IMPAIRED MICROSOMAL OXIDATION OF THE ATYPICAL ANTIPSYCHOTIC AGENT CLOZAPINE IN HEPATIC STEATOSIS

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Abbreviations: CLZ, clozapine; CLZ N-oxide, clozapine N-oxide; CYP, cytochrome P450; fu, unbound fraction; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; NASH, nonalcoholic steatohepatitis; norCLZ, norclozapine (N-desmethylclozapine); OA, orotic acid.

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Abstract

Hepatic lipid infiltration (steatosis) is a complication of the metabolic syndrome and can progress to non-alcoholic steatohepatitis (NASH) and severe liver injury. Microsomal cytochrome P450 (CYP) drug oxidases are downregulated in experimental steatosis. In this study we evaluated the separate and combined effects of lipid accumulation and CYP downregulation on the microsomal oxidation of the antipsychotic agent clozapine (CLZ), the use of which is associated with an increased incidence of the metabolic syndrome. Several important drug oxidizing CYPs were downregulated and the formation of \(N\)-desmethyl-CLZ (norCLZ) and CLZ \(N\)-oxide was decreased in microsomal fractions from orotic acid (OA)-induced early steatotic rat liver. Inclusion of lipids extracted from steatotic, but not control, liver decreased the free concentration of CLZ in microsomes and suppressed norCLZ formation; CLZ \(N\)-oxidation was unchanged. Triglycerides increased in steatotic liver to 15-fold of control, while increases in the monounsaturated oleic acid to 10-fold of control and total polyunsaturated and saturated fatty acids to four- and fivefold of control also occurred. Addition of triglycerides containing esterified \(\omega\)-6 and \(\omega\)-3 fatty acids inhibited the microsomal formation of norCLZ, but not CLZ \(N\)-oxide; triglycerides esterified with unsaturated and monounsaturated fatty acids were inactive. Thus, drug oxidation may be suppressed in steatosis by CYP downregulation and the accumulation of polyunsaturated fatty esters. In contrast, the activity of the flavin-containing monooxygenase that mediates CLZ \(N\)-oxidation, was unimpaired. Lipid deposition in livers of patients with the metabolic syndrome may necessitate dosage adjustments for toxic drugs, including CLZ.
Introduction

Hepatocellular lipid accumulation, or steatosis, is a central feature of non-alcoholic steatohepatitis (NASH). NASH is the hepatic component of the metabolic syndrome that is associated with obesity and type II diabetes. The metabolic syndrome is becoming increasingly prevalent in western society and is characterized by impaired glucose and lipid metabolism (Marchesini et al., 2001). Initially believed to be a benign lesion, it is now considered that early hepatic steatosis precedes the development of NASH, which may progress to cirrhosis in up to 25% of patients. Steatosis may also be induced by a number of hepatotoxic drugs (Berson et al., 1998). In recent studies it has emerged that therapy with certain antipsychotic agents, most notably the atypical antipsychotic clozapine (CLZ), is associated with an increased risk of developing hypertriglyceridemia and other manifestations of the metabolic syndrome (Lamberti et al., 2006; Meyer and Koro 2004).

Although the role of steatosis in the pathogenesis of chronic liver disease is now accepted, the detailed effects of lipid infiltration on important aspects of hepatic function, especially drug biotransformation, remain underexplored. CYP dysregulation has been well documented in animal models of steatosis, obesity and NASH (Leclercq et al., 1998; Su et al., 1999; Su et al., 2005). Suppression of important xenobiotic and steroid hydroxylating CYPs from the 2C and 3A subfamilies was proportional to the extent of hepatic lipid deposition in rats (Su et al., 1999) and force-fed geese (Leclercq et al., 1998). Drug clearance may also be impaired in humans with obesity, early steatosis and NASH (Fiatarone et al., 1991; Blouin and Warren 1999; Cheymol, 2000). However, changes in the extrahepatic distribution of lipophilic drugs in obese subjects may alter their pharmacokinetic and pharmacodynamic profiles and necessitate dose adjustments (Casati and Putzu, 2005). Sequestration in adipose tissue of obese subjects may decrease the unbound concentration of basic drugs that are available for biotransformation by CYP enzymes (McLure et al., 2000). This issue is of particular importance for drugs such as CLZ, which is associated with a high
incidence of neutropenia, cardiotoxicity and a range of other organ toxicities (Maggs et al., 1995; Pacher and Kecskemeti, 2004).

