GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Ferroptosis Affects the Progression of Nonalcoholic Steatohepatitis via the Modulation of Lipid Peroxidation—Mediated Cell Death in Mice

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Oxidative stress and its associated lipid peroxidation play a key role in nonalcoholic steatohepatitis (NASH). Ferroptosis is a recently recognized type of cell death characterized by an iron-dependent and lipid peroxidation—mediated nonapoptotic cell death. We demonstrate the impact of ferroptosis on the progression of NASH induced by methionine/choline-deficient diet (MCD) feeding for 10 days. RSL-3 (a ferroptosis inducer) treatment showed decreased hepatic expression of glutathione peroxidase 4 (GPX4) and conversely increased 12/15-lipoxygenase, and apoptosis-inducing factor, indicating that ferroptosis plays a key role in NASH-related lipid peroxidation and its associated cell death. Consistently, levels of serum biochemical, hepatic steatosis, inflammation, and apoptosis in MCD-fed mice were exacerbated with RSL-3 treatment. However, MCD-fed mice treated with sodium selenite (a GPX4 activator) showed increase of hepatic GPX4, accompanied by reduced NASH severity. To chelate iron, deferoxamine mesylate salt was used. Administration of deferoxamine mesylate salt significantly reduced NASH severity and abolished the harmful effects of RSL-3 in MCD-fed mice. Finally, treatment with liproxstatin-1 (a ferroptosis inhibitor) repressed hepatic lipid peroxidation and its associated cell death, resulting in decreased NASH severity. Consistent with the *in vivo* findings, modulation of ferroptosis/GPX4 affected hepatocellular death in palmitic acid—induced *in vitro* NASH milieu. We conclude that GPX4 and its related ferroptosis might play a major role in the development of NASH. (*Am J Pathol* 2020, 190: 68–81; <https://doi.org/10.1016/j.ajpath.2019.09.011>)

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases, and the incidence has recently increased worldwide. It ranges in severity from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. Of these, nonalcoholic steatohepatitis (NASH) is the more severe form of NAFLD and is characterized by lobular inflammation, hepatocellular lipid accumulation, and ballooning degeneration in patients who do not abuse alcohol.¹ Numerous preclinical or clinical studies have been conducted to demonstrate the precise mechanism of NASH development from simple steatosis and its progression.^{2,3} However to date, the detailed mechanism of the progression from NAFLD to NASH has not been fully understood. Recent interesting insight beyond the two-hit theory that provides a possible and reliable mechanism on NASH development has indicated that multiple factors,⁴ such as genetic or environmental factors, gut–liver axis

dysfunction, and adipose tissue—derived adipokines, are involved in the pathogenesis of NASH. However, steatosis and its associated lipotoxicity induced by saturated free fatty acids, including palmitic acid (PA), still seem to represent the onset of NASH development.^{5–7} Furthermore, saturated free fatty acid—induced activation of toll-like receptors results in consequent increase of the inflammatory responses in macrophages.⁸ Because lipotoxicity is closely related to hepatocyte (HP) dysfunction, insulin resistance, inflammation, and fibrogenesis, modulation of lipotoxicity and its

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related oxidative stress may be powerful strategies to improve NASH severity.⁹

In contrast to other regulated cell death, such as apoptosis, necroptosis, pyroptosis, and autophagic cell death, ferroptosis is not involved in caspase activation, adenosine triphosphate depletion, increased intracellular levels of Ca^{2+} , and Bax/Bak, providing that ferroptosis is a unique form of regulated cell death.¹⁰ Although the role of ferroptosis has focused on tumor-killing effects in response to specific compounds, recent findings of ferroptosis are closely associated with the pathogenesis of nonmalignant cellular injury accompanied by lipid peroxidation.¹¹ Glutathione peroxidase 4 (GPX4) is one of the most important selenium-dependent GPXs, because of its unique function, which is antioxidative activity by reducing phospholipid hydroperoxides in membranes, resulting in reduced membrane damage.¹² Recently, the additional role of GPX4 has been identified, indicating that GPX4 acts as the critical regulator of ferroptosis characterized by iron-dependent production of reactive oxygen species, and consequent nonapoptotic form of cell death.¹³

Recently, it has been demonstrated that activation of GPX4 with the use of allosteric activator suppressed inflammation by modulating lipoxygenase-associated oxidative arachidonic acid and NF- κ B pathway, as well as reducing intracellular reactive oxygen species and consequently suppressing ferroptosis.¹⁴ Considering that hepatic oxidative stress and inflammation play key roles in the progression of NASH in patients with simple steatosis,¹⁵ we hypothesized that ferroptosis and/or GPX4 might be associated with the development and/or progression of NASH. Furthermore, depletion of GPX4 in mice or cells showed apoptosis-inducing factor (AIF)-mediated cell death induced by 12/15-lipoxygenase (12/15-Lox)-derived lipid peroxidation.¹⁶ Therefore, the aim of the present study was to demonstrate the role of ferroptotic cell death in NASH pathogenesis.

