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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.

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

effects on the levels of TG, LDL-VLDL or HDL. Addition of cholesterol to the HF diet (HFC) had similar effects to HF alone on plasma levels of TG, total cholesterol and HDL. Although the plasma levels of LDL-VLDL in HFC mice were higher than in the CHC group, there were no significant differences in any of these parameters between the HF and HFC groups (all $P>.2$).

3.2. Effects on levels of TG and cholesterol and histology of the liver

HF feeding alone increased the liver TG level to approximately 3-fold the level of CH-fed mice without changing cholesterol content (Fig. 1A). While addition of cholesterol to the HF diet increased the hepatic levels of total cholesterol (1.9-fold, $P<.01$) and free (1.6-fold, $P<.01$) cholesterol, it did not influence the effect of HF feeding on liver TG contents. Removal of cholesterol from the HFC diet for 5 weeks returned levels of liver cholesterol to those seen in HF-fed mice, without altering the TG content. To assess concurrent histological changes, H&E-stained sections of the liver were examined. Inclusion of cholesterol in the CH diet did not induce hepatosteatosis, as indicated by both TG content and histological appearance (Fig. 1A and B). In contrast, HF alone induced moderate microvesicular steatosis within hepatocytes, while HFC induced both microvesicular and macrovesicular steatoses. Withdraw of cholesterol from the HFC diet reduced the severity of the microvesicular and macrovesicular steatoses.

3.3. Effects of dietary fat and cholesterol on oxidative stress and ER stress

As oxidative stress and ER stress have been suggested to be important components of the second "hit" to induce NASH [14,15], we next measured biochemical markers for oxidative stress and levels of the key proteins involved in ER stress pathway. Dietary fat and cholesterol did not affect the expression or activity of oxidative stress markers, such as protein carbonyls, GSH, MDA content and SOD activity in the liver (Fig. 2). We did not observe significant changes in ER stress markers including protein levels of phospo-eIF2α, CHOP and GRP78, except a mild suppression of IRE1 phosphorylation in the HFC group.

3.4. Effects on key features of NASH

To examine the effects of dietary cholesterol on liver damage and inflammation (the key features of NASH), we measured the plasma ALT level as an indicator of liver damage, the mRNA expression of inflammatory and fibrotic markers. The results showed that HF feeding alone did not affect the ALT levels in plasma or the mRNA expression of tumor necrosis factor α (TNFα), interleukin 1β (IL1β), monocyte chemotactic protein 1 (MCP1) and cluster of differentiation 68 (CD68) (Fig. 3A and B). Addition of cholesterol to the HF diet significantly increased the plasma ALT level (1.8-fold, $P<.01$) together with a 2-fold increase in mRNA expression of TNFα, IL1β, CD68 and MCP1 compared with the HF diet alone. In line with increased mRNA expression of inflammatory cytokines, western blotting indicated that cholesterol in the HF diet increased phosphorylation of cJUN, a downstream target of TNFα activation (Fig. 3C). The changes in inflammatory cytokines were accompanied by the increase in mRNA expressions of toll-like receptor 4 (TLR4) and IRF3 (a downstream target of TLR4) and elevated NLRP3 inflammasome levels (Fig. 3D and E). Apart from NLRP3 levels, withdrawing cholesterol from the HF diet effectively attenuated the abovementioned effects. Addition of cholesterol to the chow diet markedly increased mRNA expression of collagen I and a similar trend was observed in HFC mice. Removal of cholesterol returned the collagen I mRNA level to the normal level (Fig. 3F).

