Mosapride citrate improves nonalcoholic steatohepatitis with increased fecal lactic acid bacteria and plasma glucagon-like peptide-1 level in a rodent model

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Okubo H, Nakatsu Y, Sakoda H, Kushiyama A, Fujishiro M, Fukushima T, Matsunaga Y, Ohno H, Yoneda M, Kamata H, Shinjo T, Iwashita M, Nishiuma F, Asano T. Mosapride citrate improves nonalcoholic steatohepatitis with increased fecal lactic acid bacteria and plasma glucagon-like peptide-1 level in a rodent model. Am J Physiol Gastrointest Liver Physiol 308: G151–G158, 2015. First published November 26, 2014; doi:10.1152/ajpgi.00198.2014.—Several lines of evidence have suggested a role of gut microbiota in the etiology of nonalcoholic steatohepatitis (NASH). NASH subjects reportedly showed a prolonged orocecal transit time coexistent with small intestinal bacterial overgrowth. We considered the possibility that enhanced gastrointestinal motility would influence gut microbiota and thus investigated the effects of the gastroprokinetic agent mosapride citrate (MC) on gut microbiota and the development of NASH using a methionine-choline deficient (MCD) diet-fed rodent model. Mice were divided into three groups, given the normal chow diet (NCD), the MCD diet, or the MCD diet containing 10 mg·kg−1·day−1 of MC (MCD plus MC) for 6 wk. NASH development was evaluated based on hepatic histochemical findings, serum parameters and various mRNA and/or protein expression levels. MC treatment suppressed MCD diet-induced NASH development, with reduced serum lipopolysaccharide and increased plasma glucagon-like peptide-1 (GLP-1) concentrations. Calculation of the relative abundance of each strain based on gut microbiota analyses indicated lactic acid bacteria specifically, such as Bifidobacterium and Lactobacillus, in feces to be decreased in the MCD, compared with the NCD group. Interestingly, the reduction in lactic acid bacteria in the MCD diet group was reversed in the MCD plus MC group. In addition, colon inflammation observed in the MCD diet group was reduced in the MCD plus MC group. Therefore, MC showed a protective effect against MCD diet-induced NASH development in our rodent model, with possible involvements of increased fecal lactic acid bacteria, protection against colon inflammation and elevated plasma GLP-1.

mosapride citrate; inflammation; gut microbiota; nonalcoholic steatohepatitis; glucagon-like peptide-1

THE INCIDENCE AND PREVALENCE of nonalcoholic fatty liver disease (NAFLD) are increasing to epidemic proportions around the world. A subset of individuals with NAFLD develops nonalcoholic steatohepatitis (NASH), characterized by hepatocellular lipid accumulation along with inflammation and varying degrees of fibrosis. Some NASH patients eventually develop cirrhosis and/or liver cancer, and at least some of these individuals will ultimately die from liver-related diseases (26, 34, 35). However, the current standard of care for NAFLD/NASH is limited to ameliorating components of the metabolic syndrome through weight loss with lifestyle interventions or surgery (22, 33).

Gut microbiota participate not only in the digestion and absorption of nutrients, but also in maintaining homeostasis of host immunity (29) and metabolism (39). Thus disruption of intestinal homeostasis and alterations of the intestinal microbiota have been considered to contribute to the pathogenesis of many disorders, including liver disease (3, 10, 37). In fact, several lines of evidence have suggested a role of gut microbiota in the etiology of NAFLD/NASH (1, 24, 28). Thus microbiota appear to be an important factor affecting the development and progression of NAFLD/NASH, possibly through several mechanisms including lipopolysaccharide (LPS) production in the gut, induction of the inflammatory cascade, modulation of insulin sensitivity, and so on (1, 5, 9). Gut microbiota composition was reported to be influenced by various factors such as genetic background, diet, drug treatment, and gut motility (13).

