

ORIGINAL ARTICLE OPEN ACCESS

Chronic Nanoplastic Exposure Promotes the Development and Progression of Metabolic Dysfunction-Associated Steatotic Liver Disease

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Received: 24 April 2025 | **Revised:** 25 June 2025 | **Accepted:** 7 July 2025

Handling Editor: Luca Valentini

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) to Youngmi Jung (no. RS2025-00517677) and NRF grant funded by MSIT to Yung Hyun Choi (RS2023-00270936).

Keywords: hedgehog signalling | hepatocytes | metabolic dysfunction-associated steatotic liver disease | nanoplastics

ABSTRACT

Background and Aims: Plastic particles are a global pollution problem, and humans are potentially exposed to them. Ingested plastic particles, microparticles (MPs) and nanoparticles (NPs), predominantly accumulate in the liver and cause hepatotoxicity through oxidative stress and metabolic dysfunction. NPs promote more toxic actions than MPs; however, the mechanisms involved in developing and progressing metabolic dysfunction-associated steatotic liver disease (MASLD) from chronic exposure to NPs remain poorly understood. Hedgehog (Hh) signalling regulates MASLD pathogenesis. Herein, we investigated the pathophysiological effects of NPs in MASLD.

Methods: Mice were orally administered NPs via drinking water while fed a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) for 12 weeks.

Results: NPs increased lipid accumulation in hepatocytes and apoptosis. Moreover, these actions were enhanced in lipotoxicity-exposed hepatocytes. Chronically exposed NPs accumulated in mice livers and aggravated CDAHFD-induced hepatic damage, especially fibrosis. Activated Hh signalling in the CDAHFD group was elevated by NP treatment. Increased Sonic Hh expression in the hepatocytes of NP-treated mice in the CDAHFD group triggered Hh signalling in hepatic stellate cells (HSCs), which promoted liver fibrosis.

Abbreviations: α -Sma, α -smooth muscle actin; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BA, bile acid; BSA, bovine serum albumin; BW, body weight; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; CM, conditioned medium; Col1a1, collagen type 1 alpha 1; Cxcl, chemokine C-X-C motif ligand; DAB, 3,3'-Diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; DCFH-DA, 2',7'-Dichlorofluorescein diacetate; FBS, fetal bovine serum; FFA, free fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, haematoxylin-eosin staining; Hh, Hedgehog; HRP, horseradish peroxidase; HSCs, hepatic stellate cells; IgG, immunoglobulin; IHC, immunohistochemistry; IHH, Indian Hh; IL-6, interleukin 6; LW, liver weight; MAFL, metabolic dysfunction-associated fatty liver; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MDA, malondialdehyde; MPs, microplastics; NAFLD, non-alcoholic fatty liver disease; NPs, nanoparticles; P/S, penicillin/streptomycin; PA, palmitic acids; PAGE, polyacrylamide gel electrophoresis; PEG, propylene glycol; pHEPs, primary hepatocyte; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SHH, sonic Hh; SMO, smoothened; TG, triglyceride; Tgf- β 1, transforming growth factor- β 1; TLB, triton lysis buffer; Tnf- α , tumour necrosis factor α ; Veh, vehicle; WT, wild-type.

Jinsol Han, Hayeong Jeong, and Chanbin Lee contributed equally.

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Conclusions: These results demonstrate that chronic exposure to NPs increases vulnerability to MASLD progression, suggesting that NPs are a potentially harmful factor in the development and progression of liver disease.

1 | Introduction

Over the past 70 years, plastic production and use have increased exponentially worldwide [1]. Moreover, the demand for plastics and the resulting plastic waste is expected to grow significantly. Only 9% of plastics are recycled, and the remaining discarded plastics cannot be decomposed, meaning they are broken into micro or nano-scaled plastic pieces that leak into the environment, such as the air, soil and ocean [2, 3]. Therefore, plastic waste should no longer be overlooked as a new environmental pollutant threatening global public health. People ingest microplastics (MPs; 1–5 μm) and nanoplastics (NPs; 1–100 nm) almost every day, and these plastic particles accumulate in various human tissues, including brain, lung, gut, intestine, kidney, and liver [4]. Indeed, the liver is one important organ that, alongside the kidneys, accumulates nanomaterials and becomes damaged [5]. NP exposure has been reported to cause hepatic oxidative stress and lipid accumulation and is especially related to dysregulated lipid metabolism in mice [6]. Polystyrene NPs were also shown to increase the severity of type 2 diabetes [7]. The harmful effects of NPs themselves are well known; however, the action of NPs in the development and progression of liver disease is unknown, even though liver injuries caused by NPs are, in fact, closely related to chronic liver disease.

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly called non-alcoholic fatty liver disease (NAFLD), is one of the most common liver diseases worldwide [8]. MASLD occurs when excessive lipid accumulation in the liver injures cells, especially hepatocytes, and is unrelated to excessive drinking [9]. MASLD encompasses various disease stages, ranging from simple steatosis to metabolic dysfunction-associated steatohepatitis (MASH), which is characterised by massive hepatocyte death followed by inflammation and fibrosis [10]. The imbalance between lipid acquisition and lipid removal results in lipotoxicity, which promotes mitochondrial dysregulation and oxidative stress, resulting in hepatocyte death [11]. Dying hepatocytes secrete various substances to induce the inflammatory and fibrotic responses [12]. Hedgehog (Hh) signalling is one of the stimulating pathways in MASLD progression [13]. Hh ligands, Sonic and/or Indian Hh, released by apoptotic hepatocytes, trigger the activation and/or proliferation of Hh-responsive cells, such as hepatic stellate cells (HSCs), immune cells, and progenitors [14]. Then, these Hh-responsive cells activate their Hh signalling in an autocrine manner. HSCs are the main cells producing collagen matrix and causing fibrosis in the liver.

MASLD is indeed related to modern diseases and has become increasingly prevalent in recent years, correlating with the rise in metabolic syndromes, such as obesity, dyslipidaemia, and type 2 diabetes, and modern lifestyles characterised by sedentary behaviour and consumption of high-calorie processed foods [15]. Considering the increasing trend in MASLD and the increasing use of plastics in contemporary society, it is necessary to investigate the impact of NPs on MASLD. However, no direct evidence exists on whether the consumption of NPs over a long time

influences MASLD progression to MASH. Since ingested NPs cause hepatotoxicity, which is considered a key driver in MASH progression, we investigated the effect of dietary exposure to NPs in MASLD. In the present study, we demonstrate that long-term treatment of NPs causes lipotoxicity in hepatocytes by promoting excessive lipid accumulation in these cells, and these damaged hepatocytes release SHH to activate HSCs and induce liver fibrosis, leading to MASLD/MASH exacerbation in mice. These findings suggest that prolonged NP exposure acts as a deleterious accelerator in the development and progression of MASLD.

2 | Methods

Methods are described in detail in the [Supplementary Methods](#) section.

3 | Results

3.1 | NPs Influx Induces Hepatocyte Injury

To investigate whether NPs exposure affected hepatocytes, 50 or 100 $\mu\text{g}/\text{mL}$ of NPs was first tested in AML 12 cells—a mouse hepatocyte cell line (Figure S2A). NPs labelled using green fluorescence were detected within AML 12 cells. However, fluorescent particles were more evident in cells administered 100 $\mu\text{g}/\text{mL}$ of NPs than in cells administered 50 $\mu\text{g}/\text{mL}$ (Figure S2B). Cell viability, which did not change at 24 h post-NP treatment, significantly decreased at 48 h post-NP treatment (Figure S2C). Cleaved CASPASE-3 expression, a cell death marker, was also elevated in AML 12 cells at 48 h after NPs treatment (Figure S2D). To check whether NP influx induced lipid accumulation in the cells, we performed Oil-red O staining after NPs treatment. NP-exposed AML 12 cells contained more lipid droplets than vehicle (Veh)-treated cells, and these lipid droplets were more apparent at 48 h than at 24 h during NP treatment (Figure S2E). In addition, the CD36 level, a lipid uptake marker, was upregulated in cells treated with NPs compared with Veh-treated cells (Figure S2D).

