DMD Fast Forward. Published on June 8, 2006 as doi:10.1124/dmd.106.009670

DMD #9670

POTENTIAL IMPACT OF STEATOSIS ON P450 ENZYMES OF HUMAN HEPATOCYTES ISOLATED FROM FATTY LIVER GRAFTS.

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Running title: P450 enzymes in fat overloaded human hepatocytes

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Text pages: 25

Tables: 3

Figures: 5

References: 38

Words in Abstract: 221

Words in Introduction: 584

Words in Discussion: 1088

Abbreviations: BROD, 7-benzoxyresorufin O-debenzylation; BUF, bufuralol 1'-hydroxylation; CH, coumarin 7-hydroxylation; CPR, NADPH-cytochrome P450 reductase; C6OH, chlorzoxazone 6-hydroxylation; D4OH, diclofenac 4'-hydroxylation; ECOD, 7-ethoxycoumarin O-deethylation; FFA, free fatty acids; GT, UDP-glucuronyltransferase; MROD, 7-methoxyresorufin O-demethylation; MID, midazolam 1'-hydroxylation; OHT, hydroxytestosterone; PCR, polymerase chain reaction; PHE, phenacetin O-deethylation; P450, cytochrome P450; RT, reversed transcription.

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ABSTRACT

Liver grafts discarded for transplantation because of macrosteatosis can constitute a valuable source of human hepatocytes for *in vitro* metabolic and pharmaco-toxicological studies or for therapeutic applications. A condition for using hepatocyte suspensions for these purposes is the preservation of their metabolic competence and, particularly, drug-metabolising enzymes. A reduction in microsomal cytochrome P450 (P450) activities was observed in fatty livers (> 40% steatosis) with respect to normal tissue. Similarly, decreased levels of 7-ethoxycoumarin O-deethylation and testosterone metabolism were observed in human hepatocyte cultures prepared from steatotic liver tissue. To clarify the potential impact of lipid accumulation on human hepatic P450 enzymes, we have used an *in vitro* model of "cellular steatosis" by incubation of cultured hepatocytes with increasing concentrations (0.25-3 mM) of long chain free fatty acids (FFA). A dose-dependent accumulation of lipids in the cytosol is induced by FFA mixture. Hepatocytes exposed to 1 mM FFA for 14 h showed lower activity values of CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 enzymes than non-treated hepatocytes (about 45-65% reduction). This treatment also produced significant decreases in CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 mRNAs to about 55-75% of mRNA levels in control cells. Our results suggest that, although human hepatocytes isolated from steatotic liver show reduced P450 activities, they are metabolically competent and can be used for drug metabolism studies.

Primary human hepatocytes are recognised as a suitable cellular model for the study of metabolism and pharmaco-toxicological effects of drugs (Gómez-Lechón et al., 2004). However, a major limitation for their expanded use is the lack of a regular supply of adequate amounts of the tissue required to sustain a research program and, related to this, the quality of the tissue. Healthy human liver is only occasionally available for experimental purposes, and this scarcity is increased by the competing demands for human cells for clinical applications, such as hepatocyte transplantation programs or artificial liver devices. Normal liver resected during therapeutic hepatectomies, remnants of livers after reduction or split-liver procedures and human cadaveric livers thought unsuitable for transplantation are potentially good sources of high-quality isolated hepatocytes (Baccarani et al., 2003; Mitry et al., 2003; Serralta et al., 2003; Barbich et al., 2004; Haghighi et al., 2004). High-grade macrosteatosis and cirrhosis are the two principal reasons for organ refusal for transplantation. Steatotic livers give better results, in terms of cell yield, than cirrhotic organs from hepatocyte isolation procedures (Baccarani et al., 2003). A key question is the functionality of cells isolated from fatty livers.

Steatosis has been considered as a condition predictive of failure of liver transplantation from cadaveric donors and, consequently, for discarding liver tissue. The implantation of livers from cadaveric donors with severe fatty infiltration is frequently associated with early hepatic dysfunction and an increased incidence of primary nonfunction after liver transplantation (Todo et al., 1989). More recent data show that even mild steatosis negatively affects graft and patient survival (Marsman et al., 1996). A clinical consensus exists that grafts with severe steatosis (>60%) should be discarded, whereas grafts with mild steatosis (<30%) may be used (Imber et al., 2002; Koneru and Dikdan 2002). However, the impact of hepatic steatosis on the outcome of hepatocyte

transplantation and/or culture has not yet been determined. The quality of isolated cells to be used for transplantation has been usually assessed on the basis of cellular viability, plating efficiency, or plasma proteins mRNA expression after a short period of culture (Baccarani et al., 2003; Mitry et al., 2003; Lloyd et al., 2004). Some discrepancies have been found in predictions of hepatocyte functionality after transplantation based on viability of isolated cells and survival in primary culture (Olinga et al., 2000; Nishitai et al., 2005). The functional competence of hepatocytes should also be used as an indication of the quality of cell preparations (Serralta et al., 2003). Among other hepatic functions, hepatocytes to be used for transplantation purposes or for *in vitro* metabolic and pharmaco-toxicologic studies must maintain their drug metabolising capability. Preservation of phase I and phase II enzyme activities, and particularly those catalysed by P450s, the major system responsible for oxidative metabolism of drugs and other xenobiotics, is a key condition for therapeutic and/or experimental applications of hepatocytes.

The aim of the present study was to investigate the potential effects of liver steatosis on drug-metabolising capacity of hepatocytes. To this end, we have developed an experimental model of cellular steatosis based on the incubation of primary cultured human hepatocytes with long chain free fatty acids, as previously reported in HepG2 cells (Feldestein et al., 2003). The effects of fat-overloading on individual P450s were analyzed at both the P450 monooxygenase activity and mRNA levels. The results indicate that *in vitro* fat-overloading of hepatocytes results in a down-regulation of several P450 enzymes involved in drug metabolism, that could explain the lower metabolic capability found in human hepatocytes isolated from steatotic livers.