In addition, NASH subjects reportedly had a prolonged orocecal transit time coexistent with small intestinal bacterial overgrowth (41). We considered the possibility that a drug affecting gastrointestinal motility would influence gut microbiota and the development of NASH, and thus investigated the effects of the gastroprokinetic agent mosapride citrate (MC) using a methionine-choline deficient (MCD) diet-induced NASH rodent model. Herein, we present evidence that MC exerts a protective effect against this MCD diet-induced NASH development, possibly involving increased fecal lactic acid bacteria, reduced serum LPS and increased glucagon-like peptide-1 (GLP-1). The results obtained employing this rodent...
model raise the possibility of MC being an effective treatment for NASH.

MATERIALS AND METHODS

Animals and treatments. C57BL/6 mice (SLC, Hamamatsu, Japan) were crossbred and kept in the same environment. They were housed in temperature and light controlled rooms with free access to food and water. At 6 wk of age, the mice were randomly divided into three experimental groups and fed normal chow diet (NCD; Oriental Yeast, Tokyo, Japan; n = 12), the MCD diet (MCD; Oriental Yeast, Tokyo, Japan; n = 12), or the MCD diet containing 10 mg·kg⁻¹·day⁻¹ MC (MCD plus MC) (n = 12) for 6 wk. MC was obtained from Dainippon Sumitomo Pharma, (Osaka, Japan). The mice were killed, and their sera, livers, and colons were collected. The fecal contents of the colon were weighed at the time death. The animals were handled in accordance with the guidelines for the care and use of experimental animals published by the Japanese Association for Laboratory Animal Science.

Histochemical studies. Paraffin-embedded liver sections were stained with hematoxylin and eosin and azan. Histological evaluations were performed employing the histological scoring system for NAFLD (21). Colon sections were immunohistochemically stained with NFkB p65 antibody, and the positive cells were counted as previously described (31).

Biochemical analysis. Hepatic total lipid was extracted and then assayed using the Folch method (16). The triglyceride content was assayed with the Triglyceride E test (Wako, Osaka, Japan). Serum alanine aminotransferase activity was determined using a Transaminase C-II test Wako kit (Wako). The liver hydroxyproline content was measured using a Hydroxyproline Colorimetric Assay Kit (BioVision, Milpitas, CA). The serum LPS concentration was determined using the LAL kit endpoint QCL-1000 (Walkersville, MD), according to the manufacturer’s instructions. The plasma active GLP-1 level was measured employing a mouse active GLP-1 ELISA kit (Shibayagi, Gunma, Japan). The plasma glucose concentration was measured using the Medisafe Mini system (TERUMO, Tokyo, Japan).

Quantitative real-time reverse transcription PCR. Total RNA was extracted from mouse livers and colons using Sepasol reagent (Nakalai Tesche, Kyoto, Japan). First-strand cDNAs were synthesized using PrimeScript reverse transcriptase with oligo (dT). Quantitative real-time reverse transcriptional PCR (qRT-PCR) was performed using SYBR Green PCR master mix (Invitrogen, Tokyo, Japan) on a CFX96 real time PCR system (Bio-Rad, Tokyo, Japan). Relative mRNA gene levels were normalized to the GAPDH mRNA level and relative expressions were determined by the comparative Ct method.

The designed primers were as follows: α-smooth muscle actin (α-SMA) forward: ACCAACTGGGACGACATGGAA, α-SMA reverse: TGTCAGCAGTGTCGGATGCTC; tissue inhibitor of metalloproteinase-1 (TIMP-1) forward: ATTCAAGGCTGTGGGAAATG, TIMP-1 reverse: CTCAGAGTACGCCAGGGAAC; transforming growth factor β (TGF-β) forward: GGAAGGACCTGGGTTGGAAG, TGF-β reverse: GGACAACTGCTCCACCTTGG; sterol regulatory element binding protein 1c (SREBP1c) forward: TAGAGCATATCCCCCAGGTG, SREBP1c reverse: GTGTCGCGCCAAGAGAAATGA; carnitine

![Graphs and figures](image-url)

Fig. 1. Mosapride citrate (MC) treatment suppressed NASH development. Mice were divided into three groups, given the normal chow diet (NCD), the methionine-choline deficient (MCD) diet or the MCD diet containing 10 mg/kg/day of MC (MCD plus MC) for 6 wk and then killed.

