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Protective role of endogenous plasmalogens against hepatic steatosis and steatohepatitis

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Abstract

Free cholesterol (FC) accumulation in the liver is an important pathogenic mechanism of nonalcoholic steatohepatitis (NASH). Plasmalogens, key structural components of the cell membrane, act as endogenous antioxidants and are primarily synthesized in the liver. However, the role of hepatic plasmalogens in metabolic liver disease is unclear. In this study, we found that hepatic levels of docosahexaenoic acid (DHA)-containing plasmalogens, expression of glyceronephosphate O-acyltransferase (*Gnpat*, the rate-limiting enzyme in plasmalogen biosynthesis), and expression of *Ppara* were lower in mice with NASH caused by accumulation of

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Potential conflict of interest

J.E.J., E.H.K. and K.-U.L. have applied for a patent on the effect of alkyl glycerol on nonalcoholic steatohepatitis.

free cholesterol (FC) in the liver. Cyclodextrin-induced depletion of FC transactivated Δ^6 desaturase by increasing *Srebp2* expression in cultured hepatocytes. DHA, the major product of Δ^6 desaturase activation, activated GNPAT, thereby explaining the association between high hepatic FC and decreased *Gnpat* expression. *Gnpat* siRNA treatment significantly decreased *Ppara* expression in cultured hepatocytes. In addition to GNPAT, DHA activated PPAR α and increased expression of *Ppara* and its target genes, suggesting that DHA in the DHA-containing plasmalogens contributed to activation of PPAR α . Accordingly, administration of the plasmalogen precursor alkyl glycerol (AG) prevented hepatic steatosis and NASH through a PPAR α -dependent increase in fatty acid oxidation. *Gnpat*^{+/-} mice were more susceptible to hepatic lipid accumulation and less responsive to the preventive effect of fluvastatin on NASH development, suggesting that endogenous plasmalogens prevent hepatic steatosis and NASH.

Conclusions—Increased hepatic FC in animals with NASH decreased plasmalogens, thereby sensitizing animals to hepatocyte injury and NASH. Our findings uncover a novel link between hepatic FC and plasmalogen homeostasis via GNPAT regulation. Further study of AG or other agents that increase hepatic plasmalogen levels may identify novel therapeutic strategies against NASH.

Keywords

free cholesterol; PPAR α ; fatty acid oxidation; glyceronephosphate *O*-acyltransferase; alkyl glycerol

Plasmalogens are a special group of phospholipids (ether lipids) synthesized in peroxisomes and the endoplasmic reticulum and widely distributed in mammalian tissues. Plasmalogens, a key structural component of the cell membrane, protect cells from oxidative stress-induced damage^(1,2) and are highly distributed in nervous tissues. Defects in plasmalogen biosynthesis are related to several inherited human diseases. Zellweger syndrome, the most severe disorder of peroxisomal biogenesis, is characterized by a lack of functional peroxisomes.⁽³⁾ This neurodegenerative disorder involves tissue accumulation of very long-chain fatty acids and low levels of phosphatidylethanolamine (PE)-plasmalogen and docosahexaenoic acid (DHA).⁽¹⁻³⁾ Rhizomelic chondrodysplasia punctata type 1 (RCDP1) is caused by defects in peroxisomal biogenesis factor 7, which encodes the cytosolic receptor for proteins carrying the peroxisomal targeting signal 2, leading to impaired peroxisomal import of proteins such as alkylglycerone phosphate synthase (AGPS) and impaired plasmalogen biosynthesis.⁽⁴⁾ Patients with RCDP1 are characterized by severe mental retardation and various degrees of skeletal dysplasia.⁽⁵⁾ RCDP type 2 and type 3 are caused by mutations in glyceronephosphate *O*-acyltransferase (GNPAT) and AGPS, respectively, the rate-limiting and first two enzymes of plasmalogen synthesis.⁽⁴⁾ RCDP type 2 and 3 patients have similar clinical presentations to type 1 patients, indicating that the defect in plasmalogens is the main cause of the disease presentation.⁽⁴⁾ In addition to neurologic diseases,^(2,4,6) alterations in plasmalogen levels may also be related to metabolic diseases, inflammatory diseases, and cancers.^(1,2,7)

Although the liver is the primary organ involved in plasmalogen synthesis, plasmalogens are transported to other tissues via lipoproteins, and the actual levels of hepatic plasmalogens are relatively low.⁽¹⁾ However, in Zellweger syndrome, severe hepatic dysfunction, fatty

liver, and liver cirrhosis are commonly observed,^(8,9) suggesting that low plasmalogen levels contribute to liver disease in these patients. In addition, a lipidomic study performed in humans showed that plasma plasmalogen levels are significantly lower in subjects with nonalcoholic steatohepatitis (NASH).⁽¹⁰⁾ We thus reasoned that impairment of plasmalogen synthesis in the liver contributes to NASH development.

In this study, we found that endogenous hepatic plasmalogens protect hepatocytes against lipid accumulation and lipid-induced injury. Administration of alkyl glycerol (AG),⁽¹¹⁾ a precursor of plasmalogen, prevented hepatic steatosis and NASH and significantly increased *Ppara* expression. PPAR α is a nuclear receptor that stimulates hepatic mitochondrial and peroxisomal fatty acid oxidation (FAO) through transcriptional regulation.^(12,13) Interestingly, animals with NASH, but not those with simple hepatic steatosis, had lower hepatic DHA-containing plasmalogen and expression of *Gnpat* and *Ppara*.

