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Long-Term Feeding of Soy Protein Attenuates Choline Deficient-Induced Adverse Effects in Wild Type Mice and Prohibitin 1 Deficient Mice Response More Sensitively.

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ABSTRACT: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, however the exact cause of NAFLD remains unknown. Methionine, an essential amino acid, is the first limiting amino acid of soy protein, and its deficiency is suggested to cause hepatocyte damage and NAFLD. The objective of this study is to examine the changes in NAFLD susceptibility with soy protein consumption and deterioration due to prohibitin 1 (PHB1) deficiency, an important protein in hepatic mitochondrial function. In this study, liver-specific *phb1* +/− mice and wild-type mice were fed a normal diet, choline-deficient diet (CDD), or soy protein diet without choline (SPD) for 16 weeks. Using hematoxylin and eosin staining, we showed that SPD attenuates symptoms of hepatocyte damage and lipid accumulation induced by CDD in mouse liver. The liver damage in mice fed the SPD was alleviated by decreasing lipogenic markers and by increasing anti-inflammatory markers. Furthermore, mRNA expression of genes involved in hepatic methionine metabolism was significantly lower in liver-specific *phb1* +/− mice fed with a SPD compared with wild-type mice fed with a SPD. These data suggest a CDD can cause non-alcohol related liver damage, which can be attenuated by a SPD in wild-type mice. These phenomena were not observed in liver-specific *phb1* +/− mice. It may therefore be concluded that SPD attenuates CDD-induced liver damage in wild-type mice, and that PHB1 deficiency blocks the beneficial effects of SPD against CDD-induced liver damage.

Keywords: non-alcoholic fatty liver disease, methionine, soy protein, prohibitin 1, isoflavone

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently the most common chronic liver disease (1). The estimated prevalence of NAFLD is approximately 10～24% in various countries (2). NAFLD comprises of various kinds of liver damage, such as simple hepatic steatosis, nonalcoholic steatohepatitis (NASH), progressive fibrosis, and cirrhosis (3). These diseases can develop into liver failure and further, hepatocellular carcinomas (4). The mechanisms responsible for NAFLD pathogenesis are complex and have not yet been fully established. Hepatic steatosis is brought about by the following mechanisms: increased uptake of free fatty acid (FFA) into the liver, increased lipogenesis, decreased β-oxidation, and impaired hepatic export of triglycerides as very low density lipoproteins (VLDL) (5). Moreover, FFA-induced reactive oxygen species (ROS) production, lipid peroxidation, apoptosis, and inflammation can cause NAFLD and hepatocyte injury (6). A methionine choline-deficient (MCD) diet has frequently been used as a nutritional model of NASH (7). The MCD diet increases amounts of alanine aminotransferases (ALT) and induces hepatic histological changes, such as steatosis, focal inflammation, hepatocyte necrosis, and fibrosis (8). These histological changes occur rapidly, and are morphologically similar to changes observed in human NASH.

Methionine, which is mainly metabolized by the liver, is an indispensable amino acid and a precursor of many dispensable amino acids such as homocysteine, cysteine, and cystine (9). Thus, methionine deficiency results in decreases synthesis of other amino acids and consequential deficiency symptoms. Studies show that methionine deficiency induces growth retardation, decreases in levels of ALT and depletion of glutathione (GSH), and contributes to development of NAFLD through lipid deposition, inflammation, necrosis, and fibrosis (8,10,11). Whereas methionine supplementation increases accumulation of

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collagen in liver tissues of fibrotic models (12). Moreover, abnormalities in methionine metabolism can lead to liver diseases. For example, methionine adenosyltransferase 1 alpha (*Mat1α*) deletion in mice reduces hepatic *S*-adenosylmethionine (SAMe) and GSH contents, resulting in oxidative stress and liver tumors (13). In patients with liver disease, enzymes such as MAT1A, glycine *N*-methyltransferase (GNMT), and cystathionine β-synthase (CBS) involved in methionine metabolism are abnormally expressed (14).

Soy protein is a good source of protein. However, the level of methionine is relatively low. Since methionine is the first limiting amino acid of soy protein (15), abnormal changes in methionine metabolism can occur when soy is the major dietary source of protein. Furthermore, protein synthesis during methionine deficiency may be limited in those whose main food is beans, or in vegetarians who consume soybean protein as their major protein source. Since methionine is the initiating amino acid in eukaryotic protein synthesis (16), cellular protein synthesis cannot be initiated in methionine-deficiency, which can lead to decreased growth (17). There are many studies suggesting that soy proteins are beneficial to health, due to encompassing hypocholesterolemic, anti-carcinogenic, bone-sparing effects, hypotensive activity, and protection against metabolic diseases such as obesity or diabetes (18-22). However, little is known about the adverse effects of soy proteins, with the exception of allergic reactions and poor their digestibility acting as a trypsin inhibitor (21,22). Despite the abundance of soy protein studies, few have examined development of NAFLD due soy protein-induced methionine deficiency. Therefore, the purpose of this study was to investigate the effect of a methionine-deficient soy protein diet on NAFLD and growth retardation in mouse.