Materials and Methods

Mice and Experimental Design

Male C57BL/6 mice purchased from Taconic Farms, Inc. (Samtako Bio Korea, O-San, South Korea) were used in this study. Mice were maintained in a pathogen-free environment in standard environmental condition with sterile water and standard control diet. All animal experiments followed the criteria of the requirements of the Animal Care and Ethics Committees of Jeonbuk National University.

Eight-week-old mice were fed a normal chow diet (ND) or a methionine/choline-deficient diet (MCD; Dyets Inc., Bethlehem, PA) for 10 days to induce the early stage of NASH, as previously described.¹⁷ For *in vivo* inhibition of GPX4, mice were intraperitoneally injected with RSL-3 (10 mg/kg; Cayman Chemical, Ann Arbor, MI) once a day for 10 days. In addition, mice were given an i.p. injection with

an iron chelator, deferoxamine mesylate salt (DFO; 100 mg/kg; Sigma, St. Louis, MO), daily during the experimental period. For *in vivo* induction of GPX4, mice were orally administered 0.158 mg/kg sodium selenite (SS; Sigma) once a day for 10 days. Finally, mice were daily injected intraperitoneally with lipoxstatin-1 (Lip-1; Cayman Chemical) at a dose of 10 mg/kg to inhibit ferroptosis, for 10 days. After 10 days, tissues were collected by standard necropsy techniques for future analysis.

Histopathologic Examination

Liver tissue was collected and fixed in 10% phosphate-buffered formalin and then embedded in paraffin according to standard procedure. Embedded tissue was cut to 4- μ m thickness with the use of a microtome (HM-340E; Thermo Fisher Scientific Inc., Waltham, MA) and placed on glass slides. Hematoxylin and eosin (H&E) staining was performed according to standard techniques.

H&E-stained liver sections were assessed according to the scoring system for NASH proposed by Kleiner et al.¹⁸ This system indicates the severity of NASH as the sum of steatosis (0 to 3) and lobular inflammation (0 to 3). The liver sections were averaged at least 5 random fields at $\times 200$ magnification for each mouse.

To detect hepatic lipid accumulation, Oil-red O staining was conducted with frozen liver sections with the use of a commercially available Oil-Red O stain kit (ScyTek, Logan, UT), according to the manufacturer's instruction. Data were expressed as percentage of Oil red O-positive area per field.

To detect cell death in the liver, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; ApopTag Peroxidase *In Situ* Apoptosis Detection Kit; Merck Millipore, Burlington, MA) assay was performed on paraffin-embedded sections according to the kit's protocols. To visualize the positive reaction, 3,3'-diaminobenzidine substrate and nuclear counterstaining (methyl green dye) were performed. A total of 10 high-power fields were analyzed from the liver tissue of each mouse. Data were expressed as the percentage of TUNEL-positive area. To confirm iron contents in paraffin-embedded liver tissues, the iron stain kit (ScyTek) was used in line with the manufacturer's protocol. Total liver section images were analyzed for each animal with the use of light microscopy (BX-51; Olympus Corp., Tokyo, Japan) and digital imaging software (analySIS TS Auto version 5.1; Olympus Corp.).

Biochemical Measurements

NASH-related liver injury was determined by measuring serum levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with the use of spectrophotometric assay kits (AM101-K; ASAN Pharmaceutical, Hwasung, Korea). Absorbance values of serum

biochemical were measured at a wavelength of 490 nm with the use of an EMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

Hepatic levels of triglycerides were determined with a spectro-photometric assay kit (AM202-K; ASAN Pharmaceutical), and absorbance values were measured at a wavelength of 550 nm with the use of an EMax spectrophotometer (Molecular Devices).

Measurement of MDA and GSH in Liver

The hepatic content of malondialdehyde (MDA) as oxidative stress marker was determined with the OxiSelect TBARS Assay Kit (STA-300; Cell Biolabs Inc., San Diego, CA) according to the manufacturer's instructions. Hepatic glutathione (GSH) contents were determined with a commercial GSH quantification kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Measurement of Total Iron Content in Liver

Hepatic total iron concentration was determined with an iron colorimetric assay kit (BioVision, Milpitas, CA) according to the manufacturer's instructions.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA from tissue was extracted with RiboEx (GeneAll, Seoul, Korea) and purified with Easy-Spin Total RNA extraction kit (GeneAll). After treatment of DNase I plus RNase inhibitor (Toyobo, Osaka, Japan) for degradation of the remaining DNA, RNA was reverse transcribed with ReverTra Ace qPCR RT Master Mix (Toyobo) to synthesize cDNA. Real-time PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with a PCR Master Mix (SYBR Green; Toyobo). After the reaction was completed, specificity was verified by melting curve analysis. Data were normalized to hypoxanthine-guanine phosphoribosyl-transferase as the internal control and analyzed with the comparative CT method. The sequences of PCR used in this study are listed in Table 1.¹⁹

