

Antagonism of central melanin-concentrating hormone 1 receptor alleviates steatohepatitis in mice

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Abstract

Blockade of brain melanin-concentrating hormone 1 receptor (MCH1R) significantly ameliorates fatty liver as well as obesity. However, the mode of action of this effect is unknown. This study examined the effect of a MCH1R antagonist in murine steatohepatitis models with and without obesity and clarified whether these pharmacological effects were attributed to anti-obesity effects. Steatohepatitis with concomitant obese phenotypes was developed after 52-week exposure to a high-fat diet, and steatohepatitis with reduced body weight was developed by exposure to a methionine- and choline-deficient diet for 10 days. Chronic intracerebroventricular infusion of a peptidic MCH1R antagonist reduced hepatic triglyceride contents and ameliorated steatohepatitis on histological observations in both mice models.

Improvement of steatohepatitis was concomitant with amelioration of obese phenotypes such as hyperinsulinemia and hyperleptinemia in the case of the obese model, whereas body weight reduction was not associated with amelioration of steatohepatitis by the antagonist in the lean model. Reduction of hepatic gene expressions encoding cytochromes P450 4A was identified by treatment with the antagonist in both the obese and lean models. These results suggest that brain blockade of MCH1R could alleviate steatohepatitis independently from anti-obesity effects. In conclusion, MCH1R antagonist could have a new therapeutic potential for the treatment of human nonalcoholic steatohepatitis.

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Introduction

With the growing prevalence of metabolic disorders, nonalcoholic fatty liver diseases (NAFLD), which range from simple steatosis to end stage liver disease, have become a common cause of chronic liver diseases (Marchesini *et al.* 2003, Caldwell & Crespo 2004, Machado *et al.* 2006). Among NAFLD, nonalcoholic steatohepatitis (NASH) is characterized by fat accumulation with various degrees of inflammation, fibrosis, and necrosis (Neuschwander-Tetri & Caldwell 2003, Caldwell *et al.* 2004, Bugianesi *et al.* 2005). Because NASH is a possible cause of cryptogenic cirrhosis and hepatocellular carcinoma, medical treatment for NASH is needed.

Animal NASH models, designed to increase the understanding of the pathogenic mechanisms in disease development, have been well characterized. Of these models, we previously reported that mice fed a high-fat diet (HFD) for 50 weeks exhibited NASH after onset of concomitant metabolic abnormalities such as adiposity, hyperinsulinemia, and hypercholesterolemia, as is also observed in human NASH patients (Ito *et al.* 2007). This animal model could be useful in addressing

the potential efficacy of anti-obesity agents in the treatment of NASH, because amelioration of obesity and related metabolic abnormalities is expected to be effective for NASH (Caldwell & Crespo 2004, Caldwell *et al.* 2004, Dixon *et al.* 2004).

Melanin-concentrating hormone (MCH) is an orexigenic neuropeptide and modulates energy expenditure (Qu *et al.* 1996, Gomori *et al.* 2003, Ito *et al.* 2003, Shimizu *et al.* 2007). We showed that blockade of the brain MCH signal by a peptidic MCH 1 receptor (MCH1R) antagonist had anti-obesity effects in diet-induced obese mice, but not in diet-induced obese *mch1r* gene knockout mice (Mashiko *et al.* 2005). Moreover, we also clarified that the MCH signal is an important factor in the development of fatty liver in mice with estrogen deficiency (Gomori *et al.* 2007). Although it remained uncertain whether the improvement of fatty liver could be simply a result of a concomitant anti-obesity effect, these effects of the MCH1R antagonist could be useful for the treatment of NASH. Therefore, both a HFD-induced obese and a methionine- and choline-deficient (MCD) diet-induced lean models of NASH would be interesting models to address the efficacy and mechanism of action of MCH1R antagonists.

This study aimed to evaluate the effect of an MCH1R antagonist in murine steatohepatitis models with and without obesity and to clarify whether these pharmacological effects were attributed to anti-obesity effects.

Materials and Methods

Materials

The peptidic MCH1R antagonist was synthesized by Banyu Pharmaceutical Co., Ltd (Ibaraki, Japan). The antagonist has high affinity for MCH1R with a K_i value of 9.9 nM and shows no significant cross-reactivity in 120 other binding and enzyme assays for targets involved in feeding regulation (Mashiko *et al.* 2005). Furthermore, the compound did not affect food intake and body weight in *mch1r* gene knockout mice (Mashiko *et al.* 2005). All other chemicals were of analytical grade.