Recent studies show that disturbance of hepatic free cholesterol (FC) homeostasis, and particularly its trafficking to mitochondria, is a major factor determining progression from simple steatosis to NASH.^(14–18) In the present study, we found that the hepatic FC level was increased in mice exposed to a methionine- and choline-deficient diet (MCDD) or high-fat high-cholesterol diet (HFHCD), and that this was associated with decreased expression of *Gnpat* and *Ppara*. Together, these results suggest that altered hepatic FC metabolism leads to decreased hepatic plasmalogen levels and subsequent induction of NASH.

Experimental procedures

Details of the experimental protocols are provided in the Supporting Experimental Procedures.

Animals

All animal use and experiment protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Seoul, Korea. Eight-week-old male C57BL/6N mice were fed a normal chow diet (ND; 12% energy from fat), high fat diet (HFD; 60% energy from fat, Research Diets, Inc., New Brunswick, NJ), MCDD (Dyets Inc., Bethlehem, PA), or HFHCD (60% energy from fat containing 2.5% cholesterol, Dyets)⁽¹⁹⁾ for the indicated times.

Gnpat-knockout (KO) mice were generated using the transcription activator-like effector nuclease method (Supporting Fig. S1).⁽²⁰⁾ Because *Gnpat*^{−/−} mice did not survive beyond 2 weeks in our colony, *Gnpat*^{+/−} mice were used for experiments. Founder mice harboring transcription activator-like effector nuclease-induced mutations were crossed with wild-type (WT) mice. Littermate control (*Gnpat*^{+/+}) and *Gnpat*^{+/−} mice were fed a ND, HFD, or MCDD. To further evaluate the effect of PPAR α activation on *Gnpat*^{+/−} mice, *Gnpat*^{+/−} mice were fed a HFD and treated with fenofibrate (0.1% or 0.5% wt/wt; Sigma-Aldrich, St. Louis, MO) for 8 weeks.

In another set of experiments, fluvastatin (Sigma-Aldrich) and 1-*O*-octadecyl-rac-glycerol (a commercial form of AG; also called batyl alcohol; Sigma-Aldrich) were used to supplement

the MCDD or HFHCD. After 8 or 12 weeks, mice were fasted for 5 h in the morning and sacrificed. The liver tissue was rapidly removed and kept frozen at -70°C for subsequent measurements.

To demonstrate that the preventive effect of AG is dependent on PPAR α activation, the effect of AG was examined in PPAR α -KO mice. PPAR α -KO mice (Stock number: 008154) were obtained from Jackson Laboratory (Bar Harbor, Maine). WT mice and PPAR α -KO mice were given a MCDD with AG for 8 weeks.

Measurement of lipid metabolites

Plasmalogen levels were determined using liquid chromatography with a tandem mass spectrometry (LC-MS/MS) system equipped with a 1290 HPLC (Agilent, Waldbronn, Germany) and QTRAP 5500 (AB Sciex, Toronto, Canada).

Statistical analyses

Where appropriate, results are expressed as the mean $\pm$ s.e.m. Differences between groups were assessed by a Student's two-tailed *t*-test or one-way ANOVA followed by Bonferroni *post-hoc* test. $P < 0.05$ was considered statistically significant.

Results

Ppara expression and plasmalogen levels are lower in the liver of MCDD-fed mice

Rodents fed a HFD or MCDD are frequently used as valuable models of nonalcoholic fatty liver disease (NAFLD).^(21,22) Administration of the HFD to C57BL/6N mice for 8 weeks caused mild hepatic steatosis without significant inflammation, whereas exposure to the MCDD for 8 weeks caused NASH and early fibrosis (Fig. 1A,B; Supporting Fig. S2A). Mice fed a HFD had significantly higher body weight and plasma insulin levels than those fed a ND. Conversely, mice fed a MCDD had lower body weight and plasma insulin levels than HFD- and ND-fed mice (Supporting Table 1).

Changes in several metabolic pathways can lead to the development of hepatic steatosis.⁽²³⁾ In the present study, the MCDD significantly decreased mRNA expression of enzymes involved in mitochondrial and peroxisomal FAO, such as carnitine palmitoyl transferase-1 (*Cpt1a*), very long-chain acyl-CoA dehydrogenase (*Vlcad*), acyl-CoA oxidase-1 (*Acox1*), and D-bifunctional protein (*Dhpl*) (Fig. 1C). The MCDD also significantly decreased the expression of *Ppara*, a nuclear receptor that stimulates hepatic mitochondrial and peroxisomal FAO through transcriptional regulation^(12,13) (Fig. 1C). Conversely, HFD feeding increased the expression of sterol regulatory element-binding protein-1c (*Srebp1c*), a key transcription factor for lipogenesis,⁽²³⁾ but did not change the expression of *Ppara* or FAO enzymes (Fig. 1C). These results are in line with previous suggestions that decreased *Ppara* expression and FAO may be important contributors to the development of NASH.^(12,13)

We next measured the expression levels of the plasmalogen biosynthetic enzymes and hepatic plasmalogen contents in mice administered the MCDD or HFD. The MCDD

significantly decreased mRNA expression of *Gnpat* and *Agps* (Fig. 1D), whereas HFD feeding did not change expression of *Gnpat* or *Agps* (Fig. 1D).

