Abstract

Objective: Non-Alcoholic Steato Hepatitis (NASH) is now recognized as a global liver disease. Medication combined with diet and exercise regimen is used to treat NASH, because NASH is associated with metabolic syndrome, such as insulin resistance or dyslipidemia. However, it currently remains unclear whether NASH affects the metabolic power and activity in the liver especially for hypo-cholesterolemic drugs. To address this issue, we measured major Cytochrome P450 (CYP) subtype activities in mice with diet-induced NASH.

Methods: C57Bl/6J mice (male, 8 weeks of age) were fed a High-Fat and High-Cholesterol (HFHC) diet or normal chow diet (control) ad libitum for 12 weeks. Plasma lipid levels and hepatic lipid contents were then measured. The mRNA expression levels of the CYP subtypes and inflammatory cytokines in the liver were assessed using quantitative RT-PCR methods. Liver microsomal CYP activities were evaluated by luminogenic CYP assays.

Results: Body weight was significantly lower and the liver weight was higher in HFHC-fed mice than in control mice. Plasma levels of AST, ALT, and ALP were higher in HFHC-fed mice. Oil red O staining and the evaluation of hepatic lipid contents revealed the accumulation of lipids in the liver of HFHC-fed mice. Furthermore, increased inflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) mRNA expression in the liver and a pathological analysis confirmed the characteristics of NASH in HFHC-fed mice. CYP1A1, 1A2, and 3A activities were higher in HFHC-fed mice than in control, which were coincident with mRNA expression levels in the liver. However, CYP2C activity was significantly lower, even though CYP2C29 mRNA expression was higher in HFHC-fed mice than in control mice.

Conclusion: These results indicated that major CYP subtypes activities in the liver were influenced in NASH mice model. It could be occurred in human, and physicians need to consider the administration of appropriate medication to patients with NASH.

Keywords: Cholesterol; cytochrome P-450 (CYP); Drug; Inflammation; Liver; Mouse model; Non-alcoholic steatohepatitis

Introduction

Non-Alcoholic Steatohepatitis (NASH) is now recognized as a global liver disease. NASH is characterized by the liver biopsy findings of alcoholic hepatitis, in addition to elevated plasma indicators of hepatic damage and accumulation of lipids in the liver reported in Non-Alcoholic Fatty Liver Disease (NAFLD). NASH progresses to mild fatty degeneration, inflammation, cell degeneration, fibrosis, cirrhosis, and ultimately cancer [1,2]. It is important to treat NASH before it progresses to a more severe stage. Medication combined with diet and exercise is used to treat NASH. For example, statins or probucol reduce hyperlipidemia, metformin and thiazolidine derivatives improve insulin resistance, and angiotensin II-converting enzyme antagonists reduce blood pressure. However, it has not yet been established whether the hepatic condition of NASH, such as the accumulation of lipids or inflammation, could influence drug metabolism.

Methionine and Choline-Deficient (MCD) diet is used to quickly produce more progressive liver pathology, the development of steatosis with inflammation and fibrosis, in rodent models. However, severe weight loss has been reported in mice or rats fed a MCD diet (due to a vastly lower caloric intake) and they do not become insulin resistant [3,4]. This represents an important difference between MCD diet-induced NASH models and human NASH, because most humans with NASH are obese and resistant to insulin. Therefore, several diets have been examined for their abilities to induce NASH in a mouse model. Recent studies reported that a high-fat and high-cholesterol (HFHC) diet containing sodium cholate could induce NASH in mice, and the pathological features of this model were similar to those of humans, including steatohepatitis, inflammation, fibrosis [5], lipid metabolism, insulin resistance, and the hepatic gene expression profiles responsible for liver pathology [6]. Sodium cholate may play several roles in the progression of NASH. In addition to the role in the digestion and absorption of dietary fats, cholate regulates lipid metabolism, especially down-regulate bile acids synthesis from cholesterol in the liver [7,8]. Dietary intake of cholate increased cholesterol levels in the liver [9,10]. Furthermore, dietary intake of cholate increased inflammatory events, which including serum amyloid A (SAA) expression, in the liver injury model [11]. These studies showed that the HFHC diet containing sodium cholate could induce a pathology that resembled human NASH more than the MCD diet, even if this diet also induced weight loss.

Few studies have examined the hepatic drug-metabolizing enzyme. Cytochrome P450 (CYP) in patients with NASH. Weltman et al. reported that the CYP2E1 protein levels were higher while the CYP3A protein levels were lower in the livers of patients with NASH than in those with normal livers [12]. Fisher et al. found changes in the expression of the mRNA, protein contents, and enzymatic activities of the major CYP subtypes in livers with progression from NAFLD to NASH [13]. Approximately three-quarters of prescribed drugs are metabolized by the CYP superfamily, especially CYP1A1, CYP2C9, CYP2D6, and CYP3A [14]. The influence of NASH on these CYP
subtypes for the metabolism of medication in patients with NASH needs to be clarified. Therefore, we herein examined the major CYP subtypes, including CYP1A1, CYP1A2, CYP2C, and CYP3A, in mice with diet-induced NASH.

