Induction of Cytochrome P450 2B6 and 3A4 Expression by Phenobarbital and Cyclophosphamide in Cultured Human Liver Slices

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Purpose. To examine the potential of cultured human liver slices to predict cytochrome P450 (CYP) inducibility, regarding global and zonal CYP expression, together with drug-induced histologic changes.

Methods. We first assessed whether CYP2B6, 3A4, and 2C9 expression was maintained in cultured liver slices. Cultured hepatocytes were used as the reference culture system. Then we tested the effects of phenobarbital and cyclophosphamide on CYP expression in both models.

Results. Morphologic features are preserved in slices. Basal CYP expression declines with time in culture in both models. Slices display the same region specificity of CYP2B6, 2C9, and 3A4 expression as intact liver. CYP2B6 and 3A4 mRNA, apoprotein, and enzyme-related activities were induced by phenobarbital and cyclophosphamide, whereas CYP2C9 apoprotein was not. Their immunoreactivities were also increased, while their zonal distribution was preserved on slice tissue sections. Microsomal enzyme induction was confirmed by histology.

Conclusions. Cultured human liver slices are an attractive alternative to hepatocyte culture for the prediction of human CYP isoenzyme induction by xenobiotics.

KEY WORDS: CYP expression and induction; human liver slices; human hepatocytes; cyclophosphamide; phenobarbital.

INTRODUCTION

Cytochromes P450 (CYPs) are a superfamily of enzymes that play a major role in the biotransformation of xenobiotics and of endogenous substances. An important feature of these enzymes is their inducibility. In drug development, CYP induction is an undesirable effect, leading to a risk of drug interaction. *In vitro* models have been developed to detect CYP inducibility early in development and to allow the selection of lead drug candidates.

Most *in vitro* studies on CYP induction have been performed using primary cultures of human hepatocytes (reviewed in Ref. 1). The major limitation of this system is the isolation of hepatocytes from their original environment, excluding interactions with other liver cell types. Precision-cut liver slices have been developed to mimic the *in vivo* situation (2). The main advantage of liver slices over isolated hepatocytes is the maintenance of cellular heterogeneity and cell–cell interactions within an original tissue matrix, making slices the only available tool for investigating the region specificity of enzyme expression and histologic alterations after *in vitro* drug exposure. Several authors have reported that cultured human liver slices express major CYP isoforms (3–7), which could be increased after treatment by prototypical CYP inducer compounds, such as methylclofenapate (3), Aroclor 1254 (3,5), rifampicin (4), TCDD (8) and β-naphthoflavone (9).

Cyclophosphamide (CPA) is currently used to treat a variety of solid tumors and also for its immunosuppressive properties in organ transplantation. CPA is a prodrug bioactivated in human liver by several CYP isoforms including CYP2B6, 3A4/5 and 2C8/9/18/19 (10,11). The induction by CPA of several CYP isoforms in primary cultures of human hepatocytes has also been reported, although with discrepancies (10,11). Chang *et al.* (10) have shown that CPA induced CYP2C8/9 and 3A4 apoprotein, but little or no increase in CYP2B6 was observed. On the other hand, Gervot *et al.* (11) have reported an increase in CYP2B6 and 3A4 mRNA after CPA treatment but no induction of the corresponding apoproteins. The latter study, however, was performed with only one human liver sample and thus failed to take into account interindividual variability. In both publications, as in many others, phenobarbital (PB), which has been proved to be inducer of CYP2B6, 3A4, and 2C9 mRNA and/or apoprotein levels (10–15), was used as a positive reference compound, except that Runge *et al.* (13) did not observe any induction of CYP2C9 apoprotein after PB treatment.

The purpose of the present study was to examine CYP expression and inducibility in human liver slices, with respect to cultured human hepatocytes. The induction potential of PB and CPA is yet uncharacterized in cultured human liver slices. In addition, neither histologic alterations related to xenobiotic treatment nor the region specificity of CYP distribution has been examined in human liver slices. Here we assessed basal expressions of CYP2B6, 3A4, and 2C9 in cultured human liver slices and inductive effects of PB and CPA. We demonstrate the region specificity of basal and induced expression of CYP isoforms using immunohistochemistry.

MATERIALS AND METHODS

Chemicals

Hanks' buffered saline solution (HBSS), Williams' E medium (WME), and fetal calf serum (FCS) were purchased from Gibco BRL (Paisley, UK). Hydrocortisone hemisuccinate was from Roussel-Uclaf (Romainville, France). Bovine serum albumin (BSA), insulin, and cyclophosphamide were from Sigma Chemicals (St Louis, MO). University of Wisconsin (UW) solution (ViaSpan®) was from Dupont Pharmaceuticals (Herts, Netherlands). Phenobarbital (RPR5515A) was from Aventis Pharma (Maisons-Alfort, France). Collagenase A was from Roche Diagnostics (Mannheim, Germany). Rabbit polyclonal antibodies specifically targeting human CYP2B6, 2C9, and 3A4 were previously described (11,16). These antibodies have been found to be specific because they

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did not recognize other CYP families and other members of their subfamilies. Polyclonal anti-CYP2B6 peptide antibody used for western blotting was described by Stresser and Kupfer (17) and provided by Gentest corporation (Woburn, MA). The manufacturer's instructions indicated that no cross reactivity with other CYP families was evident with enhanced chemiluminescent (ECL) detection. All other reagents were of analytic grade.