Prohibitin1 (PHB1) is a pleiotropic protein that has been preserved for a long time. PHB1 functions in various ways dependent on the cell type and subcellular location (23). The role of PHB1 in cancer remains controversial, however it has been reported that PHB1 acts as a tumor suppressor in the liver. Ko et al. (24) did not observe cristae or mitochondrial abnormalities in 3-week-old mice, whereas oxidative stress and hepatocellular carcinomas were observed in 8-month-old liver-specific *Phb1* knockout mice. In addition, five-month-old male *Phb1* heterozygous mice developed steatohepatitis, and upregulated expression of pro-inflammatory cytokines after feeding on an MCD diet for 3 weeks (25). Expression of *Phb1* is closely related to liver disease susceptibility, and a number of relevant studies are currently in progress. Studies conducted in our laboratory have so far revealed that *Phb1* deficiency likely increases susceptibility to hepatotoxicity due to a variety of external challenges. When *Phb1* is deficient, mice show a higher sensitivity to alcohol toxicity, combined with increases inflammation and abnormal lipid metabolism (data not yet published). We therefore also investigated whether a methionine-deficient soy protein diet contributes to the pathogenesis of NAFLD, and if NAFLD deteriorates when liver-specific *Phb1* is deficient.

MATERIALS AND METHODS

Diet compositions

Three diets were used in this study to evaluate the contribution of methionine, the essential nutritional component. The first was the normal diet (Pico 5053) as a control. The second was a choline-deficient diet (CDD), custom-made with a modified AIN-93G diet, with no added choline bitartrate product. The third was an experimental soy protein diet (SPD), custom-made with a modified AIN-93G diet and soy protein, with no added choline bitartrate product. The SPD replaced casein protein, the source of protein in the normal diet and CDD, with soy protein. Detailed amino acid compositions of the diets are shown in Table 1. The normal diet and CDD contained 0.62% and 0.51% methionine, respectively, while SPD contained 0.22% methionine. The food was stored at 4°C prior to consumption.

Table 1. Amino acid composition of experimental diets

Amino acid	Composition (g% protein)		
	Normal diet	CDD	SPD
Essential amino acids			
Histidine	0.53	0.45	0.47
Isoleucine	0.86	0.75	0.88
Leucine	1.57	1.57	1.45
Lysine	1.18	1.31	1.12
Methionine	0.62	0.51	0.22
Phenylalanine	0.91	0.84	0.92
Threonine	0.78	0.72	0.67
Tryptophan	0.24	0.21	0.24
Valine	0.97	0.92	0.90
Non-essential amino acids			
Alanine	1.19	0.51	0.75
Arginine	1.29	0.59	1.35
Aspargine	0.00	0.70	0.00
Aspartic acid	2.19	0.51	2.06
Cysteine	0.36	0.42	0.20
Glutamine	0.00	1.71	0.00
Glutamic acid	4.18	2.08	3.39
Glycine	0.97	0.30	0.75
Proline	1.31	1.76	0.90
Serine	0.98	0.99	0.92
Tyrosine	0.60	0.91	0.67

CDD, choline-deficient diet; SPD, soy protein diet.

Animal experiments

For liver-specific *Phb1* knockout mice, it is impossible to examine the sensitivity of liver disease due to severe liver damage from birth. Therefore, it is necessary to use liver-specific *Phb1* heterozygous mice to conduct efficient research. Liver-specific *Phb1* +/− mice and wild type mice were raised in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Male mice (3 ~4 weeks of age) were fed with a normal diet, CDD, or SPD for 16 weeks. Body weight and food intake were measured twice a week. Mice were anesthetized with 1% isoflurane (Piramal Critical Care Inc., Bethlehem, PA, USA) and sacrificed. Blood was collected by cardiac puncture, and the liver was removed. Serum and liver samples were stored at −80°C until analysis. All experimental animals were handled and treated in compliance with IACUC standards of Ewha Womans University (approval number 18-021).

Serum ALT and aspartate aminotransferase (AST)

The activities of ALT and AST, which are used as liver injury index, were assessed using an ALT and AST kit, according to manufacturers' instructions (Asan Pharm Co., Ltd., Hwaseong, Korea).