After confirming the deleterious effect of NPs in AML 12 cells, we further examined their impact on primary hepatocytes (pHEPs) isolated from healthy mice (Figure 1A). Fluorescence images illustrated that the NPs were deposited mostly in the cytoplasm of pHEPs at 24 and 48 h (Figure 1B). Cell viability was significantly alleviated during NP treatment and was reduced more in the cells administered 100 $\mu\text{g}/\text{mL}$ of NPs than 50 $\mu\text{g}/\text{mL}$ (Figure 1C). The level of cleaved CASPASE-3 was also significantly elevated and tended to increase at 24 and 48 h in pHEPs treated with 100 $\mu\text{g}/\text{mL}$ of NPs (Figure 1D). As assessed by Oil Red O staining, NPs enhanced lipid accumulation in pHEPs (Figure 1E). Lipid droplets and CD36 expression were more evident in 100 $\mu\text{g}/\text{mL}$ NP-treated pHEPs than in 50 $\mu\text{g}/\text{mL}$ NP-treated pHEPs (Figure 1D,E). In addition, the intracellular reactive oxygen species (ROS) concentrations and malondialdehyde (MDA) concentration, a marker

Summary

- Prolonged exposure to nanoplastics—tiny plastic pollution found in the environment—leads to their accumulation in the liver and worsens fat-related liver damage.
- In animal studies, these particles did not cause much harm on their own when the animals ate a normal diet.
- However, when the animals had an unhealthy diet putting lipotoxic stress on the liver, nanoplastics significantly made the damage and scarring much worse.
- This suggests that nanoplastics may pose a greater risk to individuals whose dietary habits increase their susceptibility to MASLD.

of oxidative stress, were markedly elevated in pHEPs exposed to NPs at 48 h compared with other groups (Figure 1F,G). Therefore, these results show that NPs accumulating within hepatocytes increase lipid deposition in these cells by upregulating lipid uptake and possibly injuring them.

3.2 | NPs Deteriorate PA-Induced Lipotoxic Damages in Hepatocytes

After confirming the hepatotoxicity of NPs per se (Figure S2 and Figure 1), we next investigated the effect of NPs on MASLD development in vitro. Mouse pHEPs were exposed to 50 μ M of palmitic acid (PA), a well-known lipotoxic inducing agent, and 100 μ g/mL of NPs for 24 and 48 h (Figure 2A). Fluorescent NPs were detected in pHEPs regardless of PA treatment, indicating that PA hardly affects NPs influx (Figure 2B). Cell viability significantly decreased in all treatment groups compared with the control group (Veh-given pHEPs), and pHEPs treated with PA + NPs (PA + NPs group) had lower cell viability than NP-exposed cells (NP group) at 24 and 48 h; notably, cell viability was lower at 48 h than in the cells administered PA (PA group) (Figure 2C). ROS production and MDA accumulation were significantly higher in the PA + NPs group than in other groups (Figure 2D,E). The cleaved CASPASE-3 level was also upregulated in the treatment groups compared with the control group and significantly higher in the PA + NPs group than in other treatment groups (Figure 2F). In addition, the PA + NPs group contained increased CD36 and lipid droplet levels compared to other groups, as assessed by Western blotting and Oil red O staining, respectively (Figure 2F,G). These results demonstrate that accumulated NPs accelerate PA-promoted hepatocyte damage by facilitating lipid deposition.

3.3 | Long-Term Exposure to NPs Exacerbates CDAHFD-Induced Liver Damage in Mice

To examine the physiological effect of NPs in MASLD development and progression into MASH, NPs were added to the drinking water of mice fed a choline-deficient, L-amino acid-defined high-fat diet (CDAHFD) for 12 weeks. The CDAHFD model reflects human MASLD, whereby after being administered the diet, the mice exhibited metabolic dysfunction-associated fatty liver

(MAFL) and MASH at 6 and 12 weeks, respectively. The body weight (BW) of each mouse and the amount of water consumed were measured weekly, and the same concentration of NPs per BW was given to each mouse for 12 weeks (Figure S1A–C). CDAHFD hardly disturbed the intake of NPs per BW throughout the diet feeding period (Figure S1D). Before sacrificing mice, the biodistribution of ingested NPs was examined using the Maestro in vivo fluorescence imaging system. In mice fed a Chow diet, NPs were detected extensively in the torso. However, in mice fed CDAHFD, NPs were mainly distributed in the gastrointestinal tract and were especially concentrated in what appeared to be the liver and small intestine (Figure S1E). We also confirmed that NPs in the liver were particularly present in hepatocytes (Figure S1F). Fluorescent signals were rarely detected in the Veh-treated mice fed Chow or CDAHFD. The data revealed that the liver was one of the predominant tissues for NPs accumulation following oral exposure. In an analysis of gross macroscopic images, Chow-fed mice seemed to have healthy livers regardless of NP treatment, but mice fed CDAHFD had enlarged and yellowish livers. Meanwhile, additional treatment of NPs caused the mice to possess a rough liver surface compared with mice in CDAHFD groups fed the vehicle (Figure S1G).

Haematoxylin and eosin (H&E) staining presented that CDAHFD-treated mice had damaged histomorphological structures with fatty hepatocytes and inflammation, and additional treatment of NPs caused more macrovesicular fat accumulation, ballooning hepatocytes (marked by arrows) and inflammation in these mice (Figure 3A, top panel). However, Chow-fed mice with or without NPs showed normal liver morphology and appeared unaffected by chronic exposure to NPs. CDAHFD significantly increased liver weight (LW)/body weight (BW) ratios and serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), but NP treatment did not provide significant changes (Figure 3B). However, higher levels of hepatic triglycerides (TG) in the CDAHFD groups than the Chow groups were significantly increased by NP treatment (Figure 3C). In addition, CD36 expression, which was upregulated in CDAHFD-fed mice compared to Chow-fed mice, was significantly elevated by NP exposure in the livers of CDAHFD-fed mice (Figure 3D). In line with severe fat deposition in the CDAHFD + NPs group, hepatic levels of ROS and MDA were significantly elevated in the CDAHFD + NPs group compared to any other groups (Figure 3E,F). The amount of active CASPASE-3 in the CDAHFD group was also higher in NPs-administered mice than in vehicle-treated mice (Figure 3A, middle panel, and 3D). Furthermore, TUNEL-positive apoptotic cells were more apparent in the liver section of NP-treated mice compared to vehicle-treated mice in the CDAHFD groups (Figure 3A, bottom panel). Increased expression of CD36 and cleaved CASPASE-3 in pHEPs isolated from CDAHFD-fed mice with NP treatment also confirmed that chronic exposure to NPs promoted increased fat accumulation and damage in the livers of CDAHFD-fed mice (Figure 3G).