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MATERIALS AND METHODS

Chemicals. Collagenase, and β-glucuronidase/arylsulfatase were obtained from Roche (Barcelona, Spain). Culture media (Ham's F-12, Lebovitz L-15), newborn calf serum, and DNase I Amplification Grade were from Gibco BRL (Paisley, UK). Nile red, oleate, palmitate, cytochrome c, 7-ethoxycoumarin, 7-hydroxycoumarin, coumarin, 7-benzoxyresorufin, chlorzoxazone, diclofenac, resorufin, testosterone, 16α- and 11β-hydroxytestosterone (OHT), androstenedione, and 4-methylumbellipherone, were purchased from Sigma (Madrid, Spain). 7-Methoxyresorufin was from Molecular Probes Europe BV (Leiden, The Netherlands). 4'-Hydroxydiclofenac, 6-hydroxychlorzoxazone, hydroxybufuralol, midazolam, 1'-hydroxymidazolam, phenacetin, acetaminophen, and 2β-, 6β-, 15β-, 16α-, 16β- and 11β-OHT were supplied by Ultrafine (Manchester, UK). All other chemicals were of analytical grade.

Preparation of microsomes from human liver tissue. Samples of human liver tissue from cadaveric organ donors were obtained in conformance to the rules of the Hospital's Ethics Committee. The tissue was obtained in the bank surgery and it was transported in a cold University of Wisconsin preservation solution. All liver samples were from donors who were not suspected of harbouring any infectious disease and tested negative for human immunodeficiency virus and hepatitis. The extent of steatosis was graded by pathological examination and only non-steatotic livers (n=10, 6 male and 4 female, patients aged between 15 and 71 years, mean 49 ± 18) or those classified as steatotic (>40%, pathologist confirmation) (n=9, 6 male and 3 female, patients aged between 19 and 69 years, mean 44 ± 21) were included in the study. After reception, liver samples were immediately dissected into small pieces, frozen in liquid nitrogen, and stored at -80º C until used. To prepare liver microsomes, tissue was homogenized in 50 mM Tris HCl pH 7.4 containing 150 mM KCl and 1mM EDTA. Homogenates

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were centrifuged at 10.000 g for 20 min at 4º C, and the supernatant obtained (S9 fraction) was subsequently centrifuged at 100.000 g for 1 h at 4º C. The microsomal pellet was resuspended in 100 mM phosphate buffer pH 7.4 containing 1 mM EDTA, quickly frozen in liquid nitrogen and stored in aliquots at -80º C. Protein content was determined by the method of Lowry et al. (1951).

Isolation and culture of human hepatocytes. A total of 17 non-steatotic (11 male and 6 female, age 48 ± 19 yr) and 16 steatotic (9 male and 7 female, age 52 ± 14) liver grafts were used for cell harvesting. Tissue samples were obtained and transported as described above for livers used for microsome preparation. Non-steatotic elective liver biopsies (1-4 g) obtained in the course of therapeutic laparotomy for non-malignant liver disease or extrahepatic disease after receiving informed consent from patients, in conformity with the rules of the Hospital's Ethics Committee were also used. None of the patients were habitual consumers of alcohol or other drugs. All liver samples, cadaveric and elective, were from donors who were not suspected of harbouring any infectious disease and tested negative for human immunodeficiency virus and hepatitis. No underlying malignant liver pathology was present in any of the cases. Hepatocytes were isolated using a two-step perfusion technique and cultured as described in detail elsewhere (Gómez-Lechón et al., 1990). Cellular viability was assessed by the Trypan blue dye exclusion test. Hepatocytes were seeded on fibronectin-coated plastic dishes. The medium was changed 1 h later to remove unattached hepatocytes. By 24 h, the cells were shifted to serum-free hormone-supplemented medium (10 nM dexamethasone and insulin).

Fat-overloading induction in cultured hepatocytes. To induce fat-overloading of cells, primary cultures of human hepatocytes prepared from elective non-steatotic liver biopsies were exposed to a mixture of free fatty acids (2:1 ratio of oleate and palmitate)

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(Feldestein et al., 2003). Stock solutions of 50 mM oleate and 50 mM palmitate prepared in culture medium containing 1% bovine serum albumin (BSA) were conveniently diluted in culture medium to obtain the desired final concentrations (0.25 to 3 mM). Long-chain free fatty acid (FFA) mixture was added to cultures 6-8 hours after medium renewal. Fat content was determined fluorimetrically by Nile Red staining (McMillian et al., 2001). Briefly, hepatocyte monolayers were washed twice with PBS and incubated for 15 min with nile red solution at a final concentration of 1 mg/ml in PBS at 37ºC. Monolayers were washed thereafter with PBS and read in a microfluorimeter (excitation 488 nm and emission 550 nm). Cytotoxicity of FFA was assessed by measuring neutral red uptake (Babich and Borenfreund, 1992).