Human Liver Samples and Experimental Design

Human liver (HL) samples were obtained from macroscopically healthy surgical waste after tumor resection (see Table I). The sampling procedure and use of this tissue, which was surplus to clinical requirements, were in agreement with current French legislation. The warm-ischemia time ranged from 0 to 15 min. Because of the small amount of human material available, both hepatocytes and liver slices could not be prepared from the same liver donor. After their preparation, hepatocytes and slices were cultured using the same media, as described below. Moreover, the small amount of liver samples implies that we were not able to examine CYP inducibility at three organization levels, i.e., mRNA, apoprotein, and activity levels.

Hepatocyte Isolation and Culture

Human hepatocytes were isolated by use of an established procedure described by Ballet *et al.* (18). Cell viability ranged between 83 and 99%. Hepatocytes were transported from the operating room to Aventis Laboratories in WME at 4°C (maximum transfer time 1 h). Hepatocytes were seeded on 59 cm^2 collagen-coated dishes (9×10^6 viable hepatocytes) in 12 ml of WME supplemented with 10% FCS, 0.2% BSA, 5 μg/ml insulin, 25 IU/ml penicillin, and 25 μg/ml streptomycin and cultured in humidified 5% CO_2/95% air. Hepatocytes were precultured for 18–20 h, first to allow hepatocyte structure, which was totally lost when collagenase was used, to recover; and second to increase CYP expression, which was decreased after enzymatic dissociation, as compared to liver samples (19). This initial decrease was followed by an increase after 24 h of culture (20). After 18–20 h, the medium was changed to fresh WME supplemented with 0.2% BSA, 5 μg/ml insulin, and 1 μmol/L hydrocortisone (defined as time T_0). Subsequently the medium was changed every 24 h.

Preparation and Culture of Human Liver Slices

Human liver samples were perfused with cold UW solution via cannulas through the portal venules and transported from the operating room to Aventis Laboratories in UW solution at 4°C (maximum transfer time 1 h). Human liver slices were prepared by using the Krumdieck tissue slicer as previously described for rat liver, except that human liver tissue often has to be dissected into 3-cm-thick slabs. Slices were precultured for 3 h in glass vials containing 6.8 ml of WME supplemented with 10% FCS, 0.2% BSA, 5 μg/ml insulin, 25 IU/ml penicillin, and 25 μg/ml streptomycin at 37°C in 40% O_2/55% N_2/5% CO_2 because 40% oxygen improved cell viability and CYP expression in human liver slices, as compared to 20% oxygen (data not shown). After preculture time allowing slices to remove any surface debris and enzymes possibly released from damaged cells (for example lactate dehydrogenase), the medium was changed to fresh WME supplemented with 0.2% BSA, 5 μg/ml insulin, and 1 μmol/L hydrocortisone (defined as time T_0). Subsequently the medium was changed every 24 h.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from hepatocytes and liver slices by the guanidium thiocyanate method. Northern blot

Table I. Characteristics of Human Liver Samples

	Sex	Age (years)	Diagnosis	Historical
Hepatocytes				
HL1	Female	43	Adenoma	None
HL2	Male	77	Adenoma	None
HL3	Female	39	Adenocarcinoma	Chemotherapy
HL4	Female	34	Adenocarcinoma	Chemotherapy
HL5	Male	62	Hilus liver tumor	Hepatitis B
HL7	Female	18	Adenoma	None
HL8			No information on this patient was available	
HL9	Male	76	Adenocarcinoma	Chemotherapy
HL10	Male	54	Adenocarcinoma	Chemotherapy
HL12	Male	51	Hydatic cyst	None
Slices				
HL22	Female	41	Adenocarcinoma	Chemotherapy
HL23	Male	65	Metastatic colon cancer	Chemotherapy, Steatosis
HL24	Male	60	Adenocarcinoma	Chemotherapy
HL25	Female	70	Adenocarcinoma	Chemotherapy
HL26	Female	67	Metastatic small intestine cancer	None
HL27	Male	62	Adenocarcinoma	Chemotherapy
HL28	Female	39	Adenoma	None
HL30	Male	46	Adenocarcinoma	Chemotherapy
HL31	Male	63	Adenocarcinoma	None
HL33	Male	68	Adenocarcinoma	Chemotherapy
HL36	Female	61	Metastatic colon cancer	Chemotherapy
HL39	Male	64	Metastatic colon cancer	Chemotherapy

analyses were performed according to Gervot *et al.* (11). An 18S oligonucleotide probe was used to control RNA loading and integrity.

Microsome Preparation and Western Blot Analysis

Cultured hepatocytes were scraped in HBSS, centrifuged for 5 min at 10,000 *g* and 4°C, and the resulting pellet was frozen at –80°C before microsomes were prepared. Slices were frozen at –80°C in Eppendorf without medium before the preparation of microsomes, according to Lakehal *et al.* (21). Microsomal proteins (10 μg for CYP3A4 and 2C9 detection; 15 μg for CYP 2B6 detection) were electrophoresed on 10% SDS-PAGE, and then resolved proteins were electrophoretically transferred to nitrocellulose filters. CYP 3A4 and 2C9 apoprotein blots were incubated with rabbit polyclonal antibodies followed by peroxidase-conjugated antirabbit immunoglobulins, according to Gervot *et al.* (11). The CYP2B6 apoprotein on the filters was detected according to the manufacturer's instructions (Gentest, Woburn, MA). Immunoreactive proteins were visualized by an ECL detection system.

