Medium-chain fatty acids suppress lipotoxicity-induced hepatic fibrosis via the immunomodulating receptor GPR84

Ryuji Ohue-Kitano, …, Junken Aoki, Ikuo Kimura


Graphical abstract

Find the latest version:
https://jci.me/165469/pdf
Medium-chain fatty acids suppress lipotoxicity-induced hepatic fibrosis via the immunomodulating receptor GPR84

Ryuji Ohue-Kitano1,2†, Hazuki Nonaka3†, Akari Nishida2†, Yuki Masujima1, Daisuke Takahashi4, Takako Ikeda1,2, Akiharu Uwamizu4, Miyako Tanaka6, Motoyuki Kohjima7, Miki Igarashi3, Hironori Katoh1,2, Tomohiro Tanaka8, Asuka Inoue9, Takayoshi Suganami6, Koji Hase4,10, Yoshihiro Ogawa7, Junken Aoki5, Ikuo Kimura1,2,3*.

Affiliations

1 Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.

2 Laboratory of Molecular Neurobiology, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.

3 Department of Applied Biological Science, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan.

4 Division of Biochemistry, Faculty of Pharmacy and Graduate School of Pharmaceutical Science, Keio University, Tokyo 105-8512, Japan.

5 Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan.

6 Department of Molecular Medicine and Metabolism, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan.
7 Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 819-0395, Japan.

8 Department of Gastroenterology and Metabolism, Nagoya City University, Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, 467-8601, Japan

9 Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3, Aoba, Aramaki, Aoba-ku, Sendai, Miyagi, 980-8578, Japan.

10 International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo (IMSUT), Bunkyo-ku, Tokyo 108-8639, Japan.

†These authors contributed equally to this work.

*Corresponding author.

Address correspondence to: Ikuo Kimura, Laboratory of Molecular Neurobiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Phone: +81 75 753 9256; Email: kimura.ikuo.7x@kyoto-u.ac.jp.

Conflict of interest

This study received funding from Nisshin OilliO Group, Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare no other competing interests.
Abstract

Medium-chain triglycerides (MCTs), which consist of medium-chain fatty acids (MCFAs), are unique forms of dietary fat with various health benefits. GPR84 acts as a receptor for MCFAs (especially C10:0 and C12:0); however, GPR84 is still considered an orphan receptor, and the nutritional signaling of endogenous and dietary MCFAs via GPR84 remains unclear. Here, we showed that endogenous MCFA-mediated GPR84-signaling protected hepatic functions from diet-induced lipotoxicity. Under high-fat diet (HFD) conditions, GPR84-deficient mice exhibited non-alcoholic steatohepatitis (NASH) and the progression of hepatic fibrosis but not steatosis. With markedly increased hepatic MCFA levels under HFD, GPR84 suppressed lipotoxicity-induced macrophage over-activation. Thus, GPR84 is an immunomodulating receptor that suppresses excessive dietary fat intake-induced toxicity by sensing increases in MCFAs. Additionally, administering MCTs, MCFAs (C10:0 or C12:0, but not C8:0), or GPR84 agonists effectively improved NASH in mouse models. Exogenous GPR84 stimulation is therefore a potential strategy for treating NASH.

Keywords
lipotoxicity, medium-chain fatty acids, medium-chain triglycerides, GPR84, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis
Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide (1–6). NAFLD includes a spectrum of well-defined stages, encompassing simple fatty liver (NAFL), which is a mostly benign condition, and non-alcoholic steatohepatitis (NASH). NASH progresses to cirrhosis and hepatocellular carcinoma (HCC) by activating inflammatory cascades and fibrogenesis (2, 3). The major risk factors of NASH include metabolic disorders such as obesity, insulin resistance, glucose intolerance or type 2 diabetes, and dyslipidemia (4, 5). Although the prevalence of NASH is rising in parallel with the global obesity pandemic, effective therapeutic strategies against the former are still under development (1, 6). Patients have to undergo liver transplantation to prevent the progression of NASH. The crucial event involved in NAFLD progression is hepatic lipotoxicity resulting from an excessive free fatty acid (FFA) influx from the peripheral tissues, mainly the adipose tissue, to hepatocytes, or from increased hepatic de novo lipogenesis (1–5). Hepatic lipotoxicity occurs when the capacity of hepatocytes to manage and export FFAs as triglycerides (TGs) is overwhelmed.

FFAs act as energy sources and affect physiological functions such as hormone secretion, immune responses, and neurotransmission via the FFA-specific receptors FFAR1, FFAR4 (for long-chain fatty acids), FFAR2, and FFAR3 (for short-chain fatty acids) (7–12). Medium-chain fatty acids (MCFAs) also have a specific receptor—GPR84 (7, 13, 14). However, GPR84 is still considered an orphan G protein-coupled receptor (GPCR) because of the low plasma levels of endogenous MCFAs (15). Medium-chain triglycerides (MCTs), which consist of MCFAs, are unique forms of dietary fat exhibiting various health benefits (7, 16, 17). MCTs are an appropriate dietary choice for individuals with high energy demands. In the elderly, MCTs counteract age-related decreased energy production, and in athletes, MCTs enhance performance. MCTs are also beneficial for individuals who have
undergone major surgeries or experience stunted growth (18–20). GPR84 is coupled with the pertussis 
toxin-sensitive Gi/o protein and is predominantly expressed in the bone marrow, lungs, and peripheral 
leucocytes (13, 14, 21). Although some studies on GPR84-deficient mice have demonstrated that 
GPR84 plays an important role in immune and metabolic responses and may mediate the crosstalk 
between immune cells and adipocytes (22–25), comprehensive and integrated data bridging the gap 
between endogenous MCFAs and GPR84 are lacking, and the molecular mechanisms underlying 
these processes remain unclear.

Here, we investigated the effects of molecular nutritional signaling by MCFAs on metabolic 
functions using GPR84-deficient mice, a model of high-fat diet (HFD)-induced obesity, and a NASH 
mouse model.
Results

