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Effects of the dietary supplements, activated charcoal and copper chlorophyllin, on urinary excretion of trimethylamine in Japanese trimethylaminuria patients

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Abstract

Trimethylaminuria (TMAU) is a metabolic disorder characterized by the inability to oxidize and convert dietary-derived trimethylamine (TMA) to trimethylamine *N*-oxide (TMAO). This disorder has been relatively well-documented in European and North American populations, but no reports have appeared regarding patients in Japan. We identified seven Japanese individuals that showed a low metabolic capacity to convert TMA to its odorless metabolite, TMAO. The metabolic capacity, as defined by the concentration of TMAO excreted in the urine divided by TMA concentration plus TMAO concentration, in these seven individuals ranged from 70 to 90%. In contrast, there were no healthy controls examined with less than 95% of the metabolic capacity to convert TMA to TMAO. The intake of dietary charcoal (total 1.5 g charcoal per day for 10 days) reduced the urinary free TMA concentration and increased the concentration of TMAO to normal values during charcoal administration. Copper chlorophyllin (total 180 mg per day for 3 weeks) was also effective at reducing free urinary TMA concentration and increasing TMAO to those of concentrations present in normal individuals. In the TMAU subjects examined, the effects of copper chlorophyllin appeared to last longer (i.e., several weeks) than those observed for activated charcoal. The results suggest that the daily intake

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of charcoal and/or copper chlorophyllin may be of significant use in improving the quality of life of individuals suffering from TMAU.
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Introduction

Trimethylaminuria (TMAU), or fish-like odor syndrome, is a genetic disease characterized by excretion of trimethylamine (TMA) (Al Waiz et al., 1987; Ayesh et al., 1993; Cashman et al., 2003). The odor of TMA is recognizable as "fishy", however at low concentrations it may only be recognized as foul or "garbage-like." In normal humans, malodorous TMA is metabolized to trimethylamine *N*-oxide (TMAO). TMAO is more polar than TMA, is non-odorous, and readily excreted in the urine (Cashman and Zhang, 2002). Patients suffering from TMAU have a reduced capacity to oxidize free TMA to TMAO. The inability to efficiently oxidize TMA may lead some individuals to emanate a body odor, that in the extreme form, may be unpleasant and fish-like. This offensive odor is caused by excess, unoxidized TMA present in the systemic circulation that makes its way into urine, sweat, and breath. Consequently, some affected individuals may express a foul or fishy malodor that leads to social problems.

TMAU is an autosomal, recessive inborn error of metabolism. The severe forms are caused by mutations in the flavin-containing monooxygenase (*FMO3*) gene. Severely deleterious FMO3 mutations are quite uncommon and non-randomly distributed in the population (Treacy et al., 1998; Zschocke et al., 1999). TMAU-affected individuals have been relatively well-documented in British, Australian, and American populations (Akerman et al., 1999; Mitchell and Smith, 2001). In addition, TMAU has been reported in Thailand (Thithapandha, 1997; Kubota et al., 2003) and Hong Kong (Lee et al., 2000), however, to date, no Japanese affected by TMAU have been reported in the literature (Mitchell and Smith, 2001). More common inter-individual FMO3 variation arises from the milder forms caused by a "spectrum" of single nucleotide polymorphic changes in the *FMO3* gene (Cashman et al., 2003; Lattard et al., 2003).

For normal individuals, the metabolic capacity of the FMO3 enzyme converts over 95% of TMA to TMAO (Cashman and Zhang, 2002; Cashman, 2002). Diagnosis has been performed by determining urinary TMA and TMAO concentrations. Individuals showing FMO3 metabolic capacity lower than 90% conversion of TMA to TMAO are considered to be suffering from TMAU (Zhang et al., 1995; Zschocke et al., 1999).

Considering the mechanism by which TMA is formed in the large intestines, it seemed possible to reduce the amount of TMA available to the liver by dietary supplements. Because the production of TMA from food constituents such as phosphatidyl choline or TMAO is mediated by an enzyme(s) present in gut flora, one method is to reduce intestinal bacteria by administration of bactericidal agents (Treacy et al., 1995; Zhang et al., 1999). The other is to sequester TMA once it is produced in the gut. We hypothesized that over-the-counter dietary supplements such as activated charcoal or copper chlorophyllin would diminish free amine compounds like TMA (Dashwood et al., 1996). Charcoal has a high surface area and is commonly used by municipal water utilities to adsorb small, odorous molecules (Suffet and Wable, 1995). In addition, copper chlorophyllin has been shown to strongly bind

amine-containing compounds in the gut (Dashwood et al., 1996). These treatments should leave only small amounts of TMA un-sequestered and lessen the burden on the FMO3 enzyme thereby reducing free TMA-related odor symptoms.

We report herein that activated charcoal and copper chlorophyllin when added to the diet of Japanese suffering from TMAU improved their apparent metabolic capacity to convert TMA to TMAO by decreasing the concentration of free TMA excreted in the urine.

Materials and methods

Volunteers and treatment

The ethics committee of Hokkaido University approved this study. Informed consent was obtained from every subject. We asked for volunteers via an Internet article describing the screening of urinary TMA and 19 males and 8 females ranging from 19 to 52 years of age responded to the request. Twenty individuals (22–56 years old; 28 $\pm$ 8 yrs: mean $\pm$ SD) constituted healthy controls.

Most of the TMAU-positive subjects in the Japanese cohort reported that they started to suffer from apparent TMAU-symptoms in their teens. The age of onset for TMAU symptoms can vary considerably (Cashman et al., 2003); however, many of the severe cases reported in the literature have childhood onset. Subject 1 (male, 28 years old) and Subject 2 (female, 19 years old) were given 3 tablets (250 mg $\times$ 3) containing active charcoal (Super Carbon Diet 20, Noguchi Medical Institute, Tokyo, Japan) twice a day according to the manufacturer's description for 10 days. Subject 1, Subject 3 (male, 29 years old), and Subjects 4 (male, 26 years old) took commercial copper chlorophyllin tablets (4 tablets; Saclophyl®, Eisai, Tokyo, Japan) after each meal three times per day (for a total of 180 mg copper chlorophyllin administered per day) for three weeks according to the maximum approved dose as an over-the-counter medication. This is also below the allowable maximum amount (300 mg/day) of copper chlorophyllin approved for use by the Food and Drug Administration (of the U.S.A.) for incontinent individuals (Federal Register, 1990).

Analysis of urine samples

After an overnight fast, the study participants collected their first urine samples which were acidified with 1 M HCl to pH $\sim$ 2–3 for this study. Urinary TMA and TMAO concentrations were determined by gas chromatography using a flame thermoionic detector as described previously (Tjoa and Fennessey, 1991; Kubota et al., 2003). Briefly, TMA concentration in the urine was directly analyzed by a head-space gas chromatography after basicifying with 10 M NaOH and preheating at 95 °C for 20 min. TMAO concentrations were calculated by subtraction the free TMA concentrations from total TMA (= free TMA + TMAO) concentrations after chemical reduction of TMAO to TMA using $TiCl_3$. Intra- and inter-assay variation for free and total TMA were within 5% using the previously described gas chromatography conditions (Kubota et al., 2003). The detection limit for TMA concentration was 0.01 μg/ml of urine.