Most plasmalogens have ethanolamine (PE-plasmalogens) or choline (PC-plasmalogens) in the polar head group and are enriched with *n*-3 or *n*-6 polyunsaturated fatty acids (PUFAs), such as DHA (22:6*n*-3) or arachidonic acid (AA, 20:4*n*-6), in the *sn*-2 position. Representative peaks of DHA-containing PC- and PE-plasmalogens in LC-MS/MS are shown in Supporting Fig. S3. MCDD feeding increased AA-containing PC-plasmalogen levels but decreased DHA-containing PC- and PE-plasmalogens (Fig. 1E). HFD feeding did not affect hepatic plasmalogen levels (Fig. 1E).

Increased hepatic FC is a major contributor to the development of NASH.^(14–18) Indeed, the hepatic FC level was significantly increased in MCDD-fed mice, but not in HFD-fed mice (Fig. 1F). SREBP-2 is a major transcription factor that promotes cholesterol synthesis and is inhibited by intracellular cholesterol.⁽²⁴⁾ Accordingly, the MCDD, but not HFD, significantly decreased mRNA expression of *Srebp2* in the liver (Fig. 1C).

Administration of the HFHCD decreases hepatic plasmalogen levels and Δ -6 desaturase expression

We next investigated possible reasons for decreased plasmalogen levels and *Gnpat* expression in MCDD-fed but not HFD-fed mice. We hypothesized that increased hepatic FC in animals with NASH decreases hepatic plasmalogen levels. To test this hypothesis, we examined hepatic plasmalogen levels in mice fed a high-cholesterol diet. In agreement with a recent study,⁽¹⁹⁾ administration of a HFHCD containing 2.5% cholesterol for 12 weeks induced NASH in C57BL/6N mice (Supporting Fig. S2B and S4). HFHCD-fed mice did not have a greater gain in body weight but had significantly higher plasma glucose and insulin levels (Supporting Table 2). Expression of *Ppara* was significantly lower in HFHCD-fed mice and was associated with decreased mitochondrial and peroxisomal FAO (Fig. 2A). HFHCD feeding did not increase total cholesterol in plasma but significantly increased the hepatic FC level, and decreased mRNA expression of *Srebp2* (Fig. 2B).

Regarding plasmalogen biosynthesis, HFHCD feeding significantly decreased mRNA expression of *Gnpat* but did not change *Agps* (Fig. 2C). The HFHCD significantly decreased the levels of DHA-containing PC- and PE-plasmalogens as well as AA-containing PC-plasmalogens (Fig. 2C).

An earlier study showed that a high-cholesterol diet decreased the activity of liver Δ -5 and Δ -6 desaturases (encoded by *Fads1* and *Fads2*, respectively), which was associated with a decrease in the percentages of AA and DHA in total and microsomal liver lipids.⁽²⁵⁾ Because PUFA composition in plasmalogens can be influenced by the activity of Δ -5 and Δ -6 desaturases,⁽²⁶⁾ we measured the expression of these enzymes. As expected, expression of *Fads1* and *Fads2* was significantly decreased in HFHCD-fed mice (Fig. 2D).

To gain further insight into the molecular hierarchy among Δ -5 and Δ -6 desaturases, GNPAT, and PPAR α , we tested the effects of siRNAs against Δ -5 and Δ -6 desaturases on the expression of the other molecules in AML12 hepatocytes. Interestingly, treatment with

siRNA against *Fads2*, but not *Fads1*, significantly decreased the mRNA expression of *Gnpat* and *Ppara* (Fig. 2E). Unlike Δ^5 desaturase, Δ^6 desaturase is required for DHA synthesis from eicosapentaenoic acid (EPA, 20:5n-3) (Supporting Fig. S5).⁽²⁷⁾ We thus hypothesized that DHA regulates GNPAT. Indeed, DHA increased *Gnpat* expression in cultured hepatocytes in a dose-dependent manner and restored *Gnpat* expression in si-*Fads2*-treated hepatocytes (Fig. 2F). In addition, DHA increased the promoter activity of *Gnpat* (Fig. 2F). Collectively, these data indicated that DHA transcriptionally activates *Gnpat*.

Molecular link between higher hepatic FC level and lower *Gnpat* expression in NASH

We next examined the molecular mechanism linking higher hepatic FC and lower *Gnpat* expression in animals with NASH. We observed that the promoter region of *Fads2* contained a *Srebp2*-binding site (Supporting Fig. S6). Cyclodextrin, a detergent that depletes cellular cholesterol content by binding and extracting cholesterol,⁽²⁸⁾ significantly increased expression of *Srebp2* and *Fads2* in cultured hepatocytes (Fig. 3A). Cyclodextrin also significantly increased promoter activity of *Fads2*, and siRNA against *Srebp2* inhibited the cyclodextrin-mediated increase in the promoter activity of *Fads2* (Fig. 3B). Together, our findings indicated that higher hepatic FC level in animals with NASH decreased *Srebp2* expression and thereby *Fads2* expression, leading to decreased production of DHA to decrease GNPAT transcription (Fig. 3C).