The present study tested the impact of lipid accumulation in early steatotic liver induced by dietary orotic acid (OA) on CYP expression and the microsomal oxidation of the lipophilic antipsychotic drug CLZ. The principal findings to emerge were that several important drug-metabolizing CYPs were downregulated and the accumulated lipids in steatotic liver markedly decreased the formation of the CYP-derived metabolite norCLZ, whilst the formation of CLZ N-oxide by the flavin-containing monooxygenase was spared. The addition of triglycerides containing esterified polyunsaturated fatty acids, but not saturated or monounsaturated fatty acids, to microsomal incubations mediated these inhibitory effects on norCLZ formation. Thus, the accumulation of specific triglycerides in steatosis exerts differential effects on oxidative pathways of CLZ elimination in liver that may necessitate dosage adjustments during drug therapy in individuals with the metabolic syndrome.
Methods

Chemicals and biochemicals. CLZ and its metabolites N-desmethyl-CLZ (norCLZ) and CLZ N-oxide, OA, 7-ethoxyresorufin, resorufin and biochemicals were from Sigma Aldrich (Castle Hill, NSW, Australia) or Roche Pty Ltd (Castle Hill, NSW, Australia). The authentic triglycerides tristearin (which contains three esterified stearoyl residues), triolein (three esterified oleoyl residues), triarachidonin (three esterified arachidonoyl residues) and tridocosahexaenoin (three esterified docosahexaenoyl residues) were purchased from Larodan Fine Chemicals AB (Malmo, Sweden). Reagents for electrophoresis were from Bio-Rad (Richmond, CA). HPLC-grade solvents were from Rhone-Poulenc (Baulkham Hills, NSW, Australia) and analytical reagents were from Ajax (Sydney, NSW, Australia). Hyperfilm-MP, Hybond-N+ filters, and reagents for enhanced chemiluminescence were from Amersham Pharmacia Biotech (Castle Hill, NSW, Australia).

Animal Treatments. Dietary manipulation studies in rats were approved by Institutional Animal Ethics Committees and followed the guidelines of the Australian National Health and Medical Research Council. The control synthetic diet contained (per kilogram) sucrose (600 g), casein (200 g), cellulose (110 g), corn oil (40 g), salt mixture (#4179 ICN Biochemicals, Seven Hills, NSW, Australia; 40 g), ICN vitamin fortification mixture (10 g), α-tocopherol (20 mg) and retinyl acetate (8.7 mg). OA (1%) was added to the control diet for the induction of hepatic steatosis. Inbred male Wistar rats (~200 g) were obtained from the institutional animal facility and received one of the experimental diets for 21 days (Su et al., 1999). Animals had free access to diet and water.

Preparation of hepatic subcellular fractions and extraction of hepatic lipid fractions. Animals were killed under enflurane anesthesia. Livers were removed and perfused with cold saline and a segment was fixed in Milloneg’s buffered formalin for histology. The remainder of the tissue was immersed in liquid nitrogen and then stored at –70°C for
subsequent lipid analysis and for the isolation of hepatic microsomal fractions (Murray et al., 1983).

Hepatic lipids were extracted with chloroform and methanol containing 1% Triton X-100, as described by Janssen and Meijer (1995), and dried over silica gel in a vacuum desiccator. Total triglyceride esters, cholesteryl esters and phospholipids in liver extracts were estimated with commercial kits (Peridochrom GPO-PAP, Chol MPR1 and PL MPR2 kits, respectively; Boehringer-Mannheim GmbH, Mannheim, Germany); whereas total free fatty acid content was determined using the Roche Half-micro kit (Roche Diagnostics, Castle Hill, NSW, Australia). The contents of individual fatty acids in hepatic extracts were quantified as follows. Extracts were hydrolyzed by treatment with 0.1 M sodium hydroxide. The resultant fatty acids were methylated with boron trifluoride and quantified by gas chromatography using authentic fatty acid methyl esters, with a C14:0 methyl ester standard (Varian 3800 GC instrument coupled to a BPX70 column; 60m, 0.25 x 0.25µm) and flame ionization detection (Weston Food Laboratories, Sydney, NSW, Australia). The limit of quantification of esterified fatty acids was at least 0.1%.

Microsomal oxidation assays. Incubations contained rat hepatic microsomes (0.05 mg microsomal protein; Lowry et al., 1951), CLZ (100 µM), NADPH (1 mM) and potassium phosphate buffer (pH 7.4, 0.1 M). Reactions were conducted for 30 min at 37°C and were terminated by the addition of 1 ml of ice-cold 0.1% formic acid. Product formation was linear under these conditions. Kinetic studies of CLZ oxidation were conducted over the concentration range 5-100 µM and analyzed by non-linear regression using Prism 4 (GraphPad Software, Inc., San Diego, CA).

Lipids extracted from control or steatotic liver were equilibrated with microsomes with gentle shaking at room temperature for 5 min and then preincubated at 37°C for 2 min, prior to the estimation of CLZ oxidation as described above. Lipid was included in
microsomal incubations at ratios of 1:1 and 25:1 with microsomal protein to reflect the proportions found in control and steatotic liver, respectively. In further experiments authentic triglycerides (100 µg and 1 mg of tristearin (18:0), and 100 and 250 µg of triolein (18:1), triarachidonin (20:4, ω-6), or tridocosahexaenoin (22:6, ω-3) were added to microsomes, in combination with a control liver lipid extract (ratio 1:1) to directly assess the effect of augmentation with esterified fatty acids on CLZ oxidation.

