

Original Article

Role of mitochondrial depolarization and disrupted mitochondrial homeostasis in non-alcoholic steatohepatitis and fibrosis in mice

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Abstract: The pathogenesis of non-alcoholic steatohepatitis (NASH) is poorly understood. Here, relationships between mitochondrial depolarization (mtDepo) and mitochondrial homeostasis were studied in a mouse model of NASH. C57BL/6 mice were fed a Western diet (high fat, fructose and cholesterol) for 2 weeks, 2 months and 6 months, and livers were harvested for histology and biochemical analysis. Hepatic mtDepo was evaluated by intravital multiphoton microscopy. After Western diet feeding, mixed hepatic micro- and macrovesicular steatosis and leukocyte infiltration occurred at 2 weeks and continued to increase afterwards. ALT release, mild necrosis, apoptosis, and ballooning degeneration were present at 2 and 6 months. Smooth muscle α -actin expression increased at 2 weeks and longer, and increased collagen-I expression and mild fibrosis occurred at 6 months. After feeding Western diet for 2 weeks and longer, mtDepo appeared in 50-70% hepatocytes, indicating mitochondrial dysfunction at an early stage of NASH. mtDepo can initiate mitophagy, and mitophagic markers increased at 2 and 6 months. Concurrently autophagic processing became impaired. Oxidative phosphorylation proteins, mitochondrial biogenesis signals, and proteins associated with mitochondrial fission and fusion decreased after 2 months and longer of Western diet. Proinflammatory and profibrotic signaling (NLRP3 inflammasome activation, expression of IL-1, osteopontin and TGF- β 1) also increased in association with mitochondrial stress/dysfunction after Western diet feeding. Taken together, we show that hepatic mtDepo occurs early in mice fed a Western diet, followed by increased mitophagic burden, suppressed mitochondrial biogenesis and dynamics, and mitochondrial depletion. These novel mitochondrial alterations in NASH most likely play an important role in promoting steatosis, inflammation, and progression to fibrosis.

Keywords: Mitochondrial depolarization, mitochondrial biogenesis, mitochondrial dynamics, mitophagy, non-alcoholic fatty liver disease

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease with excessive hepatic fat accumulation in patients without alcohol abuse (< 20 g alcohol/day). The prevalence of NAFLD in general populations in Western countries is 20-30% [1-4]. However, prevalence rises markedly to up to 75% in patients with obesity or type 2 diabetes [5]. Due to the pandemic of obesity and metabolic syndrome, NAFLD is sharply increasing, becoming the leading cause of chronic liver disease and a significant issue in public health [6, 7]. Up to 30% of NAFLD patients will progress from simple steatosis to

inflammation (non-alcoholic steatohepatitis, NASH), some of whom will develop fibrosis and cirrhosis [8-10]. Moreover, up to 13% patients with NASH-related cirrhosis will develop hepatocellular carcinoma, a highly malignant cancer and the second leading cause of cancer-related death worldwide [11, 12]. It is estimated that cirrhosis and liver cancer collectively cause ~2 million deaths every year globally [7]. Patients with NAFLD also have a markedly higher risk of atherosclerosis and cardiovascular disease [13].

Mechanisms of NAFLD/NASH pathogenesis remain incompletely understood. Insulin resis-

tance and conditions associated with insulin resistance, such as obesity, physical inactivity, and dyslipidemia, all are associated with NAFLD/NASH [14-19]. A Western diet with high fat, high fructose and high cholesterol is well recognized to cause obesity and NAFLD [3, 20, 21]. Gut microbiota alterations, Kupffer cell activation, oxidative stress, endoplasmic reticulum stress, and inflammatory signaling also contribute [15-18, 22, 23]. Although clinical trials are ongoing, no proven effective pharmacotherapies are currently available for NAFLD. Weight loss is the only proven treatment [5].

Although the progress of NASH is usually slower than alcoholic steatohepatitis (ASH), the histopathology of the two diseases is very similar. Our previous study showed that mitochondrial depolarization (mtDepo) occurs *in vivo* in the liver after ethanol consumption in response to ethanol metabolism to acetaldehyde. This mtDepo leads to accumulation of fat droplets and liver injury [24]. Other studies show that mitochondrial production of reactive oxygen species (ROS) also increases in NAFLD, which could promote mitochondrial dysfunction [25, 26]. Moreover, expression of cyclophilin D, a component of the mitochondrial permeability transition pore, increases in NAFLD, and mitochondria isolated from *ob/ob* mice or mice fed a high fat diet exhibit increased sensitivity to calcium-induced mitochondrial swelling *in vitro* [27-29]. ROS also cause lipid peroxidation, leading to formation of aldehydes like malondialdehyde and 4-hydroxynonenal that might potentially cause mtDepo. However, whether mtDepo occurs in NAFLD *in vivo* and its relation to other mitochondrial alterations remains unknown.

In the face of various stresses, mitophagy, mitochondrial biogenesis, and mitochondrial dynamics are important processes maintaining mitochondrial function, homeostasis, quality control and ultimately cell survival [30]. Impairment of mitochondrial homeostasis likely contributes to various hepatic pathologies, including steatosis, cell death, inflammation, and fibrosis [31-35]. In this study using a model of NAFLD/NASH produced by Western diet feeding in mice, we examined alterations of mitochondrial membrane potential, mitophagy, mitochondrial biogenesis and mitochondrial

dynamics and related these mitochondrial alterations to the progression of disease.

Methods

Animals

Male C57BL/6J mice (7-8 weeks) obtained from Jackson Laboratory were fed a powder diet with high fat (26.2% w/w as corn oil, 50% of calories), high fructose (35.8% w/w, 29% of calories), and high cholesterol (0.5% w/w) (Dyets, Bethlehem, PA), which mimics the Western diet, for up to 6 months. Remaining calories were derived from protein (casein). Control mice (CTR) were fed a regular chow diet with low fat (5%, w/w, 13% of calories), low fructose (0.27%, w/w), and low cholesterol (0.02% w/w) for 6 months. Other carbohydrates in the chow diet provided 56.7% of calories and proteins provided ~29% of calories. All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Measurement of serum alanine aminotransferase and blood glucose

After 2 weeks, 2 months and 6 months of feeding, the abdomen was opened under pentobarbital anesthesia (80 mg/kg, *i.p.*), and blood was collected from the inferior vena cava. Alanine aminotransferase (ALT) was measured using a kit from Pointe Scientific (Canton, MI). Glucose in blood from the tail vein was detected at 2 weeks and 2 months after feeding CTR or Western diet using a ReliOn Prime Blood Glucose Monitoring System (Walmart, Bentonville, AR) according to the manufacturer's instructions. Mice were fasted for 7-8 h before blood glucose measurement.