Cytochrome P450 Enzyme Activities

Testosterone 16β- and 6β-hydroxylations were determined in intact hepatocytes and liver slices as indices of CYP2B6 and 3A4 activity, respectively (22,23). Briefly, hepatocytes and slices were incubated in WME containing 500 μM testosterone and 0.1% BSA on culture dishes and in six-well culture plates (two slices per well), respectively. Incubation was carried out with gentle agitation for 30 min at 37°C in 95% air/5% CO_2. The medium was collected and stored at –20°C for HPLC analysis. Hepatocytes and slices were solubilized in 1 N NaOH for protein determination. The internal standard 11α-hydroxyprogesterone (21.0 nmol/ml) was added to the media before extraction. Formation of testosterone metabolites was measured by HPLC on a 5-μm-particle Nucleosil C18 column (150 × 4.6 mm), with monitoring at 245 nm.

Histology

Four human liver slices (at T_0 and after 24, 48, and 72 h of culture) prepared from HL25, 26, and 30 have been fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Two sections of each paraffin-embedded slice were stained with hematoxylin–eosin–saffron for histologic examination.

Immunohistochemistry

Isolated hepatocytes were seeded in collagen-coated eight-well culture slides at 1000 cells/100 μl/well. At the end of the treatment period, the medium was drawn off, and the hepatocytes were washed twice with cold HBSS and then fixed for 10 min in cold acetone at 4°C. These slides were stored at –20°C. Hepatocyte slides and four sections of liver slices were processed for immunohistochemistry. After inhibition of endogenous peroxidase activity and saturation of nonspecific binding sites, the sections were incubated at 4°C overnight with rabbit polyclonal antibodies specifically targeting human CYP2B6, 2C9, and 3A4 (1:50, 1:400, and 1:400 dilution in Tris-buffered saline, respectively). Primary antibodies were detected by incubation at room temperature for 60 min with peroxidase-conjugated antirabbit immunoglobulin (Dako, Denmark; 1:50 dilution in Tris-buffered saline). Peroxidase activity was developed with 0.025% (w/v) diaminobenzidine and 0.05% (v/v) H_2O_2. Controls, obtained by omitting the primary antibodies, were all negative.

RESULTS

Histology of Human Liver Slices in Culture: Effects of PB and CPA

The viability of human liver slices in culture was evaluated by histologic examination of slices from HL25, 26, and 30. At T_0, the lobular pattern in liver slices was easily recognizable, with central vein and portal triads (Fig. 1A). After 48–72 h of culture, tissue structure was well preserved in all samples (Fig. 1B–D). Occasional necrosis affecting only hepatocytes and never exceeding 20% of slice surfaces was observed. In addition, mild diffuse hepatocytic clear cell change (interpreted as glycogen infiltration) was noted in slices. All the other cell types were remarkably well preserved after 72 h of culture. We demonstrated that treatment for 72 h with 2 mM PB or 500 μM CPA was not toxic for human liver slices by the MTT reduction assay (data not shown). Figure 1E,F shows that PB induced a moderate hypertrophy of centrilobular and midzonal hepatocytes, which sometimes assume a pale, ground glass appearance. Ground glass hypertrophic hepatocytes are usually related to smooth endoplasmic reticulum proliferation and associated with microsomal enzyme induction (24). After 72 h of treatment with 500 μM CPA, histologic changes were similar to those observed with 2 mM PB but were less marked (Fig. 1G,H).

CYP Expression in Human Hepatocytes and Liver Slices in Culture

Figure 2 illustrates the levels of CYP2B6, 3A4, and 2C9 apoproteins obtained in human hepatocyte and liver slice cultures. Four different donor samples were used for hepatocyte and slice preparations. In hepatocyte cultures, CYP2B6 apoprotein levels fell very rapidly during the first 48 h of culture [mean of 38.7% (13.7–76.4%) of T_0 values at 24 h and 25.8% (15.4–38.5%) at 48 h]. These levels remained stable up to 72 h [mean of 26.1% (6.8–40.6%) of T_0 values]. CYP3A4 apoprotein levels were markedly reduced over the 72-h culture period [mean of 62.9% (43.2–79.5%) of T_0 values at 24 h, 32.5% (12.9–47.3%) at 48 h, and 17.9% (10.5–30.3%) at 72 h]. By contrast, CYP2C9 apoprotein levels were better maintained than the two other CYP isoforms [mean of 71.7% (59.3–89.1%) of T_0 values at 24 h, 60.2% (54.2–71.7%) at 48 h, and 49.6% (36.9–57.0%) at 72 h]. In human liver slice cultures, CYP 2B6, 3A4, and 2C9 apoprotein levels were almost unchanged at 24 h [mean of 114.5% (80.7–136.3%), 89.9% (84.7–96.2%), and 97.6% (82.9–113.8%) of T_0 values, respectively]. After 48 h of culture, CYP 2B6, 3A4, and 2C9 apoprotein levels varied around 77.6% (37.7–115.6%), 58.8% (41.7–78.6%), and 70.7% (48.9–107.9%) of T_0 values, respectively. At 72 h, CYP 2B6, 3A4, and 2C9 apoprotein contents in cultured liver slices represented 69.2% (18.8–102.0%), 27.7% (4.7–43.0%), and 61.5% (24.7–94.7%) of T_0 values,