Table 1 Primer Sequence of Quantitative Real-Time PCR

Gene	Forward	Reverse
<i>CHOP</i>	5'-CCACCACACCTGAAAGCAGAA-3'	5'-AGGTGAAAGGCAGGGACTCA-3'
<i>XBP1</i>	5'-TGGCCGGGTCTGCTGAGTCCG-3'	5'-GTCCATGGGAAGATGTTCTTGG-3'
<i>XBP-1s*</i>	5'-CTGAGTCCGAATCAGGTGCAG-3'	5'-GTCCATGGGAAGATGTTCTTGG-3'
<i>WFS1</i>	5'-TCCGAGTGACCGAGATCGAC-3'	5'-GCCGTGGACGTGTTACCAGA-3'
<i>BiP</i>	5'-GAACACTGTGGTACCCACCAAGAA-3'	5'-TCCAGTCAGATCAAATGTACCCAGA-3'
<i>ATF3</i>	5'-GCTGCTGCCAAGTGTGCGAA-3'	5'-CGGTGCAGGTTGAGCATGTATATC-3'
<i>ATF4</i>	5'-CGGCTGGTCTGTCACCTATAAAGTA-3'	5'-GGTAACTGTGGCGTTAGAGATCGT-3'
<i>HPRT</i>	5'-TTGTTGTTGGATATGCCCTTGACTA-3'	5'-AGGCAGATGGCCACAGGACTA-3'

*Active spliced form of *XBP1* from Jiang et al.¹⁹

Immunoblot Analysis

Protein was extracted from liver tissues or cells by homogenization on ice with an extraction buffer (T-PER or RIPA buffer; Thermo Fisher Scientific Inc.), followed by centrifugation at $13,000 \times g$ for 15 minutes at 4°C. Supernatant was collected, and the protein concentration was determined with Bradford's reagent (Thermo Fisher Scientific Inc.). Equal amounts of protein were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) that contained 0.05% Tween-20 at room temperature for 1 hour. The membrane was then probed with anti-GPX4 antibody (ab125066; Abcam, Cambridge, UK), 12/15-Lox antibody (Abcam), anti-AIF antibody (Abcam), and anti-β-actin (Cell Signaling Technology, Danvers, MA) at 4°C overnight. To detect antigen–antibody complexes, anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Enzo Life Sciences, New York, NY) were diluted in a blocking solution and incubated for 2 hours at room temperature. Protein bands were visualized with enhanced chemiluminescence detection system with the use of ImageQuant LAS 500 (GE Healthcare Life Science, Pittsburgh, PA). Image analysis for determining relative intensity was performed with ImageQuantTL software version 8.1 (GE Healthcare Life Science).

Enzyme-Linked Immunosorbent Assay

Hepatic protein levels of IL-1β, tumor necrosis factor (TNF)-α, and IL-6 were measured with commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Absorbance of sample was measured with an EMax spectrophotometer (Molecular Devices).

Isolation of Primary HPs

Primary HPs were isolated from mice, as previously described.²⁰ Briefly, mouse livers were perfused (1 mL/min) with collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) via cannulation of the vena cava. Liver

cell suspension was suspended and centrifuged at $50 \times g$ for 3 minutes. After centrifugation, the pellet representing HPs was re-suspended, filtered, and washed several times with Dulbecco's Modified Eagle Medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 100 nmol/L dexamethasone (Sigma), antibiotics-antimycotic (Thermo Fisher Scientific Inc.). Trypan blue (Sigma) exclusion test was performed to determine cell viability in a hemocytometer. Only HPs with viability $>85\%$ were used for further experiments. The isolated HPs were plated onto collagen-coated plates (Sigma). After incubation for cell adhesion at 37°C , media were replaced with fresh serum-free media and incubated overnight at 37°C in a $5\% \text{ CO}_2$ incubator, for further experiments.

To establish an *in vitro* NASH model, primary HPs were treated with 0.4 mmol/L PA, as previously described.²¹ Stock solutions of RSL-3, Lip-1, and SS were prepared and stored at -20°C . DFO was freshly prepared and dissolved in culture medium. Primary HPs were treated with the indicated concentration of these compounds.

Cell Cytotoxicity Assays

Primary HPs were cultured in 12-well plates (2×10^5 cells/well). Then, the cells treated with or without 0.4 mmol/L PA were incubated with RSL-3, Lip-1, DFO, and SS for 24 hours. To inhibit 12/15-Lox, $1 \mu\text{mol/L}$ PD-146176 (Cayman Chemical) was used. Hepatocellular cytotoxicity was evaluated by measuring the amount of lactate dehydrogenase (LDH) leaked into the culture medium with the use of Cytotoxicity Detection Kit^{PLUS} (Sigma) according to the manufacturer's instruction. A wavelength of 490 nm was used to detect absorbance of the sample, using an EMax spectrophotometer (Molecular Devices).