GNPAT is upstream of PPAR α

Because GNPAT is localized in peroxisomes,⁽¹⁾ we reasoned that *Gnpat* expression may also be regulated by PPAR α . Contrary to our expectations, *Ppara* siRNA (si*Ppara*) treatment did not affect *Gnpat* gene expression in AML12 hepatocytes (Fig. 4A). Similarly, fenofibrate, a PPAR α ligand, did not change *Gnpat* gene expression (Supporting Fig. S7), although it significantly increased expression of PPAR α target genes. Conversely, *Gnpat* siRNA (si*Gnpat*) treatment significantly decreased *Ppara* expression (Fig. 4A), and siRNA against *Gnpat* and *Ppara* significantly decreased expression of *Cpt1a*, *Vlcad*, *Acox1*, and *Dbp1* (Fig. 4A). The si*Gnpat* also decreased mitochondrial and peroxisomal FAO and significantly increased cell apoptosis (Fig. 4B), suggesting that changes in GNPAT are upstream of changes in PPAR α signaling.

DHA activates PPAR α in hepatocytes

We next examined the mechanism by which changes in GNPAT affect PPAR α signaling. DHA can be selectively targeted to plasmalogens, and this targeting occurs during *de novo* synthesis of plasmalogens.⁽²⁹⁾ Because DHA-containing plasmalogens were decreased in the liver of animals with NASH (Fig. 1E, Fig. 2C), we reasoned that plasmalogens or DHA in DHA-containing plasmalogens may activate PPAR α . Therefore, we examined the effect of the plasmalogen precursor AG or DHA on the expression and transcriptional activity of PPAR α in cultured hepatocytes.

HEPG2 cells were transfected with human PPAR α expression plasmid along with PPRE-Luc (kindly provided by Dr. S.H. Um, Sungkyunkwan University School of Medicine, Suwon, Korea) and PPAR α promoter-Luc to assess the effect of DHA on PPAR α transcriptional and promoter activity, respectively.

Interestingly, DHA dose-dependently increased mRNA expression of *Ppara* and its target genes in HEPG2 cells (Fig. 4C). DHA also significantly increased both transcriptional and promoter activity of PPAR α , suggesting regulation of PPAR α transcription by PPAR α itself.⁽³⁰⁾ Conversely, AG did not change transcriptional and promoter activity of PPAR α (Supporting Fig. S8).

Changes in hepatic metabolism in *Gnpat*-KO mice

We next examined hepatic metabolism of *Gnpat*-KO mice (Supporting Fig. S1). Because homozygous KO animals in our colony did not survive beyond 2 weeks, we examined the metabolic phenotype in *Gnpat*^{+/-} (heterozygous KO) mice. Changes in body weight were similar between *Gnpat*^{+/-} and WT (*Gnpat*^{+/+} littermate) mice, but the *Gnpat*^{+/-} mice showed higher levels of plasma glucose and insulin (Supporting Fig. S9), increased susceptibility to HFD-induced systemic insulin resistance and adipose tissue inflammation (Ko MS et al., unpublished data).

Gnpat expression in the liver of *Gnpat*^{+/-} mice was ~50% that of WT mice (Fig. 5A). Interestingly, HFD-fed *Gnpat*^{+/-} mice showed significantly higher lipid accumulation in the liver than WT mice (Fig 5B, Supporting Fig. S2C), even though there was no difference in hepatic inflammation. On the other hand, no significant differences were noted between WT and *Gnpat*^{+/-} mice fed the MCDD (Fig. 5B).

Gnpat and *Ppara* expression differed in WT mice fed HFD or MCDD and was significantly lower in the *Gnpat*^{+/-} mice fed the HFD but not MCDD (Fig 5C, 5D). Hepatic DHA-containing plasmalogen levels were significantly lower in HFD-fed *Gnpat*^{+/-} mice than in WT mice, whereas they were not significantly different between MCDD-fed *Gnpat*^{+/-} and WT mice (Fig. 5C,D).

Plasmalogens are important for intracellular cholesterol and membrane trafficking.^(31,32) Therefore, reduced mitochondrial function in *Gnpat*^{+/-} mice may be due to disruption of the mitochondrial network rather than a specific defect in PPAR α signaling. We thus examined the morphology of mitochondria in the liver of *Gnpat*^{+/-} mice by electron microscopy, but did not find alterations in mitochondrial structure (Supporting Fig. S10).

We next examined the effect of PPAR α activation on hepatic steatosis in HFD-fed *Gnpat*^{+/-} mice. Fenofibrate treatment significantly attenuated hepatic steatosis in HFD-fed *Gnpat*^{+/-} mice (Fig. 5E,F, Supporting Fig. S2C). Taken together, these results suggested that decreased PPAR α signaling led to hepatic steatosis in HFD-fed *Gnpat*^{+/-} mice independent of the mitochondrial dysfunction.

