The combined effect of a farnesoid X receptor agonist and dipeptidyl peptidase-4 inhibitor on hepatic fibrosis

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Running title: Combined effects of FXR agonist and DPP4-I on NASH

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Abstract

Aim: Nonalcoholic steatohepatitis (NASH) has a broad clinicopathological spectrum (inflammation to severe fibrosis). The farnesoid X receptor agonist obeticholic acid (OCA) ameliorates the histological features of NASH; satisfactory anti-fibrotic effects have not yet been demonstrated. Here, we investigated the combined effects of OCA + a dipeptidyl peptidase-4 inhibitor (sitagliptin) on hepatic fibrogenesis in a rat model of NASH.

Methods: Fifty Fischer 344 rats were fed a choline-deficient L-amino-acid-defined (CDAA) diet for 12 weeks. The *in vitro* and *in vivo* effects of OCA+sitagliptin were assessed along with hepatic fibrogenesis, lipopolysaccharide (LPS)-toll-like receptor 4 (TLR4) regulatory cascade and intestinal barrier function. Direct inhibitory effects of both OCA+sitagliptin on activated hepatic stellate cells (Ac-HSCs) were assessed *in vitro*.

Results: OCA+sitagliptin potentially inhibited hepatic fibrogenesis along with Ac-HSC proliferation and hepatic transforming growth factor (TGF)-β1, α1(I)-procollagen and tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA expressions and hydroxyproline levels. OCA inhibited hepatic TLR4 expression and increased hepatic matrix metalloproteinase-2 (MMP-2) expression. OCA decreased intestinal permeability by ameliorating CDAA diet-induced zonula occludens-1 disruption, whereas sitagliptin directly inhibited Ac-HSC proliferation. The *in vitro* suppressive effects of OCA+sitagliptin on TGF-β1 and α1(I)-procollagen mRNA expressions and p38 phosphorylation in Ac-HSCs were almost consistent. Sitagliptin directly inhibited the regulation of Ac-HSC.

Conclusions: OCA+sitagliptin synergistically affect hepatic fibrogenesis by

counteracting endotoxemia induced by intestinal barrier dysfunction and suppressing Ac-HSC proliferation, respectively. Thus, OCA+sitagliptin may be a promising therapeutic strategy for NASH.

Keywords: hepatic stellate cells, nonalcoholic steatohepatitis, obeticholic acid, sitagliptin

Introduction

Prevalence of nonalcoholic steatohepatitis (NASH) is increasing globally. Patients with NASH risk developing cirrhosis and hepatocellular carcinoma[1]. The presence and severity of liver fibrosis observed in liver biopsy are relevant long-term prognostic histologic features in patients with NASH and liver fibrosis[2 3]. However, no Food and Drug Administration (FDA)-approved therapeutic agent is currently available for NASH[4]. Therefore, developing treatment regimens for patients with NASH and severe fibrosis is crucial[5].

Recently, NASH pathogenesis progression has been identified to involve 'multiple parallel hits', including the production of gut-derived endotoxin, which is very closely related to toll-like receptor 4 (TLR4) signalling in promotion of liver fibrosis promotion[6]. Bile acids play a pivotal role in the physiological defence of macroorganisms against bacterial endotoxins[7]. The farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily and is a bile acid receptor representing important targets for treating NASH[8,9]. It contributes bile acid homeostasis maintenance for the orchestration of proinflammatory responses mainly in the liver and intestine[10,11]. FXR agonist monotherapy significantly inhibited liver fibrosis in experimental models[12,13,14]. The FXR Ligand NASH Treatment (FLINT) study demonstrated that obeticholic acid (OCA) improves the histological features of NASH[9] and surrogate fibrosis markers[15]; however, satisfactory effects on hepatic fibrosis in humans with NASH were not noted. This may be because FXR was able to critically determine fibrotic responses in mice liver[10] and because FXR expression was low in human hepatic stellate cells (HSCs) and periductal myofibroblasts[16]. Although monotherapy exerted significant inhibitory effects on liver fibrogenesis in a rat model of

steatohepatitis[17,18], completely suppressing the cumulative development of liver fibrosis using a single agent may be difficult clinically[19]. Therefore, pharmacotherapeutic approaches combined with OCA (INT747) monotherapy are urgently required for treating NASH patients with fibrosis.

