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The GPR40 full agonist SCO-267 improves liver parameters in a mouse model of

nonalcoholic fatty liver disease without affecting glucose or body weight

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Running title: GPR40 full agonist for nonalcoholic fatty liver disease

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AUC, area under the curve; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet;

DPP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; GPR40, G protein-coupled

receptor 40; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis;

SGLT2, sodium glucose co-transporter 2; TBARS, thiobarbituric acid reactive substances

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Abstract

Full agonism of G-protein-coupled receptor 40 (GPR40)/free fatty acid 1 (FFA1) receptor improves glycemic control in diabetic rodents. However, the effects of GPR40 full agonism on liver parameters are largely unknown. In the present study, we examined the effects of a GPR40 full agonist, SCO-267, on liver parameters in a non-diabetic mouse model with early-stage nonalcoholic fatty liver disease (NAFLD). SCO-267 was orally administered to mice, which were fed a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD), a mouse model for NAFLD. An oral dose of SCO-267 increased levels of circulating glucagon and glucagon-like peptide-1 in CDAHFD-fed mice. In a chronic dose experiment, effects of SCO-267 were compared with those of a dipeptidyl peptidase-4 inhibitor (alogliptin) and a sodium glucose co-transporter 2 inhibitor (dapagliflozin). SCO-267 decreased liver triglyceride content, weight, and collagen content, and plasma alanine aminotransferase (ALT) levels without affecting food intake or glucose levels in CDAHFD-fed mice. Furthermore, SCO-267 decreased levels of liver thiobarbituric acid reactive substances (TBARS), markers of oxidative stress. Alogliptin and dapagliflozin had no effect on liver weight or levels of triglyceride, collagen, plasma ALT, and liver TBARS. SCO-267 elevated mRNA levels of molecules with roles in mitochondrial function and beta-oxidation, while inhibiting those with roles in lipogenesis, inflammation, reactive oxygen species generation, and fibrosis in the liver, all of which were less evident with alogliptin and dapagliflozin. This

is the first study to show that the GPR40 full agonist, SCO-267, improves liver parameters

without affecting glucose or body weight in a mouse model of NAFLD.

Significance Statement

Full agonism of GPR40/FFA1 receptor signaling stimulates islet and gut hormone secretions.

The present study is the first to show the treatment effects of GPR40 full agonism on liver

parameters in a mouse model for nonalcoholic fatty liver disease.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of chronic liver diseases from non-inflammatory isolated steatosis, characterized by triglyceride accumulation in hepatocytes, to nonalcoholic steatohepatitis (NASH), a more advanced form of the disease, which is characterized by steatosis, inflammation, and hepatocyte cell ballooning associated with liver fibrosis (Arab et al., 2018). NAFLD is a major cause of liver disease worldwide and will likely emerge as the leading cause of end-stage liver disease in the future. To date, weight loss and lifestyle changes are considered to be effective ways to overcome NAFLD (Wattacheril et al., 2018). In addition, due to the lack of approved drugs, there is an urgent need for development of effective pharmacological treatments for patients with NAFLD (Wattacheril et al., 2018). The pathogenesis of NAFLD is complex and still unclear. Although understandings of the molecular mechanisms regulating disease development and progression has grown significantly over recent years, the detailed contribution of genetic and environmental factors, as well as that of intrahepatic and extrahepatic events, in determining the disease conditions remains poorly defined (Musso et al., 2016). These factors hinder the development of new drugs for the treatment of NAFLD.

G-protein-coupled receptor 40 (GPR40)/free fatty acid 1 (FFA1) receptor primarily couples with the Gq/11 protein, facilitating phospholipase C-mediated hydrolysis of

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phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-triphosphate

(Ghislain and Poitout, 2017). GPR40 is expressed in pancreatic islet and intestinal endocrine

cells and regulates hormone secretions from both sites (Mancini and Poitout, 2013; Pais et al.,

2016). The recent discovery of synthetic GPR40 full agonists confirmed the significant role

of GPR40 activation in stimulating glucagon, an islet hormone, and glucagon-like peptide-1

(GLP-1), a gut hormone (Ueno et al., 2019). GPR40 activation is confirmed to be effective in

alleviating diabetes in rodents and humans (Kaku et al., 2016; Ueno et al., 2019). Recently,

clinical trials investigating glucagon-like peptide-1 (GLP-1) receptor agonists for the

treatment of NAFLD have shown therapeutic potential (Armstrong et al., 2016). In addition,

injectable GLP-1 receptor/glucagon receptor dual agonists are being investigated for the

treatment of NAFLD (Patel et al., 2018), indicating the therapeutic potential of combining

islet and gut hormones to treat NAFLD. The ability of GPR40 full agonists to increase levels

of glucagon and GLP-1 warrants further investigation into the effects of this class of

compounds on NAFLD.

This study is the first to report the therapeutic potential of a GPR40 full agonist, SCO-267

(Ueno et al., 2019), on liver parameters in non-diabetic mouse models of early-stage NAFLD.

In the current study, we utilized a choline-deficient, L-amino acid-defined, high-fat diet

(CDAHFD)-induced mouse model of NAFLD (Matsumoto et al., 2013). Initially, the

glucagon- and GLP-1-elevating effects of SCO-267, a GPR40 full agonist, were evaluated in

CDAHFD-fed mice. Subsequently, the effects of chronic dosing of SCO-267 were evaluated

in CDAHFD-fed mice. In addition, certain key effects induced by this GPR40 full agonist

were compared to those of a dipeptidyl peptidase-4 inhibitor (alogliptin) (Keating, 2015) and

a sodium glucose co-transporter 2 inhibitor (dapagliflozin) (Filippatos et al., 2015). Both

these drugs represent different classes of orally available antidiabetic drugs used for the

treatment of metabolic dysfunction, a risk factor for NAFLD (Kothari et al., 2019), and have

been reported to have glucose-independent therapeutic effects (Uchii et al., 2016; McMurray

et al., 2019). However, the roles of these drugs in reversing non-diabetic NAFLD are poorly

understood.

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Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (Tokyo, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), or Cayman Chemical (Michigan, USA) unless otherwise indicated. SCO-267, alogliptin, and dapagliflozin were obtained from SCOHIA PHARMA, Inc. For *in-vivo* studies, compounds were suspended in 0.5% (w/v) methylcellulose (MC) solution (FUJIFILM Wako).

Animals

All animals were housed in a room with controlled temperature (23 °C), humidity (55%), and lighting (12 h light/dark cycle). All animals were provided *ad libitum* access to standard laboratory chow diet (CE-2, 3.4 kcal/g, 12 kcal% fat, 59 kcal% carbohydrate, and 30 kcal% protein, CLEA Japan, Inc.) and tap water during the acclimation period. The care of the animals and use of the experimental protocols in the current studies were approved by the Institutional Animal Care and Use Committee in Shonan Health Innovation Park accredited by the American Association for Accreditation of Laboratory Animal Care. We used 0.5% methylcellulose as the vehicle in animal studies. Blood samples were obtained from the tail vein of the animals. Blood samples were centrifuged (13,000 × g, 4 °C, 5 min) and plasma samples were used for subsequent analyses.