Because hepatic inflammation indicates the degree of liver injury, hepatic inflammation was examined in this mouse model. The RNA expression of proinflammatory genes, including *tumour necrosis factor α* (*Tnf- α*), *interleukin 6* (*Il-6*), *chemokine (C-X-C motif) ligand* (*Cxcl1*) and *Cxcl2*, was significantly upregulated in the liver of CDAHFD-fed mice than in Chow-fed mice; NP treatment further elevated the expression of these genes in

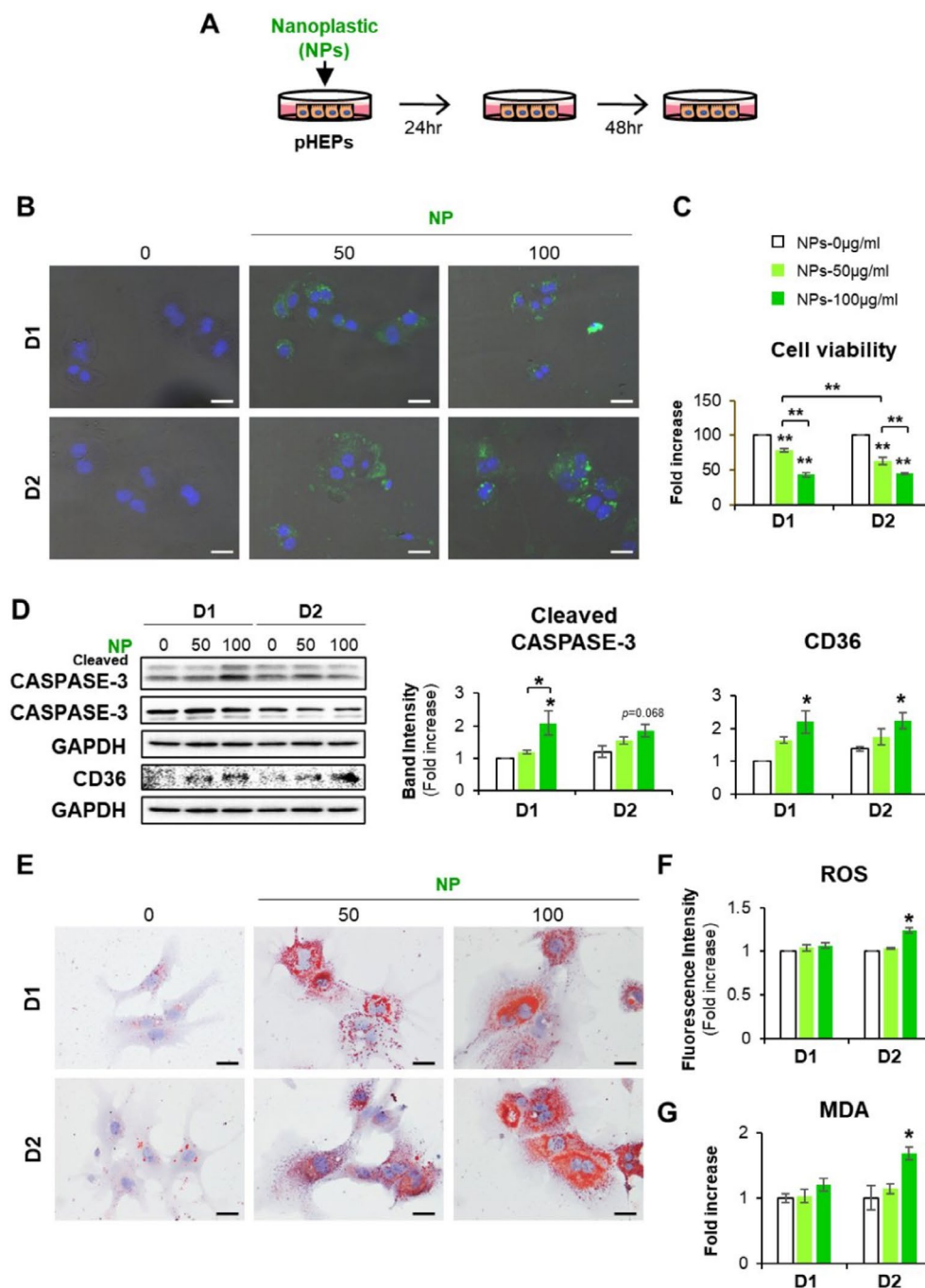


FIGURE 1 | NPs brings to cell damage and lipid accumulation in mouse primary hepatocytes. (A) A scheme of in vitro experiments in which primary hepatocytes (pHEPs) were treated with 50 or 100 µg/mL of Fluoresbrite-labelled NPs for 24 or 48 h. (B) Representative fluorescent images of accumulated NPs (green) in these cells (scale bar, 50 µm). DAPI (blue) was used as nuclear counterstaining. (C) Cell viability in these cells was analysed using MTS assay. (D) Western blot analysis and cumulative densitometric analysis of cleaved CASPASE-3, pro-CASPASE-3, CD36. GAPDH was used as internal control. (E) Representative images of Oil Red O stained pHEPs (scale bar, 50 µm). (F) The levels of intracellular reactive oxygen species (ROS) and (G) malondialdehyde (MDA) in these cells. All data shown represent one of three experiments with similar results and are presented as the mean ± SEM (* $p < 0.05$, ** $p < 0.005$ vs. own control).

the CDAHFD-fed mice (Figure S3A). In addition, immunostaining for CD68, a macrophage marker, showed that CDAHFD results in the accumulation of CD68-positive cells in the livers of mice, and these cells were more apparent in the livers of NP-exposed mice fed CDAHFD (Figure S3B). Taken together, these results show that ingested NPs are specifically accumulated in the liver and participate in liver lipotoxicity caused by CDAHFD, leading to severe liver injury.

3.4 | Accumulated NPs Increase Hepatic Fibrosis by Impacting Hh Signalling in CDAHFD-Fed Mice

Massive hepatocyte death accompanied by inflammation is a characteristic of MASH, which has the risk of progressive fibrosis leading to cirrhosis [16, 17]. Because long-term exposure to NPs worsens CDAHFD-induced hepatocyte damage with inflammation, we assessed the fibrotic response in the