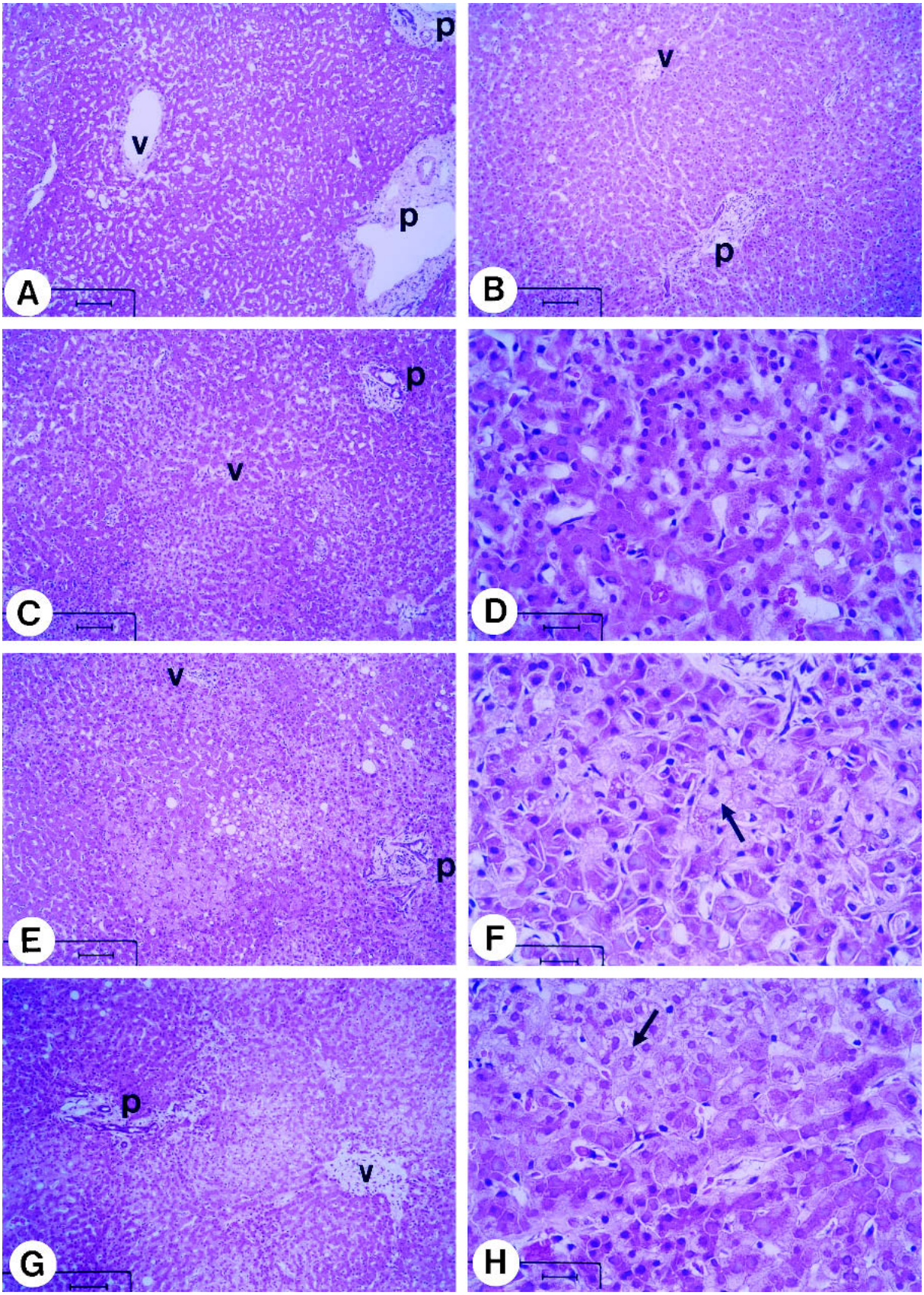


Fig. 1. Histology of cultured human liver slices (HL30). Hematoxylin–eosin–saffron staining of control slices at T_0 (A) and after 48 h (B) and 72 h of culture (C,D). Slices treated with 2 mM PB for 72 h (E,F) or with 500 μM CPA for 72 h (G,H). *Bars* represent 100 μm (A,B,C,E,G) or 25 μm (D,F,H). Lobular architecture was well preserved in all samples; central veins (v) and periportal areas (p) are easily recognized. *Arrows* indicate hypertrophic hepatocytes with a ground glass cytoplasm.

