Triglyceride peroxidation progression in lipid droplets of hepatocytes in nonalcoholic steatohepatitis

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Graphical Abstract

Abstract

Objective: Nonalcoholic steatohepatitis is a chronic liver disease caused by the progression of hepatocellular death and inflammation from simple steatosis. However, the pathogenesis of this disease remains unclear. Lipid peroxidation is one of the most critical factors in the development of nonalcoholic steatohepatitis; however, oxidised lipids – the products of lipid peroxidation – are insufficiently analysed. Here, we comprehensively analysed oxidised lipids in the liver during nonalcoholic steatohepatitis development in a choline-deficient, l-amino acid-defined, high-fat diet-fed mouse model.

Methods: Liver from C57BL/6J mice, fed a standard diet or a choline-deficient l-amino acid-defined high-fat diet for 1, 3, or 6 weeks, were collected to evaluate fibrosis, steatosis, inflammation, liver injury, and oxidised lipid production and to observe the suppression of these parameters upon vitamin E administration. In addition, organellar localisation of lipid peroxidation was assessed using fluorescence imaging. Finally, a mitochondria-targeted antioxidant was administered to model mice to investigate the mechanism underlying lipid peroxidation.

Results: We found an accumulation of oxidised triglycerides in the early stages of nonalcoholic steatohepatitis. Furthermore, our data indicate that oxidised triglycerides are generated by lipid peroxidation in lipid droplets due to mitochondria-derived reactive oxygen species.

Keywords

- nonalcoholic steatohepatitis
- lipid peroxidation
- oxidised triglycerides
- 4-hydroxy nonenal
- lipid droplet
**Conclusion:** These results suggest the importance of lipid droplet peroxidation in the progression of nonalcoholic steatohepatitis and may contribute to the development of therapeutic methods for nonalcoholic steatohepatitis in the future.

**Significance statement**

We demonstrate the specific and early occurrence of lipid peroxidation in nonalcoholic steatohepatitis pathogenesis and propose a previously unknown mechanism of disease progression.

**Introduction**

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease characterised by hepatic steatosis that affects approximately 25% of the general population (Younossi et al. 2016, Loomba et al. 2021). NAFLD includes a wide range of pathological conditions, from relatively mild hepatic steatosis (NAFL) to a more aggressive disease state (nonalcoholic steatohepatitis, NASH) with progressive hepatocellular death and enhanced inflammation (Singh et al. 2015). NASH is a progressive disease, and 20% of patients with NASH progress to end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma, which require hepatectomy and transplantation (Loomba et al. 2020). Therefore, it is extremely important to prevent the exacerbation of NASH. The development of treatment and prevention methods as well as elucidation of the mechanism of NASH progression, which can serve as a fundamental principle for the development of such methods, are required (Friedman et al. 2018).

Conventionally, the ‘two-hit theory’ has been proposed as the molecular mechanism involved in NASH pathogenesis (Day and James, 1998). This theory suggests that after steatosis, which is the ‘first hit’, oxidative stress promotes inflammation and fibrosis as the ‘second hit’ (Peverill et al. 2014). Lipid peroxidation (LPO) is an important oxidative stress factor in NASH progression. Lipids, particularly those containing polyunsaturated fatty acids (PUFAs), are extremely susceptible to oxidation. Free radical-mediated LPO enhances the formation of numerous LPO metabolites, which individually promote cell death and inflammation (Ayala et al. 2014, Di Gioia et al. 2020). Electrophilic aldehydes produced by LPO, such as 4-hydroxynonenal (4-HNE) and acrolein, modify functional proteins and induce hepatocellular death, inflammatory responses, and fibrosis (Dalleau et al. 2013, Pizzimenti et al. 2013, Bekyarova et al. 2019). Indeed, 4-HNE-modified proteins accumulate in the liver tissues of patients with NASH (Seki et al. 2002). Furthermore, ferroptosis, recently identified as a mechanism for LPO-dependent programmed cell death, has been reported to occur in the early stages of NAFLD and to trigger the transformation from NAFL to NASH (Dixon et al. 2012, Tsurusaki et al. 2019, Li et al. 2020). Vitamin E (vitE), clinically indicated for the treatment of NASH, is a lipophilic antioxidant that inhibits LPO (Sanyal et al. 2010).

