EFFECTS OF ACUTE RENAL FAILURE ON THE PHARMACOKINETICS OF CHLORZOXAZONE IN RATS

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ABSTRACT:

The purpose of this study is to report the changes of CYP2E1, CYP1A2, CYP2B1/2, CYP2C11, CYP3A23, and CYP3A2 expression and pharmacokinetics and tissue distribution of chlorzoxazone (CZX) and 6-hydroxychlorzoxazone (OH-CZX) in rats with acute renal failure induced by uranyl nitrate (U-ARF), and the role of CYP3A23 and CYP3A2 in the formation of OH-CZX in rats with U-ARF. In rats with U-ARF, CYP2C11 decreased to 20% of control, whereas CYP2E1 and CYP3A23 increased 2.3 and 4 times, respectively, compared with control. But expression of CYP1A2 and CYP2B1/2 was not changed by U-ARF. After i.v. administration of CZX at a dose of 20 mg/kg to rats with U-ARF, the areas under the plasma concentration-time curve from time 0 to time infinity (AUCs) of CZX and OH-CZX were significantly smaller and greater, respectively, than those in control rats. In rats with U-ARF, CZX was below the detection limit at 120 min in all rat tissues studied, whereas it was detected in all tissues of control rats at both 30 and 120 min. However, in control rats, OH-CZX was below the detection limit at both 30 and 120 min in all rat tissues except kidney, whereas it was detected in all tissues of rats with U-ARF at both 30 and 120 min. Based on results from supporting experiments with DDT and 2,2-bis(4-chlorophenyl)1,1-dichloroethylene treatment of rats, the contribution of CYP3A23 and CYP3A2 to the enhanced formation of OH-CZX in rats with U-ARF is likely to be negligible.

Chlorzoxazone [5-chloro-2(3H)-benzoxazolone; CZX1], once used as a skeletal muscle relaxant for the treatment of painful muscle spasms (Chen and Yang, 1996), primarily undergoes hydroxylation to form 6-hydroxychlorzoxazone (OH-CZX) catalyzed mainly by CYP2E1 (Conney and Burns, 1960; Peter et al., 1990). OH-CZX is then rapidly glucuronidated and excreted in urine (Conney and Burns, 1960; Desiraju et al., 1983). CZX has been suggested for use as a chemical probe to assess the activity of CYP2E1 in vitro and in vivo due to the good correlation between formation rate of OH-CZX and CYP2E1 activity in rats (Rockich and Blouin, 1999).

The pharmacokinetic changes of many drugs (mainly excreted either via renal excretion or by hepatic metabolism) have been reported in rats with acute renal failure induced by uranyl nitrate (U-ARF). For example, the total area under the plasma concentration-time curve from time 0 to time infinity (AUC) was significantly greater, and the time-averaged total body (CL), renal (CLR), and/or nonrenal (CLNR) clearances were significantly slower after i.v. administration of the following drugs (or compounds) to rats with U-ARF: methotrexate (Park et al., 1996); vancomycin (Engineer et al., 1981); DA-1131, a new carbapenem (Kim SH et al., 1998); azosemide (Park et al., 1998); diltiazem (Lee et al., 1992); amiodarone (Fruncillo et al., 1981); tetraethylammonium bromide and para-aminohippurate (Lin and Lin, 1988); salicylic acid (Liu et al., 1996); and M1, an active metabolite of a new anthracycline, DA-125 (Kim YG et al., 1996). The pharmacokinetic differences of the above-mentioned drugs (or compounds) have mainly been explained due to differences in the formation of conjugates or liver and/or kidney impairments in rats with U-ARF based on plasma and urine chemistry data and/or tissue microscopy (Kim SH et al., 1998, and references therein). However, the AUC and CL of cyclosporin (Lee and Ku, 1998), l-propranolol...
(Lee and Ku, 1999), YJA-20379-8, a new proton pump inhibitor (Kim HJ et al., 1998), doxorubicin (Adriamycin) (Lee et al., 1996), and tacrolimus (Son et al., 2000) were not significantly different between control rats and rats with U-ARF.

It has been reported that although CYP2E1 is the major form metabolizing CZX to OH-CZX, human CYP1A2 and CYP3A4 are involved in CZX hydroxylation in vitro (Carriere et al., 1993; Ono et al., 1995; Gorski et al., 1997; Shimada et al., 1999). Hence, the changes of CYP2E1, CYP1A2, CYP2B1/2, CYP2C11, CYP3A2, and CYP3A23 in rats with U-ARF were evaluated. We report that the increase in formation of OH-CZX in rats with U-ARF after i.v. administration of CZX at a dose of 20 mg/kg, a substrate of CYP2E1, could be mainly due to induction of CYP2E1 in the rats.