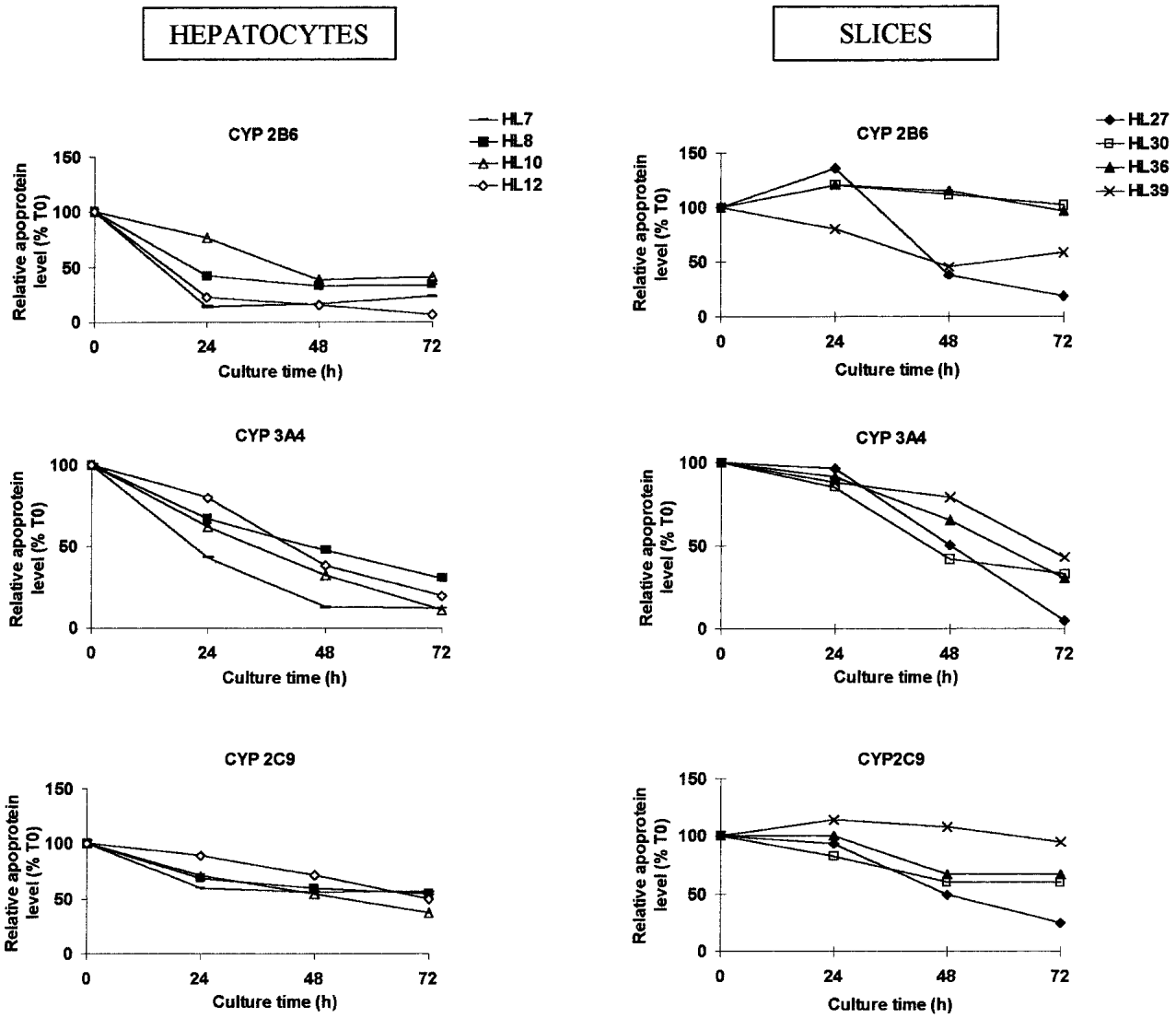


Fig. 2. CYP apoprotein levels in cultured hepatocytes and liver slices. After preparation, hepatocytes and slices were precultured for 18–20 h and 3 h, respectively. Microsomes were prepared from hepatocytes and liver slices after preculture time (T_0) and after 24, 48, or 72 h of culture. CYP2B6, 3A4, and 2C9 apoprotein contents were measured by immunoblotting as described in Methods. Data are expressed as a percentage of CYP amount at T_0. CYP2B6, 3A4, and 2C9 apoprotein levels at T_0 are, respectively, 10.5, 59.0, and 19.8 (HL7); 11.6, 68.4, and 25.4 (HL8); 12.2, 87.5, and 29.7 (HL10); 18.8, 45.5, and 37.8 (HL12); 7.1, 41.0, and 47.9 (HL27); 4.2, 27, and 30.4 (HL30); 6.4, 65.2, and 39.8 (HL36); 8.9, 15.5, and 18.9 (HL39) pmol/mg protein.

respectively. Overall, these data showed that CYPs apoprotein levels decline with time in culture in both models. Additionally, on the basis of the limited number of samples used, we showed a high interindividual variability in CYP2B6, 3A4, and 2C9 expression in human liver slices.

One of the main advantages of liver slices over hepatocytes is the possibility of examining the region specificity of CYP expression and induction. At T_0, CYP2B6- and 2C9-immunoreactive hepatocytes were randomly distributed throughout the parenchyma (Fig. 3A,E). In contrast, hepatocytes positive for CYP3A4 protein showed a centrilobular distribution (Fig. 3C). No immunoreactivity was observed in any other cell types. After 48 h of culture, despite decreases in CYP2B6 and 3A4 immunoreactivities as expected from western immunoblotting findings, their respective distribution within liver parenchyma was well maintained (Fig. 3B,D). At 72 h, CYP2B6 and 3A4 immunoreactivities were too low to be detected by immunohistochemistry. CYP2C9 immunoreactivity was undetectable from 48 h of culture (Fig. 3F).

