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Dietary cholesterol induces hepatic inflammation and blunts mitochondrial function in the liver of high-fat-fed mice

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Abstract

The present study investigated the role of dietary cholesterol and fat in the development of nonalcoholic fatty liver disease, a common liver disease in metabolic disorders. Mice were fed a diet of regular chow (CH), chow supplemented with 0.2% w/w cholesterol (CHC), high fat (HF, 45 kcal%) or HF with cholesterol (HFC) for 17 weeks. While both HF and HFC groups displayed hepatic steatosis and metabolic syndrome, only HFC group developed the phenotype of liver injury, as indicated by an increase in plasma level of alanine transaminase (ALT, by 50–80%). There were ~2-fold increases in mRNA expression of tumor necrosis factor α, interleukin 1β and monocyte chemotactic protein 1 in the liver of HFC-fed mice (vs. HF) but no endoplasmic reticulum stress or oxidative stress was observed. Furthermore, cholesterol suppressed HF-induced increase of peroxisome proliferator-activated receptor γ coactivator 1α and mitochondrial transcription factor A expression and blunted fatty acid oxidation. Interestingly, after switching HFC to HF diet for 5 weeks, the increases in plasma ALT and liver inflammatory markers were abolished but the blunted of mitochondrial function remained. These findings suggest that cholesterol plays a critical role in the conversion of a simple fatty liver toward nonalcoholic steatohepatitis possibly by activation of inflammatory pathways together with retarded mitochondrial function.
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Keywords: Dietary cholesterol; High fat diet; Inflammation; Mitochondrial function; Nonalcoholic fatty liver disease

1. Introduction

The term 'nonalcoholic fatty liver disease' (NAFLD) encompasses a broad range of liver disorders including hepatic steatosis, nonalcoholic steatohepatitis (NASH) and cirrhosis. It has become a worldwide health problem that is estimated to affect at least 1.46 billion obese adults largely due to the increasing occurrence of obesity [1]. In obese individuals, hepatic steatosis is caused by storage of excess energy in the form of triglyceride (TG) in the liver. This is the early stage of NAFLD and it is usually benign without clinical symptoms. However, 30% of people with hepatic steatosis develop to NASH, which is characterized by hepatocyte injury and inflammation [2]. NASH presents with elevated liver enzymes including aspartate transaminase and alanine transaminase (ALT) in the plasma. In the later stage, NASH can progress to serious and irreversible liver diseases such as cirrhosis, liver failure and even hepatocarcinoma.

Although it is well known that excess accumulation of TG in the liver causes hepatic steatosis, the etiology of NASH is more complex and not fully understood. In 1998, Day and James first suggested a two-"hit" theory for the pathogenesis of steatohepatitis, namely steatosis (first "hit") and oxidative stress (second "hit") in the liver [3]. Since then, a growing body of evidence has suggested that multiple factors may constitute the second "hit" in the progression from hepatic steatosis to NASH. These factors may include inflammation, oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and insulin resistance [2,4]. Proinflammatory cytokines play an important role in inflicting the inflammatory reaction in NASH and they can induce apoptosis and oxidative stress [5]. Mitochondria are involved in oxidative stress [6] and perturbation of mitochondrial function can impair fatty acid oxidation, which is known to result in insulin resistance and NAFLD [7,8].

Dietary compositions have important impacts on the development of the metabolic syndrome including NAFLD [1]. A diet rich in fat [8,9] or fructose [10,11] is well known to cause lipid accumulation in the liver (hepatic steatosis), obesity and insulin resistance. However, there is little evidence to indicate that they are sufficient by themselves to result in liver injury and hepatic inflammation, the hallmarks that differentiate hepatic steatosis from NASH. Therefore, it is important to investigate what dietary components may induce the second "hit".

Dietary cholesterol has been widely investigated for its key role in the pathogenesis of atherosclerosis. For example, cholesterol contributes to

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the formation of low-density lipoprotein (LDL), foam cells and plaque formation that induces the release of inflammatory cytokines in the artery [12]. As dietary cholesterol is directly transported from gut to liver after absorption [13], we speculate that it may be a potential dietary factor that initiates inflammatory responses in the liver. Thus, the first aim of the present study was to investigate whether a typical level of dietary cholesterol (0.2% w/w) plays a role in the progression of hepatic steatosis to NASH by inducing a second "hit". Since insulin resistance, oxidative stress [14], ER stress [15], inflammation [16] and mitochondrial dysfunction [7] have been implicated in the pathogenesis of NAFLD, our second aim was to investigate their possible role in the second "hit" in the transition from hepatic steatosis toward NASH. Reported here are our findings showing that, in the presence of hepatic steatosis, cholesterol resulted in liver injury and inflammation within the liver. In addition, cholesterol also persistently suppressed the expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) and mitochondrial transcription factor A (TFAM) and mitochondrial fatty acid oxidation. These findings provide novel insight into how a typical level of dietary cholesterol may convert simple hepatic steatosis to NASH as a trigger of the second "hit" in the pathogenesis of NAFLD.