Material and Methods

Animals and experimental design

Male C57BL/6J mice were purchased from CLEA Japan Inc. (Tokyo, Japan) at 7 weeks of age. After 1 week of acclimation, 20 mice were divided into the following 2 groups: (1) 10 mice given normal chow (CRF-1, Charles River Laboratories Japan), (2) another 10 mice given a HFHC diet to induce NASH. The compositions of each diet are shown in Table 1. These diets were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). To evaluate the efficacy of drugs in NASH, mice were fed a HFHC diet for 12 weeks, and administered pravastatin (20 mg/kg) dissolved in H2O, atorvastatin (20 mg/kg) dissolved in DMSO, or each vehicle via intragastric injection for the last five days of the treatment regimen. Mice were housed in cages with a 12-hour light/dark cycle, and were given food and water ad libitum. All animal experiments were conducted with the approval of the National Institute of Health and Nutrition laboratory animal ethics committee.

Plasma chemistry

After 12 weeks of feeding, mice were killed under isoflurane anesthesia after overnight fasting. Blood samples were collected from the heart, and plasma samples were immediately prepared. Plasma levels of lipids (Triglycerides (TG), Total Cholesterol (TC), and HDL-cholesterol), and liver functional markers (Asparagine Aminotransferase (AST), Alanine Aminotransferase (ALT), and Aspartate Aminotransferase (ALT), Alkaline Phosphatase (ALP)) were measured by enzymatic methods (Wako Pure Chemical Industries, Osaka, Japan).

Measurement of hepatic lipids content

Hepatic lipids were extracted with chloroform/methanol (2:1) according to the Folch method [15]. The liver (approx. 100 mg) was homogenized in 6 mL chloroform/methanol (2:1) with a polytron homogenizer. Lipids were extracted by an overnight incubation and filtered to remove insoluble residues, and adjusted to 10 mL with chloroform/methanol. The 1 mL of each sample was moved to microtube and filtrated to remove insoluble residues, and adjusted to 10 mL with H2O. The residue was dissolved in 50 mM Tris-HCl buffer (pH7.4), and used as the liver microsome fraction. The protein concentration in the liver microsome fraction was determined by the Lowry method using DC protein assay reagents (Bio-Rad Laboratories, Inc., Hercules, CA).

Histological examination

Livers were removed and a part of each liver was immediately fixed with 10% buffered formalin solution. Liver samples were embedded in paraffin and sectioned at a thickness of 3 µm for hematoxylin and eosin (H&E) and Masson’s trichrome staining, or 10 µm for Oil Red O staining. These morphological analyses were outsourced to Biosafety Research Center, Foods, Drugs, and Pesticides (Anpyo Center, Shizuoka, Japan).

Quantitative RT-PCR

Total RNA was extracted from the liver using the TRIzol Plus RNA Purification System (Life Technologies, Carlsbad, CA), and reverse transcribed with PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed on 96-well plates with SYBR Green PCR Master Mix and a Thermal Cycler Dice Real Time System Single (Takara Bio Inc.). Results are expressed as the copy number ratio of the target mRNA to GAPDH mRNA. The following mouse-specific primer pairs were used:

Table 1: Composition of experimental diets.
measured by a luminescent method using the P450-Glo™ CYP1A1 System (Luciferin-CEE) Assay, CYP1A2 System (Luciferin-1A2) Assay, CYP2C9 System (Luciferin-H) Assay, CYP3A4 System (Luciferin-PX) Assay, and NADPH Regeneration System with GloMax-Multi+ Detection System (Promega Co., Madison, WI). CYP activity was adjusted according to the protein concentration and the results obtained were represented as a percentage of the control.

### Statistical Analyses

Data are presented as the mean ± SEM. Statistical analyses were conducted using an unpaired Student's t-test (PASW Statistics Base 18, IBM, Armonk, NY). A P value <0.05 was considered significant.

### Results

Body weight, liver weight, and plasma chemistry, C57BL/6J mice (male, 8 weeks of age) were fed HFHC or normal chow (as control) for 12 weeks. In the first 2 weeks of experimental diet feeding, body weight was reduced in HFHC-fed mice even though they ate 1.42 g (9.5 k calories)/day, and was increased in control mice that ate 2.53 g (9.8 k calories)/day. After 12 weeks of feeding, body weight was significantly lower in HFHC-fed mice than in control mice; however, liver weight was significantly higher in HFHC-fed mice (Table 2). Plasma TG levels were significantly lower and TC and non-HDL-cholesterol levels were significantly higher in HFHC-fed mice. AST, ALT, and ALP were higher in HFHC-fed mice than in control mice (Table 2).