Dipeptidyl peptidase 4 (DPP4/CD26) is a 110-kDa cell-surface glycoprotein from the serine protease family that is expressed on the surface of reactive fibroblasts, including activated HSCs (Ac-HSCs)[17,20]. We previously demonstrated that the DPP4 inhibitor (DPP4-I) sitagliptin[21] at a clinically comparable dose successfully ameliorated liver fibrosis by inhibiting platelet-derived growth factor-BB-induced phosphorylation of Smad2/3, p38 MAPK and ERK1/2 in As-HSCs[17]. However, two randomised controlled trials recently showed that sitagliptin alone does not ameliorate liver fibrosis and liver fat in NAFLD patients[22,23]. Accordingly, OCA+sitagliptin treatment may be beneficial for patients with NASH fibrosis. This study evaluated the effects of OCA+sitagliptin on hepatic fibrogenesis and assessed the possible underlying mechanisms using a rat model of NASH.

Methods

Animals and reagents

The study included 50 male Fischer 344 (F344) rats aged 6 weeks (CLEA Japan, Inc., Tokyo, Japan). Rats were individually housed in hanging, wire-mesh, stainless steel cages under controlled conditions of temperature, 23°C±3°C; relative humidity, 50%±20%; air changes, 10–15/h; and light illumination, 12 h/day. They were allowed access to tap water ad libitum. OCA was provided by Intercept Pharmaceuticals Inc. (New York, NY) and Sumitomo Dainippon Pharma Co. Ltd. (Tokyo, Japan). Sitagliptin was purchased from Merck Ltd. (Tokyo, Japan), conventional chemical reagents from Nacalai Tesque (Kyoto, Japan) and choline-deficient (CDAA) and choline-sufficient (CSAA) L-amino acid-defined diets from CLEA Japan Inc. (Tokyo, Japan).

Animal treatment

All experiments were performed over a 12-week period. Rats were randomly divided into five groups (10 rats each). Rats in Group (G) 1 (negative control group) were fed a CSAA diet and provided distilled water as a vehicle and those in G2–5 were fed a CDAA diet. Rats in G2 were provided phosphate-buffered saline (PBS) through daily oral gavage for 12 weeks; those in G3, G4 and G5 were provided clinically equivalent doses of OCA (30 mg/kg/day), sitagliptin (150 mg/kg/day) and both OCA and sitagliptin through daily oral gavage, respectively. Food intake did not differ among the groups. At the end of the experimental period, rats were anaesthetised with isoflurane and several indices were investigated. All experiments were performed according to the guidelines for the proper care and use of laboratory animals and approved by the Animal Care and Use Committee of Nara Medical University.

Histological and immunohistochemical analyses

In all experimental groups, 5-μm-thick sections of formalin-fixed and paraffin-embedded liver specimens were processed for haematoxylin–eosin and sirius red staining to assess hepatic fibrosis. α-smooth muscle actin (α-SMA) represents a marker of fibroblastic cells, i.e., Ac-HSCs, whereas desmin is a marker of quiescent HSC and stains many pericentral fat-storing cells in normal rat liver [24]. Moreover, immunohistochemical staining for α-SMA (Dako, Kyoto, Japan) and desmin (nichirei, Tokyo, Japan) was performed [25, 26 27]. Stained sections were analysed using ImageJ software (Version 1.47, https://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD). Six microscopic visual fields (magnification ×40) per specimen from 10 rats were used for ImageJ analysis, as previously described[25, 28]. Histological features were semi-quantitatively assessed according to the NAFLD scoring system as previously described[29].

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was extracted from powdered frozen liver and intestinal tissues and isolated Ac-HSCs using the RNeasy mini kit (Qiagen, Tokyo, Japan). Total RNA (2 μg) from each sample was reverse transcribed to complementary DNA (cDNA) using the High-Capacity RNA-to-cDNA kit (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. mRNA expressions of transforming growth factor-β1 (TGF-β1), α1(I)-procollagen, TLR4, lipopolysaccharide (LPS)-binding protein (LBP), FXR, peroxisome proliferator-activated receptor-γ (PPAR-γ), small heterodimer partner

