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Original Article

Differential expression of Cytochrome P450 1A2 in simple fatty liver and steatohepatitis

Azusa Kawashima[1], Naoto Uramaru[1], Toshiyasu Mikuma[2], Makoto Osabe[1] and Toshiyuki Higuchi[1]

[1]Division of Pharmaceutical Health Biosciences, Nihon Pharmaceutical University, 10281, Komuro, Ina-machi, Kitaadachi-gun, Saitama 362-0806
[2]Division of Physical and Analytical Sciences, Nihon Pharmaceutical University, 10281, Komuro, Ina-machi, Kitaadachi-gun, Saitama 362-0806

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ABSTRACT — Clarifying the cytochrome P450s (CYPs) changes in non-alcoholic fatty liver disease (NAFLD) is important for optimizing drug therapy. This study compared the expression of CYP isoforms in rat models of nonalcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). An NAFL model without tissue damage or inflammation was established by feeding rats a high-fat diet (HFD) for a relatively short period of 4 weeks. Feeding rats with a methionine-choline-deficient diet (MCDD) for 4 weeks produced steatohepatitis-like NASH. Here, the mRNA and protein expression levels of several CYP enzymes in NAFL and NASH models were compared with those in rats fed a control diet (CD). CYP1A2 expression and activity were upregulated in the NAFL model and downregulated in the NASH model, suggesting a reversal of CYP1A2 expression between NAFL and NASH. Differential expression of CYP1A2 in the NAFL and NASH models was observed in primary rat hepatocytes. These findings suggest that CYP1A2 expression/activity vary from the early stages of NAFL to NASH and that monitoring the pharmacokinetics of CYP1A2-metabolized drugs in humans with NAFL and NASH is necessary.

Key words: Cytochrome P450s, Non-alcoholic fatty liver disease, Non-alcoholic fatty liver, Non-alcoholic steatohepatitis, Lipid accumulation, Drug interaction

INTRODUCTION

The efficacy of drugs is greatly affected by the ability of the liver to metabolize drugs because orally administered drugs are subject to first-pass effects in the small intestine, portal vein, and liver (Tsunoda *et al.*, 2021). Cytochrome P450s (CYPs) play a major role in the metabolism of xenobiotics in the liver, including drugs and food ingredients (Zhao *et al.,* 2021). Drug metabolism in the liver varies depending on various factors, such as age, sex, and disease, which can be caused by variations in the expression levels of CYP enzymes (Zanger and Schwab, 2013). The metabolism of orally administered drugs in the liver depends on CYP enzyme expression. Previous studies have demonstrated that when drugs such as nifedipine (Kleinbloesem *et al.*, 1986), atorvastatin (Lipitor® [package insert]. 2022), and propranolol (Kalam *et al.*, 2021) were orally administered to patients with liver cirrhosis, it was reported that the plasma levels of these drugs were higher than those in patients without liver cirrhosis. In addition, mild or moderate liver disease decreases the activities of CYP2C19 (Adedoyin *et al.*, 1998). Moreover, cholestatic cirrhosis decreases CYP1A2, CYP2C9, and 2E1 (George *et al.*, 1995). Thus,

Correspondence: Toshiyuki Higuchi (E-mail: higuchi@nichiyaku.ac.jp)

liver disease has been postulated to affect drug metabolism, pharmacokinetics, and pharmacology.

Non-alcoholic fatty liver disease (NAFLD) includes non-alcoholic fatty liver (NAFL), a simple fatty liver characterized by lipid accumulation in hepatocytes, and non-alcoholic steatohepatitis (NASH) with inflammation that can progress to fibrosis, cirrhosis, and hepatocellular carcinoma (Torres *et al.*, 2012). The two-hit theory (Day and James, 1998) was proposed by adding secondary stresses such as oxidative stress and inflammatory cytokines. The multiple-parallel hit hypothesis (Buzzetti *et al.*, 2016) involves multiple factors simultaneously in the development of NASH from NAFL. NAFLD is a phenotype of metabolic syndrome of the liver and is associated with obesity, glucose intolerance, dyslipidemia, and hypertension (Rector *et al.*, 2008; Marchesini *et al.*, 2003). Therefore, patients with NAFLD may take multiple medications, suggesting alterations in the drug metabolism and pharmacokinetics of NAFLD (NAFL/NASH) should be considered.

Animal model studies have demonstrated that fat accumulation and/or inflammation in the liver alter the expression and activity of CYPs. Feeding a methionine-choline-deficient diet (MCDD) produces fatty acid accumulation and inflammation or tissue injury (assumed for NASH) in the liver, which markedly decreases the expression of CYP2B1 and CYP2E1 compared with feeding a normal diet (Cho *et al.*, 2016; Stärkel *et al*., 2003). Similarly, the activity of most CYP enzymes in the livers of MCDD-induced NASH model mice was lower than that in normal diet-fed mice (Na *et al.*, 2018). In addition, liver damage caused by carbon tetrachloride (Sasame *et al.*, 1968) and acetaminophen (Bao *et al.*, 2022) leads to a similar decrease in the expression and activity of CYP enzymes. Feeding mice a high-fat diet (HFD) increased the expression levels of CYP1a1, 1a2, 2b10, and 2c29 and decreased CYP3a11 in the liver (Chiba *et al.*, 2016). Thus, the changes in the expression of CYP isoforms in simple fatty livers (assumed for NAFL) produced by feeding HFD seem to differ from those in the livers of MCDD-fed mice. Regulation of CYP expression may be affected by the presence or absence of inflammation; however, the details remain unclear. In addition to the CYP superfamily, we previously demonstrated that the expression of hepatic UDP-glucuronosyltransferases 1A1 and 1A6 is altered in the livers of HFD-fed rats (Osabe *et al.*, 2008). As mentioned previously, patients with NAFL are expected to take therapeutic drugs for glucose tolerance, dyslipidemia, and hypertension. Therefore, it is necessary to clarify the differences in drug metabolism and pharmacokinetics between NAFL and NASH to optimize drug therapies for NAFL. However, there is little information on the changes in the expression of CYP enzymes in NAFL, a simple fatty liver, and studies comparing NAFL and NASH models.

This study aimed to clarify the differences in regulating CYP subfamily expression in fatty livers with and without tissue damage/inflammation. To achieve this objective, NASH and NAFL models were developed by subjecting rats to MCDD and HFD, respectively, and the expression of CYP enzymes was compared between the NAFL and NASH models.