Administration of AG prevents NASH

We next examined the effect of the plasmalogen precursor AG, which enters the ether lipid synthetic pathway downstream of the peroxisomal pathway and acts as a plasmalogen precursor independent of peroxisomal function (Supporting Fig. S11).⁽¹¹⁾ Treatment with AG in MCDD-fed mice did not change body weight or plasma insulin levels (Supporting Table 1), but did prevent hepatic steatosis and inflammation (Fig. 6A, Supporting Fig. S12A,B). Relative to their untreated counterparts, MCDD-fed mice treated with AG also had

significantly higher plasmalogen levels (Fig. 6B) and restoration of hepatic mitochondrial and peroxisomal FAO, as well as *Ppara* and target gene expression (Fig. 6C). Interestingly, the preventive effect of AG on hepatic steatosis and inflammation was abolished in PPAR α -KO mice (Fig. 6D, Supporting Fig. S2D), indicating that the preventive effect of plasmalogen on the development of NASH is dependent on PPAR α activation.

AG treatment also ameliorated NASH development in HFHCD-fed mice (Supporting Fig. S12C), restored mRNA expression of *Ppara* and enzymes involved in mitochondrial and peroxisomal FAO (Supporting Fig. S12D, E) and significantly increased the peroxisomes as estimated by the immunofluorescence detection of peroxisomal proteins catalase and PEX14 as described previously (Supporting Fig. S13).^(33,34) These results are in accordance with a recent study that showed that PPAR α activation increases peroxisomal biogenesis.⁽³⁵⁾

We next examined several possibilities other than PPAR α activation by which AG treatment prevents development of NASH. The conversion of cholesterol to bile acids is the predominant pathway for cholesterol catabolism.⁽³⁶⁾ One of the prime functions of peroxisomes is bile acid synthesis,⁽³⁷⁾ and defective bile acid synthesis may cause cholesterol accumulation in hepatocytes. Indeed, livers of HFHCD-fed mice contained significantly lower amounts of several bile acid subspecies compared to control mice (Supporting Fig. S14A). HFHCD feeding also profoundly decreased the expression of cholesterol 7 α -hydroxylase (*Cyp7a1*), the rate-limiting enzyme of bile acid synthesis (Supporting Fig. S14B),⁽³⁶⁾ suggesting that this mechanism may contribute to the increased FC levels in these mice. However, AG did not significantly increase bile acids or *Cyp7a1* expression (Supporting Fig. S14). As increased cellular plasmalogen level has been reported to reduce cholesterol biosynthesis,⁽³⁸⁾ we tested whether AG reduces hepatic FC level. However, AG did not change the hepatic FC level in MCDD-fed mice (Fig. 6E). Taken together, the data suggest that plasmalogens do not protect hepatocytes from injury by increasing bile acid synthesis or decreasing the intracellular FC level.

Free fatty acids (FFAs) are involved in hepatic inflammation.⁽³⁹⁾ Because FAO is decreased in the liver of MCDD-fed mice, we hypothesized that hepatic FFA levels would be higher in these mice. However, hepatic FFA levels were not increased in animals with NASH (Supporting Table 3), in agreement with a previous study.⁽¹²⁾ Moreover, the level of hepatic ceramide, another important lipotoxic metabolite,⁽³⁹⁾ and the expression of serine palmitoyl transferase-2 (*Spt2*), the rate-limiting enzyme of ceramide biosynthesis, were significantly higher in MCDD-fed mice (Supporting Fig. S15), suggesting that increased ceramide levels may be involved in the development of NASH.⁽³⁹⁾ Interestingly, administration of AG did not affect hepatic ceramide levels or *Spt2* expression (Supporting Fig. S15), suggesting that the effect of plasmalogen is independent of alterations in ceramide metabolism. Collectively, these results suggest that AG prevents NASH through activation of PPAR α signaling.

The preventive effect of fluvastatin on NASH is partially abrogated in GNPAT^{+/-} mice

Statins are widely used lipid-lowering drugs that competitively inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. Previous studies in animals found that statins reversed hepatic FC accumulation and prevented NASH development.^(40,41) Administration of fluvastatin abolished NASH in

MCDD- and HFHCD-fed mice (Fig. 7A–C, Supporting Fig. S16). As expected, the increased hepatic FC level in MCDD-fed mice was significantly decreased by fluvastatin treatment (Fig. 7D). Fluvastatin significantly increased gene expression levels of Δ^6 desaturase, *Gnpat*, and *Ppara*, as well as the plasmalogen levels in the liver (Fig. 7E,F).

To further establish the role of endogenous plasmalogens in mediating the effects of fluvastatin, we examined the effect of fluvastatin in *Gnpat*^{+/-} mice fed the MCDD. Even though hepatic FC level was decreased by fluvastatin treatment (Fig. 7D), the preventive effect of fluvastatin on the development of NASH was partially abrogated in MCDD-fed *Gnpat*^{+/-} mice (Fig. 7A–C; Supporting Fig. S2E). Moreover, the fluvastatin-induced increases in the plasmalogen levels and expression of *Gnpat* and *Ppara* were ameliorated in *Gnpat*^{+/-} mice fed MCDD (Fig. 7E,F).

