhibited higher levels of TG, total cholesterol, and ALT in plasma than those fed with HP diet ($p<0.05$). The ALT level of the LP treatment was 116% higher than NP treatment and 176% higher than HP treatment ($p<0.05$). The result of this study supports our argument that ALT is a good marker of fatty liver development caused by dietary protein deficiency applicable without sacrificing the animals.

The LP diet increased the concentrations of total lipid and TG in the liver more than the NP or HP diet ($p<0.05$). Our results were consistent with the previous findings that an increase in dietary protein levels decreased lipid content of the liver in chicks (Rosebrough et al., 1990; Rosebrough et al., 1996; Adams and Davis, 2001; Rosebrough et al., 2002). They used soy protein isolate or soy bean meal as a protein source. Because soy protein contains isoflavones and unknown compounds that exhibit hypolipidemic and hypocholesterolemic effects in blood, the effects of these compounds were implicated in the results of their studies. In the present study, our results were more sensitive to dietary protein level than the previous findings with soy protein, since casein was used as a protein source free of isoflavones- and hypolipidemic compounds.

In avian species, the liver was known as the main tissue for the de novo synthesis of fatty acids (Goodridge, 1968). The enzymes that produced NADPH were ME, ICDH, and glucose-6-phosphate dehydrogenase in the liver of rats and mice (MacDonald, 2002). ICDH activities were the highest among the three enzymes for hepatic NADPH production in rodents. The hepatic ME is believed to supply NADPH for fatty acids synthesis, and the activities of ME are known to be positively correlated with the rate of fatty acid synthesis and the percentage of body fat and abdominal fat in chicks (Tanaka et al., 1983; Winberry et al., 1983; Ma et al., 1990). Unlike the previous findings, the activities of ME in our study did not differ among the dietary protein levels. Hepatic ICDH activities, however, increased with the increasing dietary protein levels ($p<0.05$). We observed that ICDH exhibited the highest enzymatic activity for NADPH production from 7.4 to 10.4 fold of those of ME in chicks. The results of our study indicated that the estimation of NADPH for de novo synthesis of fatty acids in the liver of chicks should be performed with the activity of ICDH as well as that of ME. And also, it is possible that the main purpose of ME and ICDH activities in chicks is not the production of NADPH but the synthesis of pyruvate and α-ketoglutarate. The previous studies have revealed that the activity of ME is increased by the injection of

