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Fish Oil Protects the Liver from Parenteral Nutrition-induced Injury via GPR120-mediated PPAR γ signaling

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Abstract

Intravenous fish oil lipid emulsions (FOLE) can prevent parenteral nutrition (PN)-induced liver injury in murine models and reverse PN-induced cholestasis in pediatric patients. However, the mechanisms by which fish oil protects the liver are incompletely characterized. Fish oil is rich in omega-3 fatty acids, which are ligands for the G-protein coupled receptor 120 (GPR120), expressed on hepatic Kupffer cells. This study tested the hypothesis that FOLE protects the liver from PN-induced injury through GPR120 signaling. Utilizing a previously described murine model of PN-induced liver injury in which mice develop steatosis in response to an oral parenteral nutrition diet, FOLE was able to preserve normal hepatic architecture in wild type mice, but not in congenic GPR120 knockout (*gpr120* $-/-$) mice. To further characterize the requirement of intact GPR120 for FOLE-mediated hepatic protection, gene expression profiles of key regulators of fat metabolism were measured. PPAR γ was identified as a gene that is up-regulated by the PN diet and normalized with the addition of FOLE in wild type, but not in *gpr120* $-/-$ mice. This was confirmed at the protein expression level. A PPAR γ expression array further identified CD36 and SCD1, both down-stream effectors of PPAR γ , to be up-regulated in PN-fed wild type mice yet normalized upon FOLE administration in wild type but not in *gpr120* $-/-$ mice. Together, these results suggest that FOLE protects the liver, in part, through activation of GPR120 and the downstream effectors PPAR γ and CD36. Identification of key genetic determinants of FOLE-

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mediated hepatic protection may provide targets for small molecule-based hepatic protection strategies.

Summary

Omega-3 fatty acid-rich intravenous fish oil lipid emulsions can be used to treat parenteral nutrition-associated liver injury and have demonstrated beneficial effects in other hepatic pathologies including non-alcoholic fatty liver disease (NAFLD). The hypothesis tested in this study is that the omega-3 fatty acid receptor GPR120 is required for fish oil to protect the liver from parenteral nutrition-associated steatotic injury. In a murine model, GPR120 was required for fish oil to protect the liver from parenteral nutrition-associated liver injury. Expression of downstream effectors of GPR120, including PPAR γ , CD36, and SCD1 was dysregulated by a parenteral nutrition diet, and normalized by the addition of fish oil in a GPR120-dependent fashion. These data suggest one potential mechanism through which fish oil protects the liver from parenteral nutrition-induced injury and diet-induced steatotic injury. These data further identify potential targets for small molecule-based therapies for intestinal failure-associated liver disease and NAFLD.

Keywords

Intestinal failure-associated liver disease; Non-alcoholic fatty liver disease; Fish oil; Omega-3 fatty acids; Lipid emulsions; GPR120; peroxisome proliferator-activated receptor-gamma

1. Introduction

Intestinal failure-associated liver disease (IFALD) is characterized by hepatic inflammation that can progress to cholestasis and end-stage liver disease in parenteral nutrition (PN)-dependent patients. The standard of care in patients who require PN includes the administration of a soybean oil intravenous lipid emulsion (SOLE) as a source of fat and essential fatty acids. In patients who develop IFALD, transition from SOLE to fish oil intravenous lipid emulsions (FOLE) can reverse acute hepatic injury and stop the progression of liver disease [1–5]. FOLE has also been shown to prevent PN-induced liver injury in animal models [6,7]. However, the mechanisms by which FOLE protects the liver remain incompletely understood.

FOLE contains an abundance of long-chain polyunsaturated omega-3 fatty acids. These essential fatty acids and their metabolites have important roles in modulating metabolism, the coagulation cascade, and the inflammatory response [8–12]. The G-protein coupled receptor 120 (GPR120) binds to long-chain fatty acids, with a particular affinity for omega-3 fatty acids (hence its alternative name, free fatty acid receptor or FFAR-4). GPR120 is expressed on the surface of adipocytes and macrophages, including hepatic Kupffer cells [13]. It has been shown that the anti-inflammatory effects of omega-3 fatty acids are mediated in part through GPR120 signaling [14]. A GPR120-dependent insulin-sensitizing effect of omega-3 fatty acids has also been demonstrated [14–17].

