약학박사학위논문

당뇨와 간경화 랫드 질병 모델에서 CYP 효소 발현 및 약동학 변화

Pharmacokinetic change of drugs in diabetic rats with liver cirrhosis: Correlation between metabolism and hepatic microsomal cytochrome P450(CYP) isozymes

2013 년 8 월

서울대학교 대학원

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지도교수 신 완 균

이 논문을 약학박사학위논문으로 제출함

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서울대학교 대학원
약학과 예방·임상약학 전공
안 충 열

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위 원 장 오 정 미 (인)

부위원장 정 석 재 (인)

위 원 이 명 걸 (인)

위 원 이 병 구 (인)

위 원 신 완 균 (인)

Abstract

Pharmacokinetic change of drugs in diabetic rats with liver cirrhosis: Correlation between metabolism and hepatic microsomal cytochrome P450(CYP) isozymes

Ahn, Choongyul

Department of Pharmcacy

The Graduate School

Seoul National University

It has been reported that protein expression of the hepatic Cytochrome P450(CYP) isozymes are altered in diabetic rats. These enzymes are involved in metabolizing many kinds of drugs. Although patients with liver cirrhosis have relatively higher prevalence of diabetes mellitus, there has been no reported studies on the correlation between CYP isoenzyme expression and pharmacokinetic changes in liver cirrhosis with diabetes mellitus rats. In this present study, pharmacokinetics and CYP protein expression changes were evaluated in rats induced to liver cirrhosis(LC) by N-dimethylnitrosamine, in rats with diabetes mellitus(DM) by streptozotocin, and liver cirrhosis with diabetes mellitus(LCD) rats.

First, chlorzoxazone (CZX) is mainly metabolized to 6-hydroxychlorzoxazone (OH-CZX) by the hepatic CYP2E1. Compared with control rats, LCD rats had significantly increased (by 124%) the protein expression of CYP2E1, but the intrinsic clearance (CL_{int}: formation of OH-CZX per milligram protein) was comparable in both groups of rats. Thus, OH-CZX formation in LCD and control rats was expected to be similar. As expected, after i.v. (20 mg/kg) and p.o. (50mg/kg) administration of CZX, the area under the curve (AUC) of OH-CZX was comparable in control and LCD rats (i.v. 571±85.8 and 578±413 μg·min/ml, respectively; p.o., 1540±338 and 2170±1070 μg·min/ml, respectively)

Second, oltipraz (OLT) is also primarily metabolized via hepatic Cytochrome P450 isozymes CYP1A1/2, 2B1/2, 2C11, 2D1 and 3A1/2 in rats. Compared with control rats, the protein expression of hepatic CYP1A increased, that of CYP2C11 and 3A decreased, but that of CYPB1/2 and 2D was not altered in LCD rats. The CL_{int} for the disappearance of OLT in hepatic microsomes were in the order, DM>control>LCD>LC rats. After i.v. or p.o. administration of OLT to control, LC, DM and LCD rats, the AUC was greater in LC rats and smaller in DM rats respectively, than that in control rats. In LCD rats (i.v. 350±132; p.o., 438±164 μg·min/ml), the AUC was partially restored to control rats(i.v. 249±32.9; p.o., 350±132 μg·min/ml).

Last, It has been reported that sildenafil was metabolized via hepatic CYP2C11 and 3A1/2 and N-desmethylsildenafil was formed via hepatic CYP2C11 in rats. The CL_{int} for the formation of N-desmethylsildenafil in hepatic microsomes were in the order, control>DM>LCD>LC rats. After i.v. (10 mg/kg) administration of sildenafil to control, LC, DM, and LCD rats, the AUCs of parent drug were 577±34.9, 1700±399, 890±92.8 and 1310±229 μg·min/ml, respectively. In addition, the AUCs of sildenafil after p.o. administration were, 64.7±22.4, 2010±1300, 202±151, 1380±909 μg·min/ml, respectively. In LC, DM, and LCD rats, significantly greater AUCs of intravenous sildenafil were due to the slower hepatic extraction of sildenafil (because of decrease in the protein expression of hepatic CYP2C11 and 3A subfamily in LC and LCD rats and CYP2C11 in DM rats). In LC and LCD rats, greater magnitude of increase in AUCs of oral sildenafil than those after the intravenous administration could be mainly due to the decrease in the intestinal extraction of sildenafil (because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats).

Key words: Chlorzoxazone, Oltipraz, Sildenafil, Pharmacokinetics, LCD rats, and Cytochrome P450

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A. ABSTRACT

Protein expression of the hepatic CYP2E1 has been reported to be increased in diabetic rats. This enzyme is the primary metabolizer of chlorzoxazone (CZX) to 6-hydroxychlorzoxazone (OH-CZX). Although patients with liver cirrhosis have a higher prevalence of diabetes mellitus, there have been no reported studies on the protein expression of CYP2E1 in rats induced to have liver cirrhosis and diabetes mellitus by injection of *N*-dimethylnitrosamine followed by streptozotocin [liver cirrhosis with diabetes mellitus (LCD) rats]. Thus, in the present study, the pharmacokinetics of CZX and OH-CZX were evaluated in LCD rats. Compared with control rats, LCD rats had significantly decreased (by 62%) total liver protein and significantly increased (by 124%) protein expression of CYP2E1, but the intrinsic clearance (CL_{int}; formation of OH-CZX per milligram protein) was

comparable in both groups of rats. As a result, the relative CL_{int} was also comparable for the two groups. Thus, OH-CZX formation in LCD and control rats was expected to be similar. As expected, after i.v. (20 mg/kg) and p.o. (50 mg/kg) administration of CZX, the area under the curve (AUC) of OH-CZX was comparable in control and LCD rats (i.v., 571 ± 85.8 and 578 ± 413 μg · min/ml, respectively; p.o., 1540 ± 338 and 2170 ± 1070 μg · min/ml, respectively). In LCD rats, the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio was similar to the value in control rats after i.v. and p.o. administration. These results indicate that OH-CZX can be used as a chemical probe to assess the activity of CYP2E1 in LCD rats.

B. INTRODUCTION

Chlorzoxazone [5-chloro-2(3*H*)-benzoxazolone; CZX], a skeletal muscle relaxant once used for the treatment of painful muscle spasms, is primarily metabolized to 6-hydroxychlorzoxazone (OH-CZX), which is subsequently glucuronidated and excreted in the urine (Conney and Burns, 1960; Desiraju et al., 1983). Formation of OH-CZX from CZX is primarily catalyzed by the hepatic microsomal cytochrome P450 (P450) enzyme 2E1 in humans (Conney and Burns, 1960) and rats (Rockich and Blouin, 1999; Moon et al., 2003). OH-CZX formation has been used as a chemical probe to assess the

activity of CYP2E1 in vitro and in vivo because of its good correlation with CYP2E1 activity in humans (Peter et al., 1990) and rats (Rockich and Blouin, 1999).

Kim et al. (2005) reported that induction of diabetes mellitus in male Sprague-Dawley rats by treatment with alloxan or streptozotocin (DMIA or DMIS rats, respectively) also increased their protein expression and mRNA level of CYP2E1. Furthermore, Baek et al. (2006) reported that the increased protein expression of CYP2E1 caused a significant increase in the formation of OH-CZX in both DMIA and DMIS rats. The association between liver disease and diabetes mellitus is well known (Vidal et al., 1994; Kwon, 2003; Moscatiello et al., 2007). Thus, we examined CZX in this study.

Wang et al. (2003) p.o. administered CZX to diabetic patients and found that the total area under the plasma CZX concentration–time curve from time 0 to infinity (AUC) was reduced by 25% in type 1 diabetic patients and by 70% in obese type 2 diabetic patients as compared with that in 20 control volunteers. Furthermore, they found that protein expression of CYP2E1 in peripheral blood mononuclear cells increased in both types of diabetic patients. However, to our knowledge, no studies on the protein expression of CYP2E1 and the pharmacokinetics of CZX and OH-CZX in diabetic rats or humans with liver cirrhosis have yet been reported.

The objectives of the current studies were to evaluate, using a rat

model, the effects of diabetes and liver cirrhosis, alone and in combination, on the pharmacokinetics of CZX and OH-CZX. Changes in the protein expression of hepatic CYP2E1 in rats with liver cirrhosis with or without diabetes were also investigated.

C. MATERIALS AND METHODS

Chemicals

CZX, OH-CZX, 3-aminophenyl sulfone [internal standard for the high-performance liquid chromatography (HPLC) analysis of CZX and OH-CZX], monoclonal anti-β-actin antibody, NADPH (tetrasodium salt), Tris buffer, EDTA (disodium salt), β-glucuronidase (type H-1, from *Helix pomatia*), streptozotocin, and Kodak X-OMAT film were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Dimethylnitrosamine was a product from Tokyo Kasei Kogyo Company (Tokyo, Japan), and ketamine hydrochloride was from Yuhan Corporation (Seoul, South Korea). Polyclonal anti-human CYP2E1 antibody was obtained from Detroit R&D (Detroit, MI), and horseradish peroxidase-conjugated goat anti-rabbit antibody was from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents were purchased from Amersham Biosciences Corporation (Piscataway, NJ). Other chemicals were of reagent or HPLC grade.

Animals

Protocols for the animal studies were approved by the Animal Center and Use Committee of the College of Pharmacy of Seoul National University, Seoul, South Korea. Male Sprague-Dawley rats (4–5 weeks old, weighing 180–200 g) were purchased from the Charles River Company Korea (Orient, Seoul, South Korea). The rats were randomly divided into three disease groups [liver cirrhosis (LC), diabetes mellitus (DM), and liver cirrhosis with diabetes mellitus (LCD)] and a control group. They were maintained in a clean room (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University) at a temperature of 22 ± 2°C with 12-h light (7:00 AM to 7:00 PM) and dark (7:00 PM to 7:00 AM) cycles and a relative humidity of 55 ± 5%. Rats were housed in metabolic cages (Tecniplast, Varese, Italy) under filtered, pathogen-free air, with food (Sam Yang Company, Pyungtaek, South Korea) and water available ad libitum.

Induction of LC in rats by N-dimethylnitrosamine injection

Freshly prepared *N*-dimethylnitrosamine (diluted to 0.01 mg/ml in 0.9% NaCl injectable solution) was injected i.p. at a dose of 0.01 mg/kg on three consecutive days per week for 4 weeks (Ohara and Kusano, 2002; Bae et al., 2006). On day 29, citrate buffer (pH 7.4; 1 ml/kg) was injected via the tail vein. On day 36, rats were treated with CZX.

Laboratory rats with *N*-dimethylnitrosamine-induced LC have clinical features similar to those of humans with LC, such as increased mortality, hepatic parenchymal cell destruction, formation of connective tissue, and nodular regeneration (Kang et al., 2002). LC in the LC rats was evident by histological analysis, which revealed extensive micronodular cirrhosis with regenerative hepatocellular changes. Bile ductular proliferation was also detected (Bae et al., 2006). It has been reported that *N*-dimethylnitrosamine-induced liver cirrhosis in rats is reproducible (Jenkins et al., 1985; Jezequel et al., 1987).

Induction of DM in rats by streptozotocin injection

A 0.9% NaCl injectable solution was injected i.p. (1 ml/kg) on three consecutive days a week for 4 weeks. On day 29, one dose (45 mg/kg) of freshly prepared streptozotocin (dissolved in citrate buffer, pH 4.5, to 45 mg/ml) was administered via the tail vein (Kim et al., 2005). The rats were treated with CZX on day 36.

Induction of LCD in rats with N-dimethylnitrosamine and streptozotocin injections

LC was induced by i.p. injection of *N*-dimethylnitrosamine as described above. Then, on day 29, DM was induced by injection of streptozotocin via the tail vein as described above. The rats were

treated with CZX on day 36.

Control rats

Rats were injected i.p. with 0.9% NaCl injectable solution (1 ml/kg) on three consecutive days a week for 4 weeks. On day 29, one dose (1 ml/kg) of citrate buffer, pH 4.5, was administered via the tail vein. The rats were treated with CZX on day 36. During the pretreatment, food and water were available ad libitum to all the rats. Immediately before the experiment, blood glucose levels in all the rats were measured using the MediSense Optium Kit (Abbott Laboratories, Bradford, MA), and rats with blood glucose levels greater than 250 mg/dl were selected as diabetic (DM and LCD rats).

Measurement of liver, kidney, and spleen function

To assess liver, kidney, and spleen function, a 24-h urine sample was collected on day 36 from LC, DM, LCD, and control rats (n = 6, each) for the measurement of creatinine levels. A blood sample was collected from the carotid artery for the measurement of the hematocrit (microprocessor pH/°C meter) (Eutek Cybernetics, Singapore, Singapore). The plasma was measured for the total protein, albumin, urea nitrogen, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), total bilirubin, direct bilirubin, phosphatase, lactate dehydrogenase (LDH), and creatinine

by the Green Cross Reference Laboratory (Seoul, South Korea). Plasma protein binding of CZX was measured using equilibrium dialysis (Shim et al., 2000). The whole liver, kidney, and spleen of each rat were excised, rinsed with 0.9% NaCl injectable solution, blotted dry with tissue paper, and weighed. Small portions of each organ were fixed in 10% neutral phosphate-buffered formalin and then processed for routine histological examination with hematoxylin and eosin staining. Each rat was exsanguinated and sacrificed by cervical dislocation.

Preparation of hepatic microsomes

The procedures used were similar to those described by Baek et al. (2006). Livers from LC, DM, LCD, and control rats (n = 5, each) were homogenized (Ultra-Turrax T25, Janke and Kunkel; IKA-Labortechnik, Staufeni, Germany) in ice-cold homogenization buffer (0.154 M KCl/50 mM Tris-HCl in 1 mM EDTA, pH 7.4). After the homogenate was centrifuged (10,000*g*, 30 min), the supernatant fraction was removed and centrifuged at high speed (100,000*g*, 90 min). The resulting microsomal pellet was resuspended in homogenization buffer and stored at –70°C (Revco ULT 1490 D-N-S; Western Mednics, Asheville, NC) until used. Protein content was measured using the Bradford method (Bradford, 1976).

Western immunoblot analysis of CYP2E1

The procedures used were similar to a reported method (Kim et al., 2001). Liver microsomes were resolved by SDS gel electrophoresis on a 7.5% polyacrylamide gel (10 µg protein/lane; n = 3 each). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) that was then blocked for 1 h in 5% milk powder in phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBS-T). For immunodetection, blots were incubated overnight at 4°C with rabbit anti-human CYP2E1 antibody (diluted 1:10,000 in PBS-T containing 5% bovine serum albumin), followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:10,000 in PBS-T containing 5% milk powder). Protein expression of CYP2E1 was detected by enhanced chemiluminescence on Kodak X-OMAT film and quantitated by densitometry with a microcomputer imaging device (model M1; Imaging Research, St. Catharine's, ON, Canada). The β-actin band was used as a loading control.

Measurement of V_{max}, K_m, and intrinsic clearance for the formation of OH-CZX in hepatic microsomes

Microsomal fractions (equivalent to 0.2 mg of protein) were mixed with 50 µl of 0.1 M phosphate buffer, pH 7.4, containing 1 mM NADPH; 10 µl of CZX dissolved in a minimal amount of 10 N NaOH

to make final concentrations of 2.5, 5, 10, 20, 50, 100, 200, 500, and 1000 μM CZX; and 0.1 M phosphate buffer, pH 7.4, sufficient to make a final volume of 0.5 ml. This reaction mixture was incubated in a water-bath shaker (37°C, 50 oscillations/min) for 20 min, at which time the reaction was terminated by addition of 1 ml of ether. The formation of OH-CZX was determined using an HPLC method (Frye and Stiff, 1996). The kinetic parameters (K_m, V_{max}) for the formation of OH-CZX were determined by fitting the unweighted kinetic data from rat liver microsomes to a single-site Michaelis-Menten equation, $V = V_{max} \times [S]/(K_m + [S])$, where [$S$] is the substrate concentration. The best-fit model was selected based on the statistical goodness of fit (Yamaoka et al., 1978); the model with the lowest Akaike information criterion was chosen. Calculations were performed using the WinNonlin software (Pharsight, Mountain View, CA). The intrinsic clearance (CL_{int}) for the formation of OH-CZX per milligram protein was calculated by dividing the V_{max} by the K_m. The relative CL_{int} for the formation of OH-CZX based on the whole rat liver was estimated by the protein expression below and expressed as a percentage of the controls (100%): total liver protein (mg) × protein expression of CYP2E1 (% relative to controls) × CL_{int} (ml/min/mg protein).

Pretreatment of rats for i.v. or p.o. study

Early in the morning on day 36, each rat was anesthetized by i.m.

injection of ketamine hydrochloride at a dose of 100 mg/kg. The jugular vein (for drug administration in the i.v. study) and the carotid artery (for blood sampling) were cannulated with a polyethylene tube (Clay Adams, Parsippany, NJ). Both cannulas were exteriorized to the dorsal side of the neck, where each cannula was terminated with a long silastic tube (Dow Corning, Midland, MI). Both silastic tubes were inserted into a wire sheath to allow free movement of the rat. Each rat then was housed individually in a rat metabolic cage (Daejong Scientific Company, Seoul, South Korea) and allowed to recover from anesthesia for 4 to 5 h before beginning the experiment. Thus, the rats were not restrained in the present study. Ketamine was used instead of ether to minimize the effect on CYP2E1 because Liu et al. (1993) reported that ether anesthesia alone increased the protein expression of CYP2E1 by 40%, as determined by assaying *p*-nitrophenol hydroxylase activity.

Intravenous study

CZX (dissolved in a minimum amount of 10 N NaOH) at a dose of 20 mg/kg was infused (total infusion volume of 2 ml/kg) over 1 min via the jugular vein to rats in each group (n = 9, 7, 7, and 8 for LC, DM, LCD, and control rats, respectively). A blood sample (approximately 0.12 ml) was collected via the carotid artery at 0 (control), 1 (at the end of the infusion), 5, 15, 30, 45, 60, 90, 120, and 180 min after the

start of the i.v. infusion of CZX. A heparinized 0.9% NaCl injectable solution (20 units/ml; 0.3 ml) was used to flush the cannula immediately after each blood sampling to prevent clotting. Each blood sample was immediately centrifuged, and a 50-µl aliquot of plasma was stored at –70°C for later analysis of CZX and OH-CZX by HPLC (Frye and Stiff, 1996). At the end of the experiment (24 h after CZX treatment), each metabolic cage was rinsed with 20 ml of distilled water, and the rinse water was combined with the 24-h urine sample. The volume of the combined urine sample was determined, and two 50-µl aliquots were stored at –70°C for later analysis. At the same time (24 h), as much blood as possible was collected via the carotid artery, and each rat was sacrificed by cervical dislocation. The abdomen then was opened, and the entire gastrointestinal tract (including its contents and feces) of each rat was removed, transferred to a beaker containing 50 ml of 0.1 N NaOH (to facilitate the extraction of CZX and OH-CZX), and cut into small pieces with scissors. After stirring with a glass rod for 1 min, two 50-µl aliquots of the supernatant were collected from each beaker and stored at –70°C for later analysis.

Oral study

CZX (the same solution used in the i.v. study) at a dose of 50 mg/kg was administered p.o. (total p.o. volume of 3 ml/kg) using a feeding

tube to rats in each group (n = 8, 8, 7, and 7 for LC, DM, LCD, and controls, respectively). Blood samples were collected at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min after p.o. administration of CZX. Other procedures were similar to those described above for the i.v. study.

Measurement of rat plasma protein binding of CZX using equilibrium dialysis

Binding of CZX to protein in fresh plasma from LC, DM, LCD, and control rats (n = 5 each) was measured using equilibrium dialysis (Shim et al., 2000). Plasma (1 ml) was dialyzed against 1 ml of isotonic Sørensen phosphate buffer, pH 7.4, containing 3% (w/v) dextran to minimize volume shift (Boudinot and Jusko, 1984) in a 1-ml dialysis cell (Fisher Scientific, Fair Lawn, NJ) fitted with a Spectra/Por 4 membrane (molecular mass cutoff of 12–14 kDa; Spectrum Medical Industries Inc., Los Angeles, CA). The initial concentrations of CZX spiked into the plasma compartment were 1, 10, and 50 μg/ml. After a 24-h incubation, two 50-μl aliquots were removed from each compartment and stored at –70°C for later HPLC analysis of CZX.

HPLC analysis of CZX and OH-CZX

Concentrations of CZX and OH-CZX in the samples were determined

using an HPLC method (Frye and Stiff, 1996). Briefly, a 0.1-ml aliquot of 0.2 M sodium acetate buffer, pH 4.75, and a 0.1-ml aliquot of isotonic Sørensen phosphate buffer, pH 7.4, containing 200 units of β-glucuronidase were added to 50 µl of sample. The mixture was mixed manually and incubated in a water-bath shaker (50 oscillations/min) for 2 h at 37°C. A 50-µl aliquot of methanol containing 10 mg/ml of 3-aminophenyl sulfone (internal standard) was then added. After the mixture was vortexed, 1 ml of diethyl ether was added, and the mixture was shaken for 10 min. After centrifugation (16,000*g*, 10 min), the upper organic layer was transferred to a clean tube and dried (Dry Thermobath; Eyela, Tokyo, Japan) under a gentle stream of nitrogen gas at 37°C. The residue was reconstituted in 0.1 ml of mobile phase [0.1 M ammonium acetate/acetonitrile/tetrahydrofuran (72:22.5:5.5, v/v/v)], and a 50-µl aliquot was directly injected onto a reversed-phase (C18) HPLC column. The mobile phase was run at a flow rate of 1.0 ml/min. An ultraviolet detector at 283 nm was used to monitor the column eluent. Unconjugated concentrations of OH-CZX were also measured in the urine samples without incubation with β-glucuronidase. The retention times of OH-CZX, 3-aminophenyl sulfone (internal standard), and CZX were approximately 6, 10, and 18 min, respectively. The detection limit for CZX and OH-CZX in the rat plasma and urine samples was all 0.05 µg/ml. The coefficients of variation of the assay

(within and between-day) were less than 8.2%.