MATERIALS AND METHODS

Chemicals

Normal rodent diet containing 10% fat calories (control diet, CD) (cat. D12450B), a high-fat diet containing 60% calories from fat (HFD) (cat. D12492), and a methionine-choline-deficient diet containing 21% calories from fat (MCDD) (Cat. A02082002B) were purchased from Research Diets, Inc. (New Brunswick, NJ, USA). Williams' medium E, L-glutamine, Ca^{2+}, Mg^{2+}-free 10 mM phosphate-buffered saline (PBS[-]), penicillin/streptomycin solution, sodium oleate (oleic acid), sodium palmitate (palmitic acid), bicinchoninic acid, bovine serum albumin (BSA), and fatty acid-free BSA of cell-culture grade (endotoxin-free) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HyClone™ fetal bovine serum (FBS) was purchased from Cytiva (Tokyo, Japan). A lipid extraction kit (Cat. STA-612) was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Collagenase, cholesterol (T-Cho) E-test Wako, non-esterified cholesterol (F-Cho) E-test Wako, triglycerides (TG) E-test Wako, non-esterified fatty acid (NEFA) C-test Wako, transaminase (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) C-test Wako and glucose (Glu) C2-test Wako were purchased from FUJIFILM Wako Pure Chemical Co. (Tokyo, Japan). A lipid assay kit (Cat. AK09F) was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). The reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). ISOGEN LS and ISOGEN II were purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). The ReverTra Ace qPCR RT Master Mix and Thunderbird SYBR NEXT qPCR Mix were purchased from Toyobo Co., Ltd. (Osaka, Japan). The polyclonal antibodies against rat CYP1A1 (cat. ab79819) were purchased from Abcam (Cambridge, UK). The monoclonal antibodies against rat CYP1A2 (cat. sc-53241), CYP2C6 (cat. sc-53245), CYP3A1 (cat. sc-53246), and CYP4A1 (cat.

sc-53248) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The polyclonal antibodies against CYP2D1 (cat. 17868-1-AP) were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse β-actin (cat. A3854) was purchased from Sigma-Aldrich. The HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were of reagent grade.

Animals

Male Wistar rats (6-week-old, weighing 120–140 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed at 22°C with a 12 hr light/dark cycle, 55% relative humidity, free tap water access, and a normal laboratory diet (CE-2) (CLEA Japan, Inc., Tokyo, Japan) for 1 week. The rats were divided into three groups based on their diets (n = 6 per group): CD, HFD, and MCDD-fed. The rats were fed CD, HFD, or MCDD for 4 weeks. All animal experiments were approved by the Animal Experimentation Ethics Committee of the Nihon Pharmaceutical University (approval number: AE2021-003, Saitama, Japan).

Preparations of liver homogenates and microsomes

Rat livers were homogenized in four volumes of 10 mM potassium/sodium phosphate buffer (pH 7.4) containing 1.15% KCl solution (homogenate buffer) using a Potter-Elvehjem homogenizer. The S9 fractions of rat livers were obtained from the homogenates via centrifugation at 9,000 × g for 20 min at 4°C. The microsomal and cytosolic fractions were obtained from the S9 fractions via centrifugation at 105,000 × g for 60 min at 4°C. The microsomal fractions were washed by resuspension in two volumes of homogenate buffer and resedimented at 105,000 × g for 60 min. The microsomal pellets were resuspended in 1 mL of homogenate buffer, equivalent to 1 g of liver. Protein content was determined using the bicinchoninic acid (BCA) method (Smith *et al.,* 1985), with bovine serum albumin as the standard. The liver homogenates and microsomal fractions were stored at -80°C until further analysis.

Plasma biochemical analysis

Blood was collected from the overnight-fasted rats after 4 weeks of CD-, HFD-, or MCDD-based diets. After adding heparin, the plasma component was extracted from the blood sample via centrifugation and stored at -80°C until further analysis. Following the manufacturer's instructions, plasma levels of T-Cho, TG, F-Cho, NEFA, AST, ALT, and Glu were quantified using enzymatic methods and the corresponding assay kits.

Liver histological analysis

The livers were dissected into 5 mm squares, fixed with 10% formalin neutral buffer solution, exchanged with 20% sucrose (overnight) and 30% sucrose (overnight), and embedded in an O.C.T. compound (Sakura Seiki, Tokyo, Japan). The sections were prepared using a cryostat at -20°C and dried overnight at 22°C. Oil Red O staining was performed using a lipid assay kit to observe fat accumulation. Briefly, the sections were washed three times with distilled water. Thereafter, it was stained with an Oil Red O-staining solution (60% isopropanol solution), which was prepared by diluting the Oil Red O stock solution to 60% with distilled water for 30 min at 22°C. The slides were washed with 60% isopropanol and distilled water, dried for 3 hr at 37°C, and mounted with glycerol. The images were captured using an Olympus IX71 inverted microscope (Olympus Co., Tokyo, Japan). To identify morphological alterations in the liver, sections were stained with hematoxylin and eosin (HE) using standard procedures to identify morphological alterations in the liver. The degree of tissue fibrosis in the liver was examined using Azan-Mallory staining.

Liver lipid contents

Lipids in the liver homogenates were extracted using a lipid extraction kit following the manufacturer's instructions. The T-Cho, F-Cho, TG, and NEFA contents in the liver were determined using the corresponding assay kits and enzymatic methods, following the manufacturer's instructions.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total mRNA was isolated from the liver homogenate using ISOGEN LS following the manufacturer's instructions. RNA was quantified on a drop plate using a Multiskan Sky High Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). To obtain cDNA, 1 µg of RNA was subjected to reverse transcription using the ReverTra Ace qPCR RT master mix (Toyobo Co., Ltd., Osaka, Japan). PCR amplification of cDNA for rat CYP 1A1, 1A2, 2B1, 2C6, 2C11, 2D1, 2D2, 2E1, 3A1, 4A1, and β-actin was performed using the Thunderbird Next SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and a StepOne™ real-time PCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The primer sequences used for PCR are listed in Table 1. The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ meth-

Table 1. Primer sequences used for quantitative PCR.