FMO3 metabolic capacity to convert TMA to TMAO was defined as the ratio of TMAO to total TMA (% of TMAO/(TMA + TMAO)). The values were shown as the averages of at least two samples obtained from first morning void urine.

To evaluate the effects of active charcoal and copper chlorophyllin, urinary concentrations of free TMA or total TMA (μmol/ml of urine) were corrected by creatinine excretion (mmol/ml) from individual subjects to compare the changes of daily TMA concentrations and to account for urine volume. Creatinine concentrations in the urine were measured by a commercially available diagnostic reagent (BML, Tokyo, Japan).

Statistical analysis

A statistical analysis for frequency distribution of the metabolic capacity to convert TMA to TMAO was done by unpaired *t* test with Welch correction ($p < 0.05$) using a software InStat (GraphPad software, San Diego, CA, USA).

Results

Urinary Ratios of TMAO to total TMA

FMO3 metabolic capacity was determined in both the self-reported TMAU-affected individuals and control subjects (Fig. 1). Frequency distribution of the FMO3 metabolic capacity to convert TMA to TMAO was significantly different as determined by an unpaired *t* test with Welch correction ($p = 0.002$) between the self-reported TMAU individuals (Fig. 1A) and the control subjects (Fig. 1B). A lower metabolic capacity (mean, 92.6%; n = 27) and larger variation (SD, 7.3) were observed for the group of self-reported TMAU-affected individuals (Fig. 1A) compared with controls (Fig. 1B). The frequency of subjects who showed less than 90% of the FMO3 metabolic capacity was 26 % (7 individuals out of 27

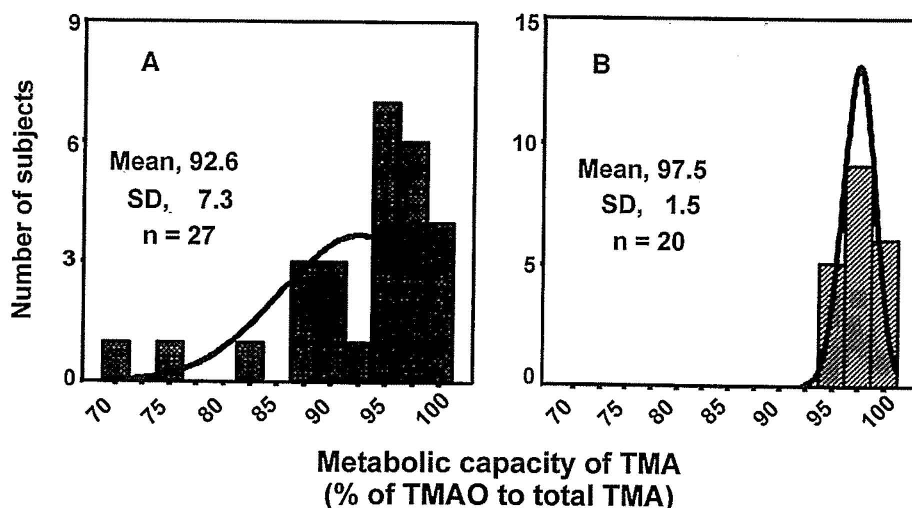

Fig. 1. Frequency distribution of 27 Japanese volunteers who suffered from self-reported malodor (A) and 20 Japanese control subjects (B). Metabolic capacity (% of TMAO/(TMA + TMAO)) was calculated by determination of urinary TMA excretion after analysis by gas chromatography. The distribution in these two groups were significantly different ($p = 0.002$) examined by unpaired *t* test with Welch correction for their different variances.

volunteers); this suggested that several individuals who thought they were TMAU-affected were actually affected (Fig. 1A). In contrast, 20 of the individuals examined were found to have a metabolic capacity of >95% for conversion of TMA to TMAO (Fig. 1). These 20 individuals were categorized as control subjects.

Effects of charcoal and copper chlorophyllin on the metabolic capacity of FMO3

To investigate whether activated charcoal or copper chlorophyllin was effective in decreasing TMA excretion in the urine, three volunteers that had low metabolic capacities to convert TMA to TMAO were asked to test these agents. The effects of charcoal ingestion on urinary TMA concentrations are shown in Fig. 2. Charcoal clearly decreased the urinary TMA concentration when subjects took 1.5 g of charcoal per day for 10 days. Concomitantly, the metabolic capacity of FMO3 to convert TMA to TMAO increased to over 90% in both Subject 1 (Fig. 2A) and Subject 2 (Fig. 2C). After terminating the

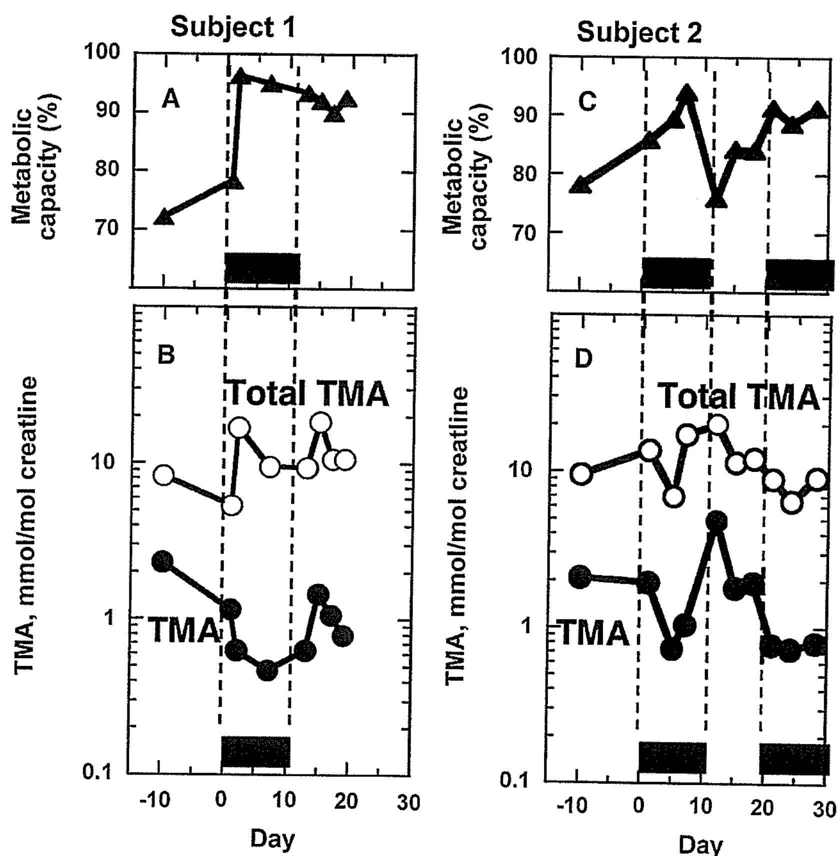

Fig. 2. Decrease of urinary TMA concentration and the increase of apparent FMO3 metabolic capacity by active charcoal in Subjects 1 and 2. Subject 2 received two runs of charcoal treatments as indicated. The metabolic capacity of FMO3 (A, C) is indicated as percent of TMAO to total TMA excreted in the urine as described in the text. Total urinary TMA and free TMA concentrations (B, D) were calculated as mmol TMA/mol creatinine. Bars indicate the periods of active charcoal treatments (10 days).