7-Ethoxyresorufin O-deethylation activity was determined in a FLUOstar fluorescence plate reader (BMG LabTech, Mt. Eliza, VIC, Australia) and cytochrome c reductase activity was measured spectrophotometrically using methods described previously (Murray et al., 1997).

Analysis of CLZ and its metabolites by HPLC. A solid phase method using Oasis HLB SPE cartridges (containing 60 mg of divinylbenzene and N-vinylpyrrolidone sorbent; Waters Corp, Milford, MA) was developed for the extraction of CLZ metabolites from microsomal incubations. The cartridges were conditioned with 1 ml of methanol, followed by 1 ml of distilled water. Following microsomal incubations, samples were diluted with 1 ml of 0.1% formic acid in water and applied to the cartridges, which were washed with 0.1% of formic acid in water (1 ml), and then two aliquots (0.5 ml) of 0.1% formic acid in methanol, followed by acetonitrile (0.3 ml). The mean recoveries of norCLZ were 96±2% (mean±SEM; at 10 µM) and 97±10% (at 1 µM), respectively, and for CLZ N-oxide were 96±11% (at 10 µM) and 100±8% (at 1 µM), respectively. Standard calibration curves were linear over the range 0.25–12.5 µM for both CLZ N-oxide (r=0.999) and norCLZ (r=0.998); interassay variability was <10%.

The HPLC system consisted of an LC-10AT isocratic pump, SIL-10A vp autosampler, SPD-10A vp dual wavelength UV-VIS detector and a C-R8A Chromatopac integrator (Shimadzu Corporation, Kyoto, Japan). Separations were achieved on a Synergy Fusion-RP
polar embedded C$_{18}$ column (250 x 4.6 mm, particle size 4 µm; Phenomenex Australia Pty Ltd, Pennant Hills, NSW) operating at 38°C (Shimadzu CTO-10 AC vp column oven). The mobile phase consisted of 3:2:5 acetonitrile:methanol:ammonium acetate buffer (20 mM, pH 5.0), containing N,N-dimethyloctylamine (0.4 ml/l), the flow rate was 1.0 ml/min, and UV detector was set at 260 nm. The retention times of authentic norCLZ, doxepin (internal standard) and CLZ N-oxide were 11.4, 13.1 and 16.9 min, respectively.

**Estimation of the unbound fraction of CLZ (f$_{u}$) in microsomal systems.** The effects of hepatic lipid on the f$_{u}$ of CLZ in microsomal fractions were assessed by ultrafiltration. Thus, lipid extracts from control or steatotic liver (0.05 or 1.25 mg total lipid) were incubated with microsomal fractions (0.05 mg) and CLZ (100 µM) for 30 min. The incubate was transferred to the sample reservoir of a Microcon YM-10 device (10 kD molecular weight cut-off; Millipore Corporation, Bedford, MA), equilibrated for 10 min and then centrifuged at 8,000 g for 30 min. CLZ in the ultrafiltrate was measured by HPLC as described above. All final estimates were corrected for non-specific binding of CLZ to the YM-10 device (19±3%).

**Sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblotting.** Microsomes (5 µg/lane) were incubated at 100°C for 5 min with 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol and electrophoresed on 7.5% polyacrylamide gels (Laemmlli 1971) with minor modifications. Proteins were transferred to nitrocellulose (Towbin et al., 1979) and incubated with anti-CYP IgGs for 120 min (3.7 µg protein/ml). The rabbit anti-CYP2E1 IgG, the anti-CYP2J2 IgG and the goat-anti-CYP1A2 antiserum were provided by Dr M. Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden), Dr D.C. Zeldin (NIEHS, Research Triangle Park, NC) and Prof M.E. McManus (University of Queensland, St Lucia, Australia), respectively, and the properties of the rabbit anti-CYP2C11 and anti-CYP3A IgGs were reported previously (Murray et al., 1992b). Immunoreactive proteins were
detected by enhanced chemiluminescence (Hyperfilm-ECL film) and analyzed by densitometry (BioRad GS-7000, Richmond CA, USA).

**Statistics.** Data are presented as means±SEM from measurements in hepatic fractions from individual animals (n=4-6/group), unless otherwise indicated. Differences between means of two groups were detected with the Student’s t-test. Differences between multiple group means were detected by one-way analysis of variance and Fisher’s PLSD test.
Results

Effect of an OA-containing steatotic diet on liver weight, hepatic lipid accumulation, microsomal CYP Expression and CLZ Oxidation in rats. Consumption of diet was not different between rats that received the OA+steatotic or OA-devoid control diet. Animals that received the OA+steatotic diet gained weight at rates that were slightly lower than those that received the control diet. Thus, rats that received the control and steatotic diets were 134±5% and 120±10% of starting body weights after 21 days of dietary manipulation (p<0.05). Serum bilirubin and γ-glutamyltranspeptidase were increased in rats with early steatosis, consistent with impaired hepatocellular function (Su et al., 1999).