3.5. Effects on fatty acid oxidation and markers of mitochondrial function

To assess mitochondrial function, we next examined the effect of cholesterol on fatty acid oxidation in liver tissues. HF feeding by itself significantly increased palmitate oxidation (~2-fold vs. CH, $P<.01$, Fig. 4A) as expected. However, this compensatory increase was blunted by the addition of cholesterol into the HF diet ($P<.01$ vs. HF, but $P>.05$ vs. CH), suggesting retarded mitochondrial function in the liver of HFC-fed mice. Consistent with this possibility, the HF-induced increase in PGC1α expression (~2-fold vs. CH, $P<.05$) was diminished in diets supplemented with cholesterol ($P>.05$ vs. CH, Fig. 4B). A similar pattern of reduction was observed in the mRNA levels of TFAM (a transcription factor regulated by PGC1α [22]) in the HFC and HFC-HF groups compared to HF alone (both $P<.05$). The content of AMPK was increased significantly in HFC-HF group (55%, $P=.03$) compared with CH, and there were similar trends in HF (37%, $P=.33$) and HFC (37%, $P=.26$) (Supplementary Figure). Compared with HF mice, AMPK phosphorylation (pAMPK/GAPDH) was increased by ~75% in HFC mice ($P<.05$) (Fig. 4C). The phosphorylation of ACC (a downstream

Table 1
Effects of high-fat and high-cholesterol diet on metabolic parameters of the mice

	CH	CHC	HF	HFC	HFC-HF
Starting body weight (g)	23.6±0.4	22.9±0.3	23.5±0.7	24.7±0.4	24.7±0.3
Final body weight (g)	31.7±0.7	30.1±0.7	38.7±1.3$^{\#\#}$	36.1±0.9$^{\#\#}$	36.0±0.6$^{\#\#}$
Epididymal fat (BW%)	1.7±0.1	1.5±0.2	5.3±0.5$^{\#\#}$	5.1±0.4$^{\#\#}$	5.5±0.3$^{\#\#}$
VO_2 (L/kg h)	3.57±0.16	3.64±0.13	3.28±0.11	3.24±0.09	n.d.
RER	0.98±0.01	0.98±0.01	0.84±0.01$^{\#\#}$	0.82±0.01$^{\#\#}$	n.d.
Basal glucose (mM)	9.4±0.7	7.4±0.4	10.2±0.5	10.4±0.5	n.d.
Basal insulin (pg/ml)	280±70	467±112	1948±351$^{\#\#}$	1515±203 $^{\#\#}$	n.d.
GTT iAUC	343±51	472±61	1397±44$^{\#\#}$	905±144$^{\#\#}$**	n.d.
GTT insulin (pg/ml)	697±144	642±78	2508±358$^{\#\#}$	1649±137$^{\#\#}$*	n.d.
Plasma TG (mM)	0.91±0.07	0.94±0.04	1.13±0.06	1.24±0.06$^{\#}$	n.d
Plasma cholesterol (mg/ml)	7.5±0.1	7.9±0.3	11.0±0.7$^{\#\#}$	11.7±0.6$^{\#\#}$	n.d
Plasma LDL-VLDL (mg/ml)	0.16±0.01	0.19±0.02	0.21±0.01	0.26±0.02$^{\#}$	n.d
Plasma HDL (mg/ml)	0.90±0.04	0.77±0.07	0.91±0.02	0.95±0.03	n.d

Male C57BL/6J mice were fed chow (CH), CH with 0.2% cholesterol (CHC), high fat (HF) or HF with 0.2% cholesterol (HFC) diet for 17 weeks. The HFC-HF group was fed with an HFC diet for 12 weeks followed by 5 weeks HF feeding ($n=7$–8/group). Oxygen consumption rate (VO_2) and respiration exchange ratio (RER) were measured after 5 weeks of feeding. ipGTT (glucose 1.5 g/kg ip) was performed after 5–7 h of fasting in week 12 and iAUC was calculated. Plasma levels of insulin during GTT are the average values of 15 and 60 min. TG and total cholesterol levels were measured at week 8 and HDL and LDL-VLDL levels were measure at week 17. n.d.: not determined.

* $P<.05$ vs. HF.

** $P<.01$ vs. HF.

$^{\#}$ $P<.05$ vs. CH group.

$^{\#\#}$ $P<.01$ vs. CH group.

