EFFECT OF THE ACUTE-PHASE RESPONSE ON THE PHARMACOKINETICS OF CHLORZOXAZONE AND CYTOCHROME P-450 2E1 IN VITRO ACTIVITY IN RATS

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ABSTRACT:

The acute-phase response is known to produce alterations in hepatic cytochrome P-450 (CYP) expression. Lipopolysaccharide (LPS), a well known inducer of acute-phase response decreases hepatic CYP2E1 in vitro activity in rats. This study was designed to determine if LPS administration produced alterations in the pharmacokinetics of chlorzoxazone (CZN), a marker for CYP2E1 expression. Sprague-Dawley rats were administered a single i.p. injection of LPS (5 mg/kg) or saline control approximately 24 h before a single i.v. bolus dose of CZN (15 mg/kg). Serial blood samples were collected over a 120-min period to quantitate CZN plasma concentrations and protein binding. In addition, livers were removed and processed for evaluating in vitro CYP2E1 protein concentrations and activity. Systemic clearance decreased by 35% in LPS-treated rats, whereas half-life and steady-state volume of distribution increased by 167 and 66%, respectively. The plasma free-fraction of CZN increased 2-fold after LPS treatment. The CZN intrinsic clearance decreased in LPS rats by 71% compared with control values. The CYP2E1 liver microsomal activity decreased between 55 and 75% along with a 41% decrease in CYP2E1 protein concentration. The CZN intrinsic clearance was significantly correlated with both the CZN and p-nitrophenol liver microsomal activity (r = 0.87 and r = 0.91, respectively). This study demonstrated that LPS administration produced expected reductions in the in vivo intrinsic clearance of CZN, and these changes were highly correlated with in vitro activity studies. In addition, LPS produced significant increases in the steady-state volume of distribution of CZN secondary to reductions in its plasma protein binding.

Gram-negative sepsis is a major cause of morbidity and mortality in the critically ill patient. Gram-negative sepsis initiates a systemic inflammatory response syndrome after systemic cytokine release. This systemic inflammatory reaction often leads to multiple organ dysfunction syndrome, which contributes to significant morbidity and mortality in these patients (Beal and Cerra, 1994).

One of the body's first defenses initiated in response to Gram-negative sepsis is the acute-phase response (APR). The APR is triggered when resident macrophages, mainly the Kupffer cells of the liver, circulating monocytes, and macrophages are stimulated to release factors known as cytokines. These cytokines are noted to trigger a large inflammatory response and are also responsible for physiologic changes to include the release of proteolytic enzymes such as C-reactive protein from hepatocytes. The known proinflammatory cytokines include interleukin (IL)-1, IL-6, and tumor necrosis factor. Changes that occur during the APR have been associated with altering the disposition and metabolism of certain drugs that are biotransformed through the cytochrome P-450 (CYP) pathway in healthy volunteers (Shedlofsky et al., 1994, 1997). In addition, hospitalized patients with viral infections were observed to have altered theophylline pharmacokinetics (Chang et al., 1978).

To assess the effect of the APR on the CYP-mediated drug metabolism, animal rodent models have been used. In rats, the administration of endotoxin [lipopolysaccharide (LPS)] resulted in a suppression of mRNA levels, apoprotein, and liver microsomal activity (Morgan, 1989, 1993; Sewer et al., 1996; Roe et al., 1998). Also, the administration of the individual cytokines, IL-1, IL-6, and tumor necrosis factor caused a similar decrease in the CYP drug-metabolizing enzymes (Chen et al., 1992, 1995; Morgan et al., 1994).

Ethanol-inducible CYP2E1 is one of the CYPs that is altered in rats after LPS administration. Levels of mRNA decreased to 20% of control 6 h after LPS administration to male Sprague-Dawley rats and did not return to baseline until 48 h (Sewer et al., 1996). Furthermore, the apoprotein and the liver microsomal activity were both decreased compared with control and did not return to baseline until 72 h (R.B., unpublished observations).