2. Material and methods

2.1. Animal study

All experiments were approved by the RMIT University Animal Ethics Committee (#1012). Male C57BL/6J mice (10 weeks old) were purchased from the Animal Resources Centre (Perth, Australia). They were kept at 22±1°C on a 12-h light/dark cycle. After 2 weeks of acclimatization, mice were fed a diet of chow (CH; 8% calories from fat, 21% calories from protein and 71% calories from carbohydrate), CH supplemented with 0.2% cholesterol (CHC), high fat (HF; 45% calories from fat, 20% calories from protein and 35% calories from carbohydrate) or HF supplemented with 0.2% cholesterol (HFC), *ad libitum* for 17 weeks. From week 13, cholesterol was withdrawn from the diet of one HFC subgroup for 5 weeks. The chow diet (Specialty Feeds, Australia) was ground into powder and mixed with cholesterol. The powdered CH, with or without cholesterol, was made as pellets. HF and HFC diets were prepared as we previously described [17,18]. The detailed composition of the HF diet is shown in Supplementary Table 1. All of the diets were stored at −20°C for less than 1 month and were changed daily during the experiments.

The whole body metabolic rate, including oxygen consumption (VO_2) and respiratory exchange ratio (RER), was measured after 5 weeks of feeding using an indirect calorimeter (Comprehensive Laboratory Animal Monitoring System; Columbus Instruments, USA) [17,19]. A glucose tolerance test (GTT) (glucose 1.5 g/kg ip) was performed after 5–7 h of fasting in week 12 using a glucometer (Accu-Chek, Australia). Blood samples were collected from the tail tip at 0, 15, 60 and 90 min for plasma insulin measurement using a radioimmunoassay kit (Abacus ALS, Australia).

For the terminal studies, additional plasma samples were collected and stored at −80°C for biochemical assays. Mice were then killed by cervical dislocation. Liver tissues were harvested and either put in 10% neutral buffered formalin (Sigma-Aldrich, Australia) for histological analysis or ice-cold buffer (pH 7.4, 250 mmol/L sucrose, 10 mmol/L Tris–HCl and 1 mmol/L EDTA) for measurement of palmitate oxidation or freeze-clamped immediately for further assessment. Epididymal fat mass was weighed using an analytical balance. ALT levels were measured using an ALT/SGPT Liqui-UV Kit (Stanbio, USA). Briefly, 20 µl of plasma was mixed with 200 µl of reagent (R1:R2, 5:1 as described in manufacturer's instructions). To determine ALT activity, absorbance at 340 nM was measured immediately by a FlexStation (Molecular Devices, USA) and then every minute for 10 min. Plasma levels of HDL and LDL-VLDL were measured using a commercial assay kit following the manufacturer's instructions (Sigma-Aldrich, Australia). Plasma levels of total TG and cholesterol were determined using Triglyceride GPO-PAP and Cholesterol CHOD-PAP kits (Roche Diagnostics, Australia).

2.2. Measurement of palmitate oxidation ex vivo

Liver tissues stored in ice-cold buffer were homogenized and used for the measurement of fatty acid oxidation *ex vivo* as described previously [19]. Briefly, liver homogenates were incubated at 30°C for 90 min in a reaction buffer (pH 7.4) containing 0.5 µCi [^{14}C]palmitate, 2 mmol/L L-carnitine and 0.05 mmol/L CoA. CO_2 produced from the reaction was captured in 1 M NaOH; 1 M perchloric acid was used to stop the reaction. Palmitate oxidation rates were determined by measuring the ^{14}C radioactivity in captured CO_2 and acid-soluble metabolites.