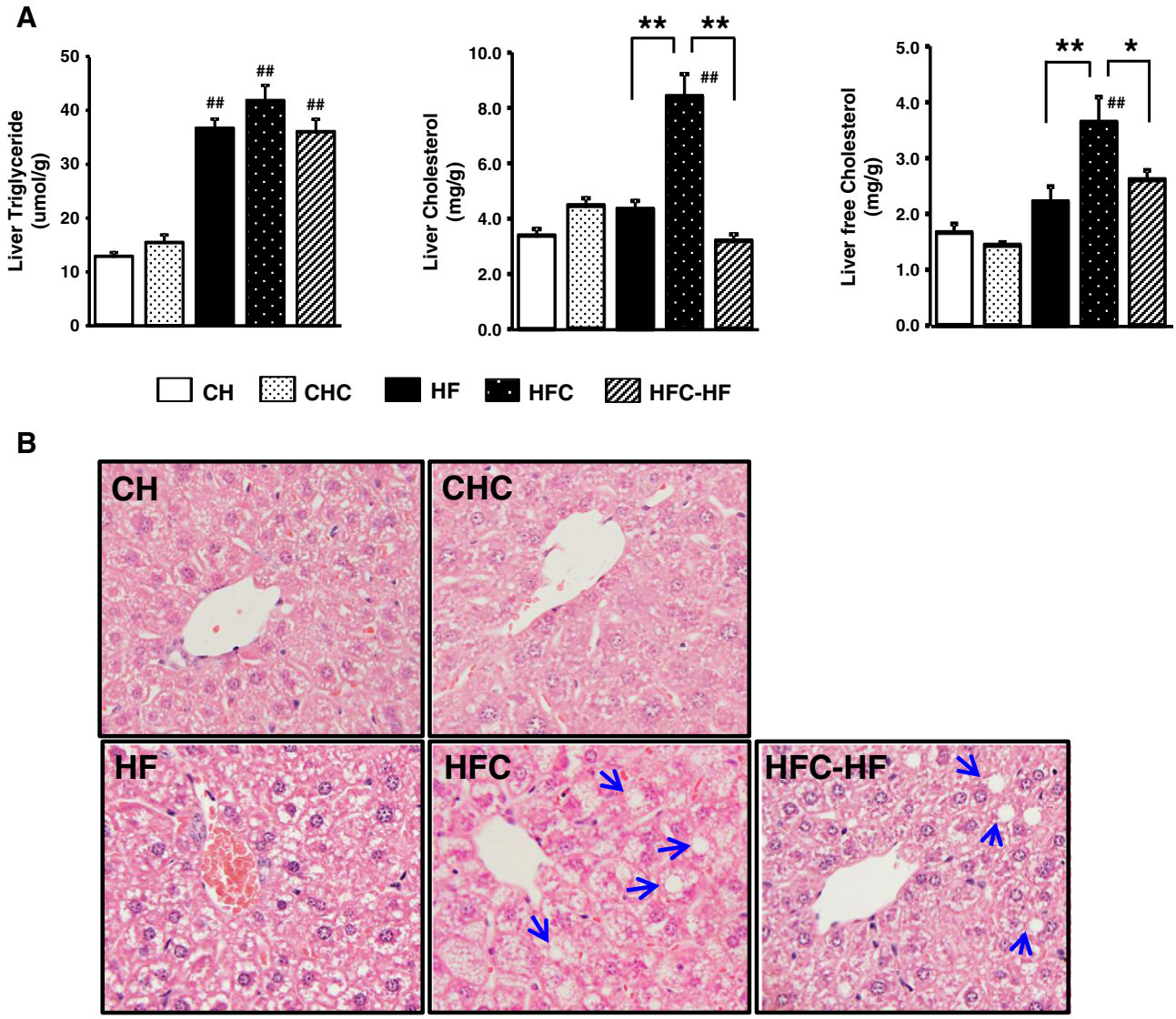

Fig. 1. Effects on liver TG and cholesterol levels. Male C57BL/6J mice were fed with chow (CH), CH with 0.2% cholesterol (CHC), high fat (HF) and HF with 0.2% cholesterol (HFC) diet for 17 weeks. The HFC-HF group was fed with HFC diet for 12 weeks followed by 5 weeks of HF feeding alone (n=7–8/group). (**A**) TG, cholesterol and free cholesterol contents in liver tissues. *P<.05, **P<.01 vs. indicated group; ##P<.01 vs. CH group. (**B**) Representative images of H&E staining of liver sections. Arrows indicate the shape of lipid droplets.