Lipid deposition in the cytoplasm of rat hepatocytes was extensive after intake of the steatotic diet for 21 days. In contrast, steatosis was not evident in liver of animals that received the control diet from which OA was excluded. The pronounced deposition of lipid in steatotic liver led to an increase in relative liver weight (7.6±0.4 versus 5.6±0.2% relative to control; p<0.001). In accord with this finding, total esterified and free fatty acid content was six-fold greater in steatotic liver compared with control (212±15 versus 35±14 mg/g liver; p<0.001). Triglycerides were strikingly increased by intake of the steatotic diet, from 9±1 mg/g liver to 135±9 mg/g liver (p<0.001), with lesser increases in cholesteryl esters (to 4.7-fold of control: 7.4±1.1 versus 1.6±0.2 mg/g liver; p<0.001) and phospholipid esters (to 2.1-fold of control: 36.2±4.3 versus 16.9±1.9 mg/g liver; p<0.001). Thus, proportionately greater quantities of triglycerides were present in steatotic liver.

Immunoblot analysis indicated that expression of rat hepatic microsomal CYP1A, CYP2E1, CYP2C11 and CYP3A proteins was decreased in early steatosis to 27±10% (p<0.05), 68±12% (p<0.05), 53±14% (p<0.01) and 50±6% (p<0.001) of respective control (Fig. 1); in contrast, CYP2J protein expression was unchanged from control.
Consistent with previous reports, when tested over the 5-100 µM concentration range, CLZ was oxidized efficiently in rat liver microsomes to norCLZ and CLZ N-oxide (Bun et al., 1999). $K_m$ values for the formation of these metabolites were determined in control microsomes to be 41±3 µM and 38±5 µM, respectively, and $V_{max}$ values were 1.08±0.06 and 0.59±0.10 nmol/mg protein/min, respectively (Table 1). The $V_{max}$ for norCLZ formation was decreased in microsomes from early steatotic rat liver ($p<0.05$) whereas, in the case of CLZ N-oxidation, the $K_m$ was increased and the $V_{max}$ was decreased. Intrinsic clearances ($V_{max}/K_m$) were decreased to 44% and 33% of control for norCLZ and CLZ N-oxide, respectively (Table 1).

**Impact of hepatic lipid extracts on microsomal CYP-mediated oxidation of CLZ and 7-ethoxyresorufin in vitro.** In the present study the impact of hepatic lipids on microsomal CLZ oxidation was evaluated. Control liver contained 1.0±0.2 mg lipid/mg microsomal protein (mean±SEM, n=6), which increased to 23±5 mg lipid/mg microsomal protein in OA+steatotic liver. Thus, lipid:microsomal protein ratios in control and early steatotic liver were ~1:1 and ~25:1, respectively. As shown in Fig. 2 the addition of a control liver lipid extract, in the ratio with protein that is present in control liver (1:1), had minimal impact on the oxidation of CLZ (100 µM) in microsomes from control liver. In contrast, addition of steatotic liver extracts to control microsomal incubations at the ratio found in steatotic liver (~25:1) substantially decreased the formation of norCLZ to 27% of the activity in the absence of exogenous lipid ($p<0.001$; Fig. 2). In microsomes from early steatotic liver the addition of steatotic lipid extracts (at the 25:1 ratio) similarly decreased norCLZ formation from 0.34±0.09 to 0.022±0.005 nmol/mg protein/min ($p<0.05$).

In contrast with these findings, neither control hepatic lipid (ratio 1:1) nor steatotic liver lipid (ratio 25:1) inhibited microsomal CLZ N-oxide formation in hepatic microsomes from control or steatotic rats (Fig. 2). Thus, there was relative preservation of CLZ N-oxide
formation over norCLZ. For comparative purposes the effects of control lipid extracts at the 25:1 ratio were evaluated and were found to decrease norCLZ and CLZ N-oxide formation to 70±4% and 50±5% of corresponding control. Thus, the nature of the accumulated lipid in early steatosis appears to be a significant determinant of the extent of inhibition. However, it is important to note that the accumulation of control lipids to these levels does not occur in liver.

Consistent with the decreased expression of CYP1A2, the microsomal O-deethylation of 7-ethoxyresorufin was decreased in steatotic liver to 55±11% of control (p<0.05). Further decreases in resorufin formation were noted in hepatic microsomes from control and steatotic rats in the presence of steatotic liver extracts (25:1 ratio) to 31% and 37% of the respective activities in the absence of lipid; control liver lipid extracts (1:1 ratio with protein) were without significant effect. Rates of microsomal cytochrome c reduction were unimpaired by exogenous lipid extracts (not shown).