Discussion

This study is the first to show the importance of plasmalogens in common hepatic disorders. We found that plasmalogens play an important role in fatty acid metabolism in the liver. Administration of AG almost completely prevented hepatic steatosis and NASH, and this was associated with restoration of the plasmalogen level, *Ppara* expression, and hepatic mitochondrial and peroxisomal FAO. Furthermore, the preventive effect of AG on hepatic steatosis and NASH was abolished in PPAR α -KO mice, suggesting that hepatic plasmalogens protect against hepatic steatosis and NASH through PPAR α -dependent activation of FAO (Fig. 8).

We found distinct changes in the levels of plasmalogen subspecies and *Gnpat* and *Agps* expression in response to different diets. While DHA-containing PC- and PE-plasmalogen levels were decreased by MCDD and HFHCD feeding, the AA-containing PC-plasmalogen level was increased by MCDD and decreased by HFHCD. Similarly, MCDD feeding decreased expression of *Gnpat* and *Agps*, whereas HFHCD feeding decreased *Gnpat* but not *Agps* expression. The cause of this variability in plasmalogen subspecies and plasmalogen synthetic enzyme levels in response to different diets is presently unknown. However, the decrease in *Gnpat* expression and DHA-containing plasmalogen levels in both the MCDD- and HFHCD-fed mice suggests that these molecules contribute to the pathogenesis of NASH.

Mammals have three distinct desaturases: stearoyl CoA desaturase-1 (SCD-1; also called Δ^9 desaturase) and Δ^5 and Δ^6 desaturases. SCD-1 catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids, whereas Δ^5 and Δ^6 desaturases are required for the synthesis of *n*-3 or *n*-6 PUFA (Supporting Fig. S5).⁽²⁷⁾ In the present study, siRNA-mediated inhibition of Δ^6 desaturase decreased *Gnpat* expression in cultured hepatocytes. However, siRNA-mediated inhibition of Δ^5 desaturase did not decrease *Gnpat* expression. Unlike Δ^5 desaturase, Δ^6 desaturase is required for DHA synthesis from EPA (20:5*n*-3) (Supporting Fig. S5).⁽²⁷⁾ These findings suggested that DHA regulates *Gnpat* expression. Indeed, DHA increased *Gnpat* expression and transcriptionally activated GNPAT in cultured hepatocytes.

Of importance in the present study was that the increased hepatic FC level in animals with NASH was associated with decreased *Gnpat* expression and DHA-containing plasmalogen levels. Regarding the molecular mechanism linking higher hepatic FC level and lower *Gnpat* expression in animals with NASH, we found that depletion of cellular cholesterol by cyclodextrin⁽²⁸⁾ significantly increased expression of *Srebp2* and *Fads2* in cultured hepatocytes. Cyclodextrin also increased promoter activity of FADS2 (Δ -6 desaturases), and siRNA against *Srebp2* inhibited the cyclodextrin-mediated increase in the promoter activity of Δ -6 desaturases. Along with the increase of GNPAT promoter activity by DHA, higher hepatic FC may decrease *Srebp2* expression and thereby *Fads2* expression, which leads to decreased production of DHA and reduced GNPAT transcription.

Long-chain fatty acids regulate energy metabolism as ligands of PPARs.⁽⁴²⁾ DHA increases hepatic FAO by regulating PPAR α ⁽⁴³⁾ and prevents development of NASH in low-density lipoprotein receptor (*Ldlr*)^{-/-} mice.⁽⁴⁴⁾ We also found that DHA activated PPAR α and increased expression of *Ppara* and its target genes. However, the meaning of this finding in the context of the present study needs further consideration (see below).

Treatment with si*Gnpat* significantly decreased expression of *Ppara* and its target genes. GNPAT does not synthesize DHA or other PUFAs, but previous studies show that DHA can be selectively targeted to plasmalogens during their *de novo* synthesis.⁽²⁹⁾ Thus DHA in the DHA-containing plasmalogens may activate PPAR α . We examined the effect of the plasmalogen precursor AG and DHA on PPAR α activity and found that DHA, but not AG, significantly increased PPAR α activity and its transcription, suggesting that DHA in the DHA-containing plasmalogens may be presented by a lipase reaction to activate PPAR α . Indeed, a previous study showed that arachidonic acid is released from ethanolamine plasmalogens in macrophages by calcium-independent phospholipase A(2).⁽⁴⁵⁾ However, the role of specific lipases in the release of DHA from DHA-containing plasmalogens in the liver is presently unknown.

Decreased FAO increases intracellular oxidative stress,⁽⁴⁶⁾ and decreased *Ppara* expression may be an important contributor to development of NASH.^(12,13) Thus, decreased plasmalogen levels in the liver of animals with NASH may decrease PPAR α -dependent FAO. In particular, increased hepatic FC levels in MCDD- or HFHCD-fed animals may suppress plasmalogen synthesis to limit FAO and increase susceptibility to hepatocyte injury. However, plasmalogens can act as endogenous antioxidants,^(1,2) and additional mechanisms independent of lipid metabolism may also protect hepatocytes from injury.