GPR84 deficiency accelerates chronic inflammation under HFD conditions

To study the role of GPR84 in the metabolic and immune systems, we generated \textit{Gpr84}\textsuperscript{-/-} mice (Supplemental Figure 1, A–C). HFD feeding in wild-type mice increases the levels of inflammatory cytokines, such as TNF-\(\alpha\), and long-term HFD exposure leads to chronic inflammation (26). Therefore, we first compared the levels of plasma TNF-\(\alpha\) as an inflammatory marker under short-term HFD feeding between wild-type and \textit{Gpr84}\textsuperscript{-/-} mice. Although plasma TNF-\(\alpha\) levels were comparable between wild-type and \textit{Gpr84}\textsuperscript{-/-} mice under normal chow (NC) feeding, HFD feeding markedly increased plasma TNF-\(\alpha\) levels in \textit{Gpr84}\textsuperscript{-/-} mice than in wild-type mice (Figure 1A; 46.24\% increase). Moreover, the hepatic expression of the \textit{Tnf} mRNA in \textit{Gpr84}\textsuperscript{-/-} mice was markedly higher than that in wild-type mice, whereas its expression in other tissues, such as the WAT (in both mature adipocytes and stromal vascular fraction), muscle, small intestine, and colon, was comparable between wild-type and \textit{Gpr84}\textsuperscript{-/-} mice (Figure 1B and Supplemental Figure 2). RNA sequencing and a Gene Ontology (GO) enrichment analysis of liver from the HFD-fed \textit{Gpr84}\textsuperscript{-/-} mice revealed a relationship between the chemokine pathway and chronic inflammation (Supplemental Figure 3, A–C). Among the differentially expressed genes (DEGs), the expression of 59 inflammation-related genes was altered compared with that of the wild-type mice (Figure 1C). In particular, the hepatic mRNA expression of the fibrosis markers \textit{Col1a}, \textit{Tgfb1}, and \textit{Acta2} was considerably higher in \textit{Gpr84}\textsuperscript{-/-} mice than in wild-type mice (Figure 1D; \textit{Col1a}: 4.03-fold increase, \textit{Tgfb1}: 2.14-fold increase, and \textit{Acta2}: 1.30-fold increase). The hepatic TG levels and mRNA expression of these fibrosis marker genes in the WAT were comparable between the groups (Supplemental Figure 4, A and B). Thus, GPR84-deficient mice exhibited chronic hepatic inflammation and fibrosis without the acceleration of hepatic fat accumulation, even under short-term HFD feeding.
Long-term HFD-fed GPR84-deficient mice exhibit NASH

To determine how GPR84 deficiency affects the liver, we induced chronic inflammation and hepatic steatosis through long-term (12 weeks) feeding of an HFD to wild-type and Gpr84−/− mice. Liver weight was markedly lower in Gpr84−/− mice than in wild-type mice (Figure 2A); the hepatic TG levels were comparable between them (Figure 2B). The hepatic levels of the inflammatory marker Tnf and the fibrosis markers Colla, Tgfb1, and Acta2 were markedly elevated in Gpr84−/− mice compared with those in wild-type mice (Figure 2C; Tnf: 2.66-fold increase, Colla: 8.46-fold increase, Tgfb1: 3.76-fold increase, and Acta2: 3.51-fold increase), whereas their levels in WAT were comparable (Supplemental Figure 4, C and D). Furthermore, HFD-fed Gpr84−/− mice showed increased numbers of F4/80-positive macrophages, levels of the fibrosis marker α-smooth muscle actin (α-SMA) (Figure 2D), and the macrophage marker gene Adgre1, Cd68, and Cd14 in the liver compared with HFD-fed wild-type mice (Figure 2E; Adgre1: 7.65-fold increase, Cd68: 6.87-fold increase, and Cd14: 6.75-fold increase). Consequently, the NAFLD activity score (NAS) for the livers of HFD-fed Gpr84−/− mice was higher than that for the livers of HFD-fed wild-type mice (Figure 2F). Thus, GPR84 deficiency accelerates the progression from HFD-induced hepatic steatosis to NASH.

HFD feeding increases endogenous MCFA levels as GPR84 ligands in liver

GPR84 has been identified as a receptor for MCFAs and is coupled with the Gi/o protein, which decreases the intracellular cAMP concentration (14). In HEK293 cells expressing mouse GPR84 (Supplemental Figure 5A), C9:0, C10:0, C11:0, C12:0, and C13:0 activated GPR84 in a dose-dependent manner, whereas such activation was not displayed by C6:0, C7:0, C8:0, and C14:0 or not observed in doxycycline-uninduced controls [Dox
non-GPR84-expressing HEK293 cells (Figure 3A and Supplemental Figure 5B). C10:0 was found to be the most potent agonist of GPR84, with an EC₅₀ of 3.5 μM; C12:0 was the second most potent agonist, with an EC₅₀ of 4.4 μM (Figure 3A).

Next, we investigated the levels of endogenous MCFAs as GPR84 ligands after HFD feeding. Upon examining the profiles of FFAs (C6:0–C14:0) including MCFAs, their levels were found to be elevated in the plasma and liver of HFD-fed mice compared with those in NC-fed mice (Figure 3B and Supplemental Figure 6A). The hepatic levels of C10:0 and C12:0 were markedly elevated in HFD-fed mice compared with those in NC-fed mice (Figure 3C). This increase in MCFA levels sufficiently activated GPR84 (Figure 3A). MCFAs were hardly detected in cecal contents under HFD conditions (Supplemental Figure 6, B and C). Comparison of the RNA sequencing data of the liver from NC- and HFD-fed mice showed that the 6 fatty acid-synthesis and β-oxidation genes were coded as MCFA synthesis-related enzymes in 34 fatty acid synthesis and metabolism-related genes of DEGs (Supplemental Figure 6D). That is, Acsl1 (acyl-CoA synthetase long-chain family member 1) and Acsm3 (acyl-CoA synthetase medium-chain family member 3) code medium-chain acyl-CoA synthetase. Acadl (acyl-coenzyme A dehydrogenase, long chain) and Acadm (acyl-coenzyme A dehydrogenase, medium chain) code medium-chain acyl-CoA dehydrogenase. Acot11 (acyl-CoA thioesterase 11) and Acot13 (acyl-CoA thioesterase 13) code medium-chain acyl-CoA thioesterase. The hepatic mRNA expression levels of these MCFA synthesis-related enzymes were considerably higher in HFD-fed mice than in NC-fed mice (Figure 3D). Thus, HFD feeding increases the levels of endogenous MCFAs, which are GPR84 ligands, and accelerates fatty acid synthesis and β-oxidation in the liver.