Unbound CLZ concentrations and CLZ oxidation in microsomal systems containing hepatic lipids. It would be expected that only the unbound fraction (f_u) of CLZ would be available for oxidation by microsomal CYP enzymes. As part of the present study we evaluated the effect of hepatic lipid accumulation on the f_u of CLZ (determined at a concentration of 100 µM). In microsomes from control and early steatotic liver the CLZ f_u was 0.88±0.03 and 0.84±0.01, respectively (Fig. 3). The f_u in control microsomes decreased in the presence of lipid extracted from control liver (tested at the 1:1 ratio found in control liver) to 0.70±0.01 (p<0.001). Steatotic liver extracts, in contrast, markedly decreased f_u CLZ to 0.14±0.01 and 0.10±0.01 (p<0.001) in control and steatotic microsomes, respectively. Thus, hepatic lipid accumulation in steatosis diminished the concentration of CLZ available for microsomal oxidation.
These $f_u$ values were applied to the apparent $K_m$ values that had been determined for microsomal norCLZ and CLZ N-oxide formation in the presence of liver lipid extracts. Thus, the measured $K_m$ values in the presence of control liver lipid (1:1) were 29±6 µM and 27±3 µM, respectively, and application of $f_u=0.70$ (as shown in Fig. 3) produced corrected values of 20±4 µM and 19±4 µM, respectively. $K_m$ values of 880±380 µM and 850±350 µM were determined for norCLZ and CLZ N-oxide formation, respectively, in the presence of steatotic liver lipid (25:1 ratio); these values were decreased to 123±53 µM and 119±49 µM, respectively, after application of CLZ $f_u=0.14$ that was determined previously (Fig. 3). Thus, $f_u$ considerations partially corrected the pronounced effects of exogenous lipids, especially from early steatotic liver, on apparent $K_m$ values for CLZ biotransformation.

**Differential accumulation of fatty acids in control and early steatotic rat liver.**

Extracts from steatotic liver had been found to be relatively enriched in triglycerides, which was confirmed by $^1$H-NMR (not shown). In further experiments we identified the major fatty acids that accumulated in early steatosis. Intake of the steatotic diet for 21 days increased the total amounts of polyunsaturated, monounsaturated and saturated fatty acids in liver. The most striking increase was in the monounsaturated fatty acid content, which increased tenfold from 9.83±0.37 to 99.1±1.7 mg/g liver (p<0.001), with 4- and 5-fold increases, respectively, in total polyunsaturated and saturated fatty acids (p<0.001; Fig 4A). Thus, the steatotic diet increased the proportion of monounsaturated fatty acids from 28.0±1.1% in control liver to 46.7±0.8%. In steatotic liver total ω-6 polyunsaturated fatty acids increased to 4.3-fold of control (42.2±2.3 versus 9.8±0.2 mg/g liver; p<0.001; Fig 4B), whereas ω-3 polyunsaturated fatty acid content was not significantly different from control.

Major quantifiable fatty acids in control liver included palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), elaidic (18:1, the 9-trans-isomer of oleic acid), linoleic (18:2 ω-6), arachidonic (20:4 ω-6) and docosahexaenoic (22:6 ω-3) acids (Table 2); several
other fatty acids were minor constituents of liver extracts. Pronounced increases in the contents of the monounsaturated palmitoleic, oleic and elaidic acids to 7-, 13- and 5.3-fold of respective control were observed in steatotic liver (p<0.001). A striking increase in the ω-6 linoleic acid to 8.6-fold of control was also noted, whereas the unsaturated palmitic acid was increased to 6.3-fold of control (p<0.001; Table 2).

**Direct effects of triglycerides on microsomal CLZ oxidation in vitro.** The possibility that the fatty acid esters that accumulated in early steatosis may contribute to the decrease in the microsomal formation of norCLZ was tested directly. In these studies microsomal incubations incorporating control lipid extracts (1:1 ratio) were augmented with authentic triglycerides that reflected the principal fatty acid classes detected in rat liver. The saturated lipid tristearin (18:0, which contains three esterified stearate residues; 100 and 1000 µg/incubation) and the monounsaturated triolein (18:1; 100 and 250 µg/incubation) produced minimal changes in the formation of norCLZ and CLZ N-oxide (Fig. 5). However, the ω-6 ester triarachidonin (20:4, which contains three arachidonate residues esterified to glycerol; 250 µg/incubation) and the ω-3-fatty acyl triglyceride tridocosahexaenoin (22:6) significantly decreased norCLZ formation. This was selective for norCLZ because the production of CLZ N-oxide was not significantly affected by any of the triglycerides (Fig. 5).
**Discussion**

Hepatic steatosis is an increasing health problem, especially in western society because of its association with obesity and type II diabetes. Early steatosis can progress in a proportion of patients to NASH. However, the consequences of extensive lipid infiltration for many important hepatic functions, including drug disposition and biotransformation are unclear. The OA-fed rat is a model of extensive hepatic steatosis that is uncomplicated by the inflammatory activity that occurs later in the progression to NASH (Su et al., 1999). Accordingly, the model is useful for the study of the consequences of early lipid infiltration on hepatic drug metabolism.

In obesity, changes in drug pharmacokinetics are often observed as a result of altered drug distribution and biotransformation. Apart from the dysregulation of microsomal CYP enzymes, lipid accumulation in adipose tissue can increase the volume of distribution and decrease drug $f_{d}$ and clearance (Blouin and Warren, 1999). In general, therapy with basic lipophilic drugs, such as benzodiazepines, anesthetics and other CNS-active drugs, is complicated in obese subjects (Abernethy et al., 1983; Casati and Putzu, 2005). Dose adjustment may be required with drugs such as CLZ because it has a highly significant toxicity profile and because close monitoring of plasma concentrations during therapy is mandatory. Obesity is frequently associated with hepatic steatosis which could also influence the hepatic distribution of CLZ and decrease its oxidative biotransformation by microsomal enzymes.