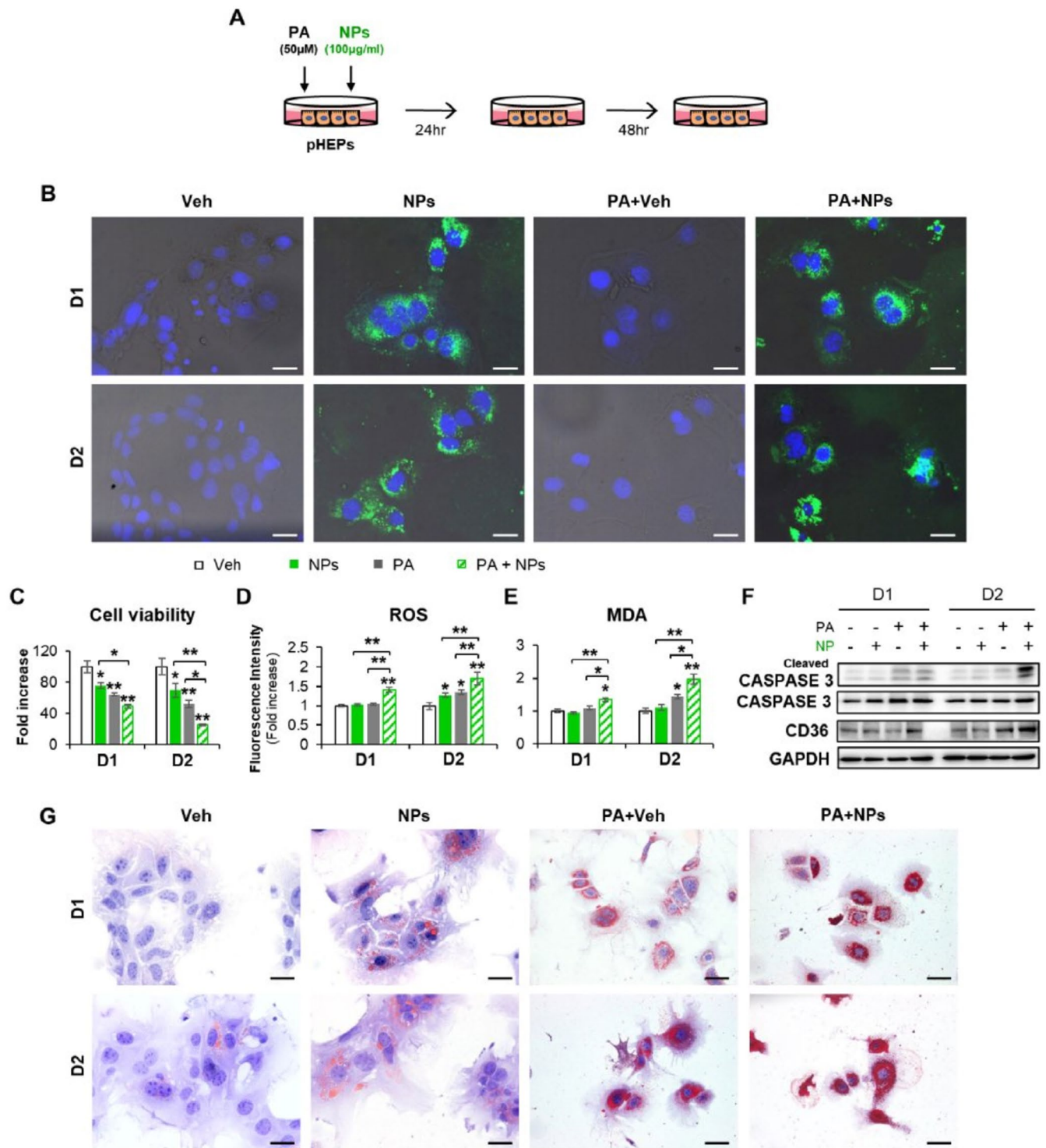


FIGURE 2 | Hepatocyte injury caused by lipotoxicity is worsened by NP treatment. (A) A scheme of cell experiments in which pHEPs were exposed to 50µM of PA and 100µg/mL of Fluoresbrite (green)-labelled NPs for 24 or 48 h. (B) Representative fluorescent images of NPs-given mouse pHEPs (scale bar, 50µm). DAPI (blue) was used as nuclear counterstaining. (C) Cell viability, (D) intracellular ROS and (E) MDA in these cells. Data are presented as the mean ± SEM (* p < 0.05 vs. own control). (F) Western blot analysis for cleaved CASPASE-3, pro-CASPASE-3, and CD36 in NP-treated mouse pHEPs with PA. GAPDH was used as internal control. (G) Oil red O staining for lipid droplets in these cells. Representative images are shown (scale bar, 50µm). All data shown represent one of three experiments with similar results.

experimental animal model. The mRNA levels of fibrotic markers, including *transforming growth factor-β1* (*Tgf-β1*), *collagen type 1 alpha 1* (*Col1a1*) and *alpha-smooth muscle actin* (*α-Sma*), were upregulated in the livers of CDAHFD-fed mice compared with Chow-treated mice, and the expression of

Col1a1 and *α-Sma* in CDAHFD-fed mice was significantly elevated by NP treatment (Figure 4A). Protein amounts of fibrotic markers, such as TGF-β1, COL1α1, α-SMA and VIMENTIN, were higher in the CDAHFD-fed groups than in the Chow-fed groups, and these protein levels were further increased in

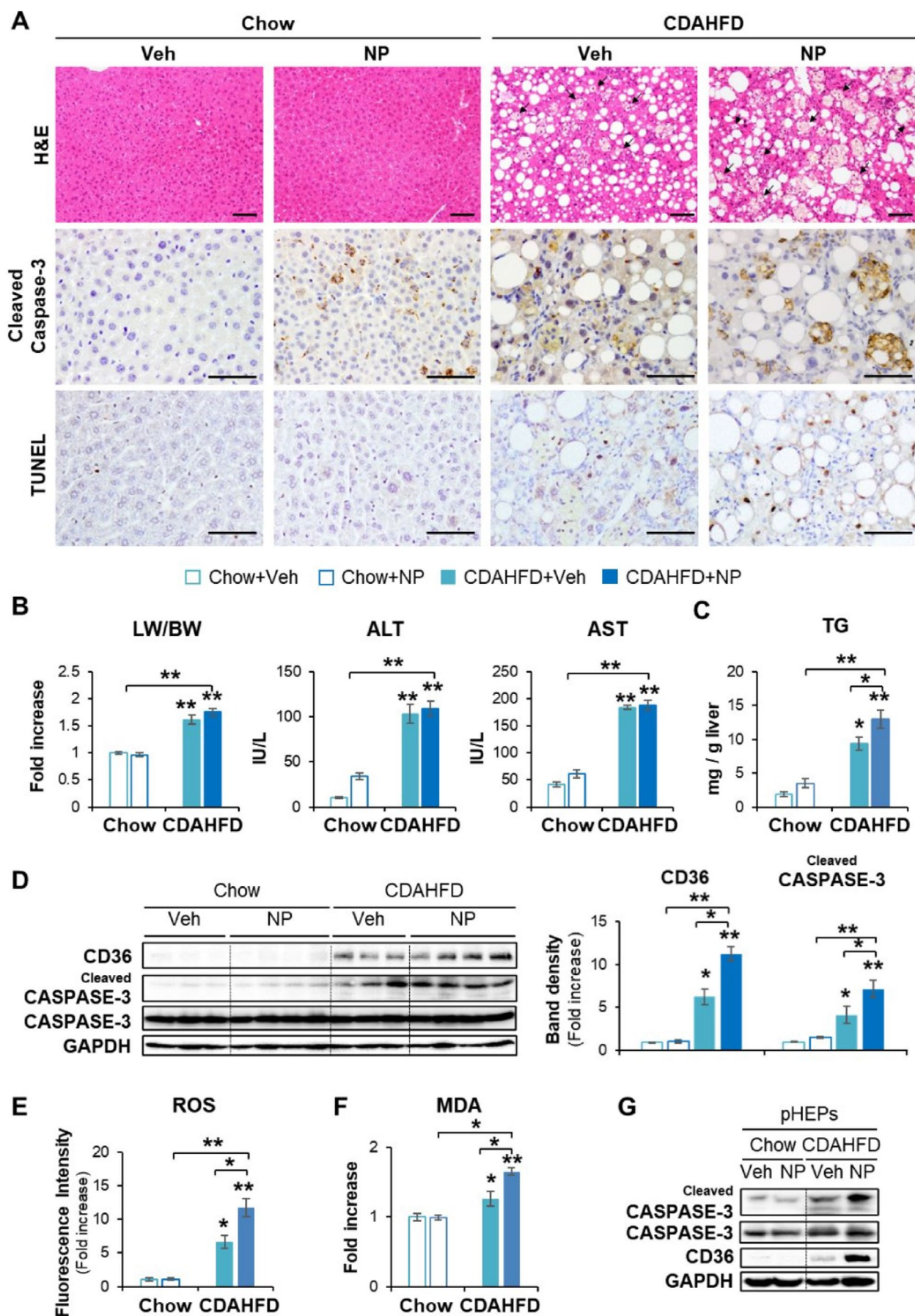


FIGURE 3 | Legend on next page.