LPO is a critical contributor to NASH progression, and the profiles of oxidised lipids can provide important insights for evaluating its mechanism. However, only a few types of oxidised lipids (such as short-chain aldehydes) have been analysed previously in NASH liver tissues, which may be due to the lack of an analytical system for oxidised lipids (Bellanti et al. 2017). In addition, because short-chain aldehydes diffuse into other intracellular organelles after formation (Català, 2009), no information about their precursor oxidised lipids and production sites is available. Therefore, the profile of oxidised lipids in NASH remains unclear. Clarification of oxidised lipid profiles and LPO mechanisms in NASH will contribute to the development of more effective therapeutic agents aimed at inhibiting LPO.

In this study, we performed a comprehensive analysis of hepatic oxidised lipids in a choline-deficient l-amino acid-defined high-fat diet (CDAHFD) mouse model using liquid chromatography-tandem mass spectrometry (LC/MS/MS)-based analytical methods. We found that oxidised triglycerides (oxTGs) levels were significantly increased in the early phase of NASH development. Furthermore, oxTGs were found to be generated in lipid droplets and may contribute to the formation of electrophilic aldehydes, which are important lipotoxic factors in NASH development. These results indicate a new physiological significance of oxTGs in NASH progression.
Materials and methods

Reagents

(+/−)-α-Tocopherol was obtained from Wako Pure Chemical Industries, Ltd. and MitoTEMPO was purchased from Sigma-Aldrich. Acetonitrile (LCMS grade, ≥99.9%), isopropanol (LCMS grade, ≥99.9%), methanol (LCMS grade, ≥99.9%), and water (LCMS grade, ≥99.9%) were purchased from Wako Pure Chemical Industries, Ltd. Ammonium formate (Optima LC/MS) was obtained from Thermo Fisher Scientific. C11-BODIPY Lipid Peroxidation Sensor, HCS LipidTOX Deep Red neutral lipid stain for cell imaging, and ProLong Glass Antifade Mountant with NucBlue Stain were sourced from Invitrogen.

Animal studies

All procedures and animal care steps were approved by the Committee on Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University and were conducted according to the guidelines of the Graduate School of Pharmaceutical Sciences, Kyushu University.

Preparation of CDAHFD-induced NASH mouse model and lipid extraction samples

Five-week-old C57BL/6J male mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All the mice were housed in a light-controlled room (12 h light:12 h darkness cycle) at 24 ± 1°C and 60 ± 10% humidity, with free access to water and CLEA Rodent Diet CE-2 (CLEA Japan, Inc.). Six-week-old mice received either a standard diet (SD) (CLEA Rodent Diet CE-2) or CDAHFD (L-amino acid diet with 60 kcal% fat and 0.1% methionine without added choline; Research Diets A06071302) for 1, 3, or 6 weeks ad libitum. To suppress CDAHFD-induced LPO, mice were administered 100 mg/kg bw/day α-tocopherol or vehicle intraperitoneally for 6 weeks. In addition, 1 mg/kg bw/day MitoTEMPO or vehicle was administered intraperitoneally for a week to evaluate the contribution of mitochondrial ROS. Mouse liver and plasma were collected and hepatic lipids were extracted using a modified Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 1 mL of extraction solution (methanol:chloroform:water = 5:2:2) containing 100 μM dibutyl hydroxytoluene and 100 μM ethylenediaminetetraacetic acid was added to a frozen tissue sample (50 mg), and the sample was homogenised using a Macro Smash homogeniser. The extraction solutions were sonicated in an ice bath for 5 min. After centrifugation (6000g, 10 min, 4°C), 700 µL of the supernatant was collected and 235 µL chloroform and 155 µL water were added to it. The organic layer was collected in a glass tube and dried under a stream of nitrogen gas; the dried residue was dissolved in methanol (200 µL) and stored at −80°C before performing the LC/MS/MS.

LC/MS/MS measurement

LC/MS/MS was performed using an LCMS-8060 mass spectrometer (Shimadzu Co., Kyoto, Japan) equipped with an electron spray ionisation source. The extracted solution was measured using LC/MS/MS in the multiple reaction monitoring mode. The LC conditions were as follows: injection volume, 10 μL; autosampler temperature, 4°C; column, InertSustain C18 column (2.1 mm × 150 mm, 3 μm particle size, GL Sciences, Tokyo, Japan); column temperature, 40°C; mobile phase 5 mM ammonium formate in acetonitrile:H2O=2:1 (A) and isopropanol:methanol=95:5 (B); flow rate, 0.4 mL/min; and gradient elution, 0–22.5 min, 0–100%; 22.5–27.5 min, 100%; 27.5–27.6 min, 100–0%; 27.6–30.0 min, 0%. The LC/MS/MS analyses were carried out using LabSolutions version 5.80 (Shimadzu Co.).