Animals

Male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used. The animals were housed individually in plastic cages kept at $23 \pm 2^\circ\text{C}$, $55 \pm 15\%$ relative humidity, and maintained on a light:darkness cycle with the lights on from 0700 to 1900 h. Water and regular diet (RD; CE-2, CLEA Japan) were made available *ad libitum*. All experimental procedures followed the Japanese Pharmacological Society Guideline for Animal Use. The experimental protocol was accepted by the in-house institutional Animal Care and Use Committee.

Intracerebroventricular (ICV) cannula implantation

Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.; Dinabot Co., Tokyo, Japan), and a sterile brain infusion cannula (28 gauge; Durect Corp., Cupertino, CA, USA) was stereotaxically implanted into the right lateral ventricle. The stereotaxic coordinates were 0.4 mm posterior to the bregma, 0.8 mm lateral to the midline, and 2.0 mm from the surface of the skull, using a flat skull position. The cannula was fixed to the skull with dental cement. The infusion cannula was connected via polyvinylchloride tubing to an osmotic minipump (model 2004; Durect Corp.) filled with 30% (v/v) propylene glycol in distilled water. The pump was implanted under the skin of the mouse's back, and antibiotic (Cefamezin, 50 mg/kg; Astellas Pharma Inc., Tokyo, Japan) was injected subcutaneously. The placement of the cannula was confirmed at the end of the experiment by injection of 0.5% (v/v) Evans blue dye.

Experimental designs

Treatment with MCH1R antagonist in obese steatohepatitis model mice fed HFD Male C57BL/6J mice (10 weeks, CLEA Japan) were used. Mice were divided into two groups matched for average body weight. Each group was fed either an RD or a HFD (D12492, Research Diets, Inc.,

New Brunswick, NJ, USA). The HFD contained 60 cal% fat (lard), 20 cal% carbohydrate, and 20 cal% protein. Body weights and food intake were monitored once daily throughout the study. About 1 year after the start of HFD loading, the ICV cannula was implanted into the lateral ventricle. After a 4-week recovery period, the mice fed the HFD were divided into two groups to match average values of daily food intake and body weight, and the implanted pump was replaced with a new pump filled with the MCH1R antagonist (7.5 $\mu\text{g}/\text{day}$; $n=7$) or its vehicle (30% (v/v) propylene glycol; $n=6$) under isoflurane anesthesia. Mice fed the RD underwent ICV infusion with vehicle ($n=5$). Body weights were monitored during the study. After 4 weeks of treatment with the MCH1R antagonist, mice were killed by collecting whole blood from the inferior vena cava under isoflurane anesthesia. Blood samples were used for measurement of plasma parameters. After exsanguination, the livers were excised and divided into three pieces. Pieces for histomorphological examinations were fixed and kept in 10% (v/v) neutral buffered formalin. The remaining specimens were snap-frozen in liquid nitrogen and stored at -80°C .

Treatment with MCH1R antagonist in lean steatohepatitis model mice fed MCD diet Male C57BL/6J mice (16 weeks, CLEA Japan) were used. A sterile brain infusion cannula was stereotaxically implanted into the right lateral ventricle. After a 2-week recovery period, the mice were divided into three groups to match average values of basal food intake and body weight and, under isoflurane anesthesia, the pump was replaced with a new pump filled with the MCH1R antagonist (7.5 $\mu\text{g}/\text{day}$; $n=12$) or its vehicle (30% (v/v) propylene glycol; $n=24$). After 4 days of the initiation of infusion, the diet was replaced with the MCD diet (960439, ICN, Aurora, OH, USA) for all mice treated with the MCH1R antagonist and half of the mice treated with vehicle. The other vehicle-treated mice were exposed to methionine- and choline-sufficient control diet (control diet; 960441, ICN). Body weights were monitored once daily throughout the study. After 10 days of the MCD or control diet, mice were killed by collecting whole blood from the inferior vena cava under isoflurane anesthesia. Blood samples were used for measurement of plasma parameters. After exsanguination, livers were excised and divided into three pieces. Pieces for pathological observation were fixed and kept in 10% (v/v) neutral buffered formalin. The remaining samples were snap-frozen in liquid nitrogen and stored at -80°C .