Measurement of Phase I and Phase II activities in human liver microsomes. P450 activities were assayed by incubating microsomes (100 μg of protein) for 15 min at 37º C in 300 μl of 100 mM phosphate buffer pH 7.4 containing NADPH-regenerating system (5 mM Cl_2Mg, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 0.3 U/ml glucose-6-phosphate dehydrogenase) and the appropriate substrate. Substrate concentrations for P450 assays were as follows: 500 μM 7-ethoxycoumarin, 10 μM 7-methoxyresorufin (CYP1A2), 50 μM coumarin (CYP2A6), 200 μM diclofenac (CYP2C9), 250 μM chlorzoxazone (CYP2E1), and 200 μM testosterone (CYP3A4). 7-Methoxyresorufin O-demethylation (MROD) assay was stopped by adding 300 μl of methanol, and the resorufin formed was determined fluorimetrically as described (Donato et al., 1993a). Coumarin 7-hydroxylation (CH) and 7-ethoxycoumarin O-deethylation (ECOD) assays were stopped with 30 μl of 25% TCA, and 7-hydroxycoumarin formation was quantified fluorimetrically (355 nm excitation and 460 nm emission) as previously described (Edwards et al., 1984). The diclofenac 4'-hydroxylation (D4OH) and chlorzoxazone 6-hydroxylation (C6OH) assays were

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stopped by adding 300 µl of acetonitrile, and the metabolites formed were analyzed by HPLC (Bort et al., 1999). Testosterone oxidation assays were stopped with 500 µl of ethylacetate, and hydroxylated metabolites were extracted and analyzed by HPLC as described elsewhere (Donato et al., 1993b). NADPH-cytochrome P450 reductase (CPR) activity was assayed using cytochrome c as substrate as described (Guzelian et al., 1977). UDP-glucuronyltransferase (GT) activity was assayed using 4-methylumbellipherone as substrate (Donato et al., 1999).

Evaluation of metabolic competence of human hepatocytes. Activity assays were performed by direct incubation of cell monolayers with the substrates. Hepatocytes were incubated at 37ºC with 800 µM 7-ethoxycoumarin, 200 µM testosterone, 10 µM methoxyresorufin, 15 µM benzoxyresorufin, 10 µM phenacetin or 100 µM coumarin for 1 h (metabolite formation was linear for at least 2 h) or with 300 µM diclofenac, 400 µM chlorzoxazone, 5 µM midazolam or 10 µM bufuralol for 3 h (linearity of the assays was > 4 h). Reactions were stopped by aspirating the incubation medium from plates and medium samples were then incubated with β-glucuronidase and arylsulfatase for 2 h at 37 ºC (Donato et al., 1993a). Metabolites formed during ECOD, MROD, CH, D4OH, C6OH, or testosterone oxidations were quantified fluorimetrically or by HPLC analysis as described above for microsomal assays. Resorufin formed during the 7-benzozyresorufin O-debenzylation (BROD) assay was determined fluorimetrically as described (Donato et al., 1993a). Analysis of metabolites formed during phenacetin O-deethyltion (PHE), midazolam 1'-hydroxylation (MID) and bufuralol 1'-hydroylation (BUF) assays was conducted by HPLC-MS/MS. The LC-MS/MS system comprised a Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization source (Micromass, USA) interfaced with an Alliance Waters 2795 liquid chromatograph (Waters, Spain). An aliquot (20 µl) was injected onto a

Teknokroma C18 column (100 mm x 2.1 mm, 3µ particle size) operated at 40º C. The flow rate was 0.4 ml/min. Mobile phase was A 0.1% formic acid in acetonitrile and B 0.1% formic acid in water. The proportion of acetonitrile was increased linearly from 0 to 90% in 6 min and then the injection column was allowed to re-equilibrate at initial conditions for 10 min. The column eluent was directed to an atmospheric pressure ionization interface without splitting, operating at 320 ºC using nitrogen as auxiliary gas (400 or 600 l/h). For quantification, the mass spectrometer was operated in the selected multiple reaction monitoring mode to monitor for detecting metabolites of phenacetin, midazolam and bufuralol selecting the 152>110, 342>324 and 278>186 m/z ion transitions, respectively. Dextromethorphan at final concentration of 0.2 pM was used as internal standard, following the ion transition 258>157. To evaluate accuracy, quality controls of each compound were prepared and used to ensure linearity and intra-interassay precision and accuracy.

Measurement of mRNAs by RT-PCR. Total RNA was extracted from cultured human hepatocytes using TRIzol RNA extraction kit (Life Technologies) following the supplier's recommendations. The amount of purified RNA was estimated by ribogreen fluorescence and its purity was assessed by the absorbance ratio 260:280 nm. RNA integrity was examined by electrophoresis in a 1% agarose gel upon ethidium bromide staining. The reverse transcription (RT) reaction mixture consisted of 1 µg of total RNA which was reverse transcribed in 20 µl of reverse transcriptase buffer, 10 mM DTT, 500 µM deoxynucleotides, 3 µM oligo d(T)14 primer, 60 U RNAse and 250 U reverse transcriptase. The reaction was allowed to proceed for 60 min at 42ºC, followed by 5 min heating at 95ºC and then rapid cooling on ice. The cDNA was stored at –20ºC until use. The polymerase chain reaction (PCR) was conducted in semi-automatic equipment (Roche, Light-Cycler®). The conditions used for the quantitative PCR, the

specific primers and the quantification strategy were as previously described (Pérez et al., 2003).

Statistical analysis. Data are expressed as mean ± S.D. Comparisons of variables between groups were performed using Student's t-test, and $p<0.05$ was assumed statistically significant.

RESULTS

Xenobiotic-metabolising activities in steatotic or non-steatotic liver grafts. The evaluation of the metabolic capacity of the tissue requires functional studies at activity level. Selected phase I and phase II drug metabolizing enzyme activities were measured in microsomes obtained from fatty livers (>40% macrosteatosis) and compared to those found in non-steatotic livers. Activity levels of CPR and different P450 enzymes were determined as representative of phase I reactions. Similar CPR activities were found in microsomes from steatotic and non-steatotic livers (Table 1). ECOD activity is catalysed by several human P450s (Waxman et al., 1991; Yamazaki et al., 1996) and is commonly measured as a representative estimation of total P450 activity (Donato et al., 1999). Lower, but not significantly lower, ECOD activity was observed in fatty livers (Table 1). Evaluation of the activity of individual P450 enzymes requires the use of appropriate substrates for each individual P450 enzyme. 7-Methoxyresorufin, coumarin, testosterone, diclofenac and chlorzoxazone were chosen as isozyme-selective substrates for the evaluation of P450 enzymes in liver microsomes (Table 1). From these five selective reactions, only MROD (CYP1A2) and 6β-OHT (CYP3A4) activities were significantly decreased in microsomes from livers with steatosis. Similar activity levels of GT activity, a phase II enzyme, were found in both groups of livers (Table 1).