Effects of PB and CPA on CYP2B6, 3A4, and 2C9 Expression in Human Hepatocytes and Liver Slices in Culture

Selected treatment conditions with PB and CPA were those producing optimal induction of CYP2B6, 2C9, and 3A4 as demonstrated in previously published studies (2 mM PB) (10,13) or earlier in our studies (500 μM CPA). Considering the high interindividual heterogeneity of responses in human models, we investigated the effects of PB and CPA on CYP2B6, 3A4, and 2C9 in cultures from three to eight separate donors without performing statistical analysis.

As illustrated in Figs. 4 and 5, the level of induction of CYP2B6 and 3A4 by 500 μM CPA was generally similar to

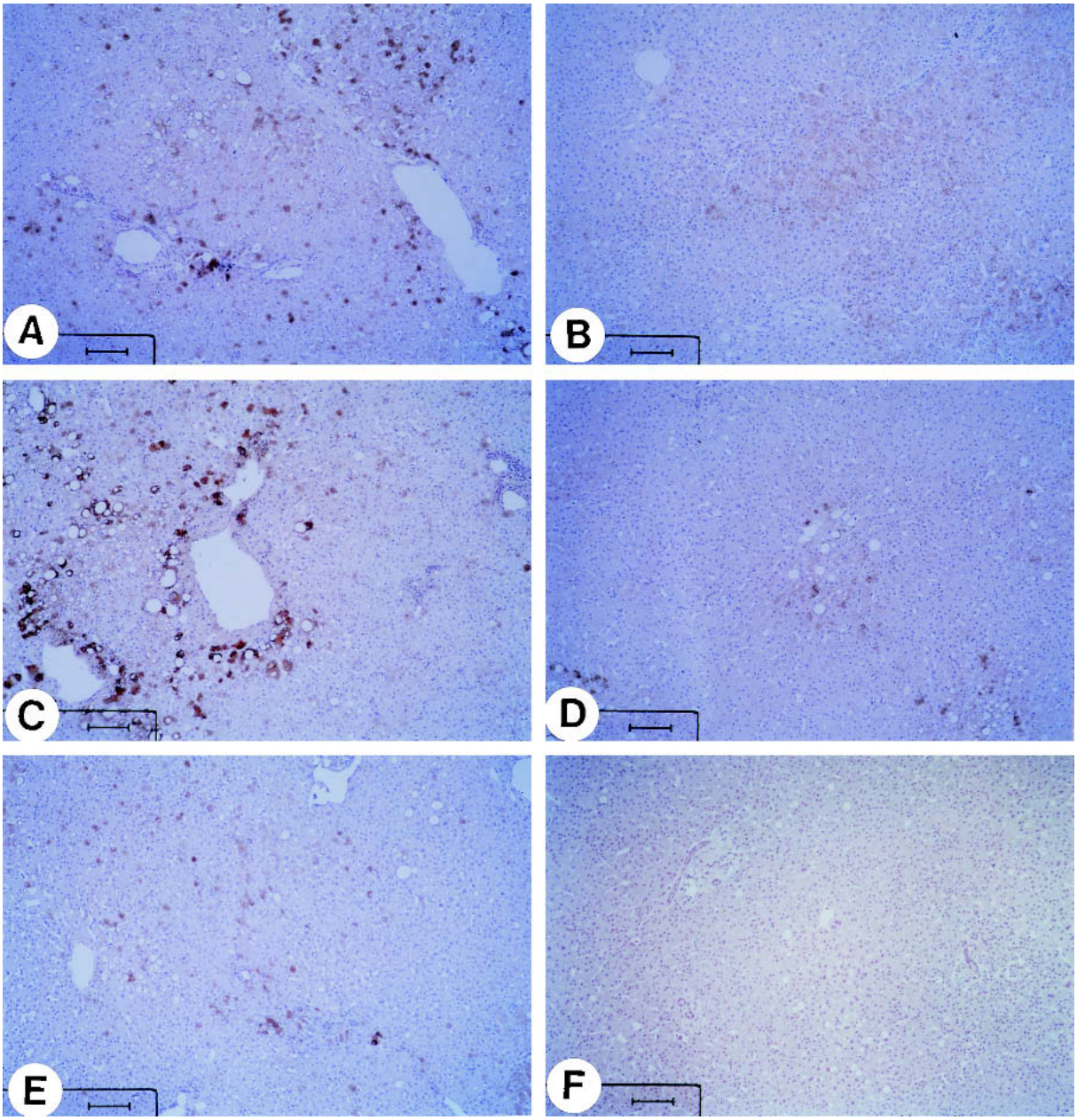
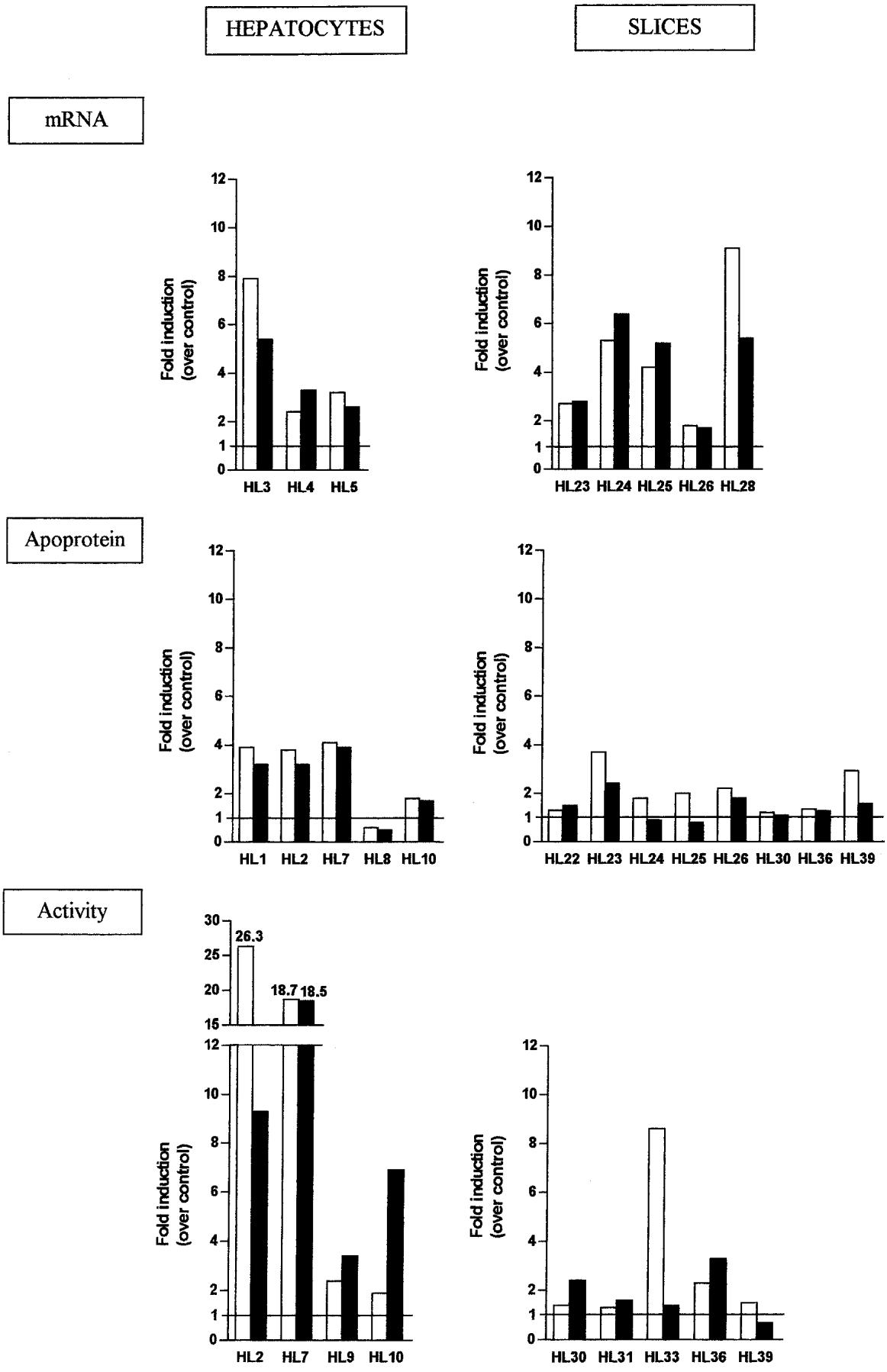


