Cytochrome P450 expression in human hepatocytes and human hepatoma cell lines. Molecular mechanisms that determine lower expression in cultured cells.

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CYP expression in human hepatocytes and hepatomas

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ABSTRACT

1. Cultured hepatic cells have reduced cytochrome P450 (CYP) activities in comparison with human liver, but the mechanism(s) that underlies this circumstance is not clear. We investigated the causes of this low CYP activity by analyzing the activity, protein, mRNA, and heterologous nuclear RNA contents of the most important CYPs involved in drug metabolism (CYPs 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) in cultured human hepatocytes and in HepG2 and Mz-Hep-1hepatoma cell lines.

2. After 24 h of culture, hepatocytes retained most of their CYP activities and protein contents, but the mRNA decreased 20-fold. However, the mRNA content of most CYPs in 24-h hepatocytes was still 400-fold higher than in hepatoma cells. When we examined the transcriptional activity of the CYP genes, this decreased during culture time in hepatocytes and it was poor in hepatoma cell lines.

3. We investigated the abundance of key hepatic transcription factors that govern CYP transcription (C/EBP-β: LAP and LIP, HNF-3α, HNF-4α, RXR-α) and observed that the expression of some of these factors was altered in the hepatoma cells.

4. In conclusion, the loss of biotransformation activity in cultured hepatic cells is caused by a decrease in CYP transcription, which correlates with an alteration in the expression of key transcription factors.

Abbreviations used:

AhR, aryl hydrocarbon receptor; arnt, aryl hydrocarbon receptor nuclear translocator; BROD, 7-benzoxyresorufin O-debenzylation; CAR, constitutive androstane receptor; C/EBP, CCAAT enhancer-binding protein; CH, coumarin 7-hydroxylation; CYP, cytochrome P450; DMSO, dimethylsulfoxide; DXOD, dextromethorphan O-demethylation; ECOD, 7-ethoxycoumarin O-deethylation; EROD, 7-ethoxyresorufin O-deethylation; HNF, Hepatocyte nuclear factors; hnRNA, heterologous nuclear RNA; LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein; MC, 3-methylcholanthrene; MROD, 7-methoxyresorufin O-demethylation; 4'OH-DIC, diclofenac 4' hydroxylation; 6β-OHT, testosterone 6β-hydroxylation; OHT, hydroxytestosterone; PB, phenobarbital; PNH, p-nitrophenol hydroxylation; PXR, pregnane X receptor; Rif, rifampicin; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate.

Introduction

Liver biotransformation mediates many of the biological effects, both toxic and therapeutic, of toxic compounds and clinical drugs (Meyer 1996). Among the drug metabolising enzymes in human liver, CYPs are the most important (Murray and Burke 1995, Paine 1995, Wrighton *et al.* 1996) and they catalyse the oxidation of many endogenous and exogenous compounds and provide the main route for xenobiotic biotransformation. Therefore, understanding the regulation of these genes and their encoded enzymes has a major clinical significance.

At the moment, human hepatocytes in culture and hepatoma cell lines are the most frequently used in vitro systems to investigate drug metabolism and toxicity. Human cultured hepatocytes contain the entire hepatic drug-metabolising enzyme system in an integrated form and they provide an in vitro model that has been successfully used to investigate drug effects on specific liver functions and hepatic drug metabolism (Maurel 1996, Gómez-lechón *et al.* 1997, Guillouzo 1998). However, cultured hepatocyte drug detoxification capacity decreases during culture. Hepatoma cell lines have an unlimited life-span, a more stable phenotype than primary cultures, and are always available, but they have a low CYP expression (Castell *et al.* 1998, Guillouzo 1998). HepG2 cells (Aden *et al.* 1979) are commonly used and Mz-Hep-1 cells (Dippold *et al.* 1985) are also human hepatoma cells. However, expression of the different CYPs has not been studied in Mz-Hep-1 cells and in HepG2 cells it has not been thoroughly characterised to date (Roe *et al.* 1993, Nakama *et al.* 1995, Sumida *et al.* 2000).

The mechanism that leads to decreased CYP activities in human cultured hepatic cells (both in primary cultures and in continuous cell lines) is still largely unknown. An inactivation of CYP enzymes, a decreased stability of the apoprotein, a decreased

mRNA production or mRNA stability etc, could all lead to the observed loss. The phenotypic instability of CYPs in primary cultures has been studied in the rat, where an early decrease in CYP mRNA contents precedes the decline in activities (Padgham *et al.* 1992, Woodcroft and Novak 1998). In human hepatocytes in culture, protein and activity contents decrease gradually, although slower, than in rat hepatocytes (Guillouzo and Guguen-Guillouzo 1992). Up to now, CYP expression in cultured human hepatocytes has not been analysed at activity, protein, and mRNA level in the same cell preparation. Hepatocyte isolation procedures and culture conditions greatly affect CYP expression, making impossible to use data from different studies to investigate at which level CYP expression is altered. Furthermore, time dependent expression of CYP mRNAs in human hepatocytes in culture has been poorly characterised and the results obtained are contradictory (Morel *et al.* 1990, George *et al.* 1997). To shed light on the regulatory mechanism that accounts for the decreased expression of the most important drug metabolising CYPs (1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) we have analysed the activity, protein, mRNA, and heterologous nuclear RNA (*hn*RNA) contents in human cultured hepatocytes and in HepG2 and Mz-Hep-1 cell lines.

Liver-specific gene expression in adult hepatocytes relies on four families of transcription factors that are liver-enriched (enhancer-binding protein (C/EBP), hepatocyte nuclear factor (HNF)-1, HNF-3 and HNF-4) (Cereghini 1996) and, although the mechanisms of liver-specific expression of the CYP genes are diverse, these liver enriched transcription factors (LETFs) govern the transcription of CYPs (Gonzalez and Lee 1996). The content of these LETFs has been related to CYP expression during primary culture (Padgham *et al.* 1993, Gómez-Lechón *et al.* 1998), therefore, we

analysed the cellular contents of some key transcription factors, namely C/EBP-β, HNF-3α and -4α, and retinoid X receptor (RXR)-α, in hepatocytes and cell lines.

The results of the present study show that the low expression of CYPs in human cultured cells is caused by a decrease in CYP gene transcription and that an alteration in the expression of LETFs could be involved in this process.

Materials and methods

Chemicals and reagents.

7-Ethoxyresorufin, 7-benzoxyresorufin and collagenase were from Roche (Mannheim, Germany). 7-Methoxyresorufin was from Molecular Probes Europe BV (Leiden, The Netherlands). Diclofenac sodium, coumarin, 7-ethoxycoumarin, 7-hydroxycoumarin, p-nitrophenol, 4-nitrocatechol, resorufin, testosterone, 16α- and 11ß-hydroxy-testosterone (OHT) androstenedione, phenobarbital (PB), rifampicin (Rif) and 3-methylcholanthrene (MC) were from Sigma (St. Louis, USA). The 6ß- and 16ß-hydroxy derivatives of testosterone were supplied by Steraloids Inc. (Wilton, USA). 2ß-Hydroxy-testosterone was from the Steroid Reference Collection (London, UK). 15ß-Hydroxy-testosterone (G.D. Searle and Co, Skokie, IL, USA) was kindly supplied by Dr. B. Blaauboer (Utrecht, Netherlands). Plastic culture plates were obtained from Nunc (Denmark). All other reagents used in this study were of analytical grade. Ham F-12, Leibovitz L-15 media, fetal calf serum and all RNA/ DNA modifying enzymes were from Lifetechnologies (Barcelona, Spain), who also custom-synthesised oligo d(T)14 primer and all the oligonucleotide primers used. Ribogreen and picogreen were from Molecular Probes, Rneasy Total RNA Kit was from Quiagen (Basel, Switzerland). The High Pure PCR Product Purification Kit was purchased from Roche.