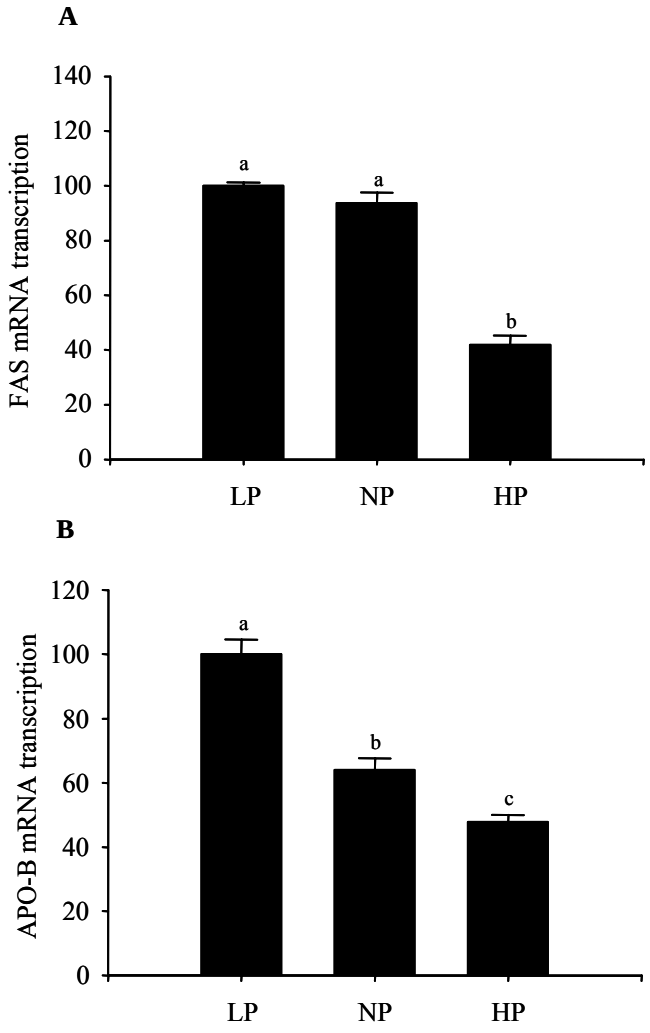

Figure 1. Effects of dietary protein level on transcriptions of FAS (A), and APO-B (B). All data are normalized by the intensity of β-actin band. The levels are the percentage of the LP dietary group (LP = 100). Data are means±SEM, n = 4. Bars in a panel without a common letter differ, $p<0.05$.

triiodothyronine, while it decreased the ICDH activity in chicks (Goodridgh and Garay, 1974; Rosebrough et al., 1990). More studies are required to understand the relations of hepatic ME and ICDH activities to various metabolic situations of animals.

The chicks fed with HP diet showed lower transcription levels of FAS and APO-B gene in the liver than those given the other treatments ($p<0.05$, Figure 1). Our result of FAS mRNA expression in the chicks fed with various levels of dietary protein agrees with the findings of previous study (Rosebrough et al., 2002). Sato at al. (1996) found that hepatic APO-B and APO-E mRNA transcriptions are inhibited, and that fatty livers are developed in rats fed with low protein diets. The protein inclusion rate was at 5%, and the sources were casein or whey protein in their study. De Jong and Schreiber (1987) have reported that protein-deficient diets decrease APO-E mRNA in the rat liver. Severe low protein diet or protein-deficient diet inhibited hepatic APO-B and APO-E mRNA expressions in rats. The LP diet in the present study did not have severely low protein content. The protein content of the LP diet was 10.95%. In the case of mild-low protein diet as in this study, the animal increased food and energy intake (Adams and Davis, 2001). The results of experiment 1 indicated that the low protein diet, even when not severely low, stimulates hepatic APO-B mRNA transcription in chicks within 48 h.

Experiment 2: Effects of dietary protein level with or without DE on lipid metabolism of chicks

In experiment 2, the protein inclusion rate of HP diet was 30% and experimental diet feeding-period was 2 days. Food intakes of the HP diet chicks were lower than those of the LP diet chicks ($p<0.05$, Table 6), but the decrease rates of food intakes between the LP and HP diet chicks diminished as compared with those of experiment 1. The body weight gains, food intakes, and food/gain ratios were affected by the dietary casein levels ($p<0.05$), but the effects of DE on these parameters were not significant ($p>0.05$).

In plasma, TG levels were affected by the dietary protein levels and also by the addition of DE at 1,000 mg/kg diet ($p<0.05$), but there were no interactions between the dietary protein level and the addition of DE ($p>0.05$). Cholesterol levels in plasma, hepatic total lipids, and TG were prominently affected by the dietary protein levels, as in the results of experiment 1. It is interesting to note that the concentrations of DE in plasma interacted between the dietary protein levels and the supplementation of DE ($p<0.05$).

In the liver, the LP diet resulted in a marked increase of total lipid and TG content ($p<0.05$), and the effect of level DE has to be considered. The activities of ICDH were affected by the addition of DE into the diets at all levels of protein ($p<0.05$). However, the concentrations of total lipid and TG in the liver were not affected by the addition of DE. This result suggests that changes in the hepatic lipid content by DE needed a relatively long-term period in contrast to the acute fatty liver that was developed by the LP diet.