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Single dose study of SCO-267 in CDAHFD-fed mice

Male C57/BL6J mice, obtained from CLEA Japan, Inc. (Tokyo, Japan), were fed a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD (Matsumoto et al., 2013)) (5.2 kcal/g, 62 kcal% fat, 20 kcal% carbohydrate, and 18 kcal% protein, and 0.1% methionine, A06071302, Research Diets, Inc.) for 1 week prior to beginning the study. For the determination of glucagon and GLP-1 levels in the non-fasted state, 12-week-old CDAHFD-fed mice were randomized into groups (n = 6) based on their body weight. The test compounds were orally administered at the indicated dose levels. Blood was collected at the indicated time points and plasma parameters were determined. A pharmacokinetic study was similarly performed using non-fasted, 15-week-old, CDAHFD-fed mice (n = 4).

Repeated dose study of SCO-267 in CDAHFD-fed mice

Male C57/BL6J mice, obtained from CLEA Japan, Inc. (Tokyo, Japan), were fed CDAHFD for 1 week before the start of the study. For the chronic dosing study, 11-week-old CDAHFD-fed mice (baseline body weight, 25.4 ± 1.6 g) were randomized into groups (n = 8) based on their body weight, plasma alanine aminotransferase (ALT), aspartate aminotransferase, and glucose levels. Mice were then orally dosed with SCO-267 (3, 10 mg/kg, BID), alogliptin (10 mg/kg, BID), dapagliflozin (1 mg/kg, BID), or vehicle alone, at

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approximately 8 to 10 AM for the first dose and 4 to 6 PM for the second dose, for 4 weeks.

Food intake and body weight were measured regularly. Male C57/BL6J mice (baseline body

weight, 25.3 $\pm$ 0.8 g) in the normal control group were fed a standard diet (CE-2, CLEA

Japan, Inc.) and received vehicle during the experimental period. Plasma glucose,

triglyceride, alanine aminotransferase, and insulin levels were determined after 4 weeks of

drug dosing. At the end of the study, the mice were euthanized with isoflurane (3 %), and their

livers were isolated, weighed, divided, and immediately frozen for measurements of liver

triglyceride, thiobarbituric acid reactive substance (TBARS), and mRNA levels.

Plasma measurements

Plasma parameters were determined using Autoanalyzer 7180 (Hitachi, Tokyo, Japan).

Enzyme-linked immunosorbent assay kits were used to measure total plasma levels of insulin

(MS301, Takara, Shiga, Japan), glucagon (10-1271-01, Mercodia, Uppsala, Sweden), and

total GLP-1 (299-75501, FUJIFILM Wako). The amount of total collagen in the liver was

quantified using a QuickZyme Total Collagen Assay kit (QuickZyme Biosciences, Leiden,

The Netherlands).

Measurement of hepatic triglyceride content

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Liver tissue was weighed and placed in a polypropylene tube. A 5-fold (v/w) volume of saline

was added to the tube, and samples were homogenized on ice. The homogenate (0.5 mL) was

mixed with 1 mL of extraction solvent (hexane:isopropanol = 3:2 v/v) and shaken for 10 min.

The samples were centrifuged ($800 \times g$, 5 min, room temperature), and the supernatant was

transferred to clean tubes. The bottom layer was again mixed with extraction solvent (0.5 mL),

and the supernatant was prepared in a similar manner. The supernatant layers containing the

extracted lipid were then mixed and dried under a stream of nitrogen gas. The dried sample was

dissolved in isopropanol. The concentrations of triglyceride were measured using a

Triglyceride E-test (FUJIFILM Wako).

Measurement of TBARS content

Lipid peroxidation is a well-established mechanism of cellular injury in animals and is used

as an indicator of oxidative stress in cells and tissues. The measurement of TBARS content is

a well-established method for monitoring lipid peroxidation (Yagi, 1998). For the

measurement of tissue TBARS levels, radioimmunoprecipitation assay buffer containing

cOmplete™ mini ethylene diaminetetraacetic acid-free protease inhibitor cocktail (4693159,

Roche) was added to liver samples. The samples were then homogenized and centrifuged

($1600 \times g$, 4℃, 10 min). TBARS levels of the samples were determined using a TBARS

(TCA Method) Assay Kit (700870, Cayman Chemical, Ann Arbor, Michigan, USA).

Measurement of mRNA levels

Liver RNA samples were prepared using QIAzol Lysis Reagent (Qiagen, Venlo, Netherlands) and RNeasy Mini Kit (Qiagen, Venlo, Netherlands). First strand cDNA was synthesized by using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative polymerase chain reaction was performed using the TaqMan gene expression assay (Supplementary Table 1, Uniprot entry included) and THUNDERBIRD® Probe qPCR Mix (Toyobo, Osaka, Japan) or primers (Supplementary Table 2, Uniprot entry included) and THUNDERBIRD® SYBR® qPCR Mix on the ABI PRISM 7900HT Sequence detector. Expression relative to the vehicle group was calculated by using the comparative CT ($\Delta\Delta$CT) method (User Bulletin #2, Applied Biosystems). For the comparative CT method, the ΔCT value was determined by subtracting the average of internal control (*Gapdh*) CT value from the average of target CT value.

Statistical analysis

Statistical significance was first analyzed using Bartlett's test for homogeneity of variances, followed by the Williams' test ($P > 0.05$) and Shirley–Williams test ($P \leq 0.05$) for dose-dependent studies. Alternatively, statistical significance was analyzed using the F test for homogeneity of variances, followed by Student's *t*-test ($P > 0.2$) or the Aspin–Welch test

($P \leq 0.2$). The Williams' and Shirley–Williams tests were conducted using a one-tailed

significance level of 2.5% (0.025). Other tests were conducted using a two-tailed significance

level of 1% (0.01) and 5% (0.05). All data are presented as mean ± standard deviation (S.D.).

Results

SCO-267 stimulated glucagon and GLP-1 secretion in CDAHFD-fed mice

To determine the effects of SCO-267 on glucagon and GLP-1 secretion in CDAHFD-fed mice, the compound was orally administered, and changes in these hormone levels were measured. Consistent with a previous report in rats (Ueno et al., 2019), SCO-267 was also found to significantly ($P < 0.025$) stimulate glucagon and GLP-1 secretion in CDAHFD-fed mice (Fig. 1A, B). Based on the effective elevation of glucagon and GLP-1 by a single dose of SCO-267, we performed the subsequent chronic dose study in CDAHFD-fed mice.

SCO-267 did not alter food intake, body weight, and glucose control in CDAHFD-fed mice

Due to the relatively short half-life of each compound in mice (Supplementary Figure 1), and based on previous reports (Zhang et al., 2011; Tahara et al., 2016), we dosed SCO-267 (3 and 10 mg/kg), alogliptin (10 mg/kg), and dapagliflozin (1 mg/kg) twice a day in a repeated dose study in CDAHFD-fed mice. As shown in Fig. 2A, SCO-267 and alogliptin did not affect food intake, whereas dapagliflozin slightly increased this parameter. Body weight changes were within a similar range for SCO-267, alogliptin, and dapagliflozin (Fig. 2B). The plasma glucose level was unchanged in SCO-267- and dapagliflozin-treated mice, whereas alogliptin slightly elevated this parameter (Fig. 2C). SCO-267, alogliptin, and dapagliflozin had no

effect on plasma triglyceride levels (Fig. 2D). Plasma insulin levels were unaltered by the treatment with SCO-267, alogliptin, and dapagliflozin (Supplementary Figure 2).