Histology and detection of fibrosis on liver sections

Under pentobarbital anesthesia, the liver was harvested, fixed and processed for paraffin sections, as described previously [36]. Liver sections were stained with hematoxylin and eosin (H&E) for histological analysis, and images were captured (Zeiss AX10 microscope, Thornwood, NY) using a 20 × objective lens. Some liver slides were stained with the Mason's

trichrome staining, which specifically reveals liver fibrosis.

Intravital multiphoton microscopy

Intravital multiphoton microscopy was performed after feeding Western or CTR diets for 2 weeks and 2 months. Mitochondrial polarization status in living mice was detected using the cationic fluorophore tetramethylrhodamine methylester (TMRM, Invitrogen, Carlsbad, CA) that is taken up electrophoretically by polarized mitochondria in response to their negative membrane potential. Fluorophore loading and preparation for intravital microscopy were performed as described previously [24]. A Zeiss LSM 880 NLO laser scanning confocal/multiphoton microscope and a 30 × 1.2 N.A. water-immersion objective lens were used for image capture. The excitation wavelength was 800 nm, and non-descanned fluorescence emission was collected through 562-633 nm band-pass filter.

Immunoblotting

Liver tissue was snap-frozen in liquid nitrogen and kept at -80°C until detection of proteins of interest by immunoblotting, as described previously [36]. Primary antibodies were against caspase-1, cleaved caspase-3 (CC3), NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and actin (Cell Signaling Technology, Danvers, MA); myeloperoxidase (MPO, DAKO Corp., Carpinteria, CA); transforming growth factor-β1 (TGF-β1) and Type I collagen (Abcam, Cambridge, MA); osteopontin (OPN), sequestosome 1 (SQSTM1), dynamin-related GTPase protein-1 (Drp-1), fission-1 (Fis-1), mitofusin-2 (Mfn-2), interleukin-1 (IL-1), NADH dehydrogenase-3 (ND3), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and PTEN-induced putative kinase 1 (PINK-1) (Santa Cruz Biotech., Santa Cruz, CA); F4/80 (Serotech, Raleigh, NC); smooth muscle α-actin (α-SMA, DAKO, Carpinteria, CA); ATP synthase-β (AS-β) and mitochondrial transcription factor-A (Tfam) (GenWay Biotech, San Diego, CA); and microtubule-associated protein 1A/1B-light chain 3 (LC3, MBL International, Des Plaines, IL) at concentrations of 1:1000 to 1:3000 overnight at 4°C.

Statistical analysis

Data shown are means ± S.E.M. (3-4 mice per group). Groups were compared using ANOVA

plus Student-Newman-Keuls posthoc test. Differences were considered significant at $P < 0.05$.

Results

Western diet feeding increases blood glucose and liver steatosis and injury in mice

In mice fed CTR diet, blood glucose was 105 ± 2.4 mg/dL, which increased to 216 ± 11.9 mg/dL ($n = 3/\text{group}$, $P < 0.05$ vs CTR) after feeding Western diet for 2 weeks. Blood glucose stayed at a similar level (191.3 ± 20.0 mg/dL, $P > 0.05$ vs 2 weeks) after 2 months on Western diet.

In livers from mice fed the CTR diet, no histopathological features were present (**Figure 1A**). After feeding Western diet for 2 weeks, mixed micro- and macrovesicular fat droplets (primarily microvesicular) developed in ~40% hepatocytes (**Figure 1A**). After feeding Western diet for 2 and 6 months, more overt steatosis developed with widespread formation of macrovesicular fat droplets that were larger in size and much more abundant than after 2 weeks (**Figure 1A**).

Serum ALT, an indicator of liver injury, was not changed compared to CTR diet after feeding Western diet for 2 weeks. By contrast, ALT increased 4.0- and 5.6-fold, respectively, after 2 and 6 months (**Figure 1B**). Mild necrosis and overt ballooning degeneration were also observed in the livers after 2 and 6 months of Western diet (**Figure 1A**), and cleaved caspase-3, an indicator of apoptosis, increased 54- and 99-fold after 2 and 6 months (**Figure 1C** and **1D**).

Western diet causes inflammatory responses in mouse liver

In the livers from mice fed the CTR diet, leukocytes were rare, as observed in H&E sections (**Figure 1A**). After feeding Western diet for 2 weeks, leukocyte infiltration increased but remained scattered. By contrast, after 2 and 6 months, leukocyte infiltration increased markedly in a progressive time-dependent fashion with development of numerous necroinflammatory foci (**Figure 1A**, arrows).

Leukocyte infiltration was further confirmed by detection of MPO, a marker of neutrophils, and F4/80, a marker of monocytes/macrophages,