	Forward (5′ → 3′)	Reverse (5′ → 3′)
β-Actin	TGTCACCAACTGGGACGATA	GCATGAGGGAGCGCGTAA
CYP1A1	ACGTGAGCAAAGAGGCTGAA	TCGTGGTCATAACGTCTGCC
CYP1A2	TCGAACCAGTCAACCAGGTG	GTCCTTGCTGCTCTTCACGA
CYP2B1	AGGACCATGGAGCCCAGTAT	AGAAATCCTCAGCTTGGCCC
CYP2C6	TTGCTCCTGCTGAAGTGTCC	AATGTCACAGGTCACCGCAT
CYP2C11	CAGTCCTAGTCCTGGTGCTCACT	CTGAAGGGTGTTTCCAATGATTG
CYP2D1	TTGGTCATTTGTCTTTGGGAGC	CACAGGCCAGTCCCATTCAG
CYP2D2	CACCTTACGGACCTGAGTGG	GGCAATCACGTTGCTCACAG
CYP2E1	TCCCCAAGTCTTTCACCAAGTT	GAGCCAAGGTGCAGTGTGAAC
CYP3A1	CCTCTGTTTGCCATCACGGA	GACAGGTTTGCCTTTCTCTTGC
CYP4A1	AGGAGCGAGGAACTGCATTG	CGGAGCTCCACAACGGAAT

od (Livak and Schmittgen, 2001) and normalized against the expression level of β-actin as an internal standard. All quantifications were performed three times independently.

Western blot analysis

The samples containing the microsomes were mixed with an equivalent volume of 2 × sample buffer (125 mM Tris-HCl, pH 8.8, 0.2% sodium dodecyl sulfate, 20% glycerol, 0.04% bromophenol, and 0.1 M dithiothreitol). The mixture was then boiled for 5 min. Subsequently, 40 μg of protein per lane was separated using 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore Sigma, Bedford, MA, USA). The membranes were incubated using 20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride (Tris-buffered saline; TBS) containing 0.05% Tween 20 and 2% skim milk as blocking solutions for 2 hr at 22°C. The membranes were incubated with the indicated primary antibodies for 2 hr at 22°C. After being washed with TBS containing 0.05% Tween 20, the membranes were incubated with the HRP-conjugated secondary antibodies for 1 hr at 22°C and visualized using an enhanced ImmunoStar LD or ImmunoStar Zeta (both FUJIFILM Wako Pure Chemical Co.) with a LuminoGraph I (ATTO, Tokyo, Japan). Densitometric analysis was performed using CS Analyzer 4 software (ATTO, Tokyo, Japan), and the data were normalized to the expression level of β-actin as an internal standard.

Assay of CYP1A2 activity

CYP1A2 activity was determined by measuring caffeine 8-oxidase activity (Kot and Daniel, 2008). Caffeine 8-oxidase activity was measured using the high-performance liquid chromatography (HPLC) method. The reaction mixture consisted of 1.0 mmol/L caffeine, 10 mmol/L NADPH, and 1.6 mg protein/mL rat liver microsomes in 0.1 M potassium/sodium phosphate buffer (pH 7.4) at a final volume of 2 mL. The mixture was incubated at 37°C for 20 min, after which 2% $ZnSO_4$ of 1.8 mL and 2 mol/L HCl of 200 μL were added to immediately terminate the reaction. The mixture was extracted twice with 5 mL ethyl acetate containing 1 μmol/L 7-(2-hydroxypropyl) theophylline (an internal standard). The extraction mixture was centrifuged, and the organic layer was collected and evaporated to dryness. The residue was dissolved in 200 μL acetonitrile, and 20 μL of the resulting sample was subjected to HPLC analysis using a GL-7450 Hitachi chromatograph equipped with a CAPCELL-PAK C18 UG120 column (particle size 5 μm, 4.6 mm × 250 mm) (Shiseido Co., Ltd., Tokyo, Japan). Analysis was performed using acetonitrile: 0.1% formic acid (5:95) as the mobile phase. The chromatography was operated at a flow rate of 1 mL/min at 40°C, with detection at 254 nm. The metabolite 1,3,7-trimetric acid (C8-oxidation product) was detected as a single peak in the extract of the incubation mixture using HPLC analysis. This peak was eliminated upon incubation with boiled rat liver microsomes or without NADPH, indicating that the metabolite was produced via a CYP1A2 enzymatic reaction. The amount of 1,3,7-trimethyluric acid was determined from peak areas. Caffeine 8-oxidase activity was expressed as 1,3,7-trimethyluric acid (nmol/min/mg protein).

Primary culture of rat hepatocytes and fatty acid (FA)-induced steatosis

Primary hepatocytes were isolated from 6-week-old male Wistar rats using the two-step collagenase perfusion method (Berry and Friend, 1969). The hepatocytes were seeded in 24-well (2×10^5 cells/well) and 6-well (1.2×10^6 cells/well) collagen-coated culture plates in Williams' medium E supplemented with 2 mM Glutamine, 100 U/mL penicillin, 100 μg/mL, and 10% FBS (10% FBS-Williams' medium E) at 37°C in 5% CO_2. To induce

Table 2. Body weights, liver weights, and relative liver weights in the liver of CD-, HFD- or MCDD-fed to rats for 4 weeks.

	CD	HFD	MCDD
Body weights (g)	243.1 ± 7.01	259.4 ± 3.63*	88.8 ± 6.07*
Liver weights (g)	7.75 ± 0.79	7.46 ± 0.28	4.70 ± 0.27*
Relative liver weights (Liver weights /Body weights %)	3.19 ± 0.37	2.88 ± 0.11	5.29 ± 0.39*

Male Wistar rats were fed the CD, HFD, or MCDD for 4 weeks. Body weights and liver weights were measured 4 weeks after the initiation of the experimental diets. Data are presented as mean ± standard deviation (SD), n = 6 in each group. *$p < 0.05$ vs. control.

steatosis, hepatocytes were cultured in FA solutions containing (bovine serum albumin BSA (fatty acid-free, endotoxin-free). Briefly, sodium oleate (OA) at a concentration of 3 mM or a combination of OA and sodium palmitate (PA) in a ratio of 2:1 (OA 3 mM: PA 1.5 mM) with a BSA (fatty acid-free, endotoxin-free) (1.5 mM) mixture was completely dissolved by applying ultrasonic waves. The dissolved FA-BSA mixtures were diluted with 10% FBS-William's medium E to the experimental concentrations, and the hepatocytes were cultured with OA (0.5 mM) or a combination of OA (0.5 mM OA and 0.25 mM PA). The degree of fat accumulation in primary cultured hepatocytes was examined via Oil Red O staining. The stained lipid droplets were dissolved in isopropanol and quantified using spectrophotometry at 540 nm. Total mRNA was isolated from hepatocytes in 24-well plates using ISOGEN II, following the manufacturer's protocol. Reverse transcription and polymerase chain reaction (PCR) amplification were performed as previously described. The PCR primer for rat IL-1β was as follows: forward 5′-ACAAGGAGAGACAAGCAACGAC-3′; reverse 5′-TCTTCTTTGGGTATTGTTTGGG-3′. The mRNA levels of IL-1β were determined as described previously. The cell proteins were collected from hepatocytes in the 6-well plates and were subjected to western blotting for CYP1A2 and β-actin.