Liver sections stained with hematoxylin and eosin.

Magnification, ×20.

NAFLD activity score

Hepatic triglyceride levels

Serum alanine aminotransferase levels

Data are presented as means ± SE. *Statistical significance P < 0.05.
were log transformed before the analysis. All statistical analyses were honestly significant difference test. Variables not normally distributed for 6 wk. Mice fed the MCD diet lost weight compared with mice were fed the NCD, the MCD diet, or the MCD plus MC. To investigate the effects of MC on the development of NASH, mice were divided into three groups, given the normal chow diet (NCD), the methionine-choline deficient (MCD) diet or the MCD diet containing 10 mg/kg/day of MC (MCD plus MC) for 6 wk and then killed. Overall, this series of data showed MC treatment to markedly suppress MCD diet-induced NASH development.

**MC suppressed liver fibrosis.** Progression to NASH is accompanied by fibrosis and cirrhosis. Azan staining performed to evaluate fibrotic changes revealed marked collagen deposition in the MCD diet group, while these fibrotic changes were suppressed in the MCD plus MC group (Fig. 1 D). In accordance with the histochemical analysis, the hepatic hydroxyproline content and the mRNA levels of fibrosis markers such as \( \alpha \)-SMA, TIMP-1, and TGF-β were elevated in the MCD diet group, while these elevations were significantly reversed in the MCD plus MC group (Fig. 2 A). These data showed MC treatment to suppress liver fibrosis.

**MC treatment suppressed the expressions of genes involved in NASH pathogenesis.** To investigate the molecular mechanisms underlying the resistance to NASH development con-
ferred by MC treatment, the mRNA expression levels of lipogenic enzyme genes such as SREBP-1c, as well as the β-oxidation enzyme genes CPT-1 and CD36, were examined by quantitative real-time PCR. The mRNA levels of SREBP-1c were elevated in the MCD diet group, while these levels were normalized in the MCD plus MC group (Fig. 3A). On the other hand, the mRNA levels of CPT-1 were slightly decreased in the MCD diet compared with the NCD group and were further decreased in the MCD plus MC group (Fig. 3B). The mRNA levels of CD36 were elevated in the MCD diet compared with the NCD group, and there was no significant difference between the MCD diet and the MCD plus MC groups (Fig. 3C).

Alterations in these lipogenic enzyme genes and fatty acid oxidation genes may explain the lack of significant differences in hepatic triglyceride contents between the MCD diet and MCD plus MC groups (Fig. 1B).

In addition to lipid accumulation in hepatocytes, increased expressions of inflammatory cytokines play an important role in the pathogenesis of NASH. The mRNA levels of TNF-α were upregulated in the MCD diet group, whereas these elevations were suppressed in the MCD diet plus MC group (Fig. 3D). Translocation of bacterial endotoxin is considered to play an important role in the pathogenesis of NASH (24). Serum concentrations of LPS were markedly elevated in the MCD diet group as previously reported (12), whereas these levels were normalized in the MCD diet plus MC group (Fig. 3D). The gut-derived hormone GLP-1 reportedly attenuates the development of NASH (38). While plasma GLP-1 levels were lower in the NCD group compared with the MCD and MCD plus MC groups (Fig. 3D), GLP-1 levels were increased in the MCD plus MC group, indicating that GLP-1 may play a role in the suppression of NASH development by MC treatment.

**Fig. 3.** Mosapride citrate (MC) treatment suppressed the expressions of genes involved in NASH pathogenesis. Mice were divided into three groups, given the normal chow diet (NCD), the methionine-choline deficient (MCD) diet or the MCD diet containing 10 mg/kg/day of MC (MCD plus MC) for 6 wk and then killed.