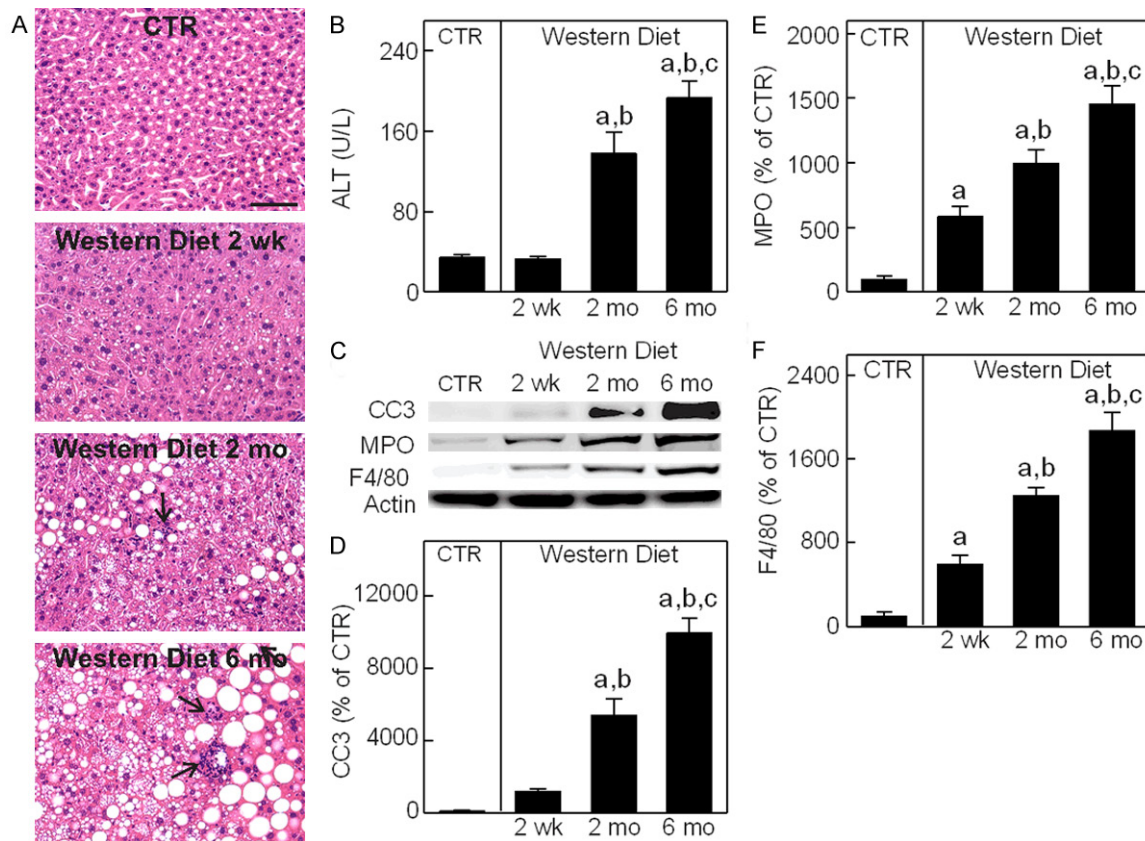


Figure 1. Western diet causes liver steatosis, injury and inflammation in mice. Mice were fed control (CTR) or Western diet for up to 6 months. A. Representative images of liver sections with H&E staining; B. Serum alanine aminotransferase (ALT) levels; C. Representative Western blots of CC3, MPO, F4/80 and actin; D. Quantification of CC3 Western blots by densitometry. E. Quantification of MPO Western blots. F. Quantification of F4/80 Western blots. Values are means \pm S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks; c, $P < 0.05$ vs Western diet for 2 months ($n = 4$ per group).

by immunoblotting. MPO and F4/80 increased 5.8- and 6-fold after feeding Western diet for 2 weeks and continued to increase progressively to 14.5- and 18.7- fold after 6 months (**Figure 1A, 1E and 1F**).

Western diet causes early fibrosis in mouse liver

Stellate cells are major effector cells of liver fibrosis. Expression of α -SMA, an indicator of stellate cell activation, increased 13-, 37-, and 45-fold after feeding Western diet for 2 weeks, 2 months and 6 months, respectively (**Figure 2A and 2B**). Excessive synthesis and deposition of extracellular matrix lead to fibrosis. After feeding Western diet, Type I collagen showed a non-significant trend to increase at 2 months (7.4-fold, $P = 0.061$) and increased significantly at 6 months (18.5 fold, $P < 0.05$) (**Figure 2A and 2C**).

After trichrome staining of liver sections from mice fed CTR diet, no fibrosis was detected (**Figure 2D** upper panels). By contrast after feeding Western diet for 6 months, blue trichrome staining increased. Fibrosis was predominantly sinusoidal and perivenular (**Figure 2D**, lower panels). Bridging fibrosis was present occasionally in some livers of Western diet-fed mice (**Figure 2D**, lower left).

Western diet causes mitochondrial depolarization in mouse liver

Previously, we observed mtDepo in a rodent model of ASH. Accordingly, we investigated whether mtDepo occurs in NAFLD after feeding Western diet using state-of-the-art intravital multiphoton microscopy to visualize directly individual polarized mitochondria by the fluorescence of TMRM, a cationic fluorophore that accumulates electrophoretically into mitochondria.

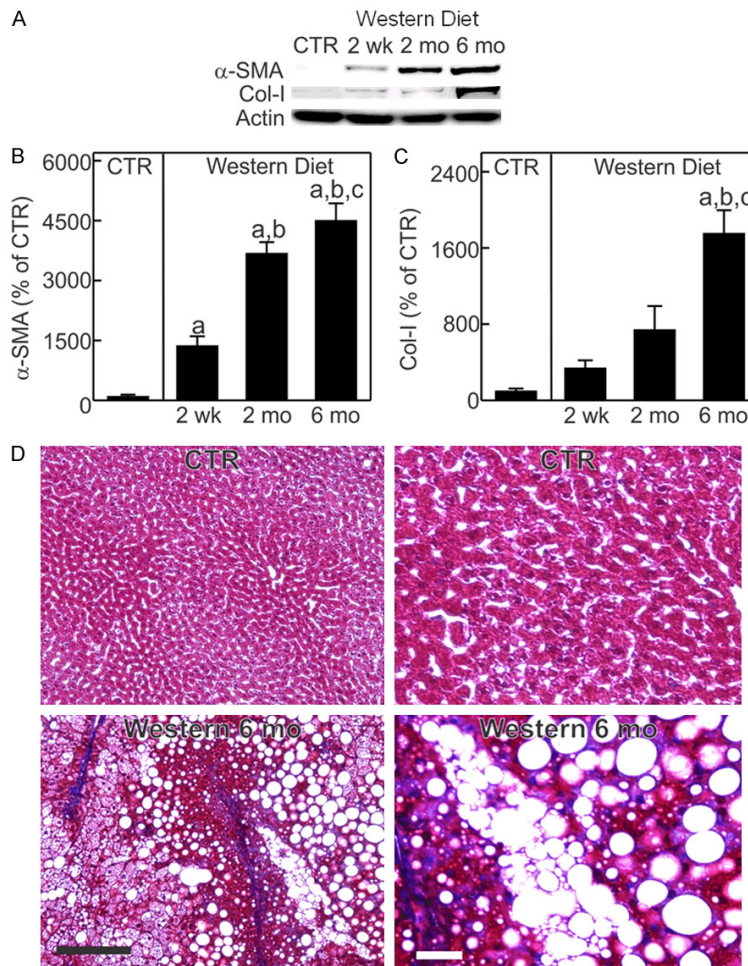


Figure 2. Western diet feeding induces stellate cell activation and fibrosis in the liver. Mice were fed control (CTR) or Western diet for up to 6 months. A. Representative Western blots of smooth muscle α -actin (α -SMA), Type I collagen (Col-I) and actin; B. Quantification of α -SMA Western blots by densitometry; C. Quantification of Col-I Western blots; D. Representative images of liver sections with Mason's trichrome staining at low and high magnification. Bars are 200 μ m (left panel) and 50 μ m (right panel). Values are means \pm S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks; c, $P < 0.05$ vs Western diet for 2 months ($n = 4$ per group).