More recently, several groups have characterized a role for GPR120 signaling via omega-3 fatty acids in amelioration of alcoholic hepatic injury and non-alcoholic fatty liver disease (NAFLD) [18,19]. Nakamoto et al. demonstrated protective effects of the omega-3 fatty acid docosahexaenoic acid (DHA) in a choline-deficient high-fat diet murine model of NAFLD. This DHA-dependent hepatic protection from liver injury was GPR120-dependent [19]. Raptis et al. reported that fish oil-mediated GPR120 signaling can attenuate hepatic ischemia-reperfusion injury [13]. While a relationship between omega-3 fatty acid-mediated GPR120 signaling and protection of the liver from inflammatory insults has been established, the downstream cellular pathways of FOLE-mediated hepatic protection that could identify targets of pharmacologic intervention have yet to be characterized.

Given the previously identified relationships between omega-3 fatty acids, GPR120 signaling, and improvements in various hepatic pathologies, the purpose of this study was to test the hypothesis that GPR120 signaling is required for FOLE to protect the liver from PN-associated liver injury. A further aim of this study was to identify additional downstream effectors of the GPR120 signaling pathway that mediate FOLE-dependent hepatic protection, which could be relevant for clinical entities including IFALD and NAFLD.

2. Materials and Methods

2.1. Mice

GPR120 knockout mice (*gpr120*^{-/-}) were obtained from the UC Davis Knockout Mouse Project Repository. These were bred with C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) to generate heterozygotes for GPR120 (*gpr120*^{+/-}). Heterozygotes were bred and *gpr120*^{-/-} and wild type littermates were utilized for experiments. Genotyping was performed by Transnetyx (Cordova, TN) to confirm genotypes.

2.2. Murine model of PN-induced liver injury

All animal experiments were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. A murine model of PN-induced liver injury, previously used by our group, was utilized for our experiments [6,7]. Six week-old male GPR120 knockout (*gpr120*^{-/-}) mice and congenic wild type littermates were administered either a standard chow diet or a liquid fat-free diet equivalent to the PN administered to patients at Boston Children's Hospital (20% dextrose, 2% amino acids, 30mEq/L sodium, 20mEq/L potassium, 15mEq/L calcium, 10mEq/L magnesium, 10mMol/L phosphate, 36.67mEq/L chloride, 19.4mEq/L acetate, pediatric multivitamins, and pediatric trace elements). Mice fed the PN diet were administered either intravenous (IV) saline, the commercial FOLE Omegaven (Fresenius Kabi, Bad Homburg, Germany), or the commercial SOLE Intralipid (Fresenius Kabi, Uppsala, Sweden). Groups for each genotype were: Chow, PN+saline, PN+FOLE, PN+SOLE. Fat was administered at a dose of 2.4g/kg/day (administered as 4.8g/kg every other day) by tail vein injection. Animals remained on their respective diets for 19 days, after which they were euthanized by carbon dioxide asphyxiation. Blood was collected by inferior vena cava puncture and centrifuged for 15 minutes at 5000 rpm at 4°C to obtain serum. Livers were procured for further analysis: one third of each liver was immediately flash frozen in liquid nitrogen for gene and protein expression analyses, one third was frozen

in Optimum Cutting Temperature medium (Fisher Scientific, Pittsburgh, PA) for histologic analysis (lipid staining), and the remaining third was fixed in 10% formalin for 18–24 hours then transferred to 70% ethanol for histologic analysis.

2.3. Histology

Formalin-fixed liver segments were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) to assess hepatic architecture. Paraffin-embedded samples also underwent immunohistochemical staining with an antibody to the macrophage cell surface marker F4/80 (AbCam, Cambridge, MA) to stain hepatic Kupffer cells. Antigen retrieval was via a heat-induced antigen retrieval method per manufacturer's instructions. Fresh frozen liver segments underwent frozen sectioning and were stained for oil red O to assess hepatic fat accumulation. Visualization of slides was performed with a Zeiss Axiophot microscope (Oberkochen, Germany). A board-certified pathologist masked to the treatment groups interpreted the slides.

2.4. Gene Expression Analysis

Previously frozen liver segments were cut into 25mg pieces, and RNA was extracted using the Qiagen AllPrep DNA/RNA/Protein kit (Gaithersburg, MD) according to manufacturer's instructions. Taqman primers (Invitrogen, Carlsbad, CA) were used for each reaction according to manufacturer's instructions using 200 ng of RNA. A 2-step cycling reverse transcription polymerase chain reaction (RT-PCR) protocol was utilized in an ABI One-Step Plus cycler (Applied Biosystems, Foster City, CA). The reverse transcription step was performed at 50°C for 30 minutes followed by 95°C for 10 minutes. The amplification step consisted of 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Target gene expression was normalized to the expression of GAPDH and compared to samples from the chow-fed wild type control mice using the 2^{-C_t} method [20].