Intake of the OA+steatotic diet led to a pronounced increase in the hepatic content of triglycerides and to lesser increases in phospholipids and cholesteryl esters. Triglyceride accumulation in this model is due to increased fatty acid synthesis and impaired lipoprotein transport through the Golgi apparatus. Significant correlations have been observed previously between the extent of hepatic lipid deposition and suppression of CYP-mediated substrate oxidation in models of fatty liver in the rat (Murray et al., 1992a; Su et al., 1999) and the
force-fed goose (Leclercq et al., 1998). The present study found significant decreases in CYP1A2, CYP2C11, CYP2E1 and CYP3A expression in steatotic liver; these are important drug metabolizing CYPs in the rat. A number of nuclear hormone receptors have been shown to regulate CYP genes in human and rodent liver (Honkakoski and Negishi, 2000). Thus, the pregnane X-receptor and constitutive androstane receptor heterodimerize with the retinoid X-receptor to stimulate the transcription of several CYPs. In previous studies we have found that ingestion of the OA-steatotic diet by rats decreases the hepatic expression of the important transcription partner, the retinoid X-receptor (Su et al., 2005). PPARα, another closely related member of the nuclear receptor superfamily, was also down-regulated in early steatosis. Similar alterations in the expression of other nuclear hormone receptors may contribute to CYP dysregulation in steatotic liver.

Decreased CYP1A2 and 3A4 expression has been noted in human liver microsomes and hepatic sections from subjects with steatosis and/or NASH (Weltman et al., 1998; Donato et al., 2006). Importantly, Donato et al., have demonstrated that several CYP mRNAs were down-regulated in hepatocytes, and the corresponding CYP activities decreased, by inclusion of millimolar concentrations of a free fatty acid mixture containing oleate and palmitate (Donato et al., 2006). However, several studies have shown that the predominant lipids that accumulate in clinical and experimental steatosis are triglycerides, in which fatty acids are esterified with glycerol; the accumulation of phospholipids and cholesteryl esters and free fatty acids is somewhat less pronounced. The present study has identified triglycerides esterified with ω-3 and ω-6 polyunsaturated fatty acids as lipids with the potential to inhibit microsomal CYP oxidases. It is noteworthy that CYP1A2 and CYP3A4 have been shown to be important enzymes in norCLZ formation in human liver (Tugnait et al., 1999; Olesen and Linnet, 2001). These findings are also in accord with the decrease in antipyrine clearance that has been reported in NASH patients (Fiatarone et al., 1991). Indeed,
antipyrine clearance in humans has been shown to be dependent on several CYPs, especially CYPs 1A2 and 3A4 (Engel et al., 1996).

In the present study CYP2E1 expression was decreased in hepatic microsomes from rats with early steatosis. Studies in humans with NASH and in the methionine-choline-deficient rat model of NASH have reported an increase in the expression and function of this enzyme (Weltman et al., 1996; Weltman et al., 1998; Emery et al., 2003). The reasons for these discrepancies are unclear but it may be significant that inflammatory activity is a feature of NASH and methionine-choline deficiency, which is absent from OA+steatotic rat liver (Su et al., 1999). Indeed, microsomal activities mediated by CYP2E1 were also decreased in several other models of fatty liver, including the obese zucker rat (Zaluzny et al., 1990), the choline-deficient rat, which exhibits steatosis but not NASH (Murray et al., 1988), and the intragastric high-fat dietary model in the mouse (Deng et al., 2005). Thus, CYP2E1 expression has emerged from a number of studies as a sensitive indicator of pathogenic processes in rat and possibly human liver (Leclercq et al., 1998).

Consistent with CYP downregulation, the oxidation of CLZ to norCLZ was decreased in microsomal incubations from steatotic liver that were conducted in the absence of lipid. The inclusion of control lipid extracts at the level found in control rat liver (1:1 lipid:protein ratio) had minimal effects on CLZ oxidation. However, incorporation of lipid extracts from steatotic liver in microsomal incubations produced further suppressive effects on microsomal CLZ oxidation. Lipid extracts also decreased CLZ fu, which would diminish the concentration of the drug able to penetrate to the CYP active site. A partial correction of apparent Km values that had been determined in the presence of exogenous lipid was produced by application of fu values. Importantly, however, the inhibitory effects of steatotic liver lipids were restricted to CYP-dependent norCLZ formation, whereas CLZ N-oxidation was spared. Rates of CLZ oxidation in early steatosis appear to be decreased because of the combined effects of CYP dysregulation and the accumulation of particular lipids. Indeed,
considerations from CYP immunoquantitation data could underestimate the effect of steatosis on drug clearance.