Pharmacokinetic analysis

The AUC was calculated using the trapezoidal rule-extrapolation method (Chiou, 1978). The area from the last datum point to time infinity was estimated by dividing the last measured plasma concentration by the terminal-phase rate constant. Standard methods (Gibaldi and Perrier, 1982) were used to calculate the following pharmacokinetic parameters using a noncompartmental analysis (WinNonlin; Pharsight Corporation): the time-averaged total body, renal, and nonrenal clearances (CL, CL_r, and CL_{nr}, respectively), the terminal half-life ($t_{1/2}$), the first moment of AUC, the mean residence time (MRT), the apparent volume of distribution at steady state (V_{ss}), and the extent of absolute oral bioavailability (F). The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were directly read from the experimental data.

Statistical analysis

A p value <0.05 was deemed to be statistically significant using an unpaired t test or a Duncan's multiple range test, with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) posteriori analysis of variance among the four means for the unpaired data. All the data are expressed as the mean ± S.D., with the exception of T_{max},

which is expressed as the median (range).

D. RESULTS

Liver, kidney, and spleen function

Body weight, blood glucose level, hematocrit, 24-h urine output, plasma chemistry data, CL_{cr}, and relative organ weights for the four rat groups are listed in Table 1. For comparison, literature values from normal (albino) rats (Mitruka and Rawnsley, 1981; Davies and Morris, 1993) are also shown.

Compared with the control rats, the LC rats had significantly decreased 24-h urine volume and plasma levels of total protein and albumin; significantly increased plasma levels of GOT, GPT, total bilirubin, direct bilirubin, alkaline phosphatase, and total cholesterol; significantly decreased relative liver weight; and significantly increased relative spleen weight. In DM rats, the blood glucose level, the 24-h urine output, the plasma levels of GPT, alkaline phosphatase, urea nitrogen, and total cholesterol, and the relative kidney weight were significantly increased compared with the control rats. In LCD rats, the blood glucose level, the plasma levels of total protein and albumin, the 24-h urine output, and the relative liver weight were significantly decreased compared with the control rats, whereas the plasma levels of GOT, GPT, total bilirubin, direct bilirubin, alkaline

Table 1 Body weight, blood glucose, hematocrit, 24-h urine output, plasma chemistry data, CL_{cr}, and relative organ weights in LC, DM, LCD, and control rats Literature values from normal (Albino) rats are shown for comparison.

Parameter[a]	Control (n = 6)	LC (n = 6)	DM (n = 6)	LCD(n=6)	Literature Values from Normal (Albino) Rats
Initial body weight(g)	216 ± 17.4	208 ± 11.5	194 ± 24.5	204 ± 19.4	
Final body weight (g)[b]	348 ± 14.7	285 ± 12.6	263 ± 15.7	264 ± 17.6	
Blood glucose (mg/dl)[c]	134 ± 11.6	139 ± 13.7	516 ± 71.4	512 ± 59.3	50–135
Hematocrit (%)[d]	52.1 ± 1.15	34.9 ± 3.78	50.1 ± 1.58	41.2 ± 1.50	44.4–50.4
Urine volume (ml/24-h)[e]	25.2 ± 5.46	13.2 ± 7.60	83.5 ± 15.5	90.2 ± 7.14	
Plasma					
Total protein (g/dl)[f]	5.50 ± 0.141	4.17 ± 0.339	5.22 ± 0.527	4.55 ± 0.543	4.70–8.15
Albumin (g/dl)[d]	3.43 ± 0.121	2.50 ± 0.310	3.28 ± 0.293	2.85 ± 0.138	2.70–5.10
GOT (IU/l)[f]	48.7 ± 11.5	169 ± 29.8	90.0 ± 27.7	223 ± 10.2	45.7–80.8
GPT (IU/l)[b]	18.8 ± 6.74	65.5 ± 16.2	64.5 ± 23.5	73.3 ± 18.9	17.5–30.2
Total bilirubin (mg/dl)[g]	BD[h]	1.08 ± 0.685	BD[h]	0.410 ± 0.313	0.00–0.55
Direct bilirubin (mg/dl)[g]	BD[h]	0.872 ± 0.553	BD[h]	0.260 ± 0.265	
Alkaline phophatase (IU/l)[i]	213 ± 24.6	550 ± 161	942 ± 267	516 ± 139	56.8–128
Lactate dehydrogenase (IU/l)[j]	161 ± 47.0	409 ± 293	201 ± 83.8	580 ± 400	61.0–121
Urea nitrogen (mg/dl)[k]	17.6 ± 2.26	19.4 ± 2.27	28.4 ± 2.03	40.2 ± 6.79	5.0–29.0
Total cholesterol (mg/dl)[b]	49.0 ± 8.44	76.3 ± 13.0	75.2 ± 4.62	79.3 ± 8.68	10.0–54.0
CL_{cr} (ml/min/kg)	3.74 ± 0.619	3.46 ± 0.733	3.30 ± 0.589	2.79 ± 0.431	5.24
Liver weight (% of body weight)[d]	3.84 ± 0.280	2.11 ± 0.419	3.50 ± 0.237	3.00 ± 0.296	4.00
Kidney weight (% of body weight)[c]	0.706 ± 0.0700	0.772 ± 0.143	1.02 ± 0.0586	1.06 ± 0.0642	0.80
Spleen weight (% of body weight)[d]	0.203 ± 0.0305	0.455 ± 0.0287	0.182 ± 0.0233	0.335 ± 0.115	0.30

[a]Data are expressed as mean ± S.D.

[b]Control group was significantly different ($p < 0.05$) from LC, DM, and LCD groups.

[c]Control and LC groups were significantly different ($p < 0.05$) from DM and LCD groups.

[d]Control and DM groups, LC group, and LCD group were significantly different ($p < 0.05$).

[e]Control group, LC group, and DM and LCD groups were significantly different ($p < 0.05$).

[f]Control and DM groups were significantly different ($p < 0.05$) from LC and LCD groups.

[g]LC group was significantly different ($p < 0.05$) from LCD group.

[h]Below the detection limit.

[i]Control group, LC and LCD groups, and DM group were significantly different ($p < 0.05$).

[j]Control and DM groups were significantly different ($p < 0.05$) from LCD group.

[k]Control and LC groups, DM group, and LCD group were significantly different ($p < 0.05$).

phosphatase, LDH, urea nitrogen, and total cholesterol and the relative kidney and spleen weights were significantly increased. However, the CL_{cr} did not differ significantly among the four groups.

These findings suggest that in LC and LCD rats, neither kidney nor spleen function was seriously impaired, whereas liver function was somewhat impaired. Consistent with this result, no significant histological findings were detected in the liver, kidney, or spleen in any rats, except that extensive hepatocellular degeneration with bridging fibrosis (precirrhotic change) was detected in the livers of LC and LCD rats.

Protein expression of CYP2E1

Compared with the control rats, the protein expression of CYP2E1 increased (by 258%) in the DM rats, decreased (by 22.0%) in the LC rats, and increased (by 124%) in the LCD rats (Figure 1).

V_{max}, K_m, and CL_{int} for formation of OH-CZX in hepatic microsomes

The rates of OH-CZX formation in liver microsomes from LC, DM, LCD, and control rats treated with varying concentrations of CZX are shown in Figure 2, and the V_{max}, K_m, CL_{int}, relative liver weight, total protein, and relative CL_{int} for each group are listed in Table 2. For the LCD rats, the V_{max} for OH-CZX formation was significantly lower than that of the DM rats (by 39.7%) and higher than that of the LC rats

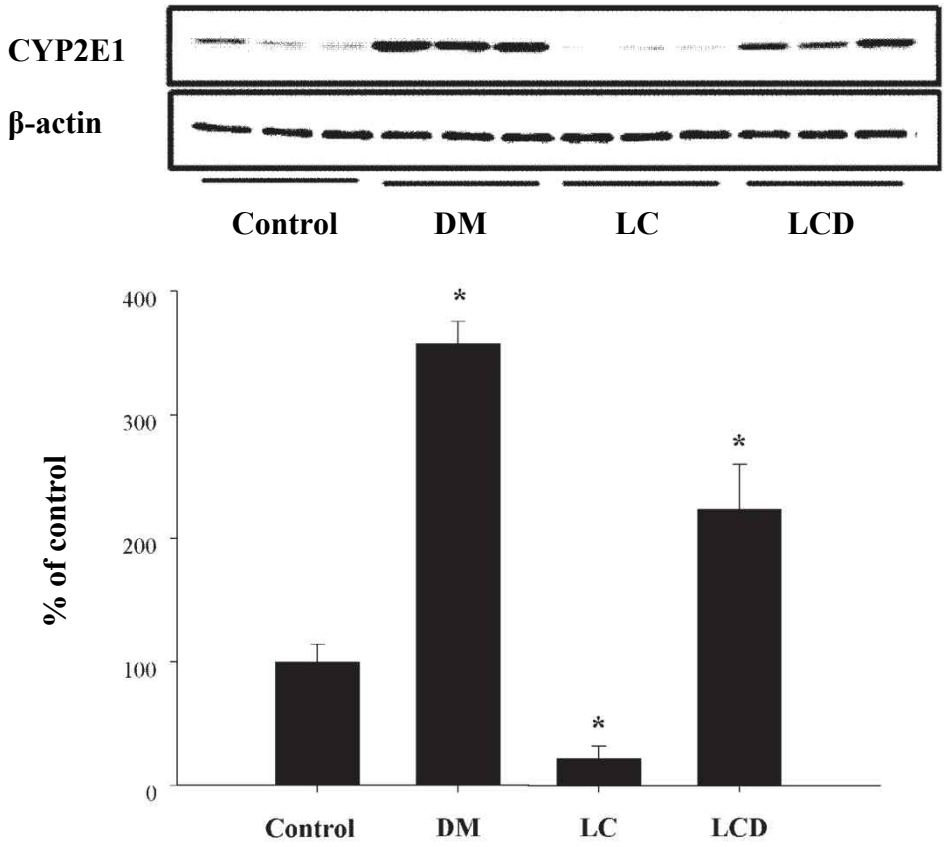

Figure 1 Hepatic protein expression of CYP2E1 in LC, DM, LCD, and control rats was quantitated by Western immunoblotting and densitometry. A, immunoblot of gel loaded with 10 μg of microsomal protein per lane. β-Actin was used as a loading control. CYP2E1 was detected by enhanced chemiluminescence on Kodak X-OMAT film. B, protein expression of CYP2E1 was quantitated by densitometry. Results are shown relative to protein expression of CYP2E1 in the control rats (control = 100%). Error bars represent S.D. *, $p < 0.05$ compared with the controls; each value was significantly different.

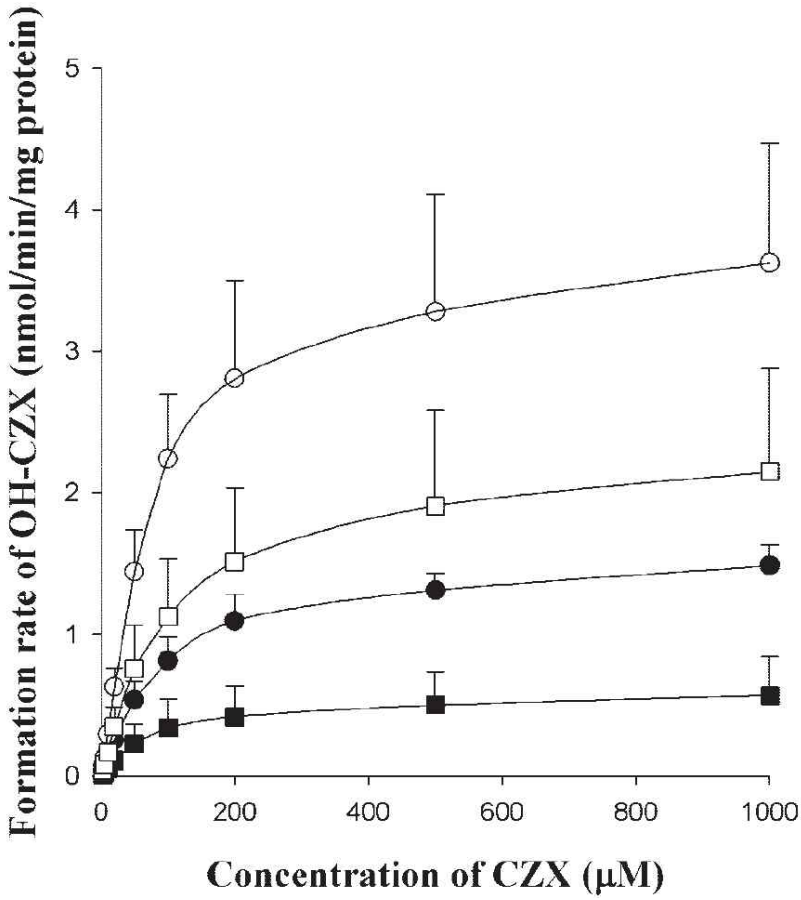

Figure 2 Kinetics for the formation rate of OH-CZX. CZX was incubated at the indicated concentrations (2.5–1000 μM) with liver microsomes from LC (■), DM (○), LCD (□), and control (●) rats (n = 5, each) at 37°C for 20 min. The kinetic data were fit to a simple Michaelis-Menten equation. Error bars represent S.D.

Table 2 V_{max}, K_m, and CL_{int} for the formation of OH-CZX in the hepatic microsomes of LC, DM, LCD, and control rats

Parameter[a]	Control (n = 5)	LC (n = 5)	DM (n = 5)	LCD(n = 5)
Final body weight(g)[b]	384 ± 17.4	292 ± 25.6	309 ± 37.3	253 ± 18.2
Liver weight (% of body weight)[c]	3.25 ± 0.418	2.55 ± 0.568	3.17 ± 0.246	3.52 ± 0.263
V_{max} (nmol/min/mg protein)[d]	1.63 ± 0.139	0.612 ± 0.270	3.95 ± 0.995	2.38 ± 0.785
K_m (μM)	110 ± 48.3	108 ± 45.2	86.8 ± 20.3	119 ± 31.8
CL_{int} (ml/min/mg protein)[e]	0.0165 ± 0.00520	0.00720 ± 0.00509	0.0461 ± 0.00970	0.0214 ± 0.00866
Total protein (mg/whole liver)[f]	373 ± 113	121 ± 24.7	239 ± 13.6	143 ± 35.7
Protein expression of CYP2E1 (%)[g,h,]	100 ± 15	22 ± 10	358 ± 22.0	224 ± 36
Relative CL_{int} (%)[f]	100	3.11	641	113

[a]Data are expressed as mean ± S.D.

[b]Control group, LCD group, and LC and DM groups were significantly different ($p < 0.05$).

[c]Control, DM, and LCD groups were significantly different ($p < 0.05$) from LC group.

[d]Control and LCD groups, LC group, and DM group were significantly different ($p < 0.05$).

[e]Control, LC, and LCD groups were significantly different ($p < 0.05$) from DM group, and LC group was significantly different ($p < 0.05$) from LCD group.

[f]Control group, DM group, and LC and LCD groups were significantly different ($p < 0.05$).

[g]Each group was significantly different ($p < 0.05$).

[h]$n = 3$ per each group.

(by 289%) but was similar to the value observed in control rats. The changes observed in the V_{max} reflect the changes observed in the amount of CYP2E1 (Table 2). This result suggests that in LCD rats, the maximum velocity for the formation of OH-CZX was similar to the value observed in control rats. However, the K_m for the formation of OH-CZX was comparable (not significantly different) among the four groups of rats, indicating that the affinity of the enzyme(s) for CZX was not changed. As a result, in LCD rats, the CL_{int} for the formation of OH-CZX per milligram protein was significantly slower than in DM rats (by 53.6%) and faster than in LC rats (by 197%) but was similar to the value observed in control rats. The total protein was significantly lower in LCD rats than in control or DM rats, but it was comparable with that in LC rats.

As discussed above (Figure 1), the protein expression of CYP2E1 differed significantly among the four groups. Because the relative liver weight, total protein per whole liver, and protein contents of CYP2E1 were not comparable among the four groups, the relative CL_{int} for the formation of OH-CZX, based on total liver protein, was calculated; in LCD rats, the value was considerably higher (by 353%) than that in LC rats and lower (by 82.4%) than that in DM rats but was similar to the value observed in control rats. This result suggests that in LCD rats, the formation of OH-CZX, based on the whole liver, may be comparable with that in control rats and that the V_{max}, CL_{int} per

milligram protein, and relative CL_{int}, based on the whole liver, were similar to values observed in control rats. Mizuno et al. (2000) reported a K_m of 73 ± 3.1 μM and a V_{max} of 1.09 ± 0.38 nmol/min/mg protein for the formation of OH-CZX in 10 control rats. These values differ somewhat from the present data (Table 2), possibly because of differences in the preparation of hepatic microsomes, in the concentrations of protein (0.1 versus 0.2 mg) and substrate (0.01–1 versus 2.5–1000 μM) used, or in the incubation time used (15 versus 20 min).

Rat plasma protein binding of CZX

The values for CZX binding of proteins in fresh plasma from the four groups of rats were 73.0 ± 4.96% (control), 62.5 ± 8.53% (LC), 67.1 ± 2.44% (DM), and 72.3 ± 2.23% (LCD), respectively; the value in LC rats was significantly lower than that in control and LCD rats. Protein binding of CZX to plasma from control rats (n = 3 each) was constant for CZX concentrations of 1, 10, and 50 μg/ml, which yielded values of 68.3 ± 3.51, 69.6 ± 1.91, and 67.3 ± 5.44%, respectively. Thus, a CZX concentration of 10 μg/ml was arbitrarily chosen for the plasma protein binding studies.

Pharmacokinetics of CZX and OH-CZX after i.v. administration of CZX

The mean arterial plasma CZX concentration–time profiles for i.v. administration of CZX (20 mg/kg) to LC, DM, LCD, and control rats are shown in Figure 3A, and the relevant pharmacokinetic parameters are listed in Table 3. In LC rats, the AUC of CZX was significantly greater; the terminal $t_{1/2}$ and MRT were significantly longer; and the CL, CL_r, and CL_{nr} were significantly lower than those in control rats. In DM rats, the AUC was significantly smaller; the terminal $t_{1/2}$ was significantly shorter; and the CL, CL_r, and CL_{nr} were significantly faster than those in control rats. Interestingly, the AUC, MRT, CL, CL_r, and CL_{nr} of CZX were similar between LCD and control rats. The contribution of the CL_r to the CL of CZX was almost negligible; the values were less than 3.63% in all the rats studied. However, the V_{ss} of CZX and the percentage of the i.v. dose of CZX excreted in the 24-h urine as unchanged drug ($Ae_{0\text{-}24\,h}$) were not significantly different among the four groups. CZX was undetectable (under the detection limit) in the gastrointestinal tract at 24 h ($GI_{24\,h}$) in all the rats. Thus, the contribution of changes in the CL_r of CZX to other pharmacokinetic changes of CZX may also be almost negligible.

For the i.v. administration of CZX to DM, LC, LCD, and control rats, the mean arterial plasma OH-CZX concentration–time profiles are shown in Figure 3B, and the relevant pharmacokinetic parameters are shown in Table 3. Formation of OH-CZX was rapid; for all four groups of rats, OH-CZX was detected in plasma at the first blood

Table 3 Pharmacokinetic parameters of CZX and OH-CZX after i.v. administration of CZX at a dose of 20 mg/kg to LC, DM, LCD, and control rats

Parameter[a]	Control (n = 8)	LC (n = 9)	DM (n = 7)	LCD(n = 7)
Initial body weight (g)	213 ± 13.6	210 ± 14.1	206 ± 16.5	208 ± 9.94
Final body weight (g)[b]	334 ± 8.21	268 ± 20.6	259 ± 19.2	236 ± 12.4
Blood glucose (mg/dl)[c]	117 ± 9.90	102 ± 20.3	397 ± 90.2	455 ± 33.6
Urine Volume (ml/24-g)[d]	21.2 ± 3.31	18.8 ± 4.49	40.2 ± 14.6	25.1 ± 9.83
Hematocrit (%)[e]	50.3 ± 3.95	34.5 ± 7.50	49.6 ± 3.07	35.4 ± 7.60
CZX				
AUC (μg·min/ml)[f]	1720 ± 442	2700 ± 429	744 ± 92.1	1740 ± 809
Terminal $t_{1/2}$ (min)[g]	28.9 ± 2.97	65.1 ± 25.0	24.2 ± 7.13	38.3 ± 19.6
MRT (min)[h]	31.9 ± 5.98	71.5 ± 30.6	16.7 ± 6.75	42.2 ± 24.4
CL (ml/min/kg)[f]	12.3 ± 3.21	7.60 ± 1.47	27.2 ± 3.27	14.4 ± 8.29
CL_r (ml/min/kg)[i]	0.358 ± 0.326	0.0989 ± 0.154	0.881 ± 0.534	0.523 ± 0.443
CL_{nr} (ml/min/kg)[f]	12.0 ± 3.38	7.50 ± 1.32	26.3 ± 3.68	13.8 ± 7.92
V_{ss} (ml/kg)	391 ± 61.1	524 ± 181	459 ± 196	466 ± 93.6
$Ae_{0\text{-}24\ h}$ (% of CZX dose)[f]	3.31 ± 3.12	1.06 ± 1.05	3.44 ± 2.44	3.42 ± 2.25
$GI_{24\ h}$ (% of CZX dose)	BD[j]	BD	BD	BD

(Table 3 continued)

Parameter[a]	Control (n = 8)	LC (n = 9)	DM (n = 7)	LCD (n = 7)
OH-CZX				
AUC (μg·min/ml)[f]	571 ± 85.8	168 ± 153	1000 ± 176	578 ± 413
Terminal $t_{1/2}$ (min)[k]	32.3 ± 6.09	34.5 ± 8.70	45.5 ± 4.04	44.3 ± 14.6
C_{max} (μg/ml)[f]	8.36 ± 1.50	2.38 ± 2.54	15.2 ± 3.24	6.55 ± 3.76
T_{max} (min)[e]	15 (15–30)	30 (15–60)	15 (5–15)	45 (15–60)
$Ae_{0\text{-}24\ h,\ total\ OH\text{-}CZX}$ (% of CZX dose)[f]	42.3 ± 14.0	18.7 ± 14.5	78.0 ± 6.32	43.0 ± 13.6
$Ae_{0\text{-}24\ h,\ free\ OH\text{-}CZX}$ (% of CZX dose)[k]	15.3 ± 6.22	10.5 ± 10.1	33.0 ± 2.26	25.6 ± 11.9
$GI_{24\ h}$ (% of CZX dose)	BD	BD	BD	BD
$AUC_{OH\text{-}CZX}$ / AUC_{CZX} (%)[f]	35.6 ± 13.0	6.41 ± 5.69	136 ± 25.6	39.1 ± 30.8

[a]Data are expressed as mean ± S.D.