GPR84 suppresses bone marrow-derived hepatic macrophages
We next investigated the molecular mechanisms underlying the protective activity of GPR84 against the progression of HFD-induced hepatic steatosis to fibrosis. The HFD increased not only hepatic endogenous MCFA production but also hepatic \textit{Gpr84} mRNA expression (Figure 4A). Hepatic \textit{Gpr84} was expressed in macrophages but not in hepatocytes, monocytes, stellate, or Kupffer cells (Figure 4B), and HFD feeding further accelerated its expression (Figure 4C). The population of macrophages in the livers of HFD-fed \textit{Gpr84}–/– mice was higher than that in HFD-fed wild-type mice (Figure 4D). In contrast, the population of macrophages in the livers of NC-fed \textit{Gpr84}–/– mice was comparable to that of macrophages in the livers of wild-type mice (Supplemental Figure 7A). Additionally, the population of Kupffer cells in the livers of both NC- and HFD-fed \textit{Gpr84}–/– mice were also comparable to that of Kupffer cells in the livers of wild-type mice (Supplemental Figure 7, A and B). Moreover, \textit{Tnf} mRNA expression was markedly higher in the hepatic macrophages of HFD-fed, but not NC-fed, \textit{Gpr84}–/– mice than in HFD-fed wild-type mice (Figure 4D and Supplemental Figure 7C). \textit{Gpr84} was mainly expressed in the bone marrow, which is the primary site of hematopoiesis (Supplemental Figure 7D). Hence, we further investigated the GPR84-mediated relationship between the bone marrow and hepatic macrophages. RNA sequencing and GO enrichment analysis of the bone marrow from HFD-fed \textit{Gpr84}–/– mice showed that its expression profile was related to the macrophage-related chemokine pathway and chronic inflammation (Supplemental Figure 8, A–C). Additionally, the transplantation of \textit{Gpr84}–/– mouse-derived bone marrow into wild-type mice caused macrophage infiltration into the liver and NASH under HFD feeding as well as the hepatic phenotype of \textit{Gpr84}–/– mice (Figure 4E). Thus, GPR84-positive bone marrow-derived macrophages may prevent hepatic fibrosis.

The mechanisms underlying this process in the liver were investigated under HFD feeding conditions using GPR84-deficient RAW264.7 macrophages. Saturated fatty acids, such as palmitic acid (C16:0), which are
abundant in HFD, induce inflammation by activating macrophages (7, 27). C16:0 stimulation upregulated the expression of the inflammatory marker Tnf and the macrophage infiltration marker C-C motif chemokine 2 (Ccl2) in RAW264.7 cells (Figure 4F and Supplemental Figure 9). C10:0 suppressed these effects and increased the expression of the anti-inflammatory M2-macrophage marker arginase 1 (Arg-1) in a dose-dependent manner; the effects of C10:0 were diminished in Gpr84+ RAW264.7 cells (Figure 4F and Supplemental Figure 9). Furthermore, C16:0 administration increased the levels of intracellular MCFAs in the mouse hepatocyte cell line, AML12 (Figure 4G). C16:0 stimulation in Gpr84+ RAW264.7 cells co-cultured with AML12 showed a marked increase in Tnf expression compared with that in RAW264.7 cells co-cultured with AML12 (Figure 4H). Thus, MCFAs suppress lipotoxicity-induced macrophage activation via GPR84 in the liver.

**GPR84 activation by MCFAs improves NASH**

Finally, we investigated whether GPR84 activation could suppress NASH progression in a NASH mouse model. A choline-deficient L-amino acid-defined HFD (CDAHFD) and CCl4 were used to establish NASH with rapidly progressive hepatic fibrosis in mice (28). Wild-type mice fed with the CDAHFD for 10 weeks exhibited signs of NASH and HCC (Figure 5A). Supplementation of dietary MCFAs (C8:0, C10:0, and C12:0) in CDAHFD-fed mice increased the plasma and hepatic levels of each MCLA (Supplemental Figure 10A). Interestingly, unlike in HFD-fed mice (Figure 3C), basal endogenous MCLA levels were comparable among NC-fed, CDAHFD-fed, and CCl4-administered mice (Supplemental Figure 10B). Although MCLA supplementation did not significantly change the liver and WAT weights, C10:0 and C12:0 supplementation in CDAHFD-fed wild-type mice effectively suppressed the signs of NASH and HCC (Figure 5, A and B).
The hepatic TG levels were comparable between CDAHFD-fed wild-type and Gpr84−/− mice supplemented with dietary MCFAs (Figure 5C). The levels of the inflammatory marker Tnf, fibrosis markers Col1a, Tgfb1, and Acta2, and macrophage marker Adgre1 were also markedly decreased by C10:0 and C12:0, but not C8:0, supplementation in the livers of CDAHFD-fed wild-type mice. The effects of C10:0 were abolished in Gpr84−/− mice (Figure 5D). Consequently, the NAS decreased considerably after C10:0 and C12:0, but not C8:0, supplementation in wild-type mice, but not in Gpr84−/− mice (Figure 5E). Thus, MCFAs, except for C8:0, markedly suppressed NASH progression via GPR84. Furthermore, among the dietary MCT oils, which are sources of MCFAs, trioctanoin (TriC8) and tridecanoin (TriC10) supplementation increased the levels of MCFAs C8:0 and C10:0 in the plasma and liver, respectively (Supplemental Figure 1A). Under TriC10 supplementation, but not TriC8, the levels of inflammatory, fibrosis, and macrophage markers markedly decreased without any changes in hepatic TG levels in CDAHFD-fed wild-type mice, but not Gpr84−/− mice (Supplemental Figure 11, B–D). The NAS markedly dropped after TriC10 supplementation (Figure 5F). Thus, GPR84 activation by dietary MCFAs (C10:0 and C12:0, but not C8:0) markedly improves NAFLD, thereby suppressing the progression of NAFL to NASH, but not to hepatic steatosis.

**GPR84 agonists are potential NASH therapeutic drugs**

We confirmed that Gpr84 expression and NASH progression increased in human livers (Figure 6A). Therefore, GPR84-selective compounds may be potential therapeutic drugs. Embelin is a known GPR84 agonist (29). In HEK293 cells expressing mouse GPR84, a tetracycline-controlled Tet-On gene expression system and transforming growth factor (TGF)-α shedding assay (30) were used to confirm that embelin activated GPR84 in a dose-dependent manner (Figure 6B). Embelin, as well as C10:0, suppressed palmitate-
induced increases in *Tnf* expression in a dose-dependent manner. The effects of embelin were abolished in *Gpr84*−/− RAW264.7 cells (Figure 6C). Hence, we administered GPR84-selective compounds in the NASH mouse model using embelin as the GPR84 agonist. Consequently, embelin markedly suppressed the levels of inflammatory, fibrosis, and macrophage markers, as well as the NAS, in both the CDAHFD-fed and CCl₄-induced NASH mouse models (Figure 6, D–F and Supplemental Figure 12, A and B). Thus, exogenous GPR84 stimulation markedly improved NAFLD.
Discussion

The exact contribution of endogenous MCFAs and the receptor GPR84 in controlling metabolic syndrome was previously unclear. Herein, MCFAs showed hepatoprotective activity against dietary fat-induced NASH progression. Under HFD feeding, NASH progression was observed in HFD-fed Gpr84−/− mice. In addition to saturated fatty acid excess-mediated macrophage activation under HFD feeding, macrophage-mediated phagocytosis of fat-accumulated hepatocytes further accelerated the inflammatory response. Thus, under lipotoxic conditions, endogenous MCFAs, which are released from hepatocytes along with long-chain fatty acids, suppressed the over-activation of macrophages via GPR84, thereby protecting hepatic functions.