Experiment 3: Effects of DE and E2 on hepatic lipid metabolism in chicks fed the low protein diet

In experiment 3, the final body weights, body weight gains, and food intakes did not differ among the dietary treatments during 7 days (Table 7). The intra-muscle injection of E2 in chicks exhibited a lower food/gain ratio than the LP dietary group ($p<0.05$). The plasma TG level of the LP+E2 treatment was higher than the other treatments ($p<0.05$). The total cholesterol concentration of plasma in chicks fed with the LP+DE diet was significantly lower than that of the LP+E2 treated chicks ($p<0.05$). Plasma

Table 6. Growth performance and total cholesterol, triglyceride, ALT, AST and daidzein of plasma, total lipid, triglyceride, enzyme activities of liver in chicks fed various levels of protein with or without daidzein in experiment 2[1]

	Low protein	Low protein + DE[2]	Normal protein	Normal protein + DE	High protein	High protein + DE	ANOVA[3]		
							P. level	DE	P. level ×DE
Body weight (g/bird, n = 3)									
Initial	98.4±0.2	98.9±0.2	98.3±0.5	97.9±0.3	98.6±0.1	98.2±0.4	-	-	-
Final	117.7±2.5	116.2±0.4	120.3±0.6	122.7±0.4	120.9±1.3	118.3±2.4	0.05	NS	NS
Gain	19.32±2.51^{ab}	17.32±0.16^{b}	21.95±0.71^{ab}	24.80±0.09^{a}	22.33±1.41^{ab}	20.07±1.95^{ab}	0.05	NS	NS
Food intake (g/bird/day, n = 3)	19.4±1.8^{a}	18.6±0.3^{ab}	17.2±0.5^{abc}	17.1±0.1^{abc}	14.9±1.0^{bc}	14.0±0.4^{c}	0.01	NS	NS
Food/gain ratio (n = 3)	2.02±0.08^{a}	2.14±0.04^{a}	1.57±0.09^{b}	1.38±0.01^{b}	1.33±0.01^{b}	1.42±0.12^{b}	0.01	NS	NS
Plasma (n = 5)[4]									
Triglyceride (mmol/L)	6.80±0.27^{a}	5.68±0.32^{ab}	5.98±0.56^{ab}	5.59±0.11^{ab}	5.49±0.34^{ab}	5.05±0.21^{b}	0.05	0.05	NS
Cholesterol (mmol/L)	5.07±0.28^{a}	5.32±0.32^{a}	4.29±0.19^{ab}	4.28±0.32^{ab}	3.65±0.22^{b}	3.79±0.17^{b}	0.01	NS	NS
ALT (IU/mL)	1.78±0.34	1.61±0.40	0.75±0.23	1.85±0.18	1.08±0.27	1.07±0.42	NS	NS	NS
AST (IU/mL)	30.72±3.28	25.47±1.07	23.65±2.85	23.77±1.56	28.55±2.72	25.57±1.01	NS	NS	NS
Daidzein (nmol/L)	28.7±18.7^{c}	722.9±88.0^{b}	15.7±15.7^{c}	707.1±64.2^{b}	21.6±13.6^{c}	1235.4±103.9^{a}	0.01	0.01	0.01
Liver (n = 5)[5]									
Total lipid (%)	7.82±0.53^{a}	7.62±0.30^{ab}	5.88±0.69^{ab}	5.04±0.63^{b}	5.67±0.54^{ab}	5.10±0.40^{b}	0.05	NS	NS
Triglyceride (μmol/g)	53.74±4.87^{a}	50.27±2.30^{a}	45.16±7.89^{ab}	42.04±8.13^{ab}	37.40±3.90^{ab}	32.02±2.34^{b}	0.05	NS	NS
ME (nmol/min/mg protein	49.28±7.14^{ab}	72.18±8.17^{a}	37.44±10.61^{b}	53.38±6.35^{ab}	53.48±3.24^{ab}	49.99±4.99^{ab}	NS	NS	NS
ICDH (nmol/min/mg protein)	230.73±8.28^{b}	209.00±13.56^{b}	350.81±29.34^{a}	273.58±22.25^{ab}	347.44±21.41^{a}	280.53±22.19^{ab}	0.01	0.01	NS

[1] Values are means±SEM. Means in a row without a common superscript differ, $p<0.05$.
[2] DE: daidzein. Daidzein was added to each protein level diet at 1,000 mg/kg diet.
[3] P. level: dietary protein level.
[4] ALT: Alanine transaminase; AST: Aspartate transaminase.
[5] ME: Malic enzyme; ICDH: Isocitrate dehydrogenase.

