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Dietary phospholipid-rich dairy milk extract reduces hepatomegaly, hepatic steatosis and hyperlipidemia in mice fed a high-fat diet

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ABSTRACT

Recent studies have suggested that milk and certain dairy food components have the potential to protect against cardiovascular disease. In order to determine whether the addition of milk-derived phospholipids to the diet results in an improvement in metabolic and cardiovascular risk factors, we studied four groups ($n = 10$) of C57BL/6 mice that were fed: (1) a normal non-purified diet (N); (2) the normal non-purified diet supplemented with phospholipid-rich dairy milk extract (PLRDME, 2.5% by wt) (NPL); (3) a high-fat semi-purified diet (HF) containing 21% butterfat + 0.15% cholesterol by wt; or (4) HF supplemented with 2.5% by wt PLRDME (HFPL). Dietary PLRDME supplementation did not have a significant effect on metabolic parameters in mice fed the N diet. In contrast, in high-fat fed mice, PLRDME caused a significant decrease in: (a) liver wt (1.57 ± 0.06 g vs. 1.20 ± 0.04 g, $P < 0.001$), (b) total liver lipid (255 ± 22 mg vs. 127 ± 13 mg, $P < 0.001$, (c) liver triglyceride (TG) and total cholesterol (TC) 236 ± 25 μmol/g vs. 130 ± 8 μmol/g ($P < 0.01$), 40 ± 7 μmol/g vs. 21 ± 2 μmol/g ($P < 0.05$), respectively); and serum lipids (TG: 1.4 ± 0.1 mmol/L vs. 1.1 ± 0.1 mmol/L, $P = 0.01$; TC: 4.6 ± 0.2 mmol/L vs. 3.6 ± 0.2 mmol/L, $P < 0.001$; and PL: 3.3 ± 0.1 mmol/L vs. 2.6 ± 0.1 mmol/L, $P < 0.01$). These data indicate that dietary PLRDME has a beneficial effect on hepatomegaly, hepatic steatosis and elevated serum lipid levels in mice fed a high-fat diet, providing evidence that PLRDME might be of therapeutic value in human subjects as a hepatoprotective or cardioprotective nutraceutical.

1. Introduction

Recent epidemiological studies have shown that increased consumption of milk and other dairy products is associated with a reduced incidence of cardiovascular risk factors, such as obesity, insulin resistance, dyslipidemia and type 2 diabetes [1,2]. These results have encouraged a search for biologically active dairy food components that might be of benefit in the prevention and treatment of cardiovascular disease (CVD). Milk proteins, minerals and fats have thus attracted recent attention as functional foods or nutraceuticals with potentially important cardioprotective properties [3,4].

Milk fat (representing 2.4–5.5% by wt of whole milk) is 98% triglyceride and is contained within spherical lipid droplets (0.1–15 μm in diameter) surrounded by a lipid and protein bilayer, called the milk fat globule membrane (MFGM) [5]. The MFGM prevents lipid droplets from aggregating and these droplets therefore remain dispersed in the milk. Only after destruction of the structure of the MFGM through mechanical force like churning, do lipid droplets aggregate and subsequently form large fat clumps (i.e., butter). As well as containing over 50 biologically active proteins and polypeptides [6], the MFGM is a rich source of phospholipids (PLs), primarily phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), with smaller amounts of phosphatidylserine (PS) and phosphatidylinositol (PI). Recent research into the biological properties of milk PL has identified a number of potential health benefits, including protection against tumour growth [7] and improvement in memory [8]. A car-

Abbreviations: ACAA, acetyl-CoA acyltransferase; ACAT, acetyl-CoA acetyltransferase; ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPT, carnitine palmitoyl transferase; CVD, cardiovascular disease; ELOVLF, elongation of very long chain fatty acids; FA, fatty acid; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HF, high fat-fed mice; HFPL, high fat-fed mice supplemented with PLRDME; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; ME, malic enzyme; LDL-R, LDL receptor; N, normal diet-fed mice; NAFLD, non-alcoholic fatty liver disease; NPL, normal diet-fed mice supplemented with PLRDME; MFGM, milk fat globule membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLRDME, phospholipid-rich dairy milk extract; PS, phosphatidylserine; SCD-1, stearoyl-CoA desaturase-1; S:M:P, saturated:monounsaturated:polyunsaturated ratio; SM, sphingomyelin; SR-BI, scavenger receptor class B type 1; TC, total cholesterol; TG, triglyceride.

