Effects of Chronic Whey Protein Supplementation on Atherosclerosis in ApoE\(^{-/-}\) Mice

Zheng ZHANG, Ru ZHANG, Zhi-Zhen QIN, Jia-Ping CHEN, Jia-Ying XU and Li-Qiang QIN

1 Department of Nutrition and Food Hygiene, School of Public Health, Soochow University, Suzhou, 215123, China
2 School of Public Health, Hebei Medical University, Shijiazhuang, 050017, China
3 Key Laboratory of Radiation Biology, School of Radiation Medicine and Protection, Soochow University, Suzhou, 215123, China
4 Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Disease, School of Public Health, Soochow University, Suzhou, 215123, China

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Summary Whey protein is associated with improvement of metabolic syndrome. This study aimed to evaluate effects of whey protein on atherosclerosis in ApoE\(^{-/-}\) mice. Male ApoE\(^{-/-}\) mice were fed with a high-fat/cholesterol diet (HFCD), or HFCD supplemented with 10% or 20% whey protein for 18 wk. At the end of experiment, serum lipid profiles and inflammatory cytokines were assayed. Livers were examined using HE staining and Oil Red O staining. Aortas were used for en face and cryosection analyses to observe aortic lesions. Western blotting analysis was used to assess relative protein expression of cholesterol metabolism in the liver and aorta. No significant differences were observed in body weight or food intake among the three groups. Liver examination demonstrated decreased lipid droplets and cholesterol content in the whey-protein-supplemented groups. En face lesion of the aorta revealed a 21.51% and 31.78% lesion reduction in the HFCD supplemented with 10% and 20% whey groups, respectively. Decreased lesion was also observed in cryosection analysis. Whey protein significantly increased the serum high-density lipoprotein cholesterol level by 46.43% and 67.86%. The 20% whey protein significantly decreased serum IL-6 (a proinflammatory cytokine) by 70.99% and increased serum IL-10 (an anti-inflammatory cytokine) by 83.35%. Whey protein potently decreased lipogenic enzymes (ACC and FAS) in the liver and NF-B expression in the liver and aorta. Whey protein significantly increased protein expression of two major cholesterol transporters (ABCA1 and ABCG1) in the liver and aorta. Thus, chronic whey protein supplementation can improve HFCD-induced atherosclerosis in ApoE null mice by regulating circulating lipid and inflammatory cytokines and increasing expressions of ABCA1 and ABCG1.

Key Words whey protein, atherosclerosis, inflammation, dyslipidemia, reverse cholesterol transport

Atherosclerosis is a complicated vascular disorder and associated with severe cardiovascular disease. Dyslipidemia triggers formation of atherosclerosis (1). Enhanced cholesterol efflux and reverse cholesterol transport are considered important targets for antiatherosclerosis (2). Atherosclerosis is a chronic inflammatory disease. Scientific interest has focused on the role of cytokines as possible therapeutic agents for atherosclerosis (3). Hence, dyslipidemia and inflammatory response have been described as “partners in atherosclerosis” (4).

Whey protein, a cheese byproduct, comprises \(\alpha\)-lactalbumin, \(\beta\)-lactoglobulin, lactoferrin, and other proteins; this byproduct is also rich in branched chain amino acids, such as leucine (5). As a functional component in milk, whey protein and its derived active peptides exert pleiotropic effects on the prevention of obesity, hypertension, nonalcoholic fatty liver, and type 2 diabetes mellitus (6–11). Obesity, hypertension, and other metabolic syndromes are strongly related to dyslipidemia and inflammatory response, which are risk factors for atherosclerosis (1, 12). Thus, atherosclerosis may be prevented using whey protein supplementation.

Apolipoprotein E (ApoE) is a ligand for receptors that clear remnants of chylomicrons and very low-density lipoproteins (VLDL) (13). Lack of ApoE is, therefore, expected to result in the over-accumulation of cholesterol-rich remnants in plasma, which would be atherogenic in the long term. Thus, the ApoE\(^{-/-}\) mouse model is considered an ideal model for researching atherosclerosis (14). In the present study, we investigated whether chronic whey protein supplementation exerted a direct effect on the aorta and liver of ApoE\(^{-/-}\) mice fed with a high-fat/cholesterol diet (HFCD). Circulating lipids,
inflammatory cytokines, and protein expression related to cholesterol metabolism in the aorta and liver were observed.