substrate of AMPK) and content of Sirt1 (another PGC1α upstream regulator) were similar among all groups.

4. Discussion

The present study investigated the role of cholesterol in the development of NAFLD and associated metabolic syndrome. Our results showed that long-term HF feeding induced obesity, glucose intolerance and benign hepatic steatosis without liver injury. Inclusion of cholesterol at a moderate level in the HF diet produced typical features of NASH indicated by liver injury and inflammation in addition to steatosis. Interestingly, these NASH phenotypes were associated with blunted mitochondrial function. Removal of cholesterol from the HF diet confirmed the role of cholesterol for the HF diet to cause liver injury. Our findings demonstrated that dietary cholesterol combined with consumption of fat plays a critical role in the development of NASH and these provide the first evidence for compromised mitochondrial function in this pathological process.

NAFLD represents a broad range of liver disorders from benign steatosis to NASH and, in some severe cases, even cirrhosis. While hepatic steatosis is regarded as the first "hit", most people in this stage are asymptomatic. In the progress of NAFLD, NASH is a turning point because of the incurred injury that precipitates the liver pathology [1]. It has been widely recognized that additional insults or a second "hit", such as inflammation, are required to progress benign steatosis to NASH [3,23,24]; the triggers of the second hit are not well understood. Our results showed that the HF diet resulted in marked hepatic steatosis, obesity and insulin resistance along with moderate increases in plasma levels of TG, cholesterol and LDL. In comparison, dietary cholesterol did not exacerbate these metabolic changes or alter HF-induced increases in plasma lipids and hepatic steatosis. However, the effects of cholesterol in combination with HF were evident in the development of NASH.

We found that addition of a moderate level of cholesterol in HF diet induced significant liver injury and this was associated with inflammation in the liver as indicated by increased mRNA expression of proinflammatory cytokines and TLR4 as well as elevated NLRP3