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diovascular benefit has not been investigated however, based on the expectation that the saturated fatty acids in milk PL would have an adverse effect on plasma and liver lipid metabolism.

In order to address this question, the aim of the present study was to investigate the effect of dietary milk-derived PL on plasma and liver lipid metabolism in experimental mice. A phospholipid-rich dairy milk extract (PLRDME) was prepared, which contained ~50% by weight PL, and it was given as a dietary supplement to experimental mice fed either a normal diet or a high-fat semi-purified diet. Despite the presence of saturated fatty acids in PL from dairy milk, dietary supplementation with PLRDME was found to have favourable effects on plasma and liver lipid metabolism.

2. Materials and methods

2.1. Animals and diets

Experiments were approved by the Animal Welfare Committee of the Sydney South West Area Health Service. Six-week old male C57BL/6 mice were obtained from the Australian Resources Centre (Perth, Australia). They were housed in standard cages (5 mice per cage) at a constant temperature of 20 °C with a 12 h light/dark cycle. They were allowed *ad libitum* access to diet and water. After 1 week of acclimatization, they were divided into four groups (n = 10 mice per group). One group was fed a normal non-purified diet (N), i.e., normal mice pellets (Specialty Feeds, Glen Forrest, Western Australia). The non-purified diet was composed of wheat, lupins, barley, soya meal, fish meal, mixed vegetable oils, canola oil, sodium chloride, calcium carbonate, dicalcium phosphate, magnesium oxide and a vitamin and trace mineral premix. It contained 4.6% total fat, 4.8% crude fibre, and 19% protein. A second group (NPL) was fed the normal diet supplemented with 2.5% by wt PLRDME. A third group (HF) was fed a high-fat diet containing 21% butterfat and 0.15% cholesterol (SF00-219, Specialty Feeds, WA). This semi-purified diet contained (g/kg): casein, 195; DL-methionine, 3; sucrose 341; wheat starch, 154; cellulose, 50; clarified butter, 210; calcium carbonate, 17.1; sodium chloride, 2.6; potassium citrate, 2.6; potassium dihydrogen phosphate, 6.9; potassium sulphate, 1.6; AIN93G trace minerals, 1.4; choline chloride (65%), 2.5; vitamins, 10; cholesterol, 1.5. A fourth group (HFPL) received the HF-diet supplemented with 2.5% by wt PLRDME. Food intake was recorded weekly and body weights were measured 3 times a week. The PLRDME was prepared, analyzed and supplied by MG Nutritionals (Melbourne, Australia) and extract components were assayed with Australian Food Standard methods. PLRDME was added to the diets as a supplement rather than as a substitution, in order to mimic the every-day human situation of the extract being consumed as a nutraceutical supplement.

2.2. Tissue processing

Mice were fed diets for 8 weeks. Food was removed the night (i.e., 16 h) before sacrifice. They were exsanguinated by heart puncture under methoxyflurane anaesthesia. Blood was allowed to clot and serum was separated by centrifugation (1500 × g, 10 min). Sera were aliquoted and stored frozen (−80 °C) until analysis. Livers were immediately excised, weighed and divided into smaller pieces for storage at −80 °C (for lipid analysis), in RNA*later*® Solution (Albion, Austin, TX) (for gene expression analysis) or in 4% paraformaldehyde for histological analysis. Epididymal, inguinal and perirenal fat pads, as well as the quadriceps muscle from one leg, were excised and weighed. Liver samples were examined histologically after embedding in paraffin, sectioning, and staining with hematoxylin and eosin. Frozen sections were also stained with Oil-Red-O.