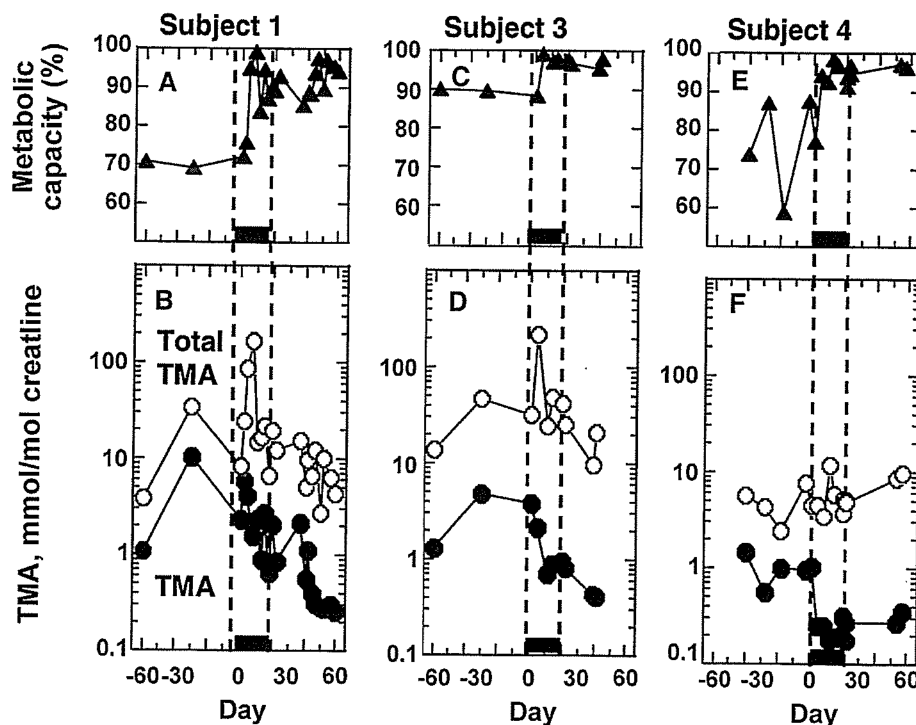

Fig. 3. Decrease of urinary TMA concentration and subsequent increase of metabolic capacity of FMO3 by copper chlorophyllin in Subjects 1, 3, and 4. Bars indicate the period of copper chlorphyllin treatment. See legend of Fig. 2 for details.

charcoal administration, the urinary concentration of TMA and the metabolic capacity of FMO3 returned to previous values especially in Subject 2 (Fig. 2C, 2D).

Copper chlorophyllin was also effective in decreasing the urinary TMA concentration and increasing the metabolic capacity of FMO3 (Fig. 3). While ingesting copper chlorophyllin, the urinary concentration of TMA was decreased in all subjects. The apparent metabolic capacity was also improved by the treatment with copper chlorophyllin (Fig. 3A, 3C, 3E). The beneficial effects of copper chlorophyllin on TMA elimination continued for several weeks after terminating its use (Fig. 3A, 3B).

Discussion

TMAU arises from the decreased capacity of the FMO3 enzyme to form TMAO from TMA. Factors that increase the substrate TMA burden include the microbial formation of TMA from dietary precursors by gut flora and an increase of intestinal absorption of TMA contained in the diet. It should be mentioned that some foodstuff components contain indoles that may competitively inhibit TMA metabolism by human FMO3 in vivo and in vitro (Cashman et al., 1999). The intake of vegetables containing indoles and/or nitrogen-containing drugs may be other factors making the situation worse for individuals affected by TMAU. The consumption of brussels sprouts was reported to inhibit the TMA N-oxidation in vivo (Cashman et al., 1999). Further detailed studies are needed to evaluate the effects of

vegetables on the metabolism of TMA. This is under investigation with some Japanese subjects in this laboratory.

TMAU is considered an autosomal recessive disorder resulting in a deficiency of the FMO3 enzyme (Cashman and Zhang, 2002). Some disease-causing factors have been reported and include: polymorphic changes of the *FMO3* gene in North American and European populations, viral infection and hormonal modulation as well as the intake of competitive FMO3 substrates or the presence of serious liver damage (Mitchell, 1999; Cashman and Zhang, 2002). The subjects of these preliminary studies, presented herein, do not appear to have the previously reported *FMO3* gene mutations that cause severe symptoms and low metabolic capacity of FMO3 (<40% TMAO). However, because of the mild symptoms presented by the individuals examined herein, the subjects may be either heterozygotes for one of the *FMO3* mutations or have one or more of the affected polymorphic changes to the *FMO3* gene (Zschocke et al., 1999; Cashman et al., 2003). Detailed analysis of *FMO3* DNA sequences for the Japanese subjects examined in this study is now under investigation in our laboratory (Fujieda et al., 2003).

In the present study, we observed a very high apparent frequency (i.e., 26% shown in Fig. 1A) for impaired FMO3 metabolic capacity in Japanese, because 7 out of 27 self-reported individuals were recruited by our internet article seeking volunteers suffered from malodor. A frequency of 26% is similar to other self-reporting populations that have been tested for TMAU (Preti et al., 1995; Cashman et al., 2003). In contrast, there were no healthy control Japanese individuals examined that showed low FMO3 activities among 20 control subjects tested (at least <5% frequency, Fig. 1B). The frequency of impaired FMO3 metabolic capacity in a normal Japanese population is not known as yet. The extent of *N*-oxidation of TMA to TMAO in various ethnic groups has been reported in previous studies (Mitchell et al., 1997; Lee et al., 2000): the incidence of deficiency in TMA *N*-oxidation varied from at least 1% (British Caucasian and Chinese) to probably 11% (New Guinean) of the population (Mitchell et al., 1997; Lee et al., 2000).

One report has appeared on the change of metabolic capacity of TMA around the time of menstruation (Zhang et al., 1996). We found 2 Japanese females in our population that showed lower FMO3 metabolic capacity to convert TMA to TMAO during menstruation (e.g., metabolic capacities of 75% versus 82% and 90% versus 98%). Further studies are needed to clarify the mechanisms responsible for the increased TMA excretion during menses.