MATERIALS AND METHODS

Treatment of animals. Six-week-old male ApoE\(^{-/-}\) mice were obtained from Shanghai Laboratory Animal Company (Shanghai, China) and housed in an air-conditioned environment (22 ± 2°C) with 60% humidity and a 12 h light/dark cycle. After 7 d of acclimatization, animals were randomly allocated to the following three dietary groups with eight mice in each group: 1) HFCD group, 2) HFCD supplemented with 10% whey protein (HFCD\,+10% whey group), and 3) HFCD supplemented with 20% whey protein (HFCD\,+20% whey group). HFCD was obtained from Research Diets Inc. (New Brunswick, NJ, D12451+1% cholesterol). Whey protein concentrate powder (81.4% protein) was obtained from Warrnambool Cheese and Butter Factory Co., Ltd. (Allansford, Australia). Whey protein was directly mixed with HFCD for intervention diets. The compositions of energy-yielding nutrients and energy are shown in Table 1. The energy ratio was higher from protein and lower from carbohydrate and fat when the whey supplemented diets were compared with the HFCD diet. The energy content in the HFCD, HFCD\,+10% whey and 20% whey diets was 4.79, 4.66 and 4.53 kcal/g, respectively. Animals were allowed access to diet and water ad libitum. During the experiment, body weights and food intake were recorded weekly. Energy intake was calculated. The experiment lasted for 18 wk. All animal studies were performed in accordance with the Guidelines in the Care and Use of Animals and with the approval of the Soochow University Animal Welfare Committee (201607A243).

Determination of serum lipids and inflammatory cytokines. At the end of the experiment, blood samples were obtained from the retrobulbar vein of mice fasted for 12–14 h. Serum was separated and stored at −80°C until assayed. Serum total cholesterol (TC) and triglyceride were determined by enzymatic methods using commercial kits (Applygen Technologies Inc., Beijing, China). Serum LDL cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and non-esterified fatty acid (NEFA) were measured with kits from Biosino Biotechnology and Science Inc. (Beijing, China). Additionally, serum non-HDL-C was obtained by subtracting HDL-C from TC. Serum levels of tumor necrosis factor α (TNF-α), monocytechemotactic protein-1 (MCP-1), interferon γ (IFN-γ), interleukin (IL)-6, IL-10, and IL-12 were determined by BD Cytometric Bead Array (BD Biosciences, Franklin Lakes, NJ). Serum C-reactive protein (CRP) level was measured with a kit obtained from R&D Systems, Inc. (Minneapolis, MN).

Management of liver and aortas. When mice were sacrificed, liver and aortic tissues were quickly removed. Liver slices were processed with standard hematoxylin and eosin (HE) staining and Oil Red O staining. Histological changes and fat accumulation were observed under an optical microscope. Parts of the liver tissue were homogenized. The hepatic levels of TC and triglyceride were extracted and analyzed with commercialized kits (Applygen Technologies Inc., Beijing, China) and normalized to protein concentration. Protein concentration was measured using a bicinchoninic acid (BCA) assay kit (Applygen Technologies Inc., Beijing, China). Aortic atherosclerotic lesions were observed using two methods: en face analysis of the entire aortic tree and cryosection analysis of aortic tissues. For en face analyses, aortas were longitudinally opened from the heart to iliac arteries. Atherosclerotic lesions were stained with Oil Red O and positive areas were quantified using Image Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD). For cryosection analysis, serial 6 mm-thick sections of mouse aorta, which begin at the aortic sinus, were collected. Sections were also stained with Oil Red O.