HFHC diet induced lipid accumulation, inflammatory cell infiltration, and fibrosis in the liver. The livers of HFHC-fed mice were larger and paler in color than those of control mice (Figure 1A). Oil red O staining revealed the accumulation of lipids in the livers of HFHC-fed mice (Figure 1B). Hepatic TC contents were also significantly higher in HFHC-fed mice (Figure 1C). On the other hand, no significant differences were observed in hepatic TG contents between two groups (Figure 1C).

In addition to advanced diffused vesiculate fatty changes, swelling of the hepatocytes, anisokaryosis, mitotic figures in the nucleus, inflammatory cell infiltration (such as lymphocytes and neutrophils), oval cell hyperplasia, and fibrosis were observed in HFHC-fed mice (Figure 1B). Fibrosis was mainly observed around the central vein or oval cell hyperplasia and disfigured hepatic plates (cords) with almost absent sinusoidal spaces due to fibrosed architecture were distributed throughout the liver (Figure 1B). These morphologies were consistent with diagnostic criteria of NASH in humans.

### Table 2: Body weight, liver weight, and plasma biomarker levels.

The mRNA expression levels of the major inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, in the liver were significantly higher in HFHC-fed mice than in control mice (Figure 2). The mRNA expressions of F4/80, a macrophage maker, and SAA1 and SAA2, inflammatory proteins, were also higher in HFHC-fed mice. These results indicated that the HFHC diet induced not only the accumulation of lipids, but also inflammation, including the infiltration of macrophages, in the liver (Figure 2).

### CYPs mRNA Expression Levels and Activities in the Liver

The mRNA expression levels of the major CYP subtypes, such as CYP1A1, CYP1A2, CYP2C, and CYP3A, were measured in the liver. CYP1A2, CYP2C9, and CYP3A11 mRNA expression levels were significantly higher in HFHC-fed mice than in control mice. CYP1A1 mRNA expression levels were also higher in HFHC-fed mice (Figure 3A). According to mRNA expression levels, CYP1A1, CYP1A2, and CYP3A activities were significantly higher in HFHC-fed mice than in control mice (Figure 3B). However, CYP2C activity was significantly higher in HFHC-fed mice than in control mice (Table 2).
lower in HFHC-fed mice than in control mice, even CYP2C29 mRNA expression levels were higher in HFHC-fed mice.

**Efficacy of Atorvastatin on NASH Model Mice**

We examined efficacy of the pravastatin (which is not metabolized by CYP3A11) and the atorvastatin (which is metabolized by CYP3A11) in HFHC-fed mice (Table 3). Pravastatin could slightly decrease plasma levels of total cholesterol and non-HDL-cholesterol, and hepatic TC and TG contents compared to its control (H2O). On the other hand, atorvastatin increased liver weight, plasma levels of TC and non-HDL-cholesterol, and hepatic lipids contents compared to its control (DMSO). In addition, atorvastatin dramatically increased plasma levels of ALT, AST, and ALP. These adverse effects of atorvastatin were not observed in mice fed a control diet (data not shown). These results indicated that atorvastatin not only failed to decrease lipid levels, but also increased its adverse effects in NASH.

**Discussion**

We herein examined changes in the activities of the major CYP subtypes in mice fed a HFHC diet. Feeding mice the HFHC diet for 12 weeks induced morphological changes in the liver that were consistent with the diagnostic criteria for NASH in humans. Under this condition, CYP1A1, 1A2, and CYP3A activities were increased, whereas CYP2C activity was decreased in the liver.

Body weight was significantly lower in HFHC-fed mice than in control mice (Table 2). Food intake was also lower by HFHC-fed mice than by control mice; however, no marked differences were observed in energy intake between two groups. Du et al. previously reported that protein intake was important for body weight gain. Although more than 10% of energy from protein did not affect body weight gain, less than 8% of energy from protein decreased it in a protein-dose dependent manner in a rat model, and may be attributed to a decrease in body fat with a lower protein diet [16]. In the present study, mice obtained 5.1% of energy from protein in the HFHC diet, and weight loss in HFHC-diet-fed mice may be attributed to lower protein intake and the subsequent decrease in body fat.