Hepatic mRNA levels of sterol regulatory element binding protein 1c (SREBP1c), carnitine palmitoyltransferase 1 (CPT-1) and CD36

The hepatic mRNA level of tumor necrosis factor α (TNF-α)

Serum lipopolysaccharide (LPS)

Plasma glucagon-like peptide-1 (GLP-1)

Data are presented as means ± SE. *Statistical significance P < 0.05.

**Fig. 4.** Intestinal transit time changes in response to mosapride citrate (MC) treatment. Mice were divided into three groups, given the normal chow diet (NCD), the methionine-choline deficient (MCD) diet or the MCD diet containing 10 mg/kg/day of MC (MCD plus MC) for 6 wk. The intestinal transit times were determined by measuring the time elapsed from initial ingestion of carmine red by oral gavage until excretion of colored feces. The colonic fecal weight was determined at the time of death.

Intestinal transit time

Fecal weight in the colon

Data are presented as means ± SE. *Statistical significance P < 0.05.
in the MCD diet than in the NCD group, interestingly, plasma GLP-1 levels were markedly elevated in the MCD plus MC group (Fig. 3D).

Intestinal transit time changes in response to MCD diet feeding and MC. The intestinal transit time was significantly longer in the MCD diet than in the NCD group. The intestinal transit time in the MCD plus MC group was significantly shorter than that in the MCD diet group, but was still longer than that in the NCD group (Fig. 4A). In addition, colonic feces were slightly more abundant in the MCD diet than in the MCD plus MC group (Fig. 4B).

Gut microbiota changes in response to MCD diet feeding and MC. Gut microbiota were examined in the three groups of mice fed the NCD, the MCD diet, or the MCD diet plus MC. The total numbers of bacteria were lower in the MCD diet than in the NCD group, but similar to those in the MCD plus MC group (Fig. 5A). The ratios of each bacterial subgroup to total bacteria are shown in Fig. 5, B and C. Calculations of the relative abundance of each strain indicated that the ratios of lactic acid bacteria, such as *Bifidobacterium* and *Lactobacillus*, in feces were markedly lower in the MCD than in the NCD-fed group, whereas the ratios of the *Bacteroides fragilis* group and *Enterococcus* were higher, as we reported previously (31). Interestingly, the reduction in lactic acid bacteria in the MCD diet group was reversed in the MCD plus MC group. Amounts of archaeaen and fungal species were not measured in this study.

MC treatment suppressed colon inflammation. Previous studies have shown MC to attenuate drug-induced gastric
mucosal damage and intestinal lesions (17, 20) and that some lactic acid bacteria suppress colon inflammation and contribute to the maintenance of barrier function (14, 19). Thus, to investigate the effects of MC on colon inflammation, we performed immunostaining employing the anti-NFκBp65 antibody. Histological analysis showed mucosal surface irregularity, goblet cell depletion, and an increase in nuclear NFκBp65 positive cells in the MCD diet group compared with the NCD group, while these changes were suppressed in the MCD plus MC group (Fig. 6).

DISCUSSION

This study aimed to investigate the effect of MC, an agonist of the 5-hydroxytryptamine (5-HT4) receptor, on MCD diet-induced NASH development. MC accelerates the release of acetylcholine from intestinal cholinergic neurons, thereby eliciting smooth muscle contraction, and thus participates in gastrointestinal motility (18, 36). Although biochemical analysis using the Triglyceride E test revealed hepatic triglyceride contents to not differ significantly between the livers of the MCD diet and the MCD plus MC groups, the numbers of large fat droplets in hepatocytes were markedly reduced in the MCD plus MC group compared with the MCD diet group, which likely reflects maintenance of the normal functions of hepatocytes of the MCD plus MC group. Furthermore, more marked differences were observed in terms of increased inflammatory cytokines and hepatic fibrosis, which were strongly suppressed in the MCD plus MC, compared with the MCD diet group. Based on these observations, our study clearly demonstrated MC treatment to attenuate MCD-diet induced NASH development.