Hepatic GSH concentration

The concentration of GSH was estimated by reducing total oxidized GSH using GSH reductase (Sigma-Aldrich Co., St. Louis, MO, USA). Liver tissue was homogenized by adding 10 times phosphate buffered saline, and centrifuged to measure the protein concentration of the supernatant. The protein was deposited from the supernatant with the same amount of 0.6 M perchloric acid. The content of GSH was assessed by mixing 0.01 mL of sample with 0.1 mL GSH reductase (10 units/mL) and 2.5 mL of reaction buffer [1.5 mM ethylenediaminetetraacetic acid (Sigma-Aldrich Co.), 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich Co.), 0.15 mM nicotinamide adenine dinucleotide (Sigma-Aldrich Co.), 50 mM $NaPO_4$ (Junsei Chemical Co., Ltd., Tokyo, Japan)], and measuring the change of absorbance at 412 nm between 0 and 60 s. From the GSH standard curve obtained using GSH (Sigma-Aldrich Co.), GSH concentrations in the samples were calculated in nmol/mg protein.

Analysis of histologic changes

In order to analyze histological changes in the liver tissues, the right lateral lobes of various nutritional groups were excised. Hematoxylin-eosin (H&E) staining was conducted to observe liver damage, and lipid content was verified by Oil red O staining.

RNA isolation and quantitative reverse-transcription polymerase chain reaction (PCR)

Trizol solution (Life Technologies Inc., Carlsbad, CA, USA) was used for total RNA isolation in liver tissue. The total RNA of the sample was synthesized by a first-strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). The cDNA was used as a template for quantitative reverse-transcription PCR (qPCR). qPCR was conducted using Maxima SYBR Green qPCR Master Mixe (Thermo Scientific). Relative quantitative analysis of target genes were calculated as expression ratios of the housekeeping gene.

Statistical analysis

Results are described as a mean±standard error of mean (SEM). Data were analyzed using SAS ver. 9.4 (SAS Institute Inc., Cary, NC, USA). The mean difference between experimental diet groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range post-hoc test, to verify statistical significant differences. Statistical significance (*P*-value) was verified at 5%.

RESULTS

Changes of body and liver weight

The body weight of all mice was increased by 14.07± 0.73 g during the experimental period (16 weeks). There was no significant difference in body weight among groups. However, liver weight, especially relative liver weight (liver weight/body weight ratio) was significantly lowered in mice fed with the CDD and SPD, compared with those receiving the normal diet (Table 2).

Changes of ALT and AST levels

Levels of biochemical serum markers of liver injury, ALT and AST level, were measured. ALT had a tendency to increase in mice fed with the CDD compared with the normal diet. The serum level of ALT decreased in mice fed with the SPD compared with the CDD. For serum AST, there were no significant changes between the experimental groups (Fig. 1A).

Changes of total hepatic GSH concentration

To evaluate the hepatic antioxidant capacity, total GSH concentrations were measured. The GSH concentration was significantly decreased in mice fed with the CDD and SPD compared with the normal diet. Mean hepatic GSH concentrations (nmole/mg protein) of each group were 191.18±12.31 (normal diet), 120.59±7.65 (CDD), and 136.30±10.25 (SPD) (Fig. 1B).

Histopathological changes in liver

For histopathological analysis, liver tissues were stained

Table 2. Physical changes of mice in experimental groups

	Wild type			Liver-specific *Phb1* +/−		
	Normal	CDD	SPD	Normal	CDD	SPD
Baseline body weight (g)	18.23±1.29	18.69±0.94	18.01±1.09	16.40±1.22	18.57±1.39	16.30±1.24
Final body weight (g)	31.16±0.56	32.79±1.20	33.29±1.63	30.40±0.96	32.48±0.21	30.25±2.66
Body weight change (g)	12.94±0.94	14.10±0.82	15.28±1.40	14.00±1.22	13.92±1.63	13.95±3.12
Liver weight (g)	1.28±0.07^{a}	1.10±0.06^{ab}	1.10±0.09^{ab}	1.28±0.03^{a}	1.06±0.05^{b}	0.91±0.07^{b}
Relative liver weight (%)	4.09±0.18^{a}	3.35±0.10^{b}	3.28±0.15^{b}	4.22±0.11^{a}	3.27±0.11^{b}	3.06±0.23^{b}
Average daily food intake (g)	4.87±0.06^{a}	3.61±0.04^{c}	3.53±0.07^{cd}	4.25±0.00^{b}	3.38±0.03^{ce}	3.27±0.17^{e}
Food efficiency[1)]	2.45±0.93^{b}	3.88±0.35^{a}	3.67±0.21^{a}	3.02±1.35^{ab}	3.78±0.46^{a}	3.85±0.84^{a}

Results are expressed as mean±standard error.
Difference letters (a-e) with thin same row indicate significant differences (P<0.05).
CDD, choline-deficient diet; SPD, soy protein diet.
[1)]Feed efficiency=[increase in body weight (g)/food intake (g)]×100.