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the frozen liver tissues using Isogen II (Nippon Gene, Tokyo, Japan). mRNA expression was measured using ReverTra Ace’ qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd., Osaka, Japan) or using the THUNDERBIRD® SYBR® qPCR Mix (TOYOBO Co., Ltd.) and the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) under the following thermal cycling conditions: one cycle at 95°C for 60 s, followed by 45 cycles at 95°C for 15 s and at 60°C for 60 s, with a final stage of melting curve analysis. GAPDH was used for normalisation, and the relative gene expression was calculated using the 2−ΔΔCt method. Primers used in this study are listed in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

Histological staining

For paraffin and frozen block preparation, the liver tissue was fixed in 4% paraformaldehyde or embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Tokyo, Japan). Paraffin-embedded liver tissues were used for
haematoxylin and eosin staining as well as for Sirius red staining. Frozen tissue was used for oil red O staining and for F4/80 and 4-HNE immunohistochemistry. The primary antibodies used were anti-F4/80 antibody (Invitrogen) and anti-4-HNE antibody (this antibody was kindly provided by K. Uchida at Tokyo University in Japan). Fluorescence in each section was visualised using BZ-X810 (Keyence Co., Osaka, Japan).

**Biochemical measurements**

Plasma aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured using an assay kit (Wako Pure Chemical Industries Ltd.). Lipids were extracted from the liver tissues using the Bligh and Dyer method, and triglyceride levels were measured using a Triglyceride Assay Kit (Wako Pure Chemical Industries, Ltd.).

**Preparation and culture of primary hepatocytes**

Hepatocytes were prepared using a previously reported perfusion method for fatty liver (Jung et al. 2020). Primary hepatocytes from 8-week-old C57BL/6j mice were perfused with collagenase (Wako Pure Chemical Industries Ltd.). The cells were cultured in DMEM (1.0 g/L glucose) with L-glutamine and sodium pyruvate, liquid (Life Technologies), 5% FBS (Invitrogen), and 1% penicillin-streptomycin (Nacalai Tesque Inc., Kyoto, Japan). After culturing for 6 h, the medium was changed to William’s Medium E (Life Technologies) containing 1% 2 mM glutamine (Nacalai Tesque, Inc.) and 1% penicillin-streptomycin and the primary cells were used in the fluorescence imaging assay.

**Fluorescence imaging of primary hepatocytes**

Primary hepatocytes from 1 week SD- or CDAHFD-fed mice were cultured with C11-BODIPY S81/S91 (2 µM) for 20 min and fixed in 4% paraformaldehyde with PBS for 15 min, followed by three washes with PBS. The cells were then incubated with LipidTOX (1:1000 dilution) for 30 min. After washing with PBS, the samples were mounted on coverslips using ProLong™ Glass Antifade Mountant with NucBlue™ Stain. Fluorescence imaging was performed using a confocal laser-scanning microscope (LSM700, Carl Zeiss Microscopy GmbH) equipped with a 63× objective lens. The following detection lasers were chosen for fluorescence imaging: Hoechst 33342, λex = 405 nm, λem = 410–470 nm; C11-BODIPY (oxidised form), λex = 488 nm, λem = 489–549 nm; and LipidTOX, λex = 639 nm, λem = 640–700 nm.

**Isolation of lipid droplets and membrane**

The isolation of lipid droplets and membranes from liver tissues was performed by modifying a previously reported method (Preuss et al. 2019). Briefly, 25 mg of liver tissue was homogenised in 500 µL of buffer A (20 mM Tris/HCl, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose, pH 7.4 2× PIC) using a Wheaton overhead stirrer (Wheaton Inc., Millville, NJ, USA) and a Potter-Elvehjem Tissue Grinder (Wheaton Inc.). Samples were transferred to centrifuge tubes, overlaid with 150 µL buffer A (3% sucrose), and centrifuged for 1 h at 100,000 g and 4°C. The floating lipid droplet layer and membrane pellet were collected, and the lipids in each fraction were extracted using the Bligh and Dyer method, as described earlier for LC/MS/MS analysis.