Hormone and blood chemistry measurements

Plasma insulin and leptin levels were measured by ELISA kits (Morinaga, Kanagawa, Japan). Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), free fatty acid (FFA), triglyceride (TG), and glucose levels were measured by an automated Clinical Analyzer 7070 (Hitachi High-Technologies Co, Tokyo, Japan).

Hepatic biochemical parameters measurements

Liver specimens were homogenized with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) in 100 mM Tris-HCl buffer (pH 7.5), containing 225 mM mannitol, 75 mM sucrose, 2 mM dithiothreitol, 100 mM NaF, and a protease inhibitor cocktail (Roche Diagnostics). Lipid fractions in homogenates were extracted with chloroform-methanol (2:1, v/v) followed by measurement of TG by a Determiner LTGII (Kyowa Medex Co., Ltd, Tokyo, Japan). Supernatants of liver homogenates after centrifugation (2000 g, 10 min at 4 °C) were used for measurement of monocyte chemoattractant protein-1 (MCP-1) levels. Hepatic MCP-1 contents were determined with an ELISA kit (R&D Systems, Minneapolis, MN, USA). The 35% (w/v) ammonium sulfate precipitate from digitonin-lysed homogenates was used for measurement of acetyl-CoA carboxylase (ACC) activity. ACC activity was determined by measurement of $H^{14}CO_3$ fixation to acid-stable products in the presence of 20 mM citrate, as previously reported (Zhou *et al.* 2001).

Histology

Portions of livers from all animals in each group were embedded in paraffin, sectioned at 3 μ m, stained with hematoxylin and eosin, and examined microscopically.

Quantitative real-time PCR

Quantitative real-time PCR using ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) was performed to determine gene expression levels in the liver and brown adipose tissue. Total RNA was purified using Isogen (Nippon Gene, Tokyo, Japan). The quantity and purity of the RNA were determined by absorbance at 260 and 280 nm. cDNA was synthesized from 1 μ g total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). Expression of 18S rRNA was measured as an internal control. Expression levels of ACC1, fatty acid synthase (FAS), sterol regulatory element-binding protein 1c (SREBP1c), FAT/CD36, cytochrome P450 (CYP) 2E1, CYP4A10, CYP4A14, and IL-1 β mRNAs were measured as previously reported (Giulietti *et al.* 2001, Mashiko *et al.* 2003, Ito *et al.* 2006, 2007). Mixtures of primers and probes for detection of mRNA encoding the tumor necrosis factor α (TNF α), type I collagen α 1 (collagen 1 α 1), MCP-1, and 18S rRNA were purchased from Applied Biosystems (TaqMan Gene Expression Assays). PCRs and analysis were performed according to the manufacturer's protocol.

Statistical analysis

Data are expressed as mean \pm S.E.M. Data, except for body weight, were analyzed by the unpaired Student's *t*-test. Differences in body weight were analyzed by repeated-measures one-way ANOVA, followed by the Bonferroni/Dunn test. *P* values less than 0.05 were considered significant.

Results

Treatment with the MCH1R antagonist in obese steatohepatitis model mice fed HFD

Body weights and liver aspects in mice fed HFD

Initial body weight of mice was 24.6 ± 0.3 g before HFD loading. Mice fed the HFD for 52 weeks were significantly heavier than mice fed the RD, with an ~ 20 g difference in body weight (59.5 ± 0.9 g vs 38.1 ± 2.0 g respectively; $P < 0.01$, Fig. 1A). The peptidic MCH1R antagonist significantly decreased body weight gain in mice fed the HFD. Body weight reduction after 4-week ICV infusion was 12.4 ± 3.8 g in the MCH1R antagonist-treated group, whereas a moderate weight gain (3.5 ± 2.1 g) was observed in vehicle-treated group (Fig. 1A). Liver weight was significantly increased in mice fed the HFD when compared with mice fed the RD (3.39 ± 0.25 g vs 1.84 ± 0.08 g respectively; $P < 0.001$), and the MCH1R antagonist significantly reduced liver weight (2.23 ± 0.17 g; $P < 0.01$). Hepatic TG content was markedly increased in mice fed the HFD, and the MCH1R antagonist significantly reduced the accumulation to less than half ($P < 0.05$, Fig. 1B). In the pathological analysis, marked vacuolation of hepatocytes and multifocal infiltrations of inflammatory cells were visible in mice fed the HFD in the vehicle treatment group (Fig. 1C). The MCH1R antagonist improved severities of both hepatocyte vacuolation and multifocal infiltrations of inflammatory cells (Fig. 1D). Although four of six mice showed slight hepatocyte single cell necrosis in the vehicle treatment group, no mice exhibited this histomorphological feature in the MCH1R antagonist treatment group.