Statistical analyses

All data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA, followed by Dunnett's post-hoc test for multiple group comparisons. Calculations were performed using the R software (R Development Core Team). Statistical significance was set at $p < 0.05$.

RESULTS

Body weights, liver weights, and relative liver weights

The body weight, liver weight, and relative liver weight (liver weight/body weight %) of rats fed CD, HFD, or MCDD for 4 weeks are shown in Table 2. The body weight of HFD-fed rats was significantly higher than that of CD-fed rats, whereas the body weight of MCDD-fed rats was lower than that of CD-fed rats. This is consistent with previous reports showing that MCDD reduces body weight by promoting energy metabolism and expenditure (Rizki *et al.*, 2006). When the relative liver weight was expressed as liver weight to body weight, the values in the MCDD-fed rats were 1.7-fold higher than those in the CD-fed rats. In contrast, the values in the CD- and HFD-fed rats were similar. These results suggest that feeding rats MCDD is more likely to cause liver enlargement relative to body weight (liver weight/body weight %) than feeding them HFD under rearing conditions for 4 weeks. This is consistent with the findings in MCDD-fed rats in a previous study (Kirsch *et al.*, 2003).

Plasma chemistry, liver lipid contents, and liver histology

Plasma levels of T-Cho, TG, F-Cho, NEFA, AST, ALT, and Glu are shown in Table 3. In rats fed an HFD for 4 weeks, the levels of T-Cho, TG, and F-Cho were reduced to 63.9%, 42.4%, and 49.6% of those in CD-fed rats, respectively. In rats fed MCDD for 4 weeks, the levels of TG, F-Cho, and Glu were reduced to 19.4%, 66.4%, and 54.7%, respectively, of those in CD-fed rats. The plasma levels of AST and ALT in MCDD-fed rats were elevated to 211% and 268% of those in CD-fed rats, respectively. In contrast, there were no changes in their levels in HFD-fed rats. These results suggested that the livers of MCDD-fed rats were inflamed, whereas those of HFD-fed rats were not.

The lipid content in the livers of CD-, HFD-, and MCDD-fed rats was measured. The TG content in the livers of HFD-fed rats for 4 weeks increased 2.1-fold compared with that in CD-fed rats. However, the T-Cho, F-Cho, and NEFA contents did not change (Fig. 1). MCDD-fed rats showed a significant increase in all measured contents, including T-Cho, TG, F-Cho, and NEFA, compared to CD-fed rats (Fig. 1). The TG content in the

Table 3. Plasma biomarker levels in the liver of CD-, HFD- or MCDD-fed to rats for 4 weeks.

	CD	HFD	MCDD
T-Cho (mg/dL)	75.4 ± 12.5	48.2 ± 3.1*	65.9 ± 12.3
TG (mg/dL)	127.8 ± 26.5	54.2 ± 3.3*	24.8 ± 3.5*
F-Cho (mg/dL)	11.9 ± 1.3	5.9 ± 0.6*	7.9 ± 1.1*
NEFA (mEq/L)	0.95 ± 0.16	0.96 ± 0.27	0.90 ± 0.14
AST (Karmen)	78.1 ± 23.2	130.1 ± 59.5	164.9 ± 20.6*
ALT (Karmen)	10.4 ± 0.9	10.4 ± 1.2	27.9 ± 4.7*
Glu (mg/dL)	119.3 ± 19.5	127.8 ± 7.1	65.2 ± 3.9*

Male Wistar rats were fed the CD, HFD, or MCDD for 4 weeks. T-Cho, TG, F-Cho, NEFA, AST, ALT, and Glu levels of plasma were measured 4 weeks after initiation of the experimental diets. Data are presented as mean ± standard deviation (SD), n = 6 in each group. *p < 0.05 vs. control.

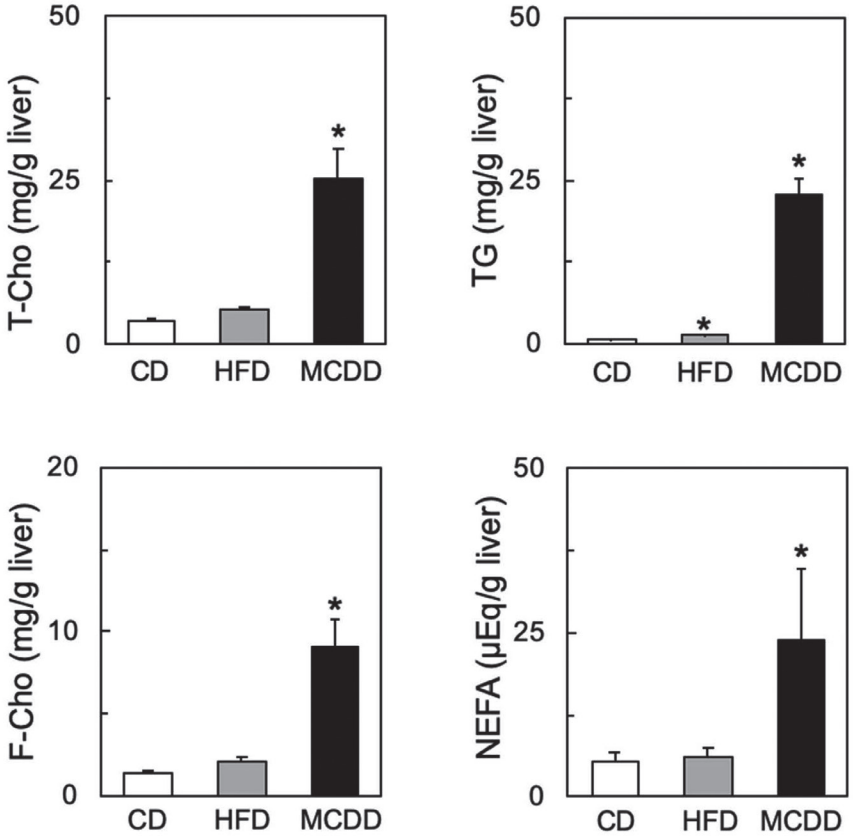

Fig. 1. Hepatic Lipid Contents in the liver of rats fed with CD, HFD, or MCDD for 4 weeks. After feeding the rats with CD, HFD, or MCDD for 4 weeks, the lipids in the livers were extracted, and the T-Cho, TG, F-Cho, and NEFA contents were measured using enzymatic methods. Data are represented as mean ± standard deviation (SD), n = 6 in each group. *p < 0.05 vs. control.