A 10-day treatment with charcoal decreased urinary TMA concentrations in TMAU-affected patients. In addition, treatment with copper chlorophyllin also decreased free urinary TMA. However, compared to charcoal, the beneficial effects of copper chlorophyllin appeared to continue longer after cessation of use. The mechanism responsible for the effects of charcoal on TMA appears to be adsorption. Because charcoal may adsorb free TMA, the decreased TMA concentration may place less of a burden on the ability of FMO3 to detoxicate TMA in TMAU-affected individuals. Copper chlorophyllin may also chemically sequester free TMA as has been previously documented for other *N*-containing compounds (Dashwood et al., 1996). The effects of copper chlorophyllin lasted for several weeks after termination of administration copper chlorophyllin. The mechanism(s) responsible for causing this prolonged effect is unclear as yet. One possible mechanism might be to retard uptake of foodstuffs that are precursors of TMA or another mechanism may be to eliminate TMA directly in the feces. However, it is also possible that copper chlorophyllin could affect the make-up of gut flora resulting in decreased formation of TMA. It is interesting to note that the total TMA excretion was increased temporally after starting the daily intake of copper chlorophyllin (Fig. 3). The mechanism for this is also unclear. Since copper

chlorophyllin is available as an over-the-counter medication in Japan and the United States, our results suggest that use may provide a significant benefit to improve the lives of TMAU-affected individuals.

Subject 1 was the first patient in our study and was involved in both courses of dietary treatments. Based on our genetic analysis (Fujieda et al., 2003), Subjects 1–4 have suffered from TMAU-related symptoms for much of their lives but do not appear to have the mutations in the *FMO3* gene seen in other non-Japanese TMAU populations. The measures of TMA excretion employed herein were done under normal dietary conditions as previously reported in other populations (Treacy et al., 1995). The other Japanese subjects examined here that possessed lower metabolic capacity to convert TMA to TMAO also had lower urinary TMA than reported by Treacy et al. (1995) (<18 mmol TMA/mol of creatinine). However, the excess urinary TMA excretion found in the subjects reported herein has still produced social problems for the TMAU-affected individuals. It is not known whether Japanese have a decreased olfactory threshold to the odor of TMA but it may differ from Western populations because of different dietary or genetic differences.

In conclusion, we were able to successfully treat Japanese individuals experiencing TMAU with dietary supplements such as activated charcoal or copper chlorophyllin. These supplements decreased the free TMA excreted in urine; consequently, our results suggest that these agents can be used to improve the quality of life of TMAU-affected individuals. In terms of treatments of TMAU-affected individuals, gut flora treatment with antibiotics or limitation of some foodstuff resulting in TMA formation has been reported (Treacy et al., 1995; Cashman et al., 2003). The dietary supplements described in the present study are recommended for use in combination with and/or following the previously published treatments for the TMAU-affected individual after consultation with the individual's physician.

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Regular Article

Effects of NO-1886 (Ibrolipim), a Lipoprotein Lipase-Promoting Agent, on Gene Induction of Cytochrome P450s, Carboxylesterases, and Sulfotransferases in Primary Cultures of Human Hepatocytes

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Summary: In the present study, the effects on expression of cytochrome P450 (CYP1A1, CYP1A2, CYP3A4 and CYP3A5), carboxylesterase (CES1 and CES2) and sulfotransferase (CHST1, CHST3, CHST4, CST, SULT2A1 and TPST2) mRNA in primary cultures of cryopreserved human hepatocytes were evaluated after exposure to NO-1886 (diethyl 4-[(4-bromo-2-cyanophenyl) carbamoyl] benzylphosphonate) for 48 hr at 2, 10, and 50 μM. Analysis was performed by RT-PCR in the presence of TaqMan probe. CYP1A1 and CYP1A2 mRNA levels after exposure to 50 μM omeprazole (positive control for CYP1As) were increased by 162 ($p<0.001$) and 37 times ($p<0.001$), respectively, compared with untreated controls. However, these mRNA levels were increased by 2 times or less after exposure to NO-1886. CYP3A4 and CYP3A5 mRNA levels after exposure to 50 μM rifampicin (positive control for CYP3As) were significantly increased by 5.8 ($p<0.01$) and 2.0 times ($p<0.01$), respectively, compared with untreated controls. The CYP3A4 mRNA level after exposure to 10 μM NO-1886 was increased by 1.3 times ($p<0.05$). Further, the CYP3A4 mRNA level after exposure to 50 μM NO-1886 was significantly increased by 3.6 times ($p<0.001$). However, the CYP3A5 mRNA level after exposure to 50 μM NO-1886 was not significantly increased. CES1 and CES2 mRNA levels after exposure to 50 μM NO-1886 were significantly increased by 1.4 ($p<0.05$) and 2.6 times ($p<0.01$), respectively, compared with untreated controls. CHST1, CST and SULT2A1 mRNA levels after exposure to 50 μM NO-1886 were significantly increased by 3.8 ($p<0.001$), 1.8 ($p<0.01$) and 4.4 times ($p<0.01$), respectively. CHST3, CHST4 and TPST2 mRNA levels after exposure to 50 μM NO-1886 were not significantly increased. This *in vitro* technique using primary cultured human hepatocytes is expected to be very useful for the preclinical evaluation of the induction of drug-metabolizing enzymes in humans.

Key words: NO-1886; mRNA induction; cryopreserved human hepatocytes; cytochrome P450; carboxylesterase; sulfotransferase

Introduction

There is a growing body of evidence showing an association between triglycerides and an increased risk of coronary heart disease.[1] Hypertriglyceridemia, which by itself is an independent risk factor, is particularly common in certain specific groups of individuals, such as people with high plasma levels of low-density lipoprotein (LDL) cholesterol.[2] Many studies have reported that exercise decreases plasma total cholesterol and triglyceride levels and increases HDL cholesterol levels.[3,4] Furthermore, it has been shown that exercise improves insulin resistance in patients with diabetes mellitus.[5] These results indicate that exercise provides

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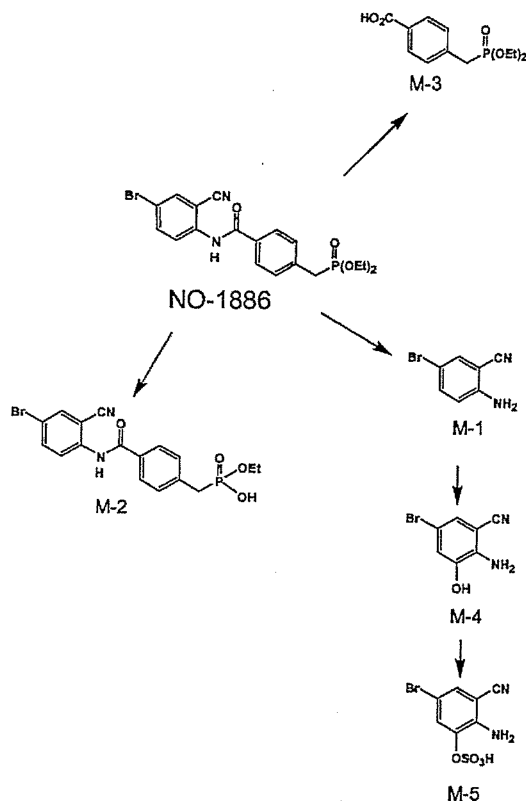