Statistical Analysis

All data are expressed as means $\pm$ SEM. Differences between the two groups were compared with a two-tailed *t*-test using Prism version 7.04 (GraphPad Software, San Diego, CA). Differences were considered statistically significant when two-tailed *P* value was < 0.05 . All experiments were repeated at least twice, and only representative results are shown in the figures.

Results

Induction of Ferroptosis Exacerbates the Severity of NASH Induced by MCD

To investigate the relation between ferroptosis and chronic liver diseases, 1.5-fold increases of hepatic GPX4 enzyme were first determined in the liver of mice, induced by Western diet for 24 weeks and type 2 diabetes (T2D) by genetic modification, respectively. Protein levels of GPX4

were significantly increased in the livers of mice with NASH or T2D, compared with each control group (Supplemental Figure S1). It is likely that ferroptotic cell death might play a critical role in the pathogenesis of NASH. To further support this notion and to explore the role of ferroptosis in NASH progression, ND or MCD-fed mice were treated with RSL-3 for the *in vivo* induction of ferroptotic cell death during diet feeding. A 1.5-fold increase of hepatic GPX4 was observed in MCD-fed mice for 10 days, although treatment of RSL-3 successfully decreased hepatic protein levels of GPX4 (Figure 1A). In parallel with the increase in GPX4, NASH-related histopathologic lesions, such as steatosis and lobular inflammation, were observed in the livers of MCD-fed mice but were more significantly exacerbated in mice treated with RSL-3 compared with untreated mice (Figure 1, B and C). To investigate whether RSL-3 treated mice corresponded to a more severe liver injury with MCD, a detailed measurement of liver injury was made. Higher lipid accumulation was found in the livers of MCD-fed mice treated with RSL-3 based on Oil-red O staining, its positive area measurements, and the levels of hepatic triglycerides (Figure 1, B, D, and F). Consistent with increased serum biochemical and hepatic inflammation, hepatocellular death was markedly increased by RSL-3 in the livers of mice with NASH, as confirmed by TUNEL staining (Figure 1, B and E). Furthermore, NASH-related liver injury was significantly augmented in the GPX4-inhibited livers, as revealed by increased ALT and AST levels (Figure 1G). Because lobular inflammation is one of the hallmarks of distinguishing between fatty liver and NASH, the protein levels of proinflammatory cytokines, including IL-1 β , TNF α , and IL-6, were next verified. The protein levels of these cytokines in RSL-3-treated mice fed with MCD were significantly higher than those in vehicle-treated mice fed with MCD (Figure 1H).

Similar to the previous finding,¹⁶ significantly increased protein levels of 12/15-Lox and AIF were observed in the livers of MCD-fed mice treated with RSL-3 (Figure 2A). Consistently, elevated NASH-related lipid peroxidation was confirmed by significant elevation of hepatic MDA contents with concurrent decrease of GSH levels in the steatohepatic livers of mice treated with RSL-3 (Figure 2, B and C). These *in vivo* findings were further supported by *in vitro* experiments that showed that RSL-3 induced cytotoxic cell damage, with concomitant increase in LDH leakage (Figure 2D) in PA-treated HPs. Furthermore, the protein levels of GPX4 in PA-treated HPs were higher than those in HPs without PA treatment. Such effects were decreased by RLS-3 with concomitant increase of protein levels of 12/15-Lox and AIF (Figure 2E). RSL-3-mediated cytotoxic effects were abrogated by the inhibition of 12/15-Lox in PA-treated HPs, showing that 12/15-Lox was a key regulator of RSL-3-induced cell death in PA-treated HPs (Figure 2F). Collectively, our results, together with *in vitro* findings, indicated that ferroptosis accelerated NASH-related

Figure 1 Inhibition of glutathione peroxidase 4 (GPX4) exacerbated the severity of nonalcoholic steatohepatitis (NASH). Mice were fed with normal chow diet (ND) or methionine/choline-deficient diet (MCD), with or without treatment of RSL-3 (a ferroptosis inducer; 10 mg/kg) to inhibit GPX4. **A**: Protein levels of GPX4 were determined by Western blot analysis and with quantification for GPX4. **B**: Liver sections were stained with hematoxylin and eosin (H&E), Oil-red O, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to assess NASH-related lesions, such as inflammation, steatosis, and apoptosis. **C**: NASH severity was evaluated based on histologic evaluation of steatosis and inflammation. **D** and **E**: The positive area of Oil-red O (**D**) and TUNEL (**E**) staining was quantified. **F**: The levels of hepatic triglycerides were determined. **G**: Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. **H**: Hepatic levels of inflammatory cytokines, including IL-1 β , tumor necrosis factor (TNF)- α , and IL-6, were determined by enzyme-linked immunosorbent assay. Data are expressed as means $\pm$ SEM per group. $n = 10$ to 11 mice (MCD-fed group); $n = 7$ to 8 mice (ND-fed group). * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μ m.