Subsequently, we performed experiments to reveal the mechanisms underlying the ameliorating effect of MC on NASH. While serum LPS was found to be elevated by MCD diet feeding as we previously reported (12), interestingly, MC treatment suppressed this MCD diet-induced rise in the serum LPS concentration (Fig. 3C). LPS is a bacterial cell wall component sensed by Toll-like receptor 4 and is regarded as an inducer of hepatic inflammation. The mice intraperitoneally injected with LPS developed hepatic lipid accumulation with SREBP1c activation (11), and elevated serum LPS concentrations were often observed in NASH patients (15). Thus protection from NASH development by MC treatment appears to be at least partially attributable to the reduced serum LPS concentration. Furthermore, plasma GLP-1 levels were shown to be lower in the MCD diet than in the NCD group, whereas they were normalized in the MCD plus MC group (Fig. 3D), observations which agree well with those in a previous report (4). GLP-1 secretion is decreased in NASH patients (6) and GLP-1 reportedly attenuates NASH development (38). In addition, a GLP-1 receptor agonist reportedly suppressed SREBP1c expressions in the liver (32), and GLP-1 treatment decreased liver inflammation (40). Thus increased plasma GLP-1 concentrations, in addition to the reduced LPS concentration, with MC administration would also contribute to the anti-inflammatory effects in the liver, ultimately providing protection from progression to NASH.
Previous reports have suggested that gut microbiota influence both GLP-1 secretion and the serum LPS concentration, and prebiotics or probiotics reportedly increase blood GLP-1 and reduce serum LPS levels (8, 42). Since prolonged oroceleal transit time is often observed in NASH subjects (41), we consider the mechanisms underlying changes in serum LPS and GLP-1 levels with MC administration to involve altered gut microbiota and a shortened oroceleal transit time.

To our knowledge, the effects of drugs designed to alter gut motility such as MC on gut microbiota have not been investigated. Although our gut microbiota analysis using RNA provided only the quantity of metabolically active bacteria rather than the semiabsolute number of bacteria, it was clearly shown that the reduction in lactic acid bacteria in the MCD diet group was reversed by the addition of MC to this diet (Fig. 5). Levels of lactic acid bacteria, such as *Bifidobacterium*, were reportedly lower in NASH patients than in healthy subjects (43), and our group and others have shown prebiotics or probiotics such as lactic acid bacteria to ameliorate NASH development with decreased endotoxemia (23, 31). Taking these prior reports into consideration, the favorable effect of MC might be attributable to increased lactic acid bacteria in the gut. In addition, given the quantity of feces in the colon, it is not unreasonable to speculate that the total amount of bacteria might be slightly larger in the guts of MCD diet group mice than in those of mice fed the MCD plus MC.

While increased intestinal permeability and disruption of the mucosal barrier would also promote translocation of microbial products from the intestinal lumen to the bloodstream, some lactic acid bacteria reportedly contribute to the normalization of tight junction proteins (2, 27) and suppress colon inflammation, while also contributing to the maintenance of barrier function (7, 19, 30). In terms of the reduction in the serum LPS concentration achieved by MC administration, the shortened intestinal transit time reduced total number of intestinal microbibiota and increases in lactic acid bacteria may all be greater, and contribute to the underlying mechanisms. In previous reports, it was shown that MC attenuated drug-induced gastric mucosal damage and intestinal lesion formation (17, 20), and the present study demonstrated MC treatment to also significantly suppress colon inflammation (Fig. 6). Thus, it is very likely that multiple mechanisms exist by which MC ultimately contributes to the suppression of NASH development (Fig. 7).

In conclusion, we have presented the first evidence that MC treatment suppresses NASH development in a rodent model. In a future study, we hope to assess whether administering MC might be an effective strategy for treating NASH.

**REFERENCES**


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