**Statistical analysis**

All data are presented as the mean ± s.d. Statistical analyses were conducted using the GraphPad Prism software (version 9.2.0, GraphPad Software, Inc.). The data were analysed using an unpaired Student’s t-test or Tukey–Kramer test, one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test, or two-way ANOVA followed by Sidak’s multiple comparison test. The number of samples used in each experiment is indicated in the figure legends. The minimum level of statistical significance was set at a P-value of 0.05. All data were expected to have a normal distribution.

**Results**

**Evaluation of NASH pathogenesis in CDAHFD-fed mice**

First, we evaluated the development of NASH pathology in CDAHFD-fed mice. Mice fed CDAHFD showed increased liver weight and liver triglyceride (TG) levels compared with those in normal mice (Supplementary Fig. 1A, B and C). Additionally, the expression of fibrogenic genes (Acta2, Tgfb1, Col1a1) was upregulated in CDAHFD-fed mice (Fig. 1A). Furthermore, morphological evaluation revealed lipid accumulation and fibrosis-like areas stained with Sirius red in the CDAHFD group (Fig. 1B, Supplementary Fig. 1D). Moreover, 4-HNE-modified proteins significantly accumulated in the liver tissue after 3 weeks (Fig. 1C). CDAHFD aggravated liver...
Figure 1
Evaluation of steatohepatitis and lipid peroxidation in choline-deficient-\-amino acid-defined high-fat diet (CDAHFD)-fed mice. (A). Hepatic mRNA levels of Acta2, Col1a1, and Tgfb1. (B) Histological observation of the liver after feeding a standard diet (SD) or CDAHFD for 1, 3, and 6 weeks (haematoxylin and eosin (H&E), Oil red O, and Sirius red). Original magnification: H&E, \times200; Oil red O, \times200; Sirius red, \times200. Scale bars: 100 µm. (C) Immunohistological analysis of 4-HNE in the liver. Comparison of 4-HNE-positive areas in the liver. (D) Plasma AST and ALT levels. (E) Hepatic mRNA levels of Ccl2, Tnfa, and Il1b. Six experiments were performed and data are presented as the mean ± s.d. P values were determined using two-way ANOVA with Sidak’s comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
injury (as indicated by ALT and AST levels) and increased expression of inflammatory genes (Ccl2, Tnfa, Il1b) was observed after 1 week (Fig. 1D and E). In addition, the abundance of F4/80-positive macrophages was remarkably increased in CDAHFD-fed mice after 6 weeks (Supplementary Fig. 1E).

**Analysis of oxidised lipids in the liver tissue of CDAHFD-fed mice**

Next, we performed a comprehensive analysis of oxidised lipids in the liver tissues of CDAHFD-fed mice. In this study, we analysed 43 oxidised lipids derived from 14 lipid species that are abundant in the liver tissue, containing phosphatidylincholines (PC), phosphatidylethanolamines (PE), and TG (Supplementary Fig. 2). Based on the oxidative modification patterns identified in previous studies, the MS/MS conditions for oxidised lipids were calculated (Matsuoka et al. 2021). The information regarding optimised MS/MS conditions, molecules, and LC separation for each oxidised lipid is summarised in Supplementary Table 2. The amount of individual oxidised lipids was normalised to those of the corresponding intact lipids. Figure 2A shows volcano plots of hepatic oxidised lipids in SD- or CDAHFD-fed mice at each week. The levels of oxTGs in CDAHFD-fed mice were significantly higher than those in the SD group. The abundance of oxTGs, especially TG16:0_18:1_18:2;O2 and TG16:0_18:1_9:1;O, increased within 1 week, and this increase was more rapid than that of oxidised phospholipids, such as PC16:0_18:2;O2, which have been reported to be involved in NASH pathogenesis (Sun et al. 2020) (Fig. 2B, C, D and E).