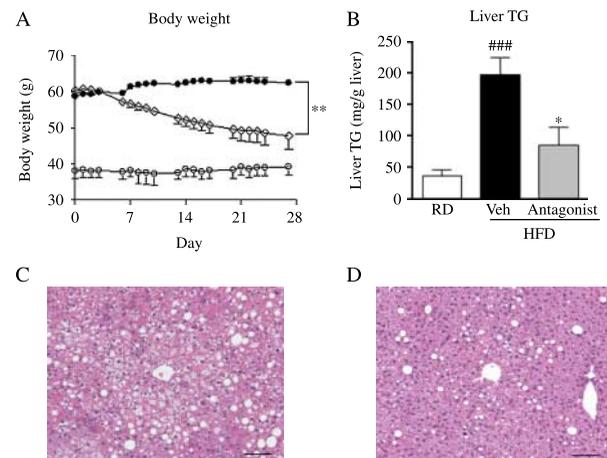


Figure 1 Effect of the MCH1R antagonist on (A) body weight, (B) hepatic TG content, and (C and D) histological changes in the liver of mice fed a high-fat diet (HFD). Open circles, mice fed a regular diet (RD); closed circles, mice fed a HFD with vehicle treatment (Veh); open diamonds, mice fed a HFD with MCH1R antagonist treatment (antagonist). Livers from HFD-fed mice with vehicle (C) or with MCH1R antagonist (D) were sectioned at 3 μ m and stained with hematoxylin and eosin. Scale bar = 100 μ m. * $P < 0.05$, ** $P < 0.01$ versus vehicle in the HFD group; *** $P < 0.001$ versus RD group.

Plasma parameters in mice fed HFD Changes in plasma parameters are summarized in Table 1. Plasma ALT levels remarkably increased 9.3-fold in mice fed the HFD, and the MCH1R antagonist significantly reduced the value by 75% ($P < 0.05$). Similar results were obtained for plasma AST and LDH levels. Mice fed the HFD exhibited significant hyperinsulinemia, hyperleptinemia, and hypercholesterolemia compared with mice fed the RD. These abnormalities were significantly improved by the MCH1R antagonist. Plasma glucose and FFA levels were not changed in mice fed the HFD. These two parameters were slightly reduced by the MCH1R antagonist, but the differences did not reach statistical significance.

Hepatic mRNA expression levels in mice fed HFD

Expression levels of the genes encoding cytokines and chemokines were measured in the liver (Fig. 2). Hepatic expressions of TNF α , IL-1 β , and MCP-1 mRNAs were significantly increased in mice fed the HFD compared with the RD ($P < 0.05$, $P < 0.001$, and $P < 0.01$ respectively). The MCH1R antagonist normalized the gene expression of TNF α and reduced the induction of IL-1 β , and MCP-1 mRNAs by more than 75% ($P < 0.05$, $P < 0.001$, and $P < 0.05$ respectively). Hepatic MCP-1 content was significantly increased in mice fed the HFD compared with mice fed the RD (625 ± 117 vs 194 ± 47 pg/g liver respectively; $P < 0.001$) and a significant reduction of hepatic MCP-1 content was observed in the MCH1R treatment group (362 ± 75 pg/g liver; $P < 0.01$). Increased expression of collagen 1a1 mRNA was observed

Table 1 Plasma parameters and hepatic mRNA levels in mice fed a high-fat diet (HFD) or regular diet (RD)