dria with a negative membrane potential. In livers of mice fed the CTR diet, mitochondria were polarized in virtually all hepatocytes, as indicated by red punctate TMRM fluorescence (**Figure 3A**). After feeding Western diet for 2 weeks, mtDepo occurred in about 50% of hepatocytes, as revealed by dim and diffuse TMRM fluorescence (**Figure 3B** and **3D**). As observed previously after ethanol feeding, mtDepo occurred in an all-or-nothing fashion within individual hepatocytes after Western diet feeding. mtDepo further increased to 69% of hepatocytes after 2 months (**Figure 3C** and **3D**).

increased autophagic burden, as well as a possible deficit of autophagic processing of autophagosomes into lysosomes for degradation. p62/SQSTM1, another indicator of autophagosome accumulation, also increased by 8- and 15-fold after 2 and 6 months of feeding (**Figure 4A** and **4E**).

Oxidative phosphorylation proteins decrease in the liver of western diet-fed mice

We explored the effects of Western diet on oxidative phosphorylation proteins. AS- β , a nuclear DNA-encoded subunit of the mitochondrial

Mitophagic burden increases but autophagic processing is disrupted in mouse liver after feeding Western diet

Type I (phosphoinositide 3-kinase [PI3K]-dependent) mitophagy as occurs in nutrient deprivation and cytoplasmic remodeling leads to mtDepo, whereas mitochondrial damage and mtDepo induces Type II (PI3K-independent) mitophagy [37, 38]. After ethanol feeding, mtDepo leads to increased hepatic mitophagy as well as steatosis [24, 39]. Accordingly, we explored whether Western diet, which we found to cause mtDepo, also increases mitophagy. PINK1 is a protein that accumulates in depolarized mitochondria and mediates mitophagy. PINK1 increased 9-fold after 2 weeks of Western diet feeding and further increased to 19- and 46-fold at 2 and 6 months, respectively (**Figure 4A** and **4B**). LC3-I/II is an indicator of autophagic flux. Overall, neither LC3-I nor LC3-II increased at 2 weeks, but both increased significantly after 2 months by 3.6- and 6.1-fold, respectively, with further increases of 16- and 19-fold at 6 months (**Figure 4A**, **4C**, and **4D**). These increases of LC3-I and LC3-II document an

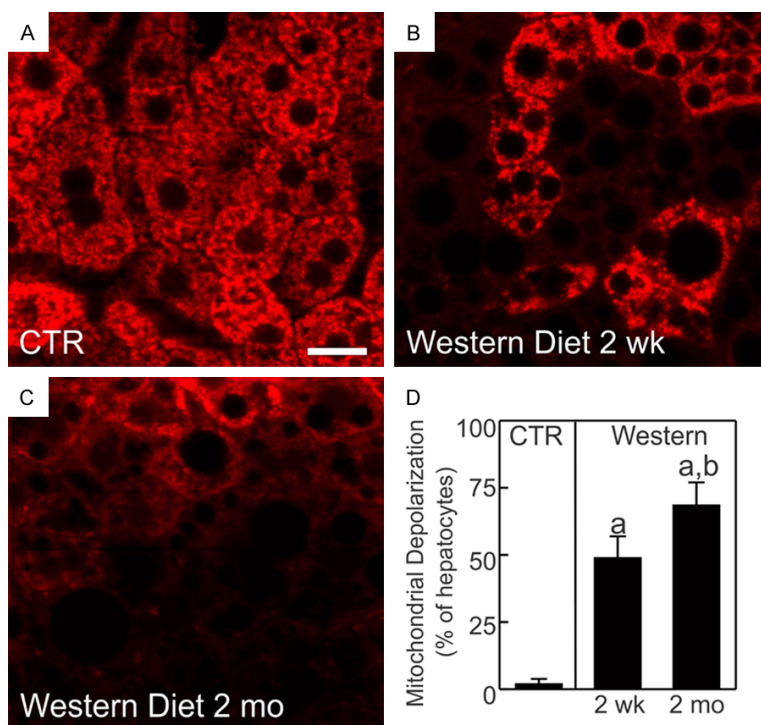


Figure 3. Western diet feeding causes mitochondrial depolarization in the liver. Mice were fed control (CTR) or Western diet for up to 2 months. A. Representative image of intravital multiphoton microscopy of TMRM fluorescence after CTR diet; B and C. Representative images of TMRM fluorescence after 2 weeks and 2 months of Western diet; D. Hepatocytes with depolarized mitochondria were counted in 10 random fields per mouse as the percentage of the total. Bar is 10 μ m. Values are means \pm S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks ($n = 4$ per group).

F_1F_0 -ATP synthase, did not change after feeding Western diet for 2 weeks but decreased by 20% and 52% at 2 and 6 months (Figure 5A and 5B). Similarly, ND3, a mitochondrial DNA-encoded subunit of respiratory complex I (NADH dehydrogenase), did not change after feeding Western diet for 2 weeks but decreased by 39% and 84% at 2 and 6 months (Figure 5A and 5C).

Western diet suppresses mitochondrial biogenesis signaling in mouse liver

Mitochondrial biogenesis is a cellular adaptation in response to increased energy demand or the need to replace damaged mitochondria [40–42]. PGC-1 α , the master regulator of mitochondrial biogenesis, did not change after 2 weeks of Western diet feeding but decreased 24% and 56% after 2 and 6 months of feeding (Figure 5A and 5D). Tfam, a transcription factor that controls the replication and transcription of mitochondrial DNA, decreased 59% and 86% at 2 and 6 months (Figure 5A and 5E). These

decreases of PGC-1 α and Tfam indicated disruption of mitochondrial biogenesis.