Materials and Methods

Chemicals. Alkaline phosphate-conjugated donkey anti-goat IgG was supplied from Invitrogen (Carlsbad, CA). The standard [3H-3-aminophenyl sulfone [an internal standard of high-performance liquid chromatography (HPLC) assay for CZX and OH-CZX] were obtained from Sigma/RBI (Natick, MA) and Aldrich Chemical Co. (Milwaukee, WI), respectively. Uranyl nitrate was used from BDH Chemicals (Poole, Dorset, UK). Other chemicals were of reagent grade or HPLC grade and, therefore, were used without further purification.

Animals. For the molecular biology and pharmacokinetics studies, 8-week-old male Sprague-Dawley rats (weighing 275–330 g) were purchased from Charles River (Atsugi, Japan). The rats were randomly divided into two groups, control rats and rats with U-ARF. All rats were provided with food (Sam Yang Company, Seoul, South Korea) and water ad libitum, and were maintained in a light-controlled room (light, 7:00 AM–7:00 PM; dark, 7:00 PM–7:00 AM) at a temperature of 22 ± 2°C and a humidity of 55 ± 5% (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University, Seoul, South Korea). Each rat was individually housed in a metabolic cage (Tecniplast, Varese, Italy) under the supply of filtered pathogen-free air and water ad libitum.

Induction of Acute Renal Failure in Rats by Uranyl Nitrate Injection. Uranyl nitrate (the uranyl nitrate powder was dissolved in 0.9% NaCl-injectable solution to make a concentration of 0.5%), 1 ml/kg (5 mg/kg), was injected once via the tail vein of each rat to induce acute renal failure (Kim SH et al., 1998, and references therein). The same volume of 0.9% NaCl-injectable solution was injected into control rats.

Preparation of Microsomal Proteins. Hepatic microsomes of rats with U-ARF and rats treated with DDE and DDT and their respective control rats were prepared by differential centrifugations at 10,000g for 30 min and then at 100,000g for 90 min. Microsomes were washed in a pyrophosphate buffer and stored in a 50 mM Tris-acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol as described previously (Kim SG et al., 1996). Protein contents were assayed (Lowry et al., 1951) and the subcellular preparations were stored in a −70°C freezer (ReuvoULT 1490 D-N; Kendro Laboratory Products, Asheville, NC) until use.

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to the previously published procedures (Kim SG et al., 1996). Microsomal proteins were separated by a 7.5% gel and electrophoretically transferred to a nitrocellulose paper. The nitrocellulose paper was incubated with anti-CYP1A2, -CYP2B1/2, -CYP2C11, -CYP2E1, and -CYP3A23 antibody (Kim SG et al., 1996), followed by incubation with alkaline phosphate-conjugated secondary antibody, and developed using 5-bromo-4-chloro-3-indolyolphosphate and 4-nitroblue tetrazolium chloride, or an ECL chemiluminescence detection kit.

Northern Blot Hybridization. Specific cDNA probes for the CYP1A2, CYP2B1, CYP2B2, CYP2C11, CYP2E1, CYP3A2, and CYP3A23 genes were amplified by reverse transcription-polymerase chain reaction using the selective primers (Kim and Novak, 1993), cloned in the pGEM+T vector (Promega), as described previously (Cho et al., 1999). Total RNA was isolated using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction according to the method of Puissant and Houdeleine (1999). Northern blot analysis was carried out according to the procedure described previously (Kim and Cho, 1996). Briefly, total RNA isolated from the liver was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formamide and transferred to a nitrocellulose paper. The nitrocellulose paper was baked in a vacuum oven at 80°C for 2 h. The blot was hybridized as described previously (Kim and Cho, 1996). Membranes were washed in 2× standard saline citrate and 0.1% SDS for 10 min at 42°C, twice, and in 1× standard saline citrate and 0.1% SDS for 10 min at 42°C, twice. Membranes were finally washed in the solution containing 0.1× standard saline citrate and 0.1% SDS for 60 min at 55°C. After quantification of mRNA levels, the membranes were stripped and rehybridized with a labeled probe complementary to 18S rRNA to quantify the amount of RNA loaded on the membranes.

Scanning Densiometry. Scanning densiometry of Northern and Western blots was performed with Image Scanning and Analysis System (Alpha Innotech Corporation, San Leandro, CA) to assess the expression of CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A2, and CYP3A23. Data were analyzed using the SigmaStat program (SPSS Science, Chicago, IL).