(SHP), bile salt export pump (BSEP), cholesterol 7-alpha-monooxygenase (CYP7A1), sodium taurocholate cotransporting polypeptide (NTCP), glucagon-like peptide-1 (GLP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-2 (MMP-2) and beta-actin (β-actin) were quantified by real-time PCR using SYBR Green on the Step One Plus sequence detection system (Applied Biosystems Inc.). PCR was performed as follows: samples were heated (20 s at 95°C) and subjected to 40 cycles (3 s at 95°C for denaturing and 30 s at 60°C for annealing). β-actin was used as an endogenous control. The primer sequences used were as follows: TGF-β1, forward 5′-CGG CAG CTG TAC ATT GAC TT-3′ and reverse 5′-AGC GCA CGA TCA TGT TGG AC-3′; α1(I)-procollagen, forward 5′-AGC TCC TGG GCC TAT CTG ATG A-3′ and reverse 5′-AAT GGT GCT CTG AAA CCC TGA TG-3′; TLR4, forward 5′-CCG CTC TGG CAT CAT CTT CA-3′ and reverse 5′-CCC ACT CGA GGT AGG TGT TTC TG-3′; LBP, forward 5′-AAC ATC CGG CTG AAC ACC AAG-3′ and reverse 5′-CAA GGA CAG ATT CCC AGG ACT GA-3′; FXR, forward 5′-TGG ACT CAT ACA GCA AAC AGA GA-3′ and reverse 5′-GTC TGA AAC CCT GGA AGT CTT TT-3′; PPAR-γ, forward 5′-AAC ATC CCC AAC TTC AGC AG-3′ and reverse 5′-TAC TGC GCA AGA ACT CAT GG-3′; SHP, forward 5′-AGA TGT TGA CAT CGC TGG CCT TCT-3′ and reverse 5′-AGA GCT GTT CCT AAG GAG CCA AGT-3′; BSEP, forward 5′-AAG GCA AGA ACT CGA GAT ACC AG-3′ and reverse 5′-TTT CAC TTT CAA TGT CCA CCA AC-3′; CYP7A1, forward 5′-CTG CAG CGA GCT TTA TCC AC-3′ and reverse 5′-CCT GGG TTG CTA AGG GAC TC-3′; NTCP, forward 5′-GCA TGA TGC CAC TCC TCT TAT AC-3′ and reverse 5′-TAC ATA GTG TGG CCT TTT GGA CT-3′; GLP-1, forward 5′-TCA TGC CAG GAG TAG GCT CT-3′ and reverse 5′-TCC ACC CAA GGC TTA TGC AG-3′; TIMP-1, forward 5′-GCA TGA TGC CAC TCC TCT TAT AC-3′ and

reverse 5′-TAC ATA GTG TGG CCT TTT GGA CT-3′; MMP-2, forward 5′-GCA TGA TGA CAC TCA TCT TAT AC-3′ and reverse 5′-TAC ATA GTG TGG CCT TTT GGA CT-3′and β-actin, forward 5′-GGA GAT TAC TGC CCT GGC TCC TA-3′ and reverse 5′-GAC TCA TCG TAC TCC TGC TTG CTG-3′.

Measuring portal endotoxin levels, intestinal permeability, hepatic inflammatory cytokines and hydroxyproline level

Portal vein serum was analysed for endotoxin using the endpoint assay method (LDL QCL 1000; BioWhittaker, Walkersville, MD). Samples were eluted in 30-ml pyrogen-free PBS at 20°C–25°C for 1 h, and dilutions were assayed using a 96-well microplate reader (Thermo Fisher Scientific Inc., MN). To assess intestinal permeability, rats received 125 mg/kg body weight of 40-kDa fluorescein isothiocyanate (FITC)-labelled dextran (Sigma-Aldrich, Oakville, ON, Canada) through oral gavage 4 h before their sacrifice. Blood samples were obtained from the portal vein. Plasma fluorescence was determined using a fluorescence spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) at excitation and emission wavelengths of 490 and 520 nm, respectively, using a series of known fluorescein isothiocyanate-dextran concentrations diluted in rat plasma. The inhibitory effects of OCA+sitagliptin on proinflammatory cytokine expressions (tumour necrosis factor-α, interferon [IFN]-γ and interleukin [IL]-6) were investigated using enzyme-linked immunosorbent assay. Hydroxyproline level was measured using a hydroxyproline detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Code No A030) according to the manufacturer's instructions.

Immunofluorescence analysis

Cryosections (10 μm) of the small intestine (terminal ileum) were prepared and fixed with 4% paraformaldehyde at 4°C for 10 min. Frozen sections were blocked with 10% normal goat serum in PBS and incubated with rabbit polyclonal anti-mouse zonula occludens-1 (ZO-1) antibody (1:100, Invitrogen Life Technologies, Carlsbad, CA) at 4°C overnight and then with donkey anti-rabbit secondary antibody conjugated with the DyLight 488 fluorochrome (Jackson Immuno Research Laboratories, West Grove, PA) at room temperature for 1 h. Sections were subsequently mounted in the VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA) with 4′,6-diamidino-2-phenylindole Fluoromount-G (DAPI) for fluorescent nucleic acid staining. Stained specimens were evaluated under a confocal scanning laser microscope (Leica TCSNT; Leica, Bensheim, Germany) equipped with a digital camera. Green fluorescence at excitation and emission wavelengths of 490 and 510 nm, respectively, corresponded with localised ZO-1 at the tight junctions of intestinal epithelial cells. Five images of each sample were randomly selected; the mean fluorescence intensity of five equally sized image regions was measured in each image using ImageJ software. The mean fluorescence intensity of negatively stained sections was subtracted from that of positively stained sections.