Western diet suppresses mitochondrial dynamics in mouse liver

After synthesis of mitochondrial components, mitochondria undergo mitochondrial fission to generate daughter mitochondria. Fis-1, a protein that mediates mitochondrial fission, did not change at 2 weeks of feeding but decreased 49% and 84% at 2 and 6 months (Figure 6A and 6B). Similar alterations were observed in Drp-1, another protein that is involved in mitochondrial fission (Figure 6A and 6C). Mfn-2, a protein that mediates mitochondrial fusion, also decreased 30% and 56% at 2 and 6 months (Figure 6A and 6D). These data indicate an impairment of mitochondrial dynamics by Western diet.

Western diet increases

NLRP3 inflammasome activation in mouse liver

Mitochondrial stresses stimulate NLRP3 inflammasomes [32, 33, 43]. We therefore explored the effects of Western diet on NLRP3 inflammasome activation and subsequent signaling. NLRP3 protein expression increased as early as 2 weeks (9-fold) of Western diet feeding and continued to rise progressively to 22-fold increase at 6 months (Figure 7A and 7B). NLRP3 inflammasome activation leads to caspase-1 activation [32, 33, 43]. Native caspase-1 p45 protein increased 2.6-fold and continued to increase progressively to 9-fold at 6 months (Figure 7A and 7C), indicating increased expression of caspase-1. Likewise, proteolytically activated caspase-1 p10 increased 14.6-fold at 2 weeks and further increased progressively to 85.3-fold at 6 months (Figure 7A and 7D). Increases in activated caspase-1 p10 far exceeded increases in caspase-1 p45, indicating markedly increased caspase-1 activation. NLRP3 inflammasomes promote the pro-

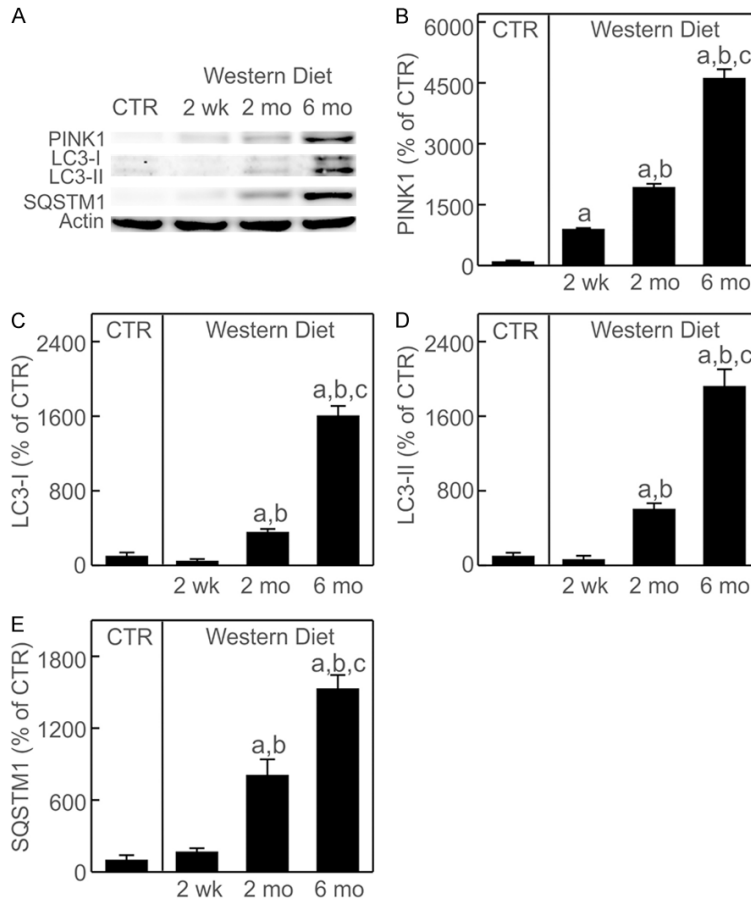


Figure 4. Western diet feeding increases mitophagic burden but disrupts autophagosome processing in the liver. Mice were fed control (CTR) or Western diet for up to 6 months. A. Representative Western blots of PTEN-induced putative kinase 1 (PINK1), microtubule-associated protein 1A/1B-light chain 3 (LC3)-I/II, sequestosome 1 (SQSTM1) and actin; B. Quantification of PINK1 Western blots by densitometry; C. Quantification of LC3-I Western blots; D. Quantification of LC3-II Western blots; E. Quantification of SQSTM1 Western blots. Values are means \pm S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks; c, $P < 0.05$ vs Western diet for 2 months ($n = 4$ per group).

teolytic maturation of inflammatory and fibrotic cytokines, such as IL-1 β . Indeed, IL-1 β increased markedly and progressively after Western diet treatment in parallel to NLRP3 inflammasome activation (Figure 7A and 7E).

Western diet increases osteopontin and TGF- β 1 expression in mouse liver

Expression of OPN, a proinflammatory and pro-fibrotic protein, has been linked to mitochondrial oxidative stress [44, 45]. A weak band of OPN expression was observed in CTR mice (Figure 7A). After Western diet feeding, OPN expression increased in a time-dependent manner, and some weak immune-responsive

bands with lower molecular weights were also detected, possibly due to different splicing of the protein or proteolytic processing. The major OPN band increased 2.6-fold at 2 weeks of Western diet feeding and progressively increased to 10-fold at 6 months (Figure 7A and 7F). Expression of the potent profibrotic cytokine TGF- β also increased markedly in a time dependent manner between 2 weeks and 6 months (Figure 7A and 7G). Interestingly, a recent study shows that mesalazine, an OPN inhibitor, decreases TGF- β expression and attenuates thioacetamide-induced liver fibrosis in rats, suggesting cross-talk between these two profibrotic mediators [46].