Table 1. The compositions of energy-yielding nutrients (g/100 g diet) and energy (kcal/g diet) in the experimental diets.

| Nutrient          | HFCD   | HFCD\,+10% whey | HFCD\,+20% whey |
|-------------------|--------|-----------------|-----------------|
| Protein           | 23.73  | 30.94           | 37.97           |
| Carbohydrate      | 40.54  | 36.00           | 31.57           |
| Fat               | 24.73  | 22.06           | 19.46           |
| Cholesterol       | 1.00   | 0.99            | 0.98            |
| Energy            | 4.79   | 4.66            | 4.53            |
Western blot analysis of liver and aortas. Aorta and liver samples were homogenized in lysis buffer and centrifuged at 13,000×g for 15 min. When supernatants were collected, protein concentration was determined according to BCA protein assay (Beyotime Institute of Biotechnology, Nantong, China). Equal amounts of protein (30–60 μg) were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane by electrophoretic transfer. Blots were incubated overnight at 48°C with the following primary antibodies: acetyl-coenzyme A carboxylase (ACC, 1:1000), fatty acid synthase (FAS, 1:2000), liver X receptor α (LXRα, 1:2,000), LDLR (1:2,000), ATP-binding cassette transporter A1 (ABCA1, 1:2000), ATP-binding cassette transporter G1 (ABCG1, 1:1000), and NF-κB (1:1000). ACC and FAS were obtained from Cell Signaling Technology (Danvers, MA). Other antibodies were purchased from Abcam (Cambridge, MA). Subsequently, blots were washed thrice, and antigen–antibody complexes were incubated for 1 h at room temperature with Peroxidase AffiniPure goat antimouse or antirabbit IgG antibody (1:4,000, Jackson ImmunoResearch Inc., West Grove, PA). Antibody reactivity was detected by chemiluminescence ECL Detection Systems (EMD Millipore, Darmstadt, Germany). Band intensity was normalized using β-actin density as internal control. To confirm reproducibility of results, each blot analysis was performed at least thrice.

Statistical analysis. All statistical parameters were calculated using an SPSS version 19.0 statistical analysis package (SPSS Inc., Chicago, IL) and expressed as a mean and standard deviation (SD). One-way ANOVA was used to analyze differences among groups, followed by the LSD post hoc test. A p<0.05 was considered statistically significant.

RESULTS

Body weight and food intake

Body weights among the three groups (approximately 21 g) were comparable at baseline. Body weight increased during the experiment and was slightly low in the whey-protein-supplemented groups before week 10 (Fig. 1A). No significant differences were observed among the three groups at any time point. During the experiments, mice in the HFCD +10% whey group had a slightly higher intake of diet (Fig. 1B). The energy intake was 17.64±0.49, 17.76±0.60 and 18.37±0.65 kcal/d in the HFCD group, HFCD +10% whey group and HFCD +20% whey group, respectively (Fig. 1C). No significant difference was observed in average food intake.
or energy intake among the three groups.

Liver staining and lipid contents

Liver HE staining showed pathological symptoms in the HFCD group with lipid droplets in the cytoplasm. These symptoms were evidently improved in the whey-protein-supplemented groups. Oil Red O staining further demonstrated that lipid droplets were remarkably observed in the HFCD group, whereas the number of lipid droplets decreased in the whey-protein-supplemented groups (Fig. 2). For hepatic lipids, whey supplementation decreased TC levels and significantly difference was observed between the HFCD group and HFCD+20% whey group (Fig. 3A). Meanwhile, there were no significant differences in hepatic triglyceride among the three groups (Fig. 3B).

Aorta Oil Red O staining and lesion area

En face lesion of the entire aorta was assessed by Oil Red O staining (Fig. 4A). Quantification analysis revealed a 21.51% and 37.78% lesion reduction in the whey-protein-supplemented groups, with a significant difference in the HFCD+20% whey group (Fig. 4B). Consistent with en face analysis, cryosection analysis of

| Table 2 | Effects of whey protein supplementation on serum lipid profiles in apoE−/− mice. |
|---------|---------------------------------------------------------------------------------|
|         | TC (mmol/L) | TG (mmol/L) | HDL-C (mmol/L) | Non-HDL-C (mmol/L) | NEFA (mEq/L) |
| HFCD    | 8.31±0.49   | 0.49±0.02   | 0.28±0.06      | 8.04±1.43          | 3.43±0.13    |
| HFCD+10% whey | 9.75±1.17   | 0.89±0.11   | 0.41±0.10*     | 9.34±1.89          | 3.53±0.19    |
| HFCD+20% whey | 8.63±0.62   | 1.04±0.13   | 0.47±0.07*     | 8.16±1.56          | 3.38±0.18    |