Table 7. Growth performance and total cholesterol and triglyceride of plasma and total lipid, triglyceride and enzyme activities of liver in chicks fed experimental diets in experiment 3[1]

	Low protein	Low protein+DE[2]	Low protein+E2
Body weight (g/bird, n = 6)			
Initial	75.9±1.2	77.0±0.9	77.2±1.0
Final	125.1±1.6	136.2±4.3	138.0±4.1
Gain	49.2±1.3	59.3±4.5	60.8±3.9
Food intake (g/bird/day, n = 6)	17.9±0.4	19.1±1.2	18.7±0.6
Food/gain ratio (n = 6)	2.56±0.10^{a}	2.28±0.06^{ab}	2.18±0.09^{b}
Plasma (n = 5)			
Triglyceride (mmol/L)	6.61±0.39^{b}	6.69±0.36^{b}	8.37±0.42^{a}
Cholesterol (mmol/L)	5.35±0.22^{ab}	4.80±0.11^{b}	6.13±0.32^{a}
Liver (n = 4)[3]			
Total lipid (%)	5.22±0.35^{b}	12.82±1.82^{a}	11.50±1.23^{a}
Triglyceride (μmol/g)	49.69±5.62^{b}	125.02±19.13^{a}	114.72±11.26^{a}
ME (nmol/min/mg protein)	27.24±6.55	25.19±10.93	18.35±5.89
ICDH (nmol/min/mg protein)	280.61±12.88^{a}	247.16±13.97^{ab}	198.90±16.80^{b}

[1] Values are means±SEM. Means in a row without a common superscript differ, $p<0.05$.
[2] DE: Daidzein; E2: 17β-estradiol.
[3] ME: Malic enzyme; ICDH: Isocitrate dehydrogenase.

concentrations of TG and cholesterol in chicks administered with E2 were higher than in those fed with the LP+DE diet ($p<0.05$).

The chicks in the LP+DE and LP+E2 treatments, as compared with the LP treatment, resulted in significantly higher concentrations of total lipids and TG in the liver ($p<0.05$). Our study agreed with the result of Hermier et al. (1996), who has reported that administration of E2 increased the concentrations of hepatic lipid, TG, and free cholesterol. ICDH activity in chicks fed with the LP diet was higher than that of the LP+E2 ($p<0.05$). Administration of E2 decreased the activity of ICDH while significantly increasing the levels of hepatic total lipid and TG ($p<0.05$) whereas the addition of DE did not significantly change ICDH level but increased total lipid and TG of the liver of the chicks that received the LP diet. This led us to conclude that DE aggravated the fatty liver, which resulted from feeding the LP diet.

Administration of DE decreased the transcriptions of hepatic FAS mRNA as compared with those of the LP diet group, and it decreased the hepatic APO-B and fructose diphosphatase mRNA expressions as compared with those