Metabolic disorders, such as obesity, insulin resistance, glucose intolerance, and type 2 diabetes, are significant risk factors of NASH (4, 5). We recently reported that MCFA-stimulated GPR84 activation maintains glucose homeostasis by insulin regulation via GLP-1 secretion (25). GLP-1 also suppresses the proinflammatory and profibrotic phenotypes of macrophages, thereby suppressing NASH development (31, 32). The regulation of GLP-1 secretion via GPR84 may thus be partly related to the suppression of NASH. Thus, GPR84 functions, including differentiation of macrophages from monocytes and filtration from bone marrow to the liver, on other organs also may influence the NASH progression. Therefore, further studies using tissue-specific GPR84-deficient mice are needed to elucidate these metabolic mechanisms.

Although it is known that MCFAs and MCTs have anti-inflammatory effects and that GPR84 is coupled with inhibitory G proteins (Gαi/o) (33, 34), recent in vitro studies have described GPR84 as a proinflammatory receptor (35–37). However, since these studies were conducted using only potent synthetic GPR84 agonists, the physiological activity of GPR84 remains unclear. In this study, we showed that endogenous or dietary MCFAs effectively suppress NASH progression through GPR84 as an “anti-inflammatory receptor,”
both in vivo and in vitro. Moreover, we confirmed the MCFA–GPR84-mediated anti-inflammatory effects under lipotoxic conditions using blinded in vitro experiments. Previous in vitro studies have reported that GPR84 stimulation weakly promotes inflammation under normal or non-lipotoxic inflammatory conditions. In contrast, we showed that GPR84 stimulation suppresses inflammation under lipotoxicity-induced hyperinflammatory conditions. This contradiction may exemplify how FFARs, including GPR84, are optimal fine-tuning receptors for maintaining homeostasis by regulating biological processes and sensing nutritional states (7). Therefore, we redefine GPR84 as an immunomodulating receptor, not simply a proinflammatory receptor.

GPR84 antagonists weakly suppress NASH, and HFD feeding in Gpr84−/− mice weakly restores fibrosis, but not steatosis and inflammation (38–40). However, actual phase II clinical trials using the selective GPR84 antagonist GLPG1205 failed to demonstrate its efficacy (35, 41, 42). Furthermore, another GPR84 antagonist (PBI-4547) also acts as a GPR40/GPR120 agonist (40). Importantly, our results indicate that, although HFD feeding induced an increase in hepatic MCFA levels (as endogenous GPR84 ligands) (Figure 3C), CDAHFD feeding and CCl₄ administration did not change hepatic MCFA levels compared with NC feeding (Supplemental Figure 10B). Furthermore, HFD-fed Gpr84−/− mice exhibited increased hepatic inflammation and fibrosis and progression to NASH compared with wild-type mice (Figure 2, C–E), whereas CDAHFD and CCl₄ did not change the basal levels of inflammatory and fibrosis markers nor the NAS between wild-type and Gpr84−/− mice (Figures 5 and 6). Therefore, Simard et al.’s HFD-fed mouse model (40) may not alter hepatic MCFA levels, and their methods, in which mice aged 10–14 weeks were fed an HFD for 14 weeks, might not be appropriate for establishing an HFD-induced metabolic syndrome mouse model. In comparison, our method involved mice aged 7 weeks, fed an HFD for 5 weeks, or aged 4 weeks, fed an HFD for 12
weeks. Although HFD induced obesity in our mouse model, neither our CDAHFD-fed or CCl$_4$-administered mice nor Simard et al.’s HFD-fed mice (40) exhibited an increase in body weight. Further studies are needed to clarify the mechanism by which endogenous MCFAs are produced under HFD feeding and that of metabolic diseases and NASH progression. Nevertheless, our results indicate that exogenous GPR84 stimulation using dietary MCTs and a GPR84 agonist is effective in suppressing progression of NASH under low endogenous hepatic MCFA levels. To validate GPR84 as a therapeutic target, we suggest that GPR84 stimulation by GPR84 agonists may be a more effective strategy than developing a substitute GPR84 antagonist.

In conclusion, GPR84 deficiency under excess dietary fat intake accelerates lipotoxicity-induced macrophage overactivation, thereby promoting hepatic fibrosis to NASH. In contrast, MCFA, MCT, and GPR84 agonist administration effectively improved NASH progression by suppressing hepatic fibrosis without influencing hepatic steatosis by fat accumulation. Hence, MCFA, either endogenously synthesized or derived from dietary MCTs, may play important roles in recognizing nutrient excess and maintaining hepatic metabolic functions through GPR84 activation. Additionally, this study formally demonstrated that orphan GPCR GPR84 is a receptor for endogenous MCFAs. Collectively, GPR84 modulation may be an effective strategy for improving the progression of NASH and HCC.
Methods

Animal study

C57BL/6J, Gpr84−/−, and congenic CD45.1 mice (Sankyo Lab Service) were housed under a 12-h light–dark cycle and fed NC (CE-2; CLEA). Gpr84−/− mice with a C57BL/6J background were generated using the CRISPR/Cas9 system (Supplemental Figure 1, A–C). For short-term treatment, 7-week-old male mice were fed NC or a HFD (D12492, 60% kcal fat; Research Diets) for 5 weeks. For long-term treatment, 4-week-old C57BL/6J male mice were fed a HFD for 12 weeks. At least three groups of littermates from each dam were analyzed in individual experiments. Chronic liver injury was induced by feeding the mice with CDAHFD (A06071302 containing 60 kcal% of fat and 0.1% of methionine; Research Diets) (43) or MCFA- or MCT-supplemented CDAHFD (Supplemental Table 1) for 10 weeks. MCT oils were purchased from the Nisshin OilliO Group, Ltd. 7–8-week-old C57BL/6J male mice were treated with CCl4 (0.6 mL/kg bodyweight, diluted in corn oil, and injected intraperitoneally every 3 days) for 8 weeks to induce hepatic fibrosis (44). CDAHFD-fed or CCl4-treated mice were administered embelin (50 mg/kg bodyweight) through oral gavage once a day for 4 weeks. All efforts were made to minimize animal suffering.

Human study

For the analysis of GPR84, TNF, and TGFB1 mRNA expression levels in human liver samples, a total of 53 liver samples were isolated from healthy subjects, NAFL, and NASH patients.

Biochemical analyses

Hepatic levels of TGs were analyzed using commercial kits (LabAssay Triglyceride; FUJIFILM
The levels of TNF-α were measured using commercial kits [Mouse TNF-alpha Quantikine ELISA Kit (R&D systems)], following the manufacturer’s instructions.