Figure 2 Inhibition of glutathione peroxidase 4 (GPX4) increased hepatocellular death by promoting lipid peroxidation via 12/15-lipoxygenase (12/15-Lox)/apoptosis-inducing factor (AIF) pathway. **A:** Protein levels of 12/15-Lox and AIF in liver were determined by Western blot analysis and with quantification for 12/15-Lox and AIF. **B:** Total amounts of hepatic malondialdehyde (MDA) were measured. **C:** Hepatic glutathione (GSH) contents were quantified. Primary hepatocytes (HPs) were treated with RSL-3 (a ferroptosis inducer; 50 nmol/L) for the inhibition of GPX4 *in vitro*. Palmitic acid (PA; 0.4 mmol/L) was used to induce the *in vitro* nonalcoholic steatohepatitis (NASH) milieu for 24 hours. **D:** The toxicity of RSL-3 in PA-treated primary HPs was measured with the lactate dehydrogenase (LDH) assay. **E:** Protein levels of GPX4, 12/15-Lox, AIF, and β -actin in HPs were measured by Western blot analysis. **F:** PD-146176 (1 μ mol/L) was used to inhibit 12/15-Lox. Then, hepatotoxicity of RSL-3 in PA-treated primary HPs was measured with the LDH assay. Data are expressed as means $\pm$ SEM per group. * P < 0.05, ** P < 0.01. Cont, control; MCD, methionine/choline-deficient diet; ND, normal chow diet.

pathologic processes, including inflammation, oxidative stress, and cellular injury at an early stage of NASH.

Increased NASH Severity Induced by RSL-3 Treatment Is Abolished by Iron Chelation Using DFO

Because iron overload promoted the progression from NAFLD to NASH,²² iron-chelating agent, DFO, which binds free iron, and is widely used to treat iron overload-associated disease, was used.²³ Consistent with the *in vitro* results showing significantly decreased PA-induced cell death by DFO treatment (Supplemental Figure S2A), treatment of DFO successfully inhibited NASH progression, as confirmed by decreased histopathologic lesions; serum levels of ALT, AST, hepatic lipid peroxidation-associated MDA; and GSH levels (Figure 3, A–D). In addition, treatment of DFO attenuated RSL-3-mediated exacerbation of NASH severity, in contrast to the results from Figures 1

and 2 (Figure 3, A–D). In accordance with these results, markedly lower lipid accumulation was seen in MCD-fed mice treated with DFO, compared with that in MCD-fed mice without DFO treatment, as revealed by Oil-red O staining and hepatic triglyceride levels (Figure 3, A, E, and F). In addition, treatment of DFO abrogated the RSL-3-mediated increment of lipid accumulation in MCD-fed mice (Figure 1, B, D, and F, and Figure 3, A, E, and F). In addition, similar patterns were found in NASH-related hepatocellular death, as confirmed by TUNEL staining (Figure 3, A and G). These results indicated that iron was closely associated with NASH progression in the normal or RSL-3-treated condition, which was consistent with previous report showing the pivotal role of GPX4 in iron-induced oxidative stress and ferroptotic cell death.¹⁴ These results were further supported by the findings that hepatic iron was markedly increased in MCD-fed mice compared with ND-fed mice, as confirmed by histologic observation

Figure 3 Iron chelation by deferoxamine mesylate salt (DFO) alleviated increased severity of nonalcoholic steatohepatitis (NASH) by glutathione peroxidase 4 inactivation. Mice were fed normal chow diet (ND) or methionine/choline-deficient diet (MCD) and administrated DFO (100 mg/kg, i.p.) for iron chelation *in vivo*. **A:** Representative images of hematoxylin and eosin (H&E)-, Oil-red O-, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-stained liver sections are shown. **B:** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed. **C:** Lipid peroxidation was determined by measuring malondialdehyde (MDA) contents in livers. **D:** Hepatic glutathione (GSH) contents were measured. **E:** The positive area of Oil-red O staining was quantified. **F:** Hepatic triglyceride levels were quantified. **G:** The positive area of TUNEL staining was determined. **H:** Hepatic protein levels of IL-1 β , tumor necrosis factor (TNF)- α , and IL-6 were determined by enzyme-linked immunosorbent assay. **I:** Hepatic protein expression of apoptosis-inducing factor (AIF) was measured by Western blot analysis with quantification for AIF based on β -actin. Data are expressed as means $\pm$ SEM per group. $n = 5$ to 6 mice (MCD-fed group); $n = 4$ mice (ND-fed group). * $P < 0.05$, ** $P < 0.01$ (two-tailed *t*-test). Scale bars = 100 μ m. RSL3, a ferroptosis inducer.