Discussion

Mitochondrial depolarization and disrupted mitochondrial homeostasis occur in NAFLD

NAFLD has become a substantial burden for public health, whereas the mechanisms of its pathogenesis remain unclear. Development of NAFLD is most likely multifactorial, but recent studies suggest that mitochondrial dysfunction may be an important contributor [25, 26, 47, 48]. Structural and molecular alterations of hepatic mitochondria occur in NAFLD [48, 49]. Mitochondria isolated from livers of mice fed a high-fat, high-fructose diet show ballooned and rounded cristae, as well as condensed matrix structures [49]. Mitochondria isolated from *ob/ob* mice and mice fed a high fat diet exhibit increased sensitivity to calcium-induced mitochondrial swelling *in vitro* [27-29]. Decreased respiratory chain activity and ATP, increased mitochondrial oxidative stress, imbalanced mitochondrial dynamics, and depletion of mitochondrial DNA are also reported [25, 26, 47, 48]. However, the sequence of events and signaling pathways that link mitochondrial alteration/dysfunction

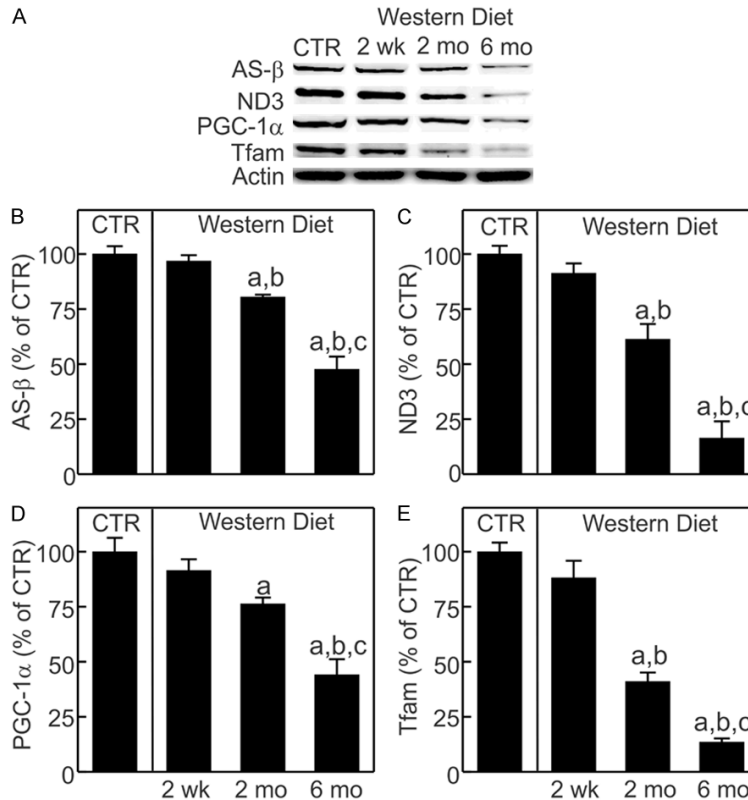


Figure 5. Western diet feeding inhibits mitochondrial biogenesis in the liver. Mice were fed control (CTR) or Western diet for up to 6 months. A. Representative Western blots of ATP synthase-β (AS-β), NADH dehydrogenase-3 (ND3), PGC-1α, Tfam and actin; B. Quantification of AS-β Western blots by densitometry; C. Quantification of ND3 Western blots; D. Quantification of PGC-1α Western blots; E. Quantification of Tfam Western blots. Values are means ± S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks; c, $P < 0.05$ vs Western diet for 2 months ($n = 4$ per group).

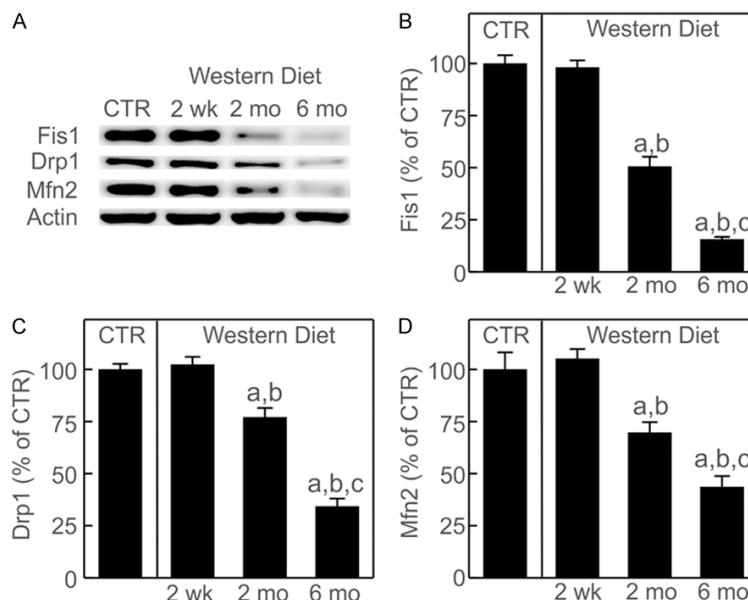


Figure 6. Western diet feeding inhibits mitochondrial dynamics in the liver. Mice were fed control (CTR) or Western diet for up to 6 months. A. Representative Western blots of Fis1, dynamin-related protein 1 (Drp1), mitofusin-2 (mfn2), and actin; B. Quantification of Fis1 Western blots by densitometry; C. Quantification of Drp1 Western blots; D. Quantification of mfn2 Western blots. Values are means ± S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks; c, $P < 0.05$ vs Western diet 2 months ($n = 4$ per group).

to NAFLD progression remain unclear. Moreover, most of the findings are based on *in vitro* or *ex vivo* studies that may not completely mirror *in vivo* alterations of mitochondria in NAFLD. In this study using intravital multiphoton microscopy technology, we demonstrate for the first time that mtDepo, a phenomenon that contributes to ASH development, also occurs *in vivo* in a Western diet model. Moreover, we showed that mtDepo occurs very early, preceding other mitochondrial alterations and likely contributing to dysregulated mitochondrial homeostasis/quality control in response to stress in NAFLD.

Many different dietary treatments have been used to induce NAFLD (e.g., high fat alone, high fat plus high fructose, high fat plus high cholesterol, high fat with high fructose and high cholesterol, high fat with high sucrose, and methionine-choline-deficient diets) [50, 51]. Here, we used a combination of high fat, high fructose and high cholesterol diet, since this combination characterizes “fast food” of Western diets. As a consequence of feeding Western