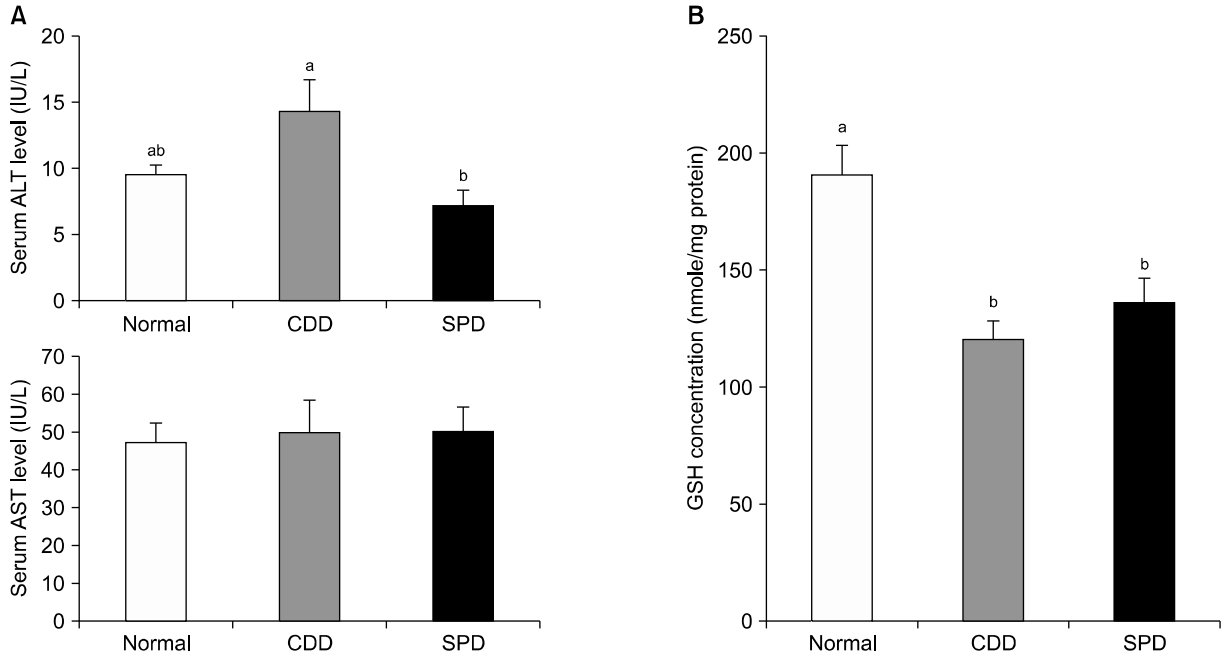

Fig. 1. Effects of the experimental diets on (A) serum alanine transaminase (ALT) and aspartate transaminase (AST) levels and (B) hepatic glutathione (GSH) concentration in mice. The choline-deficient and soy protein diets (CDD and SPD, respectively) decrease GSH concentration compared to normal diet. Each bar represents the mean±SEM. Different letters (a,b) indicate significant differences among diets (P<0.05).

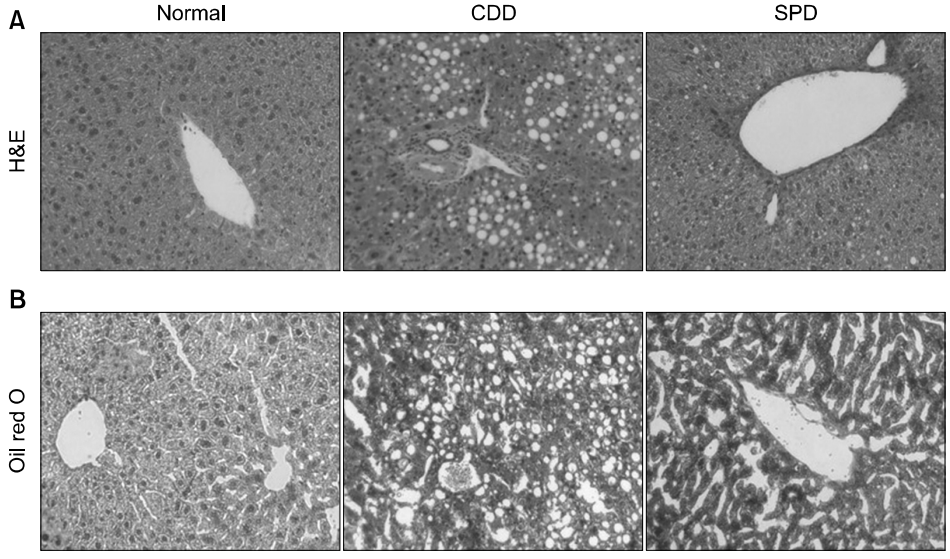

Fig. 2. Histopathological changes in the livers of mice fed the normal, choline-deficient diet (CDD), or soy protein diet (SPD). Liver tissues were stained with (A) hematoxylin and eosin (H&E) and (B) Oil red O (Original magnification 200×). CDD triggers steatosis and inflammatory cell infiltrations but SPD attenuates the symptoms.