The relative preservation of CLZ N-oxide formation is consistent with the possibility that some microsomal enzymes may be less susceptible to membrane perturbing effects of polyunsaturated fatty acids esterified in triglycerides. It has been established that the activities of certain CYPs, notably CYP3A enzymes in reconstituted systems, are sensitive to the nature of the lipid environment, possibly because of interactions with other microsomal proteins to facilitate electron transfer during substrate oxidation (Imaoka et al., 1992). Previous findings in this model of early steatosis suggest that CYP3A activity is highly susceptible to changes in hepatic lipid composition produced by dietary means (Su et al., 1999). However, there is little information on how lipids influence the flavin-containing monooxygenase that supports CLZ N-oxidation. In addition, in the present study we did not observe any effect of exogenous lipid or triglycerides on microsomal cytochrome c reduction. However, it remains a possibility that the spatial relationship of CYPs and the flavoprotein NADPH-CYP-reductase may be altered by accumulation of esterified polyunsaturated fatty acids in the vicinity of the endoplasmic reticulum and could result in altered electron flow from NADPH to CYPs.

Quantification of major fatty acids in liver extracts indicated that the proportion of monounsaturated fatty acids increased markedly in early steatotic liver relative to polyunsaturated and saturated fatty acids. By 1H-NMR, monounsaturated fatty acids have also been shown to accumulate in preference to polyunsaturated fatty acids in liver of patients with acute hepatic failure (Pollesello et al., 1996) and non-alcoholic fatty liver disease (Araya et al., 2004). The ratio of monounsaturated:polyunsaturated fatty acids also increased in liver extracts from obese Zucker rats (Serkova et al., 2006). Taken together, the accumulation of monounsaturated fatty acids observed in the present study appears to be common to several forms of hepatic steatosis in humans and experimental animals.
Although the proportion of polyunsaturated fatty acids decreased in steatotic liver, the sixfold increase in total lipid deposition led to an increase in the hepatic content of \(\omega-6\) fatty acids to 4.3-fold of control. This was due principally to the marked accumulation of linoleic acid. Direct testing of authentic lipids indicated that triglycerides esterified with the \(\omega-6\) fatty acid arachidonic acid (triarachidonin), which was used as a model \(\omega-6\)-containing triglyceride, selectively decreased the formation of norCLZ (and not CLZ N-oxide) by rat hepatic microsomes. The triglyceride tridocosahexaenoin, which contains three molecules of the \(\omega-3\) fatty acid docosahexaenoic acid esterified to the triacylglycerol backbone, was also inhibitory toward norCLZ formation in rat liver microsomes. In contrast, even the extensive augmentation of microsomal lipids with unsaturated (tristearin) and saturated (triolein) fatty acids had minimal impact on CLZ oxidation. These findings established the capacity of \(\omega-6\) polyunsaturated fatty acids esterified in triglycerides to selectively decrease CYP activity in rat liver.

Sequestration of drug substrates in subcellular compartments is increasingly recognized as a major limiting factor in rates of drug oxidation and elimination \textit{in vivo}. Drug substrates entering the hepatocyte must permeate the cytoplasm to reach the endoplasmic reticulum and protein and lipid factors may sequester drug nonspecifically. Ultrastructural findings have indicated that, after short-term OA feeding, the accumulated lipids are in close proximity to the endoplasmic reticulum (Novikoff and Edelstein, 1977), which is the principal location of CYPs in the hepatocyte. In the present study it was found that lipids that accumulate in early hepatic steatosis may decrease the \(f_u\) of CLZ, which could decrease the availability of the drug for oxidation by CYPs. Direct evaluation of the inhibitory actions of authentic triglycerides on CLZ oxidation suggested that \(\omega-6\) polyunsaturated fatty acyl esters may be potent inhibitory agents that accumulate in steatosis. The data also support the contention that the activity of certain microsomal enzymes may be refractory to the effects of
these esterified fatty acids, because of the preservation of CLZ N-oxidation. In view of these findings it is now important to evaluate in greater detail how lipid accumulation in human steatosis influences drug efficacy and safety.
Acknowledgements

The gifts of anti-CYP2E1 IgG (M. Ingelman-Sundberg, Karolinska Institute), anti-CYP2J2 IgG (D.C. Zeldin, NIEHS) and goat anti-CYP1A2 antiserum (M.E. McManus, University of Queensland) are gratefully acknowledged. The advice on lipid analysis provided by Dr Colin Duke and the technical assistance of Dr Gloria Su and Ms Rachel Sefton with aspects of CYP expression is also acknowledged.
References


Footnotes

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Legends for Figures

Figure 1. (A) Representative immunoblots of CYP1A, CYP2J, CYP2C11, CYP3A and CYP2E1 proteins in hepatic microsomal fractions from rats that received the control and steatotic diets for 21 days. (B) Microsomal CYP expression in steatotic rat liver relative to control (n=5-6 animals per group). Different from control: *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Impact of hepatic total lipid extracts on biotransformation of CLZ (100 µM) in control and steatotic microsomes to (A) norCLZ and (B) CLZ N-oxide. Lipid was extracted from control and steatotic rat liver and introduced into microsomal systems (0.05 mg protein) in 1:1 or 25:1 lipid:protein ratios, respectively, reflecting the ratios found in control and steatotic liver, respectively. Different from control (no lipid): ***p<0.001, **p<0.01 or different from steatotic (no lipid): †p<0.05.