**RNA isolation and quantitative reverse transcriptase (qRT)-PCR**

Total RNA was extracted using an RNAiso Plus reagent (TAKARA). cDNA was transcribed using RNA as templates and Moloney murine leukemia virus reverse transcriptase (Invitrogen). qRT-PCR analysis was performed using SYBR Premix Ex Taq II (TAKARA) and the StepOne real-time PCR system (Applied Biosystems), as described previously (10, 11). The PCR protocol was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 1 min. Each sample was tested in duplicate for the average Ct value. Relative mRNA expression was calculated after normalization to the 18S rRNA reference gene using the 2-ΔΔCt method. Primer sequences for the targeted mouse genes were as follows: Gpr84, 5′-AGGTGACCCGTATGTGCTTC-3′ (forward) and 5′-GTTCATGGCTGCATAGAGCA-3′ (reverse); 18S, 5′-CTTAGAGGGACAAGTGGCG-3′ (forward) and 5′-ACGTAGCCAGTCAGTGTA-3′ (reverse); Colla, 5′-CCTCAGGATTGCTGGACAAAC-3′ (forward) and 5′-ACCACCACTTCAGAGGGGCCTTT-3′ (reverse); Tgfb1, 5′-CCTGAGTGGCTGTCTTTTGACG-3′ (forward) and 5′-AGTGAGCGCTGAATCGAAAG-3′ (reverse); Acta2, 5′-GTTCAGTGCTGTCTTTTGACG-3′ (forward) and 5′-ACTGGGACGACATGGAAAAG-3′ (reverse); Tnf, 5′-GGCAGGTCTACTTTGGAGTC-3′ (forward) and 5′-TCGAGGGCTCCAGTAGTAATTCG-3′ (reverse); Adgre1, 5′-GATGTGGAGGATGGAGATG-3′ (forward) and 5′-ACAGCAGGAAGGCT TatGAGATG-3′ (reverse); Cld68, 5′-TCCAAGATCCCTCCACTGTTG-3′ (forward) and 5′-ATTGGAATTGGGCTTGAG-3′ (reverse); Cld14, 5′-
GGCGCTCCGAGTTGTGACT-3’ (forward) and 5’-TACCTGCTTCAGCCCCAGTG-3’ (reverse); 
*Ccl2*, 5’-AATCTGAAAGCTATGCATCC-3’ (forward) and 5’-GTGTGAATCTGGATCACA-3’ (reverse); 
*Arg1*, 5’-AAAGCTGGTCTGCTGGAAA-3’ (forward) and 5’-ACAGACCGTGAGTTCTTCAC-3’ (reverse); 
*Acsll*, 5’-TGCGAGCTGATGGACATTC-3’ (forward) and 5’-GGCATACAGAGGTGGTGAG-3’ (reverse); 
*Acsm3*, 5’-CTTTGCCCCAGCAGTAGT-3’ (forward) and 5’-GGCTGTCACTGGCAATTCT-3’ (reverse); 
*Acadl*, 5’-TTCTCCTCGGAGCATGATTTTT-3’ (forward) and 5’-GCCAGGCTTTCCCAGACCT-3’ (reverse); 
*Acadu*, 5’-CGAGAGGAGATTA_CCCA-3’ (forward) and 5’-ACAACAATACGCAAATCTT-3’ (reverse); 
*Acotll*, 5’-AGATCATGGCTTGGATGGAG-3’ (forward) and 5’-AAAGGCGTTATTCAGATGG-3’ (reverse); 
*Aco13*, 5’-AGGACAGCATGACCAACTCTC-3’ (forward) and 5’-GGAGCGTGCCCAGTTAA-3’ (reverse). Primer sequences for the targeted human genes were as follows: 
*GPR84*, 5’-TTCAGCCCTTCTCTGGACA-3’ (forward) and 5’-TGCAGAACACCTTCTCTGGGACA-3’ (reverse); 
*TNF*, 5’-CACCTGCTGCTGAAATC-3’ (forward) and 5’-GAGGAAGGCTAAGGTCG-3’ (reverse); 
*TGFB1*, 5’-CCAGCATCTGCAAAGCTC-3’ (forward) and 5’-GTCAAAGGTCACTGCCG-3’ (reverse); 
and 18S, 5’-CGCCGCTAGGAGGAAATC-3’ (forward) and 5’-CCAGTCGGCATCTATGG-3’ (reverse).

**Histological analysis**

The liver was excised and fixed overnight at 4°C in 4% paraformaldehyde. The fixed tissues were embedded in OCT-compound (Sakura Finetek) and sectioned into 8-μm thick sections using a cryo-
microtome (Leica). H&E staining was performed using standard techniques. The lipid contents in hepatocytes were visualized using Oil Red O staining. Immunohistochemical analysis was performed using antibodies against F4/80 (1:1000; Abcam, ab6640) and αSMA (1:300; Cell Signaling Technology, 19245), and the nuclei were stained with DAPI (1:5000; Roche, 10236276001), as previously described (9). Quantification of liver macrophage was quantified by counting F4/80 positive cells (green fluorescence), and total number of cells was counted based on the DAPI nuclear staining, using BZ-X710 (Keyence). The sections were washed with PBS, blocked with 5% bovine serum albumin (BSA) in PBS, and permeabilized with 0.1% Triton X-100 (Sigma). Next, the sections were incubated with primary antibodies, followed by incubation with secondary antibodies conjugated with a fluorescent marker. Immunoreactive signals were developed using 3,3’-diaminobenzidine (DAB) staining with the Peroxidase Stain DAB Kit (Nacalai Tesque), and the sections were counterstained with Meyer’s hematoxylin (FUJIFILM Wako). A histopathological evaluation of NASH was performed based on the NAFLD activity score (NAS) and steatosis, lobular inflammation, and ballooning degeneration scores. Steatosis, lobular inflammation, and ballooning degeneration were scored on 0–3, 0–3, and 0–2 scales, respectively. Total NAS was scored as follows: 1–3, 4/5, and 6–8. NAS is shown in Supplemental Table 2.

RNA sequencing

RNA was extracted from the liver and bone marrow of NC- and HFD-fed mice using an RNAiso Plus reagent (TAKARA) and RNeasy mini kit (Qiagen). RNA sequencing libraries were generated with the TruSeq RNA Library Prep Kit (Illumina) and sequenced on an Illumina platform. Approximately four Gb paired-end reads of length 100 bp per sample were obtained. The RNA sequencing data were pre-
processed using Trimmomatic to remove adapters or poor-quality reads (45). The quality of the trimmed sequences was then assessed using FastQC (46). The reads were aligned to the mouse reference genome (mm10) using HISAT2 (47) with the Bowtie2 aligner (48). The aligned reads were assembled using StringTie (49). The raw read counts were subjected to relative log expression normalization to obtain DEGs from all comparisons. The data were expressed as fold change using nbinomWaldTest with DESeq2. DEGs were identified based on the following two criteria: false discovery rate (FDR)-adjusted $p$-value $< 0.05$ (using the Benjamini-Hochberg procedure) and $|\log_2$ (fold change)$| > 0.5$. A Gene Set Enrichment Analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). The GO terms of molecular function, biological process, cellular component, and pathway were considered.