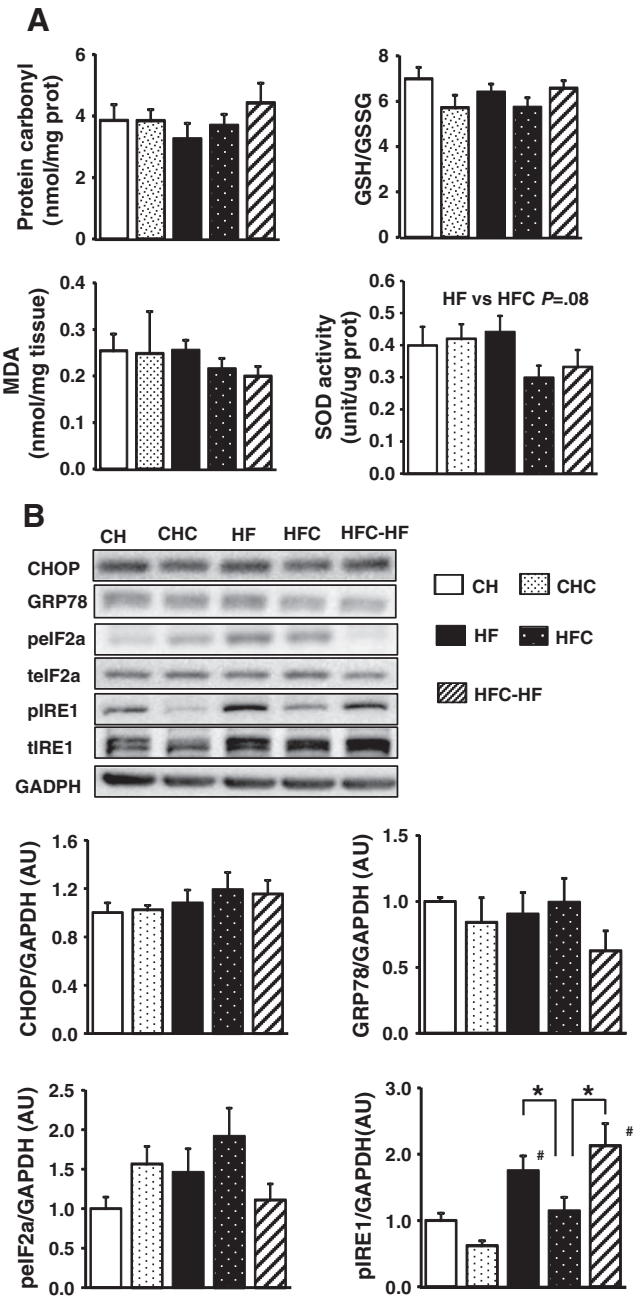

Fig. 2. Effects on oxidative stress and ER stress pathway in the liver. CH, CHC, HF and HFC groups were fed their corresponding diet for 17 weeks. HFC-HF group was fed the HFC diet for 12 weeks followed by 5 weeks of HF feeding (n=7–8/group). (**A**) Protein carbonyl, GSH/GSSG, MDA content and SOD activity were determined using commercial assay kit. (**B**) The protein levels were measured by immunoblotting and normalized by GAPDH. *P<.05 vs. indicated group; #P<.05 vs. CH group.

inflammasome levels. We have noted some previous reports of liver injury and/or inflammation in HF-fed mice [25–29]. For example, one study reported hepatic inflammation in HF-fed mice [26]. However, in that study, 72% of the total calories were present as fat; this resulted in severe steatosis (~10× increase in liver TG content) [26]. In our study, the HF diet contained less fat (45% of total calories), which resembles more closely the diet consumed by humans, and the degree of hepatic steatosis was less severe (3-fold increase in TG content). It is possible that differences in HF diet composition and feeding duration have different effects on inflammation [25,26,28,29]. Regardless of these considerations, our results demonstrated an important role of