Cell culture

Surgical liver biopsies were obtained in conformity with the rules of the Hospital´s Ethics Committee and after informed consent from patients undergoing programmed cholecystectomy. Patients had no known liver pathology, were not habitual

consumers of alcohol and other drugs and they did not receive any medication during the weeks before surgery. Human hepatocytes were isolated using a two-step perfusion technique (Gómez-Lechón *et al.* 1990) and seeded on fibronectin-coated plates (3.6 $\mu g/cm^2$) at a density of $8x10^4$ cells/cm^2 in an appropriate volume of the medium. The culture medium was Ham F-12/Leibovitz L-15 (1/1, v/v), supplemented with 2 % newborn calf serum, 5 mM glucose, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.2 % bovine serum albumin and 10^{-8} M insulin. The medium was changed one hour later to remove unattached hepatocytes. By 24 hours, the cultures were shifted to serum-free medium and 10^{-8} M dexamethasone was added. Thereafter, the medium was renewed daily. HepG2 and Mz-Hep-1 cells were plated in Ham's F-12/Leibovitz L-15 (1/1, v/v) supplemented with 7% newborn calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin. HeLa cells were grown in DMEM supplemented with 10% newborn calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin. For subculturing, cells were harvested after trypsin/EDTA (0.05%/0.02%) treatment at 37º C. Cells were used at the 75% monolayer confluency.

Treatment of cultures

Human hepatocytes and cell lines were exposed to inducers 24 h after plating. PB was prepared as an aqueous solution and added directly to cultures at a final concentration of 1 mM. MC and Rif were dissolved in DMSO and added to the culture medium to give a final concentration of 2 µM and 50 µM, respectively. The DMSO concentration in culture medium never exceeded 0.1 % (v/v). Control cultures were treated with the same concentration of the respective solvent.

Isolation, quantification and purification of total RNA from cell cultures

Total RNA was extracted from 6 cm diameter culture plates of human hepatocytes and HepG2 and Mz-Hep-1 cell lines using "Rneasy Total RNA Kit". The amount of purified RNA was estimated by ribogreen fluorescence and its purity by the absorbance ratio at 260/280 nm. RNA was incubated for 15 min at 23ºC with DNase I (1 unit/µg) according to the recommendations of the supplier, followed by thermal inactivation of the enzyme (65ºC for 10 min) in the presence of 2.5 mM EDTA and rapid cooling to 4ºC.

Measurement of mRNAs by RT-PCR

The reverse transcription (RT) reaction mixture consisted of 1 µg of total RNA which was reverse transcribed in 20 µl of 1x reverse transcriptase buffer, 10 mM DTT, 500 µM dNTPs, 3 µM oligo d(T)14 primer, 60 U Rnase OUT, and 250 U RTase H. 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 conditions used for the quantitative polymerase chain reaction (PCR), the specific primers and the strategy of quantification, have been previously described (Rodríguez-Antona *et al.* 2000).

Intron-specific RT-PCR assay

1 µg of total RNA was reverse transcribed as described before but using an intron-specific reverse transcription primer corresponding to the intron 2 of the human *CYP3A4* gene, 5´-CCT TCT TTC TGG CGC GCT CAC A-3´, together with the oligo d(T) 14 primer. PCR analysis of the CYP3A4 *hn*RNA used an upstream primer located on the intron 1 of *CYP3A4*, 5´-ATC TCA TCA TCA CTG CGG AAG A-3´ and a downstream primer located on the intron 2 of *CYP3A4*, 5´-AGA GCC CTT GGG TAA

ACA TTG C-3´. The CYP3A4 *hn*RNA fragment, which is 686 bp long, was amplified using the same PCR conditions described before (Rodríguez-Antona *et al*. 2000).

Measurement of drug-metabolising protein

Human hepatocytes that had been cultured on 6 cm diameter fibronectin-coated plates were solubilised in approximately 1 ml of 20 mM potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 0.1 mM EDTA, 0.02% (v/v) Nonidet P-40, 1% (w/v) sodium cholate and 20% (v/v) glycerol. Insoluble material was removed by centrifugation (100 000 x g for 30 min at 4°C) and the protein content of the supernatant was determined as previously described (Lowry *et al*. 1951). Samples were subjected to immunoblotting as described previously using anti-peptide antibodies which specifically recognised CYP2C forms (CYP2C8, CYP2C9, CYP2C19), CYP2E1, and CYP3A4 (Edwards et al. 1998).

The relative intensity of immunoreactive bands was determined by laser densitometry using an LKB UltroScan XL Enhanced Laser Densitometer (Amersham Pharmacia Biotech, Little Chalfont, UK) and analysed using FigP 6.0a software (Biosoft, Cambridge, UK).

Measurement of transcription factor protein

The immunoblotting analysis of transcription factors was performed as described previously (Jover *et al*. 1998). In brief, protein extracts were obtained from cells in suspension, electrophoresed in a SDS-polyacrylamide gel (20 µg protein /lane). Proteins were transferred to Immobilon-P membranes (Millipore) and sheets were incubated with the first polyclonal antibody (Santa Cruz Biotechnology, Santacruz, USA). After washing, blots were developed with horseradish peroxidase-labelled IgG, using an

Enhanced Chemiluminence Kit (Amersham Pharmacia Biotech).

Measurement of drug-metabolising activities

CYP activities were measured by incubating intact cell monolayers with specific substrates, namely 8 µM 7-ethoxyresorufin, 10 µM 7-methoxyresorufin, 15 µM 7-benzoxyresorufin, 50 µM coumarin, 800 µM 7-ethoxycoumarin, 200 µM dextormethorphan, 500 µM p-nitrophenol, 300 µM diclofenac, or 250 µM testosterone (Gomez-Lechon *et al.* 2001). Reactions were stopped by aspirating the incubation medium from plates and medium samples were then incubated with β-glucuronidase/arylsulfatase for 2 h at 37 ºC (Donato *et al*. 1993). Resorufin formed during 7-ethoxyresorufin O-deethylation, 7-methoxyresorufin O-demethylation (EROD and MROD, CYP1A1/2 and CYP1A2, respectively) and 7-benzoxyresorufin O-debenzylation (BROD, CYP2B6) assays and 7-hydroxycoumarin formed in the coumarin 7-hydroxylase (CH, CYP2A6) and 7-ethoxycoumarin O-deethylase (ECOD, various CYPs) assays were quantified fluorimetrically as previously described (Edwards *et al*. 1984, Donato *et al*. 1993). The 4-nitrocatechol formed during the p-nitrophenol hydroxylase (PNH, CYP2E1) assay was determined spectrophotometrically as previously described (Dicker *et al*. 1990). The products of diclofenac 4'-hydroxylation (4'OH-DIC, CYP2C9) and dextromethorphan O-demethylation (DXOD, CYP2D6) were measured by HPLC analysis (Kronbach *et al*. 1987, Bort *et al*. 1996). Hydroxylated metabolites formed from testosterone were extracted and analysed by HPLC as previously described (Donato *et al*. 1995).

Statistical analysis

Statistical analysis was done by Student's t-test.

Results

CYP expression during primary culture of human hepatocytes

Hepatocytes were isolated from four different human liver biopsies and cultured as described in Materials and Methods. After different culture times we determined CYP expression by measuring the mRNA contents and the specific catalytic activities of CYPs 1A1, 1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4 and the apoprotein levels of CYPs 2C9, 2E1 and 3A4. The reason for quantifying the protein only in three CYPs was that the amount of material required for this analysis is large and with the material available only the most abundant CYPs could be assayed.

Protein and activity levels generally declined in parallel (figure 1). Whereas all of the CYPs declined with time, the rate of decline was not the same. For some enzymes, after 48 h of culture, residual protein and/or activity levels were > 40% of those at 4 h (i.e. CYP2C9, CYP2D6, CYP2E1 and CYP3A4), whereas for other isoforms residual levels at this time were < 20% of those at 4 h (i.e. CYP1A2, CYP2A6 and CYP2B6).

Protein and activity levels have been expressed relative to those at 4 h of culture and hence it is not appropriate to compare the absolute percentage changes in protein level or CYP activity with those in mRNA expression. However, it is possible to compare the rates of decline and it becomes apparent that, for all of the CYPs, the decline in mRNA levels after 4 h was much more rapid than in either protein or activity levels. The CYP mRNA contents declined rapidly during the first few hours after hepatocyte isolation and after 4 h of culture the mRNA contents of all of the CYPs studied were <15% of those in fresh liver. After 24 h of culture mean mRNA content was 5.2% of that in liver and all enzymes had mRNA levels lower than 10%. The

mRNA content continued to decrease during culture and at 72 h of culture, mean CYP mRNA content was < 0.5% of that in liver (figures 1 and 2).