[b]Control group, LC and DM groups, and LCD group were significantly different ($p < 0.05$).

[c]Control and LC groups, DM group, and LCD group were significantly different ($p < 0.05$).

[d]Control, LC, and LCD groups were significantly different ($p < 0.05$) from DM group.

[e]Control and DM groups were significantly different ($p < 0.05$) from LC and LCD groups.

[f]Control and LCD groups, DM group, and LC group were significantly different ($p < 0.05$).

[g]Control group, DM and LCD groups, and LC group were significantly different ($p < 0.05$).

[h]Control and DM groups, control and LCD groups, and LC group were significantly different ($p < 0.05$).

[i]Control and LC groups, control and LCD groups, and LCD and DM groups were significantly different ($p < 0.05$).

[j]Below the detection limit.

[k]Control and LC groups were significantly different ($p < 0.05$) from DM and LCD groups.

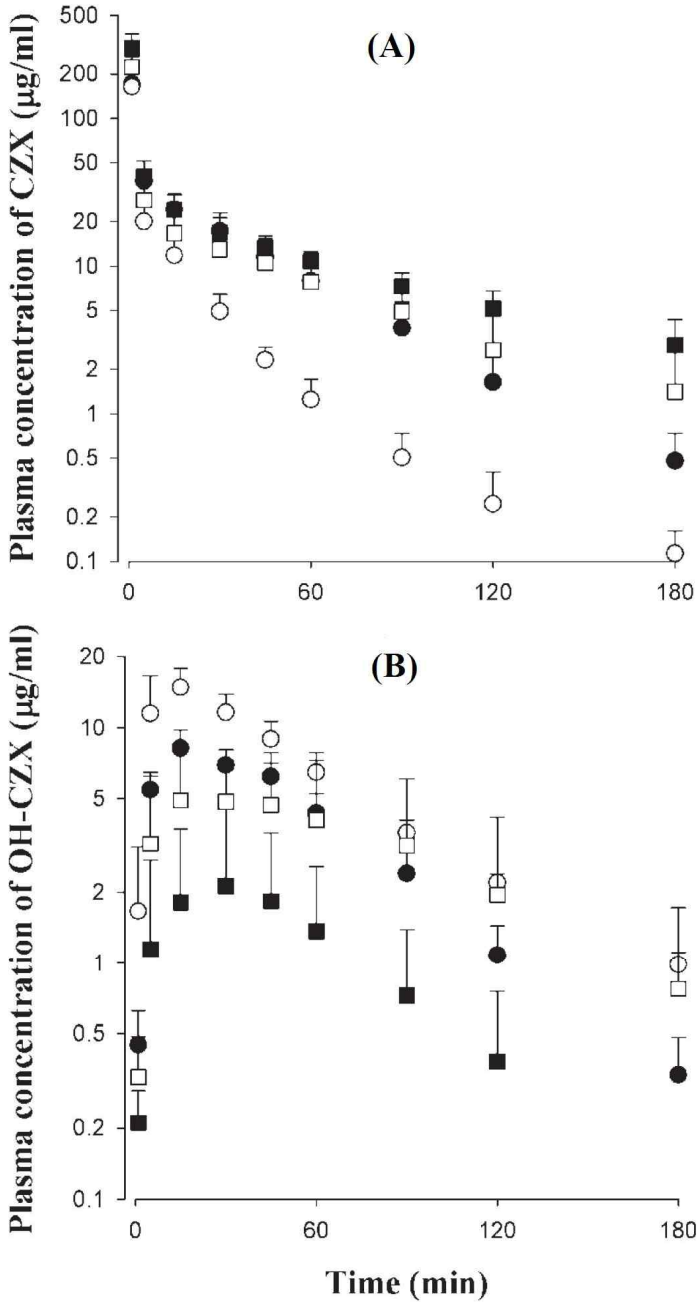

Figure 3 Mean arterial plasma concentration-time profiles of CZX (A) and OH-CZX (B) after i.v. infusion of CZX (20 mg/kg over 1 min) to LC (■; *n* = 9), DM (○; *n* = 7), LCD (□; *n* = 7), and control (●; *n* = 8) rats. Error bars represent S.D.

sampling time (1 min), and it rapidly reached T_{max} within 5 to 60 min. In LC rats, the AUC of OH-CZX was significantly smaller, the C_{max} was significantly lower, the T_{max} was significantly longer, the $Ae_{0\text{-}24\ h}$ of total OH-CZX was significantly smaller, and the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio was significantly smaller than those in controls. In DM rats, the AUC of OH-CZX was significantly greater, the terminal $t_{1/2}$ was significantly longer, the C_{max} was significantly higher, the $Ae_{0\text{-}24\ h}$ of both total and free OH-CZX was significantly larger, and the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio was significantly greater than those in controls. Interestingly, in LCD rats, the AUC, C_{max}, $Ae_{0\text{-}24\ h}$ of total OH-CZX, and $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio were similar to those in control rats. OH-CZX was also undetectable in $GI_{24\ h}$ for all the rats studied.

The ratios of $Ae_{0\text{-}24\ h}$, conjugated OH-CZX to $Ae_{0\text{-}24\ h}$, total OH-CZX were 0.638, 0.439, 0.577, and 0.405 for the control, LC, DM, and LCD rats, respectively, suggesting that formation of conjugated OH-CZX decreased considerably in LC and LCD rats compared with control and DM rats. CZX was excreted in the 24-h urine samples as the free (unconjugated) form.

Pharmacokinetics of CZX and OH-CZX after p.o. administration of CZX

The mean arterial plasma CZX concentration–time profiles for the p.o.

administration of CZX (50 mg/kg) are shown in Figure 4A, and the relevant pharmacokinetic parameters are listed in Table 4. After p.o. administration, CZX was rapidly absorbed; in all four groups of rats, it was detected in plasma at the first blood sampling time (5 min) and rapidly reached T_{max} within 5 to 45 min. In LC rats, the AUC of CZX was significantly greater, and the terminal $t_{1/2}$ was significantly longer than in the control rats. In DM rats, the AUC of CZX was significantly smaller, the terminal $t_{1/2}$ was significantly shorter, the CL_r was significantly faster, and the $Ae_{0\text{-}24\ h}$ was significantly greater than in the control rats. Interestingly, in LCD rats, the AUC, terminal $t_{1/2}$, C_{max}, and CL_r of CZX were similar to those in the controls.

The mean arterial plasma OH-CZX concentration–time profiles for the p.o. administration of CZX are shown in Figure 4B, and the relevant pharmacokinetic parameters are listed in Table 4. OH-CZX formed rapidly after p.o. administration of CZX; in all four groups of rats, it was detected in plasma at the first blood sampling time (5 min) and rapidly reached T_{max} within 5 to 90 min. In LC rats, the terminal $t_{1/2}$ of OH-CZX was significantly longer, the C_{max} was significantly lower, the T_{max} was significantly longer, the $Ae_{0\text{-}24\ h}$ of both total and free OH-CZX was significantly smaller, and the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio was significantly smaller than those in controls. In DM rats, the AUC of OH-CZX was significantly greater, the C_{max} was significantly higher, the $Ae_{0\text{-}24\ h}$ of both total and free OH-CZX was significantly

Table 4 Pharmacokinetic parameters of CZX and OH-CZX after p.o. administration of CZX at a dose of 50 mg/kg to LC, DM, LCD, and control rats

Parameter[a]	Control (n = 7)	LC (n = 8)	DM (n = 8)	LCD (n = 7)
Initial body weight (g)	214 ± 7.87	213 ± 8.86	212 ± 9.61	211 ± 10.3
Final body weight (g)[b]	349 ± 15.7	257 ± 22.8	243 ± 17.3	232 ± 12.2
Blood glucose (mg/dl)[c]	95.4 ± 9.61	103 ± 29.2	470 ± 25.8	474 ± 25.9
Hematocrit (%)[d]	52.0 ± 1.54	38.2 ± 7.89	50.0 ± 3.81	37.0 ± 4.91
CZX				
AUC (μg·min/ml)[e]	2500 ± 509	5700 ± 1280	960 ± 386	2860 ± 1750
Terminal $t_{1/2}$ (min)[f]	124 ± 41.6	159 ± 24.6	75.1 ± 27.7	101 ± 15.9
CL_r (ml/min/kg)[g]	0.0352 ± 0.0375	0.112 ± 0.0700	0.909 ± 0.772	0.219 ± 0.181
C_{max} (μg/ml)[h]	23.1 ± 8.59	35.3 ± 16.8	19.8 ± 11.1	25.6 ± 11.5
T_{max} (ml/min/kg)	15 (15–30)	15 (5–45)	15 (5–30)	15 (5–30)
$Ae_{0\text{-}24\ h}$ (% of CZX dose)[i]	0.167 ± 0.159	0.276 ± 0.266	1.56 ± 0.874	0.914 ± 0.490
$GI_{24\ h}$ (% of CZX dose)	BD[j]	BD	BD	BD
F (%)	58.1	84.4	51.6	65.7

(Table 4 continued)

Parameter[a]	Control (n = 7)	LC (n = 8)	DM (n = 8)	LCD (n = 7)
OH-CZX				
AUC (μg·min/ml)[k]	1540 ± 338	804 ± 397	2860 ± 982	2170 ± 1070
Terminal $t_{1/2}$ (min)[l]	104 ± 25.6	161 ± 68.2	131 ± 21.0	125 ± 31.3
C_{max} (μg/ml)[e]	12.6 ± 4.64	3.85 ± 2.88	27.1 ± 11.3	12.3 ± 4.95
T_{max} (min)[m]	30 (15–90)	90 (30–90)	22.5 (5–30)	45 (5–90)
$Ae_{0\text{-}24\ h,\ total\ OH\text{-}CZX}$ (% of CZX dose)[e]	32.1 ± 7.49	9.75 ± 3.22	62.6 ± 13.7	42.6 ± 21.2
$Ae_{0\text{-}24\ h,\ free\ OH\text{-}CZX}$ (% of CZX dose)[n]	14.1 ± 4.48	4.36 ± 1.59	27.4 ± 8.93	23.1 ± 13.1
$GI_{24\ h}$ (% of CZX dose)	BD	BD	BD	BD
$AUC_{OH\text{-}CZX}$ / AUC_{CZX} (%)[e]	63.9 ± 20.9	15.0 ± 7.78	323 ± 109	83.8 ± 40.5

[a]Data are expressed as mean ± S.D.

[b]Control group, DM and LC groups, and DM and LCD groups were significantly different ($p < 0.05$).

[c]Control and LC groups were significantly different ($p < 0.05$) from DM and LCD groups.

[d]Control and DM groups were significantly different ($p < 0.05$) from LC and LCD groups.

[e]Control and LCD groups, DM group, and LC group were significantly different ($p < 0.05$).

[f]Control and LCD groups, DM and LCD groups, and LC group were significantly different ($p < 0.05$).

[g]Control, LC, and LCD groups were significantly different ($p < 0.05$) from DM group.

[h]DM group was significantly different ($p < 0.05$) from LC group.

[i]Control and LC groups, LCD group, and DM group were significantly different ($p < 0.05$).

[j]Below the detection limit.

[k]Control and LC groups, control and LCD groups, and LCD and DM groups were significantly different ($p < 0.05$).

[l]Control group was significantly different ($p < 0.05$) from LC group.

[m]Control, DM, and LCD groups were significantly different ($p < 0.05$) from LC group.

[n]Control group, DM and LCD groups, and LC group were significantly different ($p < 0.05$).

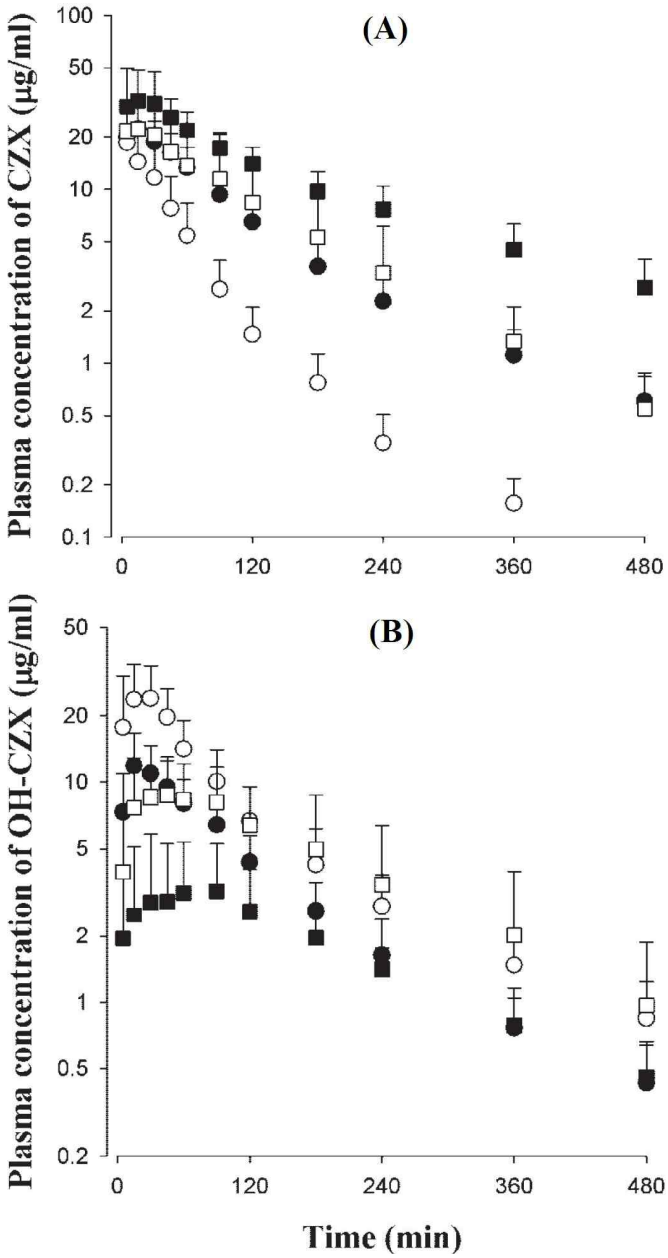

Figure 4 Mean arterial plasma concentration–time profiles of CZX (A) and OH-CZX(B) after p.o. administration of CZX (50 mg/kg) to LC (■; n = 7), DM (○; n = 8), LCD (□; n = 8), and control (●; n = 7) rats. Error bars represent S.D.

greater, and the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio was significantly greater than those in controls. Interestingly, in LCD rats, the AUC, terminal $t_{1/2}$, C_{max}, T_{max}, and $Ae_{0\text{-}24\ h}$ of total OH-CZX and the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio were similar to those in the control rats.

The ratios of $Ae_{0\text{-}24\ h}$, conjugated OH-CZX to $Ae_{0\text{-}24\ h}$, total OH-CZX were 0.561, 0.553, 0.562, and 0.458 for the control, LC, DM, and LCD rats, respectively, suggesting that the formation of conjugates of OH-CZX decreased considerably in LCD rats compared with the other rats. CZX was also excreted in the 24-h urine sample as the free (unconjugated) form.

E. DISCUSSION

The presence of LC and DM in LCD rats was apparent by their significantly decreased body weight gain; significantly higher blood glucose level; significantly larger 24-h urine output; significantly higher plasma levels of GOT, GPT, alkaline phosphatase, and LDH; and significantly lighter and heavier relative liver and kidney weights, respectively, compared with control rats (Tables 1, 3, and 4). Liver cirrhosis was also proven, based on histology, as explained under *Results*.

Baek et al. (2006) reported that the CL_{nr} of CZX could represent the metabolic clearance of the drug in rats. Additionally, the CL_{nr} of CZX

listed in Table 3 could represent the hepatic metabolic clearance of CZX. Thus, changes in the CL_{nr} of CZX could represent changes in hepatic metabolism of CZX via CYP2E1 in rats.

Pathological conditions such as diabetes (Figure 1) (Kim et al., 2005) and starvation (Johansson et al., 1990) induce CYP2E1. Pathological production of ketone bodies may be responsible for this induction of CYP2E1, presumably as the result of an adaptive response (Tu et al., 1983; Lieber, 1997). Nevertheless, ketone body production does not completely account for CYP2E1 induction. A previous study showed that, in the absence of insulin, ketone bodies at concentrations up to 10 mM failed to affect or produced a decrease in mRNA levels of CYP2E1 (Woodcroft et al., 2002), which supports the concept that the induction of CYP2E1 in diabetes or during fasting is not the result of elevated circulating ketone bodies levels. Another study indicated that alterations in energy metabolism (e.g., mitochondrial dysfunction) were associated with induction of CYP2E1 (Chung et al., 2001). We found that the hepatic CYP2E1 level was moderately greater in LCD rats than in control rats (Figure 1). However, the relative CL_{int} of CZX was comparable for the two groups (Table 2), which may be because of the accumulation of extracellular matrix in combination with a decrease in liver parenchymal cells.

After i.v. administration of CZX, the AUC of CZX was

significantly greater in LC rats (57.0% increase) than in the control rats, possibly as a result of the significantly slower CL of CZX (38.2% decrease) in the LC rats (Table 3). The slower CL was attributable to a significantly slower CL_{nr} of CZX (37.5% decrease) in the LC rats because the two groups had comparable CL_r values (Table 3). The AUC of OH-CZX was significantly smaller (70.6% decrease) in LC rats than in controls (Table 3). These results could have been caused by a significantly slower (96.9% decrease) relative CL_{int} for the formation of OH-CZX, based on total liver, because both the content and total liver protein of CYP2E1 were considerably decreased (by 78 and 67.6%, respectively, compared with the controls) (Table 2; Figure 1). The significantly smaller formation (AUC) of OH-CZX in LC rats could also be supported by their smaller $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio (82.0% decrease) (Table 3).

In contrast to LC rats, DM rats exhibited a significant (57%) decrease in AUC of CZX after i.v. administration of CZX compared with the controls, possibly because the CL in DM rats was significantly faster (by 121%) than in the controls (Table 3). The faster CL was attributable to a significantly faster (by 119%) CL_{nr} of CZX than in the controls (Table 3). Although CL_r of CZX was significantly faster (by 146%) in DM rats than in the control rats, the contribution of the CL_r to the CL of CZX was almost negligible, constituting only 3.24% in DM rats (Table 3). The AUC of OH-CZX was significantly greater

(by 75.1%) in DM rats than in the controls (Table 3). These results could have been caused by an increased (by 541%) relative CL_{int} for the formation of OH-CZX, based on total liver, which could have been the result of the significantly higher (by 258%) content of CYP2E1 in the DM rats, despite their significantly lower (by 35.9%) total liver protein, compared with the controls (Table 2; Figure 1). Thus, the contribution of the increased content of CYP2E1 to CZX metabolism and OH-CZX formation was greater than that of the decreased total liver protein. The significantly greater formation of OH-CZX (AUC) in DM rats could also be supported by the significantly greater (by 282%) $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio compared with that in the control rats (Table 3).

Similar results for the pharmacokinetics of CZX and OH-CZX and for CL_{int} per milligram protein have been reported for other rat studies (Baek et al., 2006). Wang et al. (2003) also reported that following p.o. administration of 500 mg of CZX to patients with type 1 diabetes or obese, type 2 diabetes, the AUC of CZX was reduced by 25 and 70%, respectively, compared with that in healthy volunteers. However, the urinary recovery of CZX did not differ significantly among the three groups. Wang et al. (2003) also reported increased mRNA levels for CYP2E1 in peripheral blood mononuclear cells in both types of diabetes.

Protein expression of CYP2E1 was significantly increased in DM

and LCD rats and was decreased in LC rats compared with the controls (Figure 1). Although the protein expression of hepatic CYP2E1 was increased (by 124%) in the LCD rats, compared with that in the control rats (Figure 1), the total liver protein in the LCD rats was significantly reduced (by 61.7%) (Table 2). As a result, the relative CL_{int} for the formation of OH-CZX, based on whole liver, in LCD rats was similar to that of the control rats, with a difference of only 13% (Table 2). Thus, some pharmacokinetic parameters of CZX and OH-CZX would be expected to be similar in LCD and control rats. As expected, the AUC, MRT, CL, CL_r, and CL_{nr} of CZX, and the AUC and C_{max} of OH-CZX, the $Ae_{0-24\ h}$ of total OH-CZX, and the $AUC_{OH\text{-}CZX}/AUC_{CZX}$ ratio did not differ significantly between control and LCD rats (Table 3).