Testosterone is metabolised in a regioselective manner by different P450 enzymes and it can be used as a substrate to investigate the activity of several P450 enzymes simultaneously. Comparative analysis of testosterone metabolic profile in both groups of livers revealed a lower oxidative metabolism in microsomes from liver grafts with steatosis (Table 2). Significant differences were observed for 6β- and 2β-OHT activities (CYP3A4). Microsomes from the steatotic group also showed lower (about 40-50% of liver group without steatosis) rates of formation of 16β-OHT and

androstenedione, representative of CYP2C9 and CYP2C19 activities, respectively, although these differences were not significant.

Metabolic competence of primary cultures of human hepatocytes prepared from steatotic or non-steatotic livers. Hepatocytes were isolated from 17 different human liver samples with steatosis (>40%) or non-steatosis (n=16). The use of steatotic livers led to a significant reduction in the yield of the isolation procedure, estimated as number of viable cells obtained per gram of liver tissue, with respect to non-steatotic livers (6.1 ± 5.0 *vs* 15.8 ± 9.5 million hepatocytes/g of tissue, $p<0.05$). No significant differences in the viability of hepatocytes freshly isolated from steatotic or normal livers were found (82 ± 23% *vs* 94 ± 5%, $p=0.1$). By 24 h of culture, functional competence of primary cultures was evaluated by measuring P450-dependent oxidations. To this end, ECOD activity was quantified as representative of total P450 activity, and 6β-OHT was evaluated as a selective probe for CYP3A4, the major P450 enzyme in human liver. For both P450 activities, high variability was observed among individual donors. As an average, cell preparations obtained from livers with steatosis showed significantly lower ECOD (12.3 ± 8.6 *vs* 19.7 ± 8.7 pmol/mg x min) and 6β-OHT (39.0 ± 34.5 *vs* 94.7 ± 63.5 pmol/mg x min) activity values than those of hepatocytes from non-steatotic samples (Fig. 1).

The oxidative metabolism of testosterone was analysed as an additional parameter to estimate drug-metabolising capacity of cultured cells. Human hepatocytes in primary culture actively convert the substrate to several hydroxylated metabolites. Total testosterone oxidation was significantly lower in hepatocytes prepared from liver grafts with steatosis than in cultures from normal liver (133 ± 75 *vs* 266 ± 151 pmol/mg x min, $p<0.05$). Similarly, the rate of formation of each testosterone metabolite was lower in hepatocytes from livers with steatosis. However, independently of these

quantitative differences, no appreciable changes in the relative rates of each testosterone hydroxylation reaction were found and, consequently, the profiles of testosterone metabolites obtained from both groups of hepatocytes were highly similar (Fig. 2). 6β-OHT (CYP3A4) and androstenedione (CYP2C19) were the major metabolites formed in all hepatocyte preparations, followed by 2β-OHT (CYP3A4), 15β-OHT (CYP3A4), 16α-OHT and 16β-OHT (CYP2C9).

P450 expression in an *in vitro* induced model of hepatic steatosis. Differences in drug metabolizing activities observed in cultures obtained from both groups of livers could be partially due to other factors than fat content of the liver. In an attempt to discriminate changes in hepatocyte P450 function due exclusively to steatosis we studied effects on metabolic capability of hepatocytes using an *in vitro* model of induced steatosis. To this end, human hepatocytes obtained from normal livers were incubated with increasing concentrations of FFA mixture (2:1 ratio of oleate to palmitate) for 14 or 36 h. Hepatocytes treated with FFA developed dose-dependent accumulation of lipid droplets in the cytosol which could be quantified using Nile Red as a vital lipophilic dye to label fat accumulation (Fig. 3A). Maximal lipid accumulation was reached after hepatocyte incubation for 14 h with 1 mM FFA, showing a relative lipid loading comparable to that of hepatocytes obtained from the liver of patients with steatosis (data nort shown). No cytotoxic effects were observed after hepatocyte incubation with FFA mixture up to 2 mM during 14 h (Fig. 3B). After longer exposure (36 h) to FFA, significant reductions in hepatocyte viability were observed at concentrations >1mM. Non cytotoxic treatments were selected for further treatment of hepatocytes to explore the potential effects of fat accumulation on functional competence of cultured cells.

Comparison of testosterone metabolite profiles of cultured hepatocytes treated with FFA mixture with those of cells maintained in control medium showed a dose-dependent reduction in testosterone oxidation rates of fat-overloaded cells, with no changes in the relative formation of each metabolite (Table 3). Significant decreases in testosterone metabolism were observed after 14 h of exposure of hepatocytes to 1 or 2 mM FFA (about 60-40% and 50-20% of control, respectively). Greater effects on P450 activities were observed after 36 h of treatment with 1 mM FFA mixture (36 h treatment with 2 mM FFA was cytotoxic, Fig. 3B). These results, in agreement with the lower testosterone oxidative capacity observed in microsomes and cultured hepatocytes prepared from liver grafts with steatosis (Table 2 and Fig. 1), suggested a general reduction in P450-dependent function in fat-overloaded hepatocytes. To confirm these findings the effects of fat-overloading on major human P450 enzymes were analysed using selective reactions, namely MROD and PHE (CYP1A2), CH (CYP2A6), BROD (CYP2B6), D4OH (CYP2C9), MID and 6β-OHT (CYP3A4), BUF (CYP2D6), and C6OH (CYP2E1). Exposure of cultured hepatocytes to 2 mM FFA for 12 h resulted in significant decreases (to 20-50% of controls) of activity levels of all individual P450 enzymes examined as well as in ECOD activity (indicative of several P450s) (Fig. 4). Significant reductions in CYP1A2 (MROD), CYP2A6, CYP2B6, CYP2C9 and CYP3A4 activities were also produced by 1 mM FFA.