with H&E and Oil red O (Fig. 2). The livers of mice fed with the CDD showed an abundance of lipid droplets and vesicle, and hepatocyte necrosis and inflammation in the liver parenchyma and portal duct. The livers of mice fed with the SPD showed small amounts of lipid droplets and lower infiltration of inflammatory cells into the area surrounding the hepatic portal vein. Since hepatic lipid deposition is a key component of NAFLD, the liver samples were processed for oil red O staining to examine the effect of experimental diets on hepatic steatosis. The CDD and SPD induced hepatic lipid accumulation.

mRNA expression of lipid metabolism-related genes in wild type mice

To evaluate whether or not the observed changes in gene expression further altered hepatic lipid metabolism, expression of liver lipid metabolism-related genes were examined (Fig. 3A). Fatty acid translocase (*Cd36*), a gene involved in the uptake of fatty acids, and carnitine palmitoyltransferase 1 a (*Cpt1a*), gene involved in the mitochondrial β-oxidation, showed higher expression in mice fed the CDD compared with the normal diet, but lower expression in mice fed the SPD compared with the CDD. The CDD increased mRNA expression of acetyl-CoA carboxylase 2 (*Acc2*) compared with the normal diet, and the SPD increased mRNA expression of *Acc2* compared with the CDD. Furthermore, the CDD decreased mRNA expression of fatty acid synthase (*Fasn*) compared with the normal diet, and the SPD increased expression of *Fasn* compared with the CDD. The CDD caused upregulation of stearoyl-CoA desaturase-1 (*Scd1*), a gene involved in the lipogenesis, compared with the normal diet. However, the SPD did not increase mRNA expression of *Scd1* compared with the normal diet.

mRNA expression of inflammation-related genes in wild type mice

To investigate the inflammatory effects of the experimental diets, mRNA expression of pro- and anti-inflammatory markers were measured (Fig. 3B). Results showed that mRNA expression of tumor necrosis factor α (*Tnf-α*) is increased in mice fed the CDD compared with the normal diet. However, the SPD did not increase mRNA expression of *Tnf-α* compared with the normal diet. In addition, mRNA expression of interleukin-10 (*Il-10*) was increased in mice fed the SPD compared with the normal diet and CDD.

mRNA expression of methionine metabolism-related genes

To further evaluate the effect of the CDD and SPD on methionine metabolism, and the effect of PHB1 on the experimental diets, mRNA expression of genes in the methionine metabolic network was examined (Fig. 4).

Mat1a, *S*-adenosylhomocysteine hydrolase (*Sahh*), and betaine-homocysteine methyltransferase (*Bhmt*) expressions were higher in wild-type mice fed with both the CDD and the SPD, than the normal diet. In liver-specific *phb1* +/− mice, *Mat1a* was upregulated in those fed the CDD compared with the normal diet; however, there was no difference in expression was observed in mice fed the SPD and the normal diet. Moreover, in liver-specific *phb1* +/− mice, expression of *Gnmt*, *Sahh*, *Bhmt*, and methionine synthase (*Ms*) showed a tendency for downregulation following consumption of the SPD compared with the CDD.

Expression of *Cbs* was not altered by the experimental diets in either wild type or liver-specific *phb1* +/− mice. Expression of cystathionase (*Cth*) was higher in wild-type mice fed both the CDD and SPD, compared with the normal diet, but no differences in *Cth* expression were recorded in liver-specific *phb1* +/− mice. Expressions of glutamate-cysteine ligase catalytic subunit (*Gclc*), glutamate-cysteine ligase modifier subunit (*Gclm*), and GSH synthetase (*Gss*), which are involved in the synthesis of GSH, were significantly lower in liver-specific *phb1* +/− mice fed the SPD compared with than the normal diet;