SCO-267 decreased liver triglyceride content, liver weight, liver collagen levels, and plasma

ALT levels in CDAHFD-fed mice

Liver-related parameters were determined at the end of the 4-week study. Of note, SCO-267 significantly ($P < 0.025$) decreased liver triglyceride content and weight in a dose-dependent manner (Fig. 3A, B). Furthermore, SCO-267 significantly ($P < 0.025$) decreased liver collagen content and plasma ALT levels in CDAHFD-fed mice in a dose-dependent manner (Fig. 3C, D). Alogliptin and dapagliflozin did not induce similar effects in this model (Fig. 3A–D). Plasma aspartate aminotransferase levels were unchanged in SCO-267-, alogliptin-, and dapagliflozin-treated mice (Supplementary Figure 3).

SCO-267 decreased oxidative stress marker TBARS levels in the liver

Excessive oxidative stress in the liver is considered an aggravating factor for NAFLD (Spahis et al., 2017). As SCO-267 significantly ($P < 0.025$) decreased liver triglyceride content, we further quantified the oxidative stress marker TBARS in the liver of this mouse model. Interestingly SCO-267 potently decreased liver TBARS levels, whereas alogliptin and dapagliflozin did not cause any significant change in liver TBARS levels (Fig. 4).

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SCO-267 restored dysregulated mRNA expression in liver

Liver samples isolated at the end of the study were further evaluated with respect to the levels

of mRNAs regulating liver functions. SCO-267 increased mRNA levels of transcription

factor A, mitochondrial (*Tfam*), a transcription factor regulating mitochondrial function (Fig.

5A), as well as peroxisome proliferator activated receptor alpha (*Ppara*) and acyl-CoA

dehydrogenase, long chain, (*Acadl*), both gene products involved in the beta-oxidation

pathway (Fig. 5B). In contrast, SCO-267 decreased mRNA levels of sterol regulatory element

binding transcription factor 1 (*Srebf1*) and CD36 molecule (*Cd36*) of the lipogenic pathway

(Fig. 5C), as well as tumor necrosis factor (*Tnf*), chemokine (C-C motif) ligand 2 (*Ccl2*,

interleukin-6 (*Il6*), and transforming growth factor β 1 (*Tgfb1*) of the inflammatory pathway

(Fig. 5D). Furthermore, SCO-267 down-regulated the expression of neutrophil cytosol factor

1 (*Ncf1*), and cytochrome b-245, beta polypeptide (*Cybb*, mRNA for NOX2) (Fig. 5E), both

of which contribute to the generation of reactive oxygen species, and collagen type 1 alpha 1

(*Col1a1*) and actin, alpha 2, smooth muscle, aorta (*Acta2*) (Fig. 5F), both fibrosis-causing

factors. SCO-267 treatment also significantly ($P < 0.025$) decreased mRNA levels of

glucose-6-phosphatase, catalytic subunit (*G6pc*), however, increased the mRNA levels of

glycogen synthase 2 (*Gys2*), both molecules of the glucose metabolism pathway

(Supplementary Figure 4). Most beneficial changes observed were more prominent in the

SCO-267 group than in the alogliptin and dapagliflozin groups (Fig. 5A–F). Lastly, we

assessed mRNA expression for GPR40 (*Ffar1*), and confirmed that *Ffar1* was not expressed

in livers obtained from CDAHFD-fed and standard diet-fed mice (Supplementary Figure 5,

Supplementary Table 3).

Discussion

GPR40 is a key receptor regulating pancreatic and gut hormone secretion. In this study, effects of SCO-267, a GPR40 full agonist, on liver-related parameters were evaluated in CDAHFD-fed mice, a non-diabetic animal model for NAFLD. To the best of our knowledge, this report is the first to demonstrate that a GPR40 full agonist improved abnormal liver-related conditions in a preclinical NAFLD disease model. Of note, SCO-267 improved liver-related parameters without affecting glucose levels or body weight. This suggests that improvement in glycemic parameters and body weight control need not be a primary driver for the improvement of liver-related parameters in SCO-267-treated CDAHFD-fed mice. Hence, GPR40 full agonism may be used for the treatment of NAFLD with/without diabetes and obesity.

The effective secretion of glucagon after SCO-267 treatment suggests that this hormone may have contributed to the treatment effect of SCO-267 in the present study. Glucagon is highly expressed in the liver and shows diverse physiological effects in the body (Svoboda et al., 1994). Besides its pivotal role in liver glucose metabolism, glucagon controls liver fat metabolism by stimulating lipid oxidation and decreasing lipid synthesis (Seghieri et al., 2018). In fact, SCO-267 treatment decreased liver mRNA levels of *Srebf1* and *Cd36* of the lipogenesis pathway, however, increased that of *Tfam* involved in mitochondria function and

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of *Ppara* and *Acadl* involved in beta-oxidation. Furthermore, a recent study demonstrated that glucagon receptor signaling is necessary for the regulation of hepatocyte survival as it exerts anti-apoptotic effects in the liver (Sinclair et al., 2008). Thus, SCO-267-induced activation of liver glucagon signaling may directly improve hepatic disease conditions in this NAFLD model.

In addition to the decreased liver fat, SCO-267 decreased inflammation and fibrosis markers in CDAHFD-fed mice. Based on the "multiple parallel hit" hypothesis, NAFLD pathogenesis is considered a sequence of events from simple steatosis to hepatic inflammation toward fibrosis and NASH-associated hepatocellular carcinoma (Cohen et al., 2011). Thus, SCO-267-induced decrease of hepatic steatosis may be the underlying cause of the reduction in hepatic inflammation/fibrosis by SCO-267 in the present study.

Oxidative stress has been recognized as a central mechanism contributing to liver lesions as it accelerates the transition from simple steatosis to NASH (Spahis et al., 2017). In fact, the PIVENS trial studying pioglitazone versus vitamin E versus placebo in adults with NASH and without T2D demonstrated that vitamin E, a lipid-soluble anti-oxidant, led to improvement in liver histological features and significant resolution of NASH (Sanyal et al., 2010). This demonstrated that reducing oxidative stress is likely to be a highly effective

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strategy to treat NAFLD in patients. The present study shows that SCO-267 decreased liver

oxidative stress levels, as evidenced by the decreased expression of mRNAs contributing to

the generation of reactive oxygen species and the reduced TBARS level in the liver of

CDAHFD-fed mice. Liver fat accumulation generates oxidative stress, which results in

hepatic endoplasmic reticulum stress and inflammation (Spahis et al., 2017). Thus, the

decrease in liver fat accumulation induced by SCO-267 treatment may have resulted in lower

oxidative stress levels in the liver.

The addition of GLP-1 receptor agonism to glucagon receptor agonism improves lipid

metabolism and hepatic steatosis when compared to GLP-1R agonism alone in rodents (Day

et al., 2009). In fact, a GLP-1 agonist and a glucagon/GLP-1 co-agonist, administered via

injection, are being evaluated for the indication of NASH in a clinical trial (Knudsen and

Lau, 2019; Patil et al., 2020). Considering the stimulation of glucagon and GLP-1 by GPR40

full agonists, orally available SCO-267 may be an attractive strategy to treat NASH.