MCDD-fed rats was 35-fold higher than that in the CD-fed rats (Fig. 1).

The livers of HFD- and MCDD-fed rats appeared whiter than those of CD-fed rats (Fig. 2A), suggesting that the HFD- and MCDD-fed rats had fatty livers. Oil Red O, HE, and Azan-Mallory staining were performed to histologically examine the degree of lipid droplet accumulation and fibrosis in the liver tissue. Oil Red O staining revealed stronger staining, which is characteristic of accumulated lipids, in HFD- and MCDD-fed rat livers than in CD-fed rats (Fig. 2B). HE staining showed remarkable ballooning, a characteristic of lipid droplets, in MCDD-fed rat livers, whereas there was no clear ballooning in HFD-fed rats, indicating that the TG content

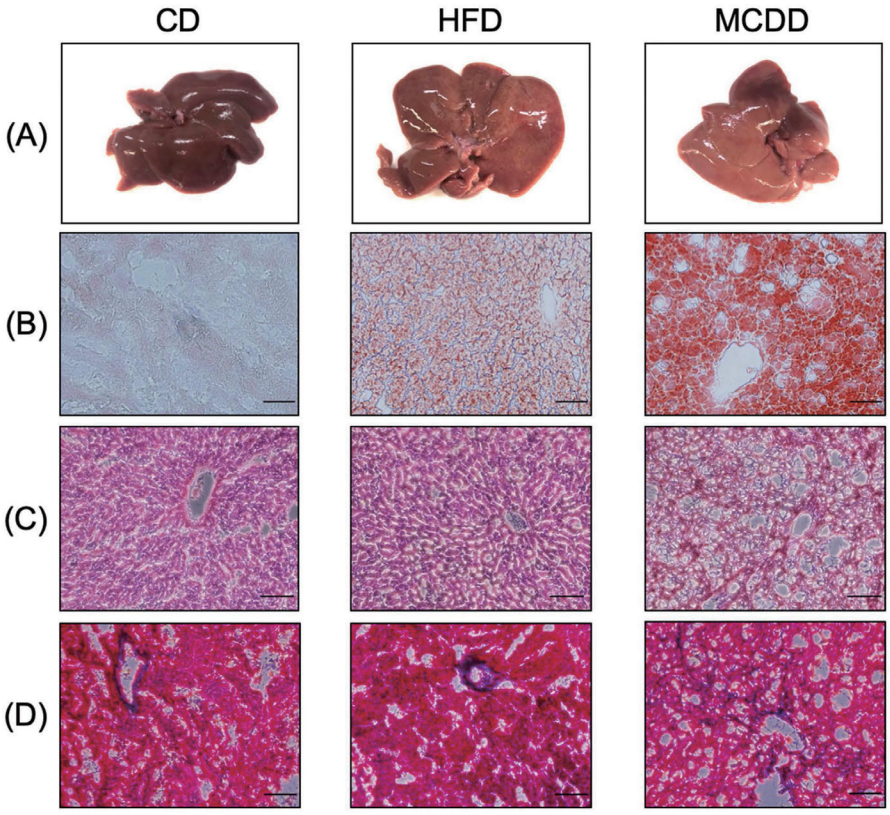

Fig. 2. Gross and microscopic findings in the livers of rats fed with CD, HFD, or MCDD for 4 weeks. (A) Gross appearance of liver from CD-, HFD-, or MCDD-fed rats. (B) Oil Red O staining of liver tissues from CD-, HFD- or MCDD-fed rats. (Magnification: 100×, scale bars: 100 μm) (C) HE staining of liver tissues from CD-, HFD-, or MCDD-fed rats. (Magnification: 100×, scale bars: 100 μm) (D) Azan-Mallory staining of liver tissues from CD-, HFD-, or MCDD-fed rats. (Magnification: 100×, scale bars: 100 μm).

in MCDD-fed rat livers was higher than that in HFD-fed rats (Fig. 2C). Azan-Mallory staining revealed significant fibrosis only in the livers of MCDD-fed rats (Fig. 2D). Additionally, plasma AST and ALT levels, which are markers of liver tissue damage, were significantly elevated only in MCDD-fed mice (Table 3). These results suggested that relatively short-term feeding of HFD and MCDD to rats resulted in an early stage of simple fatty liver and tissue fibrosis caused by inflammation, respectively (Table 3, Figs. 1 and 2). Based on these results, we speculated that the livers of the HFD-fed rats were NAFL models with a simple fatty liver, and the MCDD-fed rat livers were NASH models with steatohepatitis.

Changes in mRNA levels and protein expressions of CYPs in the livers of CD-, HFD- or MCDD-fed rats

To clarify the differences in the expression of CYP isoforms in NAFL and NASH models, the mRNA and protein expression levels of the major CYP isoforms in the livers of HFD- and MCDD-fed rats were compared with those in CD-fed rats (Figs. 3 and 4). When rats were fed an HFD for a short period of 4 weeks to develop simple fatty liver (NAFL), only the mRNA levels of CYP1A2 in the livers were significantly 1.4-fold higher than those in CD-fed rat livers (Fig. 3). In addition, western blot analysis revealed that CYP1A2 protein levels in the NAFL models were significantly higher than those in the normal livers of CD-fed rats (Fig. 4). In the MCDD-induced NASH model, the mRNA and protein levels of CYP1A1 and 4A1 were increased, whereas those of CYP1A2, 2C6, 2C11, 2D1, 2D2, 2E1, and 3A1 were decreased com-