In vitro primary HSC assay

HSCs were isolated by sequentially digesting the liver with pronase and collagenase, as previously described[28]. Freshly isolated HSCs were plated (density, 5×10^5 cells/ml) on uncoated plastic dishes. After culture for 5 days, HSCs showed myofibroblast-like characteristics with reduced lipid vesicles and increased α-SMA expression. After HSC activation through culture for 7 days on plastic dishes, all cells showed uniform

distribution and α-SMA positivity. The effects of OCA+sitagliptin on Ac-HSC proliferation were colorimetrically assessed (Roche Applied Science, Laval, Canada) based on the cleavage of a tetrazolium salt (WST-1) by mitochondrial dehydrogenases to form formazan in viable cells. Briefly, Ac-HSCs were seeded in 96-well plates (5,000/well) with 100-µl foetal bovine serum-free media and incubated with/without OCA (2×10^{-6} M) and/or sitagliptin (10^{-6} M) at 37°C for 48 h. WST-1 (10 µl) was then added; after incubating for 1 h, absorbance was measured at 450 nm using a microplate reader (reference wavelength, 630 nm).

Cell signalling assay

The effects in Ac-HSCs of OCA (2×10^{-6} M) and sitagliptin (2×10^{-6} M) on the phosphorylation of mitogenactivated protein kinase (MAPK), including ERK1/2, p38, and Mothers Against Decapentaplegic Homolog 2/3 (Smad2/3), were measured by ELISA (Ray-Biotech, Inc., Norcross, GA, USA). Moreover, the effect of OCA+sitagliptin on the phosphorylation of Smad2/3 was evaluated by ELISA (R&D Systems, Inc., Minneapolis, MN USA). The cell lysate in each experiment was prepared in the presence or absence of OCA (2×10^{-6} M) and/or DPP4-I (2×10^{-6} M). The total and phosphorylated ERK1/2, p38 and Smad2/3 were assessed by ELISA according to the manufacturer's instructions.

Statistical analysis

Data were analysed using one-way ANOVA with Tukey's post hoc tests. All tests were two-tailed. All statistical analyses were conducted with GraphPad Prism version 6.04 (GraphPad Software, Inc., La Jolla, CA); $P < 0.05$ was considered statistically

significant.

Results

General findings

Data from all experimental groups are presented in Table S1. The final body weight was lesser in G2–G5 than that in G1. Relative liver weights were greater in G2–G5 than those in G1. The serum alanine aminotransferase (ALT) level was significantly higher in G2–G5 than that in G1 and significantly lower in G3 and G5 than that in G2. Plasma glucagon, serum glucose and insulin levels and quantitative insulin sensitivity check index were unaltered after sitagliptin treatment at the current dose. Similarly, no significant differences in hepatic glucagon-like peptide-1 (GLP-1) mRNA expression levels were observed among the experimental groups (Fig. 3A), indicating that local GLP-1 alteration was not involved in the anti-fibrotic effects of sitagliptin.

Changes in the NAFLD activity score

Microscopic examination showed significant reductions in steatosis, lobular inflammation and hepatocellular ballooning in G3 and G5 than in G2 (Fig. 7 and Table 2). These changes were accompanied by a significant decrease in the alanine aminotransferase level, indicating that OCA, but not sitagliptin, had cytoprotective and anti-inflammatory effects on hepatocytes.

Inhibitory effects of OCA and sitagliptin on hepatic fibrogenesis

Marked liver fibrosis with collagen fibre deposition was noted in G2. Hepatic fibrogenesis was significantly inhibited in G3 and G4 compared with that in G2 (Fig. 1A

and B). Hepatic fibrogenesis inhibition was greater in G5 than that in G3 and G4. No hepatic fibrosis was found in G1. Immunohistochemistry revealed that the number of α-SMA-positive Ac-HSCs significantly reduced with OCA+sitagliptin treatment (Fig. 2A). Computer-assisted semi-quantitative analysis showed that the number of α-SMA-positive cells significantly decreased in G5 than that in G3 and G4 (Fig. 2B). OCA treatment significantly increased the number of desmin-positive quiescent-HSCs (Supplementary figure S1A). The ratio of total HSC-(Desmin- and α-SMA-) positive areas to Ac-HSC-(α-SMA-) positive areas was marginally higher in G3 than in G2 (Supplementary figure S1B). Moreover, hepatic TGF-β1, α1(I)-procollagen and TIMP-1 mRNA expressions and hydroxyproline levels were significantly decreased in G5 than in G3 and G4 (Fig. 3B–E, respectively). These inhibitory effects were matched with the reduction in the fibrosis area. Hepatic MMP-2 expression was significantly augmented in G3 and G5 than in G2 (Fig. 3F).