However, the contribution of SCO-267-induced glucagon and GLP-1 stimulation to the

alleviation of liver disease warrants further study.

The present study compares the effects of SCO-267 on liver-related parameters with those of

alogliptin or dapagliflozin, both clinically available antidiabetic drugs. In contrast to

SCO-267, 4-week dosing of alogliptin or dapagliflozin failed to improve fatty liver and related liver parameters. Although mRNA levels of a subset of the molecules of interest were regulated by alogliptin or dapagliflozin, effects induced by each treatment were not as prominent as those induced by SCO-267 treatment. These results suggest that neither dipeptidyl peptidase-4 inhibition nor sodium glucose co-transporter 2-inhibition may be effective methods to improve the current NAFLD condition associated with normal glycemic levels.

Recently, RLA8, a quadruple agonist for PPAR-α/γ/δ and GPR40, was shown to have therapeutic efficacy in NASH mouse models (Li et al., 2019). This suggests that a strategy to activate multiple targets will likely prove to be an effective strategy to treat NASH. Unlike GPR40 partial agonist, full agonism by SCO-267 has the ability to secrete glucagon and GLP-1, in addition to other islet and gut hormones (Ueno et al., 2019). Through these actions, SCO-267 likely activates multiple targets. Thus, full agonism of GPR40 may serve as a novel strategy to activate multiple targets for the treatment of NASH.

The primary limitation of this study is the lack of a long-term investigation of SCO-267 using the NAFLD mouse model. The present study evaluated the effects of 4-week SCO-267 treatment in CDAHFD-fed mice for 5 weeks. Thus, the effects of SCO-267 on a more

advanced disease condition remain unclear. Matsumoto et al. reported that up to 6-12 weeks of feeding with CDAHFD was necessary to induce histochemically observable fibrosis in CDAHFD-fed mice (Matsumoto et al., 2013), implying that the present study evaluated the treatment efficacy of SCO-267 in an early phase of NAFLD. Hence, longer study duration would be essential to fully understand how GPR40 full agonists act on the disease spectrum of a preclinical NAFLD model. In addition, although SCO-267-mediated stimulation of glucagon and GLP-1 secretion likely contribute to the treatment efficacy, the detailed mechanisms linking GPR40 full activation to the observed efficacy remain unclear, and this should be investigated in the future.

In conclusion, the GPR40 full agonist, SCO-267, stimulated glucagon and GLP-1 secretion in a mouse model of NAFLD. This is the first study to demonstrate that repeated administration of SCO-267 results in improved liver conditions in a non-diabetic mouse model of early-stage NAFLD. Notably, these effects were induced without any effects on glucose levels or body weight. Taken together, our findings demonstrate that SCO-267-mediated full activation of GPR40 was effective in alleviating NAFLD in mice and may induce similar treatment effects in patients.

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Authorship Contributions

Participated in research design: Ookawara, Watanabe, Moritoh

Conducted experiments: Ookawara, Matsuda

Performed data analysis: All authors

Wrote or contributed to the writing of the manuscript: Moritoh

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diabetic mice. *Diabetes Obes Metab* **13**:337-347.

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Footnotes

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Figure Legends

Figure 1. Effects of SCO-267 on glucagon and GLP-1 secretion in CDAHFD-fed mice.

(A) Glucagon levels and (B) GLP-1 levels after drug dosing. SCO-267 stimulated glucagon

and GLP-1 secretion in CDAHFD-fed mice. Values are presented as mean ± S.D. (n = 5). †

and ‡ $P < 0.025$ vs. vehicle by one-tailed Williams' test and one-tailed Shirley–Williams test,

respectively. AUC_{0-2h}, area under the curve from 0 to 2 h; CDAHFD, choline-deficient,

L-amino acid-defined, high-fat diet; SCO, SCO-267.

Figure 2. Effects of repeated doses of SCO-267 on metabolic parameters in

CDAHFD-fed mice.

(A) Daily food intake, (B) change in body weight during the study, (C) plasma glucose, and

(D) plasma triglyceride at the end of the 4-week study. Baseline body weight for CDAHFD

group was 25.4 ± 1.6 g. SCO-267 did not affect food intake, body weight, plasma glucose,

or plasma triglyceride levels. Values are presented as mean ± S.D. (n = 8). * $P < 0.05$ vs.

vehicle by Student's t-test. CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet;

Veh, vehicle; SCO, SCO-267; Alo, alogliptin benzoate; Dapa, dapagliflozin; SD, standard

diet.

Figure 3. Effects of repeated doses of SCO-267 on liver parameters in CDAHFD-fed mice.

(A) Liver triglyceride content, (B) liver weight, (C) liver collagen content, and (D) plasma alanine aminotransferase levels at the end of the 4-week study. SCO-267 decreased liver triglyceride content, liver weight, liver collagen content, and plasma alanine aminotransferase levels. Values are presented as mean ± S.D. (n = 8). †$P < 0.025$ vs. vehicle by one-tailed Williams' test. CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Veh, vehicle; SCO, SCO-267; Alo, alogliptin benzoate; Dapa, dapagliflozin; SD, standard diet.

Figure 4. Effects of repeated doses of SCO-267 on liver TBARS levels in CDAHFD-fed mice.

Liver TBARS levels at the end of the 4-week study. SCO-267 decreased liver TBARS levels. Values are presented as mean ± S.D. (n = 8). ‡ $P < 0.025$ vs. vehicle by one-tailed Shirley–Williams test. CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Veh, vehicle; SCO, SCO-267; Alo, alogliptin benzoate; Dapa, dapagliflozin; SD, standard diet; TBARS, thiobarbituric acid reactive substances.

Figure 5. Effects of repeated doses of SCO-267 on liver mRNA levels in CDAHFD-fed mice.

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Liver levels of mRNAs related to (A) mitochondrial function, (B) beta-oxidation pathway,

(C) lipogenesis pathway, (D) inflammation pathway, (E) reactive oxygen species generation,

and (F) fibrosis pathway at the end of the 4-week study. SCO-267 elevated mRNA levels of

molecules with roles in mitochondrial function and beta-oxidation and inhibited those with

roles in lipogenesis, inflammation, reactive oxygen species generation, and fibrosis in the

liver. Values are presented as mean ± S.D. (n = 7-8). † and ‡ $P < 0.025$ vs. vehicle by

one-tailed Williams' test and Shirley–Williams test, respectively. * and ** $P < 0.05$ and $P <$

0.01 vs. vehicle by Student's t-test. # and ## $P < 0.05$ and $P < 0.01$ vs. vehicle by

Aspin–Welch test. CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Veh,

vehicle; SCO, SCO-267; Alo, alogliptin benzoate; Dapa, dapagliflozin; SD, standard diet;

Tfam, transcription factor A, mitochondrial; *Ppara*, peroxisome proliferator activated

receptor α; *Acadl*, acyl-CoA dehydrogenase, long chain; *Srebf1*, sterol regulatory element

binding transcription factor 1; *Cd36*, CD36 molecule; *Tnf*, tumor necrosis factor; *Ccl2*,

chemokine (C-C motif) ligand 2; *Il6*, interleukin-6; *Tgfb1*, transforming growth factor β 1;

Ncf1, neutrophil cytosol factor 1; *Cybb*, cytochrome b-245, beta polypeptide; *Col1a1*,