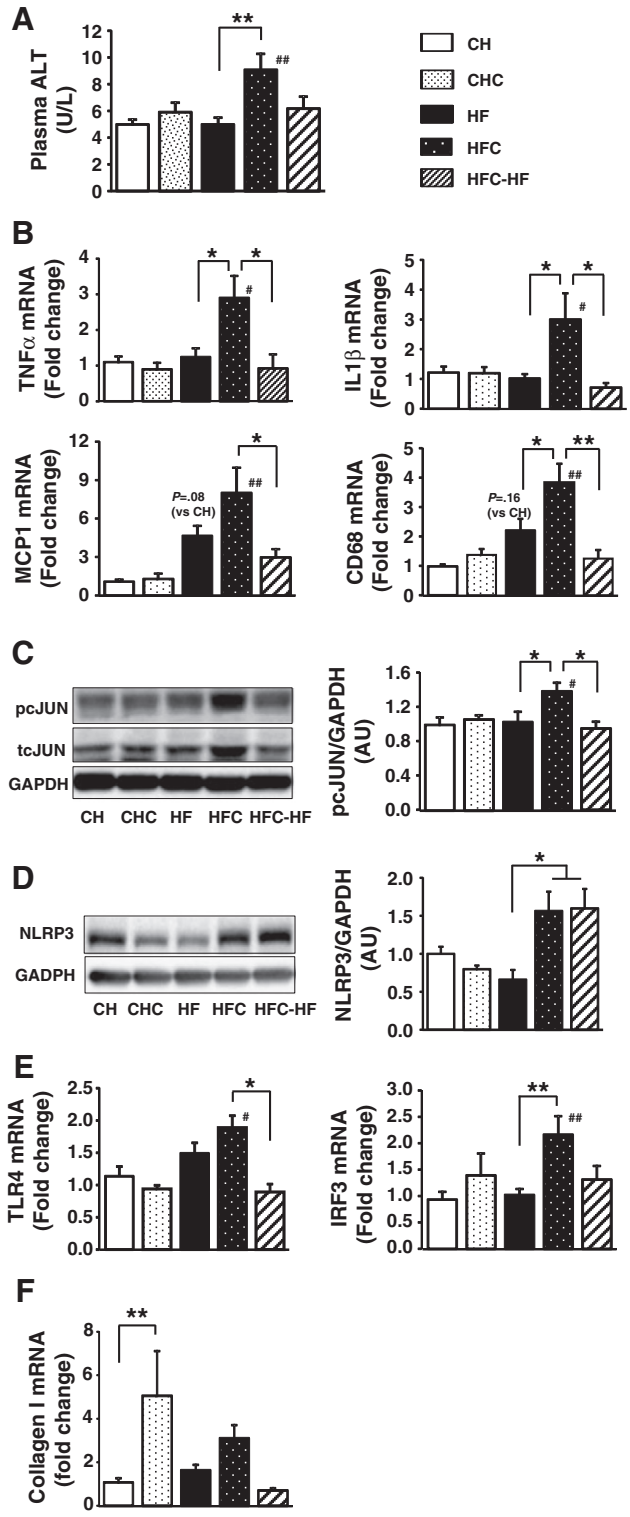

Fig. 3. Effects on plasma ALT and NASH makers. CH, CHC, HF and HFC groups were fed their corresponding diet for 17 weeks. HFC-HF group was fed the HFC diet for 12 weeks followed by 5 weeks of HF feeding (n=7–8/group). (**A**) At the end of the study, plasma samples were collected for the measurement of ALT levels. (**B**) mRNA expression levels of inflammatory markers were determined by quantitative real-time PCR. (**C**) Phospho-cJUN protein levels were measured by immunoblotting and normalized by GAPDH. (**D**) NLRP3 protein level was determined by the immunoblotting and normalized by GAPDH. (**E**) TLR4 and IRF3 mRNA expression levels were measured by quantitative real-time PCR. (**F**) Collagen I mRNA expression levels were measured by quantitative real-time PCR. *P<.05, **P<.01 vs. indicated group; #P<.05, ##P<.01 vs. CH group.