	RD	HFD	
	Vehicle	Vehicle	MCH1R antagonist
Plasma parameters			
ALT (IU/l)	25 \pm 3	233 \pm 52 [†]	58 \pm 11*
AST (IU/l)	44 \pm 5	267 \pm 54 [†]	96 \pm 5*
LDH (IU/l)	218 \pm 7	1260 \pm 201 [§]	640 \pm 34*
Insulin (ng/ml)	1.5 \pm 0.5	4.1 \pm 0.6 [†]	2.2 \pm 0.4*
Leptin (ng/ml)	9.4 \pm 3.0	71.6 \pm 6.5 [§]	25.3 \pm 6.5 [†]
Glucose (mg/dl)	218 \pm 14	225 \pm 25	184 \pm 21
FFA (μ Eq/l)	594 \pm 67	579 \pm 45	514 \pm 28
Hepatic mRNA levels			
FAS	0.68 \pm 0.06	1.08 \pm 0.09 [†]	0.67 \pm 0.06 [†]
ACC1	0.71 \pm 0.04	1.11 \pm 0.12 [†]	0.82 \pm 0.07*
SREBP1c	0.68 \pm 0.07	1.02 \pm 0.07 [†]	0.69 \pm 0.13*
FAT/CD36	0.51 \pm 0.05	1.32 \pm 0.11 [§]	0.62 \pm 0.12 [†]
CYP4A10	1.34 \pm 0.24	1.09 \pm 0.27	0.37 \pm 0.13*
CYP4A14	0.67 \pm 0.15	0.79 \pm 0.08	0.30 \pm 0.13 [†]
CYP2E1	1.04 \pm 0.12	0.79 \pm 0.08	0.73 \pm 0.09

* $P < 0.05$, [†] $P < 0.01$ versus vehicle in the HFD group; [‡] $P < 0.01$, [§] $P < 0.001$ versus vehicle in the regular diet group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; FFA, free fatty acid; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase1; SREBP1c, sterol regulatory element-binding protein 1c.

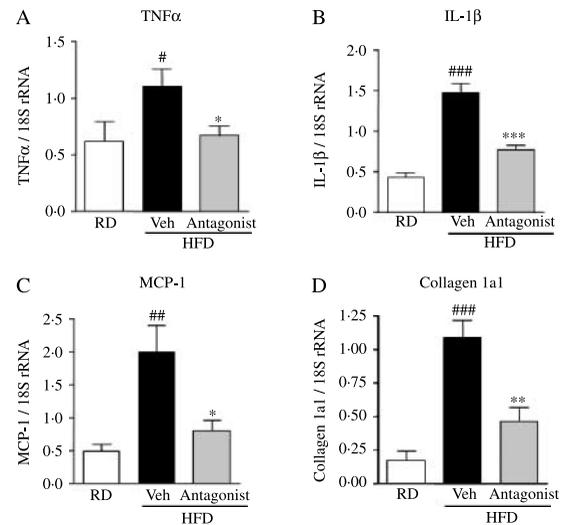


Figure 2 Expression levels of (A) TNF α , (B) IL-1 β , (C) MCP-1, and type I collagen $\alpha 1$ (collagen 1a1, D) mRNAs in the liver after treatment with the MCH1R antagonist (antagonist) or vehicle treatment (Veh) in mice fed a high-fat diet (HFD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle in the HFD group; [†] $P < 0.05$, [‡] $P < 0.01$, [§] $P < 0.001$ versus vehicle in the regular diet (RD) group.

in mice fed the HFD compared with the RD ($P < 0.001$), and the MCH1R antagonist significantly suppressed this induction ($P < 0.01$, Fig. 2D).

Expression levels of mRNA encoding lipogenic enzymes such as FAS, ACC1, and their transcriptional factor, SREBP1c, were significantly upregulated in mice fed the HFD compared with the RD (Table 1). The MCH1R antagonist significantly suppressed expressions of these mRNAs. In support, reduction of hepatic ACC activity was also confirmed in the MCH1R antagonist treatment group (1.6 ± 0.2 vs 0.83 ± 0.08 nmol/min per mg protein respectively; $P < 0.01$). Expression levels of FAT/CD36 mRNA were increased by HFD loading, but reduced by the MCH1R antagonist. Expression levels of the genes encoding CYP4A10, CYP4A14, and CYP2E1, which are known to produce lipid peroxides, were also measured. Although HFD exposure did not change the expression levels of CYP4A10 and CYP4A14 mRNAs, the MCH1R antagonist significantly decreased them by more than 60% (Table 1). Significant changes were not observed in hepatic CYP2E1 expression levels by either HFD loading or MCH1R antagonist.