Levels of mRNAs corresponding to CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 were also quantified in fat-overloaded hepatocytes. As seen in Fig. 5, human hepatocytes exposed to 1 mM FFA mixture for 14 h showed lower mRNA content corresponding to the six P450 enzymes examined than did control hepatocytes.

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DISCUSSION

The aim of the present study was to determine the suitability of fatty liver tissue as a source of metabolically competent hepatocytes to be used for therapeutic or research purposes. To this end, P450 activities were comparatively measured in primary cultures of hepatocytes isolated from human liver tissue with or without macrosteatosis. Reductions in ECOD (catalysed by several P450 enzymes) and 6β-OHT (CYP3A4) reactions were observed in hepatocyte cultures prepared from steatotic livers (Fig. 1). These results are in agreement with the decrease in microsomal P450 activities found in fatty livers relative to normal tissue (Table 1). We observed significant reductions in CYP3A4 (6β-OHT) and CYP1A2 (MROD) activities in liver tissue with steatosis, whereas minor changes or no effects were observed in other activities. Unless individual P450 enzymes were equally affected by steatosis, altered pattern of P450 activities would be found in hepatocytes isolated from fatty livers. Consequently, notable differences in the metabolic profile of a particular drug could be found as a function of the grade of steatosis present in the tissue used for cell harvesting. Potential effects of fat accumulation on individual drug-metabolising enzymes are difficult to analyse in human liver as high interindividual variability in P450s is commonly found (Gómez-Lechón et al., 2004). A number of factors such as age, sex, pathological status (infections, inflammation, cirrhosis, carcinoma, fibrosis) or drug intake (chemotherapy, alcohol consumption, smoking habits) are known to contribute to differences between donors (Kraul et al., 1991; Pelkonen and Breimer, 1994). Increased levels of CYP2E1, together with a reduction in CYP3A, have been found in patients with non-alcoholic steatohepatitis (Weltman et al., 1998). However, up to now, the potential role of liver steatosis on the regulation of major human P450s has not been exhaustively examined and the possible mechanisms responsible for these changes remain unknown. Previous

studies undertaken in nutritional models of hepatic steatosis in experimental animals suggested an association between lipid deposition and impaired P450 function (Weltman et al., 1996; Leclercq et al., 1998; Su et al., 1999). Decreased P450 activities and protein levels were observed with the appearance of steatosis, and, moreover, significant correlations between P450 activities and liver fat content have been found (Leclercq et al., 1998).

In the present study we have used an *in vitro* model of fat-overloaded hepatocytes to investigate the impact of steatosis in P450 enzymes excluding other factors that could influence hepatocyte behaviour. Hepatocytes from normal (non-steatotic) liver samples were treated with or without FFA mixture. FFA treated hepatocytes developed "cellular steatosis" (Fig. 3) and drug metabolising capability was compared with that of control hepatocytes obtained from the same liver sample. A significant reduction in the activity and mRNA levels of some individual P450 enzymes was found in fat-overloaded hepatocytes (Fig. 4 and 5), which confirmed the lower metabolic capability observed in hepatocytes prepared from steatotic liver tissue (Fig. 1). Our results provide evidence of a reduction in several P450s involved in drug metabolism. The impairment of P450 activities in *in vitro* fat-overloaded hepatocytes can be explained, at least in part, by the reduction in specific mRNA levels of P450 enzymes. These findings support the hypothesis that down-regulation of P450s could occur at a pretranslational level by interfering with transcriptional activation of the genes or by increasing mRNA degradation. However, other mechanisms could be involved in the observed reduction in P450 function due to fat accumulation. Modification of the lipid composition of microsomal membranes could affect optimal association between P450 and NADPH-cytochrome P450 reductase and/or cytochrome

b5, altering the functional capacity of the P450 system (Leclerq et al., 1998; Su et al., 1999).

Previous studies in animal models of induced steatosis suggested reductions in CYP2C, CYP3A and, to a minor extent, CYP2A enzymes (Weltman et al., 1996; Leclercq et al., 1998; Su et al., 1999). However, conflicting data on the regulation of CYP2E1 in liver steatosis have been reported. Increases in CYP2E1 have been observed in rodent models of liver steatosis with inflammation (Weltman et al., 1996; Leclercq et al., 2000), as well as in patients with NASH (Weltman et al., 1998), whereas a reduction in CYP2E1 expression has been reported in animal models of hepatic steatosis in the absence of inflammation (Leclercq et al., 1998). It was suggested that CYP2E1 increases observed in steatohepatitis are not directly linked to fat accumulation but could be more closely related to the inflammatory process (Leclercq et al., 1998). Several cytokines released during the inflammatory process have been shown to down-regulate the expression of different hepatic P450 enzymes, whereas others seemed to be involved in the up-regulation of CYP2E1 (Abdel-Razzak et al., 1993; Donato et al., 1993b; Guillen et al., 1998). Our results showed that changes in CYP2E1 mRNA content and catalytic activities observed in fat overloaded human hepatocytes are similar to those found in the other P450 enzymes studied, suggesting a similar mechanism of down regulation for the different P450s (Fig. 4 and 5). To our knowledge this is the first study in which seven different human P450 enzymes have been comparatively examined in a model of induced steatosis.