2.3. Histological analysis of liver

Liver tissues stored in 10% neutral buffered formalin were dehydrated using a Leica tissue processor (Leica, Australia). The dehydrated samples were embedded in paraffin and cut into 4-µm-thick sections. Mayer's hematoxylin and eosin (H&E) staining was performed and images were taken from Olympus BX41 microscope with a 20× objective lens using an Olympus DP72 digital camera (Olympus, Australia).

2.4. Biochemical assays of liver

Lipids were extracted from the freeze-clamped liver tissue by the method of Bligh and Dyer [20]. TG and cholesterol levels were determined using Triglyceride GPO-PAP and Cholesterol CHOD-PAP kits (Roche Diagnostics, Australia). Free cholesterol levels were tested by a Free Cholesterol kit (Wako, Japan). Protein carbonyl contents were measured using protein carbonyl content assay kit (Abcam, UK) according to the manufacturer's instruction. Glutathione (GSH)/GSH disulfide (GSSG, the oxidated product of GSH) and malondialdehyde (MDA) levels were determined using the commercial kit from Cayman (USA). Superoxide dismutase (SOD) activity was tested using the SOD activity kit (Enzo Life Sciences, USA).

2.5. Western blotting

Western blotting was performed as described previously [21]. Proteins prepared in Laemmli buffer were separated by SDS-PAGE, then transferred to PVDF membranes (Bio-Rad Laboratories, USA) and blocked in 3% BSA. Membranes were probed with the following primary antibodies. Total- and phospho-cJUN, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), total- and phospho-eukaryotic initiation factor 2α (eIF2α), total- and phospho-AMP-activated protein kinase (AMPK), sirtuin 1 (Sirt1), total- and phospho-acetyl-CoA carboxylase (ACC) and glucose-regulated protein 78 kDa (GRP78) antibodies were purchased from Cell Signaling (USA). Total- and phospho-inositol-requiring kinase 1 (IRE1) antibodies were obtained from Abcam (UK). C/EBP homologous protein (CHOP) antibody was purchased from Santa Cruz (USA). Western blot membranes were incubated with secondary antibodies from Santa Cruz (USA) that were conjugated to horseradish peroxidase (HRP) and developed using enhanced chemiluminescence HRP substrate from Perkin Elmer (USA). Images of the membranes were taken with the ChemiDoc system and densitometry analysis was performed using Image Lab software (Bio-Rad Laboratories, USA).

2.6. Quantitative real-time PCR

RNA was extracted using TRIzol Reagent and genomic DNA was digested using amplification grade DNase (Invitrogen, Australia). RNA extract was reverse-transcribed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Australia) according to manufacturer's instructions. Primers (GeneWorks, Australia) and SYBER green supermix (Bio-Rad Laboratories, USA) were used for quantitative real-time PCR. All reactions were performed at 50°C for 2 min, 95°C for 3 min, 40 cycles of 95°C for 15 s, 72°C for 30 s and followed by measurements of melt curve using QIAGEN Rotor-Gene Q PCR system (Germany). 18s was used as the normalizing control gene and results were analyzed by the ΔΔCt method. Sequences of the primers are shown in Supplementary Table 2.

2.7. Statistical analyses

Data were calculated as means±S.E. One-way analysis of variance was used for comparison of groups. When significant differences were found, the Tukey's multiple comparisons test was applied. Differences at $P<.05$ were considered statistically significant.

3. Results

3.1. Effects on whole body metabolism

HF diet alone increased the body weight of mice by 20% ($P<.01$) and 3-fold more epididymal fat mass compared to the CH control group (Table 1). Inclusion of cholesterol (0.2% w/w) in either CH or HF diet did not result in any additional effects on body weight or epididymal fat mass. Compared to the CH control group, incremental area under the curve (iAUC) and average insulin levels during GTT in the HF group increased by approximately 3-fold. Dietary cholesterol-attenuated HF induced glucose intolerance as indicated by 35% reduction in iAUC ($P<.01$) and 34% reduction in insulin levels ($P<.05$). To assess whole body energy expenditure, at week 5 of the dietary interventions, mice were placed in metabolic cages. Compared to the CH control group, RER values of HF-fed mice were reduced (15%, $P<.05$). Addition of cholesterol in either CH or HF diet did not affect the VO_2 and RER of the mice. Addition of cholesterol in chow diet (CHC) did not have significant effects on plasma levels of TG, total cholesterol, LDL-VLDL and HDL. HF feeding alone (HF) increased plasma level of total cholesterol by ~45% ($P<.01$, vs. CH group) but did not have significant

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