The changes after LPS administration in the CYP drug-metabolizing enzymes, which includes CYP2E1, is well documented in the rodent model. A very important interpretation of this model to critically ill patients would be to understand how the APR could alter the pharmacokinetics of drugs whose metabolism is mediated by the CYP enzymes. Patients that experience an APR, such as sepsis, trauma, and burn patients, typically receive medications that are metabolized by CYP enzymes. This could result in drug concentrations reaching the toxic range, which would further complicate these already critically ill patients. At present, it has not been determined how the in vitro changes in the CYP enzymes in the rodent model would effect the...
pharmacokinetic parameters of a drug that is metabolized by the CYP enzymes. Therefore, the main objectives of this study was to determine the pharmacokinetic changes of a probe drug for CYP2E1 after LPS administration and also to determine if there is a correlation between the in vitro and the in vivo changes.

Experimental Procedures

Male Spague-Dawley rats (body weight, 375–390 g) were obtained from Harlan (Indianapolis, IN). The animals were maintained on a 12-h light/dark cycle and were given free access to food (Purina Rodent Chow) and drinking water. The animals were acclimated for a minimum of 5 days before being randomly assigned to an experimental group. On the first day of the study, a pair of rats was placed under general anesthesia with a mixture of a 3:1 ratio of ketamine and xylazine (i.v.; d., 0.51 mm; o., 0.94 mm) was surgically inserted into the right jugular vein of each rat. On day 2, animals randomized to the treatment group received an i.p. injection of LPS at a dose of 5 mg/kg at 9:00 AM and control animals received an i.p. injection of an equal volume of saline. Food was removed at 12:00 AM from the animals but free access to water was permitted. A solution of chlorozoxazone (CZN) was prepared as described previously (Chen and Yang, 1996). On day 3, a bolus dose of CZN (15 mg/kg) was administered at 9:00 AM to each animal via the jugular cannula. Approximately 0.6 ml of blood was taken from the jugular cannula before and at 10, 30, 60, 90, and 120 min. Plasma was removed and stored at -20°C until analysis. Twenty-eight hours post-LPS administration, animals were placed under light anesthesia with 3:1 ketamine and xylazine (i.v.) and sacrificed by exsanguination by cardiac stick in which plasma was obtained for CZN protein-binding analysis.

Materials. Chemicals and reagents were purchased from suppliers as follows: Fort Dodge Laboratories (Fort Dodge, IA) ketamine; Butler Company (Columbus, OH) xyline; Difco Laboratories (Detroit, MI) LPS B E. coli 055:B5/LD50 = 28.7 mg/kg; Sigma Chemicals (St. Louis, MO) CZN, umbel-liferone, butylated hydroxytoluene, and p-nitrophenol; Fischer Scientific (Fair-lawn, NJ) acetic acid, acetonitrile, and diethyl ether; Research Biochemicals International (Natick, MA) 6-hydroxy-CZN.

Microsomes were prepared from livers that were excised and placed in cold 0.154 M KCl/0.25 M potassium phosphate buffer to remove blood. Liver samples were homogenized in 4 volumes of cold 0.154 M KCl/0.25 M potassium phosphate, pH 7.4, with the addition of butylated hydroxytoluene (10 μg/l tissue) as an antioxidant before homogenization. Livers were homogenized using a Teflon grinder and spun to separate the microsomal fraction. The resulting microsomal pellet was resuspended in 0.25 M sucrose/0.02 M Tris buffer, pH 7.4, and stored at -80°C until analysis. Total protein concentration in rat liver microsomes was determined by the method of Lowry et al. (1951) using BSA as the standard.

The microsomal fractions were used in the determination of total CYP concentrations by spectral analysis. The concentration of CYP in these fractions was determined spectrophotometrically by the method of Omura and Sato (1964) and based on an extinction coefficient of 91 mM⁻¹ cm⁻¹.