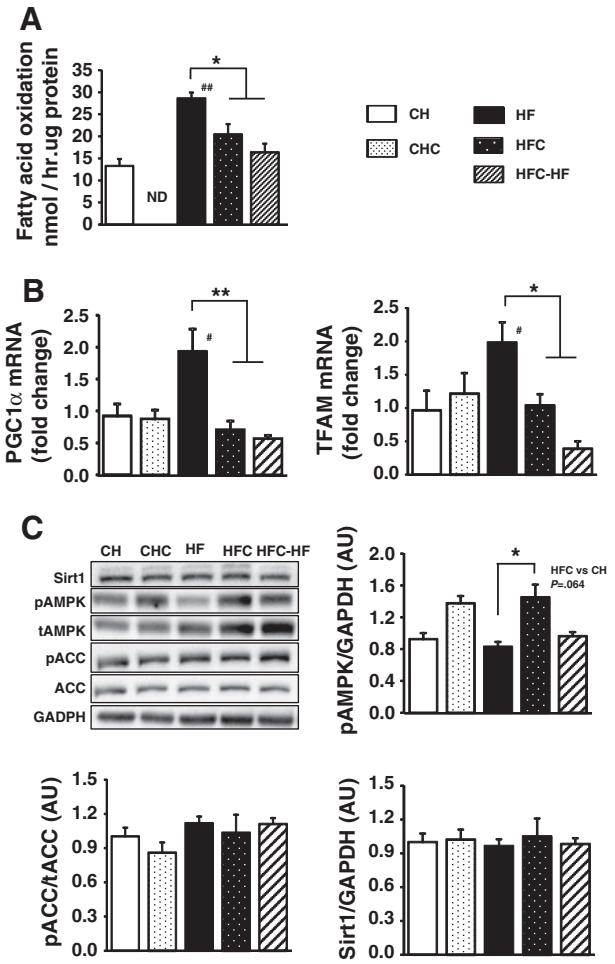

Fig. 4. Effects on liver mitochondrial phenotype. CH, CHC, HF and HFC groups were fed their respective diets for 17 weeks. The HFC-HF group was fed the HFC diet for 12 weeks followed by 5 weeks of HF feeding. (**A**) Fatty acid oxidation in isolated fresh liver homogenates measured by incubation with 0.5 μCi [^{14}C]palmitate (n=5–7/group). (**B**) PGC1α and TFAM mRNA expressions quantified by real-time PCR. (**C**) Protein content of Sirt1 and the phosphorylation of AMPK and ACC measured by immunoblotting (n=7–8/group). ND: not determined (tissue not available due to an incident). *P<.05, **P<.01 vs. indicated group; #P<.05, ##P<.01 vs. CH group.

cholesterol in converting simple hepatic steatosis to NASH while on an HF diet and with moderate hepatic steatosis. A recent study showed that addition of 0.2% cholesterol to an HF diet (45 kcal%, composed of high fat and trans-fat) induced robust NASH in mice when supplemented with sucrose in the drinking water [30]. Our results clearly showed that neither dietary cholesterol nor HF alone produced the NASH phenotype. More importantly, our study revealed, for the first time, the involvement of retarded mitochondrial biology in the transition from steatosis to NASH.

Although mitochondrial dysfunction has previously been implicated in the development of NASH [7], HF diets alone have been shown to increase mitochondrial capacity in skeletal muscle and the liver [11,31]. The present study showed that the HF-induced increase in the expression of PGC1α and TFAM was inhibited by the addition of cholesterol in the HF diet. PGC1α is a transcription cofactor that regulates the genes in mitochondrial metabolism [32] whereas TFAM is a key activator of mitochondrial transcription. Blunted expression of these key regulators of mitochondrial biogenesis may lead to a reduced mitochondrial capacity of the liver to compensate for the increased lipid influx from the HF diet. Indeed, fatty acid oxidation in the liver of HF-fed mice was impaired when cholesterol was added into the HF diet. These data together indicate that cholesterol abolishes the capacity of the liver to increase mitochondrial metabolism in response to the HF diet. These novel findings are consistent with a recent study showing that free cholesterol induces mitochondrial injury in isolated hepatocytes from mice [33].

We next investigated whether the AMPK pathway may be responsible for the increased expression of PGC1α and fatty acid oxidation. The results showed a trend of moderate increase in the content of AMPK in all HF mice independent of cholesterol. As chronic hyperinsulinemia and/or hyperleptinemia can increase the expression of AMPK [34,35], the observed changes in AMPK levels may be sue to hyperinsulinemia (shown in this study) and hyperleptinemia as previously shown by one of us [36]. Interestingly, we found an overall increase in the AMPK phosphorylation (pAMPK/GAPDH) in HFC-fed mice and the mechanism for the increased AMPK phosphorylation is, however, not currently known to us. Despite this, these data indicate that the decreased PGC1α expression and fatty acid oxidation in HFC mice do not result from an inhibition to AMPK. By a similar analogy, the blunted mitochondrial function is not attributable to Sirt1 in the absence of its change.