After p.o. administration of CZX, the AUC of CZX was significantly greater in LC rats and smaller in DM rats than in control or LCD rats (Table 4). However, this finding was not likely the result of an increase or decrease in gastrointestinal absorption of CZX found in LC and DM rats, respectively, compared with the control and LCD rats because the $GI_{24\ h}$ values were undetectable for both groups after i.v. and p.o. administration of CZX (Tables 3 and 4). CZX was stable in rat gastric and intestinal fluids (Baek et al., 2006). Thus, CZX was almost completely absorbed in all the groups of rats. Similar results were obtained from our i.v. studies (Table 3), especially for the AUC

values of CZX and OH-CZX (Table 4). The *F* of CZX in LC rats was considerably greater than in the control, DM, and LCD rats (by 45.3, 63.6, and 28.5%, respectively; Table 4). This result could have primarily been because of the decreased hepatic metabolism of CZX in LC rats.

Although CYP2E1 is the major enzyme that metabolizes CZX to OH-CZX, human CYP1A1/2 and/or CYP3A4 (Carriere et al., 1993; Shimada et al., 1993; Gorski et al., 1997; Ono et al., 1997) and rat CYP1A1 and CYP3A1/2 (Jayyosi et al., 1995) have also been reported to carry out CZX hydroxylation. The role of CYP3A2 in the formation of OH-CZX in rats was measured by treatment with DDT (an inducer of CYP3A2) (Sierra-Santoyo et al., 2000). The V_{max}, K_m, and CL_{int} for the formation of OH-CZX were not significantly different for the DDT-treated versus the control rats, indicating that the effect of CYP3A2 on the formation of OH-CZX was almost negligible (Sierra-Santoyo et al., 2000). Li et al. (1995) reported that treatment of rats with DDT had no effect on CYP2E1. We recently found (our unpublished data) that the protein expression of both CYP1A and CYP3A was increased in DM rats and decreased in LC rats, but in LCD rats, CYP1A was increased, whereas CYP3A was decreased.

In summary, after i.v. (Table 3) and p.o. (Table 4) administration of CZX, the AUC of OH-CZX was significantly smaller in LC rats than

in control rats because protein expression of CYP2E1 and total liver protein were both decreased in the LC rats (Figure 1). However, the AUC of OH-CZX was significantly greater in DM rats than in control rats because of the increased protein expression of CYP2E1 in DM rats (Figure 1). The LCD and control rats had comparable values for AUC of OH-CZX, which may have resulted from the decrease in total liver protein in LCD rats despite their increase in protein expression of CYP2E1; as a result, the relative CL_{int} for the formation of OH-CZX, based on total liver, was comparable with that in the controls. These results suggest that OH-CZX could be used as a chemical probe to assess the activity of hepatic CYP2E1 in LC, DM, and LCD rats.

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PART II. PHARMACOKINETICS OF OLTIPRAZ IN DIABETIC RATS WITH LIVER CIRRHOSIS.

A. ABSTRACT

The incidence of diabetes mellitus is increased in patients with liver cirrhosis. Oltipraz is currently in trials to treat patients with liver fibrosis and cirrhosis induced by chronic hepatitis types B and C and is primarily metabolized via hepatic cytochrome P450 isozymes CYP1A1/2, 2B1/2, 2C11, 2D1 and 3A1/2 in rats. We have studied the influence of diabetes mellitus on pharmacokinetics of oltipraz and on expression of hepatic, CYP1A, 2B1/2, 2C11, 2D and 3A in rats with experimental liver cirrhosis.

Oltipraz was given intravenously (10 mg/kg) or orally (30 mg/kg) to rats with liver cirrhosis induced by *N*-dimethylnitrosamine (LC rats) or with diabetes, induced by streptozotocin (DM rats) or to rats with both liver cirrhosis and diabetes (LCD rats) and to control rats, and pharmacokinetic variables measured. Protein expression of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A was measured using Western blot analysis.

After i.v. or p.o. administration of oltipraz to LC and DM rats, the AUC was significantly greater and smaller, respectively, than that in

control rats. In LCD rats, the AUC was that of LC and DM rats (partially restored towards control rats). Compared with control rats, the protein expression of hepatic CYP1A increased, that of CYP2C11 and 3A decreased, but that of CYP2B1/2 and 2D was not altered in LCD rats.

In rats with diabetes and liver cirrhosis, the AUC of oltipraz was partially restored towards that of control rats.

B. INTRODUCTION

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione], a synthetic dithiolthione, was developed by Rhône-Poulenc (Vitry-sur-Seine, France) for the treatment of schistosomiasis (Bueding et al., 1982). After single p.o. administration of oltipraz to normal subjects and subjects with increased risk for colorectal carcinoma, the terminal half-lives ranged from 4.16 h to 11.1 h (Gupta et al., 1995) and from 22.7 h to 9.3 h (O'Dwyer et al., 2000) respectively. After 6 months p.o. administration of oltipraz to patients, the side-effect profile was: gastrointestinal problems, photosensitivities/heat intolerance, and neurological problems (Benson et al., 2000). Based on the therapeutic effects of oltipraz on rats with liver cirrhosis (LC rats) (Kang et al., 2002; Bae et al., 2006a), oltipraz is being evaluated in phase II clinical trial in South Korea as an p.o. agent to treat patients with liver fibrosis

and cirrhosis induced by chronic hepatitis types B and C.

Bae et al. (2005a) reported that oltipraz is primarily metabolized via hepatic cytochrome P450 (CYP) 1A1/2, 2B1/2, 2C11, 2D1 and 3A1/2, but not via CYP2E1, in male Sprague–Dawley rats. Kim et al. (2005) reported that in rats with diabetes mellitus induced by streptozotocin (DM rats), the protein expression and mRNA levels of hepatic CYP1A2, 2B1/2 and 3A1 increase, whereas those of CYP2C11 decrease. Similar results have also been reported in other studies (Yamazoe et al., 1989; Shimojo et al., 1993; Raza et al., 2000; Sindhu et al., 2006). Sakuma et al. (2001) reported that the protein expression of CYP2D1 is not altered in DM rats. However, to our knowledge, no studies on changes in hepatic CYP isozymes in LC rats, with or without diabetes mellitus, have yet been reported.

Intestinal CYP isozymes have also been reported to be changed in DM rats. For example, activity of intestinal CYP1A and 2B increased (Al-Turk et al., 1981) but that of CYP3A1/2 decreased (Borbás et al., 2006). In rat intestine, mostly CYP1A and 3A are expressed (Kamninsky and Zhang, 2003), whereas CYP2D is little expressed (Aiba et al., 2003) and CYP2B2, 2C11 and 3A2 are not detectable (Kamninsky and Zhang, 2003). The pharmacokinetics of oltipraz are changed in DM rats (Bae et al., 2006b) and LC rats (Bae et al., 2004; 2006a) but there is no data on pharmacokinetics in LCD rats. Bae et al. (2005b) reported that the hepatic first-pass effect of oltipraz after

absorption into the portal vein is 40% and intestinal first-pass effect is 32% in rats. The hepatic first-pass effect of 40% is equivalent to 25% of the oral dose considering the 32% of the intestinal first-pass effect.

The association between liver disease and diabetes mellitus is well known (Vidal et al., 1994; Kwon, 2003; Moscatiello et al., 2007) and the overall prevalence of diabetes mellitus in patients with liver cirrhosis is significantly higher than that expected.

Pharmacokinetic studies on oltipraz in patients with diabetes mellitus and liver cirrhosis, alone or combined, have not been performed. Hence, the objectives of the current studies were to evaluate, using a rat model, the effects of DM and LC, alone and in combination (LCD), on the pharmacokinetics of oltipraz. Changes in the protein expression of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A in rats with LC and DM, alone and in combination (LCD) using Western blot analysis, were also investigated.

C. MATERIALS AND METHODS

Chemicals

Oltipraz was donated by the R & D Center of Pharmaceuticals, Institute of Science & Technology, CJ Corporation (Ichon, South Korea). NADPH, streptozotocin, β-actin, primary monoclonal antibody for β-actin and Kodak X-OMAT film were purchased from

Sigma–Aldrich Corporation (St. Louis, MO). Polyethylene glycol 400 (PEG 400) and *N*-dimethylnitrosamine were products from Duksan Chemical Company (Seoul, South Korea) and Tokyo Kasei Kogyo Company (Tokyo, Japan) respectively. Polyclonal rabbit antihuman CYP1A, 2B1/2, 2C11, 2D and 3A antibodies and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from Detroit R&D (Detroit, MI) and Bio-Rad Laboratories (Hercules, CA) respectively. Enhanced chemiluminescence reagents were products from Amersham Biosciences Corporation (Piscataway, NJ). Other chemicals were of reagent or HPLC grade.

Animals

The protocols for the animal studies were approved by the Animal Center and Use Committee of the College of Pharmacy of Seoul National University, Seoul, South Korea. Male Sprague–Dawley rats (4–5 weeks old, weighing 180–200 g) were purchased from the Charles River Company Korea (Orient, Seoul, South Korea). They were randomly divided into three disease groups (LC, DM and LCD) and a control group. They were maintained in a clean room (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University) at a temperature of 22 ± 2°C with 12 h light (0700–1900 h) and dark (1900–0700 h) cycles and a relative humidity of 55 ± 5%. Rats were housed in metabolic cages (Tecniplast, Varese,

Italy) under filtered pathogen-free air and with food (Sam Yang Company, Pyungtaek, South Korea) and water available *ad libitum*.

Induction of LC in rats by N-dimethylnitrosamine injection

Freshly prepared *N*-dimethylnitrosamine (diluted to 0.01 mg/mL in 0.9% NaCl-injectable solution) was injected i.p. at a dose of 0.01 mg/kg on three consecutive days per week for 4 weeks (Ohara and Kusano, 2002; Bae et al., 2004; 2006a). On day 29, one dose of citrate buffer (pH 7.4; 1 mL/kg) was injected via the tail vein. On day 36, the rats were treated with oltipraz. Laboratory rats with *N*-dimethylnitrosamine-induced liver cirrhosis have clinical features similar to those of humans with liver cirrhosis such as increased mortality, hepatic parenchymal cell destruction, formation of connective tissue and nodular regeneration (Kang et al., 2002). Liver cirrhosis in LC rats was evident by liver histological analysis, which revealed extensive micronodular cirrhosis with regenerative hepatocellular changes, and bile duct proliferation was also detected (Bae et al., 2004; 2006a). It has also been reported that *N*-dimethylnitrosamine induced liver cirrhosis in rats is a reproducible effect (Jenkins et al., 1985; Jezequel et al., 1987; Kang et al., 2002).

Induction of DM in rats by streptozotocin injection

A 0.9% NaCl-injectable solution was injected i.p. (1 ml/kg) on three

consecutive days per week for 4 weeks. On day 29, one dose (45 mg/kg) of freshly prepared streptozotocin [dissolved in citrate buffer (pH 4.5) to 45 mg/ml was administered via the tail vein (Kim et al., 2005). On day 36, the rats were treated with oltipraz.

Induction of LCD in rats with N-dimethylnitrosamine and streptozotocin injections

Liver cirrhosis was induced by intraperitoneal injection of *N*-dimethylnitrosamine as described above. Then, on day 29, diabetes mellitus was induced by injection of streptozotocin via the tail vein as described above. On day 36, the rats were treated with oltipraz.

Control rats

Control rats were injected intraperitoneally with 0.9% NaCl injectable solution (1 ml/kg) on three consecutive days per week for 4 weeks. On day 29, one dose (1 ml/kg) of citrate buffer (pH 4.5) was administered via the tail vein. On day 36, the rats were treated with oltipraz. During the pretreatment, food and water were available *ad libitum* to all rats. Immediately before the experiment, blood glucose levels in all rats were measured using the Medisense Optium kit (Abbott Laboratories, Bedford, MA), and rats with blood glucose levels greater than 13.9 mmol/L were chosen as diabetic rats (DM and LCD rats).

Preparation of hepatic and intestinal microsomes

The procedures used for the preparation of hepatic (Bae et al., 2004; 2005a; 2006b) and intestinal (Peng et al., 2004) microsomes from LC, DM, LCD and control rats (n = 4–6, each) were similar to reported methods. Hepatic and intestinal microsomes were stored at -70°C until use. Protein contents in hepatic and intestinal microsomes were measured using the method of Bradford (1976).

Immunoblot analysis of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A

The procedures used were similar to a reported method (Kim et al., 2001). Hepatic microsomes were resolved by sodium dodecyl sulphate (SDS) gel electrophoresis on a 7.5% polyacrylamide gel (10 mg protein per lane; n = 3, each). Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) that was then blocked for 1 h in 5% milk powder in phosphate-buffered 0.9% NaCl-injectable solution containing 0.05% (v/v) Tween 20 (PBS-T). For immunodetection, blots were incubated overnight at 4°C with rabbit anti-human CYP1A, 2B1/2, 2C11, 2D and 3A antibodies (diluted 1:10000 in PBS-T containing 5% bovine serum albumin), followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1 : 10,000 in PBS-T containing 5% milk powder). Hepatic CYP1A, 2B1/2, 2C11, 2D and 3A were detected by enhanced chemiluminescence on Kodak X-OMAT film and quantified by densitometry with a microcomputer

imaging device (model M1; Imaging Research, St. Catharines, Ontario, Canada). The β-actin band was used as a loading control. We performed calibration assays in some of the replicate blots to ensure the linearity of band intensity quantification.

Measurement of V_{max}, K_m, and intrinsic clearance for the disappearance of oltipraz in hepatic and intestinal microsomes

The procedures used were similar to a reported method (Ahn et al., 2008). The V_{max} (the maximum velocity) and the K_m (the apparent Michaelis–Menten constant; the concentration at which the rate is one-half of the V_{max}) for the disappearance of oltipraz in LC, DM, LCD and control rats (n = 4–6, each) were determined after incubating the above microsomes (equivalent to 0.5 and 0.1 mg protein for hepatic and intestinal microsomes respectively), a 5 µl aliquot of dimethylsulphoxide containing final oltipraz concentrations of 2.5, 5, 10, 20, 50 and 100 µM (hepatic microsomes) or 1, 2.5, 5, 7.5, 10 and 20 µM (intestinal microsomes), and 0.1 M phosphate buffer (pH 7.4) containing a 50 and 25 µL aliquot(for hepatic and intestinal microsomes respectively) of 1 mM NADPH in a final volume of 0.5 and 0.25 mL (for hepatic and intestinal microsomes respectively) by adding 0.1 M phosphate buffer (pH 7.4) in a water-bath shaker [kept at 37°C, 50 oscillations/min (opm)]. All of the above microsomal incubation conditions were linear. The reaction was terminated by adding 1 mL of acetonitrile after 5 min incubation for both hepatic and

intestinal microsomes. Oltipraz was determined using a reported HPLC method (Bae et al., 2001). The kinetic constants (K_m and V_{max}) for the disappearance of oltipraz were calculated using a non-linear regression method (Duggleby, 1995). The intrinsic clearance for the disappearance of oltipraz (CL_{int}) was calculated by dividing the V_{max} by the K_m.

i.v. study

The procedures used for the handling of rats including the cannulation of the carotid artery (for blood sampling) and the jugular vein (for drug administration in the intravenous study) were similar to methods previously reported (Kim et al., 1993).

Oltipraz, suspended in PEG 400: distilled water (40:60, v/v) (Bae et al., 2004; 2005a–c; 2006a,b) at a dose of 10 mg/kg was infused (total infused volume 2 ml/kg) for 1 min via the jugular vein of rats in each group (n = 7, 8, 11 and 8 for the LC, DM, LCD and control rats respectively). A blood sample (approximately 0.12 ml) was collected via the carotid artery at 0 (control), 1 (end of the infusion), 5, 15, 30, 60, 90, 120, 180, 240 and 360 min after the start of the i.v. infusion of oltipraz. Each blood sample was immediately centrifuged and a 50 μl aliquot of each plasma sample was stored at -70°C for later analysis by HPLC. The procedures used for the preparation and handling of the 24 h urine ($Ae_{0–24\ h}$) samples and gastrointestinal tract (including its

contents and faeces) samples at 24 h ($GI_{24\,h}$) were similar to reported methods (Bae et al., 2004; 2005a–c; 2006a,b).

Oral study

Oltipraz (the same suspension used in the i.v. study) at a dose of 30 mg/kg was administered orally (total orally administered volume 3ml/kg) using a feeding tube to rats in each group (n = 8, 9, 4 and 8 for the LC, DM, LCD and control rats respectively). A blood sample was collected at 0, 15, 30, 60, 90, 120, 180, 240, 360, 480, 600, 720 and 960 min after the p.o. administration of oltipraz. Other procedures were similar to those described above for the i.v. study.

Measurement of rat plasma protein binding of oltipraz using equilibrium dialysis

Binding of oltipraz to fresh plasma from LC, DM, LCD and control rats (n = 5, each) was measured using equilibrium dialysis (Bu et al., 2001). Plasma (1 ml) was dialyzed against 1 ml of isotonic Sørensen phosphate buffer (pH 7.4) containing 3% (w/v) dextran ('the buffer') to minimize volume shift (Boudinot and Jusko, 1984) in a 1 ml dialysis cell (Fisher Scientific, Fair Lawn, NJ) fitted with a Spectra/Por 4 membrane (molecular mass cutoff 12–14 kDa; Spectrum Medical Industries Inc., Los Angeles, CA). It took 8 h incubation to reach equilibrium between 'the buffer' and plasma

compartments, and the binding values were not influenced up to 24 h incubation. Thus, 24 h incubation was employed in the present study. The binding of oltipraz to 4% human serum albumin was independent of oltipraz concentrations ranging from 1 to 100 mg/ml. Thus, a 10 mg/ml was chosen in the present plasma protein binding study. Total protein contents in plasma from control, LC, DM and LCD rats were 5.5 ± 0.14%, 4.2 ± 0.34%, 5.2 ± 0.53% and 4.6 ± 0.54%, respectively, and the corresponding values for albumin were 3.4 ± 0.12%, 2.5 ± 0.31%, 3.3 ± 0.30% and 2.9 ± 0.14% respectively (Ahn et al., 2008).

HPLC analysis of oltipraz

Concentrations of oltipraz in the samples were determined using an HPLC method (Bae et al., 2001). Briefly, a 100 μl aliquot of acetonitrile was added to deproteinize (Chiou et al., 1978) a 50 μl aliquot of sample. After vortex-mixing and centrifugation (16,000× *g* for 1 min), a 50 μl aliquot of the supernatant was directly injected onto a reversed-phase (C_{18}) HPLC column. The mobile phase, acetonitrile: 0.5 mM ammonium acetate [55:45 (v/v) for both rat plasma and gastrointestinal tract samples, and 45:55 (v/v) for the rat urine samples], was run at a flow rate of 1.5 ml/min, and the column eluent was monitored using an ultraviolet detector at 305 nm at room temperature. The retention time of oltipraz was approximately 5.8 min in both rat plasma and gastrointestinal tract samples, and 8.6 min in rat

urine samples. The detection limits of oltipraz in rat plasma and urine samples were 20 and 50 ng/ml respectively. The mean within-day coefficients of variation (CV) in rat plasma and urine samples were 2.29% and 1.01%, respectively, and the corresponding between-day CVs of the analysis of the same samples on consecutive 3 days were 3.37% and 1.51% respectively. As oltipraz in solution was reported to be photodegraded (Christensen and Malone, 1992), all samples in the present study were covered (or wrapped) with aluminum foil or kept in the dark during the experiment or when they are not in use.

Pharmacokinetic analysis

The total area under the plasma concentration-time curve from time zero to time infinity (AUC) was calculated using the trapezoidal rule-extrapolation method (Chiou, 1978). The area from the last datum point to time infinity was estimated by dividing the last measured plasma concentration by the terminal-phase rate constant. Standard methods (Gibaldi and Perrier, 1982) were used to calculate the following pharmacokinetic parameters using a non-compartmental analysis (WinNonlin®; Pharsight Corporation, Mountain View, CA): the time-averaged total body clearance (CL), the terminal half-life ($t_{1/2}$), the first moment of AUC (AUMC), the mean residence time (MRT), the apparent volume of distribution at steady state (V_{ss}) and the extent of absolute oral bioavailability (F) (Kim et al., 1993). The

peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly read from the experimental data.

Statistical analysis

A *P*-value < 0.05 was deemed to be statistically significant using a Duncan's multiple range test with the Statistical Package for the Social Sciences (SPSS) *posteriori* analysis of variance (ANOVA) among the four (or three) means for the unpaired data. All data are expressed as mean ± SD.

D. RESULTS

Protein expression of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A

The protein expression of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A in control, DM, LC and LCD rats is shown in Figure 5 and 6. In DM rats, the protein expression of CYP1A, 2B1/2, 2D and 3A was significantly increased, but that of CYP2C11 was significantly decreased, compared with control rats. In LC rats, the protein expression of CYP1A, 2B1/2, 2C11, 2D and 3A was significantly decreased, compared with control rats. In LCD rats, the protein expression of CYP1A was significantly increased, that of CYP2C11 and 3A was significantly decreased, and that of CYP2B1/2 and 2D was comparable to control values.