Fig. 1. Chemical structures of NO-1886 and its metabolites.

protection against the development of atherosclerosis. It has been reported that the novel compound NO-1886 (ibrolipim) **(Fig. 1)** is an effective lipoprotein lipase activator and that long-term administration of NO-1886 also protects against the development of experimental atherosclerosis in animals.[6]

The pharmacokinetics and metabolic pathways **(Fig. 1)** of NO-1886 were investigated in previous studies by Morioka *et al.*,[7)9] who reported that NO-1886 is metabolized by cytochrome P450, carboxylesterase and sulfotransferase. The *in vivo* metabolic pathways of NO-1886 in the rat and cynomolgus monkey have been identified as the O-deethylation of phosphonate and the hydrolysis of the amide bond.[7,9] The main metabolite is monoethyl phosphonate (M-2), which has been reported to account for 70% and 94% of all metabolites in the rat[7] and cynomolgus monkey,[9] respectively. It has been demonstrated that the O-deethylation of phosphonate is performed by liver P450 enzymes and that the amino bond is hydrolyzed by liver microsomal carboxylesterase (CES).[8] In addition, rat plasma CES (CES1C isozyme) is mainly responsible for amide hydrolysis in the rat.[9] Since plasma CES1C is absent in humans and monkeys, the amide hydrolysis of NO-1886 is reduced in the cynomolgus monkey *in vivo*.[9] In contrast, human liver microsomes exhibited only O-deethylation of NO-1886.[8] Both CYP3A4 and CYP2C8 are major P450 enzymes involved in the phosphonate O-deethylation of NO-1886 in the human liver.[8] The level of amide hydrolysis was 5% that of O-deethylation in the human liver homogenate 9000 g supernatant.[8] Therefore, human CES contributes only slightly to the amide hydrolysis of NO-1886 in comparison with the rat and cynomolgus monkey. Furthermore, O-deethylation by P450 enzymes showed almost no first-pass metabolism of NO-1886 in the liver, and NO-1886 was eliminated from the body slowly.[8]

Drug-drug interactions are an important consideration in drug development. Human hepatocytes are used for the evaluation of gene induction of drug-metabolizing enzymes after exposure to drugs.[10,11] We previously reported a method for evaluating the gene induction of drug-metabolizing enzymes after exposure to model drugs such as rifampicin and omeprazole[12] in primary cultures of human hepatocytes. Rifampicin is a potent inducer of CYP3As in primary cultures of human hepatocytes[10] and omeprazole is a potent inducer of CYP1As in such cells.[11]

In the present study, we conducted preclinical evaluations of the effects of NO-1886 on gene induction of drug-metabolizing enzymes such as cytochrome P450s (CYP1A1, CYP1A2, CYP3A4 and CYP3A5), carboxylesterases (CES1 and CES2) and sulfotransferases (CHST1, CHST3, CHST4, CST, SULT2A1 and TPST2), which are related to the metabolism of NO-1886, using primary cultures of cryopreserved human hepatocytes.

Materials and Methods

Materials: The NO-1886 used in this study was synthesized at Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) and had a chemical purity of 99.9%. Omeprazole and rifampicin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cryopreserved human hepatocytes (Lot 100, sex: female, age: 74 years, race: Caucasian) were purchased from *In Vitro* Technologies, Inc. (Baltimore, MD, USA). Trypan blue was purchased from Flow Laboratories, Ltd. (Irvine, UK). Hepatocyte Culture Medium (CC-3198) was purchased from BioWhittaker, Inc. (Walkersville, MD, USA). Adult human total RNA from the liver was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA, USA). An Rneasy® Mini Kit and QIAshredder™ were purchased from QIAGEN (Hilden, Germany). Yeast tRNA was purchased from Life Technologies, Inc. (Rockville, MD, USA). TaqMan One-Step RT-PCR Master Mix Reagents, TaqMan GAPDH Control Reagents, TaqMan β-actin Control Reagents, Micro Amp® Optical 96-well Reaction Plates, Optical Adhesive Covers, and Optical Cover Compression Pads were

purchased from Applied Biosystems (Foster City, CA, USA).

All other chemicals and reagents used were of analytical reagent grade.

Monolayer culture of hepatocytes: The cryopreserved human hepatocytes were suspended in Hepatocyte Culture Medium. The hepatocytes were centrifuged (45g, 4°C) for 3 min and resuspended in the same medium. The number of cells was counted using a Coulter Counter. Cell suspensions with viability rates of 85% to 90% as assessed by trypan blue dye exclusion were used for the experiments. The cell suspensions were diluted to a final concentration of 2.5×10^5 viable cells/mL using Hepatocyte Culture Medium, and inocula of 1×10^5 viable cells/0.4 mL/well were introduced into 24-well plates that had been coated with type I collagen. The cells were cultured for 3 hr after inoculation under 5% CO_2 and 95% air at 37°C. The medium was then replaced with fresh medium, and the cells were cultured for 21 hr under 5% CO_2 and 95% air at 37°C. The medium was then replaced with fresh medium without human epidermal growth factor (hEGF), gentamicin, or amphotericin B, and the cells were cultured for 24 hr under 5% CO_2 and 95% air at 37°C. The cells were used for experiments at 48 hr after inoculation.

Experiments using monolayer cultures of hepatocytes: At 48 hr after inoculation, the hepatocytes were treated with a number of known inducers for an additional 48 hr. Media with the inducers but without hEGF, gentamicin, or amphotericin B were changed daily during treatment and the cells were cultured under 5% CO_2 and 95% air at 37°C. The effects of NO-1886 at concentrations of 2, 10 and 50 μM were studied. The effects of rifampicin and omeprazole at a concentration of 50 μM were also studied. All inducers were dissolved in DMSO at a final vehicle concentration of 0.1% (v/v). Controls were also exposed at DMSO at a final vehicle concentration of 0.1% (v/v). Total RNA was extracted from the hepatocytes using the Rneasy® Mini Kit and QIAshredder™.

Oligonucleotides: The forward and reverse primers and TaqMan probes used for quantitative analysis were the same as those employed in our previous study.[12] The primers and TaqMan probes were synthesized by QIAGEN (Tokyo, Japan). The TaqMan probes contained 6-carboxyfluorescein (FAM) at the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end and were designed to hybridize to a sequence located between the PCR primers.

TaqMan RT-PCR conditions: Human total RNA was diluted with yeast tRNA to 50 μg/mL. The RT-PCR assay was performed using 50 μL of TaqMan One-Step RT-PCR Master Mix Reagents containing 300 nM forward primer, 900 nM reverse primer, 200 nM TaqMan probe and about 20 ng of total RNA. Amplification and detection were performed using the ABI PRISM 7700 Sequence Detector system (Applied Biosystems) with the following profile: 1 cycle of 48°C for 30 min, 1 cycle of 95°C for 10 min and 50 cycles each of 95°C for 15 sec and 60°C for 1 min. For GAPDH, 200 nM forward primer, 200 nM reverse primer and 100 nM TaqMan probe were used; for β-actin, 300 nM forward primer, 300 nM reverse primer and 200 nM TaqMan probe were used.