In our *in vitro* model of cellular steatosis a lower testosterone oxidation capability was found (relative to control cells) with no appreciable changes in the relative metabolite profile. Similar results were observed when comparing testosterone metabolism in hepatocytes isolated from steatotic liver grafts with those obtained from

normal liver tissue. These results suggest that, despite their reduced levels of P450 activities, human hepatocytes obtained from donors with liver steatosis are metabolically competent. This finding becomes interesting as steatotic liver grafts that are considered unacceptable for ortothopic transplantation could be used for hepatocyte isolation. Severe steatosis is an absolute contraindication for liver graft transplantation, since many problems have been reported including; strong correlations of high levels of macrovesicular steatosis and primary non function after liver transplantation, susceptibility to ischemic injury, and impaired regeneration (Loinaz and Gonzalez, 2000; Selzner and Clavien, 2000; Koneru and Dikdan, 2002; Imber et al., 2002). Increased hepatic fat content is frequently found in potential donors. Data from Spain show that approximately 16% of recovered livers are rejected for transplantation and 42% of those rejections are due to hepatic steatosis (Loinaz and Gonzalez, 2000). Although fatty livers render lower cell viability, lower yield of isolation procedure (Mitry et al., 2003; Gómez-Lechón et al., 2004), and lower P450-dependent activities (Fig. 1) than non-steatotic livers, nevertheless they can be used to obtain metabolically competent human hepatocytes. Therefore, liver grafts discarded because of macrosteatosis could constitute a valuable source of the large quantities of functional hepatocytes required for experimental purposes.

ACKNOWLEDGEMENTS. The authors acknowledge the expert technical assistance of Epifanía Belenchón.

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Footnotes.

This work was supported by the ALIVE Foundation, and funds of the Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III of Spain, grant 03/0339, and the European Commission, grant LSHB-CT-2004-504761.

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FIGURE LEGENDS.

FIG. 1. ECOD and 6β-OHT activities in human hepatocyte cultures prepared from liver tissue from individual with and without steatosis. After 24 h in culture, activities were assayed in hepatocytes obtained from 17 liver grafts with steatosis and from 16 liver grafts without steatosis (normal group). ECOD (A) and 6β-OHT (B) activities are expressed as pml of corresponding metabolite formed/min x mg of cell protein. The solid line represents the mean value for the group.

FIG. 2. Oxidative testosterone metabolism in human hepatocyte cultures prepared from liver tissue with and without steatosis. Testosterone was incubated with 24 h-old cultured hepatocytes prepared from steatotic or normal (non steatotic) liver grafts. Formation of 6β-, 2β-, 15β-, 16α-, and 16β-OHT, and androstenedione (A) were analysed by HPLC. Results are expressed as percentages with respect to total testosterone oxidation rates and represent the mean ± SD of 16 (normal group) or 17 (steatosis group) hepatocyte preparations.

FIG. 3. Fat overloading of cultured human hepatocytes exposed to free fatty acid mixture (FFA). Six hours after seeding, primary hepatocytes were exposed to increasing concentrations (0.25 to 3 mM) of FFA mixture. After 14 h or 36 h of treatment, fat content was quantified fluorimetrically using Nile Red as described in Materials and Methods (A) and cytotxicity of FFA mixture was assayed by measuring neutral red uptake (B). Results are expressed as a percentage of control cultures (untreated cells). Data are the mean ± SD of 3 different hepatocyte preparations. * $p < 0.05$ with respect to control.

DMD Fast Forward. Published on June 8, 2006 as DOI: 10.1124/dmd.106.009670
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FIG. 4. P450 activities in fat-overloaded hepatocytes. After 14 h of incubation with 0.5-2 mM FFA mixture, P450 activities were assayed in intact hepatocyte monolayers using selective substrates. Results are expressed as percentage of activity in control cultures (untreated cells): 1.7 ± 0.4 (PHE), 1.5 ± 0.3 (MROD), 11.7 ± 2.3 (CH), 0.36 ± 0.08 (BROD), 49 ± 10 (D4OH), 1.4 ± 0.3 (MID), 88 ± 18 (6β-OHT), 12.1 ± 2.3 (BUF), 127 ± 27 (C6OH), and 27 ± 6 (ECOD) pmol/mg x min. Data are the mean ± SD of 3-5 different hepatocyte preparations. * $p < 0.05$ with respect to corresponding control.

FIG. 5. mRNA levels of major hepatic P450 enzymes in fat-overloaded hepatocytes. Specific P450 mRNA levels were quantified by RT-PCR after 14 h of incubation of human hepatocyte cultures with 0.5 or 1 mM FFA mixture. Results are expressed as percentage of corresponding P450 mRNA/β-actin mRNA content in control cells: 0.11 ± 0.02 (CYP1A2), 0.019 ± 0.002 (CYP2A6), 0.20 ± 0.04 (CYP2C9), 0.87 ± 0.19 (CYP2E1), 0.086 ± 0.013 (CYP2D6) or 0.36 ± 0.04 (CYP3A4). Data are the mean ± SD of 3-4 different hepatocyte preparations. * $p < 0.05$ with respect to control.

Table 1. Phase I and phase II activities in microsomes prepared from steatotic and non-steatotic human liver grafts.

Activity[a]	CYP	Non-steatosis[b]	Steatosis	%[c]	Non-steatosis[d]	Steatosis	%[c]
Phase I							
CPR		72 ± 20	66 ± 23	92			
ECOD	several	417 ± 240	300 ± 88	72	6.1 ± 3.8	4.9 ± 0.9	80
MROD	1A2	25 ± 16	11 ± 5*	44	0.35 ± 0.19	0.16 ± 0.08*	46
CH	2A6	296 ± 164	370 ± 130	125	3.7 ± 2.2	5.1 ± 2.1	139
D4OH	2C9	1.8 ± 1.0	1.4 ± 0.4	78	28 ± 19	23 ± 8	82
C6OH	2E1	6.9 ± 5.8	5.7 ± 3.0	83	110 ± 98	96 ± 34	87
6βOHT	3A4	727 ± 403	360 ± 255*	50	10.8 ± 5.7	6.0 ± 4.0*	55
Phase II							
GT		6.1 ± 2.2	8.7 ± 4.1	143			

(a) CPR: NADPH-cytochrome P450 reductase; ECOD: ethoxyrcoumarin O-deethylase; MROD: methoxyresorufin O-demethylase; CH: coumarin 7-hydroxylase; D4OH: diclofenac 4'-hydroxylase; C6OH: chlorzoxazone 6-hydroxylase; 6βOHT: testosterone 6β-hydroxylase; GT: UDP-glucuronyltransferase.