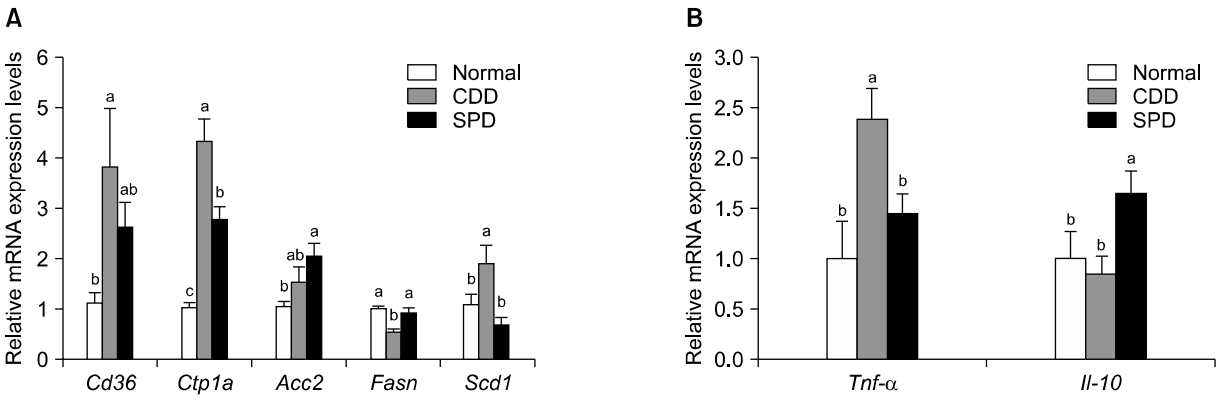

Fig. 3. mRNA expression levels of (A) lipid metabolism genes and (B) inflammatory cytokines in the livers of wild type mice fed the normal, choline-deficient diet (SDD), or soy protein diet (SPD). The expression of *Cd36, Ctp1a, Acc2, Fasn, Scd1, Tnf-α,* and *Il-10* was determined by quantitative reverse-transcription-polymerase chain reaction. Each bar represents the mean±SEM. Different letters (a-c) indicate significant differences among diets (P<0.05).

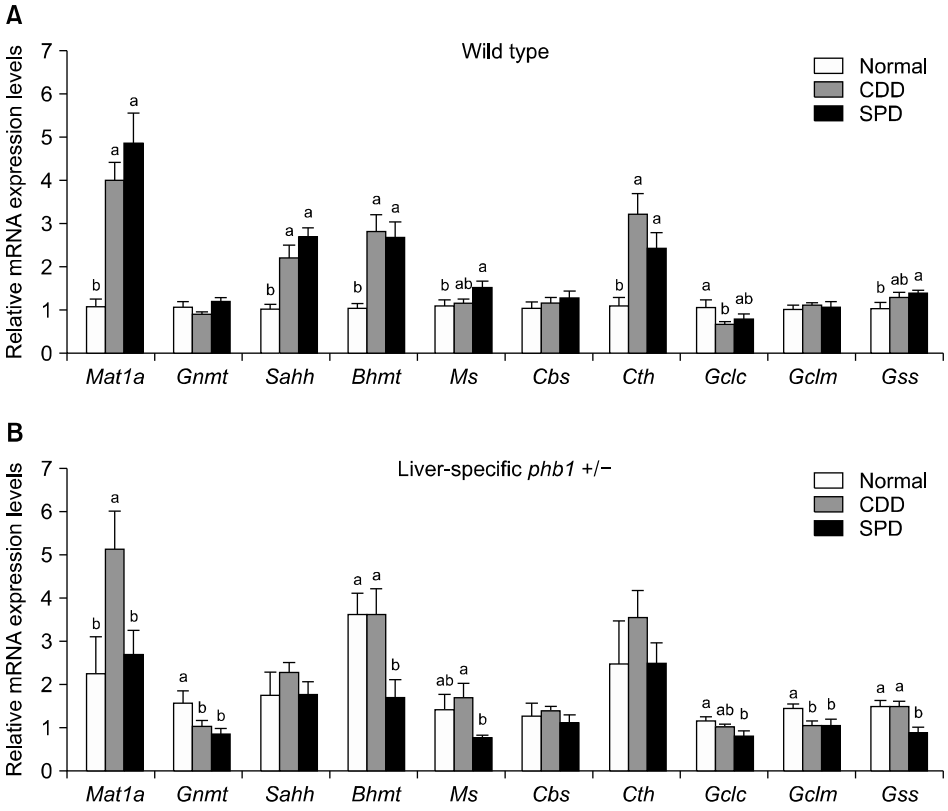

Fig. 4. mRNA expression levels of methionine metabolism genes in the livers of (A) wild type mice and (B) liver specific Phb1 heterozygous mice fed the normal, choline-deficient diet (SDD), or soy protein diet (SPD). The expression of *Mat1a*, *Gnmt*, *Sahh*, *Bhmt*, *Ms*, *Cbs*, *Ggl*, *Gclc*, *Gclm*, and *Gss* was determined by quantitative reverse-transcription-polymerase chain reaction. Each bar represents the mean± SEM. Different letters (a,b) indicate significant differences among diets (P<0.05).

no differences in expression of these genes were observed in wild-type mice.