To investigate the contribution of these oxidised lipids to NASH progression in CDAHFD-fed mice, vitE was administered to CDAHFD-fed mice (Fig. 3A) and its effects on pathology and oxidised lipid formation were evaluated (Fig. 3). CDAHFD-induced NASH pathology, fibrosis, liver injury, and inflammation were significantly suppressed by vitE treatment (Fig. 3B, C, D, and E, Supplementary Fig. 3A, B and C). In addition, vitE attenuated the accumulation of 4-HNE-modified proteins (Fig. 3F). Furthermore, the

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**Figure 2**

Oxidative lipidomics analysis of the liver in nonalcoholic steatohepatitis model mice. (A) Volcano plots for comparative analysis of the profiles of oxidised lipids (oxPC, oxPE, and oxTG) produced in the liver of mice fed a standard diet (SD) or choline-deficient l-amino acid-defined high-fat diet (CDAHFD) for 1, 3, and 6 weeks using LC/MS/MS. The MS peak areas of oxidised lipids were normalised to those of non-oxidised lipids, each of which is a substrate. Volcano plots were prepared using log2 (fold-change values) and multiple t-tests (n = 6 independent experiments). (B-D) Oxidised lipids derived from TG or PC. (B) TG16:0_18:1_18:2;O2. (C) TG16:0_18:1_9:1;O. (D) PC16:0_18:2;O2. (E) PC16:0_9:1;O. P values were determined using two-way ANOVA with Sidak’s comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
Figure 3
Vitamin E suppresses the pathogenesis of choline-deficient -amino acid-defined high-fat diet (CDAHFD)-induced steatohepatitis and accumulation of oxidised lipids. (A) Experimental setup. A daily dose of vitamin E or vehicle (100 mg/kg bw) was administered to mice fed a standard diet (SD) or CDAHFD for 6 weeks. (B) Histology of the liver after feeding CDAHFD with or without vitE for 6 weeks (HE, Sirius red). (C) Hepatic mRNA levels of Acta2 and Col1a1. (D) Plasma ALT and AST levels. (E) Hepatic mRNA levels of Ccl2 and Tnfa. (F) Immunohistological analysis of 4-HNE in the liver. Comparison of 4-HNE-positive areas in the liver. (G-J) Formation of oxidised lipids in the liver. Five or six experiments were performed and data represent mean ± s.d. P values were determined using one-way ANOVA with Tukey's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001 vs SD. P < 0.05, **P < 0.01, ***P < 0.001 compared with CDAHFD-fed mice; ns, not significant.
generation of oxidised lipids was significantly decreased (Fig. 3G, I and J). In contrast, vitE had no effect on the abundance of TGs in the liver tissue (Supplementary Fig. 3D). These results suggest that the formation of LPO products, especially oxTGs, may play an important role in the pathogenesis of NASH in CDAHFD-fed mice.

**LPO occurred in lipid droplets of hepatocytes in the early stages of CDAHFD-induced NASH**

Next, we examined the sites in hepatocytes that produce oxTGs during the early stages of NASH progression. In the liver tissue, TGs are stored in the endoplasmic reticulum membrane and lipid droplets (Mashek, 2021). Therefore, we separated the mouse liver into lipid droplet and membrane fractions to analyse the oxidised lipids in each fraction and found that oxTGs were mostly present in the lipid droplet fraction (Fig. 4A). In addition, we prepared primary hepatocytes from mice in each diet group and performed a fluorescent imaging assay using an LPO detection probe (C11-BODIPY) to determine the organelles in which LPO occurs in hepatocytes. The fluorescence emission derived from oxidised C11-BODIPY was particularly high in the CDAHFD group. Furthermore, LPO staining sites were co-stained with lipid droplets (LipidTOX) (Fig. 4B).

These results indicated that LPO occurred in the lipid droplets of hepatocytes in CDAHFD-fed mice, resulting in a significant accumulation of oxTGs.

**Mitochondrial ROS trigger LPO in lipid droplets**

Mitochondria are primary ROS-producing organelles (Murphy, 2009). Mitochondrial damage is exacerbated in hepatocytes during NASH progression and is associated with excess leakage and spread of ROS into the cytosol and other organelles (Begriche et al., 2013). Thus, we hypothesised that mitochondria-derived ROS trigger LPO in lipid droplets. We administered MitoTEMPO (a mitochondria-targeting antioxidant) to CDAHFD-fed mice and evaluated oxidised lipid production in the 1-week model. As expected, MitoTEMPO significantly inhibited the generation of hepatic oxTGs in CDAHFD-fed mice (Fig. 5A). In this study, MitoTEMPO administration did not ameliorate liver injury in the 1-week group but suppressed the upregulation of inflammatory marker expression (Fig. 5B and C).