Measurement of Vmax, Km, and CLmax for the Formation of OH-CZX in Hepatic Microsomes. On the 5th day, the livers of control rats (n = 4) and rats with U-ARF (n = 4) were homogenized in an ice-cold buffer of 0.154 M KC1/50 mM Tris-HCl in 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 9700g for 30 min and the supernatant fraction was further centrifuged at 100,000g for 90 min. The microsomal pellet was resuspended in a buffer of 154 M KC1/50 mM Tris-HCl in 1 mM EDTA, pH 7.4. Protein content was measured using the reported method (Lowry et al., 1951). The Vmax (the maximum velocity) and Km (the Michaelis-Menten constant, the concentration at which the rate is one-half of Vmax) for the formation of OH-CZX were determined after incubating the above microsomal fraction (equivalent to 0.2 mg protein), 10 μl of CZX (to have substrate concentrations of 1, 2.5, 5, 10, 50, 100, 125, 200, and 500 μM), and 1 mM NADPH in a final volume of 1 ml of 0.1 M Tris-HCl, pH 7.4, in a water bath shaker kept at 37°C and at a rate of 50 oscillations per minute. The reaction was terminated by the addition of 0.1 ml of ice-cold 20% trichloroacetic acid after a 20-min incubation. The OH-CZX formed was determined by HPLC analysis (Frye and Stiff, 1996). The kinetic constants (Km and Vmax) for the formation of OH-CZX were calculated using the Lineweaver-Burk plot (Lineweaver and Burk, 1934) by linear regression and the method of least squares. Intrinsic OH-CZX formation clearance (CLmax) was calculated by dividing the Vmax by the Km.

A CYP3A2 inducer, DDE (dissolved in corn oil), 1 mg/kg/day, was administered (total oral volume was 0.6 ml) once using a feeding tube (Sierra-Santoyo et al., 2000), and a CYP3A1/23 inducer, DDE (dissolved in corn oil), 100 μg/kg/day, was administered (total oral volume was 0.6 ml) for 7 consecutive days using a feeding tube (You et al., 1999). The same volume of vehicle was administered to each control rat. On the 2nd day (DDE-treated rats, n = 3, and their controls, n = 5) and 8th day (DDE-treated rats, n = 6, and their controls, n = 5), hepatic microsomal fractions were collected. Similar experiments were also performed at CZX concentrations of 2, 5, 10, 50, and 100 μM.

Pretreatment of Rats. In the early morning on the 4th day after i.v. administration of uranyl nitrate or 0.9% NaCl-injectable solution, the jugular vein and the carotid artery of each rat were cannulated with polyethylene tubing (Clay Adams, Parsippany, NJ) while each rat was under light ether anesthesia. Both cannulas were exteriorized to the dorsal side of the neck where each cannula terminated with long Silastic tubes (Dow Corning, Midland, MI). Both Silastic tubes were inserted into a wire sheath to allow free movement of the rat. Each rat was housed individually in a rat metabolic cage (Daejong Scientific Company, Seoul, South Korea) and was allowed to recover from light ether anesthesia for 24 h (to minimize the effect of ether on CYP2E1 activity) before the study began. It has been reported that ether anesthesia alone increased expression of CYP2E1 by 40% as determined by p-nitrophenol hydroxylase activity (Liu et al., 1993), and altered expression of CYP2E1 by solvents completely returned to that of control 24 h after solvent withdrawal (Roberts et al., 1994). Each rat was not restrained during the experimental period.
Intravenous Study. The following study was performed at the 5th day (n = 5 for each group). The 24-h urine was collected for the measurement of creatinine level with cationization of blood vessels. Plasma was collected for the measurement of total proteins, albumin, urea nitrogen, creatinine, glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) levels (analyzed by Green Cross Reference Laboratory, Seoul, South Korea). The whole kidney and liver of each rat were excised, rinsed, or perfused 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-eosin staining.