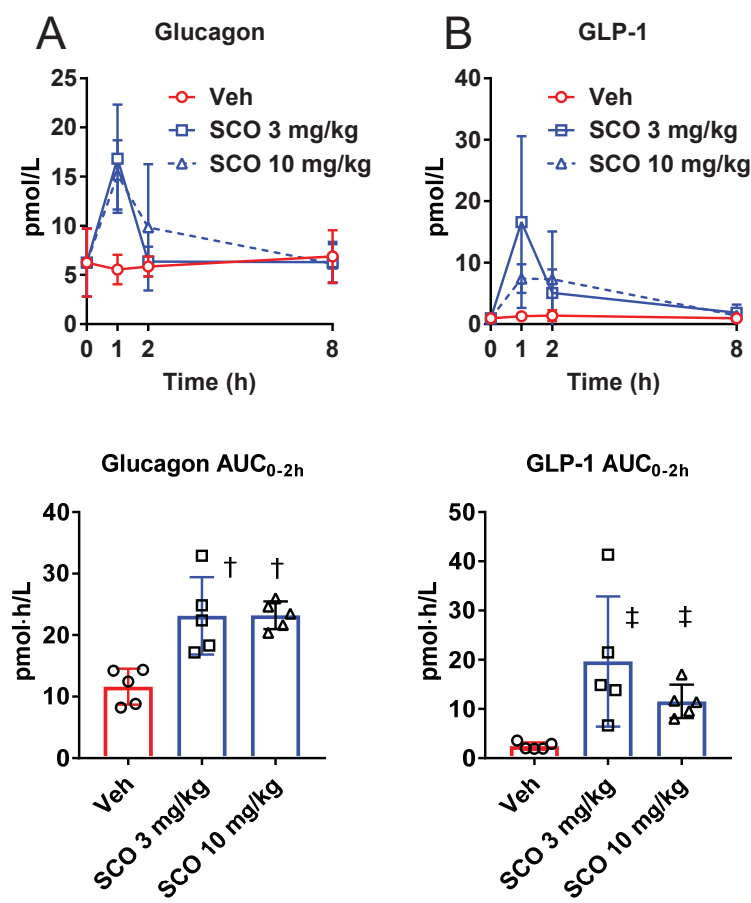collagen type 1, alpha 1; *Acta2*, actin, alpha 2, smooth muscle, aorta.
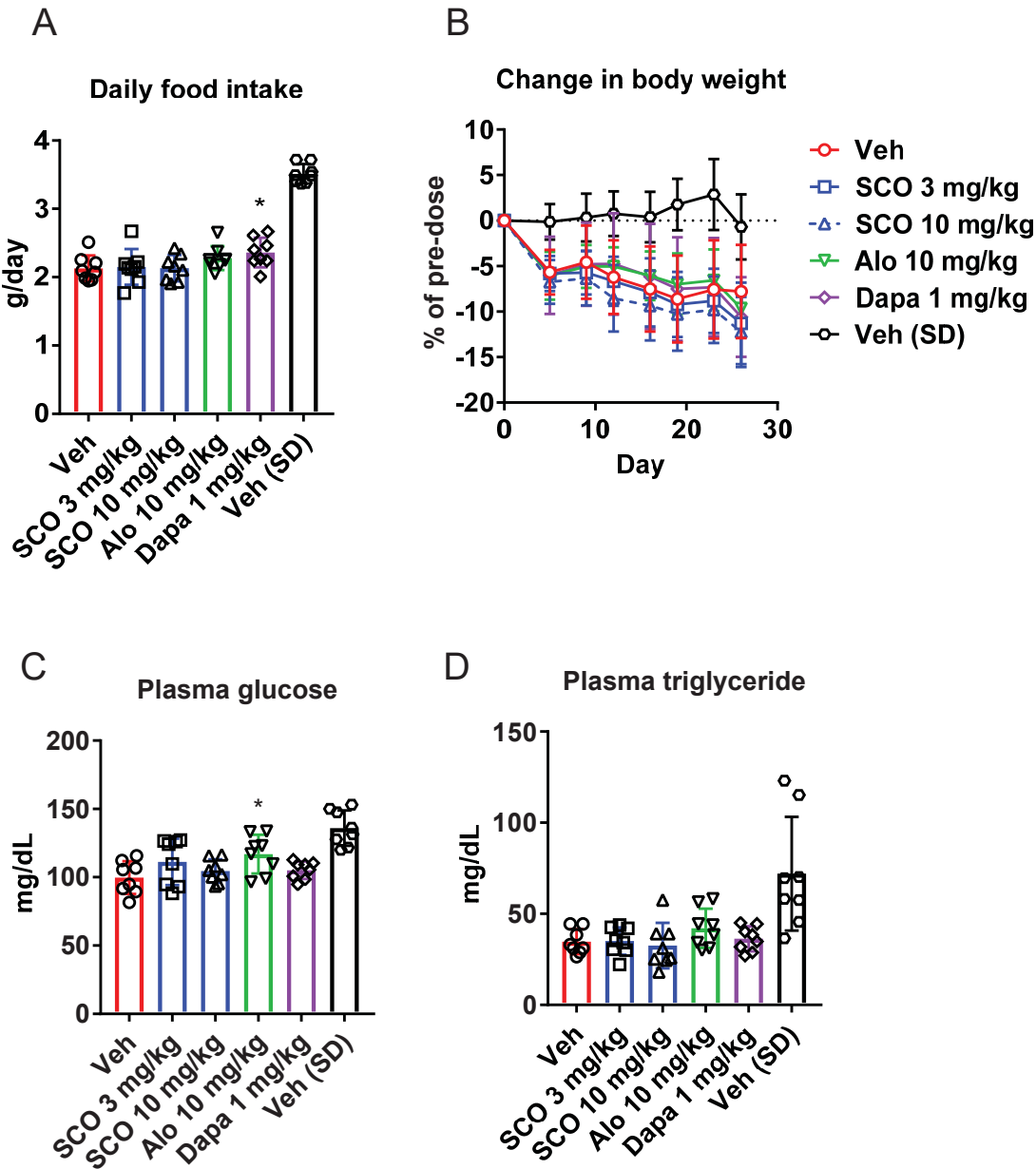
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Figure 1