*p<0.01, significantly different from the HFCD group.

| Table 3 | Effects of whey protein supplementation on inflammatory cytokine in apoE−/− mice. |
|---------|---------------------------------------------------------------------------------|
|         | IL-6 (pg/mL) | IL-10 (pg/mL) | IL-12 (pg/mL) | TNF-α (pg/mL) | MCP-1 (pg/mL) | CRP (mg/L) |
| HFCD    | 29.85±9.33   | 10.33±3.14   | 28.48±7.18    | 20.31±3.62     | 50.79±7.54   | 19.15±2.14 |
| HFCD+10% whey | 18.49±5.82*  | 13.88±2.79a  | 24.88±6.44    | 17.45±3.57     | 45.48±6.87   | 19.08±2.53 |
| HFCD+20% whey | 8.66±4.48bc  | 18.94±3.28bc | 22.14±7.54    | 17.76±3.46     | 44.11±5.21   | 19.06±1.58 |

*p<0.05, b*p<0.01 significantly different from the HFCD group, c*p<0.01 significantly different from the HFCD+10% whey group.
aortas also showed that whey protein supplementation partially improved fat accumulation in the aortic vascular wall (Fig. 4C).

**Serum lipids and inflammatory cytokines**

At the end of the experiment, serum HDL-C levels measured 0.28±0.06, 0.41±0.10, and 0.47±0.07 mmol/L, with significant increases of 46.43% and 67.86% in the HFCD+10% whey group and HFCD+20% whey group, respectively (both p<0.01). Serum levels of triglyceride, TC, non HDL-C, and NEFA were comparable among the three groups (Table 2). For serum inflammatory cytokines, serum IL-6 levels decreased by 38.06% and 70.99% in the HFCD+10% whey group and HFCD+20% whey group, respectively. Correspondingly, serum IL-10 levels increased by 34.37% and 83.35%. IL-6 and IL-10 levels in the HFCD+10% whey group (both p<0.05) and HFCD+20% whey group (both p<0.01) significantly differed from those of the control group. In general, whey protein supplementation resulted in decreased serum levels of IL-12, TNF-α, and MCP-1 without significant differences. No significant difference was observed in CRP among the three groups (Table 3).

**Expression of cholesterol metabolism in liver and aorta**

To investigate the molecular evidence for the role of whey protein in cholesterol metabolism, relative protein expression was measured in the liver and aorta. Protein expression levels of ACC in the liver significantly decreased by 42.17% and 73.21% in the HFCD+10% whey group and HFCD+20% whey group, respectively. Significantly decreased FAS expression in the liver by whey protein was also observed. NF-κB expression in the liver and aorta was also significantly decreased by whey protein supplementation. However, protein expression levels of ABCA1 and ABCG1 significantly increased in both supplemented groups. For example, ABCA1 expression in the aorta increased by 1.53 and 3.16 times compared with that of the HFCD group. Western blotting analysis showed that whey protein supplementation exerted no effect on LDLR or LXRα in the liver or aorta (Fig. 5).

**DISCUSSION**

The present study primarily showed that long-term whey protein supplementation played an anti-atherosclerotic role in ApoE<sup>−/−</sup> mice. Preventive effects of
whey protein were observed as improved atherosclerotic lesions in aortas and decreased fat accumulation in the liver. Whey protein decreased circulating IL-6, increased IL-10, and regulated protein expression of cholesterol metabolism in the liver and aorta.

Beneficial effects of whey protein on cardiovascular disease, especially hypertension, have been extensively researched (8, 9, 15). Nevertheless, the effect of whey protein on atherosclerosis, which can lead to severe cardiovascular disease or even death, remains unknown. Atherosclerotic prevention aims to reduce lesion progression. In the present study, we first observed that whey protein suppressed atherosclerosis in ApoE<sup>−/−</sup>-mice, with decreased accumulation of cholesterol in aortas. In Nabi’s study, atherogenic rabbits were established and subsequently administered with traditional fermented cheese whey for 4 wk. This whey reduced formation of atherosomatic plaques on the aortic endothelium (16). The antiatherosclerotic effect in that established model is attributed to probiotic property. Our study provided further evidence that whey protein itself exerts a preventive effect on atherosclerosis.