On the 5th day, CZX (CZX powder was dissolved in a minimum amount of 1 N NaOH at a dose of 20 mg/kg was administered by i.v. infusion over 1 min via the jugular vein (total injection volume was approximately 0.6 ml) of another set of control rats (n = 9) and rats with U-ARF (n = 9). An approximately 0.12-ml aliquot of blood sample was collected via the carotid artery at 0 (to serve as a control), 1 (at the end of the infusion), 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 min after i.v. administration of the drug. After centrifugation, a 0.05-ml aliquot of plasma sample was stored in a −70°C freezer until HPLC analysis of CZX and OH-CZX (Frye and Stiff, 1996). An approximately 0.3-ml aliquot of heparinized 0.9% NaCl-injectable solution (20 units/ml) was used to flush the cannula immediately after each blood sampling to prevent blood clotting. At the end of 8 h, each metabolic cage was rinsed with 20 ml of distilled water, and the rinses were combined with the 8-h urine. After measuring the exact volume of the combined urine, two 0.05-ml aliquots of combined urine samples were stored in a −70°C freezer until HPLC analysis of CZX (Frye and Stiff, 1996). At the same time, as much blood as possible was collected via the carotid artery, and each rat was sacrificed by cervical dislocation. At the same time, the entire gastrointestinal tract (including its contents and feces) was removed, transferred into a beaker containing 50 ml of 0.1 N NaOH (to facilitate the extraction of CZX), and cut into small pieces using scissors. After stirring with a glass rod, two 0.2-ml aliquots of the supernatant were collected from each beaker and stored in a −70°C freezer until HPLC analysis of CZX (Frye and Stiff, 1996).

Tissue Distribution After Intravenous Administration. On the 5th day, CZX (the same solution as used in the i.v. study) at a dose of 20 mg/kg was also administered intravenously to control rats (n = 5 for each time) and rats with U-ARF (n = 5 for each time). At 30 and 120 min after the end of i.v. infusion of the drug, as much blood as possible was collected via the abdominal artery, and each rat was sacrificed by cervical dissection. Blood samples were centrifuged immediately and plasma was collected. Approximately 1 g of each kidney, heart, muscle, lung, stomach, small intestine, liver, mesentery, large intestine, fat, and spleen was excised, rinsed with cold 0.9% NaCl-injectable solution to minimize blood remaining in the tissues, blotted with paper tissue, and homogenized with 4 volumes of distilled water using a tissue homogenizer (Ultra-Turrax T25 Janke and Kunkel; IKA-Labortechnik, Staufen, Germany). After centrifugation, two 0.05-ml aliquots of the 9000g supernatant fraction were stored in the freezer until HPLC analysis of CZX and OH-CZX (Frye and Stiff, 1996). All the procedures were conducted at 4°C in an ice-bath.

HPLC Analysis of CZX. The concentrations (or amounts) of CZX and OH-CZX in the above biological samples of control rats were analyzed by the reported HPLC method (Frye and Stiff, 1996). Briefly, a 0.1-ml aliquot of 0.2 M sodium acetate buffer (pH 4.75) and 200 units of β-glucuronidase dissolved in 0.1 ml of isotonic Sorenson phosphate buffer (pH 7.4) were added to a 0.05-ml aliquot of biological sample. The mixture was manually mixed and incubated in a water bath shaker kept at 37°C and at a rate of 50 oscillations per minute for 2 h. After incubation, a 0.05-ml aliquot of methanol containing 10 μl of 3-aminophenyl sulfone (the internal standard) was added and vortex-mixed, and a 1-ml aliquot of diethyl ether was added. The mixture was shaken for 10 min and then centrifuged at 2000g for 10 min. The upper organic layer was transferred to a clean tube and evaporated at 37°C under a stream of nitrogen. The residue was reconstituted with a 0.1-ml aliquot of the mobile phase, and a 0.05-ml aliquot was injected directly onto the HPLC column. The mobile phase, 0.1 M ammonium acetate/acetoniiterile/tetrhydrofuran (72:22:5.5, v/v/v) was run at a flow rate of 1 ml/min. A UV detector set at 283 nm monitored the column effluent. The retention times of OH-CZX, 3-aminophenyl sulfone, and CZX were approximately 6, 10, and 18 min, respectively. The detection limits of CZX and OH-CZX in plasma were both 0.1 μg/ml. The

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 5)</th>
<th>U-ARF (n = 5)</th>
</tr>
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<tbody>
<tr>
<td>Initial body weight* (g)</td>
<td>291 ± 7.42</td>
<td>283 ± 6.71*</td>
</tr>
<tr>
<td>Final body weight* (g)</td>
<td>313 ± 13.0</td>
<td>268 ± 4.47**</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total proteins (g/dl)</td>
<td>5.88 ± 0.172</td>
<td>6.00 ± 0.529</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.42 ± 0.0420</td>
<td>2.27 ± 0.252</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>16.8 ± 1.19</td>
<td>107 ± 23.5**</td>
</tr>
<tr>
<td>GOT (IU/l)</td>
<td>85.6 ± 12.4</td>
<td>194 ± 13.4**</td>
</tr>
<tr>
<td>GPT (IU/l)</td>
<td>33.8 ± 4.96</td>
<td>21.0 ± 6.19</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.440 ± 0.0490</td>
<td>3.50 ± 0.866**</td>
</tr>
<tr>
<td>Kidney weight (% of body weight)</td>
<td>0.736 ± 0.0608</td>
<td>1.01 ± 0.0671**</td>
</tr>
<tr>
<td>Liver weight (% of body weight)</td>
<td>3.39 ± 0.0990</td>
<td>2.89 ± 0.1577**</td>
</tr>
<tr>
<td>CL(Cr) (ml/min/kg)</td>
<td>5.46 ± 0.455</td>
<td>0.0592 ± 0.391*</td>
</tr>
</tbody>
</table>