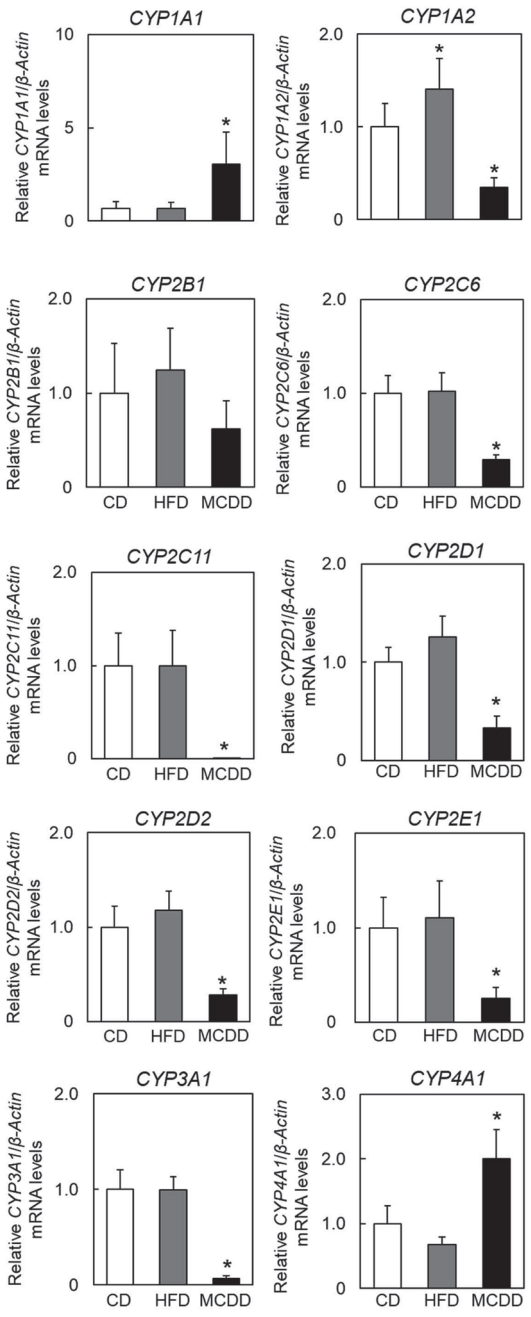

Fig. 3. Changes in mRNA levels of CYP isoforms in the livers of rats fed with CD, HFD, or MCDD for 4 weeks. After feeding the rats with CD, HFD, or MCDD for 4 weeks, they were sacrificed, total RNA was extracted from the CD-, HFD-, or MCDD-fed rat livers, and the mRNA expression levels of several CYP isoforms were measured using quantitative PCR. Relative mRNA levels were determined after normalization with β-actin. Data are represented as mean ± standard deviation (SD), n = 6 in each group. *$p < 0.05$ vs. control.

pared to those in CD-fed rats (Figs. 3 and 4). The upregulation or downregulation of CYP isoforms in the MCDD-induced NASH model was similar to that reported in previous studies (Stärkel *et al.,* 2003; Liu *et al.,* 2019). The mRNA and protein levels of CYP1A2 in the MCDD-induced NASH model were significantly reduced compared to those in the CD-fed rats, though it was induced in the NAFL model (Figs. 3 and 4). These results suggested that CYP1A2-dependent drug-metabolizing ability may be enhanced at an early stage of liver disease without tissue damage/inflammation (NAFL) and attenuated in steatohepatitis (NASH).

Changes in activities of CYP1A2 in liver microsomes of CD-, HFD-, and MCDD-fed rats

As mentioned above, we discovered that CYP1A2 expression in the rat liver was reversed in the NAFL and NASH models. To confirm this, we evaluated the enzymatic activity of CYP1A2 in NAFL and NASH models. CYP1A2 enzymatic activity was assessed based on caffeine 8-hydroxylation activity in the liver microsomes of CD-, HFD-, and MCDD-fed rats.

The caffeine 8-oxidase activities in HFD-induced NAFL microsomes were significantly higher than those in CD-fed rats. In contrast, a significant decrease in the activities was observed in MCDD-induced NASH microsomes compared with those of the control rats (Fig. 5), indicating the up- or downregulation of CYP1A2 expression in these livers (Figs. 3 and 4).

Changes in CYP1A2 expression in steatosis-induced rat primary hepatocytes

The expression and activity of CYP1A2 in rat liver microsomes increased in the NAFL model and decreased compared with those in the normal rat liver in the NASH model (Figs. 3, 4, and 5). Furthermore, we investigated changes in CYP1A2 expression levels in simple fatty liver and steatohepatitis models using primary cultured rat liver cells. Since OA, a monounsaturated fatty acid, mainly induces fat accumulation, and PA, a saturated fatty acid, mainly induces an inflammatory effect (Ricchi *et al.*, 2009), simple fatty liver and steatohepatitis were induced by OA alone or a combination of OA and PA at a molar ratio of 2:1. When primary rat hepatocytes were cultured with OA (0.5 mM) alone or in combination with OA (0.5 mM) and PA (0.25 mM) for 24 hr, both treatments induced significant fat accumulation compared to that in untreated cells (Fig. 6A). Additionally, the combination of OA and PA for 12 hr increased the mRNA levels of the inflammatory cytokine IL-1β (Fig. 6B). Based on these findings, hepatocytes cultured with OA and a com-