We further investigated whether the retarded mitochondrial function is attributable to oxidative stress [37,38], ER–mitochondria interaction [39,40] or inflammation [41] as has been suggested. None of the oxidative stress markers (protein carbonyl, GSH, MDA or SOD activity) were significantly changed with the inclusion of cholesterol in HF diet. Neither was there an indication of an increase in ER stress (GRP78, IRE1 and eIF2α). However, our data clearly indicated the presence of significant inflammation (increases in TNFα, IL1β, MCP1 and CD68 mRNA expression) when cholesterol was added to the HF diet, suggesting a link between inflammation and the blunted mitochondrial function. The increased expressions of TLR4 and its downstream target IRF3 in HFC-fed mice, but not in CH- or HF-fed mice alone, indicate the synergistic effects of cholesterol and fatty acids to amplify the response to TLR4 ligands. As free cholesterol accumulation in macrophages has been shown to activate TLR4 [42], we speculate that the increased expression of TLR4 in HFC-fed mice may result from the accumulation of free cholesterol that was dramatically exacerbated by HF diet. This interpretation is also consistent with the fact that HFC also markedly increased NLRP3 inflammasome, an intracellular complex that is well known to be induced by cholesterol crystal [43]. The substantial accumulation of cholesterol in the liver in HFC mice (as compared with CHC mice) is probably due, at least in part, to the increased absorption and decreased excretion of cholesterol in the presence of HF diet [44,45]. In addition, HF-diet-derived fatty acids may serve as the ligands of TLR4 to stimulate the inflammatory pathway.

Intriguingly, blunted mitochondrial function persisted along with the increased protein level of NLRP3 inflammasome after withdrawing cholesterol despite the fact that the levels of proinflammatory cytokines (TNFα, IL1β and MCP1) had subsided. The concurrent changes in mitochondrial function and NLRP3 inflammasome are consistent with recent studies suggesting that NLRP3 activation is associated with mitochondrial dysfunction [46,47] and impaired mitochondrial function may not immediately recover from injury [48,49]. It has been shown that significant increased cholesterol deposition in the liver can induce inflammation independently of steatosis [50]. This may explain the development of NASH in the HFC mice but not in CHC or HF mice because neither dietary cholesterol nor HF alone significantly affected liver cholesterol levels, whereas in combination, they dramatically increased the deposition of cholesterols (~2-fold for free cholesterol). Further studies are required to establish the causal relationship between inflammatory cytokines, NLRP3 inflammasome and mitochondrial function. Apart from the combined effects with HF diet, dietary cholesterol may independently promote the fibrosis (indicated by collagen I mRNA expression) of the liver.

Another interesting finding was that addition of cholesterol to the HF diet attenuated glucose intolerance. Although hepatic steatosis is generally associated with insulin resistance, the direct causes of impaired insulin action are lipid intermediates such as ceramide and diacylglycerol [51,52]. It has been suggested that sequestration of these lipid intermediates within lipid droplets can reduce their interference with the insulin signaling in the cytosol without reducing hepatic steatosis [53,54]. Clearly, addition of cholesterol into the HF diet enlarged lipid droplets in the liver. Whether this may sequester lipid intermediates to reduce their inhibition of insulin action remained to be investigated. Another possibility for the attenuated glucose intolerance by cholesterol might be due to increased SCD1 (Supplementary Figure) as previously observed [55]. As unsaturated fatty acids are relatively benign to insulin action [56], increased SCD1 may attenuate insulin resistance by converting detrimental saturated fatty acids [57] to unsaturated fatty acids.

In summary, the present study clearly demonstrated that a moderate level of dietary cholesterol (0.2% w/w) exacerbated simple hepatic steatosis into NASH in HF-fed mice. Furthermore, our data revealed the involvement of inflammation and retarded mitochondrial function in the liver during this process but not oxidative stress or ER stress. We also found, for the first time, that the blunted mitochondrial function in the liver persisted after the inflammation response and plasma ALT had subsided. These findings suggest that cholesterol plays a critical role in the conversion of hepatic steatosis toward NASH possibly by activation of inflammatory pathways along with blunted mitochondrial function.

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Author contributions

JMY and AX conceived the study and provided reagents, materials and analytical tools. JMY, SL and XYZ designed the study. SL performed most of the experiments. XYZ, XZ, HW and EJ contributed to analysis and interpretation of the research data. SRR contributed to liver morphology analysis and advised on drafts of the manuscript. JMY and SL analyzed data and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interests in this study.

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