Inhibitory effects of OCA and sitagliptin on TLR4 signalling

The LPS-TLR4 signalling cascade probably contributes to hepatic fibrogenesis. Hepatic TLR4 mRNA expression was significantly increased in G2 compared to that in G1 and reduced in G3 and G5 compared to that in G2 (Fig. 3G). However, intestinal TLR4 mRNA expression did not differ between the experimental groups (Fig. 3H). PPARγ is involved in the maintenance of a quiescent-HSCs phenotype, the development of which is promoted by the FXR-SHP regulatory cascade[30]. OCA induces a SHP-dependent upregulation of PPAR-γ in HSCs[30]. Hepatic FXR mRNA expression was not different among the experimental groups (Fig. 3I). Hepatic PPAR-γ mRNA expression was significantly suppressed in G2 than in G1, whereas hepatic SHP mRNA expression did

not differ between G1 and G2 (Fig. 3J and K). Hepatic PPAR-γ and SHP mRNA expressions were significantly augmented in G3 and G5 than in G2 (Fig. 3J and K).

Inhibitory effects of OCA and sitagliptin on LBP, intestinal permeability, portal endotoxin levels and hepatic inflammatory cytokines

Hepatic LBP mRNA expression, intestinal permeability and portal endotoxin levels were significantly augmented in G2 than in G1 and were significantly suppressed in G3 and G5 than in G2 (Fig. 3L and M and Table 1). TNF-α and IL-6 levels in the liver and IFN-γ levels in the liver and intestine significantly increased in G2 than in G1 and were significantly decreased in G3 and G5 than in G2 (Fig. 4A–D). Thus, the inhibitory effect of OCA on hepatic fibrogenesis may be mediated by cytoprotective and anti-inflammatory effects during hepatic inflammatory responses triggered by LPS.

Semi-quantitative determination of intestinal ZO-1 expression

The apical plasma membrane of epithelial cells showed ZO-1 immunoreactivity (Fig. 5A). Semi-quantitative immunofluorescence microscopy revealed lower ZO-1 expression in G2 than in G1 (Fig. 5B) and higher ZO-1 expression in G3 and G5 than in G2. Thus, the inhibitory effect of OCA on hepatic fibrogenesis in CDAA-induced NASH might be mediated, at least in a large part, by reducing the intestinal permeability through the restoration of intestinal tight-junction protein (TJP) expression and the reduction of LPS translocation.

Inhibitory effects of OCA and sitagliptin on Ac-HSCs *in vitro*

Microscopically, Ac-HSC proliferation after OCA+sitagliptin treatment showed no

relevant morphological changes in HSCs *in vitro* among the experimental groups. Sitagliptin alone and OCA+sitagliptin significantly inhibited Ac-HSC proliferation (Fig. 6A). However, TGF-β1 and α1(I)-procollagen mRNA expressions were suppressed by OCA+sitagliptin (Fig. 6B and C). OCA+sitagliptin treatment exerted more suppressive effect than either agent alone. The bile acid sensor FXR maintains bile acid homeostasis in HSCs and represses HSC activity via an FXR-SHP regulatory cascade[10]. OCA induces the expression of SHP and BSEP and represses the expression of the NTCP and CYP7A1, both of which are essential for the synthesis of bile acids from cholesterol[10]. FXR-dependent suppression of CYP7A1 is mediated by the transcriptional repressor SHP, which is an atypical orphan nuclear receptor lacking a DNA-binding domain[31]. OCA+sitagliptin significantly augmented SHP and BSEP mRNA expressions (Fig. 6D and E) and reduced NTCP and CYP7A1 mRNA expressions in Ac-HSCs (Fig. 6F and G). However, TLR4 and FXR mRNA expressions did not differ between the experimental groups (Fig. 6H and I). Thus, hepatic fibrogenesis attenuation may be mediated, at least largely, by sitagliptin-inhibited proliferation of HSCs.