Specificity of RT-PCR between CYP3A4 and CYP3A5: A cross-reactivity assay was performed to determine whether our assay system could quantify the target molecule specifically. Total RNA was extracted from *Escherichia coli* transfected with human CYP3A4[13] or CYP3A5 (Kamataki, T. unpublished observation). Measurements of CYP3A4 and CYP3A5 gene expression were obtained individually using CYP3A4- and CYP3A5-specific primer sets and probes.

Statistical analysis: The data were analyzed using the ABI PRISM Sequence Detector System software (Sequence Detector Ver. 1.9.1). The relative expression of each mRNA was calculated by the ΔCt method (where ΔCt is the value obtained by subtracting the Ct value of β-actin mRNA from the Ct value of the target mRNA), which was employed in our previous study.[14] Specifically, the amount of target mRNA relative to β-actin mRNA was expressed as $2^{-(\Delta Ct)}$. Data are expressed as the ratio of the target mRNA to β-actin mRNA. Further, the values are given as results relative to controls with 0.1% DMSO. Statistical analysis was performed using the Student's *t*-test with a significance level of $p<0.05$.

Results

Quantitative analysis and specificities: The forward and reverse primers and TaqMan probes used for the quantitative analysis were the same as those employed in our previous study.[12] Analysis was performed by RT-PCR using the ABI PRISM 7700 Sequence Detector System in the presence of the TaqMan probe. The RT-PCR method is widely used for the analysis of mRNA expression, and the most important issue in this method is its selectivity for the target gene. Both the primers and probes were homology searched by an NCBI BLAST search to ensure that they were specific for the target mRNA transcript. Further, in order to study the selectivity for CYP3A4 and CYP3A5 mRNA, a cross-reactivity assay was performed to determine whether our assay system could quantify the target molecule specifically. Measurements of CYP3A4 and CYP3A5 gene expression in *Escherichia coli* transfected with human CYP3A4[13] and CYP3A5 (Kamataki, T. unpublished observation) were obtained individually using CYP3A4- and CYP3A5-specific primer sets and probes.

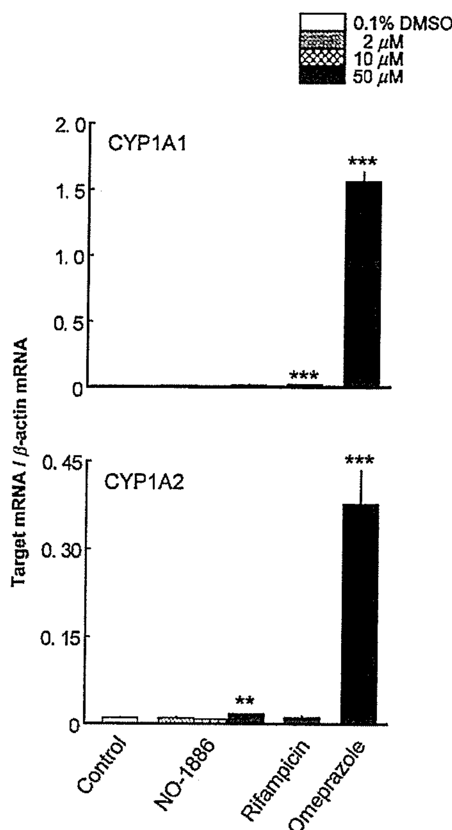

Fig. 2. Effects on CYP1A1 and CYP1A2 mRNA expression in primary cultures of human hepatocytes after exposure to drugs at various concentrations for 48 hr. Data are expressed as the ratio of the target mRNA to β-actin mRNA. Experiments (in cultures of human hepatocytes) were performed in triplicate, and data are shown as mean ±SD. $^{**}p<0.01$ and $^{***}p<0.001$ vs. controls at 0.1% DMSO.

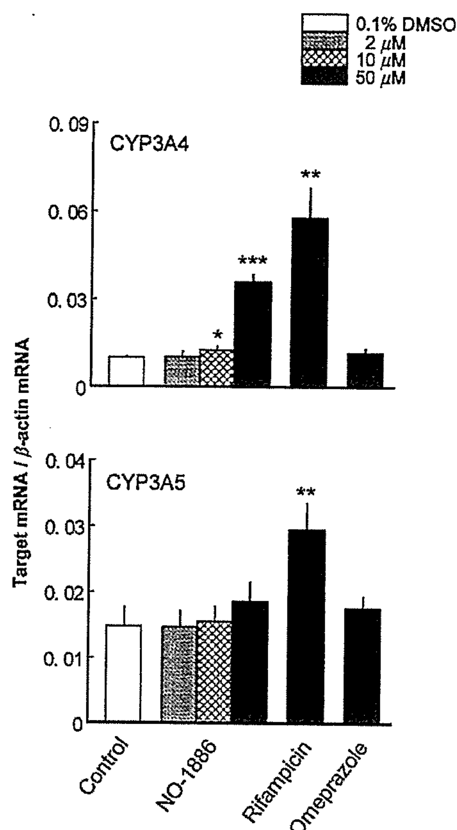

Fig. 3. Effects on CYP3A4 and CYP3A5 mRNA expression in primary cultures of human hepatocytes after exposure to drugs at various concentrations for 48 hr. Data are expressed as the ratio of the target mRNA to β-actin mRNA. Experiments (in cultures of human hepatocytes) were performed in triplicate, and data are shown as mean ±SD. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. controls at 0.1% DMSO.

The ability of this method to discriminate between CYP3A4 gene expression and CYP3A5 gene expression was clearly confirmed, with cross contamination of less 1×10^{-6} (data not shown). The ability of this method to discriminate between CYP1A1 gene expression and CYP1A2 gene expression has already been reported in the literature.[15)]

Effects of NO-1886 on mRNA expression: In the present study, the levels of gene expression of cytochrome P450s (CYP1A1, CYP1A2, CYP3A4 and CYP3A5), carboxylesterases (CES1 and CES2) and sulfotransferases (CHST1, CHST3, CHST4, CST, SULT2A1 and TPST2) were evaluated in primary cultures of cryopreserved hepatocytes after 48-hr exposure to NO-1886 (2, 10 and 50 μM) and the typical known enzyme inducers rifampicin (50 μM) and omeprazole (50 μM). All experiments were performed in the presence of 0.1% DMSO. None of the treatments showed any identifiable toxic effects such as cell death or loss of adhesion of hepatocytes at the study concentrations employed, as assessed by microscopic examination. The levels of GAPDH mRNA, which were normalized to those of β-actin mRNA, showed no changes after exposure to NO-1886, omeprazole or rifampicin compared with untreated controls (data not shown). Therefore, it is considered that the expression of both GAPDH and β-actin mRNA after exposure to NO-1886, omeprazole or rifampicin did not change to the levels in untreated controls. **Figure 2** shows the levels of CYP1A1 and CYP1A2 mRNA expression. The levels of CYP1A1 and CYP1A2 mRNA in human hepatocytes after exposure to omeprazole, which was the positive control for CYP1As, were increased by 162 ($p<0.001$) and 37 times ($p<0.001$), respectively, compared with untreated controls. However, these mRNA levels were increased by 2 times or less after exposure to NO-1886 compared with untreated controls. **Figure 3** shows the levels of mRNA expression of CYP3A4 and CYP3A5. The levels of CYP3A4 and CYP3A5 mRNA after exposure to 50 μM rifampicin, which was the positive control for CYP3As, were