CYP expression in cell lines compared to hepatocytes

The activities and mRNA contents of the major drug metabolising CYPs were determined in human cultured hepatocytes and HepG2 and Mz-Hep-1 cell lines 24 h after seeding. CYP activities were examined using specific substrates and CYP mRNA contents were quantitatively determined by RT-PCR. In general, HepG2 cells showed higher CYP expression than Mz-Hep-1 cells, but these values were significantly lower than those of human hepatocytes (tables 1 and 2). EROD activity (CYP1A1) was the highest with 20 and 9% of that in 24 h cultured hepatocytes (HepG2 and Mz-Hep-1, respectively). CYP1A1 mRNA was 7 and 1 % of that in 24 h cultured hepatocytes (HepG2 and Mz-Hep-1, respectively). BROD (CYP2B6) and MROD (CYP1A2) activities presented also quite high levels and CYP3A5 and CYP2B6 mRNA contents in HepG2 were among the highest (1 and 0.2 % of that in 24 h cultured hepatocytes, respectively). CH (CYP2A6), PNP (CYP2E1) and 6β-OHT (CYP3A4) activities were < 1.0 % of that in 24 h cultured hepatocytes and mRNA contents of CYPs 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 were < 0.25 %.

Testosterone metabolism profiles by primary hepatocytes and cell lines

CYP functionality was assessed by measuring the profile of testosterone metabolism. Testosterone is metabolised in a regioselective manner by different CYPs and can be used as a multi-enzymatic substrate to investigate the activities of multiple enzymes simultaneously (Komori *et al*. 1988, Waxman *et al*. 1991). The major

metabolites produced by hepatocytes cultured for 4 h were 6β-OHT (CYP3A4) and androstenedione, followed by 2β- and 15β-OHT (CYP3A4-5), whereas 16α- and 16β-OHT were less abundant. The profile of metabolites released by hepatocytes cultured for longer periods was similar to that observed after 4 h of culture. The HepG2 and Mz-Hep-1 testosterone metabolic profiles were also determined and only formation of 6β- and 16α-OHT metabolites was observed (figure 3).

CYP inducibility in primary hepatocyte culture and cell lines

A relevant pharmaco-toxicological issue is CYP induction by drugs, which can have important clinical consequences by influencing their therapeutic efficacy (Li *et al*. 1996, Li 1997). CYP inducibility in HepG2 and Mz-Hep-1 cells was compared with that of cultured hepatocytes by measuring mRNA content and activity. The inducers were added to 24 h cultured cells and were incubated for 48 h as described in Methods. Non treated cells were used as controls. MC induction was assessed by measuring MROD activity and CYP1A2 mRNA content, PB induction was determined by BROD activity and by CYP2B6 mRNA content and Rif induction was assessed by measuring 6β–OHT activity and CYP3A4 mRNA content.

The results are summarized in table 3 and show that in all three cell systems MC induced MROD activity (mean 10.7 fold induction) and CYP1A2 mRNA (mean 10.3 fold). Whereas PB induction of BROD activity and CYP2B6 mRNA was not significant. Rif induced 6β-OHT activity in hepatocytes and HepG2 cells and CYP3A4 mRNA levels were increased in hepatocytes (15.3 fold) and Mz-Hep-1 cells (2.6 fold).

CYP3A4 hnRNA expression in human hepatocytes and cell lines

The decreased CYP mRNA contents that we observed in the cultured hepatic cells could be caused by decreased CYP transcription, an altered RNA processing, or a mRNA instability. The determination of CYP *hn*RNA expression constitutes a reliable method for the measurement of the gene transcriptional activity (Elferink and Reiners 1996, Backlund *et al.* 1997). The expression of the CYP3A4 gene at the transcriptional level was determined by RT-PCR-based determination of CYP3A4 *hn*RNA using a 686-bp fragment corresponding to exon 2 and parts of the flanking intron sequences of the CYP3A4 gene as a target. CYP3A4 mRNA was quantified by RT-PCR using primers specific for cDNA. In the same RT preparations CYP3A4 *hn*RNA and CYP3A4 mRNA were quantified and β-actin mRNA was used as an internal standard. The assay of *hn*RNA expression revealed that CYP3A4 *hn*RNA decreased in hepatocytes with culture time in a similar way as CYP3A4 mRNA and that the levels in hepatoma cell lines were far lower than those in liver (figure 4).

Expression of transcription factors in human hepatocytes, HepG2, Mz-Hep-1, and HeLa cells

The expression of several LETFs and nuclear receptors involved in CYP transcription was analysed by Western blot analysis in a fresh suspension of human hepatocytes, HepG2 and Mz-Hep-1 (hepatic cell lines) and HeLa (non hepatic cell line). As a quality test of the samples used we studied the expression of the nuclear receptor STAT-3α which should be similar in all the cells tested (Runge *et al.* 1999). As shown in figure 5, STAT-3α levels were similar in the different cells. HNF-4α exhibited the highest levels in HepG2, followed by hepatocytes and HeLa cells, but was not detected in Mz-Hep-1 cells and the expression of nuclear receptor RXR-α was the same in the

different cells. The *C/EBP-β* gene produces different isoforms by alternative initiation of translation, the full length isoform liver-enriched transcriptional activator protein (LAP) and the truncated isoform liver-enriched transcriptional inhibitory protein (LIP), which lacks most of the transactivation domain. As shown in figure 5, LAP was detected in all the cells tested and was highest in HepG2 cells. LIP (the inhibitory isoform of C/EBP-β) expression was not detected in hepatocytes and the highest levels were detected in Mz-Hep-1 cells. HNF-3α was only detected in the non hepatic cell line HeLa, and in similar experiments HNF-1α was not detected in any of the cell lines used.

Discussion

The expression of many hepatic specific proteins (including CYPs), decline during the primary culture of hepatocytes and the cells dedifferentiate becoming more similar to hepatic cell lines. The decrease in CYP activities could be caused by enzymatic inhibition (CYP protein and mRNA contents would remain unchanged), by a decrease in protein stability (mRNA contents should be unaltered) or by a decrease in mRNA synthesis or stability (mRNA and protein levels should be diminished). The study of mRNA, protein and activity will provide information about the regulatory mechanism(s) that is altered resulting in decreased CYP expression.

In our study, CYP protein contents and activities of all CYPs examined declined in parallel, thus indicating that intact apoprotein in hepatocytes remains in the form of holoenzyme during culture. Similar results have been described with cultured cells (Wu *et al*. 1990) and with cultured human liver slices (Renwick *et al*. 2000). In contrast to CYP protein content and activity, the mRNA declined substantially, reaching very low levels after 24 h of culture. These results differ from those of George *et al*. (George *et al*. 1997) in which the protein content of CYPs 1A2, 2C9, and 3A4 decreased, whereas mRNA contents increased. Our results resemble those reported for cultured rat hepatocytes (Padgham *et al*. 1993, Wang *et al*. 1997, Woodcroft and Novak 1998) and support the conclusion that a decreased CYP mRNA content is responsible for the decline of human CYP activities during culture.

When we compared CYP mRNA contents and activities of cultured hepatocytes with those of HepG2 and Mz-Hep-1 cells, we found substantially reduced levels in the hepatoma cells (tables 1 and 2). The data showed again that a decrease in the CYP mRNA content is responsible for the low enzymatic activities in hepatoma cells, clearly

indicating that human hepatocytes are a much better in vitro system for drug-metabolism studies than cell lines.

To investigate if the low mRNA contents were consequence of a decreased transcription of the *CYP* genes rather than a posttranscriptional mechanism (processing of the RNA in the nucleus and mRNA stability) we measured the levels of CYP3A4 *hn*RNA. The results obtained were similar to those of CYP3A4 mRNA (figure 4), indicating that transcription of the *CYP* genes is the cause of the decreased mRNA levels.