**Cell culture**

All cell lines were cultured at 37°C with 5% CO$_2$. To generate Flp-In T-REx HEK293 cells (Invitrogen) expressing murine GPR84, HEK293 cells were transfected with a mixture of pcDNA5/FRT/TO-HA-mGPR84 and pOG44 using Lipofectamine reagent (Invitrogen) (Supplemental Figure 5A). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 μg/mL blasticidin S (Funakoshi), 100 μg/mL hygromycin B (Gibco), and 10% fetal bovine serum (FBS). For the localization analysis, the cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature then permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. After washing with PBS, the cells were pre-incubated with 1% BSA in PBS for 1 h then probed with the primary anti-HA high-affinity antibodies (1:1000; Roche, 3F10) in 1% BSA/PBS for 1 h at room temperature. The cells were washed twice with PBS, incubated with Alexa Fluor 488-conjugated
secondary antibodies (1:200; Invitrogen, A11006), and observed under a fluorescence microscope. For cAMP determination, GPR84-expressing HEK293 cells were seeded in 24-well plates (1 × 10^5 cells/well), cultured for 24 h, and treated with or without doxycycline (10 μg/mL; Sigma) for 24 h. The cells were treated with 2 μM forskolin (Sigma) and 500 μM of 3-isobutyl 1-methylxanthine (Sigma) to upregulate the cAMP levels. They were then stimulated with individual MCFAs (C6:0–C14:0; Nu-Chek Prep, Inc.) or embelin (Cayman Chemical) for 10 min. The cAMP levels were determined using the cAMP EIA kit (Cayman Chemical), following the manufacturer’s instructions.

The TGF-α shedding assay was performed as described previously (50). HEK293 cells were seeded in 6-well plates (2 × 10^5 cells/well) and cultured for 48 h. Plasmid transfection was performed with a mixture of 500 ng AP-TGF-α-encoding plasmid and 200 ng GPR84-encoding plasmid with or without 100 ng Ga13-encoding plasmid. After 1 day, the transfected cells were harvested by trypsinization, pelleted by centrifugation at 190 × g for 5 min, and washed once with Hank’s Balanced Salt Solution (HBSS) containing 5 mM HEPES (pH 7.4). After centrifugation, the cells were resuspended in the HEPES-containing HBSS. The cell suspension was seeded in a 96-well culture plate and incubated for 30 min at 37°C and 5% CO₂. The cells were treated with GPR84 ligands diluted in HBSS containing 5 mM HEPES (pH 7.4) and 0.01% (w/v) BSA (fatty acid-free and protease-free grade; FUJIFILM Wako) for 1 h. AP reaction solution [10 mM p-nitrophenylphosphate (p-NPP), 120 mM Tris–HCl (pH 9.5), 40 mM NaCl, and 10 mM MgCl₂] was dispensed into the cell plates. Absorbance at 405 nm (Abs405) of the plates was measured using a microplate reader (Multiskan GO, Thermo Scientific) before and after a 1-h incubation period at room temperature. Ligand-induced AP-TGF-α release was calculated as described previously (50).

RAW264.7 cells [mouse macrophage cell line; American Type Culture Collection (ATCC)] were
cultured in DMEM supplemented with 1% penicillin-streptomycin solution (Gibco) and 10% FBS (51). The GPR84-deficient RAW264.7 cells (RAW-KO cells) were generated using a CRISPR/Cas9-mediated homology-independent knock-in system. Single guide RNA (sgRNA) targeting Gpr84 (5′-ttgtcccaagctccgaacc-3′) was designed based on a previous report (52) and cloned into the sgRNA expression vector peSpCAS9(1.1)-2xsgRNA (Addgene, plasmid #80768). RAW264.7 cells were plated in 60-mm dishes (2.5 × 10^5 cells/dish) and co-transfected with the recombinant peSpCAS9(1.1)-2xsgRNA and pDonor-tBFPNLS-Neo (Addgene, plasmid #80766) using Lipofectamine 2000 (Invitrogen). On day 2 post-transfection, the cells were cultured in medium containing 250 μg/mL G418 (FUJIFILM Wako) to select the recombinant cells. At day 10 post-selection, colonies grown from single cells were isolated. RAW264.7 and RAW-KO cells were stimulated with capric acid (C10:0; 0.01, 0.1, and 1 mM; Nu-Chek Prep, Inc.) or embelin (0.1, 1, 10, 50, and 100 μM; Cayman Chemical) in the presence of palmitic acid (C16:0) for 12 h. Before stimulation with these samples, researchers were blinded to the sample origin. The cells were then harvested to isolate their RNA.

AML12 cells (mouse hepatocyte cell line; ATCC) were maintained in DMEM:HAM-F12 (1:1, 3.15 g/L glucose) (Sigma) supplemented with 1% penicillin-streptomycin solution, 10% FBS, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, and 40 ng/mL dexamethasone (53). To measure the cellular MCFA contents, AML12 cells were treated with palmitic acid (C16:0) for 48 h and harvested for liquid chromatography-mass spectrometry (LC-MS/MS) analysis. For co-culture studies, LCFA-stimulated AML12 cells were co-cultured with RAW264.7 or RAW-KO cells for 72 h and harvested for RNA isolation.

### MCFA determination
MCFA levels in the plasma, liver, adipose tissue, muscle, cecum, and NC and HFD samples were determined following a previously described protocol with modifications (12). The samples containing an internal control (C19:0) were homogenized in methanol and mixed with chloroform and water to extract lipids. The samples were centrifuged at 2000 × g and 17°C for 10 min. The supernatant containing MCFAs was collected and dried. The samples were resuspended with chloroform:methanol (1:3, v/v) and subjected to LC-MS/MS analysis using an ultra-performance LC system (Waters, UPLC) equipped with an Acquity UPLC system coupled to a Waters Xevo TQD mass spectrometer (Waters). The samples were separated on an ACQUITY UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm; Waters) using a methanol gradient in 10 mM ammonium formate aqueous solution.