(b) Activities were measured in liver microsomes using selective substrates and expressed as picomoles of corresponding metabolite formed per minute and per milligram of protein, except in the case of CPR, D4OH, C6OH and GT, which were expressed in nanomoles per minute and per milligram of protein. Results are mean ± SD of different microsomal preparations from non-steatotic (n=10) or steatotic (n=9) livers.

(c) Percentage with respect to the corresponding activity value in non-steatosis group.

(d) For each micrsomal preparation phase I activities were normalized respect to CPR activity values. Results are mean ± SD of different microsomal preparations from non-steatotic (n=10) or steatotic (n=9) livers.

* $p < 0.05$ with respect to non-steatosis group.

Table 2. Testosterone oxidation by microsomes prepared from steatotic and non-steatotic human liver grafts.

Metabolite	CYP[a]	Non-steatosis[b]	Steatosis	%[c]
15β-OHT	3A4	178 ± 130	98 ± 73	55
6β-OHT	3A4	631 ± 283	360 ± 255*	57
16α-OHT	2B?	26 ± 36	25 ± 34	98
16β-OHT	2C9	2.2 ± 2.7	1.4 ± 1.9	64
2β-OHT	3A4	389 ± 225	159 ± 95*	41
Androstenedione	2C19	669 ± 713	310 ± 271	46
Total		1895 ± 1048	955 ± 467*	50

(a) Major P450s involved in the formation of the hydroxylated metabolites are indicated (Donato et al., 1999).

(b) Activities are expressed as picomoles of the corresponding product formed per minute and per milligram of microsomal protein. Results are mean ± SD of nine different microsomal preparations from non-steatotic or steatotic livers.

(c) Percentage with respect to the corresponding activity value in non-steatosis group.

* $p < 0.05$ with respect to non-steatosis group.

Table 3. Testosterone oxidation in primary human hepatocytes exposed to fatty acid mixture (FFA).

Treatment		Testosterone metabolite formation[a]						
		15β	6β	16α	16β	2β	A	total
14 h	Control	12.0 ± 2.3 (5.0)[b]	78.9 ± 15.3 (33)	5.5 ± 0.9 (2.3)	3.6 ± 0.7 (1.5)	42.4 ± 6.9 (18)	97.4 ± 18.1 (41)	240 ± 29
	FFA 0.5 mM	11.1 ± 2.5 (5.4)	63.3 ± 12.6 (31)	5.0 ± 0.1 (2.4)	3.3 ± 0.3 (1.6)	41.6 ± 9.5 (20)	82.7 ± 34.7 (40)	207 ± 30
	FFA 1 mM	8.3 ± 2.3 (5.3)	48.3 ± 10.2* (31)	3.7 ± 0.5* (2.3)	2.6 ± 0.7 (1.7)	30.1 ± 2.1* (19)	64.8 ± 9.0* (41)	158 ± 28*
	FFA 2 mM	6.8 ± 1.1* (5.7)	37.4 ± 10.6* (32)	2.4 ± 1.7* (2.0)	1.6 ± 0.8* (1.3)	26.5 ± 6.0* (22)	43.6 ± 10.7* (37)	118 ± 32*
36 h	Control	9.4 ± 2.5 (5.2)	55.2 ± 15.1 (31)	4.1 ± 0.7 (2.3)	3.3 ± 0.7 (1.9)	27.8 ± 4.8 (16)	79.0 ± 18.3 (44)	179 ± 24
	FFA 0.5 mM	8.0 ± 1.9 (5.4)	43.2 ± 11.5 (29)	3.0 ± 0.5 (2.0)	2.4 ± 0.8 (1.6)	24.0 ± 4.5 (16)	68.2 ± 18.6 (46)	149 ± 22
	FFA 1 mM	4.5 ± 1.5* (4.4)	25.2 ± 9.8* (25)	2.1 ± 0.6* (2.1)	1.8 ± 0.5* (1.8)	22.3 ± 2.9 (22)	45.2 ± 10.1* (45)	101 ± 23*

(a) 15β, 6β, 16β, 16α and 2β: formation of the corresponding hydroxylated metabolite of testosterone; A: formation of androsternedione. Activities are expressed as picomoles of product formed per minute and per milligram of total cell protein. Results are mean ± SD of 4-5 different cultures.

(b) Values in parentheses are the percentage of metabolite formation with respect to total testosterone oxidation.

* $p < 0.05$ with respect to control hepatocytes.