Steatosis is associated with lesions of early atherosclerosis, which is independent from traditional cardiovascular risk factors (17, 18). Thus, we performed hepatic examination and observed that HFCD-induced fat accumulation was decreased by whey protein. Our results were similar to those of Hamad’s study: these researchers fed rats with fatty liver with whey protein isolate (WPI) and whey protein hydrolysate (WPH) and observed that whey protein improved steatosis and caused less fatty infiltration of hepatocytes (10). They also found that whey protein significantly decreases hepatic triglyceride: but the content of hepatic TC was not determined in that study (10). Additionally, hydrolyzed whey peptide ameliorates hepatic ischemia-reperfusion injury in rat with nonalcoholic fatty liver and prevents progression of liver cirrhosis in rat with cirrhosis (19, 20). Mechanistically, a high-fat diet directly or indirectly increases activity of lipogenic enzymes and accumulates triglyceride in hepatocytes (21). Our findings demonstrated that fat reduction by whey protein may result from inhibition of fatty acid biosynthesis due to decreased expression of two lipogenic enzymes, namely, ACC and FAS. This result was partially supported by the result of our previous study, which showed that leucine, which is rich in whey protein, significantly decreases hepatic TC and lipogenic enzymes in C57BL/6J mice fed with a HFCD diet for 24 wk (22). Whey protein also significantly decreases activity of hepatic lipogenic enzymes and their mRNA expression in rats without high-fat feeding under both sedentary and exercise-trained conditions (23). Interestingly, downregulated lipogenic enzyme at the mRNA level by whey protein was also observed in a human intestinal cell line, namely, NCI-H716 (24). However, we did not observe the decrease of serum or hepatic triacylglycerol levels following the decreased expression of ACC and FAS by whey protein intervention. This discrepancy reflected the complexity of fat accumulation involved in lipogenesis and lipolysis (25). Thus, lipolytic enzymes such as adipose triglyceride lipase and hormone-sensitive lipase should be investigated in further studies.

Dyslipidemia is an important risk factor for atherosclerosis. Increased HDL-C and decreased triglyceride are considered beneficial for atherosclerotic prevention (1). We observed that whey protein significantly increased serum HDL-C but exerted no effect on triglyceride level. In another study, supplementation of WPI, WPH, or α-lactalbumin, a component of whey protein, for 13 wk significantly increased plasma HDL-C levels in Zucker diabetic fatty rats; however, the plasma triglyceride level also increased simultaneously (26). Human trials also demonstrated the effect of whey protein on the lipid profile. Whey protein consumption for 8 wk significantly decreased circulating triglyceride and TC levels in subjects with prehypertension and mild hypertension (8). Decreased triglyceride was also observed in an acute trial, wherein WPI intake after 6 h significantly decreased the area under the curve of triglyceride in overweight, postmenopausal women (6). It has been reported that serum non-HDL-C, including VLDL, LDL and intermediate density lipoprotein particles, was increased in ApoE<sup>−/−</sup> mice (27). High non-HDL levels in circulation are well established risk factors for atherosclerosis. However, in the present study, we found there is no difference for serum non-HDL-C levels among the three groups. Thus, the effects of whey protein on individual indices of lipid profile depend on intervention strategies and observational subjects.