FIGURE 3 | NP accumulation aggravates hepatic damage in mice fed CDAHFD. (A) Representative images of H&E-, active Caspase-3 and TUNEL-stained liver sections from Chow or CDAHFD-fed mice supplemented with either vehicle (Veh) or NPs for 12 weeks (Scale bar, 50 μ m). Black arrows in H&E-stained images indicate ballooned hepatocytes. (B) The ratio of LW to BW, the serum levels of ALT & AST, and (C) hepatic TG amount in these experimental mice. All data represent the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (D) Western blot analysis and cumulative densitometric analysis of CD36, cleaved CASPASE-3, and pro-CASPASE-3 from representative mice per each group. GAPDH was used as an internal control. (E) Hepatic level of ROS and (F) MDA in these mice. All data represent the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (G) Western blot of CD36, cleaved CASPASE-3, and pro-CASPASE-3 in primary hepatocytes isolated from these mice. GAPDH was used as an internal control. All data shown represent one of three experiments with similar results and are presented as the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh).

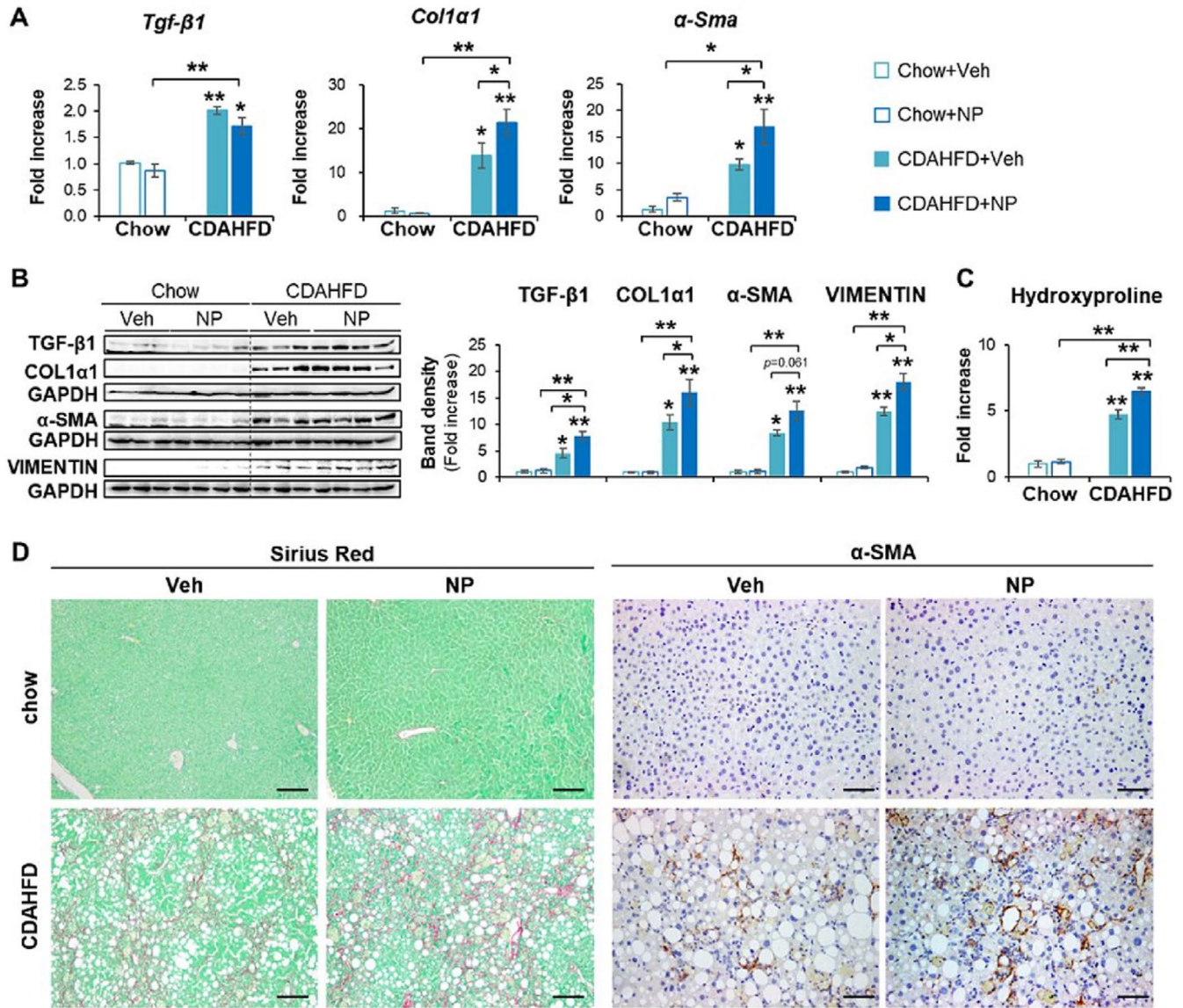


FIGURE 4 | Chronical exposure to NPs elevates fibrosis in the livers of CDAHFD-fed mice. (A) qRT-PCR analysis for hepatic *Tgf-β1*, *Col1α1*, and *α-Sma* in Chow or CDAHFD-fed mice treated with either Veh or NPs for 12 weeks. Data present the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (B) Western blot analysis and band cumulative densitometric analysis for hepatic TGF-β1, COL1α1, α-SMA and VIMENTIN in these mice. GAPDH was used as an internal control. All data shown represent one of three experiments with similar results and are presented as the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (C) Hepatic hydroxyproline content in liver tissue from representative mice per each group. Data present the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (D) Representative images of Sirius Red- and α-SMA-stained liver sections from these mice (Scale bar, 50 μ m).

the CDAHFD-fed groups by NP treatment, as examined by Western blot analysis (Figure 4B). Hydroxyproline contents, a quantitative analysis of collagen amounts, were also markedly increased after being fed CDAHFD compared with the Chow

diet, and exposure to NPs enhanced hydroxyproline contents in the CDAHFD-treated mice (Figure 4C). In addition, Sirius red staining and immunostaining for α-SMA presented an increased accumulation of collagen fibrils and α-SMA-positive