Treatment with MCH1R antagonist in lean steatohepatitis model mice fed MCD diet

Body weights and liver aspects in mice fed MCD diet

Before dietary replacement to the MCD diet, body weight was almost unchanged in the absence or presence of MCH1R antagonist treatment (Fig. 3A). After the dietary change to the MCD diet, body weight was significantly decreased in mice with vehicle treatment and the difference in body weight was nearly 7 g when compared with that in mice fed the control diet

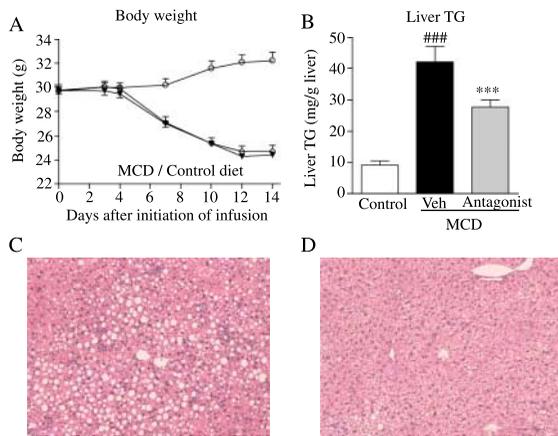


Figure 3 Effect of the MCH1R antagonist on (A) body weight, (B) hepatic TG content, and (C and D) histological changes in the liver of mice fed a methionine- and choline-deficient (MCD) diet. The MCD or control diet was started on day 4 after initiation of MCH1R antagonist (antagonist) infusion or vehicle (Veh). Open circles, mice fed a control diet (control); open diamonds, mice fed a MCD diet with vehicle treatment; closed triangle, mice fed a MCD diet with MCH1R antagonist treatment. Mice fed a MCD diet with (C) vehicle or (D) MCH1R antagonist. Livers were sectioned at 3 μ m and stained with hematoxylin and eosin. Scale bar = 100 μ m. * P <0.05, ** P <0.01, *** P <0.001 versus vehicle in the MCD diet group; ### P <0.001 versus control diet group.

(24.7 ± 0.5 g vs 32.2 ± 0.7 g respectively; P <0.001). Further reduction of body weight was not observed by treatment with the MCH1R antagonist. MCD diet exposure showed a 4.6-fold increase in hepatic TG content and treatment with the MCH1R antagonist prevented the accumulation of hepatic TG by 34% (Fig. 3B). Histomorphological evaluation showed that severity of both multifocal infiltrations of inflammatory cells and cellular necrotic damage was alleviated by treatment with the MCH1R antagonist (Fig. 3C and D).

Plasma parameters in mice fed MCD diet The MCD diet exposure significantly increased plasma ALT, AST, and LDH levels to 323 ± 56 IU, 440 ± 97 IU, and 505 ± 89 IU respectively when compared with the control diet (Table 2). Treatment with the MCH1R antagonist significantly suppressed the elevation of plasma AST and LDH levels (P <0.05 respectively). The MCH1R antagonist also reduced ALT levels by 30%, but the change was not statistically significant. Plasma glucose and leptin levels were markedly decreased by MCD diet intake. Treatment with the MCH1R antagonist did not change plasma glucose levels, but significantly decreased plasma leptin levels (P <0.05). Plasma insulin levels were under measurable levels (<0.313 ng/ml) in the MCD diet-fed groups.

Hepatic mRNA expression levels in mice fed MCD diet MCD diet exposure significantly increased expression of TNF α , IL-1 β , and collagen 1a1 mRNAs in the liver when

Table 2 Plasma parameters and hepatic mRNA levels in mice fed a methionine- and choline-deficient (MCD) diet or control diet

	Control diet	MCD diet	
	Vehicle	Vehicle	MCH1R antagonist
Plasma parameters			
ALT (IU/l)	15 ± 3.3	$323 \pm 56^{\dagger}$	231 ± 23
AST (IU/l)	42 ± 7.5	$440 \pm 97^{\dagger}$	$216 \pm 21^*$
LDH (IU/l)	103 ± 8.9	$505 \pm 89^{\dagger}$	$331 \pm 32^*$
Insulin (ng/ml)	0.90 ± 0.17	ND	ND
Leptin (ng/ml)	6.9 ± 0.7	$1.3 \pm 0.2^{\ddagger}$	$0.9 \pm 0.1^*$
Glucose (mg/dl)	246 ± 6.5	147 ± 12	167 ± 6.5
FFA (μ Eq/l)	319 ± 111	255 ± 124	243 ± 63
Hepatic mRNA levels			
FAS	0.55 ± 0.08	0.62 ± 0.11	0.57 ± 0.08
ACC1	0.47 ± 0.05	$0.81 \pm 0.06^{\ddagger}$	0.83 ± 0.05
SREBP1c	0.22 ± 0.02	0.21 ± 0.02	0.18 ± 0.02
FAT/CD36	0.17 ± 0.01	$0.39 \pm 0.08^{\ddagger}$	0.27 ± 0.03
CYP4A10	0.34 ± 0.04	$1.24 \pm 0.10^{\ddagger}$	$0.94 \pm 0.09^*$
CYP4A14	0.47 ± 0.09	0.72 ± 0.07	$0.52 \pm 0.06^*$
CYP2E1	0.33 ± 0.02	0.54 ± 0.04	0.53 ± 0.04