A

Daily food intake

B

Change in body weight

C

Plasma glucose

D
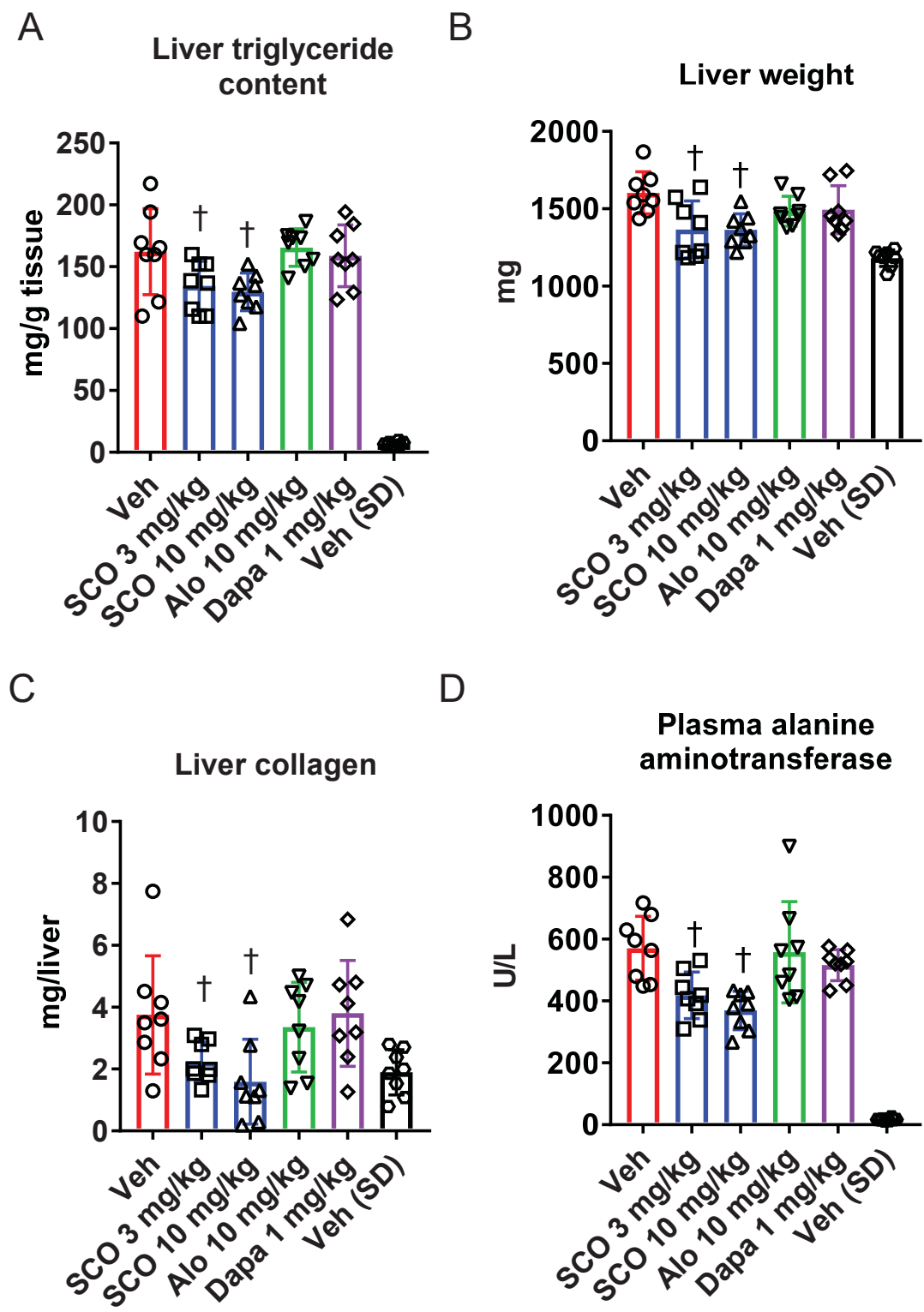
Plasma triglyceride
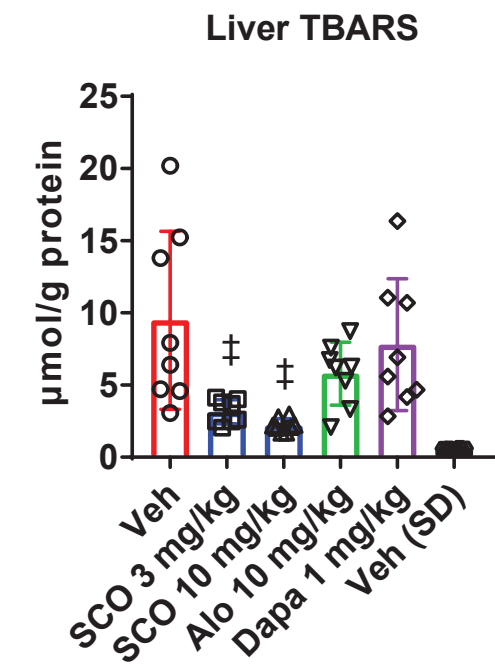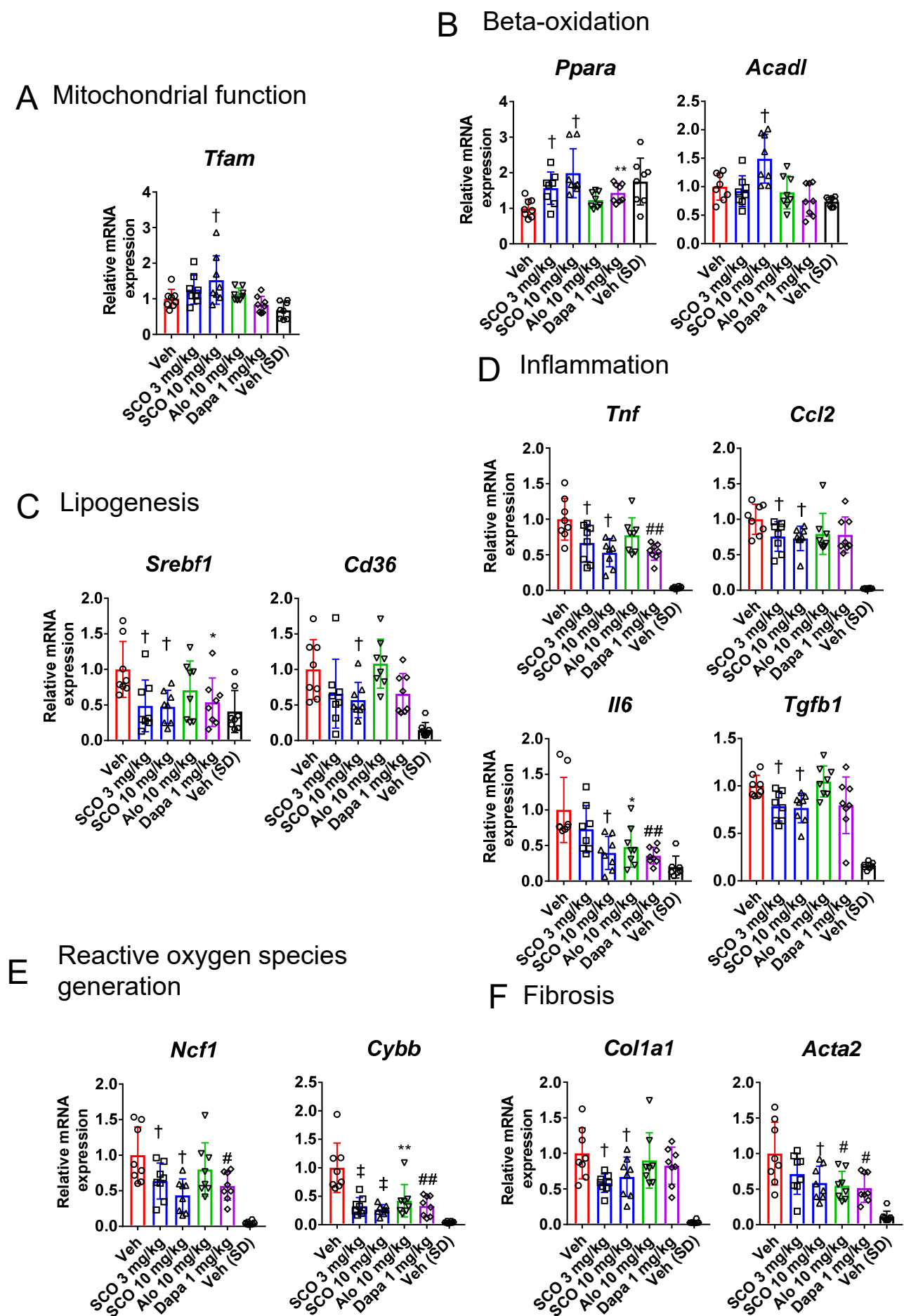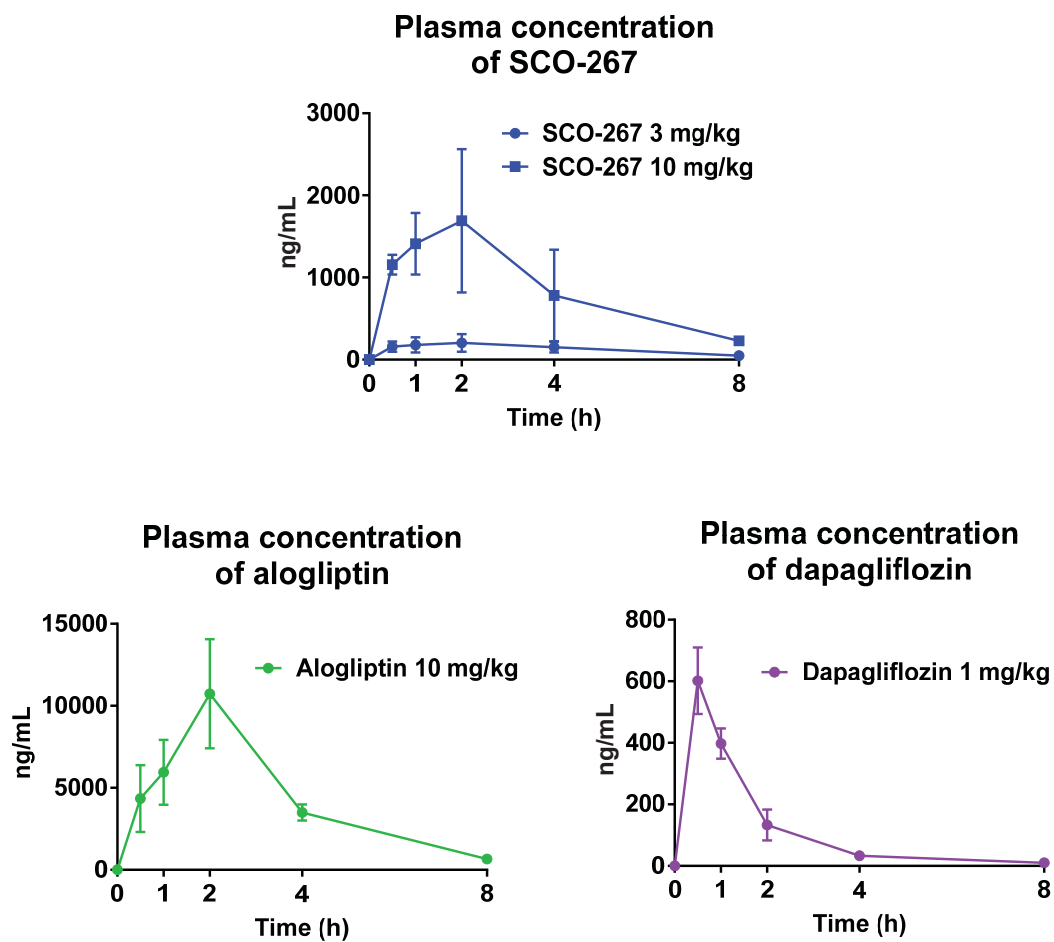