The rat plasma concentration of CZN was measured by a modified HPLC method by Peter et al. (1990). Briefly, the HPLC system was a Shimadzu SIL-6B autoinjector with UV detection. The mobile phase was isocratic that consisted of 78% of a 0.25% acetic acid solution and 22% acetonitrile. All solutions were of HPLC grade. The flow rate was set at 1 ml/min. The UV detector wavelength was set at 287 nm. The CZN and umbelliferone (internal standard, 0.078 mM) were separated using a LC-18 reversed phase (5 μm, 15 cm × 4.6 cm) column (Supelco, Bellefonte, PA). The extraction method was modified from Frye and Stiff (1996). Briefly, 0.1 ml of plasma from each sample was used for analysis and 1 ml of 10 mM sodium acetate buffer, pH 4.5, was added to the plasma. Diethyl ether (4 ml) was added to each sample and standard and vortexed for 1 min. The samples were centrifuged and the organic phase was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase and 20 μl was injected onto the HPLC system.

Protein binding of CZN in rat plasma was determined by an ultrafiltration technique. A 1.5 ml plasma sample was spiked to give a final concentration of 50 μg/ml. The samples were incubated for 15 min at 37°C and 1 ml aliquots were placed into an ultrafiltration device (Amicon, Cambridge, MA) equipped with an ultrafiltration membrane. The samples were centrifuged at 3200 rpm at 37°C for 30 min or until 250 μl filtrate was collected. Concentration of CZN in the filtrate and the aliquot for total CZN concentration was determined by the HPLC method described above.

The p-nitrophenol hydroxylase activity was determined spectrophotometrically by measuring the increase in absorbance at 546 nm and using the extinction coefficient ε546 = 9.53 mM⁻¹ cm⁻¹ as described by Koop (1986).

The formation of 6-OH CZN was determined from the method of Peter et al. (1990) with modifications from Jayyosi et al. (1995). The microsomal protein was incubated and 6-OH CZN was measured as described previously (Warren et al., 1999).

Liver microsomal samples were analyzed for CYP2E1 by noncompetitive enzyme linked immunoassortment assay. Our laboratory has shown that rat liver microsomes produce a single band on a Western blot analysis and comigrated the same distance with purified CYP2E1 (R. B., unpublished data). Liver microsomes were diluted in phosphate carbonate/bicarbonate buffered saline pH 9.6. Subsequently, 0.25 μg of total microsomal protein and known concentrations of microsomal standards (Gentest, Woburn, MA) were plated onto a 96-well flat bottom plate (Corning Glass Works, Corning, NY). The microsomal standards produced a signal that was linear through the concentration range of 62.5 to 1,000 pmol/ml. Proteins were blocked and incubated for 1 h with 50% horse serum and 50% Tris-buffered saline with 0.1% Tween 20. The plate was washed and incubated with polyclonal goat anti-rat antibody for CYP2E1 (Gentest, Woburn, MA). Next, the plate was washed and incubated with alkaline phosphatase-conjugated monoclonal rabbit anti-goat IgG antibody. After the end of the incubation, the plate was washed and p-nitrophenol phosphate substrate (ELISA Technologies, Lexington, KY) was added. The plate was analyzed at 405 nm over 30 min at 37°C with a Biotek EL340 microplate reader for color formation. Samples were quantified by extrapolation from the fitted standard curve.

Data Analysis. The plasma concentration-time curves for CZN were fitted to a one-compartment model and were analyzed by nonlinear least-squares regression analysis using the computer program PK Analysis (Micromath Scientific, Salt Lake City, UT).

\[ C_t = C_0 e^{-kt} \]  
\[ V_{ss} = \frac{D \cdot \text{AUMC}}{\text{AUC}^2} \]  

where AUMC represents the area under the moment curve. All results are given as means ± S.D.