* Measured just before the injection of uranyl nitrate or 0.9% NaCl-injectable solution.
** Measured just before starting the experiment (on the 5th day after the injection of uranyl nitrate or 0.9% NaCl-injectable solution).
* P < 0.01 and * P < 0.001 compared with control.

Pharmacokinetic Analysis. The AUC was calculated by the trapezoidal rule-extrapolation method; this method utilized the logarithmic trapezoidal rule (Chiou, 1978) for the calculation of the area during the declining plasma-level phase and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to time infinity was estimated by dividing the last measured plasma concentration by the terminal rate constant.

Standard methods (Gibaldi and Perrier, 1982) were used to calculate the CL, area under the first moment of the plasma concentration-time curve, mean residence time (MRT), and apparent volume of distribution at steady state (Vss) (Kim et al., 1993). The harmonic mean method was employed to calculate the mean values of Vss (Chiou, 1979), terminal half-life (Eatman et al., 1977), and CL (Chiou, 1980).

Gluomerlar filtration rate was calculated by measuring creatinine clearance (CL(Cr)) assuming that kidney function was stable during the 8-h experimental period. The CL(Cr) was calculated by dividing the total amount of creatinine excreted in 8-h urine by AUCU-Cr, 8 h of creatinine in plasma.

Statistical Analysis. For the molecular biology studies, one-way analysis of variance was used to assess significant difference (P < 0.05) between two groups of rats. For each significant effect, the Newman-Keuls test was used for comparisons of each group mean. For the pharmacokinetic studies, a P value of less than 0.05 was considered to be statistically significant using the unpaired t test. All data were expressed as mean ± S.D.

Results

Induction of Acute Renal Failure in Rats. In rats with U-ARF, impaired kidney function was observed; the serum levels of urea nitrogen (537% increase) and creatinine (695% increase) were significantly higher, kidney weight (percentage of body weight) was significantly heavier (37.2% increase), and CL(Cr) was significantly slower (98.9% decrease) than those in control rats (Table 1). Impaired kidney function in rats with U-ARF was also supported by kidney microscopy; acute tubular necrosis (proximal tubules) was observed. Hepatic function also seemed to be impaired in rats with U-ARF; the plasma level of GOT was significantly higher (127% increase) and liver weight was significantly lighter (14.7% decrease) than those in control rats (Table 1). In rats with U-ARF, mild liver impairment seemed to be observed by liver microscopy; mild regeneration
changes were observed. However, no significant changes were observed in both kidney and liver of control rats based on tissue microscopy. Note that body weight gain decreased significantly in rats with U-ARF (from 283 to 268 g) compared with that in control rats (from 291 to 313 g) (Table 1).

Effect of ARF on CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A2, and CYP3A23. In rats with U-ARF, the expression of CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A2, and CYP3A23 was monitored. Immunoblot analysis showed that expression of CYP2C11 decreased to 20% of control, whereas CYP2E1 and CYP3A23 increased 2.3 and 4 times, respectively, as compared with control (Fig. 1). The expression of CYP1A2 and CYP2B1/2 was not changed by U-ARF (Fig. 1). Northern blot analysis also revealed that in rats with U-ARF, the CYP2E1 mRNA increased three times and the CYP2C11 mRNA decreased to 25% of control; however, those of CYP1A2, CYP2B1, CYP2B2, and CYP3A2 were comparable to control levels (Fig. 2). These results were consistent with those of Western blot analysis. Interestingly, however, the CYP3A23 mRNA was not increased in rats with U-ARF (Fig. 2). Hence, the induction of CYP3A23 by ARF may result from protein stabilization (i.e., a decrease in protein turnover).