In this study, administration of fluvastatin ameliorated MCDD- or HFHCD-induced NASH. This is in line with previous studies showing that statins decrease the progression of steatohepatitis in HFHCD-fed mice,⁽⁴⁰⁾ although this has not been proven in human subjects.⁽⁴⁷⁾ The reduction in hepatic FC level by fluvastatin was associated with increased plasmalogen levels and increased expression of *Ppara* and its target genes in the liver. The fluvastatin-induced increase in the expression of *Ppara* and its target genes was partially abrogated in the *Gnpat*^{+/-} mice, suggesting that a fluvastatin-mediated increase in hepatic plasmalogen ameliorates the development of NASH by activating PPAR α signaling. However, statins prevent synthesis of isoprenoid intermediates as well as cholesterol.⁽⁴⁸⁾ In

particular, statins cross-talk with PPAR α via the isoprenoid pathway.⁽⁴⁹⁾ Therefore, although fluvastatin treatment clearly decreased hepatic FC levels in the present study, fluvastatin may exert its protective role though changes in other metabolites, including isoprenoids. The findings that fluvastatin ameliorated NASH in HFHCD-fed mice also suggest that the pleiotropic effect may be more important than the cholesterol lowering effect of statins in prevention of NASH. Finally, a recent study showed that *Ppara* expression in human liver decreased with progression of NASH.⁽⁵⁰⁾ Future studies are warranted to examine whether *Gnpat* expression is similarly decreased in human subjects with NASH.

In summary, our results highlight a novel role for plasmalogens in protection against hepatic steatosis and NASH via a PPAR α -dependent mechanism. AG or other molecules with similar properties that increase hepatic plasmalogen levels may serve as scaffolds for the development of new therapeutic agents and strategies against NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	arachidonic acid
AG	alkyl glycerol
AGPS	alkylglycerone phosphate synthase
ALT	alanine aminotransferase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAO	fatty acid oxidation
FC	free cholesterol
GNPAT	glyceronephosphate <i>O</i> -acyltransferase
H&E	hematoxylin and eosin
HFD	high-fat diet

HFHCD	high-fat high-cholesterol diet
MCDD	methionine- and choline-deficient diet
MT	Masson's Trichrome
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
ND	normal chow diet
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PPARα	peroxisome proliferator-activated receptor α
SREBP	sterol regulatory element-binding protein
TG	triglyceride

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FIG. 1.

Ppara expression and the plasmalogen levels in the liver of animal models of nonalcoholic fatty liver disease (NAFLD). (A) Representative hematoxylin & eosin (H&E) (top) and Masson's Trichrome (MT) staining (bottom) of livers of mice fed the normal diet (ND), high-fat diet (HFD), and methionine- and choline-deficient diet (MCDD) for 8 weeks. Scale bars, 50 μ m. (B) Plasma alanine aminotransferase (ALT) level. (C) Relative mRNA expression levels of genes responsible for inflammation and fibrosis, specifically, *Tnfa*, *Ccl2*, *Il6*, *Tgfb*, and α -*Sma* (encoded by *Acta2*). (C) Relative mRNA expression levels of *Ppara* and genes involved in mitochondrial and peroxisomal FAO, and *Srebp1c* and *Srebp2*. (D) Relative mRNA expression levels of *Gnpat* and *Agps*. (E) Levels of hepatic phosphatidylcholine (PC)- and ethanolamine (PE)-plasmalogens in ND-, HFD-, and MCDD-fed mice. (F) Free cholesterol (FC) levels in the liver of mice fed the ND, HFD and MCDD. $n = 8$ each. Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus ND-fed mice; # $P < 0.05$ versus HFD-fed mice.

FIG. 2.

HFHCD feeding decreases hepatic plasmalogen levels and Δ^6 desaturase (*Fads2*) gene expression. (A) Relative *Ppara* mRNA expression and FAO in mitochondria and peroxisomes. (B) Plasma total cholesterol and hepatic FC levels, and relative mRNA expression of *Srebp2*. (C) Relative gene expression levels of *Gnpat* and *Agps*, and hepatic plasmalogen contents. (D) Relative mRNA expression levels of *Fads1* and *Fads2* in HFHCD-fed mice ($n = 8$ each). Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus ND-fed mice (E) Relative gene expression levels of *Fads1*, *Fads2*, *Gnpat*, and *Ppara* in siFads1- or siFads2-transfected AML12 hepatocytes ($n = 6$). (F) DHA restores *Gnpat* expression in siFads2-transfected AML12 hepatocytes, and increases promoter activity of *Gnpat* ($n = 5$).

Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus control, # $P < 0.05$ versus control siRNA (siCON), § $P < 0.05$ versus *Fads2* siRNA.

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FIG. 3.

Molecular link between higher hepatic FC level and lower *Gnpat* expression in NASH. (A) Effect of cholesterol depletion by cyclodextrin (CDX) on *Srebp2* and *Fads2* mRNA expression in AML12 cells. (B) Cyclodextrin increased FADS2 promoter activity, and knockdown of *Srebp2* by siRNA inhibited cyclodextrin-induced *Fads2* transcription activity. Data are shown as the mean $\pm$ s.e.m. $n = 5$ each. * $P < 0.05$ versus vehicle, # $P < 0.05$ versus siCON. (C) Schematic flow that explains how cholesterol depletion increases GNPAT transcription activity.

FIG. 4.