Figure 3. Unbound fraction (f_u) of CLZ in microsomal fractions from control and steatotic rat liver. Different from unbound CLZ concentrations in control microsomes (no added lipid): ***p<0.001, or steatotic microsomes (no added lipid): †††p<0.001.

Figure 4. Effect of dietary conditioning on the hepatic contents of (A) total saturated (closed bars), monounsaturated (open bars) and polyunsaturated (shaded bars) fatty acids, and (B) total ω-3 (closed bars) and ω-6 (open bars) polyunsaturated fatty acids. Different from control: ***p<0.001.

Figure 5. Effects of authentic triglycerides on norCLZ and CLZ N-oxide formation in control rat microsomes. Microsomal fractions (0.05 mg protein) were augmented with control liver lipid extract (0.05 mg) containing the
indicated microgram quantities of triglycerides. Metabolite formation was monitored by HPLC as described in Materials and Methods. Different from control microsomes containing control liver lipid extract alone (1:1 ratio with protein): ***p<0.001, *p<0.05.
TABLE 1

Kinetic parameters for the microsomal oxidation of CLZ in liver of control and steatotic rats.

Data are mean ± SEM of estimates from three to four individual livers.

<table>
<thead>
<tr>
<th>CLZ metabolite</th>
<th>norCLZ</th>
<th>CLZ N-oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet</strong></td>
<td><strong>K_m</strong></td>
<td><strong>V_max</strong></td>
</tr>
<tr>
<td>Control</td>
<td>41±3</td>
<td>1.08±0.06</td>
</tr>
<tr>
<td>Steatosis</td>
<td>35±11</td>
<td>0.41±0.11*</td>
</tr>
</tbody>
</table>

Different from Control*p<0.05

Units: K_m, µM; V_max, nmol product/mg protein/min; K_m/V_max nmol product/mg protein/min/M.
TABLE 2

Major fatty acids in rat liver after dietary manipulation.

Data are means±SEM of measurements in three pooled extracts, each prepared from pairs of individual rat livers.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control mg/g liver</th>
<th>Steatotic mg/g liver</th>
<th>OA+/OA- relative content</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0 (palmitic)</td>
<td>8.88±0.13</td>
<td>56.00±2.14</td>
<td>6.3</td>
</tr>
<tr>
<td>16:1 (palmitoleic)</td>
<td>2.11±0.06</td>
<td>14.71±0.07</td>
<td>7.0</td>
</tr>
<tr>
<td>18:0 (stearic)</td>
<td>4.23±0.13</td>
<td>8.63±0.82</td>
<td>2.1</td>
</tr>
<tr>
<td>18:1 (oleic; 9-cis)</td>
<td>5.38±0.30</td>
<td>69.72±0.39</td>
<td>13.0</td>
</tr>
<tr>
<td>18:1 (elaidic; 9-trans)</td>
<td>2.25±0.02</td>
<td>12.02±0.14</td>
<td>5.3</td>
</tr>
<tr>
<td>18:2 ω-6 (linoleic)</td>
<td>3.83±0.07</td>
<td>33.12±0.94</td>
<td>8.6</td>
</tr>
<tr>
<td>20:4 ω-6 (arachidonic)</td>
<td>5.25±0.21</td>
<td>6.22±1.14</td>
<td>1.2</td>
</tr>
<tr>
<td>22:6 ω-3 (docosahexaenoic)</td>
<td>1.09±0.07</td>
<td>1.49±0.64</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Different from control: **p<0.01, ***p<0.001
Figure 1
<table>
<thead>
<tr>
<th>Added Lipid</th>
<th>Microsomes</th>
<th>Control</th>
<th>Steatotic</th>
<th>Control</th>
<th>Steatotic</th>
<th>Steatotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.50 ± 0.1</td>
<td>1.35 ± 0.1</td>
<td>0.50 ± 0.1</td>
<td>0.50 ± 0.1</td>
<td>0.50 ± 0.1</td>
<td>0.50 ± 0.1</td>
</tr>
<tr>
<td>control (1:1)</td>
<td>1.50 ± 0.1</td>
<td>1.25 ± 0.1</td>
<td>0.45 ± 0.1</td>
<td>0.45 ± 0.1</td>
<td>0.45 ± 0.1</td>
<td>0.45 ± 0.1</td>
</tr>
<tr>
<td>steatotic (25:1)</td>
<td>0.50 ± 0.1</td>
<td>0.40 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0.25 ± 0.1</td>
<td>0.25 ± 0.1</td>
</tr>
</tbody>
</table>

Figure 2
Figure 3
Figure 4

A

mg/g liver

control

steatotic

***

B

mg/g liver

control

steatotic

***
Figure 5