Figure 4 Activation of glutathione peroxidase 4 (GPX4) attenuated the development and progression of nonalcoholic steatohepatitis (NASH). Mice were fed with normal chow diet (ND) or methionine/choline-deficient diet (MCD), with or without oral administration of sodium selenite (SS; 0.158 mg/kg) to activate GPX4. **A**: Protein levels of GPX4 were determined by Western blot analysis and with quantification for GPX4. **B**: Hematoxylin and eosin (H&E), Oil-red O, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining were performed to assess NASH-related lesions, such as inflammation, steatosis, and apoptosis. **C**: Nonalcoholic fatty liver disease activity scores were evaluated based on the histologic evaluation of steatosis and inflammation. **D** and **E**: Positive area of Oil-red O (**D**) and TUNEL (**E**) staining was quantified. **F**: The levels of hepatic triglycerides were measured. **G**: NASH-related serum biochemistry was measured. **H**: Hepatic levels of inflammatory cytokines, including IL-1 β , tumor necrosis factor (TNF)- α , and IL-6, were determined by enzyme-linked immunosorbent assay. Data are expressed as means $\pm$ SEM per group. $n = 5$ to 6 mice (MCD-fed group); $n = 4$ mice (ND-fed group). * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μ m. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

and measuring hepatic iron contents. In addition, hepatic iron levels in mice fed with MCD or MCD plus RSL-3 were drastically reduced by treatment of DFO, all of which suggested that DFO treatment attenuated severity of NASH by reducing hepatic iron accumulation in mice (Supplemental Figure S3). Although no significant difference was found in the production of inflammatory cytokines by DFO, RSL-3-mediated increase of hepatic inflammation was abolished in MCD-fed mice treated with DFO (Figure 3H). Protein analysis showed that reduced AIF protein levels by treatment of DFO were observed in livers. In addition, RLS-3-mediated increased protein levels of AIF (Figure 2A) were abolished by treatment of DFO in the livers of MCD-fed mice (Figure 3I). Consistently, treatment of DFO obviously reduced protein levels of AIF in PA-treated HPs (Supplemental Figure S2B) or the livers of mice, regardless of diet type. Furthermore, increased RLS-3-mediated LDH leakage (Figure 2D) was abolished in PA-treated HPs cultured with iron-deficient media (Supplemental Figure S4). Taken together, it could be concluded that iron chelation has beneficial effects on NASH progression.

Activation of GPX4 Ameliorates the Severity of NASH

Because SS increased the levels of mRNA, protein, and activity of GPX4,²⁴ mice were orally administered with SS to activate hepatic GPX4 *in vivo*. Hepatic protein levels of GPX4 were slightly, but not significantly, increased in ND-fed mice (Figure 4A). However, MCD-fed mice showed significantly increased hepatic GPX4 by SS treatment. As expected, increased GPX4 by SS was closely associated with lower severity of NASH in the livers of MCD-fed mice than those of MCD-fed mice without SS treatment, as confirmed by histopathologic observation with the use of H&E, Oil-red O, and TUNEL staining (Figure 4B). Consistently, MCD-fed mice administered with SS showed markedly reduced NASH-related histologic lesions, such as steatosis and lobular inflammation in the livers (Figure 4, B and C). In addition, MCD-fed mice treated with SS showed lower lipid accumulation in the livers based on Oil-red O staining, its positive area measurements, and the levels of hepatic triglycerides (Figure 4, B, D, and F). Furthermore, reduced TUNEL-positive area by SS was observed in the NASH milieu (Figure 4, B and E). In addition, significantly lower serum ALT and AST was found in MCD-fed mice administered with SS (Figure 4G). Similar to these findings, protein levels of inflammatory cytokines, including IL-1 β and IL-6, in SS-treated mice fed with MCD were significantly lower than those in vehicle-treated mice fed with MCD (Figure 4H). Of interest, treatment of SS significantly decreased the protein levels of 12/15-Lox in the livers of MCD-fed mice (Figure 5A). In addition, markedly reduced protein levels of AIF were observed in the livers of MCD-fed mice treated with SS (Figure 5A). Reduced NASH-related lipid peroxidation was confirmed by

significant reduction of hepatic MDA contents with concurrent increase of GSH levels in the livers of MCD-fed mice treated with SS (Figure 5, B and C). Consistently, treatment of SS induced cell survival with concomitant decrease in LDH leakage (Figure 5D) in PA-treated HPs. Furthermore, treatment of SS reduced protein levels of 12/15-Lox and AIF, accompanied by increased protein levels of GPX4, in PA-treated HPs (Figure 5E). Overall, these results suggested that the modulation of GPX4 is one of the promising therapeutic strategies to treat NASH.

Inhibition of Ferroptosis Attenuates the Severity of NASH

Supporting the involvement of ferroptosis in NASH pathogenesis as indicated in the section above, the results mentioned above showed that treatment of Lip-1, ferroptosis inhibitor,¹¹ successfully significantly decreased NASH severity based on histopathologic examination (Figure 6, A and B). Treatment of Lip-1 significantly reduced hepatic lipid accumulation in MCD-fed mice, as confirmed by Oil-red O staining, its positive area measurements, and the levels of hepatic triglycerides (Figure 6, A, C, and D). In addition, significantly lower serum levels of ALT and AST were found in MCD-fed mice administered with Lip-1 (Figure 6E). Consistent with these findings, protein levels of inflammatory cytokines, including IL-1 β and TNF α , were significantly decreased in the livers of MCD-fed mice treated with Lip-1 (Figure 6F). In addition, increased hepatic GSH (Figure 7A), reduced lipid peroxidation (Figure 7B), and its related cell death (Figure 7C) were observed in the NASH milieu by the treatment of Lip-1. Similar results in hepatocellular death were confirmed by decreased AIF protein in the NASH milieu with treatment of Lip-1 (Figure 7D). *In vitro* results also showed that the treatment of Lip-1 exerted beneficial effects on cell survival with concomitant decrease in LDH leakage and AIF protein (Figure 7, E and F) in PA-treated HPs. Taken together, these data implicated ferroptosis as a critical contributor in NASH-related hepatocellular injury and pathologic processes.