GNPAT regulates *Ppara* expression. (A) Effects of siRNA-mediated knockdown of *Ppara* (si*Ppara*) and *Gnpat* (si*Gnpat*) versus control (siCON) in AML12 cells. Relative *Ppara* and *Gnpat* mRNA expression and FAO-related gene expression. (B) Mitochondrial and peroxisomal FAO measured by ^{14}C -palmitate oxidation and total cell death in si*Gnpat*-treated cells ($n = 6$ each). Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus siCON. (C) DHA increases *Ppara* and its target genes expression. Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus vehicle. (D) DHA increases PPAR α transcription activity and promoter activity. Data are shown as the mean $\pm$ s.e.m. $n = 5$ each. * $P < 0.05$ versus vehicle, # $P < 0.05$ versus vehicle-treated PPAR α expression plasmid.

FIG. 5.

Changes in hepatic metabolism in *Gnpat*-knockout (KO) mice. (A) Relative *Gnpat* mRNA expression of whole liver tissue in wild-type (WT; *Gnpat*^{+/+} littermates) and *Gnpat*^{+/-} mice ($n = 8$). (B) Representative H&E staining of the livers of WT and *Gnpat*^{+/-} mice fed the high-fat diet (HFD, top) or methionine- and choline-deficient diet (MCDD, bottom) for 8 weeks. Scale bars, 50 μm. (C,D) Relative *Gnpat* and *Ppara* mRNA expression (left) and hepatic plasmalogen levels (middle and right) in HFD-fed (C) and MCDD-fed (D) WT and *Gnpat*^{+/-} mice ($n = 8$ each). Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus ND-fed WT mice; # $P < 0.05$ HFD-fed WT versus *Gnpat*^{+/-} mice. (E) Fenofibrate treatment prevented hepatic steatosis in HFD-fed *Gnpat*^{+/-} mice. WT and *Gnpat*^{+/-} mice were fed a HFD supplemented without or with 0.1% or 0.5% (wt/wt) fenofibrate for 8 weeks ($n = 5$). (F) Fenofibrate treatment increases hepatic mRNA expression of *Ppara* target genes. * $P < 0.05$ versus HFD-fed WT mice, # $P < 0.05$ HFD-fed *Gnpat*^{+/-} mice without Fenofibrate treatment.

FIG. 6.

Administration of AG prevents steatosis and NASH in MCDD-fed mice. (A) H&E and MT staining of the livers of MCDD-fed mice with or without AG supplementation (100 mg/kg/day) for 8 weeks. Scale bars, 50 μm . (B) Plasmalogen contents in the liver of ND- and MCDD-fed mice with or without AG treatment. (C) Mitochondrial and peroxisomal FAO, and relative expression of *Ppara* and its target genes involved in FAO ($n = 8$). Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus ND-fed mice; # $P < 0.05$ versus MCDD-fed mice. (D) Abolishment of the preventive effect of AG on hepatic steatosis and inflammation in *Ppara*-KO mice ($n = 5$). (E) Hepatic FC is not decreased by AG treatment in MCDD-fed mice. Data are shown as the mean $\pm$ s.e.m. * $P < 0.05$ versus ND-fed mice.

FIG. 7.

The preventive effect of fluvastatin on NASH is partially abrogated in *Gnpat*^{+/-} mice. (A) Representative H&E and MT staining of the livers of WT mice fed the MCDD, and WT and *Gnpat*^{+/-} mice fed MCDD with fluvastatin (15 mg/kg/day), for 8 weeks. Scale bars, 50 μm. (B) Plasma ALT levels of WT and *Gnpat*^{+/-} mice fed the ND and MCDD with or without fluvastatin. (C) Relative *Tnfa* and *Ccl2* mRNA expression levels. (D) Liver free cholesterol. (E) *Gnpat* and *Ppara* mRNA expression levels. (F) Hepatic plasmalogen levels in each experimental group (*n* = 8 each). Data are shown as the mean ± s.e.m. *P < 0.05 versus ND-fed mice, #*P* < 0.05 versus MCDD-fed mice, §*P* < 0.05 WT versus *Gnpat*^{+/-} mice.

FIG. 8.

Conceptual model for the role of hepatic FC and DHA-plasmalogens in the pathogenesis of NASH. (A) HFD increases TG synthesis to induce hepatic steatosis. However, NASH does not develop in this model if hepatic FC, plasmalogen levels, and *Ppara* expression are unaltered. (B) MCDD and HFHCD increase hepatic FC, which decreases *Srebp2* expression to decrease *Fads2* expression and DHA synthesis. DHA transcriptionally activates GNPAT and PPARα to increase plasmalogen synthesis and FAO, respectively. DHA is selectively targeted to plasmalogens during *de novo* synthesis of plasmalogens, and DHA liberated from DHA-containing plasmalogens may be responsible for increased PPARα-dependent FAO. Collectively, MCDD and HFHCD increase hepatic FC, which is responsible for decreased hepatic DHA-containing plasmalogens and development of NASH. (C) AG treatment increases DHA-plasmalogen levels to activate PPARα-dependent FAO. In addition, plasmalogens can act as endogenous antioxidants. Taken together, these mechanisms result in the prevention of NASH development.