ND, not detected. * P <0.05 versus vehicle in the MCD diet group; $^{\dagger}P$ <0.01, $^{\ddagger}P$ <0.001 versus vehicle in the control diet group; *P <0.05 vs vehicle in the control diet group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; FFA, free fatty acid; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; SREBP1c, sterol regulatory element-binding protein 1c.

compared with the control diet (P <0.01, P <0.01, and P <0.05 respectively, Fig. 4). The MCH1R antagonist suppressed the induction of these mRNAs by 60% (P <0.05), 68% (P <0.05), and 55% (P <0.05) respectively (Fig. 4). The MCH1R antagonist did not affect the expression of MCP-1 mRNA. Expression of ACC1 mRNA was significantly increased by the MCD diet (P <0.01), but FAS and SREBP1c mRNA levels were not changed (Table 2). The antagonist did not affect these expression levels. Expression of FAT/CD36 mRNA was approximately doubled by the MCD diet. The MCH1R antagonist suppressed the induction of FAT/CD36 expression by 50%, but the effect did not achieve statistical significance. Gene expression levels of CYP2E1, CYP4A10, and CYP4A14 were increased by the MCD diet. The MCH1R antagonist significantly suppressed the expression of CYP4A10 (P <0.05) and CYP4A14 (P <0.05), but did not change the CYP2E1 expression levels.

Discussion

In this study, we evaluated the effects of pharmacological blockade of the central MCH pathway in two types of murine NASH models. In an obese NASH model, chronic ICV infusion of a MCH1R antagonist clearly ameliorated obese phenotypes such as adiposity, hyperinsulinemia, and hyperleptinemia as well as significantly reduced hepatic TG contents, as previously observed (Mashiko *et al.* 2005, Gomori

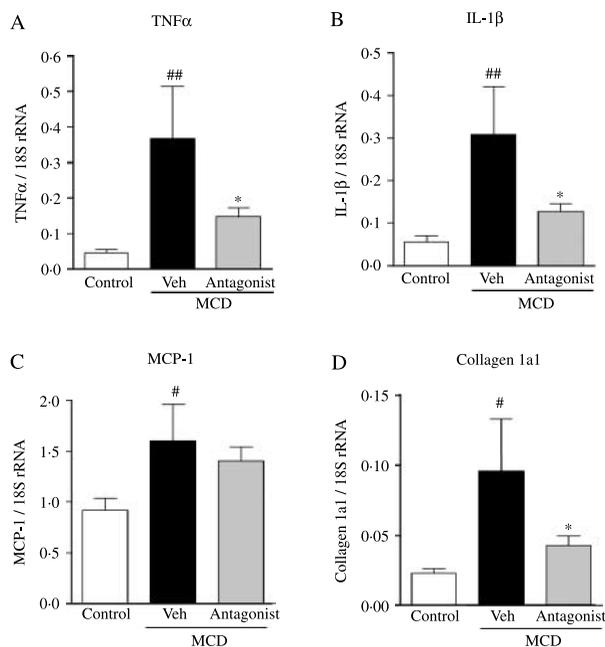


Figure 4 Expression levels of (A) TNF α , (B) IL-1 β , (C) MCP-1, and (D) type I collagen α 1 mRNAs in the liver after treatment with the MCH1R antagonist (antagonist) or vehicle (Veh) in mice fed a methionine- and choline-deficient (MCD) diet. * $P < 0.05$ versus vehicle in the MCD diet group; # $P < 0.05$, ## $P < 0.01$ versus vehicle in the control diet group (control).