Discussion

Embryonic lethality is observed in GPX4-deficient mice in contrast to other GPX-deleted mice,²⁵ providing that GPX4 plays an essential role in embryonic development. Furthermore, several lines of evidence indicate that the conditional ablation of GPX4 in the neuron induces the accumulation of lethal lipid reactive oxygen species and consequent lipid peroxidation, leading to neuron degeneration and its related pathologic process.^{16,26,27} In line with these findings, GPX4 is involved in the progression of neurodegeneration, including Parkinson's disease, secondary brain injury after intracerebral hemorrhage, multiple sclerosis, and experimental autoimmune encephalomyelitis,^{28–30} showing that

Figure 5 Lipid peroxidation and cell death were reduced by glutathione peroxidase 4 (GPX4) activation. **A:** Protein levels of 12/15-lipoxygenase (12/15-Lox) and apoptosis-inducing factor (AIF) in liver were confirmed by Western blot analysis and with quantification for 12/15-Lox and AIF. **B:** Hepatic malondialdehyde (MDA) was measured to determine lipid peroxidation. **C:** The levels of glutathione (GSH) in liver were determined. Primary hepatocytes (HPs) were treated with sodium selenite (SS; 5 μmol/L) for activation of GPX4. *In vitro* nonalcoholic steatohepatitis was induced by treatment of palmitic acid (PA; 0.4 mmol/L) for 24 hours. **D:** The toxicity of primary HPs was measured with lactate dehydrogenase (LDH) measurement. **E:** Protein expressions of GPX4, 12/15-Lox, AIF, and β-actin in HPs were measured by Western blot analysis. Data are expressed as means ± SEM per group. **P* < 0.05, ***P* < 0.01. Cont, control; MCD, methionine/choline-deficient diet; ND, normal chow diet.

GPX4 and its associated lipid peroxidation are the driving force of disease progression. Furthermore, GPX4-lacking T cells showed rapid accumulation of membrane lipid peroxides, leading to ferroptotic cell death, resulting in abnormal T-cell-mediated immune responses.³¹ From these findings, we speculated that GPX4 exerts numerous pathophysiological functions in a cell type- or tissue-specific manner. Consistent with this notion, a study has shown that HP-specific GPX4-deficient mice exhibited hepatocellular degeneration, resulting in the early death of mice, indicating that GPX4 is critical for HP survival and proper liver function, via protection from harmful cellular lipid peroxidation.³² Because liver is one of the tissues most highly expressing GPX4,³³ GPX4 might be involved in the pathogenesis of various acute and chronic liver diseases.

This study demonstrated that ferroptotic cell death plays a pivotal role in the development and progression of NASH. The modulation of GPX4 affected hepatic lipid peroxidation via affecting 12/15-Lox-AIF-related cell death pathway. These findings are consistent with a previous study that showed that hydroperoxy-phosphatidylethanolamines as ferroptotic death signals were produced by 12/15-Lox, and

their reduction to hydroxy-metabolites was mediated by GPX4.³⁴ Thus, the balance of these two reactions is involved in the decision between cell survival and ferroptotic cell death.³⁴ Furthermore, a study has shown that 12/15-Lox-mediated endoplasmic reticulum stress, followed by nuclear translocation of mitochondrial AIF, is closely associated with organelle damage-related cell death, leading to the increase of stroke-induced brain damage.³⁵ In accordance with this result, chronic 12/15-Lox activation and its associated endoplasmic reticulum stress were observed in mice with obesity, which can exacerbate inflammation and metabolic dysfunction in metabolic tissues.³⁶ Consistently, disruption of hepatic 12/15-Lox alleviates steatosis, liver injury, and inflammation in a murine model of hyperlipidemia-induced liver disease.³⁷ From these findings, it also was observed that the modulation of GPX4 can affect hepatic 12/15-Lox levels and subsequent expression levels of endoplasmic reticulum stress-related genes in livers (Supplemental Figure S5).