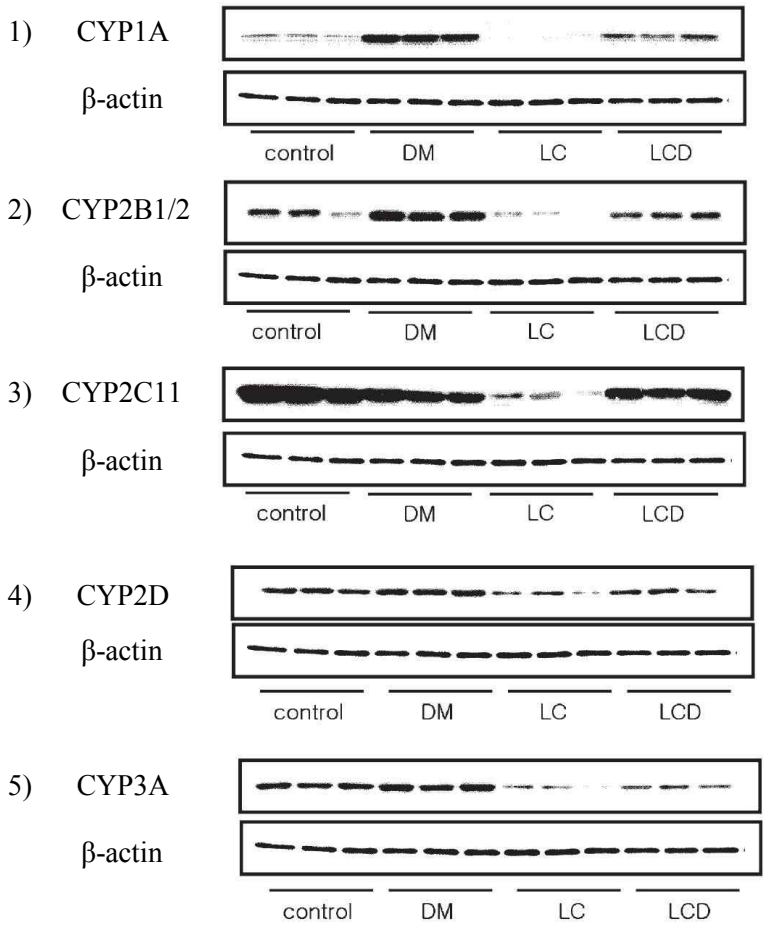

Figure 5 Immunoblotting of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A in LC, DM, LCD and control rats. Representative Western blots for each hepatic CYP isoform studied. The β-actin band was used as a loading control.

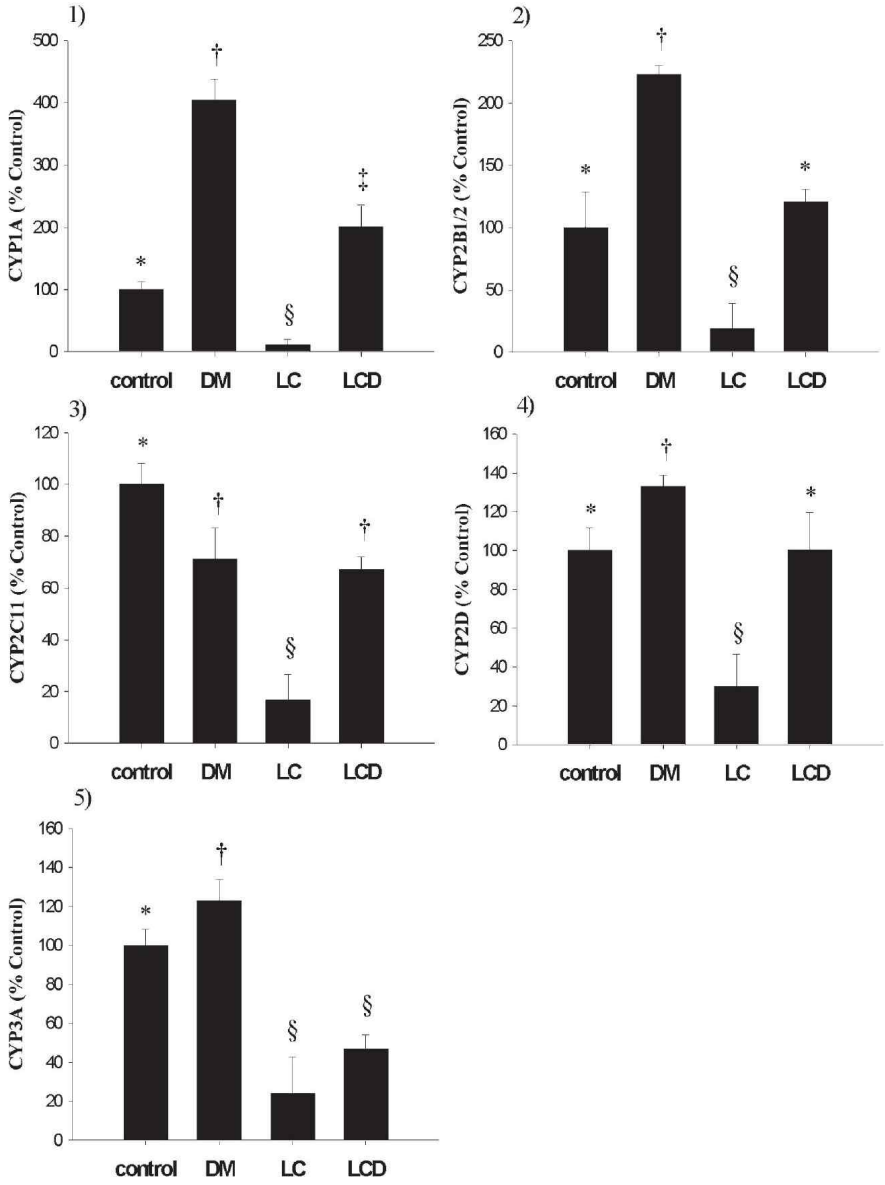

Figure 6 Summary data from the Western blots. The protein expression in LC DM and LCD groups was expressed in terms of that in control rats, set to 100%. Data are presented as mean ± SD and values with different marks (*, †, §, $^{\#}$) are significantly different ($P < 0.05$). (i) For CYP1A, all three experimental groups were different from control and each group was significantly different; (ii) For CYP2B1/2, only the DM group and the LC group were significantly different from control and the LCD groups; (iii) For

CYP2C11, all three experimental groups were different from control and the LC group was different from the DM and LCD groups; (iv) For CYP2D, the DM and the LC groups were significantly different from control and the LCD groups ($P < 0.05$); and (v) For CYP3A, all three experimental groups were different from control (*$P < 0.05$) and the DM group was significantly different from the LC and LCD groups.

V_{max}, K_m, and CL_{int} for disappearance of oltipraz in hepatic and intestinal microsomes

The V_{max}, K_m and CL_{int} for the disappearance of oltipraz in hepatic microsomes from the four groups of rats are listed in Table 5. The V_{max} was significantly different in each group, increasing in the order LC < LCD < control < DM. However, the K_m values were not significantly different among the four groups of rats. Thus, the affinity of the hepatic enzyme(s) for oltipraz in the liver is not changed in DM, LC or LCD rats. As a result, the CL_{int} showed the same trends as shown in V_{max}, and the overall formation of oltipraz metabolite(s) in the liver (Bieder et al., 1983) was the greatest in DM rats and the lowest in LC rats. Total hepatic protein was significantly different among control rats, DM rats, and LC and LCD rats. The V_{max}, K_m and CL_{int} for the disappearance of oltipraz in intestinal microsomes from four groups of rats are also listed in Table 5. Compared with control rats, the V_{max} was significantly slower in LCD rats (60% decrease). Compared with control rats, the K_m was significantly lower in DM rats (60% decrease). The CL_{int} was significantly slower (31% and 44% decrease respectively) in LC and LCD rats (not significantly different between LC and LCD rats), but was significantly faster (35% increase) in DM rats, all relative to control values. This suggests that the formation of oltipraz metabolite(s) in the intestine increased in DM rats, but decreased in LC and LCD rats. The total intestinal protein

Table 5 V_{max}, K_m, and CL_{int} for the disappearance of oltipraz in hepatic and intestinal microsomes from LC, DM, LCD and control rats

Parameter[a]	Control	LC	DM	LCD
Hepatic				
V_{max} (nmol/min/mg protein)[b]	5.38 ± 0.382	1.84 ± 0.480	9.49 ± 1.22	2.91 ± 0.497
K_m (μM)	29.3 ± 5.59	46.4 ± 34.5	38.2 ± 5.88	24.8 ± 6.12
CL_{int} (ml/min/mg/protein)[b]	0.184 ± 0.0129	0.0530 ± 0.0228	0.250 ± 0.0125	0.119 ± 0.0111
Total protein (mg/whole liver)[c]	373 ± 113	121 ± 24.7	239 ± 13.6	143 ± 35.7
Intestinal				
V_{max} (nmol/min/mg protein)[d]	0.523 ± 0.231	0.268 ± 0.169	0.257 ± 0.137	0.212 ± 0.182
K_m (μM)[d]	6.41 ± 2.60	4.60 ± 2.24	2.54 ± 1.76	4.28 ± 2.38
CL_{int} (ml/min/mg/protein) [c]	0.0807 ± 0.00444	0.0556 ± 0.00950	0.109 ± 0.0307	0.0451 ± 0.0122
Total protein (mg/whole intestine)	6.93 ± 1.23	5.37 ± 2.99	7.13 ± 2.90	4.83 ± 3.29

[a]Data are expressed as mean ± S.D.

[b]Each group was significantly different ($p < 0.05$).

[c]Control and DM groups were significantly different ($p < 0.05$) from LC and LCD groups.

[d]Control group was significantly different ($p < 0.05$) from LC, DM, and LCD groups.

was comparable among the four groups of rats.

Rat plasma protein binding of oltipraz

The binding values of oltipraz to fresh plasma from four groups of rats (n = 5, each) are 88 ± 2% (control rats), 70 ± 5% (LC rats), 83 ± 3% (DM rats) and 80 ± 3% (LCD rats). Only the value in LC rats was significantly different from those in the other groups and this could be at least partly due to significantly lower albumin concentrations in plasma from LC rats (Ahn et al., 2008).

Pharmacokinetics of oltipraz after i.v. administration

The mean arterial plasma oltipraz concentration-time profiles for the i.v. administration of oltipraz (10 mg/kg) to LC, DM, LCD and control rats are shown in Figure 7. The relevant pharmacokinetic parameters are listed in Table 6. Compared with control rats, the AUC was significantly greater, CL was significantly slower, terminal $t_{1/2}$ and MRT were significantly longer, and V_{ss} was significantly smaller in LC rats. The CL was significantly faster in DM rats than that in control rats. Interestingly, CL, terminal $t_{1/2}$ and MRT were similar for LCD and control rats, but V_{ss} in LCD rats was significantly smaller than that in control rats. Oltipraz was below the detection limit in the 24 h urine samples ($Ae_{0-24\ h}$) and gastrointestinal tract (including its contents and faeces) samples at 24 h ($GI_{24\ h}$) for all rats.

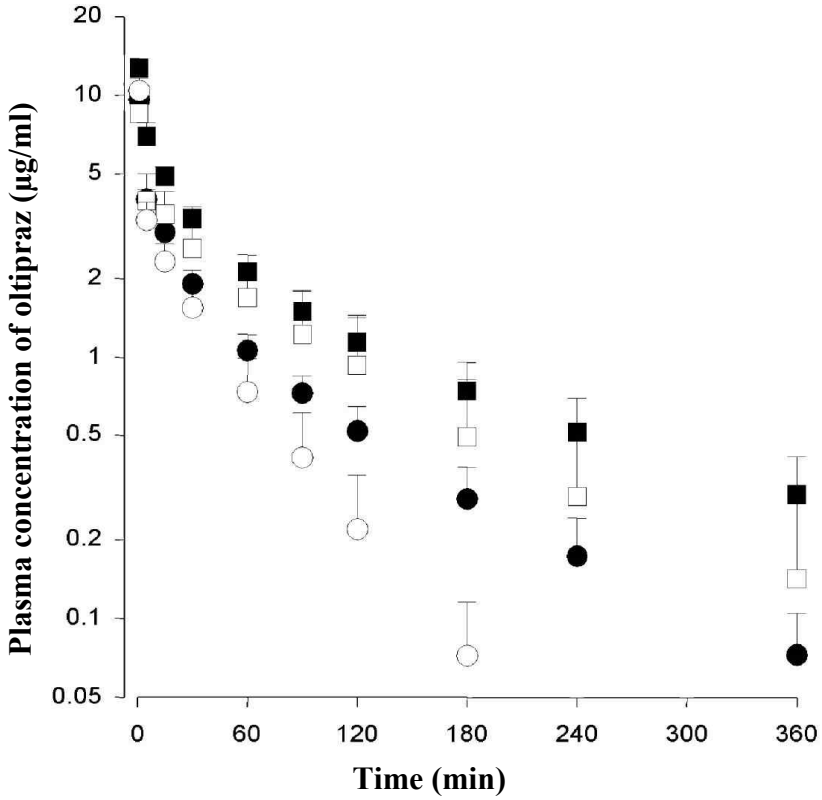

Figure 7 Mean arterial plasma concentration-time profiles of oltipraz after i.v. infusion at a dose of 10 mg/kg to LC (■; n = 9), DM (○; n = 7), LCD (□; n = 7) and control (●; n = 8) rats. Error bars represent S.D.

Table 6 Pharmacokinetic parameters of oltipraz after i.v. administration at a dose of 10 mg/kg to LC, DM, LCD, and control rats

Parameter[a]	Control (n = 8)	LC (n = 7)	DM (n = 8)	LCD (n = 11)
Initial body weight (g)	188 ± 10.3	198 ± 9.94	199 ± 12.5	194 ± 8.50
Final body weight (g)[b]	376 ± 24.7	299 ± 9.45	282 ± 13.2	250 ± 26.9
Blood glucose (mg/dl)[c]	106 ± 5.66	104 ± 13.5	360 ± 80.0	344 ± 83.2
AUC (μg·min/ml)[d]	249 ± 32.9	531 ± 103	157 ± 34.2	350 ± 132
CL (ml/min/kg)[e]	40.8 ± 6.03	19.3 ± 3.06	65.3 ± 15.5	34.1 ± 18.6
Terminal $t_{1/2}$ (min)[f]	80.5 ± 11.9	116 ± 20.0	33.2 ± 5.65	68.9 ± 27.4
MRT (min)[e]	84.1 ± 17.1	132 ± 25.8	39.9 ± 9.04	90.0 ± 37.2
V_{ss} (ml/kg)[g]	3350 ± 351	2500 ± 391	2570 ± 278	2700 ± 901
$Ae_{0\text{-}24\ h}$ (% of dose)[h]	BD	BD	BD	BD
$GI_{24\ h}$ (% of dose)[h]	BD	BD	BD	BD

[a]Data are expressed as mean ± S.D.

[b]Control group, LC and DM group, and LCD group were significantly different ($p < 0.05$).

[c]Control and LC groups were significantly different($p < 0.05$) from DM and LCD group.

[d]Control and DM groups, LC group, and LCD group were significantly different ($p < 0.05$).

[e]Control and LCD groups, DM group, and LC groups were significantly different ($p < 0.05$).

[f]Each group was significantly different ($p < 0.05$).

[g]Control group was significantly different ($p < 0.05$) from LC, DM, and LCD groups.

[h]Below the detection limit.

Pharmacokinetics of oltipraz after p.o. administration

The mean arterial plasma oltipraz concentration-time profiles for oltipraz following oral administration of 30 mg/kg to LC, DM, LCD and control rats are shown in Figure 8. The relevant pharmacokinetic parameters are listed in Table 7. Compared with control rats, the AUC was significantly greater, terminal $t_{1/2}$ was significantly longer, and C_{max} was significantly lower in LC rats. Compared with control rats, the AUC was significantly smaller and C_{max} was significantly lower in DM rats. Interestingly, the terminal $t_{1/2}$ in LCD rats was similar to that in control rats and AUC in LCD rats was in between LC and DM rats. The *F* values were in the order, LC > LCD, control > DM groups.

E. DISCUSSION

Bae et al. (2005c) reported that the pharmacokinetic parameters of oltipraz (such as AUC, terminal half-life, MRT, V_{ss}, CL and/or CL_R) were dose-independent after i.v. (at doses of 5–20 mg/kg) and p.o. (at doses of 25–100 mg/kg) administration to rats. Thus, the 10 mg (for intravenous study) and 30 mg (for oral study) of oltipraz were chosen for this study. Bae et al. (2005b) also reported that the non-renal clearance of oltipraz could represent the metabolic clearance of the drug in rats. In the present study, oltipraz was below the detection limit in the 24 h urine samples (Table 6). Thus, the CL of oltipraz

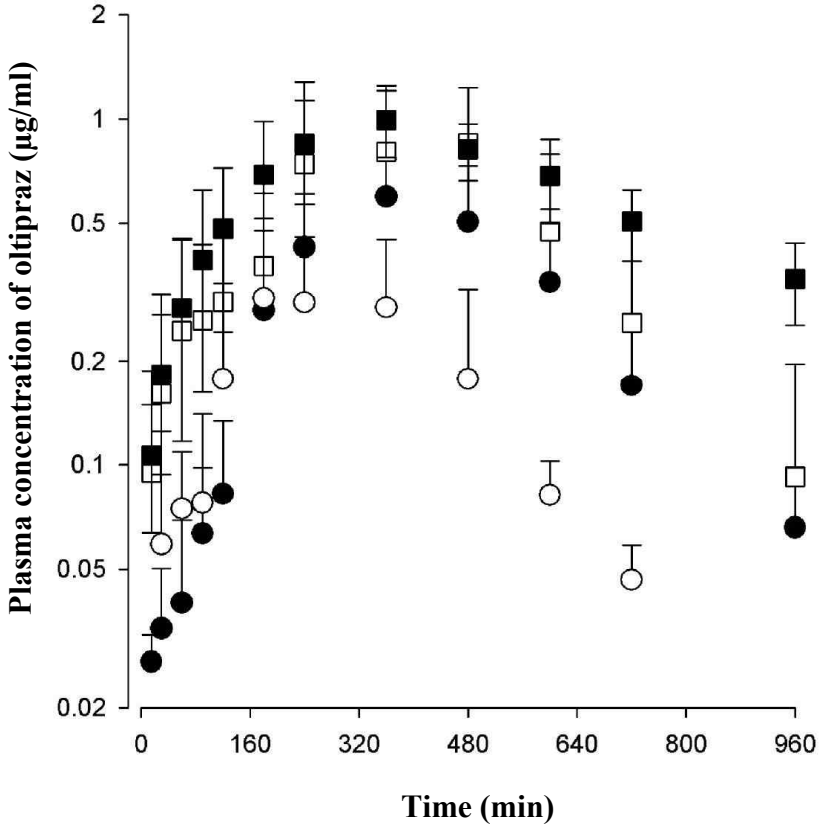

Figure 8 Mean arterial plasma concentration-time profiles of oltipraz after p.o. administration at a dose of 30 mg/ml to LC (■; *n* = 7), DM (○; *n* = 8), LCD (□; *n* = 8) and control (●; *n* = 7) rats. Error bars represent S.D.

Table 7 Pharmacokinetic parameters of oltipraz after p.o. administration at a dose of 30 mg/kg to LC, DM, LCD, and control rats

Parameter[a]	Control (n = 8)	LC (n = 8)	DM (n = 9)	LCD (n = 6)
Initial body weight (g)	214 ± 16.9	223 ± 9.64	201 ± 7.66	216 ± 13.6
Final body weight (g)[b]	334 ± 10.3	246 ± 16.2	285 ± 13.2	221 ± 22.0
Blood glucose (mg/dl)[c]	97.6 ± 9.57	101 ± 10.5	294 ± 46.7	304 ± 63.1
AUC (μg·min/ml)[b]	286 ± 78.7	812 ± 188	134 ± 29.1	438 ± 164
Terminal $t_{1/2}$ (min)[d]	168 ± 46.6	423 ± 111	157 ± 58.8	126 ± 29.8
C_{max}[e]	0.689 ± 0.166	1.06 ± 0.227	0.440 ± 0.148	1.16 ± 0.322
T_{max}	368 ± 113	308 ± 74.8	267 ± 124	370 ± 134
$GI_{24\ h}$ (% of dose)	3.68 ± 3.85	4.26 ± 6.17	3.12 ± 4.46	5.63 ± 4.63
F (%)	38.3	51.0	28.5	41.7

[a]Data are expressed as mean ± S.D.

[b]Each group was significantly different ($p < 0.05$).

[c]Control and LC groups were significantly different($p < 0.05$) from DM and LCD groups.

[d]LC group was significantly different ($p < 0.05$) from control, DM, and LCD groups.

[e]Control group, LC and LCD groups, and DM group were significantly different ($p < 0.05$).

listed in Table 6 could represent the metabolic clearance of the drug in rats. Additionally, changes in the CL of oltipraz could be due to the changes in the metabolism of the drug.

In LC rats, the AUC of i.v. oltipraz was significantly greater (113% increase) than that in control rats, possibly as a result of the significantly slower CL of oltipraz (52.7% decrease) (Table 6), as reported earlier (Bae et al., 2004). The slower CL could be supported by significantly slower (71% decrease) hepatic CL_{int} for the disappearance of oltipraz (Table 5) and slower hepatic blood flow rate (Goeting et al., 1986) than those in control rats (Wilkinson and Shand, 1975), because oltipraz has an intermediate hepatic extraction ratio in rats (Bae et al., 2005b). Goeting et al. (1986) reported that the hepatic blood flow was lower in rats with liver cirrhosis induced by carbon tetrachloride. The slower hepatic CL_{int} was attributable to a decreased protein expression of hepatic CYP1A, 2B1/2, 2C11, 2D and 3A in LC rats (Figure 5). Moreover, the hepatic protein content in LC rats was significantly lower (68% decrease) than in control rats (Table 5). However, in LC rats, the free fraction of oltipraz in plasma was clearly greater (150% increase) than that in control rats.