**Flow cytometry**

To isolate hepatic mononuclear cells and Kupffer cells, the excised livers were cut into small pieces using a razor blade and subjected to enzymatic digestion in a digestion solution (3 mM CaCl₂, 1 mg/mL collagenase I, and 1.5% BSA in HBSS) for 2 h at 37°C. The cell suspension was passed through a 70-μm nylon mesh cell strainer. The cells were isolated using Percoll density gradient centrifugation (54). Single-cell suspensions were blocked with an Fc receptor CD16/CD32 (BioLegend; 93) at 4°C for 10 min. For flow cytometric sorting, hepatic mononuclear cells and Kupffer cells were stained with Brilliant Violet (BV) 510-conjugated anti-CD45 (BioLegend; 30-F11), BV711-conjugated anti-Ly-6C (BioLegend; HK1.4), Alexa Fluor 488-conjugated anti-F4/80 (BioLegend; BM8), PE-conjugated anti-CX3CR1 (BioLegend; SA011F11), and APC-conjugated anti-CD11b (BioLegend; M1/70) antibodies for 30 min at 4°C. The cells were then washed with FACS buffer (1 × PBS containing 2% FBS and 2 mM EDTA). For the transplantation studies, hepatic mononuclear cells were obtained using
collagenase digestion and Percoll density gradient centrifugation. The samples were stained with PE-Cy7-conjugated anti-CD45.1 (BD Biosciences; A20), APC-Cy7-conjugated anti-CD45.2 (BD Biosciences; 104), PE-conjugated anti-Ly-6C (BioLegend; HK1.4), FITC-conjugated anti-F4/80 (BioLegend; BM8), and APC-conjugated anti-CD11b (BD Biosciences; M1/70) antibodies. The cells were sorted using a FACSaria III cell sorter (BD Biosciences) and FACSMelody (BD Biosciences). The purity of the sorted cells was at least 95%. Flow cytometric data were analyzed using FlowJo v10 software (BD Biosciences).

**Bone marrow cell transplantation**

C57BL/6J-CD45.1 mice were lethally irradiated with a dose of 10 Gy. A total of $1 \times 10^7$ cells obtained from C57BL/6J or Gpr84<sup>−/−</sup> (CD45.2) mice were intravenously injected into the irradiated recipient mice. The mice were bred with water supplemented with 1 g/L neomycin and 1 g/L ampicillin for 2 weeks after transplantation. Mice with chimeric bone marrow were fed the HFD (D12492 diet; Research Diets) for 8 weeks. The hepatocytes were isolated, and the proportion of lymphocytes and myeloid cells was calculated using flow cytometry.

**Statistical analysis**

All values are presented as mean ± standard error of mean. The violin plots depict the median, quartiles, and data range. The normality of the data was assessed by Shapiro-Wilk test, followed by Student's $t$ test or the Mann-Whitney U test for statistical significance at two groups, whereas those between multiple groups ($\geq 3$ groups) were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test or Kruskal
Wallis test followed by the Dunn's post-hoc test. Differences were considered significant at $P < 0.05$. The FDRs of RNA sequencing data were estimated using the Benjamini–Hochberg procedure.

Study Approval

All experimental procedures involving mice were performed according to the protocols approved by the Committee on the Ethics of Animal Experiments of the Kyoto University Animal Experimentation Committee (Lif-K21020) and the Tokyo University of Agriculture and Technology (permit number: 28–87). All mice were sacrificed under deep anesthesia using isoflurane. All studies were approved by the institutional review board of Kyushu University (approval number: 29-476, 2021-71) and performed in accordance with relevant guidelines. Informed consent was obtained from patients at the time of recruitment, and their records were anonymized and de-identified.

Data and Materials availability

The source data presented in Figures 1–6, Supplemental Figures 1–12, Supplemental Tables 1–2, and RNA sequencing have been deposited into the Dryad repository (doi:10.5061/dryad.m37pvmd36). All other data that support the findings of this study are available from the corresponding author upon reasonable request.
**Author Contributions**

R.O-K. performed the experiments, interpreted data, and wrote the paper; H.N. performed the experiments, interpreted data, and wrote the paper; A.N. performed the experiments and interpreted data; Y.M. performed the experiments; D.T. performed the experiments and interpreted data; T.I. performed the experiments; A.U. performed the experiments; M.T. interpreted data; M.K. performed the experiments; M.I. interpreted data; H.K. performed the experiments and interpreted data; T.T. interpreted data; A.I. interpreted data; T.S. interpreted data; K.H. interpreted data; Y.O. interpreted data; J.A. interpreted data; I.K. supervised the project, interpreted data, and wrote the paper; I.K. had primary responsibility for the final content. All authors read and approved the final manuscript.
Acknowledgments

This work was partly supported by research grants from the AMED (JP21gm1010007 to IK), JSPS KAKENHI (JP21H04862 to IK), JST-OPERA (JPMJOP1833 to IK), and JST-Moonshot R&D (JPMJMS2023 to IK), the Smoking Research Foundation (to IK), and Nisshin OilliO Group, Ltd (to IK).
References


Figure 1. GPR84 deficiency accelerates high-fat diet-induced chronic inflammation. (A) TNF-α levels (NC-fed group, n = 5; HFD-fed group, n = 6–7). (B) Expression of Tnf in liver, Epi, muscle, small intestine, and colon (n = 4 independent experiments). Data are represented as relative to the gene expression in WT mice. Epi, epididymal white adipose tissue. (C) RNA-Seq transcriptome profiling in liver in WT and Gpr84<sup>−/−</sup> mice fed the HFD for 5 weeks. Heatmap shows results of two-dimensional hierarchical clustering of 59 genes related to inflammation (n = 5 per group). (D) Expression of fibrosis related genes – Col1a (left), Tgfb1 (middle), and Acta2 (right), in the liver (n = 8–9). Data are represented as relative to the gene expression in WT mice. **P < 0.01; *P < 0.05 (Mann-Whitney U test: A, B, D). All data are presented as the mean ± standard error of mean (SEM).
Figure 2. HFD-fed Gpr84−/− mice exhibit NASH. (A) Liver weight (n = 8 tissues per group). (B) Oil Red O staining (left) and hepatic TG levels (right) (n = 8 tissues per group). Scale bars: 25 µm. (C) Expression of Tnf and fibrosis marker genes [Col1a (left), Tgfβ1 (middle), and Acta2 (right)] in WAT
(n = 8 independent experiments). Data are represented as relative to the gene expression in WT mice. 