DISCUSSION

The aims of this study were to investigate if soy protein diet impacts NAFLD, and to identify if deficiency of *Phb1* in mouse liver affects the pathogenesis of NAFLD. Because choline is a dietary methyl donor and is involved in remethylation (26), the CDD lowers methionine formation in animal livers by 20∼25% and induces fatty liver (27). Therefore, we used CDD as a negative control for NAFLD deterioration when the methionine-deficient soy protein diet was ingested with choline removed.

Previous studies suggest the MCD and SPD cause growth retardation in rats (8,28). Furthermore, that liver-specific *Phb1* knockout mice have retarded growth (24). However, in the experimental period of the present study, weight loss was not observed, and any differences in final body weight between groups were not significant (Table 2). These results may be explained by the nutritional content of the diets. Complete methionine deficiency triggers loss of body weight; however, the methionine content of soy protein is less than 50% of casein protein used in the normal diet and CDD (Table 1). Soy protein showed no significant effect on the body weight of mice when they were fed the SPD for 16 weeks, which indicates supplementation of methionine at approximately 50% of normal levels does not influence growth retardation. Furthermore, mouse body weight did not correlate with genotype; the liver-specific *Phb1* knockout mice used in this experiment were heterozygotes, suggesting that presence of at least one *Phb1* allele does not affect growth. However, a previous study showed liver weight loss in mice fed the SPD (28). Relative liver weight was significantly lowered in mice fed the CDD and SPD compared with the normal diet (Table 2). This increase in relative liver weight reflects the hepatomegaly caused by hepatitis, hepatotoxins, and liver tumors (29). However, the results of this study indicate that mice fed the CDD and SPD are not affected by severe liver disease.

Serum ALT and AST levels were additionally assessed (Fig. 1A). Although levels of ALT significantly differed in wild type mice by diet consumed, the magnitude of changes remained within the normal range for ALT in mice (30). Therefore, levels of ALT and AST were not affected by the experimental diets.

Since antioxidant functioning affects physiological metabolism in animals, it is important to assess the biological antioxidant capacity. The hepatic concentration of GSH is one of the major indicators to protect the liver against oxidative stress, such as ROS, and determines susceptibility to liver injury (31). To investigate the effect of soy protein on liver function, hepatic GSH concentrations were measured. Intracellular hepatic GSH concentrations were significantly reduced in mice fed the CDD and SPD compared with the normal diet (Fig. 1B), which

is in agreement with recent studies of mice fed the CDD and MCD (10). Hepatic GSH levels are decreased in NAFLD patients (32). In the present study, no significant difference in hepatic GSH concentrations were observed between mice fed the CDD and SCD. Thus, it can be suggested that choline deficiency, and not methionine deficiency in the soy protein, is responsible for reductions in levels of GSH.

Lipid metabolism is important to the pathogenesis of NAFLD. Obvious differences in histopathological lipid content were observed between mice fed the normal diet and the other two diets, shown using Oil red O staining. In mice fed the CDD and SPD, the liver tissue was stained red, confirming lipid accumulation (Fig. 2B). However, a greater amount of lipid droplets and indicators of necrosis were shown in the liver of mice fed the CDD compared with the SPD (Fig. 2A). Although no major histological differences were observed between the CDD and SPD using Oil red O staining; however, the SPD attenuated substantial hepatocellular damage caused by CDD, observed using H&E staining.

Following on from these histological results, expression of key lipid metabolic genes were examined (Fig. 3A). Expression of *Cd36* and *Cpt1a*, genes involved in uptake of fatty acids (FAs) and β-oxidation, respectively, were increased by the CDD and SPD compared with the normal diet. As influx of FAs into hepatocytes is increased or decreased, FA oxidation occurs. Even if FA oxidation is increased by the CDD and SPD, it would not be able to compensate for diet-induced increases in hepatocyte FAs. Of genes involved in lipid accumulation, ACC2 regulates FAs β-oxidation by inhibiting CPT1, and FASN is an enzyme of *de novo* lipogenesis. Moreover, SCD1 plays a key role in the synthesis of TG. Expression of *Scd1* was increased in mice fed the CDD compared with the normal diet, but was not increased in mice fed the SPD compared with the normal diet. According to previous studies, consumption of a casein protein diet increases levels of serum insulin and hepatic sterol regulatory element-binding protein 1 (*Srebp-1*) and induces fatty liver in rats. However, consumption of a soy protein diet decreases serum insulin and hepatic *Srebp-1* levels, and reduces fatty liver (33). SREBP-1 serves as a transcription factor to regulate expression of SCD-1 (34). Therefore, soy protein may attenuate lipid accumulation induced by choline deficiency in the diet in mice.