In LC rats, the AUC of p.o. oltipraz was also significantly greater (184% increase) than that in control rats (Table 7), as reported in other studies (Bae et al., 2004; 2006a). However, this was not likely due to the increased gastrointestinal absorption of oltipraz in LC rats. The

'true' fractions of the dose unabsorbed ('F_{unabs}') after p.o. administration of oltipraz were estimated using the equation of Lee and Chiou (1983). The 'F_{unabs}' values thus estimated were 3.68% and 4.26% for control and LC rats respectively. The 184% increase in p.o. AUC (Table 7) was considerably greater than 113% increase in i.v. AUC (Table 6). This could have been due to inhibited intestinal metabolism of oltipraz in addition to inhibited hepatic metabolism of oltipraz, compared with control rats. The decreased intestinal metabolism of oltipraz in LC rats could be supported by significantly slower (31% decrease) intestinal CL_{int} than that in control rats (Table 5), because oltipraz is close to a low intestinal clearance drug in rats (Bae et al., 2005b). The above results could explain somewhat greater F value in LC rats than that in control rats (Table 7).

In DM rats, the AUC of i.v. oltipraz was significantly smaller (36.9% decrease) than that in control rats, possibly as a result of the significantly faster CL (60.0% increase) in DM rats (Table 6), as reported in other studies (Bae et al., 2006b). The faster CL of oltipraz could be supported by significantly faster (35.9% increase) hepatic CL_{int} for the disappearance of oltipraz (Table 5) and higher hepatic blood flow (Sato et al., 1991) than those in control rats. The faster hepatic CL_{int} was attributable to an increased protein expression of hepatic CYP1A, 2B1/2, 2D and 3A, relative to that in control rats (Figure 5). Note that CYP2C11 decreased in DM rats (Figure 5), as

has been found earlier (Yamazoe et al., 1989; Shimojo et al., 1993; Kim et al., 2005; Sindhu et al., 2006). The free fraction of oltipraz in plasma was comparable between control and DM rats.

In DM rats, the AUC of p.o. oltipraz was also significantly smaller (53.1% decrease) than that in control rats (Table 7), as has been reported (Bae et al., 2006b). Again, this was not likely to be due to the decreased gastrointestinal absorption of oltipraz in DM rats. The estimated 'F_{unabs}' values were 3.68% and 3.12% for control and DM rats respectively. In DM rats, the 53% decrease in p.o. AUC (Table 7) was somewhat greater than 37% decrease in i.v. AUC (Table 6). This could have been due to an increased intestinal metabolism of oltipraz in addition to increased hepatic metabolism of oltipraz in DM rats. The increased intestinal metabolism of oltipraz in DM rats could be supported by the significantly faster (35.1% increase) intestinal CL_{int} than that in control rats. The faster intestinal CL_{int} of oltipraz in DM rats could have been due to increased intestinal CYP1A (Al-Turk et al., 1981), because CYP3A is decreased in DM rats (Borbás et al., 2006). The above results could explain why the F value was lower in DM rats, than that in control rats (Table 7).

In LCD rats, the AUC of i.v. oltipraz was significantly greater (40.6% increase) than that in control rats, possibly as a result of the significantly slower CL (16.4% decrease) in LCD rats (Table 6). The slower CL of oltipraz in LCD rats could be supported by significantly

slower (35.3% decrease) hepatic CL_{int} for the disappearance of oltipraz (Table 6) than that in control rats. The slower hepatic CL_{int} could be attributed to a significantly decreased protein expression of CYP2C11 and 3A compared with that in control rats, because expression of CYP1A protein increased, but that of CYP2B1/2 and 2D was comparable to that in control rats (Figure 5). The free fractions of oltipraz in plasma were comparable between control and LCD rats. No studies on changes in hepatic blood flow rate in LCD rats have yet been reported.

In LCD rats, the AUC of p.o. oltipraz was also significantly greater (53.1% increase) than that in control rats (Table 7). Again, this was not likely due to an increased gastrointestinal absorption of oltipraz in LCD rats. The estimated 'F_{unabs}' values were 3.68% and 5.63% for control and LCD rats respectively. In LCD rats, the 53% increase in p.o. AUC (Table 7) was somewhat greater than 41% increase in i.v. AUC (Table 6). This could have been due to decreased intestinal metabolism of oltipraz in addition to decreased hepatic metabolism of oltipraz in LCD rats. The decreased intestinal metabolism of oltipraz in LCD rats could be supported by significantly slower (44% decrease) intestinal CL_{int} than that in control rats (Table 5).

After i.v. administration of oltipraz to LC and LCD rats, the V_{ss} was significantly smaller than that in control rats (25% and 19% decrease respectively; Table 6). Although the exact reason is not clear, this was

not likely due to a decrease in free fractions of oltipraz in plasma from LC and LCD rats; the free fractions were rather greater than that in control rats (the free fractions of 12%, 30% and 21% for the control, LC and LCD rats respectively).

Changes in the CYP isozymes in patients with liver cirrhosis are somewhat different from those reported here in LC rats. For example, Elbekai et al. (2004) reported that in patients with liver cirrhosis, CYP1A and 3A levels and related enzyme activities are usually reduced and CYP2B and 2C are mostly unaltered. Yang et al. (2003) reported that in patients with liver cirrhosis, the enzyme activity, protein expression and mRNA level of CYP3A was reduced. Frye et al. (2006) reported that in patients with liver disease, CYP2C11, 1A2, 2D6 and 2E1 decreased. Only antipyrine metabolism was reported in patients with type I diabetes mellitus. For example, Sotaniemi et al. (2001) reported that in patients with insulin-responsive, untreated type I diabetes, antipyrine metabolism (markers of CYP1A2, 2B6, 2C and 3A) was clearly increased. However, changes in CYP isozymes in patients with liver cirrhosis and type I diabetes mellitus did not seem to be reported. Moreover, Wiwi and Waxman (2004) reported that endogenous regulation of CYPs showed pronounced species differences, particularly of CYP2C isoforms. Thus, the present experimental data in rats should be extrapolated with care to the human situation.

In conclusion, compared with control rats, the protein expression of CYP1A and 3A increased and decreased, respectively, and that of CYP2B1/2 and 2D was comparable in LCD rats (Figure 5). Thus, it could be expected that the pharmacokinetic parameters of oltipraz in LCD rats would be fully or partially (in between DM and LC rats) restored to the values in control rats. This could be supported by the following; in LCD rats, the CL, terminal $t_{1/2}$ and MRT of oltipraz after i.v. administration (Table 6), and terminal $t_{1/2}$ and F of oltipraz after p.o. administration (Table 7) were fully restored to those in the control rats. In LCD rats, the *in vitro* V_{max} and CL_{int} for the disappearance of oltipraz (Table 5), and AUC of oltipraz after i.v. (Table 6) and p.o. administration (Table 7) were in between LC and DM rats (partially restored to those in control rats).

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PART III. PHARMACOKINETICS OF SILDENAFIL AND ITS METABOLITE, N-DESMETHYLSILDENAFIL, IN RATS WITH LIVER CIRRHOSIS AND DIABETES MELLITUS, ALONE AND IN COMBINATION

A. ABSTRACT

Pharmacokinetics of sildenafil and its metabolite, *N*-desmethylsildenafil, in humans and rats with liver cirrhosis(LC) and diabetes mellitus (DM), alone and in combination (LCD) did not seem to be reported.

Sildenafil was administered intravenously (10 mg/kg) and orally (20 mg/kg) to control, LC, DM, and LCD rats. Expression of intestinal CYP isozymes in those rats was also measured.

In LC, DM, and LCD rats, the areas under the curve (AUCs) of intravenous sildenafil were significantly greater (by 195%, 54.2%, and 127%, respectively) than controls. In LC and LCD rats, AUCs of oral sildenafil were significantly greater (3010% and 2030%, respectively) than controls.

In LC, DM, and LCD rats, significantly greater AUCs of intravenous sildenafil were due to the slower hepatic extraction of sildenafil

(because of decrease in the protein expression of hepatic CYP2C11 and 3A subfamily in LC and LCD rats, and CYP2C11 in DM rats). In LC and LCD rats, greater magnitude of increase in AUCs of oral sildenafil than those after the intravenous administration could be mainly due to the decrease in the intestinal extraction of sildenafil (because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats).

B. INTRODUCTION

Sildenafil (a weak basic compound with a p*K*a of 6.5) is an inhibitor of the cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5) found in the human corpus cavernosum. Sildenafil citrate (Viagra®) is an effective oral agent for the treatment of male erectile dysfunction. The following pharmacokinetics of sildenafil has been reported in male Sprague-Dawley rats (Shin et al., 2006). The total areas under the plasma concentration–time curve (AUCs) of intravenous sildenafil (10, 30, and 50 mg/kg) from time 0 to infinity were dose-proportional. However, its oral AUCs increased more than proportional to the dose increases (10, 30, and 100 mg/kg). After the intravenous, oral, intraportal, intragastric, or intraduodenal administration of sildenafil (30 mg/kg), the unabsorbed fraction up to 24 h was 0.00626 of the

oral dose, its extent of absolute oral bioavailability (*F*) was 0.146, its hepatic first-pass extraction ratio after absorption into the portal vein was 0.49, and its intestinal first-pass extraction ration was 0.71 of the oral dose. The hepatic first-pass extraction ratio of 0.49 after absorption into the portal vein was equivalent to 0.137 of the oral dose considering that orally administered sildenafil was first undertaken (0.71 of the intestinal first-pass extraction ratio) before reaching the liver.

Sildenafil (UK-92,480) is metabolized to *N*-desmethylsildenafil (UK-103,320; M10) via piperazine *N*-demethylation (11% in rats), UK-150,564 (M9) via piperazine *N*,*N*-deethylation (16% in rats), UK-95,340 via pyrazole *N*-demethylation (20% in rats), UK-331,849 (M8A) via ring open of piperazine (12% in rats), and others (Walker et al., 1999). It has been reported that sildenafil was metabolized via hepatic cytochrome P450 (CYP) 2C11 and 3A1/2, and *N*-desmethylsildenafil was formed via hepatic CYP2C11 in male Sprague-Dawley rats (Bae et al., 2009).

In rats with liver cirrhosis (LC) induced by *N*-dimethylnitrosamine (LC rats), the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 83.1% and 75.8%, respectively) compared with controls (Ahn et al., 2009). In rats with diabetes mellitus (DM) induced by streptozotocin (DM rats), the protein expression of hepatic CYP2C11 and 3A subfamily significantly

decreased (by 28.8%) and increased (by 23.0%), respectively, compared with controls (Ahn et al., 2009). Similar results on CYP2C11 (Thummel and Schenkman 1990; Kim et al., 2005), 3A subfamily (Barnett et al., 1990), 3A1 (Kim et al., 2005), and 3A2 (Thummel and Schenkman 1990) have also been reported. In rats with LC with DM induced by *N*-dimethylnitrosamine and streptozotocin (LCD rats), the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 32.8% and 52.9%, respectively) compared with controls (Ahn et al., 2009). However, in DM, LC, and LCD rats, the changes in the protein expression of intestinal CYP2C11 and 3A subfamily using western blot analysis compared with controls did not seem to be reported.

In patients with type I DM, the increase in CYP2E1 in peripheral blood mononuclear cells (Wang et al., 2003) and CYP1A2 activity (Matzke et al., 2000) has been reported. In insulin-dependent adults with untreated type I DM, antipyrine metabolism (markers of hepatic CYP1A2, 2B6, and 2C and 3A subfamilies) increased (Sotaniemi et al., 2002). In patients with LC, the mRNA levels of hepatic CYP1A2 and CYP1A immunoreactive proteins, mRNA levels of CYP2C9 and CYP2C proteins, and mRNA and protein level of CYP2E1 were reduced compared with control subjects (George et al., 1995). In patients with LC, mRNA level of CYP3A4 also decreased (by 68.8%) in the blood compared with control subjects (Horiike et al., 2005).

However, in patients with LC and DM, the hepatic CYP isozyme changes did not seem to be reported.

The association between liver disease and DM is well known (Vidal et al., 1994; Kwon 2003; Moscatiello et al., 2007); the overall prevalence of DM in patients with LC is significantly higher than that expected. The erectile dysfunction has been reported in patients with LC (Toda et al., 2005) and DM (Musicki and Burnett 2007). The pharmacokinetic studies of chlorzoxazone (Ahn et al., 2008) and oltipraz (Ahn et al., 2009) in rats with LC and DM, alone and in combination (LCD) have been reported. However, no pharmacokinetic studies on sildenafil and *N*-desmethylsildenafil in patients and rats have yet been reported except in patients with LC (Muirhead et al., 2002). Thus, in the present study, sildenafil was selected and the pharmacokinetics of sildenafil and *N*-desmethylsildenafil were evaluated after the intravenous and oral administration of sildenafil to LC, DM, and LCD rats. The protein expression of intestinal CYP isozymes using western blot analysis was also examined.

C. MATERIALS AND METHODS

Chemicals

Sildenafil citrate, *N*-desmethylsildenafil (purity; 98%), and DA-8159

(purity; 99.5%) [Udenafil; Zydena®; internal standard for the liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis of sildenafil and *N*-desmethylsildenafil] were products from APIN Chemical (Oxfordshire, UK), Toronto Research Chemicals (North York, ON, Canada), and Dong-A Pharmaceutical Company, Ltd. (Yongin, South Korea), respectively. The reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH; as a tetrasodium salt), ethylenediaminetetraacetic acid (EDTA; as a disodium salt), streptozotocin, tris[hydroxymethyl]aminomethane (Tris®)-buffer, β-actin, primary monoclonal antibody for β-actin, and Kodak X-OMAT film were all purchased from Sigma-Aldrich Corporation (St. Louis, MO). Monoclonal anti-rat CYP1A1/2, 2B1/2, 2C11, CYP2D subfamily, 2E1, and 3A subfamily antibodies were products from Oxford Biomedical Research (Metamora, MI) and Detroit R&D (Detroit, MI). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies, and enhanced chemiluminescence reagents were purchased from Bio-Rad Laboratories (Hercules, CA) and Amersham Life Science Inc. (Piscataway, NJ), respectively. *N*-Dimethylnitrosamine was a product from Tokyo Kasei Kogyo Company (Tokyo, Japan). Other chemicals were of reagent or high-performance liquid chromatographic (HPLC) grade.

Animals

The protocols for the animal study were approved by the Animal Care and Use Committee of College of Pharmacy, Seoul National University, Seoul, South Korea. Male Sprague-Dawley rats (4–6 weeks old, weighing 160–210 g) were purchased from Charles River Company Korea (Orient, Seoul, South Korea). The procedures used for housing and handling the rats were similar to the reported methods (Bae et al., 2004, 2009; Ahn et al., 2008, 2009).

Induction of LC in rats by N-dimethylnitrosamine injection

Freshly prepared *N*-dimethylnitrosamine (diluted in 0.9% NaCl-injectable solution) was injected intraperitoneally at a dose of 0.01 mg (1 mL)/kg on three consecutive days per week for 4 weeks (Ohara and Kusano 2002; Bae et al., 2004; Ahn et al., 2008, 2009). On Day 29, one dose (1 ml/ kg) of citrate buffer (pH 4.5) was injected via the tail vein. On Day 36, the rats were treated with sildenafil citrate. Laboratory rats with *N*-dimethylnitrosamineinduced LC have clinical features similar to those of human with LC, such as increasing mortality, destruction of hepatic parenchymal cell, formation of connective tissue, and regeneration of nodule (Kang et al., 2002). Liver cirrhosis in LC rats was evident by liver histological analysis (Ohara and Kusano 2002). It has been reported that *N*-dimethylnitrosamine-induced LC in rats was reproducible (Jezequel et

al., 1987; Kang et al., 2002).

Induction of DM in rats by streptozotocin injection

A 0.9% NaCl-injectable solution was injected intraperitoneally (1 ml/kg) on three consecutive days per week for 4 weeks. On Day 29, one dose [45 mg (1 ml)/kg] of freshly prepared streptozotocin [dissolved in citrate buffer (pH 4.5)] was administered via the tail vein (Kim et al., 2005; Ahn et al., 2008, 2009). On Day 36, the rats were treated with sildenafil citrate. Diabetes mellitus in DM rats was evident by higher blood glucose level, greater 24-h urine output, and decrease in body weight gain (Ahn et al., 2008).

Induction of LCD in rats with N-dimethylnitrosamine and streptozotocin injections

LC was induced by intraperitoneal injection of *N*-dimethylnitrosamine as described above. Then, on Day 29, DM was induced by injection of streptozotocin via the tail vein as described earlier. On Day 36, the rats were treated with sildenafil citrate. The presence of LC and DM in LCD rats was evident based on the blood chemistry data as well as liver histological analysis (Ahn et al., 2008).

Control rats

Rats were injected intraperitoneally with 0.9% NaCl injectable solution (1 ml/kg) on three consecutive days per week for 4 weeks. On

Day 29, one dose (1 ml/kg) of the citrate buffer (pH 4.5) was administered via the tail vein. On Day 36, the rats were treated with sildenafil citrate. During the pretreatment, food and water were available *ad libitum* to all rats. Immediately before the experiment, blood glucose levels in all rats were measured using the Medisense Optium kit (Abbott Laboratories, Bedford, MA), and rats with blood glucose levels higher than 250 mg/dl were chosen as being diabetic (DM and LCD rats).

Preparation of hepatic microsomes

The procedures used for preparation of hepatic microsomes form control, LC, DM, and LCD rats (n = 4–6, each) were similar to the reported methods (Ahn et al., 2008, 2009; Choi et al., 2008). Then, the hepatic microsomes were stored at −70°C (Revco ULT 1490 D-N-S; Western Mednics, Ashville, NC) until use. Protein contents in hepatic microsomes were measured using a reported method (Bradford 1976).

Western immunoblot analysis of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily

The procedures used for preparation of intestinal microsomes and immunoblot analysis of intestinal CYP isozymes were similar to a reported method (Lee and Lee 2008; Ahn et al., 2009). Intestinal microsomes were resolved by sodium dodecyl sulfated-

polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel (10 μg protein per lane; n = 3, each). Proteins were transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI) that was then blocked for 2 h in the Tris®-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T). For immunodetection, blots were incubated overnight at 4°C with anti-rat CYP isozyme antibodies (diluted 1:10,000 in TBS-T containing 5% bovine serum albumin), followed by incubation for 2 h at room temperature with a secondary antibody conjugated to horseradish peroxidase (diluted 1:10,000 in TBS-T containing 5% milk powder). The protein expression of the CYP isozymes were detected by enhanced chemiluminescence on Kodak X-OMAT film and quantitated by densitometry with a microcomputer imaging device (model M1; Imaging Research, St. Catharines, Ontario, Canada). The β-actin was used as a loading control.

Measurement of V_{max}, K_m, and intrinsic clearance for the disappearance of sildenafil in hepatic microsomes

The procedures used were similar to the reported methods (Ahn et al., 2008, 2009). The V_{max} (maximum velocity) and K_m (the apparent Michaelis–Menten constant; the concentration at which the rate is one-half of the V_{max}) for the disappearance of sildenafil in control, LC, DM, and LCD rats (n = 5–6, each) were determined after incubating the above microsomes (equivalent to 0.2 mg protein), 5 μl of methanol

containing final sildenafil base (the same solution used in the intravenous study) concentrations of 1, 2, 4, 10, 20, 40, 100, and 200 μM, and 50 μl of 0.1 M phosphate buffer (pH 7.4) containing 1 mM NADPH. The volume was adjusted to 0.5 ml by adding 0.1 M phosphate buffer (pH 7.4), and the components were incubated at 37°C using a thermomixer (Eppendorf, Hamburg, Germany) at a rate of 50 oscillations/min (opm). Incubation times were 1, 5, 15, 30, and 60 min, respectively, in the preliminary study. All of the above microsomal incubation conditions were within the linear range of the reaction rate. The reaction was terminated by addition of 1 ml of acetonitrile after 5-min incubation. The kinetic constants (K_m and V_{max}) for the disappearance of sildenafil were calculated using a nonlinear regression method (Duggleby 1995). The intrinsic clearance (CL_{int}) for the disappearance of sildenafil was calculated by dividing the V_{max} by K_m.

Pretreatment of rats for i.v. or p.o. study

The procedures used for pretreatment of rats including the cannulation (early in the morning on Day 36) of the jugular vein (for drug administration in the intravenous study) and the carotid artery (for blood sampling) were similar to the reported methods (Kim et al., 1993; Ahn et al., 2008, 2009).

Intravenous study

Sildenafil base (sildenafil citrate was dissolved in distilled water in a minimum amount of 10 N NaOH) at a dose of 10 mg (2 ml)/kg was manually infused over 1 min via the jugular vein of rats in each group (n = 9, 10, 9, and 8 for control, LC, DM, and LCD rats, respectively). Blood samples (~ 0.12 ml, each) were collected via the carotid artery at 0 (control), 1 (end of infusion), 5, 15, 30, 60, 90, 120, 180, and 240 min after the start of the intravenous infusion of sildenafil citrate. A heparinized 0.9% NaCl-injectable solution (20 units/ml; 0.3 ml) was used to flush the cannula immediately after each blood sampling to prevent blood clotting. Each blood sample was immediately centrifuged and 50 μl of each plasma sample was stored at −70°C until used for the LC/MS/MS analysis of sildenafil and *N*-desmethylsildenafil. The procedures used for preparation and handling of 24-h urine sample ($Ae_{0–24\ h}$) and gastrointestinal (GI) tract (including its contents and faeces) sample at 24 h ($GI_{24\ h}$) were similar to the reported methods (Kim et al., 1993; Ahn et al., 2008, 2009).