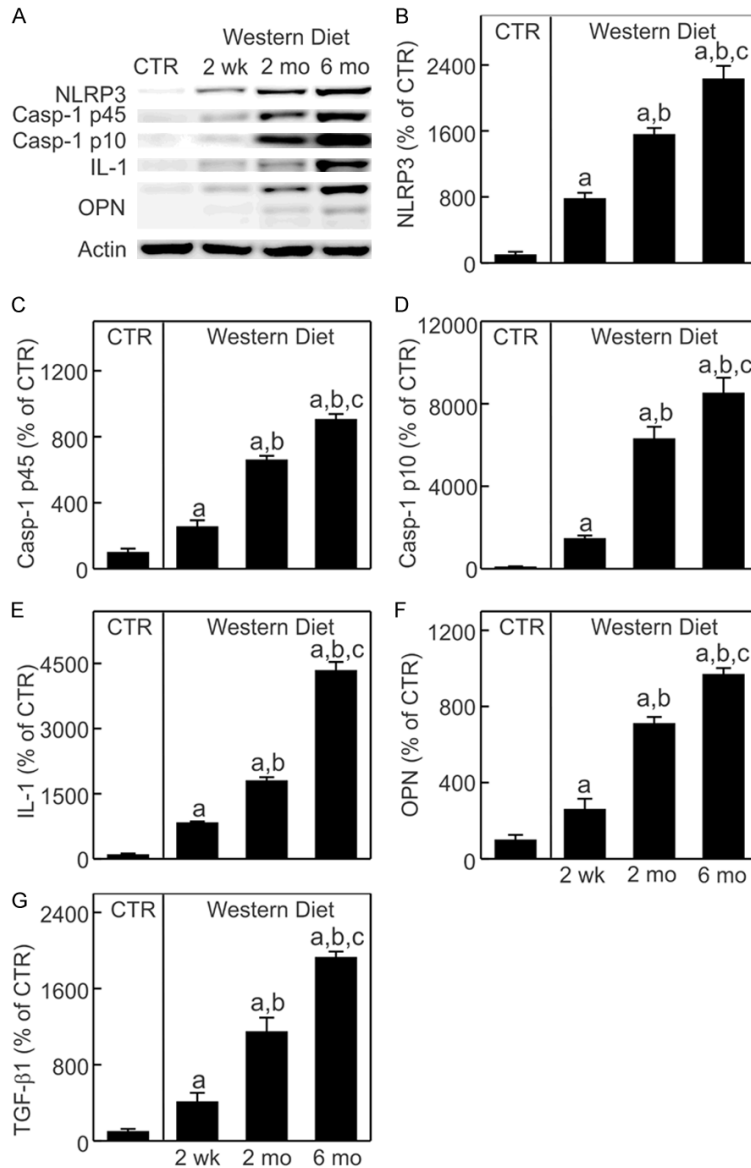


Figure 7. Western diet feeding increases NLRP3 inflammasome activation, osteopontin and transforming growth factor- β 1 expression in the liver. Mice were fed control (CTR) or Western diet for up to 6 months. A. Representative Western blots of NACHT, LRR and PYD domains-containing protein 3 (NLRP3), caspase-1 p45 (Casp-1 p45), caspase-1 p10 (Casp-1 p10), interleukin-1 (IL-1), osteopontin (OPN), transforming growth factor- β 1 (TGF- β 1), and actin; B. Quantification of NLRP3 Western blots by densitometry; C. Quantification of Casp-1 p45 Western blots; D. Quantification of Casp-1 p10 Western blots; E. Quantification of IL-1 Western blots; F. Quantification of the first band of OPN Western blots; G. Quantification of TGF- β 1 Western blots. Values are means \pm S.E.M. a, $P < 0.05$ vs CTR; b, $P < 0.05$ vs Western diet for 2 weeks; c, $P < 0.05$ vs Western diet for 2 months ($n = 4$ per group).

diet, both mtDepo and mitophagy developed (Figures 3, 4).

Removal of damaged, effete and superfluous mitochondria via mitophagy is critical for main-

taining cellular homeostasis and function [52, 53]. In Type I mitophagy, preautophagic structures wrap around individual functional mitochondria to form double membraneous mitophagosomes. Mitophagosome acidification then occurs, and mtDepo follows. By contrast, in Type 2 mitophagy, mtDepo initiates autophagic sequestration of mitochondria [38]. Mitochondria ordinarily import and proteolytically degrade PINK1 in a $\Delta\Psi$ -dependent fashion. After mtDepo, PINK1 fails to degrade and instead accumulates in the outer membrane. This increase of PINK1 in turn promotes association of parkin, an E3 ubiquitin ligase, and subsequent recruitment of autophagy receptor proteins (e.g., p62/SQSTM-1) and the formation of LC3-containing autophagosomes [38]. In this way, Type 2 mitophagy removes depolarized and integrity-disrupted mitochondria. Insufficient mitophagy is associated with mitochondrial dysfunction and the pathogenesis of many diseases, whereas excessive mitophagy may lead to mitochondrial loss and bioenergetic deficit as in the hepatotoxicity of cadmium [53, 54]. Lipids stored in fat droplets can also be taken up by autophagosomes and delivered to lysosomes for degradation to release free fatty acids. Intracellular lipids themselves regulate levels of autophagy. Impaired lipophagy is considered a potential contributor to ASH and NASH [55, 56]. Unlike Type II mitophagy,

lipophagy is not associated with parkin/PINK1 changes.

After feeding Western diet for as short a time as 2 weeks, mtDepo occurred in ~50% of hepa-

toocytes, which further increased to ~70% after 2 months (**Figure 3**). PINK-1 and LC3-I were unchanged after 2 weeks of Western diet feeding but increased substantially after 2 and 6 months of feeding (**Figure 4**). These data indicate that mtDepo is an early event after Western diet feeding, which precedes development of mitophagy. The sequence of first mtDepo and then mitophagy is consistent with the conclusion that mtDepo initiates Type 2 mitophagy in NAFLD. However, although mitophagic sequestration increases after feeding Western diet, hepatic lysosomal processing of mitophagosomes/autophagosomes appears to become impaired, as indicated by accumulation of LC3-II and p62/SQSTM-1 (**Figure 4**), consistent with previous reports of p62-positive inclusion body formation in patients with NASH [57]. These data also indicate that in the face of increased mitophagic burden by mtDepo, lysosomal processing of mitophagosomes becomes insufficient or is suppressed in the later stage. Although previous studies show mitochondrial structural and functional alterations in NAFLD, typically after 4 weeks or longer of feeding, our study suggests that mtDepo, which occurs very early and may be the primary event that triggers subsequent mitochondrial alterations with disruption mitochondrial homeostasis. Strikingly, a similarly increased mitophagic burden and impaired lysosomal processing occur in ASH caused by chronic ethanol exposure [39, 58-60].