Measurement of $V_{\text{max}}$, $K_m$, and CL$_{\text{int}}$ for the Formation of OH-CZX in Hepatic Microsomes. In rats with U-ARF, the $V_{\text{max}}$ (59.7% increase) for the formation of OH-CZX in hepatic microsomal fraction was significantly faster than that in control rats; however, the $K_m$ values were comparable between two groups of rats (Table 2). Hence, the CL$_{\text{int}}$ for the formation of OH-CZX in the hepatic microsomal fraction was significantly faster (42.3% increase) in rats with U-ARF (Table 2), suggesting that formation of OH-CZX could be faster in rats with U-ARF. In rats treated with DDE, only CL$_{\text{int}}$ was significantly faster (30.9% increase) than that in control rats (Table 2). However, in rats treated with DDT, the $V_{\text{max}}$, $K_m$, and CL$_{\text{int}}$ were comparable to those in control rats (Table 2).

Pharmacokinetics of CZX and OH-CZX After Intravenous Administration of CZX. After i.v. administration, the plasma concentrations of CZX declined in a polyexponential fashion for both groups of rats with significantly lower levels in rats with U-ARF (Fig.
control rats (120 min after i.v. administration of CZX at a dose of 20 mg/kg to its glucuronide conjugates. the elimination of CZX could be the formation rate of OH-CZX and control rats (Table 2). Hence, in control rats, the rate-limiting step for after i.v. administration of CZX was relatively short, 39.5 min, in rats was not measured in the present study, the half-life of OH-CZX unchanged CZX excreted in 8-h urine and recovered from gastrointestinal tract at 8 h were below the detection limit for both groups of rats, suggesting that the contribution of CL_{int} to CL of CZX was negligible and contribution of gastrointestinal excretion (including biliary excretion) of CZX to CL_{NR} of CZX was also negligible. CZX was stable for up to a 3-h incubation (in a water bath shaker kept at 37°C and at a rate of 50 oscillations per minute) in human gastric juices obtained from five patients before surgery at Seoul National University Hospital (Seoul, South Korea) having pH values of 6.77, 6.2, 1.8, 2.72, and 5.1 at a CZX concentration of 5 μg/ml (the gastric juice samples were not incubated with β-glucuronidase); more than 95.2% of the spiked amount of CZX was recovered after a 3-h incubation. Hence, the CLs of CZX listed in Table 3 could represent the metabolic clearances of CZX in rats. Therefore, it could be concluded that the significantly faster CL of CZX in rats with U-ARF could be due to faster metabolism of CZX to form OH-CZX in the rats.

Formation of OH-CZX after i.v. administration of CZX was fairly rapid for both groups of rats; the OH-CZX was detected in plasma from the first blood sampling time (1 min) and reached its peak at approximately 30 min (Fig. 3B). After reaching its peak, the plasma concentrations of OH-CZX declined in an apparent monoexponential fashion for both groups of rats (Fig. 3B). The plasma concentrations of OH-CZX in rats with U-ARF were significantly higher (Fig. 3B), the percentages of i.v. dose of CZX declined in 8-h urine and recovered from gastrointestinal tract at 8 h (due to gastrointestinal excretion including biliary excretion, Gh_{int}) was 0.583 and 1.81% (expressed in terms of i.v. dose of CZX) for control rats and rats with U-ARF, respectively (Table 3). Although the terminal half-life of OH-CZX after i.v. administration of OH-CZX to rats was not measured in the present study, the half-life of OH-CZX after i.v. administration of CZX was relatively short, 39.5 min, in control rats (Table 2). Hence, in control rats, the rate-limiting step for the elimination of CZX could be the formation rate of OH-CZX and its glucuronide conjugates.

Tissue Distribution After Intravenous Administration. The mean tissue-to-plasma (T/P) ratios of CZX and OH-CZX at 30 and 120 min after i.v. administration of CZX at a dose of 20 mg/kg to control rats (n = 5, each time) and rats with U-ARF (n = 5, each time) are shown in Figs. 4 and 5, respectively. In control rats, the T/P ratios of CZX were less than unity in all the tissues studied except liver and kidney at 30 min and liver, kidney, and mesentery at 120 min (Fig. 4), suggesting that affinity of rat tissues studied to CZX was not consid-

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**TABLE 2**

<table>
<thead>
<tr>
<th>Control</th>
<th>U-ARF</th>
<th>DDT-treated</th>
<th>DDE-treated</th>
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<tbody>
<tr>
<td>V_{max} (mmol/min/mg protein)</td>
<td>3.45 ± 0.281</td>
<td>5.51 ± 0.620</td>
<td>4.31 ± 0.214</td>
</tr>
<tr>
<td>Km (μM)</td>
<td>137 ± 33.9</td>
<td>149 ± 16.4</td>
<td>203 ± 56.2</td>
</tr>
<tr>
<td>CL_{int} (ml/min/mg protein)</td>
<td>0.0260 ± 0.0005</td>
<td>0.0370 ± 0.00172</td>
<td>0.0216 ± 0.00079</td>
</tr>
</tbody>
</table>