Statistical Analysis. A two-tailed t test was used assuming equal variances for all comparisons except for the protein binding analysis where unequal variances were assumed. The alpha value was set a priori at p < .05. The correlations were determined by using a nonlinear least-squares regression analysis software program (Prism; GraphPad Software, San Diego, CA.).

Results and Discussion

The mean semilog plot of the plasma CZN concentration-versus-time profile followed a simple monoexponential decline for both the LPS and control groups (Fig. 1). This clearly illustrates that the administration of LPS to male Sprague-Dawley rats significantly alters the pharmacokinetics of CZN. Table 1 lists the pertinent pharmacokinetic parameters for the control and treated groups. The decrease in liver microsomal activity as determined by CZN (75%) and p-nitrophenol (55.3%) plus a decrease in the CYP2E1 protein (41%) accounted for a decrease of 35 and 71% in CIs and intrinsic clearance of CZN, respectively. This decrease in CIs agrees with similar results in which LPS administration to rats significantly decreased the CIs for
antipyrine and metronidazole (Kokwaro et al., 1993) and also R and S-pindolol (Hasegawa et al., 1989). However, the liver microsomal activity was not determined in either of these studies. Because CZN is a restrictively cleared (low E) drug, it was important to measure the change, if any, in the protein binding of CZN. In the present study, the free-fraction in the LPS-treated animals increased compared with control values. Protein binding parameters in control animals from this study were similar to what has been shown in the literature (Yasuhara and Levy, 1988). These results may explain why only a 35% change in the Cls was observed whereas the liver microsomal activity decreased by 55 to 75%. On the other hand, the intrinsic clearance of CZN decreased by approximately 71% in the LPS animals compared with control, which falls within the range of the 55 to 75% decrease in the liver microsomal activity.

The LPS-treated group had a 66% increase in the Vss of CZN compared with controls. This may be explained by the 2-fold increase of the free-fraction in the LPS treatment group. This is supported by the result in Fig. 2D, which shows a strong correlation between the Vss and the plasma CZN free-fraction ($r = 0.95$, $p < .05$). It has not been reported in the literature as to which protein CZN is bound in the systemic circulation. However, preliminary results from our laboratory determined that CZN is highly bound to albumin (R.B., unpublished observation). In the present study, the albumin decreased by 13% in LPS animals. Although this is a modest decrease in the albumin concentration, it has been determined that albumin has a decrease in binding capacity and number of binding sites ($n$) after LPS administration (Nadai et al., 1993; Wang et al., 1993). Nadai et al. (1993) showed that the extracellular fluid of rats does not change after LPS administration; this suggests that the change in $V_{ss}$ is a result of a change in binding. The influence of protein binding on the $V_{ss}$ was

![Fig. 1](image1.png)

*Fig. 1. The semilog plot of the CZN plasma concentration-versus-time curve in control (▲) and LPS (■) treated rats after an i.v. bolus dose of CZN (15 mg/kg). Each point represents the mean ± S.D. ($n = 7$).

![Fig. 2](image2.png)

*Fig. 2. A, plot of the CZN intrinsic clearance versus the CZN liver microsomal activity in both control and LPS rats ($r = 0.97; p < .05$). B, plot of the CZN intrinsic clearance versus the $p$-nitrophenol liver microsomal activity in both control and LPS rats ($r = 0.91; p < .05$). C, plot of the CZN intrinsic clearance versus the CYP2E1 protein concentration in both control and LPS animals ($r = 0.64; p > .05$). D, plot of the CZN Vss versus the plasma CZN free-fraction in both control and LPS rats ($r = 0.95; p < .05$). Each of the lines represents the least-squares regression analysis.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cls (ml/min)</td>
<td>3.51 ± 0.24</td>
<td>2.3 ± 0.58*</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td>30.5 ± 1.78</td>
<td>81.4 ± 17.4*</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>0.39 ± 0.04</td>
<td>0.66 ± 0.12*</td>
</tr>
<tr>
<td>Cli (ml/min)</td>
<td>35.3 ± 4.04</td>
<td>10.2 ± 2.36*</td>
</tr>
<tr>
<td>$f_v$</td>
<td>0.1 ± 0.01</td>
<td>0.22 ± 0.04*</td>
</tr>
</tbody>
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Data are expressed as means ± S.D. of $n = 7$ animals.