Fig. 3. Immunoreactivity of CYP2B6 (A,B), 3A4 (C,D), and 2C9 (E,F) in human liver slice sections (HL30). At T_0 (A,C,E), the distribution of CYP2B6 and of 2C9 was random throughout the lobule, and the distribution of CYP3A4 was predominantly centrilobular. After 48 h of culture (B,D,F), CYP2B6 and 3A4 immunoreactivities were markedly decreased, but their distribution was preserved, and CYP2C9 immunoreactivity was undetectable.

that produced by 2 mM PB at the mRNA, apoprotein, or activity level. Liver slices appeared to reproduce the inductive effects of PB and CPA obtained with cultured hepatocytes, albeit with some differences. Indeed, the induction levels of CYP2B6 and 3A4 by PB and CPA achieved in liver slices were generally lower than those observed in hepatocytes. Moreover, the interindividual variability in the induction of CYP3A4 among the different slice cultures appeared to be

Fig. 4. Effects of phenobarbital and cyclophosphamide on CYP2B6 mRNA, apoprotein, and activity levels in cultured hepatocytes and liver slices. Hepatocytes and liver slices prepared from different human samples were incubated for 72 h with 2 mM PB *(open bars)* or with 500 μM CPA *(closed bars)*. The mRNA and apoprotein contents and activity were measured by northern and western blotting and by testosterone assay, respectively, as described in Methods. Data are expressed as fold induction relative to the control maintained in plain medium. CYP2B6 apoprotein levels in control cultures are respectively: 0.5 (HL1), 3.3 (HL2), 2.4 (HL7), 3.9 (HL8), 4.9 (HL10), 2.5 (HL22), 23.2 (HL23), 12.7 (HL24), 8.0 (HL25), 9.8 (HL26), 4.3 (HL30), 6.2 (HL36), and 5.2 (HL39) pmol/mg protein. CYP2B6 activity levels in control cultures are, respectively, 0.9 (HL2), 0.4 (HL7), 1.2 (HL9), 5.2 (HL10), 0.9 (HL30), 1.3 (HL31), 0.2 (HL33), 0.4 (HL36), and 0.8 (HL39) pmol/min/mg protein.