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**TABLE 3**

Mean ± S.D. body weight, terminal half-life, AUC (μg · min/ml), CL (ml/min/kg), and Glu_{int} (% of an i.v. dose) at ASPET Journals on March 31, 2017 dmd.aspetjournals.org Downloaded from d}

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![Graph](image-url)
erable. In rats with U-ARF, the T/P ratios of CZX were significantly greater in heart, lung, stomach, and large intestine, and significantly smaller in small intestine and fat at 30 min (Fig. 4). Interestingly, in rats with U-ARF, the CZX was below the detection limit at 120 min in all rat tissues studied; however, it was detected in all tissues of control rats at both 30 and 120 min (Fig. 4). The above data suggested that formation of OH-CZX increased in rats with U-ARF compared with that in control rats. In control rats, the OH-CZX was below the detection limit in all rat tissues studied except kidney, whereas it was detected in all rat tissues studied at both 30 and 120 min in rats with U-ARF (Fig. 5). The above data suggested that formation of OH-CZX increased in rats with U-ARF more than in control rats.

Discussion

As mentioned earlier, the changes of the reported pharmacokinetic parameters of many drugs (or compounds) in rats with U-ARF were explained mainly due to differences in the formation of conjugates or impaired kidney and/or liver function. However, the relationship between pharmacokinetic changes of drugs and changes in P450 isozymes in rats with U-ARF seemed not to be published. Recently, it was found from our laboratories that the concentrations of CZX and OH-CZX in plasma after i.v. administration of CZX were unconjugated CZX (not CZX glucuronides) and conjugated OH-CZX, respectively. The percentage of hepatic extraction ratio (hepatic first-pass effect) was roughly estimated by dividing the nonrenal clearance (as mentioned earlier, the CL of CZX in Table 3 could represent the metabolic clearance of CZX) by the reported hepatic plasma flow in rats assuming that nonrenal clearance of CZX was attributed solely to the liver (hence the percentage represents maximal possible value) (Lee and Chioi, 1983). The hepatic plasma flow in rats was estimated based on the reported hepatic blood flow of 30.4 ml/min/kg (Davies and Morris, 1993) and hematocrit of approximately 45% (Mitruka and Rawnsley, 1981) in rats. The hepatic extraction ratio of CZX in control rats was approximately 53.6%, indicating that CZX is an intermediate hepatic extraction ratio drug. Hence, its hepatic clearance depends on CLint, free (unbound in plasma protein) fraction of CZX, and hepatic blood flow (Wilkinson and Shand, 1975). The significantly faster CL of CZX in rats with U-ARF could be due to both significantly faster CLint (42.3% increase, Table 3) and greater unbound fraction (54.5% increase; the plasma protein binding values of CZX were 75.6 and 62.3% for

![Graph showing T/P ratios of CZX at different time points and tissues](image-url)
control rats and rats with U-ARF, respectively, based on equilibrium dialysis technique).

It is well known that CZX is mainly metabolized to OH-CZX primarily by CYP2E1 in rats (Conney and Burns, 1960; Peter et al., 1990). Since the mRNA levels of CYP2E1 increased significantly (three times) in rats with U-ARF (Fig. 2), it was expected that the formation of OH-CZX could increase in the rats. This was supported by the following results. First, the plasma concentrations of CZX were significantly lower (Fig. 3A) and the resultant AUC of CZX was significantly smaller (Table 3) in rats with U-ARF, and this could be due to significantly faster CL of CZX in the rats (Table 3). Second, the plasma concentrations of OH-CZX were significantly higher (Fig. 3B) and the resultant AUC of OH-CZX was significantly greater (Table 3) in rats with U-ARF. Moreover, the AUC<sub>OH-CZX</sub>/AUC<sub>CZX</sub> fraction increased from 0.374 in control rats to 7.54 in rats with U-ARF. However, the effect of acute renal failure-induced accumulation of OH-CZX on the increase in the ratio could not be totally ruled out, since acute renal failure reduces urinary excretion of drugs (compounds). Third, in rats with U-ARF, the CZX was below the detection limit at 120 min in all rat tissues studied, whereas it was detected in all tissues of control rats at both 30 and 120 min (Fig. 4). However, in rats with U-ARF, OH-CZX was detected in all rat tissues studied at both 30 and 120 min, whereas it was below the detection limit at both 30 and 120 min in all tissues of control rats except kidney (Fig. 5). Finally, the in vitro CL<sub>int</sub> in hepatic microsomal fraction was significantly faster in rats with U-ARF (Table 2). The above data indicated that formation of OH-CZX increased in rats with U-ARF due to increase in CYP2E1 in the rats. It has also been reported (Chung et al., 2002) that in rats with U-ARF, CYP2E1 was induced two to four times compared with that in control rats.