(D) Immunohistochemistry of F4/80 and αSMA stained with DAB or fluorescent staining in sections of liver (left; F4/80, green; αSMA, red; DAPI, blue). F4/80-positive cell numbers (right; n = 4 tissues per group). Scale bars: 25 μm (DAB staining) or 100 μm (fluorescent staining). (E) Expression of Adgre1, Cd68, and Cd14 (n = 8 tissues per group). Data are represented as relative to the gene expression in WT mice. (F) NAFLD activity score (NAS). **P < 0.01; *P < 0.05 (Mann-Whitney U test: A, B, C, D; Student’s t-test: E, F). All data are presented as the mean ± SEM. N.S., not significant.
Figure 3. Affinity of MCFAs for GPR84, and RNA-Seq transcriptome profiling of liver under NC- and HFD-feeding. (A) cAMP inhibition assay for C8:0, C9:0, C10:0, C11:0, and C12:0 using mouse-GPR84-expressing HEK293 cells. Cells were cultured for 24 h then treated with or without 10 μg/mL of Doxycycline (Dox; n = 6 independent cultures with Dox, from two biological replicates; n = 6 independent cultures without Dox, from two biological replicates). All data are presented as relative
to forskolin (Fsk)-induced cAMP levels. Closed symbols represent values from cells treated with Dox, and open symbols denote untreated groups. (B) Heatmap of relative MCFA contents among liver, muscle, adipose tissue, and plasma of WT mice after 5-week HFD-feeding. (C) Measurement of MCFA concentration (NC-fed group, n = 6–9; HFD-fed group, n = 7–9 tissues). (D) Fatty acid synthesis- and β-oxidation-related genes were determined by qPCR (n = 5 from 5 per group). Data are represented as relative to the gene expression in NC-fed mice. **P < 0.01; *P < 0.05 (Mann-Whitney U test). All data are presented as the mean ± SEM.
Figure 4. GPR84 suppresses bone marrow (BM)-derived hepatic macrophages. (A) Expression of Gpr84 in the liver after HFD feeding for 5 weeks (n = 7 tissues per group). Data are represented as relative to the gene expression in before HFD-fed mice. (B) Gpr84 expression in BM-derived
monocytes, hepatic macrophages, Kupffer cells, hepatic stellate cells, and hepatocytes isolated from WT mice fed NC for 12 weeks (n = 5–6 per group). Data are represented as relative to the gene expression in macrophage. (C) Change of Gpr84 expression after HFD feeding (NC- vs. HFD-fed group, n = 5–6 per group). Data are represented as relative to the gene expression in before NC-fed mice. (D) Flow cytometric analysis of BM-derived hepatic macrophage population and Tnf expression in WT and Gpr84−/− mice fed the HFD for 12 weeks (n = 4 per group). BM-derived hepatic macrophage, CD45Ly6C−F4/80−/CD11bhigh CX3CR1+. (E) Flow cytometric analysis showing hepatic cell profile in BM-chimeric mice fed the HFD for 8 weeks (n = 8–9 per group) and NAFLD activity score (NAS) (n = 4 per group). WT recipient mice (CD45.1) after BM transplantation from WT or Gpr84−/− donor mice (CD45.2). (F) Anti-inflammatory effect of MCFA-stimulated GPR84 (n = 8 per group; independent experiments). Data are represented as relative to the gene expression in untreated cells. (G) Intracellular MCFA production in AML12 cells (mouse hepatocyte cell line) treated with palmitic acid for 48 h (n = 8 per group; independent experiments). (H) Tnf expression in RAW264.7 cells co-cultured with AML12 pre-stimulated by palmitic acid (C16:0; n = 6 per group; independent experiments). **P < 0.01; *P < 0.05 [Mann-Whitney U test: A, C, D, E (upper), G; Student’s t-test: E (lower); Kruskal Wallis with Dunn’s post-hoc test: F, H]. All data are presented as the mean ± SEM.
Figure 5. MCFA intake improves NASH progression via GPR84. (A) Anatomy of the liver in WT after CDAHFD feeding and MCFA (C8:0, C10:0, and C12:0)-supplemented CDAHFD feeding for 10 weeks (representative images from n = 8 tissues per group). (B) Tissue weight in WT and Gpr84−/−.
mice (WT, \(n = 8\); \(Gpr84^{-/-}\), \(n = 8\) tissues per group). Epi, epididymal; peri, perirenal; sub, subcutaneous; BAT, brown adipose tissue. (C) Oil Red O staining and hepatic TG levels (WT, \(n = 6\); \(Gpr84^{-/-}\), \(n = 4\) tissues per group). Scale bars: 50 \(\mu m\). (D) Representative H&E staining images of the liver, and immunohistochemistry of F4/80 (green), \(\alpha SMA\) (red), and DAPI (blue) performed in sections of the liver (upper left, WT; upper right, \(Gpr84^{-/-}\)). Scale bars: 50 \(\mu m\). Expression of inflammation- and fibrosis-related genes in the liver (lower left and right; WT, \(n = 6\); \(Gpr84^{-/-}\), \(n = 4\) samples per group). \(\alpha SMA\), \(\alpha\)-smooth muscle actin. Data are represented as relative to the gene expression in CDAHFD-fed mice. (E, F) Component of NAFLD activity score (NAS), (E) after MCFA (C8:0, C10:0, and \(C12:0\))-supplemented CDAHFD feeding for 10 weeks, (F) MCT (TriC8 and TriC10)-supplemented CDAHFD feeding for 10 weeks. **\(P < 0.01\); *\(P < 0.05\) (Kruskal Wallis with Dunn's post-hoc test: D; ANOVA with Dunnett's test: E, F). All data are presented as the mean ± SEM.
Figure 6. GPR84 activation improves NASH. (A) GPR84 expression in human liver (Control, $n = 5$; NAFL, $n = 9$; NASH, $n = 39$). Pearson correlation coefficient between the expression levels of
**GPR84** and **TNF** or **TGFβ1** (*n* = 53). (B) Ligand affinity of embelin for GPR84 using Tet-On/Off (*n* = 6 independent cultures with Dox, from two biological replicates; *n* = 6 independent cultures without Dox, from two biological replicates) and TGF-α shedding assay (*n* = 6 independent cultures with GPR84-encoding plasmid; *n* = 6 independent cultures with mock). (C) Anti-inflammatory effect of embelin-stimulated GPR84. RAW264.7 cells and RAW-KO cells were pretreated with palmitic acid (C16:0; 200 µM) and followed by stimulation of embelin for 3 h. (*n* = 6 per group; independent experiments). Data are represented as relative to the gene expression in untreated cells. (D) Improvement of inflammation and fibrosis in the liver of mice fed the CDAHFD for 10 weeks. Expression of inflammation- and fibrosis-related genes in the liver [WT (*n* = 8); Gpr84−/− (*n* = 4) tissues per group per group]. Data are represented as relative to the gene expression in control mice (untreated with embelin). (E) Suppression of inflammation and fibrosis in carbon tetrachloride (CCL4)-accelerated NASH. Expression of inflammation- and fibrosis-related genes in the liver [WT (*n* = 8); Gpr84−/− (*n* = 4) tissues per group per group]. Data are represented as relative to the gene expression in control mice (untreated with embelin). (F) Component of NAFLD activity score (NAS) after administration of the GPR84 agonist embelin. **P < 0.01; *P < 0.05 [Kruskal Wallis with Dunn's post-hoc test: C; Mann-Whitney U test: D, E, F (CCL4); Student’s *t*-test: F (CDAHFD)]. All data are presented as the mean ± SEM.