Two different mechanisms could account for the decreased transcription rate: i) a decreased expression of activatory transcription factors needed for *CYPs* transcription, or ii) an increase in the expression of inhibitory factors. The LETFs are responsible for the hepatic specific transcription of CYPs in liver (i.e. HepG2 cells stably transfected with C/EBPα have increased CYPs levels (Jover *et al.* 1998), a decrease in the expression of key LETFs appears to cause the down-regulation of CYP expression during hepatocyte culture (Padgham *et al.* 1993, Gómez-Lechón *et al.* 1998, Jover *et al.* 1998). Interestingly, in our study the highest CYP mRNA levels in hepatoma cells corresponded to CYPs 1A1 and 3A5 (table 2), which are not liver specific (Guengerich 1995, Haehner *et al.* 1996). Our hypothesis was that the decreased transcription of *CYPs* in cultured hepatic cells could be caused by changes in the expression of LETFs implicated in CYP regulation. Therefore, we carried out a detailed examination of activatory (HNF-4α, RXR-α, and LAP) and inhibitory (LIP and HNF-3α) transcription factors.

According to the stimulatory effect of HNF-4α on expression of HNF-1 (Kuo *et al.* 1992) and some CYPs (Jover *et al.* 2001) as well as many other hepatic genes (Späth

and Weiss *et al*. 1997), the absence of HNF-4α in Mz-Hep-1 cells correlates with its lower CYP expression. However, the presence of the largest amounts of HNF-4α in HepG2 cells would suggest that HNF-4α alone does not determine CYP expression. CYP induction by PB and Rif requires heterodimerization of the nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) with RXR (Savas *et al*. 1999, Waxman *et al*. 1999). The similar RXR-α contents found in hepatocytes and hepatoma cells, suggest that the lack of response of hepatoma cell lines to PB and Rif treatments (table 3) is not due to differences in this protein. C/EBP-β has been related to the expression of CYPs (Lee *et al*. 1997, Gómez-Lechón *et al*. 2001) and can be transcribed from the same gene as a full length isoform (LAP) and a truncated isoform (LIP). The LIP isoform lacks the transactivatory domain and antagonizes the transactivation of C/EBP-α and LAP (Descombes and Schibler 1991, Ossipow *et al*. 1993). In our study LAP expression, though similar in the different cells, was largest in HepG2 cells, which does not correlate with CYP expression. However, the inhibitory factor LIP was highest in Mz-Hep-1 cells, followed by HepG2 and HeLa cells and no expression in freshly isolated hepatocytes. As a moderate increase in the LAP/LIP ratio can result in a significantly higher transcriptional activation of an appropriate target gene (Descombes and Schibler 1991), the LAP/LIP ratio found in hepatocytes and hepatoma cells correlates with their differentiation state and CYP expression. HNF-3 plays an important role in the expression of liver-specific genes and different *CYP2Cs* genes (Legraverend *et al*. 1994, Shaw *et al*. 1994, Vallet *et al*. 1995, Delesque-Touchard *et al*. 2000). In our study the only cells with a detectable expression of HNF-3α were HeLa cells. It has been reported that HNF-3α acts as a negative regulator with antagonistic transcriptional regulatory functions with HNF-3β (Duncan *et al*. 1998),

which is in accordance with the HeLa non-hepatic phenotype.

In summary, as a step towards further understanding the mechanisms leading to low CYP activity in the cultured hepatic cells, our results provide evidence that: a) human hepatocytes constitute a useful liver model for drug biotransformation and CYP regulation studies whereas HepG2 and Mz-Hep-1 are not; b) the low drug metabolising capacity of human cultured hepatic cells is the consequence of a decrease in CYP gene transcription; and c) the expression of key inhibitory and activatory LETFs is altered in the hepatoma cell lines and could be linked to decreased CYP expression and functionality of these cells.

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References

ADEN, D.P., FOGEL, A., PLOTKIN, S., DAMJANOV, I. and KNOWLES, B.B., 1979, Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature*, **282**, 615-616.

BACKLUND, M., JOHANSSON, I., MKRTCHIAN, S. and INGELMAN-SUNDBERG, M., 1997, *Journal of Biological Chemistry*, **272**, 31755-31763.

BORT, R., PONSODA, X., CARRASCO, E., GÓMEZ-LECHÓN, M.J. and CASTELL, J.V., 1996, Metabolism of aceclofenac in humans. *Drug Metabolism and Disposition*, **24**, 834-841.

CASTELL, J.V., JOVER, R., BORT, R. and GÓMEZ-LECHÓN, M.J., 1998, The challenge of using hepatic cell using cell lines for drug metabolism studies. In Proceedings of COST B1 European Symposium on the prediction of drug metabolism in man: Progress and problems (Luxemburg, European Commission) pp. 77-92

CEREGHINI, S., 1996, Liver-enriched transcription factors and hepatocyte differentiation. *FASEB Journal*, **10**, 267-282.

DELESQUE-TOUCHARD, N., PARK, S.H. and WAXMAN, D.J.,2000, Synergistic action of hepatocyte nuclear factors 3 and 6 on CYP2C12 gene expression and suppression by growth hormone-activated STAT5b. Proposed model for female specific expression of CYP2C12 in adult rat liver. *Journal of Biological Chemistry*, **275**, 34173-34182.

DESCOMBES, P. and SCHIBLER, U., 1991, A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell*, **67**, 569-579.

DICKER, E., MCHUGH, T. and CEDERBAUM, A.I., 1990, Increased oxidation of p-nitrophenol and aniline by intact hepatocytes isolated from pyrazole-treated rats. *Biochimica et Biophysica Acta*, **1035**, 249-256.

DIPPOLD, W.G., DIENES, H.P., KNUTH, A., SACHSSE, W., PRELLWITZ, W., BITTER-SUERMANN, D. and MEYER ZUM BUSCHENFELDE, K.H., 1985, Hepatocellular carcinoma after thorotrast exposure: establishment of a new cell line (Mz-Hep-1). *Hepatology*, **5**, 1112-1119.

DONATO, M.T., CASTELL, J.V. and GÓMEZ-LECHÓN, M.J., 1995, Effect of model inducers on cytochrome P450 activities of human hepatocytes in primary cultures. *Drug Metababolism and Disposition*, **25**, 553-558.

DONATO, M.T., GÓMEZ-LECHÓN, M.J. and CASTELL, J.V., 1993, A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Analytical Biochemistry*, **213**, 29-33.

DUNCAN, S.A., NAVAS, M.A., DUFORT, D., ROSSANT, J. and STOFFEL, M., 1998, *Science*, **281**, 692-695.

EDWARDS, A.M., GLISTAK, M.L., LUCAS, C.M. and WILSON, P.A., 1984, +/-Ethoxycoumarin deethylase activity as a convinient measure of liver drug metabolizing enzymes: regulation in cultured rat hepatocytes. *Biochemical Pharmacology*, **33**, 1537-1546.

EDWARDS, R.J., ADAMS, D.A., WATTS, P.S., DAVIES, D.S. and BOOBIS, A.R., 1998, Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in human. *Biochemical Pharmacology*, **56**, 377-87.

ELFERINK, C.J. and REINERS, J.J.J, 1996, Quantitative RT-PCR on CYP1A1 heterogeneous nuclear RNA: a surrogate for the in vitro transcription run-on assay. *Biotechniques*, **20**, 470-477.

GEORGE, J., GOODWIN, B., LIDDLE, C., TAPNER, M. and FARRELL, G.C., 1997, *Journal of Laboratory and Clinical Medicine*, **129**, 638-648.

GÓMEZ-LECHÓN, M., JOVER, R., DONATO, M.T., PONSODA, X., RODRIGUEZ, C., STENZEL, K.G., KLOCKE, R., PAUL, D., GUILLÉN, I., BORT, R. and CASTELL, J.V., 1998, Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. *Journal of Cellular Physiology*, **177**, 553-562.

GÓMEZ-LECHÓN, M.J., DONATO, M.T., PONSODA, X., FABRA, R., TRULLENQUE, R. and CASTELL, J.V., 1997, Isolation, culture and use of human hepatocytes in drug research. In in vitro Methods in Pharmaceutical Research (San Diego, Academic Press), pp. 129-154.