Inhibitory effects of OCA and sitagliptin on the phosphorylation of ERK1/2, p38 and Smad2/3 in the Ac-HSCs

We next examined the effects of DPP4-I on the MAPK and Smad2/3 pathway in the Ac-HSCs. The p38 phosphorylation was individually suppressed both by OCA in Ac-HSCs and by sitagliptin. Combined treatment exerted a stronger inhibitory effect than that of either agent alone (Fig. 6K). The administration of sitagliptin alone or combined therapy significantly reduced ERK1/2 and Smad2/3 phosphorylation, whereas OCA alone had

no significant effect on the phosphorylation of either ERK1/2 or Smad2/3 (Fig. 6J and M).

Discussion

Here, we demonstrated that clinically equivalent doses of OCA+sitagliptin successfully ameliorate hepatic fibrosis and suppress Ac-HSCs in CDAA diet-induced NASH model rats. OCA+sitagliptin markedly and synergistically inhibited hepatic fibrogenesis. The anti-fibrotic effect was stronger in combination treatment than with either agent alone.

DPP4 exhibits pro-fibrotic behaviour in carbon tetrachloride (CCl4)-induced liver fibrosis[32]. However, αSMA expression showed no difference between the CCl4-treated wild-type and DPP4 gene-knockout mice[32]. DPP-4 inhibitor alleviates hepatic fibrosis by reducing the number of α-SMA-positive Ac-HSCs in rats[17]. This difference may be partly because DPP4 is linked to multiple signalling pathways associated with liver fibrosis, glucose tolerance and immune activation[20].

The liver and intestine are the dominant sites of TLR4-mediated inflammatory responses against gut bacteria-derived products, such as endotoxins that cross the gut barrier and involve several layers that defend against invading microbial pathogens. The LPS-TLR4 signalling cascade in the liver is essential for CDAA-induced hepatic fibrogenesis. However, consistent with our previous findings, intestinal TLR4-mediated signalling was not involved in hepatic fibrogenesis[6, 28, 33]. Translocated LPS derived from gut microflora mediates TLR4 activation in the liver; however, TLR4 in the intestine is required for this process[34]. However, we previously showed that TLR4-mediated signalling in intestinal epithelial cells regulated hepatic fibrogenesis in porcine serum-administered diabetic rats[33]. TLR4 activation in the intestine induces

intestinal mucosal inflammation by mediating extracellular regulated kinase and c-Jun NH2-terminal kinase phosphorylations in high-fat diet-fed Sprague–Dawley rats[35]. Moreover, OCA reduces bacterial translocation along with intestinal mucosal TLR4 signalling and inhibits intestinal inflammation in a CCl4-induced hepatic cirrhosis rat model[11]. The reason for the difference in the role of intestinal TLR4 signalling between these models remains indiscernible but may be related to the fact that intestinal epithelial cell expression of TLR4 differs among strains and species. The *in vitro* experiment showed that OCA prevented the LPS-associated downregulation of ZO-1 expression in intestinal epithelial cells, whereas OCA did not significantly alter FXR expression (data not shown), suggesting that TJP was not directly regulated by OCA in intestinal epithelial cells. These findings support the notion that OCA restores impaired intestinal barrier function associated with a reduction in the expression of TJP by downregulating IFN-γ expression in the intestine[14].

The effects of DPP4-Is on intestinal permeability have not been clarified. We found that sitagliptin had no significant effect on either intestinal epithelial permeability/TJP. It modulates the immunoreactivity and subcellular distribution of TJP by reducing high blood–retinal barrier permeability in diabetic rats[36]. Conversely, the DPP4-I MK0626 triggers damaged mucosal barrier repair by facilitating increases in glucagon-like peptide-2 receptor levels and intestinal epithelial cell proliferation in mice[37]. These discrepancies may partly be explained by differences in DPP4 activity among different organs and tissues. Furthermore, DPP4-Is exhibit marked differences in their chemical structures, offering structure-activity relationships associated with variations in pharmacodynamic and pharmacokinetic profiles[38].