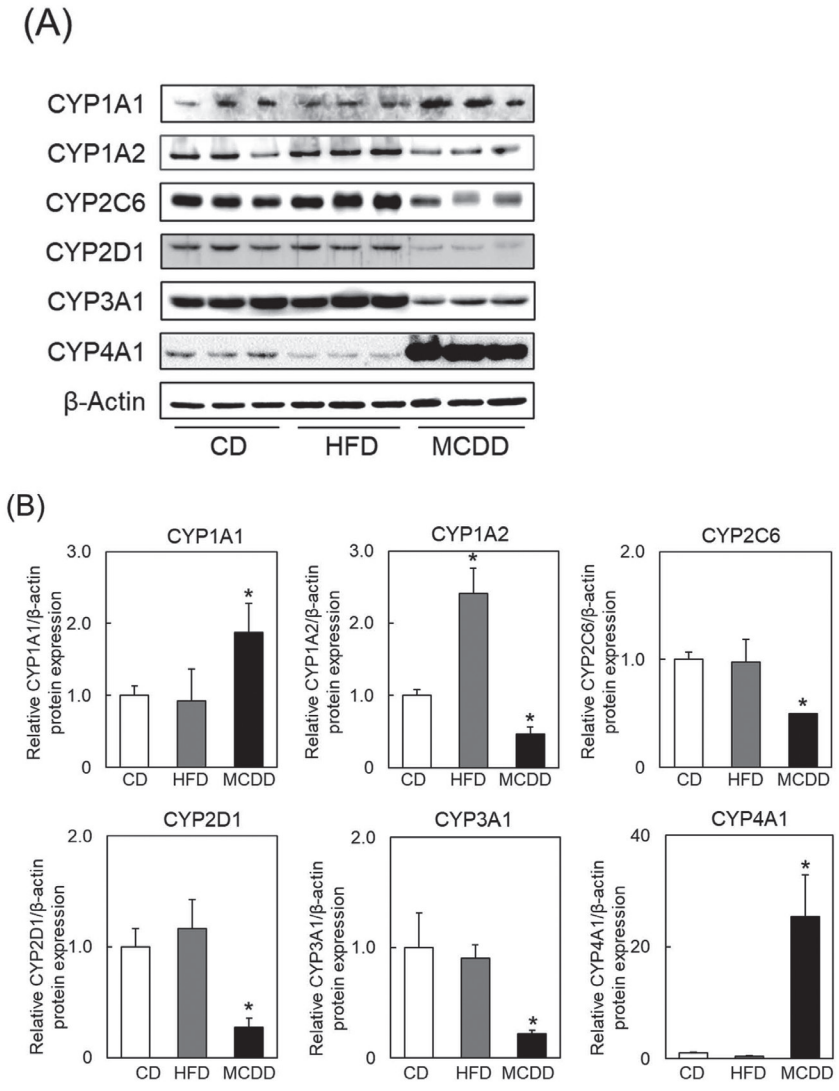

Fig. 4. Changes in expression levels of CYP isoforms in the livers of rats fed with CD, HFD, or MCDD for 4 weeks. After feeding the rats CD, HFD, or MCDD for 4 weeks, the rats were sacrificed, and liver microsomes were prepared from CD-, HFD-, or MCDD-fed rat livers. The protein levels of CYP isoforms in rat liver microsomes were measured using western blot analysis. (A) Typical image obtained via blotting. (B) Relative protein expression was determined after normalization with β-actin. Data are represented as mean ± standard deviation (SD), n = 6 in each group. *$p < 0.05$ vs. control.

bination of OA and PA were assumed to have simple fatty liver and steatohepatitis, respectively. CYP1A2 expression increased in OA-treated cells, whereas it decreased in cells treated with a combination of OA and PA (Fig. 7). These results were similar to the inverted changes in CYP1A2 expression observed in rat NAFL and NASH models (Figs. 3, 4, and 5).

DISCUSSION

Drug metabolism and pharmacokinetics can be altered in patients with liver disease. Traditionally, the expression of the drug-metabolizing enzyme CYPs is believed to be downregulated in liver diseases associated with tissue damage/inflammation. This study provides the novel finding that rat CYP1A2 is upregulated in early-stage simple fatty liver disease and downregulated in NASH with tis-

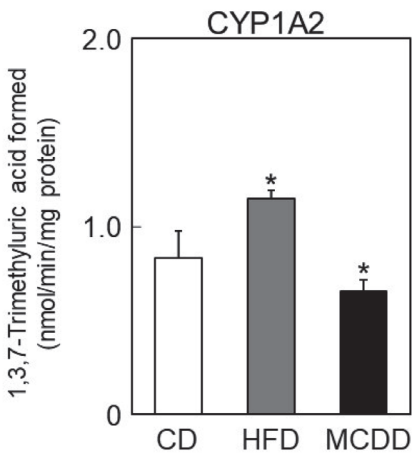

Fig. 5. Changes in CYP 1A2 activities in the livers of rats fed with CD, HFD, or MCDD for 4 weeks. The caffeine 8-oxidase activities catalyzed by CYP1A2 in rat liver microsomes from CD-, HFD-, or MCDD-fed rats were determined by measuring the formed 8-hydroxy caffeine using HPLC, as described in the Materials and Methods section. Data are represented as mean ± standard deviation (SD), n = 6 in each group. *p < 0.05 vs. control.

sue damage or inflammation.

The rats were fed HFD and MCDD for 4 weeks to establish NAFL and NASH rat models. Feeding rodents an HFD for over 8 weeks produces pro-inflammatory abnormalities in the liver (Poulsen *et al.*, 2012; Park *et al.*, 2012). However, our study showed that feeding rats an HFD for 4 weeks resulted in fatty liver formation without tissue damage or inflammation (Table 3, Figs. 1 and 2). This result is consistent with a previous report that rats fed an HFD for 4 weeks showed no changes in AST/ALT (Kai *et al.,* 2017). This suggests that a relatively short-term HFD feeding for 4 weeks was sufficient to establish an NAFL model, a simple fatty liver. In contrast, MCDD-fed rats showed greater weight loss and a more severe fatty liver than in HFD-fed rats. As a result of this mechanism, it is well known that MCDD-fed mice induce NASH by promoting fat uptake into the liver due to the impaired synthesis of very low-density lipoproteins and lipid peroxidation (Rinella *et al.*, 2008). In addition, MCDD-fed rats exhibited pathophysiological properties such as hepatocellular injury, fibrosis, and inflammatory recruitment, similar to those observed in human NASH (Brunt *et al*., 2015). Based on the increase in liver TG content with considerable ballooning by HE staining and hepatic leakage of AST and ALT, rats fed MCDD for 4 weeks produced fatty livers with tissue damage/inflammation (Table 3, Figs. 1 and 2). These