2.3. Biochemical analyses

Liver enzymes – alanine aminotransferase (ALT) and aspartate aminotransferase (AST) – were measured in serum with colorimetric endpoint assays using commercial reagents (Teco Diagnostic, Anaheim, CA). Blood glucose was measured in whole blood with a glucose meter (Accu-Chek Integra, Roche Diagnostics). Serum insulin was measured by ELISA (Ultra Sensitive ELISA Kit, Crystal Chem Inc.). Serum triglyceride (TG), total cholesterol (TC) and free fatty acid concentrations were measured by enzymatic methods, using GPO-PAP and CHOD-PAP (Roche Diagnostics) and Wako NEFA C (Wako Pure Chemicals, Osaka, Japan) kits, respectively. Serum phospholipid levels were measured enzymatically according to the method of Takayama et al. [9]. Serum HDL was separated by polyethylene glycol precipitation of apoB-containing lipoproteins (50 μL of polyethylene glycol (200 mg/mL) added to 50 μL of serum). Serum apoA-I concentration was measured by competitive ELISA, whereby goat anti-mouse apoA-I polyclonal antibody was used as the capture antibody, rabbit anti-mouse apoA-I polyclonal antibody was used for detection, and goat anti-rabbit IgG HRP conjugated was used for quantification (Biodesign International, Saco, Maine). Total liver lipids were determined gravitometrically after extraction by the method of Bligh and Dyer [10]. Individual hepatic lipids were quantitated enzymatically (as described above) after solubilization in isopropanol.

2.4. Gene expression analysis

Hepatic mRNA levels were measured by real-time PCR. Total RNA was isolated by selective binding to a silica gel-based membrane following the lysis and homogenisation of liver samples in a denaturing guanidine thiocyanate buffer (RNeasy kit, Qiagen). RNA (100 ng) was reverse transcribed into cDNA using random primers provided with the iScript cDNA Synthesis Kit (Bio-Rad). Selected genes were amplified using iQ SYBR Green Supermix (Bio-Rad) in an iCycler system (Bio-Rad) with 12 pmol of both forward and reverse primers. PCR conditions were as follows: 1 cycle of 95 °C for 3 min, 50 cycles of 95 °C for 30 s, 55–60 °C for 30 s and 72 °C for 30 s, followed by 1 cycle of 95 °C for 1 min. Purity of PCR products was assessed by melt curve analysis. Relative gene expression was calculated by normalizing cycle threshold (Ct) values for genes of interest with Ct values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or cyclophilin using the delta–delta Ct method. Primer sequences were as follows: ACAA (forward: 5′-ATGAACTGAAGCGTCGTGG-3′; reverse: 5′-TCTGTAGCGTCCCTCCTGG-3′); ACAT2 (forward: 5′-GACTTGGTGCA-ATGGACTCG-3′; reverse: 5′-GGTCTTGCTTGT AGAATC TGG-3′); ACC (forward: 5′-TTCTGAATGTGGCTATCAAGACTGA-3′; reverse: 5′-TGCTGGGTGAACTCTCTGAACA-3′); ACO (forward: 5′-TTTGTTGTC-CCTATCCGTGAGA-3′; reverse: 5′-CCGATATCCCCAACAGTGATG-3′); CPT2 (forward: 5′-ATCCCCTGGATATGTCCCAATA-3′; reverse: 5′-CA-TCACGACTGGGTTTGGGTAT-3′); CYCLOPHILIN (forward: 5′-CAA-ATGCTGGACCAAACACAA-3′; reverse: 5′-CCATCCAGCCATTCAGT CTTG-3′); CYP7a (forward: 5′-CAGGGAGATGCTCTGTGTTCA-3′; reverse: 5′-AGGCATACATCCCTTCCGTGA-3′); ELOVL5 (forward: 5′-GGTGGCTGTTCTTCCAGATT-3′; reverse: 5′-CCCTTCAGGTGGTC-TTTCC-3′); GAPDH (forward: 5′-GGCATCACTGCAACTCAGAA-3′; reverse: 5′-TTCAGCTCTGGGATGACCTT-3′); FAS (forward: 5′-ATC-CTGGAACGAGAACACGATCT-3′; reverse: 5′-AGAGACGTGTCACTCC-TGGACTT-3′); HMGCR (forward: 5′-CTTGTGGAATGCCTTGTG-ATTG-3′; reverse: 5′-AGCCGAAGCAGCACATGAT-3′); LDL-R (forward: 5′-CTGTGGGCTCCATAGGCTATCT-3′; reverse: 5′-GCGGTCCAGGGTCATCTTC-3′); malic enzyme (forward: 5′-CATAT-CTCAGCAAGTGTCAGATAAACAC-3′; reverse: 5′-AAACGCCTCGAAT-GGTATTCA-3′); SCD-1 (forward: 5′-GATAGAGCAAGTCCCCGTTG-