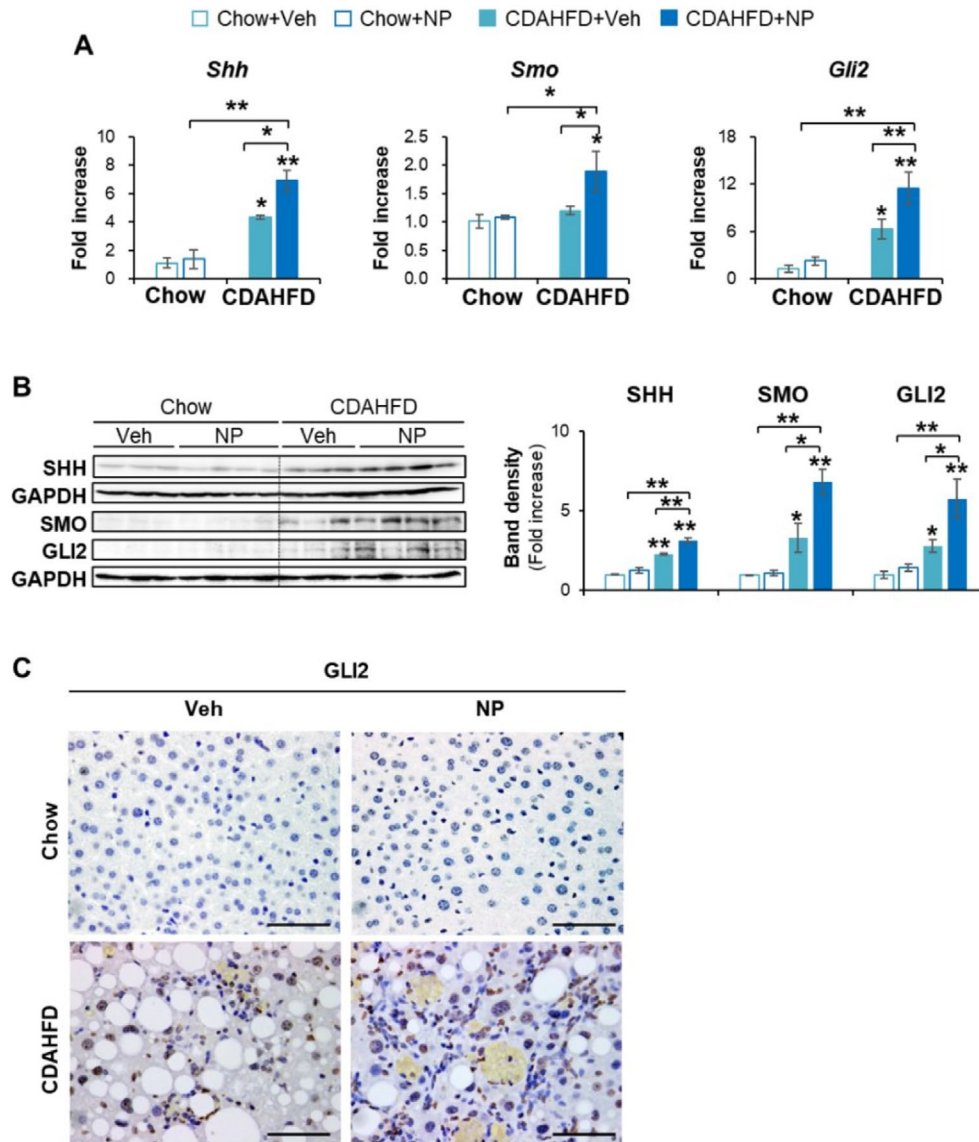


FIGURE 5 | Activated Hh signalling in mice with MASLD is reinforced by NP accumulation. (A) qRT-PCR analysis for hepatic *Shh*, *Smo*, and *Gli2* in Chow or CDAHFD-fed mice receiving either Veh or NPs for 12 weeks. Data present the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (B) Western blot analysis and band cumulative densitometric analysis for hepatic SHH, SMO and GLI2 in these mice. GAPDH was used as an internal control. All data shown represent one of three experiments with similar results and are presented as the mean $\pm$ SEM ($n \geq 3$ /group, * $p < 0.05$, ** $p < 0.005$ vs. Chow+Veh). (C) Representative images of GLI2-stained liver sections from these mice (Scale bar, 50 μ m).

cells in the CDAHFD groups, which was upregulated by NP treatment (Figure 4D). These data show that hepatotoxic NPs promote hepatic fibrosis in mice fed CDAHFD.

Given that the Hh pathway is a well-known regulator of liver fibrosis and NPs promote liver fibrosis, we examined the activation of Hh signalling in the in vivo model [14]. As expected, the mRNA expression of *Shh*, a Hh ligand, and *Gli2*, a transcriptional activator of Hh, was upregulated in the CDAHFD groups compared with the Chow group (Figure 5A). Chronic exposure to NPs further increased their expression in CDAHFD-fed mice. The mRNA level of *smoothed* (*Smo*), a receptor, was higher only in the CDAHFD+NPs group than in any other group. The mRNA data were confirmed by protein expression, showing higher expression of Hh signalling in NP-treated mice than in mice fed the vehicle in the CDAHFD groups (Figure 5B). In

addition, GLI2-positive cells were observed in CDAHFD-fed mice, and deposition of these cells was more apparent in the livers of mice fed with NPs than in the other groups (Figure 5C). These findings suggest prolonged NP exposure enhanced liver fibrosis by stimulating the Hh pathway during MASH progression.

3.5 | NPs Enhance SHH Production in Damaged Hepatocytes, Which Activate HSCs by Stimulating Hh Signalling

Dysregulated interaction of liver cells, such as resident immune cells, hepatocytes, sinusoidal endothelial cells and HSCs, is involved in MASLD pathogenesis [12, 18]. Among their interactions, SHH released from apoptotic hepatocytes is known

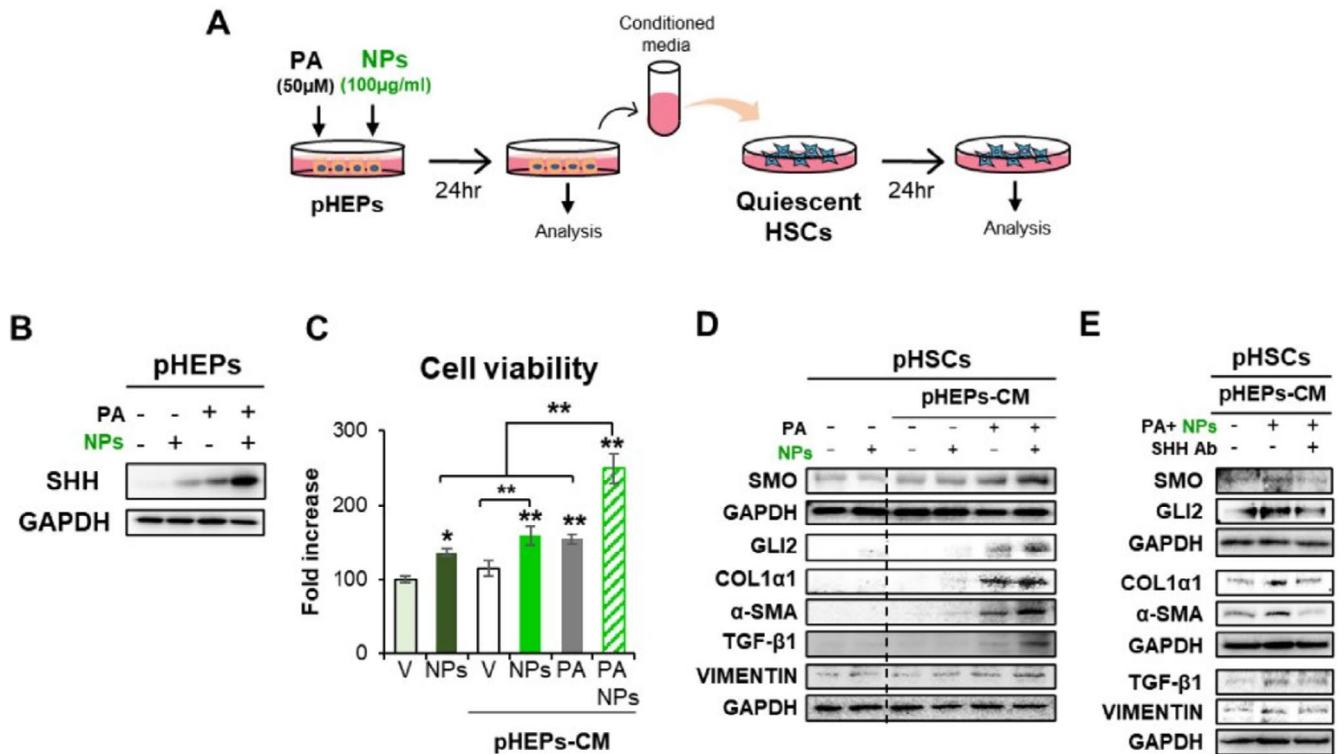


FIGURE 6 | Higher SHH production from NPs + PA-given hepatocytes triggers Hh signalling in HSCs, leading to HSC activation. (A) A schematic description of quiescent (q)HSCs cultured in CM obtained from pHEPs treated with NPs, PA, or NPs + PA for 24 h. (B) The protein levels of SHH in pHEPs exposed to vehicle, NPs, PA, or NPs + PA. GAPDH was used as an internal control. (C) Cell viability of HSCs exposed to vehicle or NPs and of HSCs cultured in CM from pHEPs treated with vehicle, NPs, PA, or NPs + PA. Data represent the mean $\pm$ SEM (* p < 0.05, ** p < 0.005 vs. Veh-treated HSCs). (D) Western blot analysis for Hh activators, SMO and GLI2, and HSC activation markers (TGF- β 1, α -SMA, COL1 α 1, and VIMENTIN) in these cells and (E) in qHSCs cultured in CM from pHEPs exposed to vehicle or NPs + PA, in the presence of control IgG or SHH-neutralising antibody. All data shown represent one of three experiments with similar results.