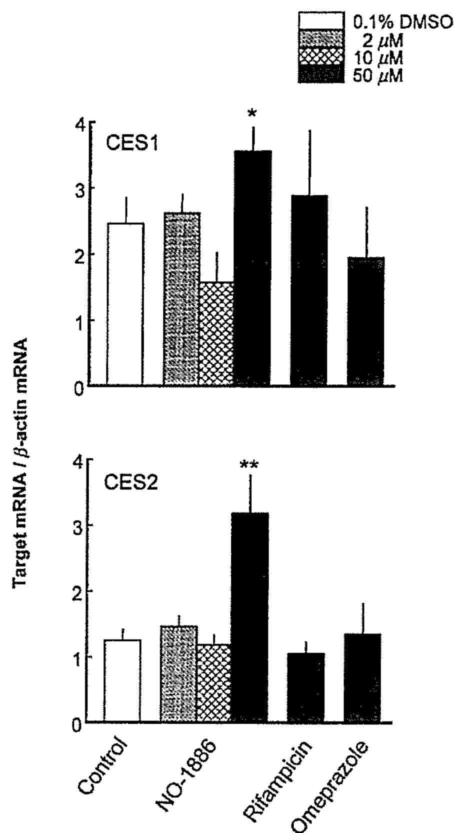

Fig. 4. Effects on carboxylesterase (CES1 and CES2) mRNA expression in primary cultures of human hepatocytes after exposure to drugs at various concentrations for 48 hr. Data are expressed as the ratio of the target mRNA to β-actin mRNA. Experiments (in cultures of human hepatocytes) were performed in triplicate, and data are shown as mean ± SD. *$p<0.05$ and **$p<0.01$ vs. controls at 0.1% DMSO.

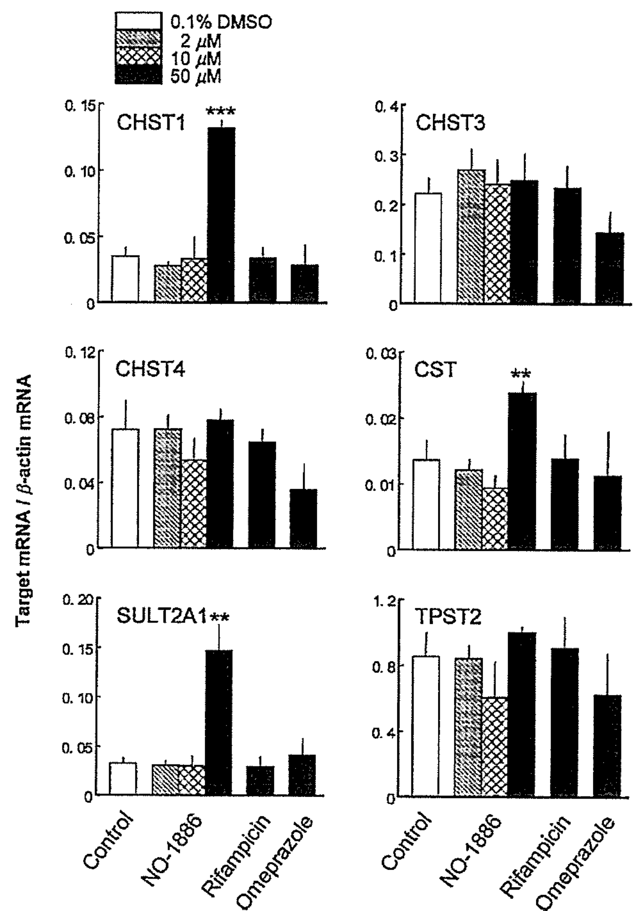

Fig. 5. Effects on sulfotransferase mRNA expression in primary cultures of human hepatocytes after exposure to drugs at various concentrations for 48 hr. Data are expressed as the ratio of the target mRNA to β-actin mRNA. Experiments (in cultures of human hepatocytes) were performed in triplicate, and data are shown as mean ± SD. **$p<0.01$ and ***$p<0.001$ vs. controls at 0.1% DMSO.

significantly increased by 5.8 ($p<0.01$) and 2.0 times ($p<0.01$), respectively, compared with untreated controls. The level of CYP3A4 mRNA after exposure to 10 μM NO-1886 was increased by 1.3 times ($p<0.05$) compared with untreated controls. Further, the level of CYP3A4 mRNA after exposure to 50 μM NO-1886 was significantly increased by 3.6 times ($p<0.001$) compared with untreated controls. However, the level of CYP3A5 mRNA after exposure to 50 μM NO-1886 showed no change compared with untreated controls. **Figure 4** shows the levels of mRNA expression of the carboxylesterases CES1 and CES2. The levels of CES1 and CES2 mRNA after exposure to 50 μM NO-1886 were significantly increased by 1.4 ($p<0.05$) and 2.6 times ($p<0.01$), respectively, compared with untreated controls. **Figure 5** shows the levels of mRNA expression of the sulfotransferases CHST1, CHST3, CHST4, CST, SULT2A1 and TPST2. The levels of CHST1, CST and SULT2A1 mRNA after exposure to 50 μM NO-1886 were significantly increased by 3.8 ($p<0.001$), 1.8 ($p<0.01$) and 4.4 ($p<0.01$) times, respectively, compared with untreated controls. The levels of CHST3, CHST4 and TPST2 mRNA after exposure to 50 μM NO-1886 showed no changes compared with untreated controls.

Discussion

In standard induction studies, the activity of drug-metabolizing enzymes is evaluated in liver samples after repeated administration to female rats. However, species differences in the induction of drug-metabolizing enzymes are frequently observed in the metabolism of xenobiotics. Therefore, the fact that there are species differences between experimental animal and human data is an important issue in pharmacological efficacy and toxicity studies conducted during drug development. For example, although rifampicin does not induce CYP3A1 in the rat, it is a potent inducer of

CYP3A4 in humans.[16] The differences observed in the results of pharmacological studies employing different species are due, at least in part, to species differences in CYP3A as assessed using liver microsomes and the expression system of CYP3A.