Figure 2

Figure 3

Liver TBARS

Figure 4

The GPR40 full agonist SCO-267 improves liver parameters in a mouse model of

nonalcoholic fatty liver disease without affecting glucose or body weight

Mitsugi Ookawara, Keisuke Matsuda, Masanori Watanabe, Yusuke Moritoh

SCOHIA PHARMA Inc., Kanagawa, Japan

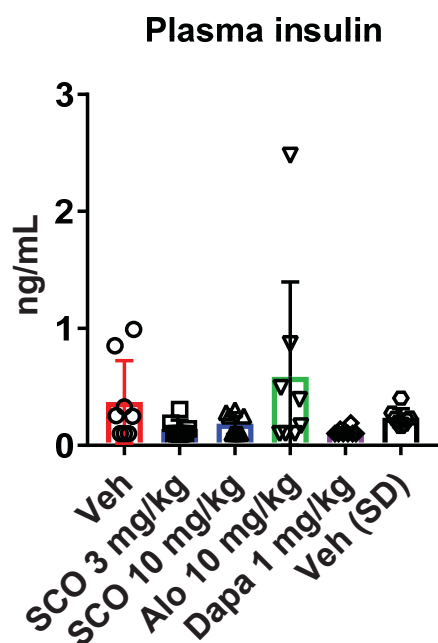*Journal of Pharmacology and Experimental Therapeutics*

Supplementary information

Supplementary Figure 1. Pharmacokinetic analysis of CDAHFD-fed mice
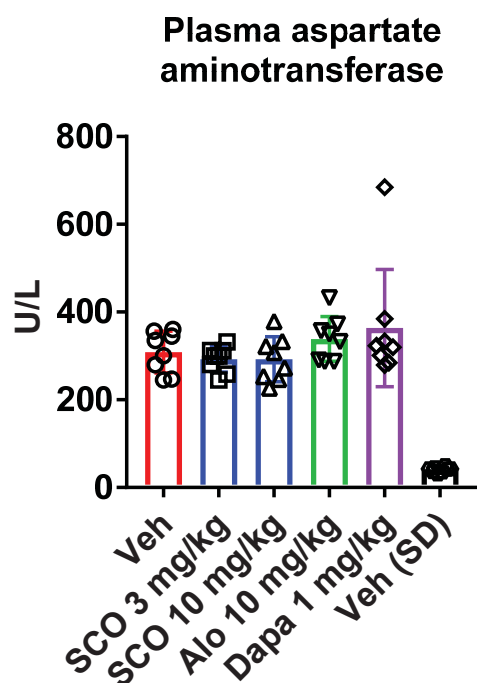
Each compound was dosed orally, and plasma concentration was determined. Values are presented as mean ± S.D. (n = 4). CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet.

Supplemental Figure 2. Plasma insulin levels in CDAHFD-fed mice

SCO-267 did not cause any significant effect on plasma insulin levels in the 4-week study of

CDAHFD-fed mice. Values are presented as mean ± S.D. (n = 8). CDAHFD, choline-deficient,

L-amino acid-defined, high-fat diet; Veh, vehicle; SCO, SCO-267; Alo, alogliptin benzoate;

Dapa, dapagliflozin; SD, standard diet.