GOMEZ-LECHON, M.J., DONATO, T., JOVER, R., RODRIGUEZ, C., PONSODA, X., GLAISE, D., CASTELL, J.V. and GUGUEN-GUILLOUZO, C., 2001, Expression and induction of a large set of drug metabolism enzymes by the highly differentiated human hepatoma cell line BC2. *European Journal of Biochemistry*, **268**, 1448-1459.

GÓMEZ-LECHÓN, M.J., LÓPEZ, P., DONATO, T., MONTOYA, A., LARRAURI, A., GIMÉNEZ, P., TRULLENQUE, R., FABRA, R. and CASTELL, J.V., 1990, Culture of human hepatocytes from small surgical liver biopsies. Biochemical characterization and comparison with in vivo. *In Vitro Cellular and Developmental Biology*, **26**, 67-74.

GONZALEZ, F. and LEE, Y., 1996, Constitutive expression of hepatic cytochrome P450 genes. *FASEB Journal*, **10**, 1112-1117.

GUENGUERICH, F.P., 1995, Human cytochrome P450 enzymes. In Cytochromes P-450: structure, mechanism and biochemistry (New York: Plenum Press), pp. 473-535.

GUILLOUZO, A. and GUGUEN-GUILLOUZO, C., 1992, In vitro approaches to hepatotoxicity studies. In In vitro methods in toxicology, (London: Academic Press Ltd), pp 133-160.

GUILLOUZO, A., 1998, Liver cell models in in vitro toxicology. *Environmental Health Perspectives*, **106**, 511-531.

HAEHNER, B.D., GORSKI, J.C., VANDENBRANDEN, M., WRIGHTON, S.A., JANARDAN, S.K., WATKINS, P.B. and HALL, S.D., 1996, Bimodal distribution of renal cytochrome P450 3A activity in humans. *Molecular Pharmacology*, **50**, 52-59.

JOVER, R., BORT, R., GOMEZ, L., MJ and CASTELL, J., 1998, Re-expression of C/EBP alpha induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS Letters*, **431**, 227-230.

JOVER, R., BORT, R., GOMEZ-LECHON, M.J. and CASTELL, J.V., 2001, Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. *Hepatology*, **33**, 668-675.

KOMORI, M., HAHIZUME, T., OHI, H., MIURA, T., KITADA, M., NAGASHIMA, K. and KAMATAKI, T., 1988, Cytochrome P-450 in human liver microsomes:

high-performance liquid chromatographic isolation of three forms and their characterization. *Journal of Biochemistry*, **104**, 912-916.

KRONBACH, T., MATHYS, D., GUT, J., CATIN, T. and MEYER, U.A., 1987, High-peformance liquid chromatographic assays for bufuralol 1'-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan O-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Analytical Biochemistry*, **162**, 24-32.

KUO, C.J., CONLEY, P.B., CHEN, L., SLADEK, F.M., DARNELL, J.E. and CRABTREE, G.R., 1992, A transcriptional hierarchy involved in mammalian cell-type specification. *Nature*, **355**, 457-461.

LEE, Y.H., WILLIAMS, S.C., BAER, M., STERNECK, E., GONZALEZ, F.J. and JOHNSON, P.F., 1997, *Molecular and Cellular Biology*, **17**, 2038-2047.

LEGRAVEREND, C., EGUCHI, H., STROM, A., LAHUNA, O., MODE, A., TOLLET, P., WESTIN, S. and GUSTAFSSON, J., 1994, Transactivation of the rat CYP2C13 gene promoter involves HNF-1, HNF-3, and members of the orphan receptor subfamily. *Biochemistry*, **33**, 9889-9897.

Li, A.P., 1997, Primary hepatocyte cultures as an in vitro experimental model for the evaluation of pharmacokinetic drug-drug interactions. In Advances in Pharmacology. Drug-Drug Interactions, Scientific and regulatory perspectives. (London: Academic Press) pp.103-129.

LI, A.P., MAUREL, P., GÓMEZ-LECHÓN, M.J., CHENG, L.C. and JURIMA ROMERT, M., 1996, Preclinical evaluation of drug-drug interaction potential : present status of the application of primary human hepatocytes in the evaluation of cytochrome P450 induction. *Chemico-Biological Interactions*, **107**, 5-16.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. and RANDAL, R.J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-75.

MAUREL, P., 1996, The use of adult human hepatocytes in primary culture and other in vitro systems to investigate drug metabolism in man. *Advanced Drug Delivery Reviews*, **22**, 105-132.

MEYER, U.A., 1996, Overview of enzymes of drug metabolism. *Journal of Pharmacokinetics and Biopharmacy*, **24**, 449-459.

MOREL, F., BEAUNE, P.H., RATANASAVANH, D., FLINOIS, J.P., YANG, C.S., GUENGERICH, F.P. and GUILLOUZO, A., 1990, *European Journal of Biochemistry*, **191**, 437-444.

MURRAY, G. I. and BURKE, D. M., 1995, Immunochemistry of drug metabolizing enzymes. *Biochemical Pharmacology*, **50**, 895-903.

NAKAMA, A., KURODA, K. and YAMADA, A., 1995, Induction of cytochrome P450-dependent monooxygenase in serum-free cultured HepG2. *Biochemical Pharmacology*, **50**, 1407-1412.

OSSIPOW, V., DESCOMBES, P. and SCHIBLER, U., 1993, CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proceedings of the National Academy of Sciences, USA.*, **90**, 8219-8223.

PADGHAM, C.R., BOYLE, C.C., WANG, X.J., RALEIGH, S.M., WRIGHT, M.C. and PAINE, A.J., 1993, Alteration of transcription factor mRNAs during the isolation and culture of rat hepatocytes suggests the activation of a proliferative mode

underlies their de-differentiation. *Biochemical Biophysical Research Communications*, **197**, 599-605.

PADGHAM, C.R., PAINE, A.J., PHILLIPS, I.R. and SHEPHARD, E.A., 1992, Maintenance of total cytochrome P-450 content in rat hepatocyte culture and the abundance of CYP1A2 and CYP2B1/2 mRNAs. *Biochemical Journal*, **285**, 929-932.

PAINE, A.J., 1995, Heterogeneity of cytochrome P450 and its toxicological significance. *Human Experimental Toxicology,* **14**, 1-7.

RENWICK, A.B., WATTS, P.S., EDWARDS, R.J., BARTON, P.T., GUYONNET, I., PRICE, R.J., TREDGER, J.M., PELKONEN, O., BOOBIS, A.R. and LAKE, B.G., 2000, Differential maintenance of cytochrome P450 enzymes in cultured precision-Cut human liver slices. *Drug Metabolism and Disposition*, **28**, 1202-1209.

RODRÍGUEZ-ANTONA, C., JOVER, R., GÓMEZ-LECHÓN, M.J. and CASTELL, J.V, 2000, Quantitative RT-PCR measurement of human cytochrome P-450s: application to drug induction studies. *Archives of Biochemistry and Biophysics*, **376**, 109-116.

ROE, A.L., SNAWDER, J.E., BENSON, R.W., ROBERTS, D.W. and CASCIANO, D.A., 1993, HepG2 cells: an in vitro model for P450-dependent metabolism of acetaminophen. *Biochemical Biophysical Research Communications*, **190**, 15-19.

RUNGE, D.M., RUNGE, D., FOTH, H., STROM, S.C., MICHALOPOULOS, G.K., 1999, STAT 1alpha/1beta, STAT 3 and STAT 5: expression and association with c-MET and EGF-receptor in long-term cultures of human hepatocytes. *Biochemical Biophysical Research Communications*, **265**, 376-381.

SAVAS, U., GRIFFIN, K.J. and JOHNSON, E.F., 1999, Molecular mechanisms of cytochrome P-450 induction by xenobiotics: an expanded role for nuclear receptors. *Molecular Pharmacology*, **56**, 851-857.

SHAW, P.M., WEISS, M.C. and ADESNIK, M., 1994, Hepatocyte nuclear factor 3 is a major determinant of CYP2C6 promoter activity in hepatoma. *Molecular Pharmacology*, **46**, 79-87.