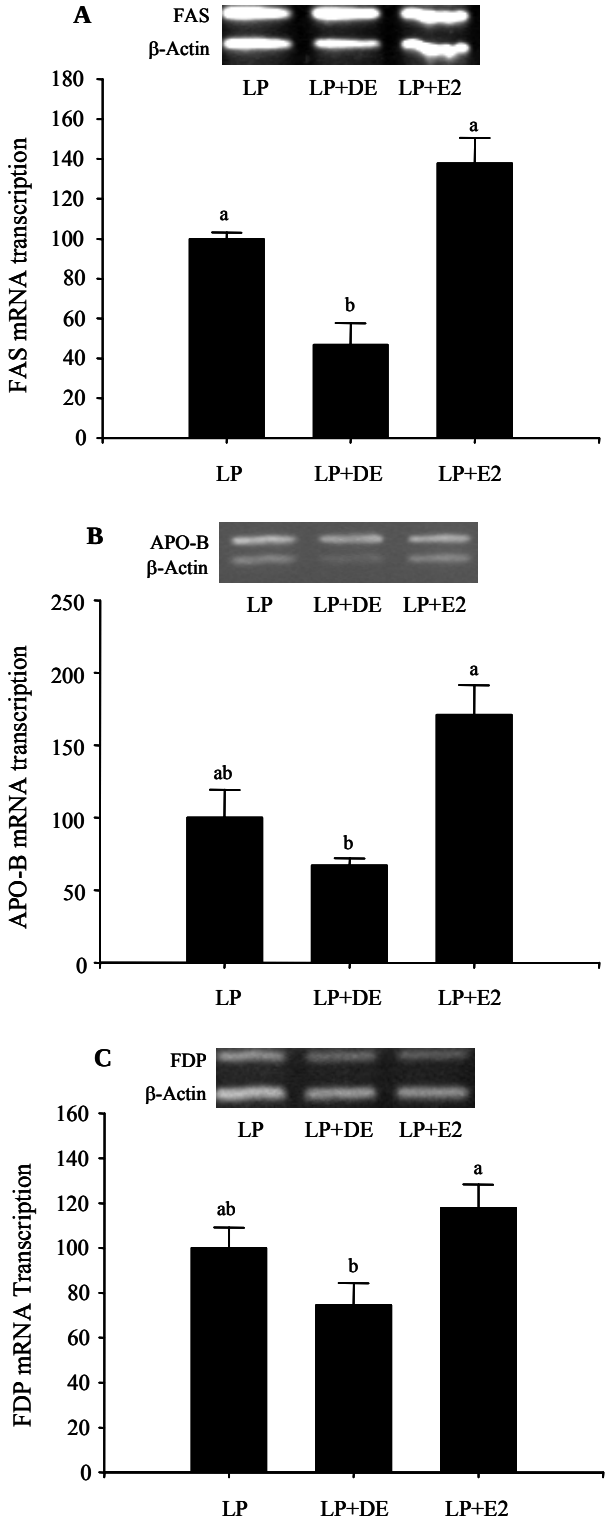

Figure 2. Effects of the administrations of DE and E2 in the LP diet on transcriptions of FAS (A), APO-B (B) and Fructose diphosphatase (C). The upper panel shows a representative photograph stained by ethidium bromide. The levels are the percentage of the LP diet (LP = 100). Data are means±SEM, n = 4. Bars in a panel without a common letter differ, $p<0.05$.

of the LP+E2 treatment (Figure 2). The previous studies have reported that DE acted agonistically against the estrogen receptor at low plasma levels while at high plasma levels it acted antagonistically (Setchell and Cassidy, 1999; Ren et al., 2001). Borradaile et al. (2002) has revealed that DE and GE decrease the excretion of APO-B and increase the LDL-receptor mRNA expression, but that APO-B mRNA levels did not significantly change in hepatocytes. We think that the decrease of excretion or transcription of APO-B and/or increase of LDL-receptor mRNA expression by DE was responsible for the increasing hepatic TG levels. Wong and Keung (1999) have revealed that isoflavones had been shown to inhibit 17β-hydoxysteroid dehydrogenase and 5-ene-4-ene isomerase in bovine adrenal. Their results meant that DE disturbs the biological activities of estrogen as well as those of progesterone, mineralocorticoid, glucocorticoid, and androgen. The results from our study could not be explained by the characteristics of estrogen antagonism of DE only, because the young male chicks could not produce estrogen actively. More studies on the steroid biochemistry are necessary to understand the reasons for the disturbance of steroid hormone metabolism by isoflavones.

In conclusion, our results suggest that DE has beneficial effects on blood cholesterol, while it aggravates the fatty liver in chicks induced by the low protein diet because lipids are accumulated through the inhibition of hepatic APO-B mRNA expression.

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