Oral study

Sildenafil base (the same solution used in the intravenous study) at a dose of 20 mg (2 ml)/kg was administered orally using a gastric gavage tube to rats in each group (n = 8, 9, 10, and 10 for control, LC, DM, and LCD rats, respectively). Blood samples were collected at 0,

5, 15, 30, 45, 60, 90, 120, 180, and 240 min after the oral administration of sildenafil citrate. Other procedures for the oral study were similar to those for the intravenous study.

Measurement of rat plasma protein binding of sildenafil using equilibrium dialysis

Protein binding values of sildenafil to fresh plasma from control, LC, DM, and LCD rats (n = 4, each) were measured using equilibrium dialysis (Shim et al., 2000). One millilitre of plasma was dialyzed against 1 ml of isotonic Sørensen phosphate buffer (pH 7.4) containing 3% (w/v) dextran ("the buffer") to minimize volume shift (Boudinot and Jusko 1984) in a 1-ml dialysis cell (Fisher Scientific, Fair Lawn, NJ) using a Spectra/Por 4 membrane (molecular weight cutoff 12–14 kDa; Spectrum Medical Industries Inc., Los Angeles, CA). To reduce equilibrium time of sildenafil between "the buffer" and plasma compartments, sildenafil was spiked into the plasma side (Øie and Guentert 1982). An initial concentration of sildenafil spiked into the plasma compartment was 5 μg/ml. After 24-h incubation, two 50 μl were collected from each compartment and stored at −70°C until used for the LC/MS/MS analysis of sildenafil.

LC/MS/MS analysis of sildenafil and N-desmethylsildenafil

Concentrations of sildenafil and *N*-desmethylsildenafil were

determined using a LC/MS/MS method (Bae et al., 2009). In brief, to 50 μl of a sample, 200 μl of acetonitrile containing 250 ng/ml of DA-8159 (internal standard) was added. After vortex-mixing and centrifugation (9,000 *g* for 10 min), the supernatant was transferred to another Eppendorf tube and a 6 μl was directly injected onto a reversed-phase HPLC column (Luna C18; 50 mm, *l* × 2.0 mm, i.d.; particle size, 3 μm; Phenomenex, Torrance, CA). The mobile phase, 10 mM ammonium acetate (pH 5.2):acetonitrile (25:75, v/v), was run at a flow rate of 0.2 ml/min using an Agilent 1200 series HPLC system (Wilmington, DE). The column and autosampler temperatures were maintained at 40°C and 4°C, respectively. A LC/MS/MS analysis was performed using a PE SCIEX API4000 LC/MS/MS system (Applied Biosystems, Foster City, CA), equipped with an electrospray ionization interface used to generate positive ions, $[M^{+}H]^{+}$. The optimized ion spray voltage and temperature were set at 5500 V and 500°C, respectively. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential, and collision cell exit potential were 60, 35, 10, and 14 V, respectively. Nitrogen gas was used for the nebulizer (NEB) gas, curtain (CUR) gas, and collision-activated dissociation (CAD) gas. Quantification was performed by multiple reactions monitoring of the protonated precursor ion and the related product ion for sildenafil and *N*-desmethylsildenafil, using the internal standard method with peak area

ratios and a weighing factor of $1/x^2$. The mass transitions used for sildenafil, *N*-desmethylsildenafil, and internal standard were *m*/*z* 475.3 → 100.1, 460.9 → 283.2, and 517.2 → 283.1, respectively, with a dwell time of 150 msec per transition. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by analyst software (version 1.4.1; Applied Biosystems). The retention times of sildenafil, *N*-desmethylsildenafil, and DA-8159 (internal standard) were approximately 1.2, 0.97, and 0.93 min, respectively. This assay was linear over concentration ranges from 5 to 1000 ng/ml with a lower limit of quantification of 5 ng/ml for both sildenafil and *N*-desmethylsildenafil. The coefficient of variation for the assay precision was less than 14.7%, and the accuracy was greater than 94.2%.

Pharmacokinetic analysis

Standard methods (Gibaldi and Perrier 1982) were used to calculate the following pharmacokinetic parameters using a non-compartmental analysis (WinNonlin®; Pharsight Corporation, Mountain View, CA): the AUC (Chiou 1978), time-averaged total body and renal clearances (CL and CL_R, respectively), terminal half-life ($t_{1/2}$), mean residence time (MRT), apparent volume of distribution at steady state (V_{ss}), and *F* (Kim et al., 1993). The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly read from the experimental data.

Statistical analysis

A *P*-value < 0.05 was considered to be statistically significant using a Duncan's multiple range test of Statistical Package for the Social Sciences (SPSS) *posteriori* analysis of variance (ANOVA) among the four means for the unpaired data. All data are expressed as mean ± standard deviation (S.D.) except median (range) for T_{max}.

D. RESULTS

V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in hepatic microsomes

The V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in hepatic microsomes from four groups of rats are listed in Table 8. In LC, DM, and LCD rats, the V_{max}s were significantly slower (by 93.0, 65.3, and 94.1%, respectively) than controls. This suggests that in LC, DM, and LCD rats, the maximum velocity for the disappearance (primarily metabolism) of sildenafil was slower than controls. In LC, DM, and LCD rats, the K_ms were significantly lower (by 82.5, 42.7, and 92.4%, respectively) than controls. This suggests that in LC, DM, and LCD rats, the affinity of enzyme(s) for the sildenafil increased compared with controls. Since the total liver weight and total liver proteins were different among four groups of rats, the CL_{int}s were calculated based on the ml/min/whole liver; the values in LC (by 71.1%; $P < 0.05$), DM

Table 8 V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in the hepatic microsomes of LC, DM, LCD, and control rats

Parameter[a]	Control (n = 6)	LC (n = 5)	DM (n = 4)	LCD(n = 5)
V_{max} (nmol/min/mg protein)[b]	13.4 ± 6.95	0.933 ± 0.569	4.65 ± 3.01	0.792 ± 0.410
K_m (μM)[c]	52.9 ± 22.0	9.28 ± 5.60	30.3 ± 13.9	4.00 ± 1.85
CL_{int} (ml/min/mg protein)[d]	0.254 ± 0.0868	0.108 ± 0.0707	0.144 ± 0.0515	0.235 ± 0.130
CL_{int} (ml/min/whole liver)[d]	22.0 ± 8.35	6.35 ± 5.27	14.4 ± 4.37	14.2 ± 8.92
Total protein (mg/whole liver)[e]	86.1 ± 9.45	62.8 ± 10.8	103 ± 15.9	62.9 ± 11.3
Liver weight (% of body weight)[f]	3.48 ± 0.396	2.68 ± 0.389	3.92 ± 0.274	3.14 ± 0.237

[a]Data are expressed as mean ± S.D.

[b]Control group were significantly different from other three groups ($p < 0.05$).

[c]Control group, DM group, and LC and LCD groups were significantly different ($p < 0.05$).

[d]Control group was significantly different ($p < 0.05$) from LC group.

[e]Control and DM groups were significantly different ($p < 0.05$) from LC and LCD group.

[f]Control and DM groups were significantly different ($p < 0.05$) from LC group, and DM group was significantly different ($p < 0.05$) from LCD group.

(by 34.5%; P = 0.134), and LCD (by 35.5%; P = 0.193) rats were slower than controls. This suggests that in LC, DM, and LCD rats, formation of metabolites of sildenafil could be decreased compared with controls.

Protein expression of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily

The protein expression of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily in control, DM, LC, and LCD rats is shown in Figure 9. In LC rats, the protein expression of CYP2B1/2, 2C11, 2D6, 2E1, and 3A subfamily significantly decreased (by 89.4%, 94.9%, 86.3%, 67.3%, and 52.4%, respectively), but CYP1A1/2 was not altered compared with controls. In DM rats, CYP1A1/2 increased (by 1,380%), 2C11 and 2D subfamily decreased (by 91.9% and 14.5%, respectively), but others were not altered compared with controls. In LCD rats, CYP1A1/2 increased (by 1,510%), 2C11 decreased (by 94.6%), but others were not altered compared with controls.

Rat plasma protein binding of sildenafil

The binding values of sildenafil to fresh plasma from four groups of rats were 78.9 ± 5.89% (control rats), 72.7 ± 4.13% (LC rats), 63.7 ± 11.9% (DM rats), and 70.5 ± 5.76% (LCD rats); the value in DM rats was significantly smaller (by 19.3%) than controls.

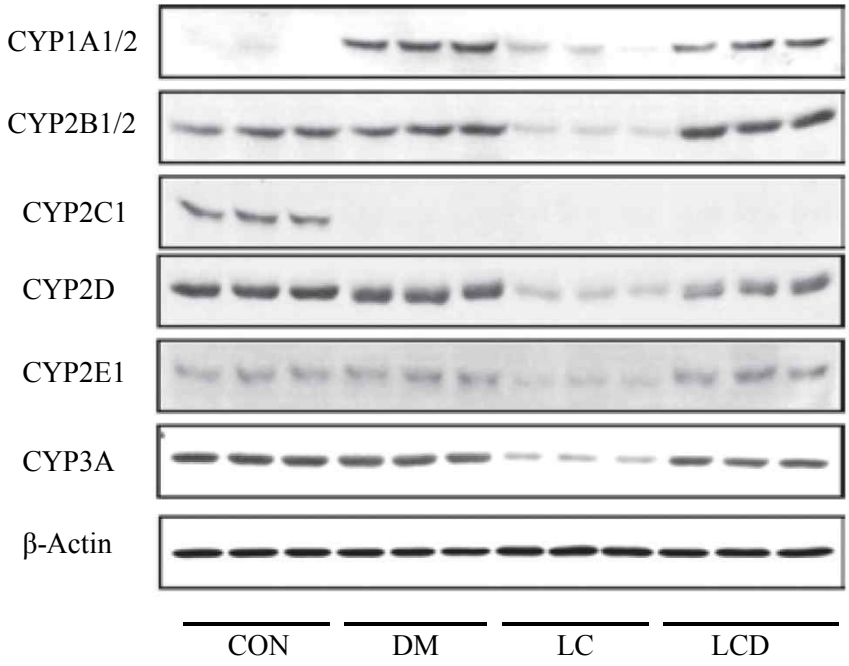

Figure 9 Immunoblotting of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily in control, DM, LC and LCD rats. The β-actin band was used as a loading control.

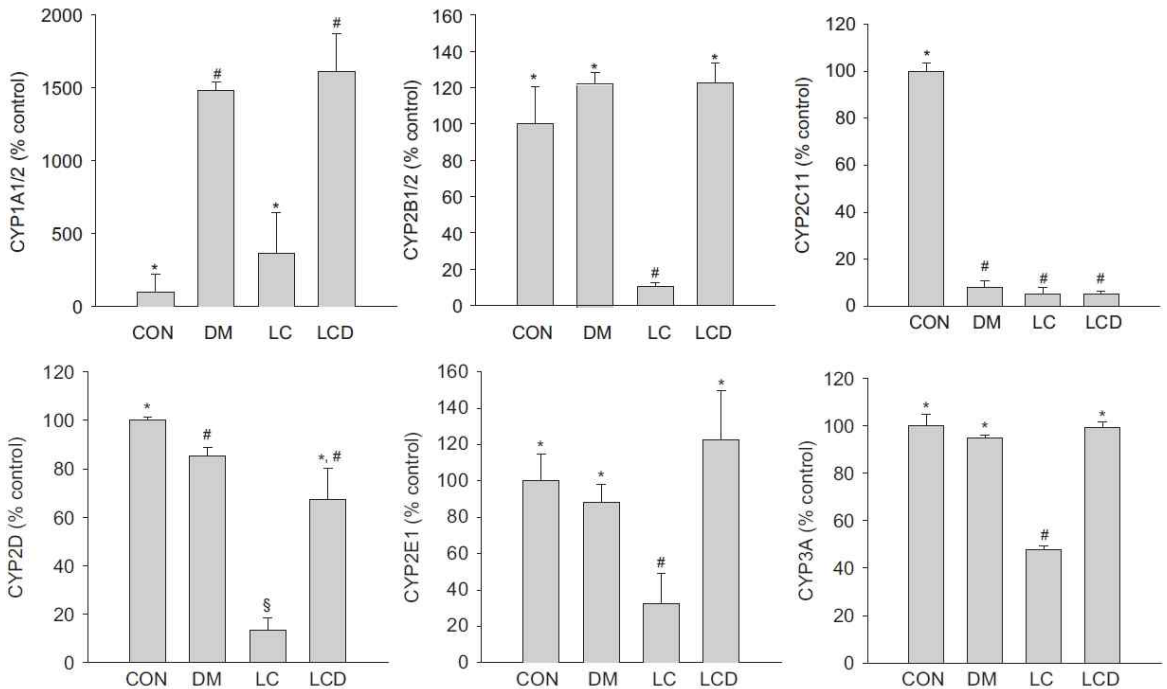

Figure 10 The protein expression was expressed in terms of % of control rats 100%. CYP1A1/2; DM and LCD groups (#) were significantly different ($P < 0.05$) from control and LC groups (*); CYP2B1/2, CYP2E1, and CYP3A subfamily; LC group (#) was significantly different ($P < 0.05$) from control, DM, and LCD groups (*); CYP2C11; DM, LC, and LCD groups (#) were significantly different ($P < 0.05$) from control group (*); CYP2D subfamily; LC group (§) was significantly different ($P < 0.05$) from other three groups (* and #), and DM group (#) was significantly different ($P < 0.05$) from control group (*). Error bars represent S.D.

It has been reported that the binding values of sildenafil in 4% human serum albumin, similar to the ratio of albumin in rat plasma (Mitruka and Rawnsley 1981), were constant, 95.0%, at sildenafil concentrations ranging from 0.01 to 10 μg/ml (Walker et al., 1999). Thus, a sildenafil concentration of 5 μg/ml was used in this plasma protein binding studies.

Pharmacokinetics of sildenafil and N-desmethylsildenafil after i.v. administration of sildenafil

After the intravenous administration of sildenafil(10mg/kg) to control, LC, DM, and LCD rats, the mean arterial plasma concentration–time profiles of sildenafil and *N*-desmethylsildenafil are shown in Figure 11A and B, respectively. The relevant pharmacokinetic parameters are listed in Table 9. In LC rats, the AUC, CL, MRT, and V_{ss} of sildenafil were significantly greater (by 195%), slower (by 64.4%), longer (by 345%), and larger (by 44.8%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 39.1%), lower (by 74.7%), and longer (by 500%), respectively; and metabolite ratio ($AUC_{N\text{-desmethylsildenafil}}/AUC_{sildenafil}$) was significantly smaller (by 78.1%) than controls. In DM rats, the AUC and CL of sildenafil were significantly greater (by 54.2%) and slower (by 34.8%), respectively; C_{max} of *N*-desmethylsildenafil was significantly lower (by 35.5%); and metabolite ratio was significantly smaller (by 42.9%) than

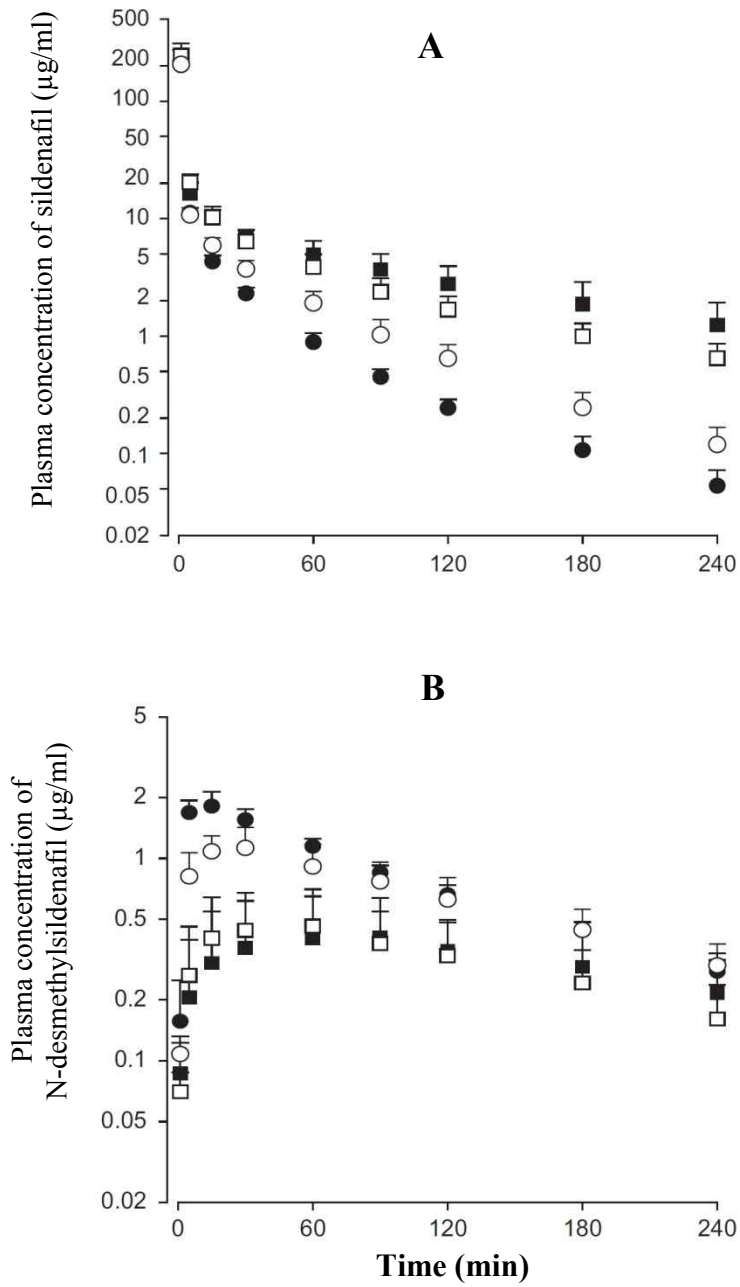

Figure 11 Mean arterial plasma concentration–time profiles of sildenafil (A) and *N*-desmethylsildenafil (B) after intravenous infusion of sildenafil at a dose of 10 mg/kg in control (●; n = 9), liver cirrhosis (LC) (■; n = 10), diabetes mellitus (DM) (○; n = 9), and liver cirrhosis with diabetes mellitus (LCD) (□; n = 8) rats. Error bars represent S.D.

Table 9 Pharmacokinetic parameters of sildenafil and N-desmethylsildenafil after i.v. administration of sildenafil at a dose of 10 mg/kg to LC, DM, LCD, and control rats

Parameter[a]	Control (n = 9)	LC (n = 10)	DM (n = 9)	LCD (n = 8)
Initial body weight (g)	178 ± 9.72	186 ± 14.5	179 ± 12.4	183 ± 14.4
Final body weight (g)[b]	377 ± 11.5	273 ± 16.2	333 ± 21.4	271 ± 16.6
Sildenafil				
AUC (μg·min/ml)[c]	577 ± 34.9	1700 ± 399	890 ± 92.8	1310 ± 229
CL (ml/min/kg) [c]	34.8 ± 2.12	12.4 ± 3.37	22.7 ± 2.47	15.7 ± 2.58
Terminal $t_{1/2}$ (min)	49.1 ± 5.74	91.6 ± 23.6	49.8 ± 11.6	69.6 ± 30.8
MRT (min)[d]	17.4 ± 2.87	77.5 ± 33.2	22.9 ± 3.66	58.9 ± 7.48
V_{ss} (ml/kg)[e]	603 ± 94.6	873 ± 218	515 ± 64.7	916 ± 140
$Ae_{0\text{-}24\ h}$ (% of sildenafil dose)	BD[f]	BD	BD	BD
$GI_{24\ h}$ (% of sildenafil dose)	BD	BD	BD	BD
N-desmethylsildenafil				
AUC (μg·min/ml)[e]	233 ± 15.0	142 ± 87.8	206 ± 47.6	113 ± 45.9
Terminal $t_{1/2}$ (min)	95.7 ± 16.6	186 ± 77.9	113 ± 13.8	145 ± 79.1
C_{max} (μg/ml)[b]	1.86 ± 0.292	0.471 ± 0.326	1.20 ± 0.262	0.500 ± 0.159
T_{max} (min)[e]	15 (5−15)	90 (15−120)	15 (5−30)	60 (5−60)

(Table 9 continued)

Parameter[a]	Control (n = 8)	LC (n = 9)	DM (n = 7)	LCD (n = 7)
$Ae_{0\text{-}24\ h}$ (% of sildenafil dose)	BD	BD	BD	BD
$GI_{24\ h}$ (% of sildenafil dose)	BD	BD	BD	BD
$AUC_{N\text{-desmethylsildenafil}}$ / $AUC_{sildenafil}$ (%)[b]	40.6 ± 3.92	8.91 ± 6.61	23.2 ± 4.93	9.29 ± 3.96

[a]Data are expressed as mean ± S.D.

[b]Control group, DM group, and LC and LCD groups were significantly different ($P < 0.05$).

[c]Each group was significantly different ($P < 0.05$).

[d]Control and DM groups, LCD group, and LC group were significantly different ($P < 0.05$).

[e]Control and DM groups were significantly different ($P < 0.05$) from LC and LCD groups.

[f]Below the detection limit.

controls. In LCD rats, the AUC, CL, MRT, and V_{ss} of sildenafil were significantly greater (by 127%), slower (by 54.9%), longer (by 239%), and larger (by 51.9%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 51.5%), lower (by 73.1%), and longer (by 300%), respectively; and metabolite ratio was significantly smaller (by 77.1%) than controls (Table 9). The $Ae_{0-24\ h}$ and $GI_{24\ h}$ (expressed in terms of the percentage of the intravenous sildenafil dose) of both sildenafil and *N*-desmethylsildenafil were below the detection limit in all rats studied. Note that in LC, DM, and LCD rats, body weight gain decreased (by 56.3, 22.6, and 55.8%, respectively) compared with controls as reported from other studies (Ahn et al., 2008, 2009).