The CYP3A subfamily is the major cytochrome P450 expressed in the human liver.[17] CYP3A is responsible for approximately 50% of the cytochrome P450-mediated metabolism of drugs[18] and is an important enzyme with respect to the side effects and actions of drugs and their metabolites. CYP3A4 is the most abundantly expressed CYP, accounting for approximately 30% to 40% of the total CYP content in the adult human liver.[17] It is generally accepted that CYP3A4 is the main member of the CYP3A subfamily expressed in the liver in the majority of humans, followed by CYP3A5, whereas CYP3A7 and CYP3A43 are expressed at much lower levels.[19] We have therefore focused on the induction of CYP3A4 and CYP3A5 in the human liver. A recent report characterizing the genetic basis of polymorphic CYP3A5 expression found that CYP3A5 was expressed in 60% of the livers of African-Americans compared with 33% for Caucasians and that CYP3A5 represented at least 50% of the total hepatic CYP3A content in people polymorphically expressing CYP3A5.[20] CYP3A5, as well as CYP3A4, may be an important contributor to interindividual and interracial differences in CYP3A-dependent drug clearance and in responses to many pharmaceutical agents. In addition, Williams *et al.*[18] reported an equal or reduced metabolic capability for CYP3A5 as compared with CYP3A4. Baune *et al.*[21] reported that both CYP3A4 and CYP3A5 metabolize halofantrine, with CYP3A4 playing a major role. Shou *et al.*[22] reported that CYP3A4 shows a 10-fold greater apparent affinity for taxotere than CYP3A5, but that both have a similar maximal velocity.

It has previously been reported that CYP3A4 is clearly induced in human hepatocytes after exposure at 2 μM or less to many drugs such as paclitaxel[10] and troglitazone.[23] In our previous study involving the primary culture of cryopreserved human hepatocytes, we also reported that CYP3A4 mRNA was clearly induced by exposure to 2 μM rifampicin.[12] Recently, the induction of CYP3A5 mRNA in the human liver has also been reported by Burk *et al.*[24] Therefore, we conducted detailed investigations into the mRNA expression profiles of CYP3A4 and CYP3A5. As shown in **Fig. 3**, the levels of CYP3A4 and CYP3A5 mRNA in human hepatocytes were increased after exposure to rifampicin. However, the induction rate of the mRNA expression of CYP3A5 was lower than that of CYP3A4. In addition, the levels of CYP3A4 mRNA after exposure to 10 μM and 50 μM NO-1886 showed only a slight increase of 1.3 times and a significant increase of 3.6 times, respectively, compared with untreated controls (**Fig. 3**), while the level of CYP3A5 mRNA after exposure to 50 μM NO-1886 was not significantly increased. In the present study, we found that CYP3A4, which was mainly responsible for metabolizing NO-1886,[8] was induced by exposure to NO-1886, and CYP3A5, which metabolized NO-1886 only slightly,[8] was not induced by exposure to NO-1886. This *in vitro* technique using primary cultured human hepatocytes is expected to be very useful for evaluating differences in the induction of CYP3A4 and CYP3A5 in humans.

It should also be noted that the induction of CYP3A4 by xenobiotics has profound clinical implications. For example, known P450 inducers such as carbamazepine and phenytoin enhance the clearance of co-administered drugs, thereby reducing their efficacy.[25] Therefore, NO-1886, which induces CYP3A4 mRNA, may enhance the clearance of co-administered drugs, thereby reducing their efficacy. Since the hepatic clearance of NO-1886 estimated from enzymatic parameters is much lower than the hepatic blood flow rate,[8] the clearance of NO-1886 itself might be increased after induction of CYP3A4. However, Morioka *et al.*[9] reported that the plasma C_{max} value of NO-1886 at a dose of 3 mg/mL in the cynomolgus monkey was about 1.5 μg/mL (3.3 μM) and protein binding in cynomolgus monkey plasma was 86.0%. The free plasma concentration of NO-1886 in the cynomolgus monkey was about 0.46 μM, which was too low to induce CYP3A4 in human hepatocytes. Based on the plasma concentration, it is thought that no induction of CYP3A4 should occur. However, it is considered that monitoring the changes in the blood concentration of NO-1886 during clinical studies may be important in evaluating the possibility of induction of CYP3A4.

CES has been found to be frequently involved in the detoxification process of several types of ester compounds. Morioka *et al.*[9] reported that the amide hydrolysis of NO-1886 to M-3 (4-[(diethoxyphosphoryl) methyl] benzoic acid) is catalyzed by CES in the rat and cynomolgus monkey but not in humans. Interestingly, in the present study, we found that CES1 and CES2 were induced in primary cultures of human hepatocytes by exposure to 50 μM NO-1886, although the levels of CES1 and CES2 mRNA after exposure to 10 μM NO-1886 were not significantly increased compared with untreated controls (**Fig. 4**). The induced CES1 and CES2 might not affect the clearance of NO-1886 itself. On the other hand, changes in CES activity have important clinical implications, since many drugs such as meperidine[26] and heroin[27] are metabolized by CES.

Morioka *et al.*[7,9] have reported that NO-1886 is biotransformed to M-5
(2-amino-5-bromo-3-cyanobenzene sulphate) *via* M-I
(2-amino-5-bromo-benzonitrile) and/or M-4

(2-amino-5-bromo-3-hydroxybenzonitrile) by sulfotransferase. There have been few reports on the induction of sulfotransferase by exposure to drugs, although it has been reported that SULT2A mRNA is induced by hydrocortisone in male rat hepatocytes[28] and that SULT2A1 mRNA and protein are induced by vitamin D in VDR-transfected HepG2 cells and Caco-2 cells.[29] In the present study, we found that the sulfotransferases CHST1, CST and SULT2A1 were induced in primary cultures of human hepatocytes by exposure to 50 μM NO-1886. However, the levels of CHST1, CST and SULT2A1 mRNA after exposure to 10 μM NO-1886 were not significantly increased compared with untreated controls (**Fig. 5**). Therefore, the disposition of NO-1886 that has been biotransformed by sulfotransferases may be influenced by the induction of these sulfotransferases after exposure to NO-1886.

NO-1886 serves as a substrate for enzymes such as cytochrome P450, CES and sulfotransferase.[7–9] In addition, NO-1886 induces mRNA expression of many enzymes such as CYP3A4, CES1, CES2, CHST1, CST and SULT2A1. Taken together, these results suggest that NO-1886 and/or co-administered drugs may exhibit altered pharmacokinetics under the conditions of a repeated drug administration schedule, indicating that careful optimization of drug dosing regimens is required.

In conclusion, this *in vitro* technique using primary cultured human hepatocytes is expected to be very useful for evaluating the induction of drug-metabolizing enzymes in humans. It is suitable for evaluation in the development of new drugs for the assessment of the *in vitro* induction of a large number of drug-metabolizing enzymes. Finally, we emphasize the important role of drug induction studies using human hepatocytes in the development of new drugs, since such studies avoid the problem of species differences between experimental animal and human data and can be expected to improve the efficacy and safety of future clinical drug therapy.

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