2.5. PPAR-gamma expression array

RNA was prepared as described above for liver samples from PN-fed wild type mice administered FOLE and PN-fed *gpr120*^{-/-} mice administered FOLE. These RNA samples were used in a microarray analysis of 84 key PPAR γ target genes (Qiagen, Gaithersburg Maryland). Positive hits were defined as genes whose expression was at least 2-fold different between the two groups. These positive hits then underwent confirmation via RT-PCR analysis as described above.

2.6. Protein Expression Analysis

Flash frozen livers were cut into 25 mg sections and homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors using a Bullet Blender and sterile stainless-steel beads. Protein concentrations were quantified using a Bradford colorimetric Assay (Bio-Rad, Hercules, CA). Ten milligrams of protein per sample were separated using a 4–12% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA), and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk for 1 hour, incubated overnight in primary antibody, then in secondary antibody for 1 hour. PPAR γ antibody was from Cell Signaling Technologies (Danvers, MA),

beta-actin antibody was from Santa Cruz Biotechnologies (Paso Robles, CA). Blots were visualized in a Bio-Rad ChemiDoc Imaging System. Quantification of protein expression was performed using the Image Lab software (Bio-Rad, Portland, ME).

2.7. Statistical Analysis

Outcomes were analyzed as fold difference with reference to chow-fed mice. Group comparisons were made by two-way analysis of variance, including one term for genotype (wild type vs. *gpr120*^{-/-}), one term for diet (chow, PN+S, PN+FO, PN+SO), and one term for their interaction. For each genotype, we compared each PN group to chow for a total of six tests and adjusted for multiple comparisons using Tukey's test procedure. All tests of significance were two-sided, with $P < 0.05$ deemed statistically significant. Statistical analysis was performed with GraphPad Prism (v.7, GraphPad Software, La Jolla, CA).

3. Results

3.1. Genetic Loss of GPR120 prevents FOLE-mediated protection from PN-induced hepatic steatosis

Both wild type and *gpr120*^{-/-} mice had normal hepatic architecture on a standard chow diet (Figure 1, first column), and both genotypes developed hepatosteatosis with a fat-free PN diet (Figure 1, second column). Neither genotype receiving the PN diet developed normal hepatic architecture with the administration of SOLE (Figure 1, fourth column). While PN-fed wild type animals had normal hepatic architecture with the administration of FOLE, hepatosteatosis still developed in PN-fed *gpr120*^{-/-} mice administered FOLE.

To assess whether GPR120 expression affects hepatic Kupffer cell presence or differentiation, liver sections were stained for the macrophage surface marker F4/80, and F4/80-expressing Kupffer cells were present in both wild type and *gpr120*^{-/-} livers (Figure S1).

3.2. PPAR-gamma expression is dysregulated by the PN diet and normalized by FOLE in a GPR120-dependent fashion

To test the hypothesis that GPR120 signaling affects FOLE-mediated protection from PN-induced steatosis through modulation of hepatic fat metabolism, the expression of regulators of hepatic fat metabolism was tested. Eight genes were chosen based on their known roles in modulating hepatic fat metabolism and the development of steatosis. Furthermore, expression of these genes increased with the PN diet for both wild type and *gpr120*^{-/-} mice. PPAR γ and sterol regulatory element-binding protein 1c (SREBP1c) were normalized by FOLE administration in wild type, but not *gpr120*^{-/-} animals. Notably, expression of these genes remained elevated in PN-fed animals of both genotypes receiving SOLE (Figure 2). Of the genes identified, only PPAR γ demonstrated a significant interaction between genotype and diet on two-factor ANOVA analysis. Thus, PPAR γ was the focus of further analysis. On protein expression analysis, PPAR γ levels increased in both genotypes with PN+Saline and PN+SOLE. While FOLE normalized PPAR γ protein levels in PN-fed wild type mice, FOLE was unable to do so in PN-fed *gpr120*^{-/-} mice (Figure 3).