In the immune system, cytokines secreted by immune cells control inflammatory responses and play important roles in cell survival, growth, and proliferation (35,36). Therefore, it is important to properly modulate the inflammatory response. The pro-inflammatory cytokine TNF-α plays important roles in liver injury (37). Expression of *Tnf-α* was increased in mice fed the CDD compared with the normal diet, but not in mice fed the SPD (Fig. 3B). It can therefore be suggested that SPD alleviates the inflammatory effect of choline deficiency. Previous studies indicate that soy protein enriched in isoflavone markedly reduces expression of *Tnf-α* in the liver of obese or nephrotic rats (38,39). In this study, expression of *Il-10*, an anti-inflammatory cytokine, was increased in mice fed the SPD compared to the other two experimental groups. This anti-inflammatory property of soy protein is due to isoflavone, which is the active compound in soy and modulates immune response (40). Therefore, soy protein inhibits induction of liver injury development.

To identify the effect of methionine-deficient soy protein on hepatic methionine metabolism and of *Phb1* deficiency on hepatic methionine metabolism, expression of genes involved in methionine metabolism were examined. Expression of genes related to the methionine cycle, including *Mat1a*, *Gnmt*, *Sahh*, *Bhmt*, and *Ms*, showed different patterns of expression between wild-type and liver-specific *Phb1* +/− mice. MAT1A catalyzes the first step in the conversion of methionine to homocysteine. Expression of *Mat1a* was increased in wild-type mice fed the CDD and SPD compared with the normal diet; there is lower extrinsic methionine to maintain homeostasis of hepatic SAMe, and lower amounts of methyl donors for re-synthesis of intrinsic methionine in mice fed the CDD and SPD. However, in liver-specific *Phb1* +/− mice, the CDD, but not the SPD, increased expression of *Mat1a*. It can be elucidated that in *Phb1* deficient mice, the SPD inhibits the increase in *Mat1a* expression in the recovery system to maintain SAMe. The subsequent genes in methionine metabolism *Gnmt*, *Sahh*, *Bhmt*, and *Ms* showed a downregulated trend in liver-specific *phb1* +/− mice fed the SPD compared with the CDD. The SPD decreases overall methionine cycle pathway reactions when liver-specific *Phb1* is deficient in mice. Expression of *Cbs* was not affected by the experimental diets or genotypes. *Cth* expression was increased in wild-type mice fed the CDD and SPD compared with the normal diet to increase cysteine, however this was not observed in liver-specific *Phb1* +/− mice. These results suggest that methionine metabolism is remarkably decreased in liver-specific *Phb1* +/− mice fed the SPD. In patients with liver disease, hepatic mRNA expression of *Mat1a*, *Gnmt*, *Bhmt*, *Cbs*, and *Ms* are significantly reduced compared with those without (41), and is coupled with abnormal methionine metabolism (42,43). Moreover, *Mat1a* (44), *Gnmt* (45,46) *Bhmt* (47), and *Cbs* (48) knockout mice, and people with mutations in genes such as *Sahh* (49,50), are vulnerable to liver disease. Thus, abnormal methionine metabolism by methionine-deficient soy protein is observed in liver-specific *Phb1* mice, and this abnormal methionine metabolism may contribute to development of NAFLD.

GSH synthesis involves the two important enzymes glutamate-cysteine ligase (GCL) and GSS. GCL is the rate-

limiting enzyme in GSH biosynthesis and is composed of catalytic and modifier subunits, GCLC and GCLM (51). *Gclc* heterozygous mice have decreased levels of GCLC protein, and of GSH (52). Thus, reductions of GSH may be caused lowered *Gclc* expression. In this study, expression of *Gclc*, *Gclm*, and *Gss* was significantly decreased in liver-specific *Phb1* +/− mice fed the SPD compared with the normal diet. Therefore, soy protein likely reduces GSH through decreasing *Gclc*, *Gclm*, and *Gss* in liver-specific *Phb1* deficient mice.

In the present study, we examined the contribution of soy protein to NAFLD induced by CDD. When wild-type mice were fed soy protein, hepatic fat accumulation and inflammation resulting from choline deficiency was relieved; this was confirmed by gene expression analysis. This preventive effect of soy protein on fatty liver may be due to the antioxidant ability of substances such as isoflavone and soy peptide in soy protein. Another important finding was that methionine metabolism was impaired in liver-specific *Phb1* +/− mice fed the SPD since the beneficial effects of soy protein for CDD-induced liver damage were diminished. Since hepatic metabolism does not occur smoothly by feeding soy protein in animals lacking *Phb1*, there is a possibility that NAFLD susceptibility may increase in those with lowered hepatic *Phb1* expression following long-term consumption of soy proteins.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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