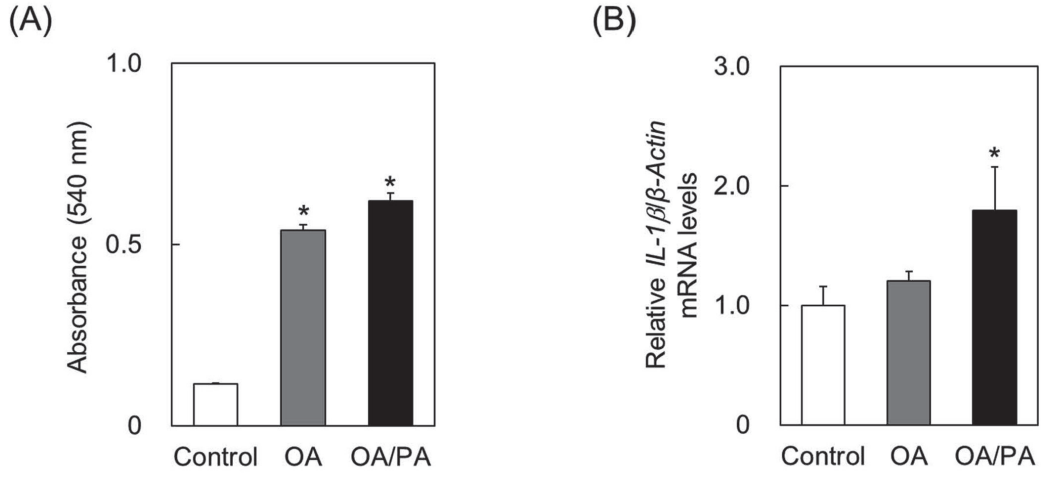

Fig. 6. Lipid accumulations and IL-1β mRNA levels in primary rat hepatocytes treated with OA or a combination of OA and PA. (A) The primary rat hepatocytes were treated with or without OA or a combination of OA (0.5 mM) and PA (0.25 mM) for 24 hr. The lipid accumulation in the primary cultured hepatocytes was examined using Oil Red O staining. The amounts of stained lipid droplets dissolved in isopropanol were quantified using spectrophotometry at 540 nm. (B) The primary rat hepatocytes were treated with or without OA or a combination of OA (0.5 mM) and PA (0.25 mM) for 12 hr. IL-1β mRNA levels were examined via quantitative PCR. Data are represented as mean ± standard deviation (SD), n = 3 in each group. *P < 0.05 vs. 10% FBS-Williams' medium E alone. OA, oleic acid; OA/PA, combination of oleic acid and palmitic acid

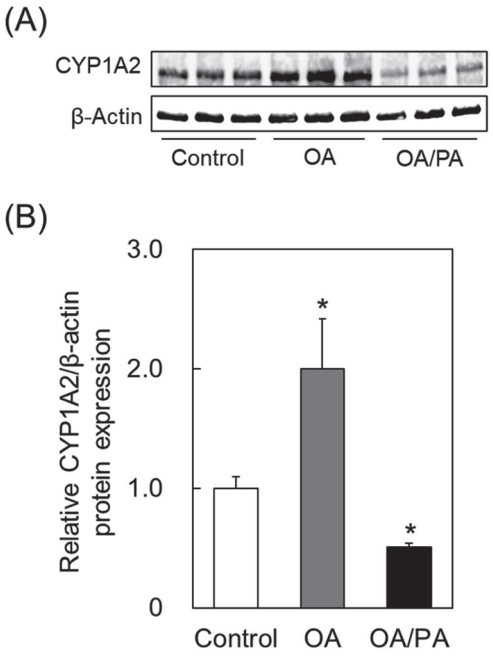

Fig. 7. Changes in expression levels of CYP 1A2 in primary rat hepatocytes treated with OA or a combination of OA and PA. Primary rat hepatocytes were treated with OA or a combination of OA (0.5 mM) and PA (0.25 mM) for 24 hr. (A) Western blots showing CYP1A2 protein in hepatocytes. (B) Quantification of CYP1A2 protein levels. Data are represented as mean ± standard deviation (SD), n = 3 in each group. *P < 0.05, vs. 10% FBS-William's medium E alone.

results were consistent with those of a previous study (Kirsch *et al.*, 2003). Thus, we established NAFL and NASH models and compared the changes in CYP expression/activity in these two models.

These data showed that the NAFL model caused only an upregulation of CYP1A2 expression compared to that in the control. However, the other isoforms did not (Figs. 3 and 4). Conversely, the NASH model revealed the downregulation of the enzyme to a level lower than that in the control. Furthermore, we demonstrated that the alterations in CYP1A2-catalyzed caffeine 8-oxidation were similar to the reversed expression of CYP1A2 in NAFL and NASH models (Figs. 3, 4, and 5). These results suggest that CYP1A2 expression in fatty liver microsomes may be altered in the absence or presence of tissue damage or inflammation. The differential expression of CYP1A2 in the NAFL and NASH models was observed in FA-treated primary rat hepatocytes (Figs. 6 and 7). Thus, feeding rats an HFD for 4 weeks, a relatively short term, will be a useful model of simple fatty liver for investigating alterations in the expression of drug metabolism enzymes, including CYP1A2. The CYP1A2 content is approximately 13% of the total CYP amount in the human liver, and 80% homology of CYP1A2 is retained between humans and rats (Martignoni *et al.*, 2006). Human and rat CYP1A2 catalyzes the metabolism of theophylline and the atypical antipsychotic clozapine, which are subjected to therapeutic drug monitoring (TDM) (Guo *et al.*, 2021; Scherf-Clavel *et al.*, 2023). Therefore, even in humans, CYP1A2-catalyzed drug metabolism may be altered during the early stages of NAFL and NASH. Therefore, the pharmacokinetics of CYP1A2-metabolizing drugs must be carefully monitored.

Our data showed that CYP1A1 and 4A1 expressions were induced in the NASH model with tissue damage and inflammation (Figs. 3 and 4). Previous studies have demonstrated that CYP1A1 expression is induced depending on the expression level of aryl hydrocarbon receptor in the NASH model and that treatment of HepG2 cells with oleic acid accelerates lipid peroxidation (Xia *et al.*, 2019). In addition, CYP1A1 expression is induced by LPS-induced NF-κB activation, which contributes to lung tissue damage and inflammation (Tian *et al.*, 2020). In contrast, the CYP4A family contributes to the ω hydroxylation of saturated straight-chain fatty acids (Johnston *et al.,* 2011; Choi *et al.,* 2018). Human and mouse CYP4A families could relate to the progression to NASH from NAFL by inducing the inflammation system, including the ω hydroxylation of fatty acids, the production of reactive oxygen species, lipid peroxidation, and the activation of the NF-κB signaling pathway (Leclercq *et al.,* 2000; Gao *et al.,* 2020). The knockout of the CYP4A14 gene (cyp4a14−/−) in mice improved MCDD-induced steatohepatitis (Zhang *et al.,* 2017). Therefore, the induction of rat CYP1A1 and 4A1 expressions may be closely related to the progression from NAFL to NASH, similar to that observed in humans and mice. The detailed mechanisms underlying the upregulation of CYP1A2 expression in NAFL, the downregulation of CYP1A2 in NASH, and the inflammation-induced upregulation of CYP1A1 and 4A1 in NASH are currently being elucidated through experiments utilizing primary cultured hepatocytes.

In this study, an NAFL model without tissue damage/inflammation was established by feeding rats an HFD for a relatively short period of 4 weeks. CYP1A2 expression/activity in the rat liver were reversed in NAFL and NASH models. These findings suggest that monitoring the pharmacokinetics of CYP1A2-metabolizing drugs may be required in human NAFL, as the expression and activity

of drug-metabolizing enzymes vary from the early phases of NAFL to NASH.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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