3.3. PPAR γ downstream effectors CD36 and SCD1 are dysregulated by PN and normalized by FOLE in a GPR120-dependent fashion, in parallel with PPAR γ

There are numerous genes on which PPAR γ acts to modulate expression [21–24]. In order to assess which genetic targets of PPAR γ are pertinent in FOLE/GPR120-mediated hepatoprotection from PN-induced steatosis, we performed a PPAR γ expression array analysis, and identified several genes whose expression was significantly different between PN-fed FOLE-treated wild type and PN-fed FOLE-treated *gpr120*^{−/−} mice (Figure 4A). Each positive hit on the gene expression array underwent confirmation testing utilizing all diet groups for both wild type and *gpr120*^{−/−} mice (Chow, PN+saline, PN+FOLE, PN+SOLE). Stearoyl-CoA Desaturase-1 (SCD1) and the scavenger receptor fatty acid translocase CD36 were identified as downstream targets of PPAR γ whose expression increased with the fat-free PN diet and normalized with FOLE in wild type, but not in *gpr120*^{−/−} mice (Figure 4B). The other 4 hits did not demonstrate this pattern of expression (Figure S2).

4. Discussion and Conclusions

This study demonstrates that the ability of FOLE to protect the liver from PN-induced injury requires intact GPR120 expression. FOLE-mediated GPR120 signaling in this model acts, at least in part, via PPAR γ . PPAR γ is a transcription factor that regulates expression and activity of several cell types and organ systems. It affects not only metabolism and insulin sensitivity, but also systemic electrolyte and water balance, bone homeostasis, angiogenesis, and inflammation [21–24]. The PPAR γ target genes that appear to be predominantly altered by the PN diet and normalized by FOLE are CD36 and SCD1. Prior studies have established roles for omega-3 fatty acid-mediated GPR120 signaling and, independently, PPAR γ in modulating hepatic lipid metabolism [25–27]. Recently, a relationship has been established between GPR120 signaling and PPAR γ activity in modulating lipid metabolism and adipocyte function [28]. CD36 is a cell surface receptor that facilitates cellular uptake of fatty acids. It is a known target of PPAR γ , and its increased activity has been identified in several models of fatty liver disease [29–32]. SCD1 catalyzes the synthesis of monounsaturated fatty acids in the liver. It is also a known target of PPAR γ , and its increased activity has been linked to the development of non-alcoholic hepatic steatosis [33,34]. The findings of this study suggest a model in which PN promotes liver injury through PPAR γ -mediated up-regulation of CD36 and SCD1; and FOLE-mediated hepatic GPR120 signaling inhibits this activity of PPAR γ to protect the liver from PN-induced injury.

Interestingly, GPR120 is expressed primarily on Kupffer cells in the liver with minimal expression in hepatocytes [35], while hepatic lipid metabolism takes place in hepatocytes. While this study does not address this specifically, one plausible hypothesis is that FOLE-mediated GPR120 signaling in hepatic Kupffer cells results in release of factors that act in a paracrine fashion on hepatocytes to attenuate PPAR γ -mediated CD36 and SCD1 activity. Future research efforts in our laboratory are aimed at answering these questions.

A limitation of this study is that the murine model of PN-induced liver injury develops steatosis, while the clinical correlate in PN-dependent patients is primarily cholestatic liver

disease. This murine model has reliably recapitulated the effects of SOLE in exacerbating, and of FOLE in preventing PN-induced liver injury [6,36–38]. Further, this model has been able to predict the effects of various intravenous lipid emulsions in patients with IFALD [7]. Studies utilizing this model have contributed to the rationale for the recent United States Food and Drug Administration's approval of FOLE for use as a fat source in PN for the treatment of IFALD. It is possible that in PN-dependent patients, hepatic inflammation and steatosis occur prior to the development of symptoms, and cholestasis and cirrhosis manifest once patients with IFALD become symptomatic. This is akin to the findings in NAFLD in which steatosis and acute inflammation precede progression to cirrhosis. Given the steatotic phenotype seen in this model, these results are highly likely to be generalizable to NAFLD and should be tested in well-established NAFLD models.

FOLE was recently approved for use in PN-dependent patients in the United States. However, potential adverse effects of FOLE, including bleeding risk, and effects on the immune system [8,39], render its use in certain patient populations controversial. Identification of key downstream modulators of PN-induced liver injury may provide a starting point for the development of alternative, more targeted therapies for PN-induced liver injury that do not rely on changes to the nutritional content of patients' PN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

A license agreement for the use of Omegaven® has been signed by Boston Children's Hospital and Fresenius Kabi. Mark Puder and Kathleen Gura hold an issued patent on the treatment of parenteral nutrition-associated liver disease. They both serve on the Scientific Advisory Board for Pronova-BASF. Kathleen Gura also serves on the Pharmaceutical Advisory Board for B. Braun USA.