SPÄTH, G.F. and WEISS, M.C., 1997, Hepatocyte nuclear factor 4 expression overcomes repression of the hepatic phenotye in dedifferentiated hepatoma cells. *Molecular and Cellular Biology*, **17**, 1931-1942.

SUMIDA, A., FUKUEN, S., YAMAMOTO, I., MATSUDA, H., NAOHARA, M. and AZUMA, J.,2000, Quantitative analysis of constitutive and inducible CYPs mRNA expression in the HepG2 cell line using reverse transcription-competitive PCR. *Biochemical Biophysical Research Communications*, **267**, 756-760.

VALLET, V., ANTOINE, B., CHAFEY, P., VANDEWALLE, A. and KAHN, A., 1995, Overproduction of a truncated hepatocyte nuclear factor 3 protein inhibits expression of liver-specific genes in hepatoma cells. *Molecular and Cellular Biology*, **15**, 5453-5460.

WANG, X.J., HODGKINSON, C.P., WRIGHT, M.C. and PAINE, A.J., 1997, Temperature-sensitive mRNA degradation is an early event in hepatocyte de-differentiation. *Biochemical Journal*, **328**, 937-944.

WAXMAN, D.J., 1999, P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Archives of Biochemistry and Biophysics*, **369**, 11-23.

WAXMAN, D.J., LAPENSON, D.P., AOYAMA, T., GELBOIN, H.V., GONZALEZ, F.J. and KORZEKWA, K., 1991, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Archives of Biochemistry and Biophysics*, **290**, 160-166.

WOODCROFT, K.J. and NOVAK, R.F., 1998, Xenobiotic-enhanced expression of cytochromes P450 2E1 and 2B in primary cultured rat hepatocytes. *Drug Metabolism and Disposition*, **26**, 372-378.

WRIGHTON, S. A., VANDENBRANDEN, M. and RING, B. J., 1996, The human drug metabolizing cytochromes P450. *Journal of Pharmacokinetics and Biopharmacy*, **24**, 461-473.

WU, D.F., CLEJAN, L., POTTER, B. and CEDERBAUM, A. I., 1990, Rapid decrease of cytochrome P-450IIE1 in primary hepatocyte culture and its maintenance by added 4-methylpyrazole. *Hepatology*, **12**, 1379-1389.

Table 1. Cytochrome P450 activity in 24h cultured human hepatocytes, HepG2 and Mz-Hep-1.

CYP activity	ECOD (CYPs)	EROD (CYP1A1/2)	MROD (CYP1A2)	CH (CYP2A6)	BROD (CYP2B6)	PNP (CYP2E1)	TEST (CYP3A4)
Hepatocytes (n=6)	24.2±11.3	4.5±2.0	5.04±1.87	121±70	2.46±1.95	104±57	253±110
HepG2 (n=5)	0.5±0.2**	0.9±0.5**	0.35±0.34**	0.05±0.04**	0.28±0.26*	< 1	0.18±0.16**
Mz-Hep-1(n=3)	< 0.1	0.4±0.3**	< 0.1	< 0.01	< 0.1	< 1	0.15±0.33**

Activities are given as pmol production/mg protein/min. Data are the mean ± SD.

* $p < 0.05$ and ** $p < 0.01$ with respect to hepatocytes

Table 2. Cytochrome P450 mRNA content in 24 h cultured human hepatocytes, HepG2 and Mz-Hep-1 cells.

CYP	1A1	1A2	2A6	2B6	2C9	2C19	2D6	2E1	3A4	3A5
Hepatocytes (n=6)	10.2±18.1	162±210	4.07±7.18	22.4±15.7	288±428	21.7±22.8	263±409	479±303	327±255	30.1±30.3
HepG2 (n=3)	0.68±0.08	0.04 ±0.02	< 0.01	0.04±0.05*	< 0.01	< 0.01	0.03±0.01	0.06±0.05*	0.10 ±0.04	0.35±0.18
Mz-Hep-1 (n=3)	0.14±0.03	0.05±0.03	< 0.01	0.02±0.01*	< 0.01	< 0.01	0.03±0.01	0.03±0.2*	0.01±0.01	< 0.01

Data are given as mRNA CYP molecules /10^3 molecule β-actin. Data are the mean ± SD.

* $p < 0.05$ with respect to hepatocytes

Table 3. Cytochrome P450 activity and mRNA induction by MC, PB and Rif in cultured human hepatocytes, HepG2 and Mz-Hep-1 cells[a]

Inducer	MC		PB		Rif	
Induction[b]	MROD	CYP1A2 mRNA	BROD	CYP2B6 mRNA	6β-OHT	CYP3A4 mRNA
Hepatocytes (n=3)	12.7±6.0*	11.1±9.9	1.5±0.6	6.1±3.8	2.5±0.8*	15.3±8.3*
HepG2 (n=3)	14.2±8.2*	11.6±5.6*	3.5±2.2	1.6±0.3	4.2±1.9*	1.3±0.5
Mz-Hep-1 (n=3)	5.2±2.7*	8.1±1.9**	nd	1.3±0.5	1.4±0.8	2.6±0.9*

[a] After 24h of culture, cells were exposed to 2 µM MC, 1mM PB or 50 µM Rif for 48 h, and, specific activities and mRNA contents were evaluated as described in Materials and Methods.

[b] Results are expressed as fold induction over control cells.

* $p < 0.05$ with respect to control

** $p < 0.01$ with respect to control

Figure Legends

Figure 1. Stability of cytochrome P450s in culture. CYPs 2C9, 2E1 and 3A4 specific activities, mRNA and protein content in human cultured hepatocytes. mRNA content (assayed by RT-PCR) was measured in a small piece of liver and at 2, 4, 16, 24, 48 and 72 h of culture. Protein content (measured by immunoblot using specific antibodies) and metabolic activities (assayed using specific substrates) were measured at 4, 16, 24, 48 and 72 h of culture. mRNA is expressed as % of that in the liver and protein contents and activities are expressed as % of that at 4h of culture. Open bars correspond to mRNA contents, grey bars correspond to activity and black bars correspond to protein content. A) CYP2C9 mRNA, CYP2C9 protein and 4´OH-Dic activity. B) CYP2E1 mRNA, CYP2E1 protein and PNP activity. C) CYP3A4 mRNA, CYP3A4 protein and 6β-OHT. Data are the mean ± SD of four different human primary cultures. *($p < 0.05$) and **($p < 0.01$) with respect to liver for mRNA or from 4h-hepatocytes for protein and activity.

Figure 2. Stability of cytochrome P450s in culture. CYPs 1A2, 2A6, 2B6 and 2D6 specific activities and mRNA were quantified in human cultured hepatocytes. mRNA content (assayed by RT-PCR) was measured in a small piece of liver and at 2, 4, 16, 24, 48 and 72 h of culture. Metabolic activities (assayed using specific substrates) were measured at 4, 16, 24, 48 and 72 h of culture. mRNA is expressed as % of that in liver and activities expressed as % of that at 4h of culture. Open bars correspond to mRNA contents and grey bars correspond to activity. A) CYP1A2 mRNA and MROD activity, B) CYP2A6 mRNA and CH activity, C) CYP2B6 mRNA and BROD and D) CYP2D6 mRNA and DXOD. Data are the mean ± SD of four different human primary cultures.