Consequently, in the early stage of NASH, mitochondria-derived ROS contribute to LPO in lipid droplets and to the accumulation of oxTGs.

**Discussion**

We found that oxTGs accumulated in the liver tissue during the early stages of NASH pathogenesis. In addition, results of the fluorescence imaging of LPO suggested that LPO occurred within lipid droplets during NASH progression. The increase in oxTGs was significantly
inhibited by vitE administration, which is known to suppress NASH development (Bril et al. 2019). Excess intracellular PUFAs are susceptible to oxidation and are stored in the form of TGs in lipid droplets where ROS are less accessible (Bailey et al. 2015). Indeed, hepatic TGs in CDAHFD-fed mice had a higher percentage of PUFA, such as FA20:4 and FA22:4, in lipid droplets than those in SD-fed mice (Supplementary Fig. 4A and B). However, under NASH conditions with excess ROS production, the accumulation of PUFA-containing TGs in lipid droplets may enhance and induce the progression of LPO.

Among the oxTGs formed in the early stages of NASH pathogenesis, dioxygenated and truncated oxTGs showed particularly high rates of increase. These molecular species might be involved in the formation of reactive aldehydes, such as 4-HNE, which have been implicated in NASH pathogenesis (Chen et al. 2020). Lipid hydroperoxides can be decomposed by intramolecular cleavage reactions in the presence of transition metals, such as iron or haeme proteins (Hock cleavage/dioxetane cleavage), resulting in free aldehydes and truncated oxidised lipids that retain glycerol structures (Supplementary Fig. 5) (Spickett, 2013). In other words, the accumulation of TG16:0_18:1_18:2;O2 and its degradation product, TG16:0_18:1_9:1;O, which was prominent in the CDAHFD model, indicated the formation of reactive aldehydes, represented by 4-HNE. However, the mechanism by which these oxidised lipid species contribute to NASH development is unknown and requires further investigation.

Furthermore, our results demonstrate that mitochondria-derived ROS contribute to the generation of oxTGs. In adipose tissue, the interconnection of mitochondria and adipose droplets via membrane contact sites is important to facilitate metabolite exchange for maintaining organelle homeostasis (Benador et al. 2018). More recently, it has also been reported that under certain physiological and pathological conditions, such as obesity, the contact between mitochondria and lipid droplets increases in hepatocytes (Ma et al. 2021). Thus, lipid droplets may be susceptible to mitochondria-derived ROS in hepatocytes of CDAHFD-fed mice.

MitoTEMPO also suppressed CDAHFD-induced expression of inflammatory genes. This finding supports the hypothesis that the generation of oxTGs and aldehydes contributes to the induction of inflammation. Future studies should investigate the interactive effects between other organelles and lipid droplets during NASH progression.

Figure 5
MitoTEMPO inhibits choline-deficient l-amino acid-defined high-fat diet (CDAHFD)-induced generation of oxidised lipids and inflammation. MitoTEMPO (MT) or vehicle (1 mg/kg bw) was administered daily to CDAHFD-fed mice for 1 week. (A) OxTG level in the liver. Levels of TG16:0_18:1_18:2;O2 and TG16:0_18:1_9:1;O were measured. (B) Plasma AST and ALT levels. (C) Hepatic mRNA levels of Ccl2 and Tnfa. Six experiments were performed and data represent mean ± s.d. P values were determined using one-way ANOVA with Tukey’s multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001 vs SD. #P < 0.05, compared with CDAHFD-fed mice; ns, not significant.
Although the importance of LPO in NASH has been mentioned, the mechanism of LPO occurrence remains largely unknown. In this study, we demonstrate that LPO in lipid droplets induced the formation of oxTGs in the early stages of NASH pathogenesis. Furthermore, mitochondria were a candidate source of ROS that induce lipid droplet LPO. These observations suggest a critical role for organelle-specific LPO progression in the development of NASH and its utility as a therapeutic target. We expect that the physiological significance of the oxidised lipid species observed in this study will be elucidated and that antioxidant therapies will be developed to treat NASH based on these findings.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/REM-22-0024.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality if the research reported.

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Author contribution statement
KS and YM designed and performed the experiments, analysed the data, and wrote the manuscript. MA and NK performed the experiments and analysed the data for histological staining experiments. KM designed the experiments. KY designed the experiments, conceived and supervised the study, and wrote and edited the manuscript.

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