Although CYP2E1 is the major form metabolizing CZX to OH-CZX, it has been reported (Curriere et al., 1993; Ono et al., 1995; Gorski et al., 1997; Shimada et al., 1999) that human CYP1A2 and CYP3A4 are involved in CZX hydroxylation. The effect of CYP1A2 on formation of OH-CZX in rats with U-ARF could be ruled out since no significant change in CYP1A2 was obtained in the rats (Figs. 1 and 2). In rats with U-ARF, the expression of CYP3A23 increased 4-fold (Fig. 1). Human CYP3A4 and rat CYP3A1 (CYP3A23) proteins have 73% homology (Lewis, 1996). It has been reported that CYP3A1 (CYP3A23) (Mahneke et al., 1997) or CYP3A2 (Debri et al., 1995; Hoen et al., 2001; Rekka et al., 2002) is the major CYP3A protein in rats. Hence, the role of CYP3A23 and CYP3A2 on the formation...
CYP3A2. Although CYP2E1 induction by ARF accompanied the increase in its mRNA, CYP3A2A3 induction in rats with ARF failed to concomitantly increase in its mRNA. This was in contrast to the effects of DDE, which we used as a comparative agent that induces both CYP2E1 and CYP3A2A3 with increases in their mRNAs. These results indicate that the induction of CYP3A2A3 by ARF may be mediated with the stabilization of the CYP3A2A3 protein instead of the transcriptional activation of the gene. In addition, we observed that ARF markedly suppressed the expression of CYP2C11, which is involved in the metabolism of testosterone as an endogenous substrate (i.e., testosterone 16α,2α-hydroxylation) (Biagini et al., 1999). Thus, the changes in the expression of P450 isofoms are likely to result in differences in the metabolic profiles of endogenous substances, therapeutic agents, and environmental chemicals.

References
CYP3A2A3 mRNA level reported (Li et al., 1995) that there was no effect on CYP2E1 in rats on the formation of OH-CZX was almost negligible in rats. It has been reported (Li et al., 1995) that there was no effect on CYP2E1 in rats treated with DDE. Multiple immunoblottings were carried out with different immunosorbent preparations. B, Northern blot analysis was performed to determine the mRNA levels for CYP2E1, CYP3A2, and CYP3A2A3 in total RNA fractions (20 μg each). Equal loading of the mRNA was confirmed by rehybridization of the blots with the probe for 18S rRNA.

of OH-CZX in rats was measured by treatment with DDT (an inducer of CYP3A2; Sierra-Santoyo et al., 2000) or DDE (an inducer of CYP3A2A3; You et al., 1999). In rats treated with DDT, the Vmax, Km, and CLint for the formation of OH-CZX were not significantly different compared to those in the control rats (Table 2), indicating that the effect of CYP3A2A3 on the formation of OH-CZX was almost negligible in rats. It has been reported (Li et al., 1995) that there was no effect on CYP2E1 in rats treated with DDE. Based on Western blot analysis, the expression of CYP3A1A2 and CYP2E1 was comparable as weighed against control (data not shown). Moreover, in rats with U-ARF, CYP3A2A3 mRNA level was not changed (Fig. 2). However, in rats treated with DDE, the CLint was significantly faster than that in control rats (Table 2). Immunoblot analysis showed that the expression of CYP2E1 and CYP3A2A3 increased 3.18 and 2.98 times, respectively, in rats treated with DDE compared with control (Fig. 6A). Northern blot analysis also revealed that in rats treated with DDE, the mRNA level of CYP2E1, CYP3A2, and CYP3A2A3 increased 3.30, 3.68, and 3.94 times, respectively, compared with control (Fig. 6B). The effect of CYP3A2 on the formation of OH-CZX in rats with U-ARF could be ruled out as explained above. Since CYP2E1 is the major form for metabolizing CZX to OH-CZX, the increased formation of OH-CZX in rats with U-ARF could mainly be due to induction of CYP2E1 in rats. However, the effect of induction of CYP3A2A3 could not be totally ruled out.

We found that either ARF or DDE induced CYP2E1 and