The expression of the major CYP subtypes is influenced by fatty liver including NAFLD and NASH. In a mouse model of NAFLD, CYP1A1 mRNA expression levels were lower, while CYP3A11 mRNA expression levels were higher in C3H mice fed the NASH-inducing diet than in control mice [17]. Furthermore, no significant differences were previously reported in CYP1A1 mRNA expression levels between mice fed a NASH-inducing diet and control mice [18]. On the other hand, both CYP1A1 and CYP3A11 mRNA expressions were higher.
fed a saturated fat rich diet [24]. mRNA and protein expression levels were markedly increased in rats (P:F:C = 20:10:70) [23]. Furthermore, although changes in dietary fat expression levels in the livers of mice more than the control diet (P:F:C = 26:13:61) increased CYP3A11 mRNA and CYP3A protein casein) [20-22]. A high-fat diet (P:F:C = 20:60:20) did not cause any influenced CYP activity. Our laboratory previously reported that CYP , mice should be considered when adjust our results to humans.

In addition to liver conditions, the composition of the diets also influenced hepatic lipid profiles between human NASH and our NASH model patients with NASH typically accumulate TG. This difference in the liver microsomes and human hepatocytes [21]. However, the livers of HFHC-fed mice, even though CYP2C9 mRNA expression levels were increased. The reason for this discrepancy may be as follows; mRNA expression was CYP2C9 definitive; on the other hand, activity was measured as total CYP2C subtype activity. Many CYP2C subtypes, including CYP2C29, CYP2C37, and CYP2C38, exist in mice. A previous study reported that CYP2C9 activity was increased and CYP2C19 activity was decreased with progressive states of liver disease in humans [13]. Therefore, the decrease observed in CYP2C activity in the present study reflected total CYP2C subtype activities.

Hepatic TC contents were significantly higher in HFHC-fed mice, whereas no significant difference was noted in hepatic TG contents between HFHC-fed mice and control mice. These results are consistent with previous findings [6]. Few studies have examined the relationship between cholesterol and CYP activity. CYP51 plays a role in cholesterol de novo synthesis in the livers of both humans and mice, whereas the expression of CYP51 is known to be regulated by cholesterol via SREBP [20]. Cholesterol also inhibited CYP3A4 activity in both human liver microsomes and human hepatocytes [21]. However, the livers of patients with NASH typically accumulate TG. This difference in the hepatic lipid profiles between human NASH and our NASH model mice should be considered when adjust our results to humans.

In addition to liver conditions, the composition of the diets also influenced CYP activity. Our laboratory previously reported that CYP, especially CYP3A, activity was induced more in mice fed a low protein diet (7% (w/w) casein) than in mice fed a control diet (20% (w/w) casein) [20-22]. A high-fat diet (P:F:C = 20:60:20) did not cause any changes in the hepatic expression of CYP3A, whereas regular chow (P:F:C = 26:13:61) increased CYP3A11 mRNA and CYP3A protein expression levels in the livers of mice more than the control diet (P:F:C = 20:10:70) [23]. Furthermore, although changes in dietary fat composition did not alter the induction of CYP2E1, hepatic CYP4A mRNA and protein expression levels were markedly increased in rats fed a saturated fat rich diet [24].

There are some limitations to adapt our results to human patients, because several crucial difference between human NASH and our model mouse. First, most of NASH patients are obese and insulin resistance, whereas body weight was decreased and we could not define insulin resistance in this mouse model. A MCD diet-induced NASH model mouse also decreased body weight. Decreased body weight is important issue in diet-induced NASH mouse model. Second, major lipids in the liver are triglycerides in human NASH, whereas it was cholesterol in our mouse model. These differences might influence on the CYP activities. However, liver pathology of this mouse model resembled to human NASH [5,6]. In addition, cholesterol accumulation in the liver was also important in the crown-like structures and activation of kupffer cells in both mice and human NASH [25], and cholesterol lowering drugs suppressed it [26]. Indeed, pravastatin slightly attenuated NASH progression in this study. However, atorvastatin exaggerated it. At this time, detail mechanism is not clear. But it is reported that atorvastatin was metabolized into para-hydroxy and ortho-hydroxy metabolites by CYP3A4 [27] and these metabolites concentration was high in patients with atorvastatin-induced myopathy [28]. Atorvastatin metabolites might be associated with its adverse effects. Increased CYP3A activity might increase atorvastatin metabolites and exaggerated NASH in our mouse model. Further investigation is needed to clarify this mechanism.

Conclusions
We showed that CYP1A1, 1A2, and CYP3A activities were increased, whereas CYP2C activity was decreased in the liver in a HFHC diet-induced NASH mouse model. These results suggest that NASH might affect medication in patients. However, diet-induced NASH model, which used in this study, could not represent human NASH morphology completely. Further investigation is needed to clarify the influence of NASH on drug efficacy in human.

Conflict of Interest
The authors have no conflict of interest directly relevant to the content of this article.

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