to activate HSCs by activating Hh signalling in HSCs [19, 20]. Given that NPs promoted damage in hepatocytes injured by lipotoxicity (Figures 2 and 3) and fibrosis (Figure 4), it seemed that hepatocytes with more severe damage following NP treatment might accelerate HSC activation by modulating Hh signalling. To prove the hypothesis, inactivated primary HSCs from healthy mice were cultured in a conditioned medium (CM) from hepatocytes exposed to NPs and PA (Figure 6A). Before examining the physiology of HSCs cultured in CM, we assessed SHH production in pHEPs treated with NPs and PA. SHH expression was rarely detected in vehicle-treated pHEPs but was apparent in the treatment groups (Figure 6B). Among the treatment groups, pHEPs treated with NPs and PA had higher SHH than cells exposed to either NPs or PA. HSCs cultured in CM from pHEPs treated with either NPs or PA showed increased cell proliferation compared with cells cultured in CM from vehicle-given pHEPs (Figure 6C). The elevated cell proliferation rate was highest under conditions where HSCs were cultured in CM from pHEPs treated with both NPs and PA. Hh signalling was also activated in these cells (Figure 6D). The SMO and GLI2 levels were increased in pHSCs cultured in CM from either PA- or NPs + PA-exposed pHEPs compared with any other groups, and their expression appeared to be higher in cells incubated in CM from NPs + PA-treated pHEPs than cells cultured in CM from PA-exposed pHEPs. As Hh signalling was activated, the expression of fibrotic markers, TGF- β 1, COL1 α 1, α -SMA, and VIMENTIN, increased in these cells, and the levels of TGF- β 1,

α -SMA, and VIMENTIN were more evident in pHSCs cultured in CM from NPs + PA-treated pHEPs than cells incubated in CM from PA-exposed pHEPs. However, the elevated expression of Hh activators and fibrotic markers in HSCs was attenuated when the SHH-neutralising antibody was added to the CM from pHEPs treated with PA + NPs (Figure 6E). These data indicate that NP treatment remarkably upregulates SHH production in lipotoxic-damaged hepatocytes, and SHH released from these hepatocytes activates Hh signalling in HSCs.

To verify these findings in vivo, primary HEPs and HSCs were isolated from the experimental mice. In line with elevated apoptosis of pHEPs in CDAHFD+NPs mice (Figure 3G), the SHH protein was more highly expressed in these cells than in the other groups (Figure 7A). Upregulated levels of SMO and GLI2 in pHSCs from CDAHFD-treated mice were remarkably elevated by NP treatment (Figure 7B). Fibrotic markers, including TGF- β 1, COL1 α 1, α -SMA and VIMENTIN, also increased in pHSCs from the CDAHFD groups, compared with cells from the Chow groups. TGF- β 1, α -SMA, and VIMENTIN expressions in pHSCs from CDAHFD-fed mice treated with NPs seem to be higher than cells from mice treated with CDAHFD, similar to the in vitro data shown in Figure 6D. Taken together, these findings demonstrate that lipotoxicity-injured hepatocytes undergo more damage by prolonged NP exposures and produce SHH, which activates HSCs by triggering Hh signalling in HSCs, eventually leading to fibrosis in mice with MASH.

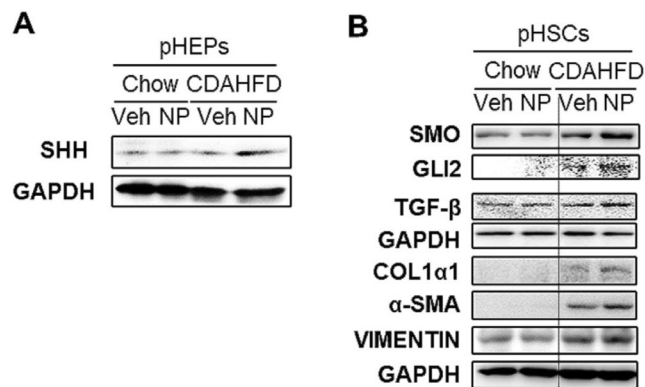


FIGURE 7 | CDAHFD+NPs-treated mice had increased SHH in hepatocytes and enhanced Hh pathway in activated HSCs. (A) The SHH expression in pHEPs isolated from Chow or CDAHFD-fed mice supplemented with either vehicle or NPs. (B) Western blot analysis for SMO, GLI2 and fibrotic markers such as TGF- β 1, COL1 α 1, α -SMA and VIMENTIN in pHSCs from these mice. GAPDH was used as an internal control. Data shown represent one of three experiments with similar results.

4 | Discussion

MPs and NPs are produced from various sources related to the production and use of plastics and are ingested into the human body through food, drink, and the environment [3]. Therefore, scientific researchers have paid much attention to the physiological effects of these plastic particles on the body. Growing evidence demonstrates that accumulated plastic particles in several human organs cause oxidative stress and metabolic disorders and impair developmental processes and reproductive ability [21–23]. MPs disrupt glucose tolerance in the liver and increase hepatic lipid contents, especially free fatty acids and TGs. Indeed, a previous study has shown that MPs could induce hepatic cholestasis by upregulating bile acid (BA) synthesis and downregulating BA excretion into blood [24]. In addition, hepatocytes injured following MP accumulation secreted double-stranded DNA, which elevated α -SMA expression by stimulating cGAS/STING signalling in HSCs, resulting in liver fibrosis in mice [25]. cGAS/STING signalling activated by DNA damage causes inflammation and fibrosis [26]. NPs, smaller in size than MPs, are much more prone to penetrate cells and tissues and accumulate within cells than MPs, meaning they can cause much more cellular damage size-dependently [27]. MPs were reported to barely enter the HL7702 cells, a human liver cancer cell line, while NPs entered these cells and caused DNA damage even at the same concentrations [25]. Banerjee et al. [28] also found that higher deposition of NPs than MPs significantly reduced cell viability compared with MPs in HepG2 cells, and toxicity caused by NPs increased in a concentration-dependent manner. In zebrafish models, NPs were shown to alleviate the survival and hatching rate of embryos and the development of primary motor neurons to promote abnormal locomotor behaviour [29]. NPs also reduced the cognitive and memory abilities of mice [30]. Chronic exposure to NPs stimulated lipolysis in white adipose tissue and led to lipid accumulation in the livers of mice fed a high-fat diet [31]. Oral administration of NPs decreased the expression of antioxidant-related genes, including *nuclear factor erythroid-2-related factor 2* and *glutathione peroxidase*, and

induced hepatocyte apoptosis and inflammation in zebrafish livers [32]. However, these physiological impacts of NPs have been based on animal studies, not human ones, because accumulated NPs in human tissues cannot be identified due to a lack of in situ detection techniques [33]. Hence, there is a critical need to develop methods for detecting NPs to confirm the harmful actions of NPs in human livers.