Here, we employed a CDAA model to examine the effects of

OCA+sitagliptin on hepatic fibrosis. The CDAA diet induces histological changes similar to those observed in human NASH[18]. However, the CDAA dietary model of steatohepatitis does not display obesity, impaired glucose tolerance and insulin resistance, which are clinical characteristics of NASH in humans. Anti-fibrotic effects of sitagliptin do not involve local GLP-1 alteration in CDAA-induced NASH. To evaluate the precise pharmacological effects of drugs, further research is needed to examine the therapeutic impact under insulin resistance. We previously elucidated the effect of combined ARB on CDAA-induced liver fibrosis development in Otsuka Long-Evans Tokushima fatty (OLETF) rats[39] who generally suffer from obesity, insulin resistance and diabetes mellitus. The effect of OCA+sitagliptin on hepatic fibrosis in CDAA-induced OLETF rats remains to be elucidated. Although ALT levels in the CDAA-fed rats were significantly more elevated than those in the controls, the inflammation in the CDAA-diet model was not as severe as that in the CCl4-induced model of liver fibrosis, especially in the early stage[18]. CDAA-diet model is widely used as a NASH model as it shows histological sequences similar to those exhibited in chronic liver diseases[40,25,6, 28, 33].

Collectively, experimental studies have shown that OCA+sitagliptin act synergistically on hepatic fibrogenesis by counteracting endotoxemia induced by intestinal barrier dysfunction and suppressing Ac-HSC proliferation, respectively. DPP-4 inhibitor, sitagliptin in combination with OCA might be more effective against hepatic fibrogenesis in patients with NASH.

Conflict of Interest

The authors declare no conflicts of interest.

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Figure Legends

Fig. 1. (a) Microphotographs of liver sections stained with sirius red (original magnification ×40). Extensive liver fibrosis with accumulation of lipid droplets is observed in G2. Marked inhibition of fibrosis is noted in G3 and G4. A more potent inhibitory effect is noted in G5 than in G3 and G4. No fibrosis is observed in G1. (b) Semi-quantitative analysis findings are consistent with histological findings. Data are presented as mean±standard deviation (bars; n=10). Asterisks indicate statistically significant differences between indicated experimental groups (*P<0.05, **P<0.01). G1, Group 1 (choline-sufficient L-amino acid-defined diet); G2, Group 2 (choline-deficient L-amino acid-defined diet [CDAA]); G3, Group 3 (CDAA+obeticholic acid [OCA]); G4, Group 4 (CDAA+sitagliptin); G5, Group 5 (CDAA+OCA+sitagliptin)

Fig. 2. (a) Immunohistochemical analysis of hepatic α-smooth muscle actin (α-SMA) expression. (b) α-SMA immunohistochemical staining is assessed using image analysis software. Hepatic α-SMA expression is lower in G3 and G4 than in G2. A more potent inhibitory effect is noted in G5 than in G3 and G4. No α-SMA-positive cells are observed in G1. Data are presented as mean±standard deviation (bars; n=10). Asterisks indicate statistically significant differences between indicated experimental groups (*P<0.05, **P<0.01).

G1, Group 1 (choline-sufficient L-amino acid-defined diet); G2, Group 2 (choline-deficient L-amino acid-defined diet [CDAA]); G3, Group 3 (CDAA+obeticholic acid [OCA]); G4, Group 4 (CDAA+sitagliptin); G5, Group 5 (CDAA+OCA+sitagliptin)

Fig. 3. Effects of obeticholic acid (OCA)+sitagliptin on hepatic and intestinal mRNA expressions, hydroxyproline levels and intestinal permeability. (a) The hepatic mRNA expression of glucagon-like peptide-1 (GLP-1) does not differ among the experimental groups. The hepatic mRNA expressions of (b) transforming growth factor (TGF)-β1, (c) α1(I)-procollagen, (d) tissue inhibitor of matrix metalloproteinases 1 (TIMP1) and (e) hydroxyproline levels are lower in G5 than in G3 and G4. (f) The hepatic mRNA expression of matrix metalloproteinase-2 (MMP-2) is higher in G3 and G5 than in G2. (g) The hepatic mRNA expression of toll-like receptor 4 (TLR4) is lower in G3 and G5 than in G2. (h) Neither the intestinal mRNA expression of TLR4 nor (i) hepatic mRNA expression of farnesoid X receptor (FXR) differs among the experimental groups. The hepatic mRNA expression of (j) peroxisome proliferator-activated receptor-γ (PPAR-γ) and (k) small heterodimer partner (SHP) is higher in G3 and G5 than in G2. (l) The hepatic mRNA expression of LBP and (m) intestinal permeability is higher in G2 than in G1 and lower in G3 and G5 than in G2. Data are presented as mean±standard deviation (bars; n=10). Asterisks indicate statistically significant differences between indicated experimental groups (*$P<0.05$, **$P<0.01$).