Decreases of OXPHOS proteins AS- β and ND3 were observed at 2 and 6 months after Western diet feeding, indicating a decrease in overall mitochondrial content as a result of mtDepo and enhanced mitophagy (**Figure 5**). Under ordinary circumstances, mitochondrial biogenesis acts to replenish mitochondria and maintain mitochondrial homeostasis [40-42]. However, after feeding Western diet for 2 months and longer, transcriptional coactivator PGC-1 α , the primary regulator of mitochondrial biogenesis, and Tfam, a transcription factor that regulates the replication and transcription of mtDNA [61-64], decreased rather than increased, indicating suppression of signaling for mitochondrial biogenesis (**Figure 5**). Together, increased mtDepo-induced mitophagy and decreased mitochondrial biogenesis appear to account for the decrease of mitochondrial content in our mouse model of Western diet-induced NAFLD.

Consistent with the present findings, humans with insulin resistance, a population in which NAFLD is very high, have decreased expression of mitochondrial biogenesis regulators (PGC-1 α and nuclear respiratory factor-1) [19, 65].

Mitochondrial quality control requires mitochondrial dynamics-fission and fusion [30, 66, 67]. Binary fission yields daughter mitochondria as mitochondria proliferate. Fission also segregates portions of larger mitochondria for later removal. Fusion is important to allow the exchange of material (e.g., protein, mtDNA) between healthy and damaged mitochondria. After feeding Western diet for 2 month or longer, fission and fusion were both suppressed as indicated by decreased Drp-1 and Fis-1 (markers of fission) and Mfn-2 (marker of fusion) (**Figure 6**). Suppression of mitochondrial dynamics by Western diet likely dampens the capability for mitochondrial repair/recovery during stress. Interestingly similar to NASH, hepatocytes isolated from chronically ethanol-fed rats also exhibit a substantial suppression of mitochondrial dynamics, and human hepatoma-derived cells with prolonged ethanol exposure show decreased mitochondrial continuity and dynamics [68].

Mitochondrial depolarization and disrupted mitochondrial homeostasis contribute to pathogenesis of NAFLD

Previous studies show that mitochondrial oxidative stress and mtDepo play a key role in the pathogenesis in alcoholic liver disease [39, 69, 70]. Here, we show that remarkably similar mitochondrial abnormalities/alterations also occur after Western diet feeding. These changes most likely also play an important role in the pathogenesis of NAFLD/NASH and account for the very similar histopathology of NAFLD/NASH and alcoholic liver disease [39]. In this study, apparent mtDepo occurred at 2 weeks, which preceded inflammation, cell death, ALT release, severe steatosis and fibrosis (**Figures 1, 2**).

Although increased fat and fructose supply from Western diet promotes steatosis, relative ATP depletion after mtDepo may further exacerbate steatosis by inhibiting acyl-CoA formation from fatty acids and mitochondrial β -oxidation [71, 72]. While not studied here, closure of voltage-dependent anion channels in the mitochondrial outer membranes occurs after expo-

sure of hepatocytes to ethanol and acetaldehyde [73, 74]. VDAC closure prevents futile ATP hydrolysis after mtDepo, but also blocks mitochondrial uptake of acyl-CoA and β -oxidation to promote steatosis. Such VDAC closure may also occur in NAFLD/NASH due to aldehydes formed after lipid peroxidation.

Although cell death increased modestly after feeding Western diet (**Figure 1**), mtDepo was much more widespread. A possible explanation is that other energy supply pathways, such as glycolysis, were adaptively increased to maintain cell survival when mitochondrial supply of ATP decreased. After ethanol treatment, widespread mtDepo also occurs, but cell death is similarly mild, possibly due to increased glycolysis and utilization of glycogen [23].

Mitochondrial stress and dysfunction also affect inflammatory and/or pro-fibrotic responses. Mitochondrial stress activates the NLRP3 inflammasome, which leads to caspase-1 cleavage and formation of mature IL-1 β and IL-18 [32, 33, 43]. Increased IL-1 β signaling can mediate inflammation, steatosis, and fibrosis [75]. In this study, activation of NLRP3 inflammasome and an increase of IL-1 β occurred as early as 2 weeks after Western diet feeding in parallel to mtDepo, which increased progressively afterwards (**Figure 7**). Mitochondrial stress/damage may also promote immune inflammatory responses by releasing proinflammatory and profibrotic mitochondrial damage-associated molecular pattern molecules (mtDAMPs) [76, 77]. Insufficient lysosomal degradation of mitophagosomal contents may lead to release of mtDAMPs [39]. In addition, oxidative stress and mitochondrial dysfunction also increases expression of OPN, a proinflammatory and profibrotic molecule, and promotes formation/activation of profibrogenic cytokine TGF- β [34, 35, 44, 45, 78]. In the present study, OPN expression and TGF- β formation increased after Western diet feeding (**Figure 7**). Similarly, inflammasome activation, mtDAMP release, OPN release and TGF- β expression also occur in alcoholic liver disease [39, 58, 79, 80].

Taken together, the present results demonstrated that as in alcoholic liver disease, extensive mitochondrial alterations occurred after Western diet feeding. mtDepo developed early and appeared to trigger mitophagy. Increased mitophagic burden, disrupted mitophagosome

processing, decreased mitochondrial biogenesis and suppressed mitochondrial dynamics then occurred. These alterations likely play an important role in triggering/exacerbating inflammatory and profibrotic responses that promote the development and progression of NAFLD/NASH.

Previous studies document increased hepatic oxidative stress after feeding high fat diets. We propose that this oxidative stress in combination with increased lipid content leads to lipid peroxidation and formation of reactive aldehydes like malondialdehyde and 4-hydroxynonenal by beta-scission. In a fashion similar to the effects of acetaldehyde formed during ethanol metabolism, these aldehydes generated from lipid peroxidation trigger mtDepo. mtDepo subsequently increases mitophagic burden with insufficient or suppressed autophagic processing, which then leads to release of mtDAMPs with inflammasome activation and subsequent expression of proinflammatory and profibrogenic mediators. Suppressed mitochondrial biogenesis in combination with mitophagy also leads to an overall relative depletion of mitochondria. All these mitochondrial alterations work together to promote progression of NASH. The molecular mechanism for mtDepo remains unclear, but multiple depolarizing mitochondrial ion channels and transporters, such as the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}), adenine nucleotide transporter, Ca²⁺ transporters, the F₀ portion of ATP synthase and uncoupling proteins, may underlie mtDepo. Future studies will be needed to identify the uncoupling circuit in mitochondria that causes mtDepo in NASH.

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Disclosure of conflict of interest

None.

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