Pharmacokinetics of sildenafil and N-desmethylsildenafil after p.o. administration of sildenafil

After the oral administration of sildenafil to control, LC, DM, and LCD rats, the mean arterial plasma concentration–time profiles of sildenafil and *N*-desmethylsildenafil are shown in Figure 12A and 12B, respectively. The relevant pharmacokinetic parameters are listed in Table 10. In LC rats, the AUC, terminal $t_{1/2}$, and C_{max} of sildenafil were significantly greater (by 3,010%), longer (by 33.0%), and higher (by 833%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 61.5%), lower (by 84.6%), and longer

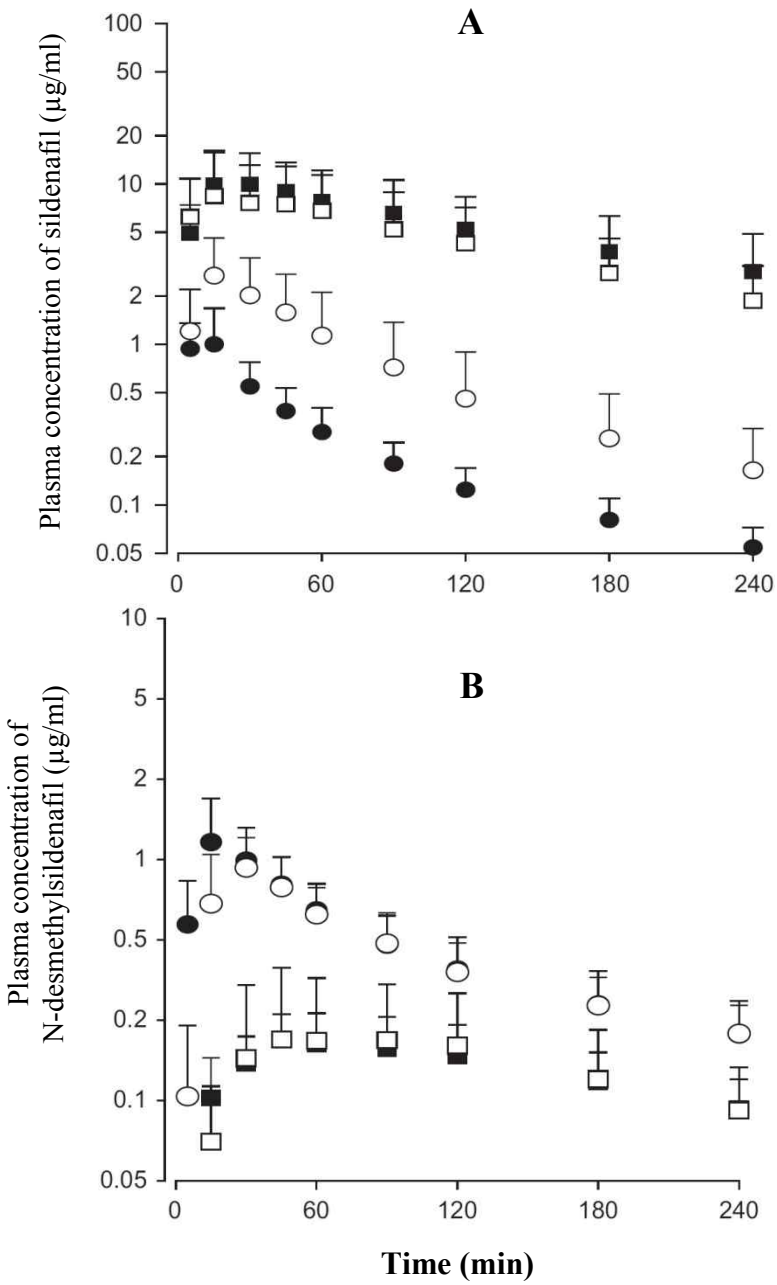

Figure 12 Mean arterial plasma concentration–time profiles of sildenafil (A) and *N*-desmethylsildenafil (B) after the oral administration of sildenafil at a dose of 20 mg/kg in control (●; $n = 8$), LC (■; $n = 9$), DM (○; $n = 10$), and LCD (□; $n = 10$) rats. Error bars represent S.D.

Table 10 Pharmacokinetic parameters of sildenafil and N-desmethylsildenafil after p.o. administration of sildenafil at a dose of 20 mg/kg to LC, DM, LCD, and control rats.

Parameter[a]	Control (n = 8)	LC (n = 9)	DM (n = 10)	LCD (n = 10)
Initial body weight (g)	169 ± 9.04	167 ± 7.55	164 ± 11.5	165 ± 7.45
Final body weight (g)[b]	328 ± 12.3	279 ± 17.2	299 ± 12.0	273 ± 32.4
Blood glucose (mg/dl)[c]	91.4 ± 8.05	103 ± 8.20	284 ± 32.1	318 ± 69.6
Sildenafil				
AUC (μg·min/ml)[d]	64.7 ± 22.4	2010 ± 1300	202 ± 151	1380 ± 909
Terminal $t_{1/2}$ (min)[e]	106 ± 31.3	141 ± 44.0	85.2 ± 13.9	104 ± 26.6
C_{max} (μg/ml)[d]	1.20 ± 0.617	11.2 ± 5.64	2.76 ± 1.85	9.55 ± 7.67
T_{max} (min)[f]	15 (5−15)	15 (5−45)	15 (15−45)	22.5 (5−45)
CL_R (ml/min/kg)	0.484 ± 0.294	0.317 ± 0.199	0.293 ± 0.120	0.511 ± 0.635
$Ae_{0\text{-}24\ h}$ (% of sildenafil dose)	0.151 ± 0.105	2.56 ± 2.12	0.242 ± 0.103	2.44 ± 2.33
$GI_{24\ h}$ (% of sildenafil dose)	BD[h]	BD	BD	BD
F (%)	5.61	59.1	11.3	52.7
N-desmethylsildenafil				
AUC (μg·min/ml)[d]	155 ± 50.5	59.7 ± 16.7	141 ± 36.2	60.0 ± 32.5

(Table 10 continued)

Parameter[a]	Control (n = 8)	LC (n = 9)	DM (n = 7)	LCD (n = 7)
Terminal $t_{1/2}$ (min)	144 ± 43.8	182 ± 39.9	141 ± 29.4	184 ± 81.5
C_{max} (μg/ml)[g]	1.34 ± 0.514	0.207 ± 0.0614	1.02 ± 0.291	0.225 ± 0.173
T_{max} (min)[d]	15 (5−45)	45 (15−120)	22.5 (15−45)	75 (15−120)
$Ae_{0\text{-}24\ h}$ (% of sildenafil dose)	BD	BD	BD	BD
$GI_{24\ h}$ (% of sildenafil dose)	BD	BD	BD	BD
$AUC_{N\text{-desmethylsildenafil}}$ / $AUC_{sildenafil}$ (%)[g]	243 ± 38.3	7.08 ± 10.4	88.5 ± 42.2	6.75 ± 7.10

[a]Data are expressed as mean ± S.D.

[b]Control group was significantly different ($P < 0.05$) from DM and LCD groups.

[c]Control and LC groups were significantly different ($P < 0.05$) from DM and LCD groups

[d]Control and DM groups were significantly different ($P < 0.05$) from LC and LCD groups.

[e]LC group was significantly different ($P < 0.05$) from other three groups.

[f]Control group was significantly different ($P < 0.05$) from LCD group.

[g]Control group, DM group, and LC and LCD groups were significantly different ($P < 0.05$).

[h]Below the detection limit.

(by 200%), respectively; and metabolite ratio was significantly smaller (by 97.1%) than controls. After the oral administration of 50-mg sildenafil to male patients with biopsy-proven LC, the AUC and C_{max} of sildenafil were also greater (by 85.0%) and higher (by 47.1%), respectively, but AUC of *N*-desmethylsildenafil was greater (by 155%) than control subjects (Murihead et al., 2002). In DM rats, the C_{max} of *N*-desmethylsildenafil was significantly lower (by 23.9%); and metabolite ratio was significantly smaller (by 63.6%) than controls. In LCD rats, the AUC, C_{max}, and T_{max} of sildenafil were significantly greater (by 2,030%), higher (by 696%), and longer (by 50%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 61.3%), lower (by 83.2%), and longer (by 400%), respectively; and metabolite ratio was significantly smaller (by 97.2%) than the controls. In LC, DM, and LCD rats, the *F* values were greater (by 953%, 101%, and 839%, respectively) than controls. The $Ae_{0–24\ h}$ and $GI_{24\ h}$ of sildenafil were almost negligible (< 2.56% of the oral dose of sildenafil) and those of *N*-desmethylsildenafil (expressed in terms of the percentage of the oral dose of sildenafil) were below the detection limit for all rats studied.

E. DISCUSSION

The hepatic CL_{int}s for the disappearance of sildenafil based on the

ml/min/whole liver were slower than controls in the order of DM, LCD, and LC rats (Table 8). This could have possibly been due to the differences in the magnitude of changes in the hepatic CYP isozymes compared with controls: in LC rats, the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 83.1% and 75.8%, respectively); in LCD rats, they were also significantly decreased (by 32.8% and 52.9%, respectively), but the magnitude was smaller than that in LC rats (Ahn et al., 2009). However, in DM rats, the CYP2C11 and 3A subfamily were significantly decreased (by 28.8%) and increased (by 23.0%), respectively (Ahn et al., 2009).

Streptozotocin has "direct" (streptozotocin *per se*) effects on the pharmacokinetic parameters of drugs due to its hepatotoxicity (Ioannides 1996). Approaches have been proposed for ensuring that observed pharmacokinetic effects in DMIS rats are due to the "indirect" (changes in CYP isozymes due to diabetes-related changes) effect of streptozotocin and not streptozotocin *per se* (Ioannides 1996). Considering the very short $t_{1/2}$ of streptozotocin (about 15 min), it is unlikely that its "direct" effects could be sustained for 7 days after administration of streptozotocin. Streptozotocin had no effect on the metabolism of model substrates *in vitro* (Ackerman and Leibman 1977), and analogues of streptozotocin, devoid of diabetogenic activity, failed to influence the metabolism of various substrates (Reinke et al., 1978). The above data suggest that the effect of

streptozotocin on Day 7 occurred through its "indirect," and not "direct," mechanism. Metabolic $t_{1/2}$ of *N*-dimethylnitrosamine was also short; < 10 min in rodents and about 20 min in non-human primates (George et al., 2001). Therefore, it is also unlikely that its "direct" effects could be sustained for more than a week after administration of *N*-dimethylnitrosamine. However, the acute toxic effects of streptozotocin and *N*-dimethylnitrosamine could not be totally ruled out.

The contribution of GI (including biliary) excretion of unchanged sildenafil to its CL did not seem to be considerable; the $GI_{24\ h}$ values of sildenafil were below the detection limit (Table 9). Sildenafil was stable in buffer solutions having pH of 3, 7, and 11 up to 12-h incubation (> 98% of the spiked amounts of sildenafil were recovered) (Wang et al., 2008). Moreover, the 24-h biliary excretion of sildenafil after its intravenous administration (20 mg/kg) to three rats with bile duct cannulation was only 0.142 ± 0.0321% of the dose in the present study. The $Ae_{0–24\ h}$ was also below the detection limit for all rats studied (Table 9). The above data suggest that intravenous sildenafil was almost completely metabolized and the CLs of sildenafil listed in Table 9 could represent its metabolic clearances.

In LC, DM, and LCD rats, the AUCs of intravenous sildenafil were significantly greater than controls, possibly as a result of the significantly slower CLs of sildenafil than controls (Table 9). Because

sildenafil is a drug with an intermediate hepatic extraction ratio [hepatic first-pass extraction ratio of 0.49 after absorption into the portal vein (Shin et al., 2006)], its hepatic clearance depends on the *in vitro* hepatic CL_{int} for the disappearance of sildenafil, the free (unbound to plasma proteins) fraction of sildenafil in the plasma, and the hepatic blood flow rate in rats (Wilkinson and Shand 1975). The significantly slower CL of sildenafil in LC rats (Table 9) could have been supported by the significantly slower hepatic CL_{int} (mL/min/whole liver) for the disappearance of sildenafil (Table 8) and slower hepatic blood flow rate than controls, because the free fraction of sildenafil in the plasma was comparable with controls. Goeting et al. (1986) reported that the hepatic blood flow rate was slower in rats with LC induced by carbon tetrachloride. In LC rats, the slower hepatic CL_{int} (Table 8) could have been due to the decrease in the protein expression of both hepatic CYP2C11 and 3A subfamily compared with controls (Ahn et al., 2009). In DM rats, the slower CL of sildenafil (Table 9) could have been due to the significantly slower CL_{int} (mL/min/whole liver) (Table 8) because the free fraction of sildenafil in the plasma was significantly greater and the hepatic blood flow rate was faster (Sato et al., 1991) than controls. In DM rats, the slower CL_{int} could be due to the decrease in the protein expression of CYP2C11 because CYP3A subfamily increased in DM rats compared with controls (Ahn et al., 2009). In LCD rat, the significantly slow CL

of sildenafil (Table 9) could at least partly have been due to the slower hepatic CL_{int} (mL/min/whole liver) (Table 8) because the free fraction of sildenafil in the plasma was comparable with controls. In LCD rats, the hepatic blood flow rate changes did not seem to be reported. In LCD rats, the slower CL_{int} (Table 9) could have been due to the decrease in the protein expression of both CYP2C11 and 3A subfamily compared with controls (Ahn et al., 2009). In LC, DM, and LCD rats, the significantly smaller metabolite ratios (by 78.1, 42.9, and 77.1%, respectively) than controls suggest that formation of *N*-desmethylsildenafil decreased compared with controls. This could at least partly have been due to the significant decrease in the protein expression of hepatic CYP2C11 compared with controls (Ahn et al., 2009).

In LC and LCD rats, the V_{ss}s of intravenous sildenafil were significantly larger than controls (by 44.8% and 51.9%, respectively; Table 9). However, this was not likely due to the increase in the free fraction of sildenafil in plasma compared with controls; the free fractions were comparable among three groups of rats. Similar results have also been reported in LC rats that the V_{ss} of theophylline was slightly larger although the protein binding value was comparable with controls (Nam et al., 1997). The exact reason for this is unclear and more studies are required.

In LC and LCD rats, the AUCs of oral sildenafil were also greater

than controls (by 3,010% and 2,030%, respectively) (Table 10). However, this was not likely due to the increased GI absorption of sildenafil compared with controls. For comparison, the "true" fractions of the oral dose of sildenafil unabsorbed ("F_{unabs}") were estimated using the reported equation (Lee and Chiou 1983). The "F_{unabs}" values thus estimated were almost negligible, suggesting almost complete absorption of sildenafil for all groups of rats. As mentioned earlier, the intestinal and hepatic first-pass extraction ratios of sildenafil were 0.71 and 0.137 of the oral dose, respectively, in rats (Shin et al., 2006). Thus, the contribution of intestinal first-pass extraction (0.71 of the oral dose) to the significantly greater AUC of oral sildenafil was greater than that of the hepatic first-pass extraction (0.137 of the oral dose). In LC and LCD rats, the magnitude of the increase in AUCs of oral sildenafil (by 3,010% and 2,030%, respectively) was considerably greater than 195% and 127% increase, respectively, after the intravenous sildenafil (Table 9). Thus, only inhibition of hepatic metabolism of sildenafil could not fully explain the considerable increase in AUCs of oral sildenafil (Table 10). This could mainly be due to the decrease in the intestinal metabolism of sildenafil because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats (Figure 10). This could be supported by the greater magnitude in the smaller metabolite ratios in LC and LCD rats after the oral administration (by 97.1% and 97.2%,

respectively) (Table 10) than that after the intravenous administration (by 78.1% and 77.1%, respectively) (Table 9). Therefore, in LC and LCD rats, the significantly greater AUCs of oral sildenafil were mainly due to the decrease in intestinal metabolism of sildenafil in addition to decrease in hepatic metabolism compared with controls. The above results could explain the greater *F* values in LC and LCD rats than controls (Table 10).

In conclusion, in LC, DM, and LCD rats, the CLs (AUCs) of intravenous sildenafil were significantly slower (greater) and the metabolite ratios were significantly smaller than controls. This could have mainly been due to the slower hepatic metabolism of sildenafil than controls. In LC and LCD rats, the magnitudes of increase in the AUCs of oral sildenafil and decrease in metabolite ratios were greater than those after the intravenous administration. This could have mainly been due to the decrease in intestinal extraction of sildenafil because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats. The changes in hepatic CYP2C and 3A subfamilies in patients with DM alone and with LC did not seem to be reported. Moreover, the pharmacokinetic studies on sildenafil in patients with diabetes with and without LC also did not seem to be reported. In patients with LC, the changes in AUC of *N*-desmethylsildenafil (Murihead et al., 2002) were different from those in the present LC rats. Thus, the present experimental data in rats

should be extrapolated with care to the human situation.

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국 문 초 록

당뇨를 유도한 랫드에서는 간의 Cytochrome P450(CYP) 효소 단백질 발현이 변화되는 것으로 알려져 있으며, 다양한 약물들이 CYP 효소에 의해 대사되는 것으로 보고되고 있다. 당뇨 환자에서는 간경화 또한 유병율이 상대적으로 높은 것으로 보고되어 있으나, 당뇨와 간경화를 동시에 유도한 랫드 모델에서의 CYP 효소 변화와 약물의 약동학 양상과의 상관성에 대한 연구가 보고되지 않았다. 본 연구에서는 Streptozotocin 으로 유발한 당뇨 랫드 모델(DM 랫드)과, N-dimethylnitrosamine 으로 유발한 간경화 랫드 모델(LC 랫드) 및 두 가지 질환을 동시에 유발한 당뇨-간경화 랫드 모델(LCD 랫드)에서의 CYP 효소 변화와 chlorzoxazone (CZX), oltipraz(OLT), sildenafil 를 투여하였을 때 약동학적 변화를 관찰하였다.

첫번째로, CZX 은 주로 간에 존재하는 CYP2E1 에 의해 6-hydroxychlorzoxazone (OH-CZX)로 대사된다. LCD 랫드의 간 CYP2E1 의 단백질 발현율이 대조군 대비 124%증가하였으나, 시험관 내 간 마이크로좀을 이용한 대사 시험계에서 OH-CZX 의 생성율을 측정한 결과 CL_{int} 는 두 군에서 유사하게 나타났다. 따라서, OH-CZX 의 생성양상은 LCD 와 대조군 랫드에서 비슷할 것으로 예상되며, CZX 를 정맥(20 mg/kg) 및 경구(50 mg/kg) 투여하여 약동학적 특성을 비교하였다. CZX 를 정맥투여시 OH-CZX 의 AUC 값은 대조군과 LCD 랫드에서 각각 571±85.8, 578±413 μg·min/ml 으로, 경구투여시 1540±338 및 2170±1078 μg·min/ml 으로 나타나 두 군에서 유사한 결과를 얻었다.

두 번째로 OLT 또한 주로 간에서 대사되며, 랫드의 CYP1A1/2, 2B1/2, 2C11, 2D1, 3A1/2 효소와 관련되어 있다고 알려져 있다. 대조군과 비교하였을 때, LCD 랫드의 CYP1A 간 효소 발현은 증가하였으나, CYP2C11 및 3A 는 감소하였고, CYP2B1/2 와 2D 는 변화가 없었다. 시험관 내 간 마이크로좀을 이용한 in vitro 대사 시험계에서 OLT 의 소실율을 측정한 결과 CL_{int} 는 DM>대조군>LCD>LC 순으로 나타났다. OLT 를 정맥 (10 mg/kg) 및 경구 (30 mg/kg) 투여하여 OLT 의 AUC 값을 비교한 결과, 대조군과 LCD 랫드에서 각각 정맥투여시 249±32.9 μg·min/ml, 350±132 μg·min/ml 으로, 경구투여시 286±78.7 및 438±164 으로 나타나 부분적으로 대조군 수치로 돌아오는 경향을 보였다.

마지막으로 sildenafil 은 주로 랫드의 간 CYP2C11 및 3A1/2 에 의해 N-desmethylsildenafil 로 대사되는 것으로 보고되었다. 시험관 내 간 마이크로좀을 이용한 대사 시험계에서 N-desmethylsildenafil 의 생성율을 측정한 결과 CL_{int} 는 대조군>DM>LCD>LC 순으로 나타났다. sildenafil 을 정맥 (10 mg/kg) 및 경구 (20 mg/kg) 투여하여 sildenafil 의 AUC 값을 비교한 결과, 대조군, LC, DM, LCD 랫드에서 각각 정맥투여시 577±34.9, 1700±399, 890±92.8 및 1310±229 μg·min/ml 으로, 경구투여시 64.7±22.4, 2010±1300, 202±151, 1380±909 μg·min/ml 으로 나타났다. 정맥 투여의 결과는 sildenafl 의 낮은 extraction ratio 특성을 고려하였을 때, LC 및 LCD 랫드에서 CYP2C11 과 3A 간효소 발현의 감소와 DM 랫드에서의 2C11 간효소 발현 감소에 기인한 것으로 판단된다. 정맥 투여 결과와 비교하였을 때, 경구 투여에서 LC, LCD 랫드의 AUC 값이 대조군에 비해 더 크게 차이가 나는 것은 위장관

CYP2C11 의 감소로 인하여 흡수과정에서 위장관내 대사가 감소되었기 때문인 것으로 판단된다.

이상에서 당뇨와 간경화를 동시에 유도한 랫드 모델에서의 CYP isozyme 의 발현 변화는 약물들의 약동학에 영향을 미친다는 것을 확인할 수 있었다.

주제어: Chlorzoxazone, Oltipraz, Sildenafil, 약동학, 간경화 랫드 모델, 당뇨–간경화 랫드 모델, Cytochrome P450

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