G1, Group 1 (choline-sufficient L-amino acid-defined diet); G2, Group 2 (choline-deficient L-amino acid-defined diet [CDAA]); G3, Group 3 (CDAA+obeticholic acid [OCA]); G4, Group 4 (CDAA+sitagliptin); G5, Group 5 (CDAA+OCA+sitagliptin); NS, not significant

Fig. 4. Effects of obeticholic acid (OCA)+sitagliptin on proinflammatory cytokine expression. (a) Tumour necrosis factor-α (TNF-α) and (b) interleukin 6 (IL-6) levels in the liver and (c and d) interferon-γ (IFN-γ) levels in the liver and intestine are higher in

G2 than in G1 and are lower in G3 and G5 than in G2. Data are presented as mean±standard deviation (bars; n=10). Asterisks indicate statistically significant differences between indicated experimental groups (*P<0.05, **P<0.01).
G1, Group 1 (choline-sufficient L-amino acid-defined diet); G2, Group 2 (choline-deficient L-amino acid-defined diet [CDAA]); G3, Group 3 (CDAA+obeticholic acid [OCA]); G4, Group 4 (CDAA+sitagliptin); G5, Group 5 (CDAA+OCA+sitagliptin); NS, not significant

Fig. 5. Effects of obeticholic acid (OCA)+sitagliptin on intestinal zonula occludens-1 (ZO-1) expression assessed with fluorescence microscopy. (a) Confocal immunofluorescence microscopy is used to evaluate the effects of OCA and sitagliptin on ZO-1 expression. (b) Semi-quantitative immunofluorescence microscopic analysis shows lower ZO-1 expression in G2 than in G1 and higher ZO-1 expression in G3 and G5 than in G2. Data are presented as mean±standard deviation (bars; n=10). Asterisks indicate statistically significant differences between indicated experimental groups (*P<0.05, **P<0.01).
G1, Group 1 (choline-sufficient L-amino acid-defined diet); G2, Group 2 (choline-deficient L-amino acid-defined diet [CDAA]); G3, Group 3 (CDAA+obeticholic acid [OCA]); G4, Group 4 (CDAA+sitagliptin); G5, Group 5 (CDAA+OCA+sitagliptin); NS, not significant

Fig. 6. Effects of obeticholic acid (OCA)+sitagliptin on activated hepatic stellate cell (Ac-HSC) in vitro. (a) Ac-HSC proliferation is inhibited by sitagliptin alone and combined with OCA. mRNA expressions of (b) transforming growth factor (TGF)-β1

and (c) α1(I)-procollagen are suppressed by both OCA+sitagliptin. Additionally, the suppressive effect is greater for combined OCA+sitagliptin than for either agent alone. The mRNA expressions of (d) small heterodimer partner (SHP) and (e) bile salt export pump (BSEP) are increased, and mRNA expressions of (f) sodium taurocholate cotransporting polypeptide (NTCP) and (g) cholesterol 7-alpha-monooxygenase (CYP7A1) are reduced by OCA alone and when combined with sitagliptin. However, (h) toll-like receptor 4 (TLR4) and (i) farnesoid X receptor (FXR) mRNA expressions are not different from the groups. (j) ERK1/2 and (l) smad2/3 phosphorylation are reduced by OCA alone and when combined with sitagliptin. (k) p38 phosphorylation is suppressed by both OCA+sitagliptin. Combined therapy exerts more potent suppression than monotherapy with either agent. Data are presented as mean±standard deviation (bars; n=8). Asterisks indicate statistically significant differences between indicated experimental groups (*P<0.05, **P<0.01).

OCA, obeticholic acid; SITA, sitagliptin; OD, optical density; NS, not significant

Fig 7. Representative images of haematoxylin–eosin staining from liver sections of different experimental groups (original magnification ×40).

Microscopic examination showed significant reductions in steatosis, lobular inflammation and hepatocellular ballooning in G3 and G5 compared to that in G2.

G1, Group 1 (choline-sufficient L-amino acid-defined diet); G2, Group 2 (choline-deficient L-amino acid-defined diet [CDAA]); G3, Group 3 (CDAA+obeticholic acid [OCA]); G4, Group 4 (CDAA+sitagliptin); G5, Group 5 (CDAA+OCA+sitagliptin)

Supplementary fig. S1

Inhibitory effect of OCA on HSC activation during hepatic fibrogenesis.

(a) The number of α-SMA-positive Ac-HSCs is significantly reduced with OCA treatment. Contrarily, OCA treatment significantly increases the number of desmin-positive quiescent-HSCs. (b) Computer-assisted semi-quantitative analysis shows that the ratio of total HSC (Desmin- and α-SMA-) positive areas to Ac-HSC (α-SMA-) positive areas is marginally higher in OCA treated group than in CDAA control group.