* $p < .05$
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References


also illustrated with R- and S-pindolol, which showed a decrease in the $V_{m}$ of both enantiomers along with a decrease in the free-fracion after LPS administration (Hasegawa et al., 1989). It is well established that pindolol is mainly bound to α-1 acid glycoprotein, which increased in this study from 13.4 to 75.3 mg/dl after LPS administration to rats (Hasegawa et al., 1989). In other reports, there was shown to be an increase in the $V_{m}$ of enprofylline after LPS administration to rats without a change in the free-fracion (Nadai et al., 1995).

Half-life increased over 2.5-fold in the LPS-treated group compared with controls. This dramatic increase in half-life is the result of the increase in $V_{m}$ and decrease in Cls, because half-life is directly proportional to $V_{m}$ and indirectly proportional to Cls (Rowland and Tozer, 1980). This is an excellent illustration of the profound effect that $V_{m}$ has on the half-life of a drug because the change in Cls was not large enough to explain the 2.5-fold increase in the half-life of CZN for this study.

The in vitro hepatic microsomal CYP changes after LPS administration were as expected. Animals that received LPS demonstrated lower total CYP concentrations [0.29 ± 0.04 nmol/mg microsomal (LPS) versus 0.67 ± 0.07 nmol/mg microsomal protein (control), $p < 0.05$, lower CYP2E1 protein concentrations [440 ± 93 pmol/mg microsomal protein (LPS) versus 748 ± 145 pmol/mg microsomal protein (control), $p < 0.05$, and lower microsomal enzyme activities for CZN [0.44 ± 0.1 nmol/min (LPS) versus 1.78 ± 0.33 nmol/min (control), $p < 0.05$] and $p$-nitrophenol [1.05 ± 0.4 nmol/min (LPS) versus 2.37 ± 0.29 nmol/min (control), $p < 0.05$, compared with controls.

One of the main objectives of this study was to determine if there was a correlation between in vitro and in vivo parameters measured in this study. When the intrinsic clearance of CZN was plotted against the liver microsomal activity, it resulted in a statistically significant correlation (Fig. 2 A and B). The liver microsomal activity measured by CZN (Fig. 2A; $r = 0.97$, $p < 0.05$) had a slightly higher correlation than did the activity measured by $p$-nitrophenol (Fig. 2B; $r = 0.91$, $p < 0.05$). CYP2E1 protein concentration was also plotted against the intrinsic clearance of CZN (Fig. 2C); however, it did not result in a statistically significant correlation ($r = 0.64$, $p > 0.05$). There are a couple of reasons why these two values may not have correlated. First, the anti-CYP2E1 antibody recognizes not only the haloprotein but also the apporoten, which is inactive. Second, there is strong evidence that not only CYP2E1 is responsible for CZN’s metabolic fate but also the subfamilies of CYP3A and CYP1A each may play a minor role in the metabolism of CZN in the rat (Carriere et al., 1993; Jayyosi et al., 1995; Yamaomaki et al., 1995).

In conclusion, this study showed that the degree of down-regulation of the CYP2E1 enzyme activity resulted in a significant change in the pharmacokinetic parameters of CZN. Furthermore, there was a strong correlation between liver microsomal activity and intrinsic clearance of CZN. This latter result may be important to investigators that utilize the animal rodent model to study changes in the CYP drug-metabolizing enzymes. Measurable changes of CYP in liver microsomal preparations, according to our results, may be highly correlated to the intrinsic clearance.