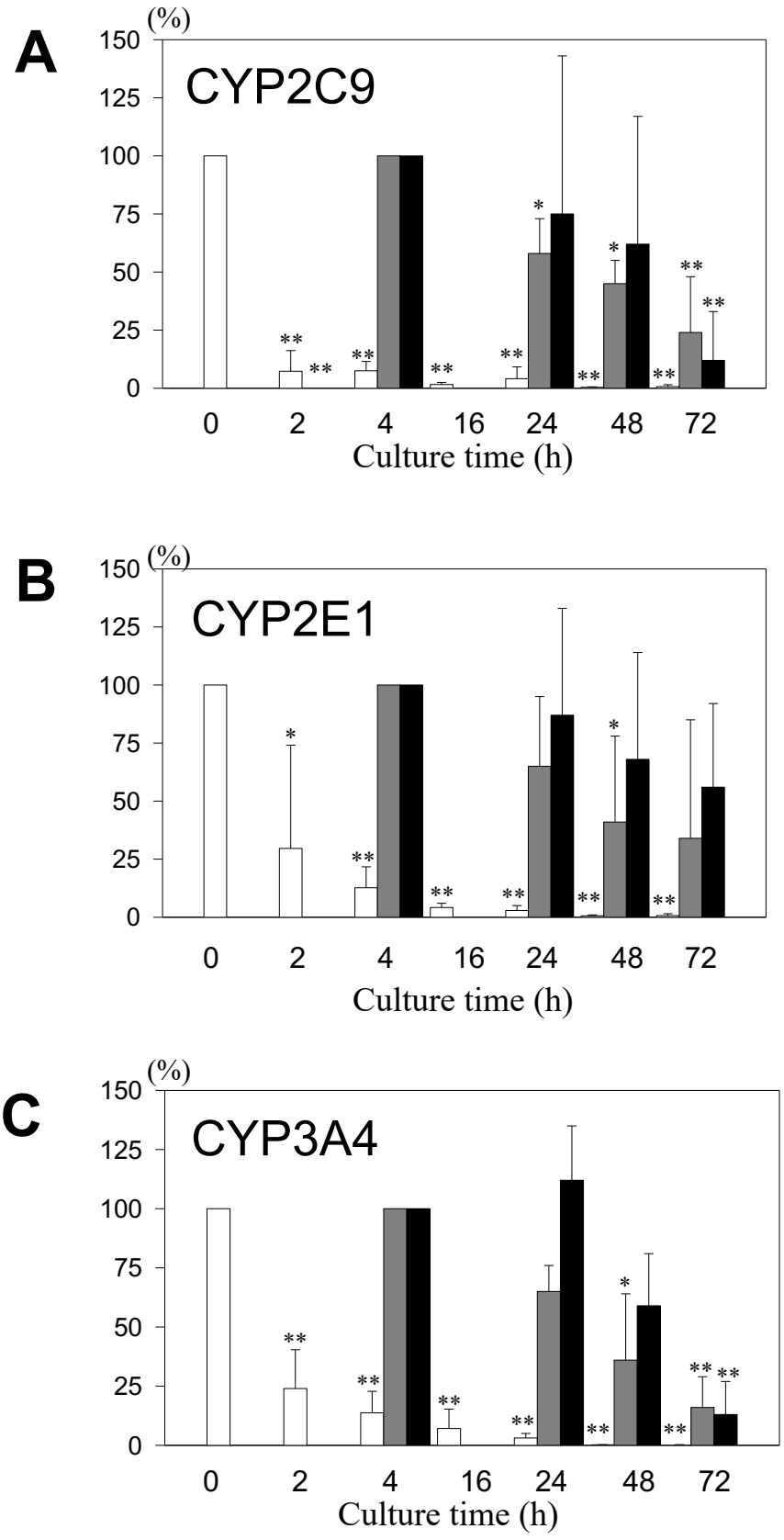
*($p < 0.05$) and **($p < 0.01$) with respect to liver for mRNA or from 4h-hepatocytes for activity.

Figure 3. Metabolite profile of testosterone by human hepatocytes and HepG2 and Mz-Hep-1 cells. A) Human hepatocytes after 4 h of culture; B) human hepatocytes after 72 h of culture; C) HepG2 cells after 24 h of culture; and D) Mz-Hep-1 cells after 24 h of culture. Cells were incubated for 1 h with 250 μM testosterone and the hydroxylated metabolites formed were extracted and analysed by HPLC. Peaks: 1, 15β-OHT; 2, 6β-OHT; 3, 16α-OHT; 4, 16β-OHT; 5, 11β-OHT (internal standard); 6, 2β-OHT; 7 androstenedione; and 8, testosterone.

Figure 4. CYP3A4 *hn*RNA levels in cultured hepatic cells. Total RNA was extracted from a piece of human liver (Hepat Liver), 4 h and 72 h human cultured hepatocytes (Hepat 4 h and 72 h), HepG2, and MzHep-1 cells. 1 μg of total RNA was reverse transcribed using both oligo(dT) and an intron-specific CYP3A4 RT primer and amplified by PCR using different specific primers for CYP3A4 *hn*RNA, CYP3A4 mRNA and β-actin mRNA. The PCR products were analysed on a 1.5 % agarose/TBE gel and visualised with UV light using ethidium bromide staining. M: 100-bp DNA ladder.

Figure 5. Western-blot of transcription factors and nuclear receptors in human hepatocytes and cell lines. Total extracts of protein were obtained from a fresh suspension of human hepatocytes, HepG2, MzHep-1, or HeLa cells and 20 μg were elecrophoresed and transfered to a membrane. LAP, LIP, HNF-3α, HNF-4α, RXR-α

and STAT-3α protein contents in the different samples were detected using specific antibodies.


A
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150
125
100
75
50
25
0
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16
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72
Culture time (h)
B
(%)
CYP2E1
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100
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25
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24
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72
Culture time (h)
C
(%)
CYP3A4
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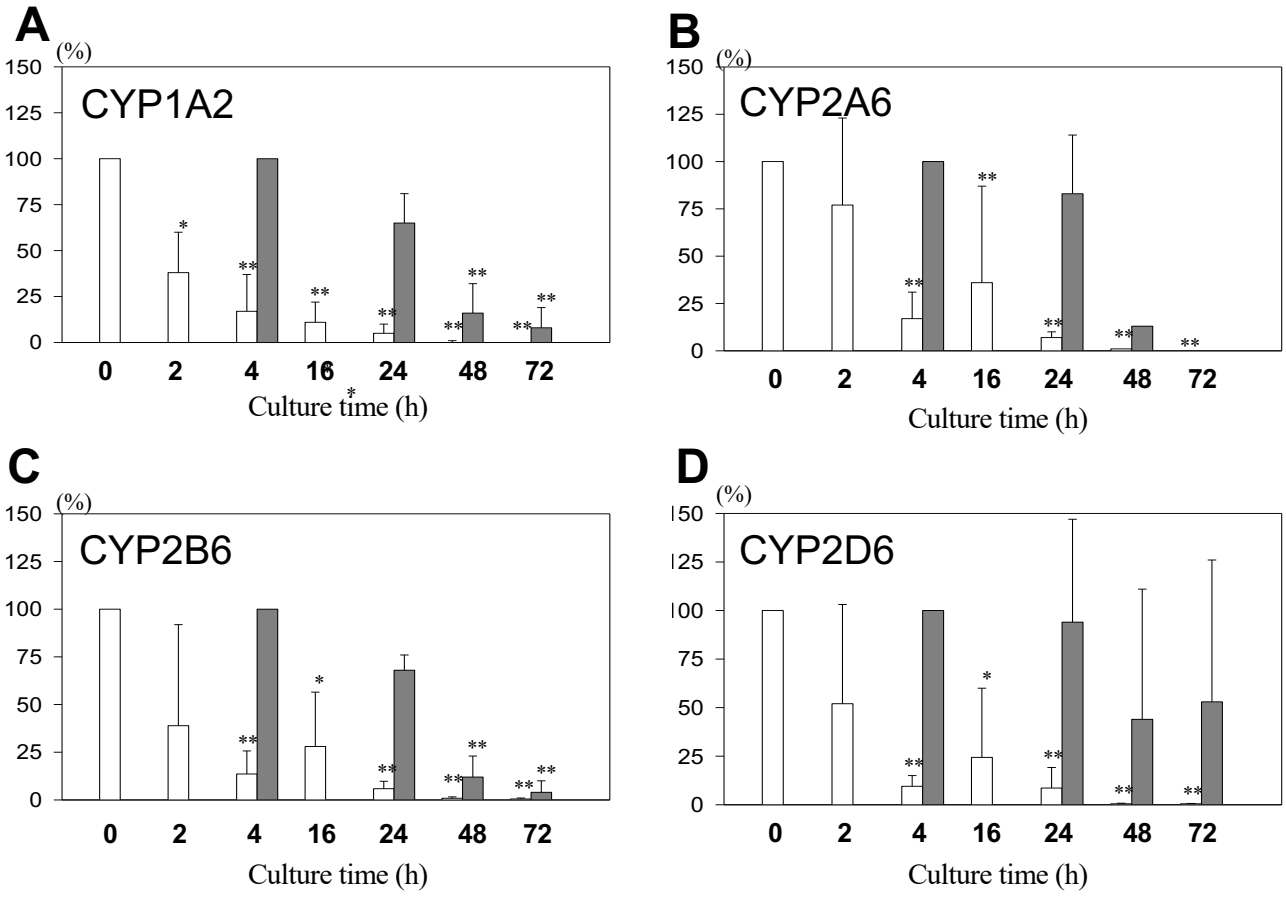
Culture time (h)