Low-grade inflammation is involved in the development of atherosclerosis (28). In the present study, we showed that whey protein supplementation significantly decreased serum IL-6 and increased IL-10 levels. This supplementation is beneficial for atherosclerotic prevention as IL-6 plays a central role in the pathogenesis of atherosclerosis, whereas IL-10 exerts its antiatherogenic effects on plaque development throughout different stages of atherosclerosis (3, 28). A similar change in inflammatory cytokines was observed in wounded and diabetic Albino rats. Oral whey protein treatment for 6 d of prewounding and 4 d of postwounding significantly increased wound closure with decreased serum IL-6 and increased IL-10 levels (29). Nevertheless, we observed no regulation of the serum CRP level, a common biomarker for cardiovascular disease. Okazaki’s prospective study, which includes 210 patients with vascular risk factors, supported our animal study. They discovered that carotid mean-maximal intima-media thickness, a marker of systemic atherosclerosis, remains significantly associated only with IL-6 level but not with CRP level (30). NF-κB is considered a central regulator/activator of the proinflammatory program (28). In the present study, decreased protein expression of NF-κB in the liver and aorta is another mechanism under atherosclerotic prevention by whey protein. Another animal study also showed that gavage with WPH and whey peptides following acute exercise significantly inhibited both serum IL-6 level and muscle NF-κB expression (31). Lactoferrin has been widely reported to possess...
anti-inflammatory and immunomodulatory properties (32). For example, Li et al. demonstrated that lactoferrin suppressed lipopolysaccharide-induced endometritis in mice via down-regulation of the NF-κB pathway (33). Despite the absence of significant differences, there is a downward trend in some pro-inflammatory cytokines, including IL-12, TNF-α, and MCP-1, in our present study. This could suggest that further studies are required to investigate the comprehensive role of whey protein in inflammation.

Reverse cholesterol transport is a pathway by which accumulated cholesterol is transported from the vessel wall to the liver for excretion: this event consequently prevents atherosclerosis. Cellular cholesterol efflux is the limiting step of reverse cholesterol transport (2). ABCA1 and ABCG1 play key roles in reverse cholesterol transport and in HDL formation (34). Deficiency in ABCA1 and ABCG1 in endothelial cells accelerates atherosclerosis in mice (35). We investigated for the first time the expression of these two major cholesterol transporters in the liver and aorta and observed a significant increase in ABCA1 and ABCG1 by whey protein in a dose-dependent manner. This increase was consistent with the serum HDL-C level due to the role of ABCA1 and ABCG1 in HDL formation. Upregulation of hepatic ABCA1 expression was proven at the mRNA level in high-fat-diet-induced obese mice fed with rice bran protein. However, this vegetable protein decreases TC and LDL-C levels and causes no increase in HDL-C levels (36). In addition to its anti-inflammatory properties, IL-10 significantly enhances HDL-induced cholesterol efflux (36). IL-10-mediated effects on cholesterol efflux were accompanied with increased IL-10-mediated expressions of ABCA1 and ABCG1 (37, 38). Therefore, increased serum HDL-C and IL-10 levels and upregulation of ABCA1 and ABCG1 expression in the liver and aorta synergistically play an antiatherosclerotic effect from whey protein. Finally, LXRs are also important regulators of cholesterol homeostasis. Although LXRs promote ABCA1 expression and inhibits the development of atherosclerosis in mice (39), it also activates lipogenic enzymes, such as FAS and ACC, in the liver, resulting in hypertriglyceridemia in mice (21). Western blotting analysis showed that whey protein exerted no effect on LXRα protein levels. These results were consistent with those of a previous study, showing that whey-based diets fed to weaned piglets for 72 d exerted no effect on LXRs either in the liver or in adipose tissues (40).

Our study had several limitations in regards to experimental design. First, although the energy content of the diet was similar among the three groups, the protein content was different between the HFCD and whey protein supplemented groups. Consequently, the whey supplemented groups had relatively lower energy from fat and carbohydrate compared to the HFCD group. Thus, it cannot be ruled out that the beneficial effects of whey protein supplementation observed in our study were due to decreased energy intake from fat and/or the differences in protein content. Second, we only analyzed the associated protein expressions in the aorta at the end of the experiment. To elucidate the potential mechanisms more clearly, protein expressions should be compared between before and after the onset of atherosclerotic lesions. Third, it would be better if we could have collected feces to measure fecal sterol excretion. Findings from other laboratories suggest whey and lactoferrin supplementation might inhibit cholesterol in the small intestine. For example, Plvi et al. demonstrated that fecal fat content was significantly greater in the whey group than in the casein group if mice were fed on a high-calcium diet (41). Nakamura et al. also demonstrated that lactoferrin significantly increased levels of fecal neutral steroids and cholesterol (42).

In conclusion, the present study demonstrated that chronic whey protein supplementation can improve HDLC-induced atherosclerosis in Apoe null mice by regulating circulating lipids and inflammatory cytokine. This improvement was ascribed to increased expression of cholesterol transporters ABCA1 and ABCG1. Further prospective studies and human trials are needed to confirm our results.

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