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Abbreviations

¹IFALD	Intestinal Failure-Associated Liver Disease
NAFLD	Non-Alcoholic Fatty Liver Disease
PN	Parenteral Nutrition
FOLE	Intravenous Fish Oil Lipid Emulsion
SOLE	Intravenous Soybean Oil Lipid Emulsion
GPR120	G-protein coupled receptor 120
PPARγ	Peroxisome Proliferator-Activated Receptor-gamma
CIDEA	Cell Death Inducing DFFA-like Effector-C

SREBP1c	Sterol Regulatory Element-Binding Protein 1c
CD36	Fatty Acid Translocase
SCD1	Stearoyl-CoA Desaturase-1
H&E	Hematoxylin and Eosin
IV	Intravenous
RIPA	Radioimmunoprecipitation Assay
RT-PCR	Reverse Transcription Polymerase Chain Reaction

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Highlights

- GPR120 is required for fish oil-mediated hepatic protection from parenteral nutrition-induced liver injury, .
- PPAR γ and its downstream effectors CD36 and SCD1 are dysregulated by a parenteral nutrition diet and normalized by intravenous fish oil in a GPR120-dependent fashion.
- GPR120-mediated PPAR γ signaling to CD36 and SCD1 activity may represent one pathway that contributes to the modulation of intestinal failure-associated liver disease.
- GPR120, PPAR γ , CD36, and SCD1 may be potential targets for small molecule-based hepatic protection strategies in intestinal failure-associated liver disease and other similar hepatic pathologies.

Figure 1:

Genetic loss of GPR120 prevents FOLE from protecting against PN-induced liver injury: A. Hematoxylin and Eosin and B. Oil red O staining of liver samples from wild type (top row) and *gpr120*^{-/-} (bottom row) mice fed chow (left column), PN+Saline (second column from the left), PN+FOLE (third column from the left), and PN+SOLE (right column). The PN diet results in steatosis in both wild type and *gpr120*^{-/-} mice (second column from the left). FOLE is able to prevent steatosis in wild type (top row third from the left) but not in *gpr120*^{-/-} mice (bottom row, third column from the left). SOLE is unable to prevent steatosis in PN-fed mice of either genotype (right column).

Figure 2:

Loss of GPR120 prevents FOLE-mediated normalization of PN-induced increases in PPAR γ gene expression. Expression of genetic regulators of hepatic fat metabolism was measured by RT-PCR. Two-factor ANOVA with Tukey's adjustment for multiple comparisons showed that PPAR γ (top left graph) had increased expression with the PN diet that was normalized with FOLE administration in wild type but not *gpr120*^{-/-} animals. PPAR α and SREBP1c demonstrated a similar pattern with no significant interaction between genotype and diet. All comparisons for a given genotype are in relation to the chow-fed animals within that genotype. * indicates p < 0.05 compared to chow-fed controls of the same genotype. n = 5 biological replicates per treatment group, each performed in technical duplicate.

Figure 3:

Loss of GPR120 prevents FOLE-mediated normalization of PN-induced increases in PPAR γ protein expression. A. Western blot images and B. quantification of PPAR γ protein expression normalized to the housekeeping gene beta-actin. PPAR γ protein expression is increased with the PN diet. FOLE suppresses protein expression of PPAR γ in PN-fed wild type, but not PN-fed *gpr120*^{-/-} mice. Two-factor ANOVA demonstrated significant differences with respect to genotype and diet, and p values are expressed based on Tukey's adjustment for multiple comparisons. n = 3 biological replicates for each group.

Figure 4:

PPAR γ expression array revealed CD36 and SCD1 as downstream effectors of PPAR γ that are increased with the PN diet and normalized with FOLE in a GPR120-dependent fashion.

A. PPAR γ expression array hits, which were those genes whose expression increased at least 3-fold different between wild type and *gpr120*^{-/-} PN-fed, FOLE-treated mice. B. On confirmation RT-PCR with greater sample sizes and utilizing all treatment groups for each of the hits, CD36 and SCD1 demonstrated increased expression with the PN diet, normalization with FOLE treatment in wild type but not *gpr120*^{-/-} mice. Two-factor ANOVA demonstrated significant differences with respect to genotype and diet. n = 5 biological replicates per group, each assayed in technical duplicate.