Figure 2


A
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Culture time (h)
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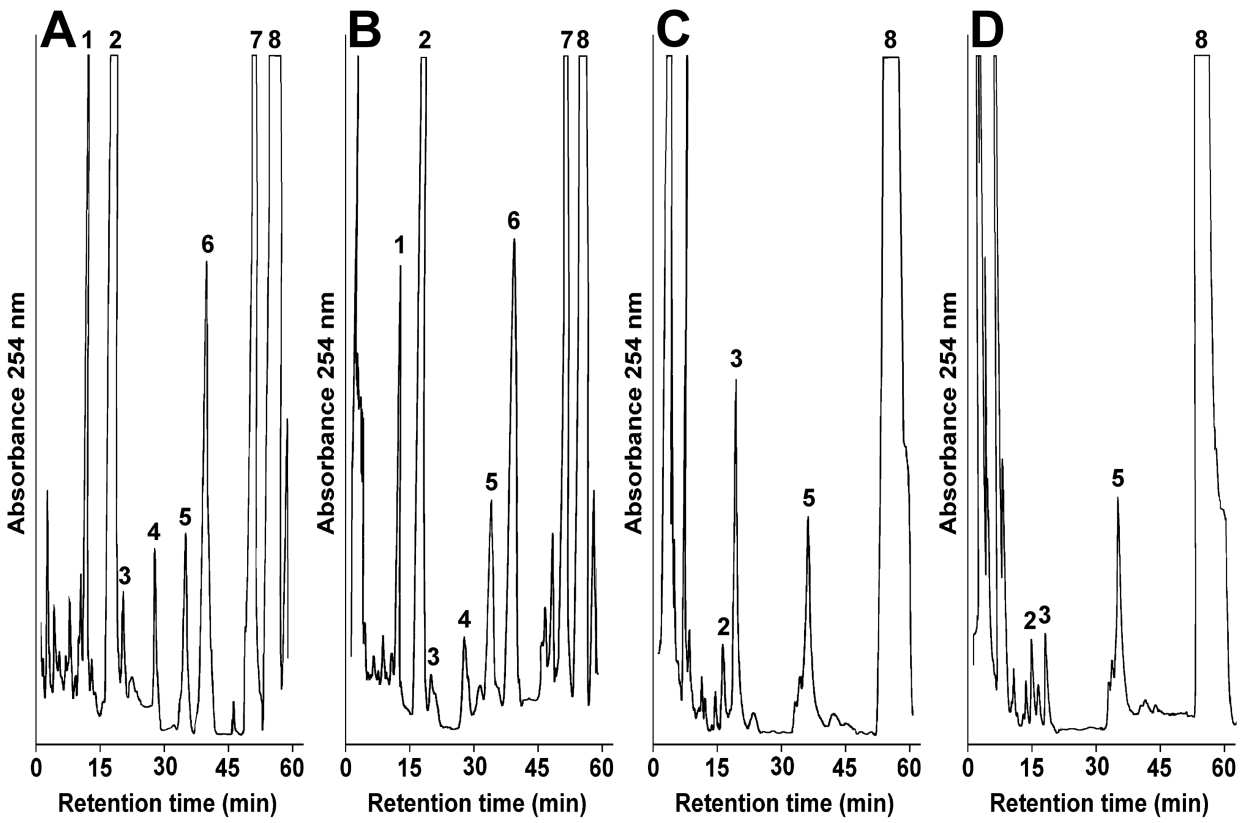
Figure 3


A
B
C
D
Absorbance 254 nm
Retention time (min)
0
15
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1
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8

Figure 4

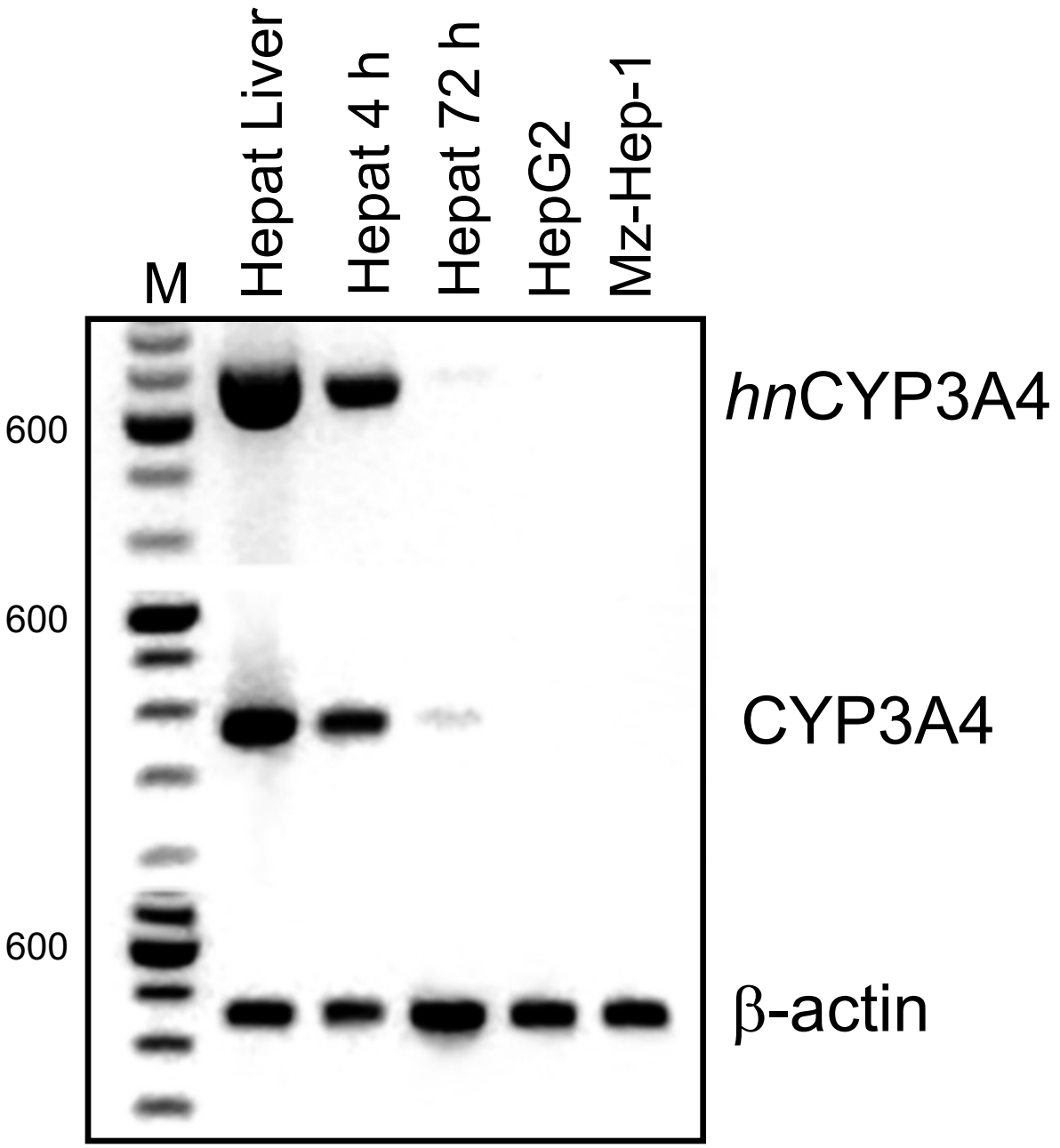
M
Hepat Liver
Hepat 4 h
Hepat 72 h
HepG2
Mz-Hep-1
600
600
600
hnCYP3A4
CYP3A4
β-actin

Figure 5