et al. 2007). Furthermore, the hepatic expression levels of genes encoding proinflammatory cytokines, and a fibrogenesis marker, type I collagen, were significantly suppressed by the treatment, suggesting improvement of inflammation and fibrogenesis in NASH. These results were consistent with histological observations showing that vacuolation of hepatocytes, cellular necrotic change, and infiltration of inflammatory cells was alleviated by the MCH1R antagonist. In contrast to the HFD-induced obese NASH model, the MCD diet-induced NASH model exhibited lean phenotypes such as lowered body weights and plasma insulin levels. Interestingly, the MCH1R antagonist also ameliorated MCD diet-induced NASH without affecting body weight. These results indicate that central blockade of MCH1R could ameliorate steatohepatitis regardless of obese phenotypes.

ICV infusion of the MCH1R antagonist also reduced hepatic TG contents without affecting body weight and expression levels of lipogenic enzymes in the lean NASH model. Pereira-da-Silva *et al.* (2005) demonstrated that ICV infusion of MCH causes peripheral insulin resistance without body weight changes. In addition, recent reports have shown that a direct connection with autonomic nerves between the hypothalamus and the liver could be important for control of energy metabolism (Uno *et al.* 2006, Lam *et al.* 2007). Furthermore, anatomical analyses suggest that the MCH-containing neuron is projected from hypothalamus to the vagal or sympathetic motor neurons (Buijs *et al.* 2001, Kreier

et al. 2006). These reports support the view that central blockade of MCH1R could affect liver function by regulating the brain–liver nerve signal. Moreover, a hormonal regulation could be a potential cause of reduced hepatic TG by the antagonist. For example, it is reported that MCH involves the regulation of the expression of growth hormone (GH) in the pituitary (Bjursell *et al.* 2005, Segal-Lieberman *et al.* 2006) and that GH stimulates hepatic mRNA expression of FAT/CD36 (Cheung *et al.* 2007), which takes up FFAs into the liver. These reports support our current data that the MCH1R antagonist tended to reduce hepatic expression of FAT/CD36 mRNA levels even in the lean NASH model. Taken together, the MCH1R antagonist reduced hepatic TG contents through multiple pathways, some of which could be independent of the anti-obesity effect. Further research is necessary to investigate a brain–liver connection in the MCH signaling pathway.

The present study provided another important finding that the MCH1R antagonist clearly suppressed gene expressions of CYP4A10 and CYP4A14, but not CYP2E1 in both models. It is interesting to note that these reductions of CYP4As were observed without body weight reduction. CYP4A10 and CYP4A14, as well as CYP2E1, produce lipid peroxides in the ω hydroxylation and have a key pathophysiological role in the development of steatohepatitis (Leclercq *et al.* 2000). Thus, reduced expression of these enzymes by the MCH1R antagonist could contribute to amelioration of steatohepatitis in lean and obese NASH models.

Recently, an interesting observation was reported that MCP-1 secreted from macrophages and hepatic stellate cells enhanced inflammation and fibrosis, and the blockade of MCP-1 signals suppressed fibrosis induced by dimethylnitrosamine (Imamura *et al.* 2005). Thus, reduced hepatic MCP-1 by the MCH1R antagonist could be one of key factors in the amelioration of inflammation in HFD-induced NASH. However, the MCH1R antagonist did not affect MCP-1 levels in the MCD diet-induced model, suggesting that MCP-1 might have a more important role in obese- than in lean steatohepatitis. On the other hand, it is reported that leptin and TNF α are also important factors in the progression of fibrogenesis or inflammation in MCD diet-induced NASH (Sahai *et al.* 2004, Koca *et al.* 2008). In accordance with these reports, we showed that plasma leptin levels and hepatic TNF α mRNAs were significantly reduced in obese and lean NASH models. These cytokines and adipokines, or a combination of them, have potential as therapeutic targets as well as diagnostic markers for the steatohepatitis.

In conclusion, blockade of MCH1R in the brain ameliorated the severity of inflammation and steatosis in both the obese and non-obese types of steatohepatitis in mice. These data suggest that an MCH1R antagonist could alleviate steatohepatitis without anti-obesity effects. Although further investigations are necessary, this study suggests that an MCH1R antagonist could have a new therapeutic potential for the treatment of human NASH.

Declaration of Interest

The authors declare that there is no conflict of interest.

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