Fig 1

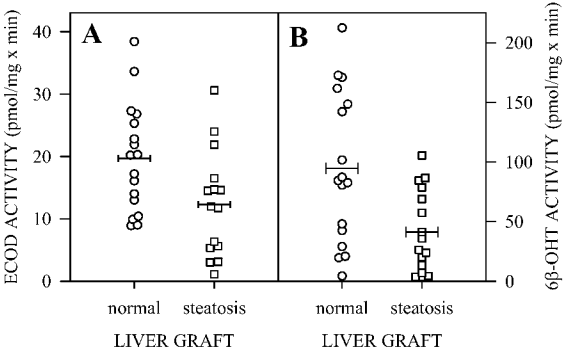

A
B
ECOD ACTIVITY (pmol/mg x min)
6β-OHT ACTIVITY (pmol/mg x min)
0
10
20
30
40
50
100
150
200
normal
steatosis
normal
steatosis
LIVER GRAFT
LIVER GRAFT

FIG 2

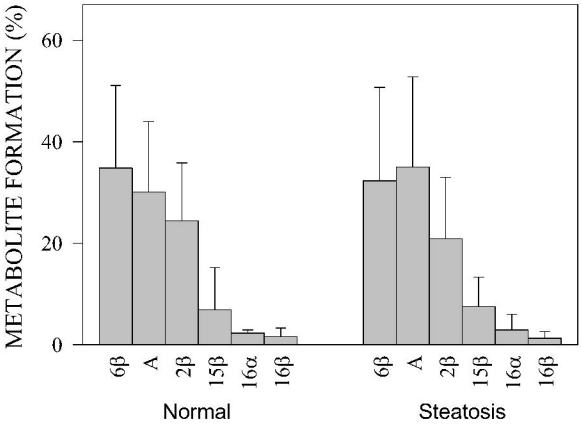

METABOLITE FORMATION (%)
60
40
20
0
6β
A
2β
15β
16α
16β
6β
A
2β
15β
16α
16β
Normal
Steatosis

Fig 3

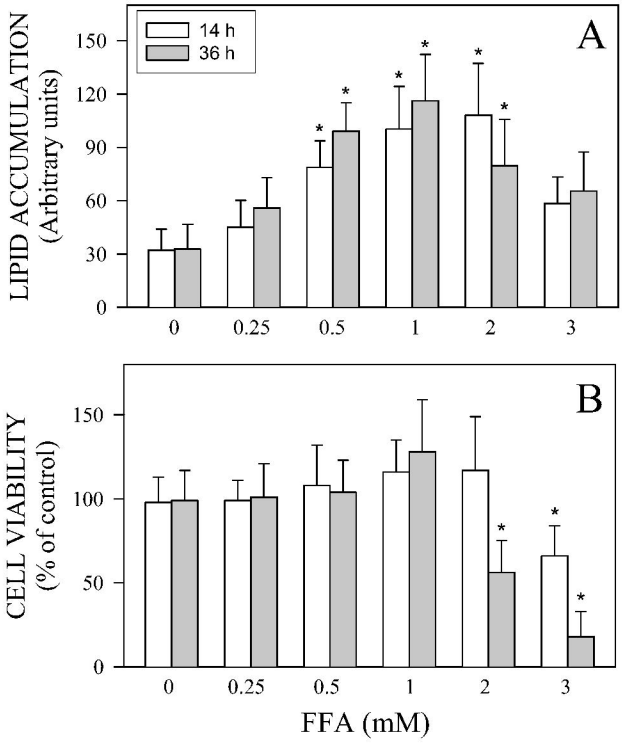

A
14 h
36 h
LIPID ACCUMULATION
(Arbitrary units)
150
120
90
60
30
0
0
0.25
0.5
1
2
3
B
CELL VIABILITY
(% of control)
150
100
50
0
0
0.25
0.5
1
2
3
FFA (mM)

FIG 4

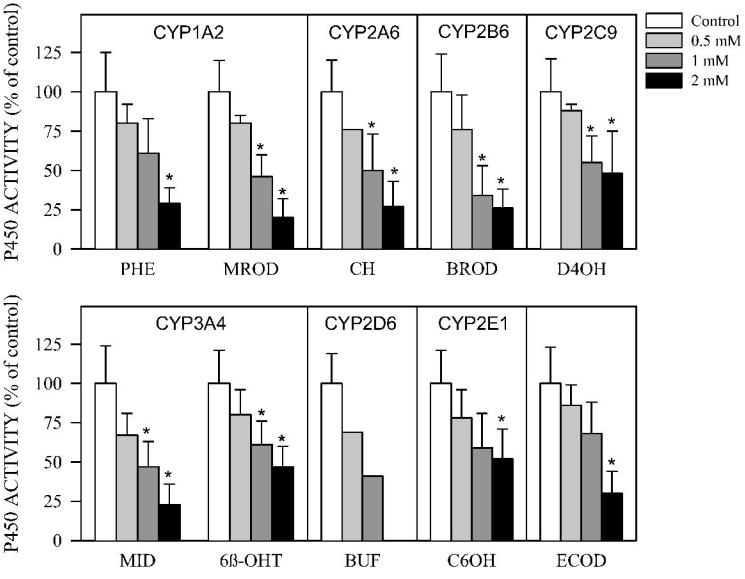
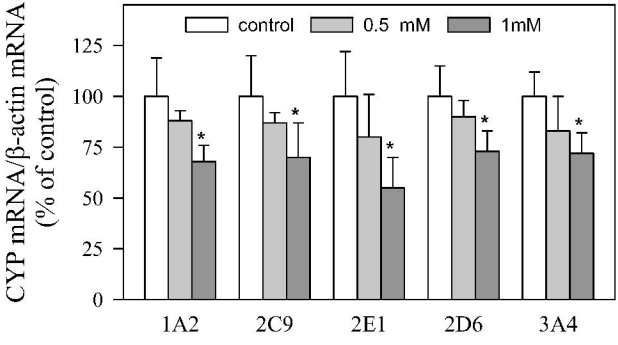

Control
0.5 mM
1 mM
2 mM
P450 ACTIVITY (% of control)
CYP1A2
CYP2A6
CYP2B6
CYP2C9
125
100
75
50
25
0
PHE
MROD
CH
BROD
D4OH
P450 ACTIVITY (% of control)
CYP3A4
CYP2D6
CYP2E1
125
100
75
50
25
0
MID
6ß-OHT
BUF
C6OH
ECOD

FIG-5


control
0.5 mM
1mM
CYP mRNA/β-actin mRNA
(% of control)
125
100
75
50
25
0
1A2
2C9
2E1
2D6
3A4
*