Supplemental Figure 3. Plasma aspartate aminotransferase levels in CDAHFD-fed mice

SCO-267 did not cause any significant effect on plasma aspartate aminotransferase levels in the 4-week study of CDAHFD-fed mice. Values are presented as mean ± S.D. (n = 8). CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Veh, vehicle; SCO, SCO-267; Alo, alogliptin benzoate; Dapa, dapagliflozin; SD, standard diet.
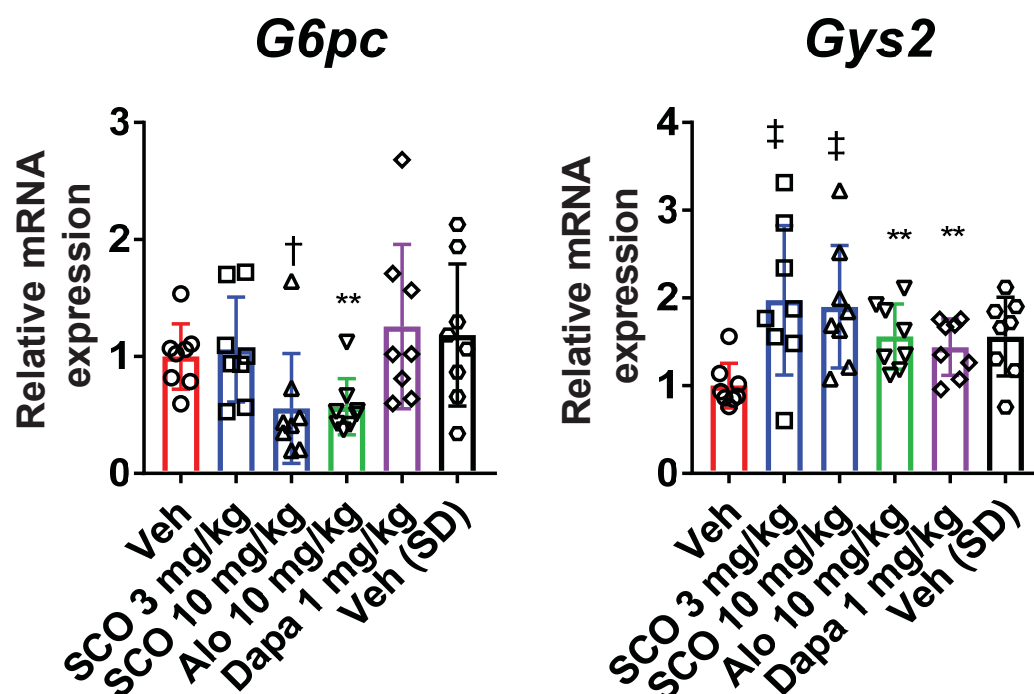
Supplemental Figure 4. Liver mRNA levels of *G6pc* and *Gys2* in CDAHFD-fed mice

Values are presented as mean ± S.D. (n = 8). CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Veh, vehicle; SCO, SCO-267; Alo, alogliptin benzoate; Dapa, dapagliflozin; SD, standard diet; *G6pc*, glucose-6-phosphatase, catalytic subunit; *Gys2*, glycogen synthase 2. † and ‡ $P < 0.025$ vs. vehicle by one-tailed Williams' test and Shirley–Williams test, respectively. ** $P < 0.01$ vs. vehicle by Student's *t*-test.
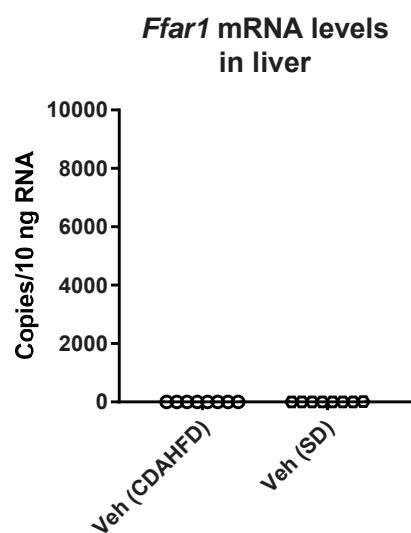
Supplemental Figure 5. Liver mRNA levels of *Ffar1*

Liver mRNA levels of GPR40 (*Ffar1*) in vehicle-treated mice fed with either CDAHFD or standard diet (n = 8). *Ffar1* mRNA was undetectable in liver of both groups. CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; Veh, vehicle; SD, standard diet.

Supplementary Table 1. Taqman Gene Expression Assays used for qPCR

Gene name	Code number	Uniprot Entry
Acta2	Mm00725412_s1	P62737
Ccl2	Mm99999056_m1	P10148
Cd36	Mm01135198_m1	Q08857
Col1a1	Mm00801666_g1	P11087
G6pc	Mm00839363_m1	P35576
Gapdh	Mm99999915_g1	P16858
Il6	Mm99999064_m1	P08505
Srebf1	Mm00550338_m1	Q9WTN3
Tgfb1	Mm01178820_m1	P04202
Tnf	Mm00443258_m1	P06804

Supplementary Table 2. Primer sequences for SYBR green qPCR

Gene name		Sequences (5′→3′)	Uniprot Entry
Acadl	Forward	TCAACAGCAGTTACTTGG	P51174
	Reverse	GACAATATCTGAGTGGAG	
Cybb	Forward	ACTCCTTGGGTCAGCACTGG	Q61093
	Reverse	GTTCCTGTCCAGTTGTCTTCG	
Gys2	Forward	CCTTGGGGTGTTTCCATCGTAC	Q8VCB3
	Reverse	CGGAGAGGTTTGTAGTCACACTGG	
Ncf1	Forward	ACATCACAGGCCCCATCATCCTTC	Q09014
	Reverse	ATGGATTGTCCTTTGTGCC	
Ppara	Forward	CGTACGGCAATGGCTTTATC	P23204
	Reverse	AACGGCTTCCTCAGGTTCTT	
Tfam	Forward	GGAATGTGGAGCGTGCTAAAA	P40630
	Reverse	TGCTGGAAAAACACTTCGGAATA	

Gene expression analysis for mouse *Ffar1*

To determine the absolute copy numbers of the mouse GPR40 mRNA (*Ffar1*), a standard curve

was generated by amplifying known concentrations of synthetic oligonucleotides and the target

gene copy number was calculated using this curve. All oligonucleotide primers and dual-labelled

(FAM-TAMRA) oligonucleotide probes synthesized by Sigma-Aldrich (Supplementary Table

3). Amplification was performed under the following conditions: initial denaturation at 95 °C for

1 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at

60 °C for 45 s on the ABI PRISM 7900HT Sequence Detector (Thermo Fisher Scientific) using

the EXPRESS qPCR supermix (Thermo Fisher Scientific).

Supplementary Table 3. Primers, probe, and standard sequence for mouse *Ffar1*

Sequence Name	Sequences (5′→3′)
Mm_*Ffar1*-F	TCAGGGCAGCTTGGGTGG
Mm_*Ffar1*-R	AGCCACATTGGAGGCATTATAGG
Mm_*Ffar1*-P	CTCTCCTCACACTCCTGCTCTGCCTGG
Mm_*Ffar1*-std	GAAGCTCAGGGCAGCTTGGGTGGCCGGAGGCGCTCTCCTCACACTC CTGCTCTGCCTGGGGGCCCTATAATGCCTCCAATGTGGCTAGTTT

Mm, mouse; F, forward primer; R, reverse primer; P, probe; std, standard oligonucleotide.