It has been documented that 12/15-Lox-mediated production of hydroperoxy-phosphatidylethanolamine is dependent on cellular non-heme iron.³⁸ Because diet-induced

Figure 6 Inhibition of ferroptosis ameliorated severity of nonalcoholic steatohepatitis. Mice were fed with normal chow diet (ND) or methionine/choline-deficient diet (MCD), with or without the treatment of lipoxstatin-1 (Lip-1; 10 mg/kg) to inhibit ferroptosis. **A:** Representative images of hematoxylin and eosin (H&E)- and Oil-red O–stained liver sections. **B:** Nonalcoholic fatty liver disease activity scores were assessed according to histologic examination of hepatic steatosis and lobular inflammation. **C:** The percentage of Oil-red O–positive area was measured. **D:** The levels of hepatic triglycerides were measured. **E:** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. **F:** Hepatic levels of IL-1 β , tumor necrosis factor (TNF)- α , and IL-6 were determined by enzyme-linked immunosorbent assay. Data are expressed as means $\pm$ SEM per group. $n = 8$ mice (MCD-fed group); $n = 5$ mice (ND-fed group). * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μ m.

Figure 7 Inhibition of ferroptosis ameliorated nonalcoholic steatohepatitis (NASH)-related lipotoxic cell death. **A:** Hepatic glutathione (GSH) levels were quantified. **B:** Hepatic malondialdehyde (MDA) contents were assessed to evaluate oxidative stress. **C:** Representative images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining are shown and with quantification for its positive area. **D:** Protein expressions of apoptosis-inducing factor (AIF) and β-actin in liver were measured by Western blot analysis, with quantification for AIF. Primary hepatocytes (HPs) were treated with lipoxstatin-1 (Lip-1; 100 nmol/L) to inhibit ferroptosis. *In vitro* NASH was induced by the treatment of palmitic acid (PA; 0.4 mmol/L) for 24 hours. **E:** The toxicity of primary HPs was measured with lactate dehydrogenase (LDH) measurement. **F:** Protein levels of AIF and β-actin in primary HPs were measured by Western blot analysis. Data are expressed as means ± SEM per group. $n = 8$ mice [methionine/choline-deficient diet (MCD)-fed group]; $n = 5$ mice [normal chow diet (ND)-fed group]. * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μm. Cont, control.

hepatic iron overload accelerates the progression of NAFLD to NASH via increased adipose tissue dysfunction, hepatic oxidative stress, and inflammation,²² iron modulation might be a beneficial therapeutic strategy against iron-associated liver diseases. Here, it was demonstrated that iron chelation with the use of DFO ameliorates NASH development. In addition, increased NASH severity in the GPX4-inhibited milieu by RSL-3 was abrogated by treatment of DFO. Consistent with the *in vivo* results, RLS-3-mediated hepatotoxic damage was abolished in HPs cultured with iron-deficient media. Furthermore, a study has provided that iron chelation attenuates Fas-induced fulminant hepatitis by reducing hepatic iron load and subsequent oxidative stress.³⁹ Therefore, hepatic iron together with 12/15-Lox promotes NASH-related lipid peroxidation and consequently induces AIF-mediated cell death, resulting in promotion of the development and progression of NASH. Finally, the therapeutic potential of NASH was assessed by inhibiting ferroptosis with the use of a ferroptosis inhibitor, Lip-1. In line with the previous result that Lip-1 attenuates ischemia/reperfusion-induced hepatic damage,¹¹ our results also showed that inhibition of ferroptosis attenuates

NASH-related pathologic processes, including steatosis, oxidative stress, inflammation, and cell death.

Although RSL-3 has been originally known to inhibit GPX4 activity,¹³ a more recent study has shown that RSL-3 affects other selenoproteins because of its high electrophilicity toward selenocysteine residues.⁴⁰ From this finding, a limitation of the present study is the lack of genetic manipulation of GPX4, which would allow for assessing the precise role of GPX4. Hence, further studies need to be performed to more clearly elucidate the role of GPX4 in NASH progression by depletion or overexpression of GPX4 in hepatocytes.

Intriguingly, in this study, the induction of GPX4 in liver of mice fed MCD and Western diet or of mice with T2D (Supplemental Figure S1) was observed. From our results, GPX4 is broadly involved in inflammatory acute and chronic liver diseases with metabolic dysfunctions. In addition, similar up-regulation of GPX4 accompanied with increased lipid peroxidation in liver and heart of mice has been found after high-fat, high-sucrose diet for 24 weeks; however, GPX4 becomes deficient in obese patients because of prolonged disease process and possible genetic causes.⁴¹

It was suggested that hepatic GPX4 adaptive response is likely to become overwhelmed or compromised in very late phase of obesity/nutrient overload in mice and patients. Therefore, the stages of NASH and T2D investigated are relatively early; thus, it is valuable to demonstrate the role of GPX4 and ferroptosis in a late phase of NASH.

Conclusions

Ferroptosis is an iron-dependent form of regulated cell death triggered by toxic lipid peroxidation, which is inhibited by GPX4 in steatohepatic livers. Hence, modulation of GPX4 might pave the way for a novel promising strategy for treating NASH and other related metabolic disorders.

Author Contributions

J.Q. and J.-W.K. performed most of the experiments and wrote the manuscript; Z.Z. helped with Western blot analysis and experiment design; C.W.L. and B.K. reviewed and edited the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.09.011>.

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