It was shown that orally administered NPs increased ROS production, which inhibited the PI3K/Akt pathway, leading to insulin resistance and hyperglycemia in mice [34]. Wei et al. [35] reported that NP treatment hardly affected the expression of *de novo* lipogenesis-associated genes, including *Srebf1*, *Fasn*, *Pparg*, *Acaca*, and *Acacb*, but elevated the levels of lipid uptake genes, such as *Fatp2* and *CD36*, in the liver. CD36 is a well-known receptor for free fatty acid (FFA) uptake into the liver [36]. The hepatic expression of CD36 was strongly enhanced in the livers of patients with MASLD compared to subjects with healthy livers [37]. In obese patients, hepatic CD36 expression was upregulated compared to healthy controls at both the protein and mRNA levels, and the mRNA expression of *CD36* was positively correlated with the hepatic lipid level [38]. Moreover, CD36 expression was related to the number of TUNEL-positive apoptotic cells in the livers of obese patients [39]. Parallel with these reports, we presented that NPs induced lipid accumulation in hepatocytes by upregulating CD36 expression (Figure S2D,E, and Figure 1D,E). In hepatocytes exhibiting additional damage by PA, NPs further upregulated CD36 and accelerated excessive ROS production and hepatocyte death (Figure 2C,D,F,G). Elevated hepatic CD36 was also confirmed in the liver and primary HEPs from CDAHFD-fed mice with NPs (Figure 3D,G). Data from hepatocytes stained using Oil-red O and TG levels in the experimental mice supported CD36-mediated lipid accumulation in hepatocytes (Figure S2E and Figures 1E, 2F and 3C). These findings demonstrate that accumulated NPs promote apoptosis of hepatocytes with lipotoxic damage by increasing CD36-modulated lipid uptake. However, it remains to be seen how NPs altered CD36 expression in the research. Yu et al. [40] demonstrated that increased ER stress by NP administration enhanced oxidative stress, which activated the PERK–ATF4 signalling pathway, leading to lipid accumulation in mouse livers. Hence, oxidative stress possibly caused by NPs may be directly related to increased CD36 expression in hepatocytes. However, further studies are essential to elucidate the detailed mechanisms through which hepatic NP deposition induces oxidative stress to lipid metabolic disturbances.

Several studies have shown that orally administered NPs are mainly distributed in the liver, and the concentration of NPs in the liver is seven times higher than that in the spleen, which has the second-highest concentration of NPs [41]. Also, significant deposition of plastic particles has been reported in the livers of patients with cirrhosis. Horvatits et al. [42] found that hepatic MPs were higher in patients with liver cirrhosis than in healthy people. Consistent with these findings, NPs administered via drinking water were accumulated primarily in the liver, especially within hepatocytes in the CDAHFD-induced MASLD model (Figure S1E,F). These results indicate that the hepatic accumulation of plastic particles is correlated with the MASH progression. It is veiled whether plastic particles induce liver disease or if the damaged liver cannot properly remove plastic particles, resulting in the accumulation of plastic particles. Lysosomal

exocytosis, an important pathway in removing internalised NPs, is interrupted by exposure to persistent and high concentrations of NPs, and NPs accumulate in cells [43]. Lipotoxicity also induces lysosomal dysfunction, and lipotoxicity may contribute to the accumulation of enhanced NPs in the MASLD liver [44]. In this current study, NPs and CDAHFD were administered simultaneously to mice for the same period, resulting in more severe liver injury than promoted by CDAHFD. Ultimately, this indicates that NPs assist in the detrimental action of CDAHFD in mice. However, further studies are essential to determine whether lysosomal dysfunction caused by lipotoxicity facilitates the accumulation of ingested plastic particles in the liver. In addition, given that chronic exposure to NPs rarely had apparent pathophysiological effects in mice, NPs could pose a greater risk to individuals whose eating habits make them more susceptible to MASLD.

MASH has more severe hepatocyte injury than MAFL, and ballooning hepatocytes characterised by enlarged hepatocytes with collapsed cytoskeleton are distinct features of MASH [45]. Hepatocellular ballooning also correlates with the severity of fibrosis in a MASH liver. Ballooned hepatocytes release various factors, such as Indian HH (IHH) and SHH, which directly activate Hh-responsive cells, specifically HSCs, in response to fibrosis [12, 20, 46]. Moore et al. [47] reported elevated IHH secretion from hepatocytes in livers with NASH. The hepatocyte-derived IHH caused liver fibrosis by activating Hh signalling in mice fed a fructose–palmitate–cholesterol diet. In MASH patients, SHH is extensively upregulated in ballooned hepatocytes but non-detectable in healthy people [46]. SHH activation was also reported to be positively correlated with the prognosis of NASH patients [48]. In mice with MASLD caused by high fat, high fructose, and high cholesterol diets, increased SHH activated SREBP1 by reducing HSP90 α ubiquitination and promoted hepatic lipid synthesis [20]. In addition, FFA-induced lipotoxicity upregulated SHH expression in hepatocytes [49, 50]. These findings indicate that SHH expression is closely related to the lipotoxic damage of hepatocytes and MASH progression. In line with these findings, the CDAHFD+NP group, which presented more damage than the CDAHFD+Veh group, showed a significant increase in SHH (Figure 5A,B). In pHEPs, as damage accumulated, cell viability decreased, and SHH expression increased (Figures 2C and 6B). Analysis of SHH in pHEPs from the experimental mice supported the upregulation of SHH in hepatocytes in the CDAHFD+NP group. Significant elevation of SHH was detected in the livers of the CDAHFD groups, whereas it was observed in the pHEPs from the CDAHFD+NP group only (Figures 5B and 7A). Given that Hh ligands act as profibrotic factors for HSCs and the CDAHFD groups were more fibrotic, HSCs activate fibrotic signals, Hh signalling, in response to SHH from hepatocytes, and activated HSCs contribute to increased production of SHH in the CDAHFD groups. Therefore, NPs promote hepatocytes to produce more SHH by adding up damage on hepatocytes and induce fibrosis, contributing to MASH progression.

5 | Conclusions

Our results show that NPs preferentially accumulate in the liver and increase lipotoxic stress in hepatocytes by elevating

lipid uptake. Chronic exposure to NPs adds to the severity of hepatocyte injury in mice fed CDAHFD, and these damaged hepatocytes produce SHH, which activates HSCs and causes liver fibrosis. These findings provide insights into the mechanism underlying the action and potential risk of NPs in the development and progression of MASLD.

Author Contributions

J.H. made contributions to data curation, methodology, investigation, validation, visualisation, and writing – revised draft. H.J. made contributions to data curation, methodology, validation, investigation, and writing – revised draft. C.L. made substantial contributions to data curation, methodology, validation, visualisation, and writing – original draft. A.S. made contributions to investigation. Y.H.C. made substantial contributions to conceptualisation and funding acquisition. Y.J. made substantial contributions to conceptualisation, project administration, resources, funding acquisition, supervision, and writing – original and revised draft. All authors read and approved the manuscript.

Acknowledgements

The authors have nothing to report.

Ethics Statement

Animal care and surgical procedures were approved by the Pusan National University Institutional Animal Care and Use Committee and carried out in accordance with the provisions of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (approval number PNU-2020-2574).

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the [Supporting Information](#) of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.