HEPATOCYTES
SLICES
mRNA
Apoprotein
Activity
Fold induction (over control)
HL3 HL4 HL5
HL23 HL24 HL25 HL26 HL28
HL1 HL2 HL7 HL8 HL10
HL22 HL23 HL24 HL25 HL26 HL30 HL36 HL39
26.3
18.7 18.5
HL2 HL7 HL9 HL10
HL30 HL31 HL33 HL36 HL39

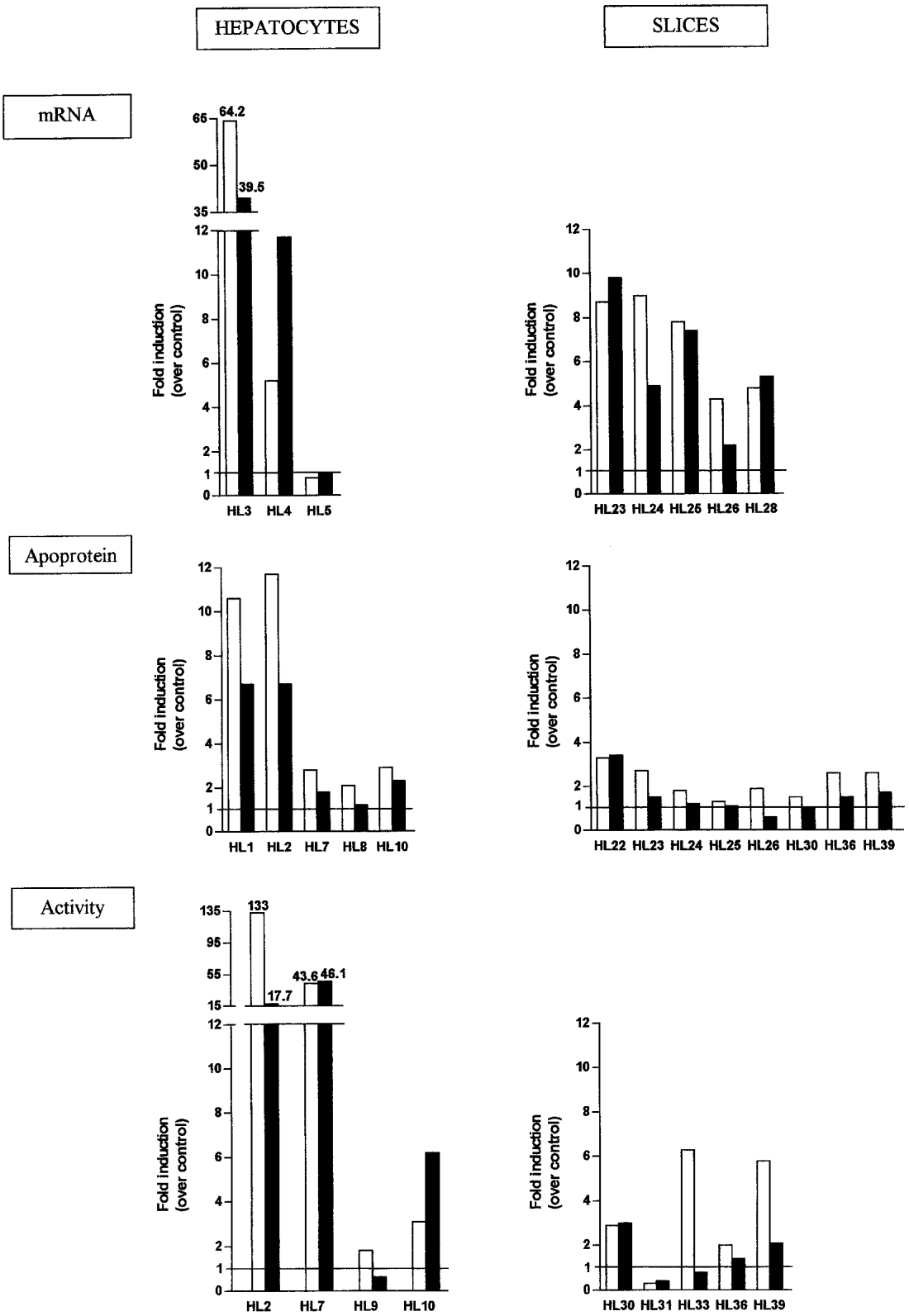


Fig. 5. Effects of phenobarbital and cyclophosphamide on CYP3A4 mRNA, apoprotein, and activity levels in cultured hepatocytes and liver slices. Hepatocytes and liver slices prepared from different human samples were incubated for 72 h with 2 mM PB *(open bars)* or with 500 μM CPA *(closed bars)*. The mRNA and apoprotein contents and activity were measured by northern and western blotting and by testosterone assay, respectively, as described in Methods. Data are expressed as fold induction relative to the control maintained in plain medium. CYP3A4 apoprotein levels in control cultures are, respectively, 3.9 (HL1), 3.8 (HL2), 6.9 (HL7), 20.7 (HL8), 9.2 (HL10), 0.5 (HL22), 5.9 (HL23), 8.3 (HL24), 14.3 (HL25), 27.9 (HL26), 8.8 (HL30), 19.8 (HL36), and 6.7 (HL39) pmol/mg protein. CYP3A4 activity levels in control cultures are, respectively, 46.8 (HL2), 16.1 (HL7), 82.1 (HL9), 13.2 (HL10), 19.8 (HL30), 32.2 (HL31), 30.7 (HL33), 54.7 (HL36), and 14.9 (HL39) pmol/min/mg protein.

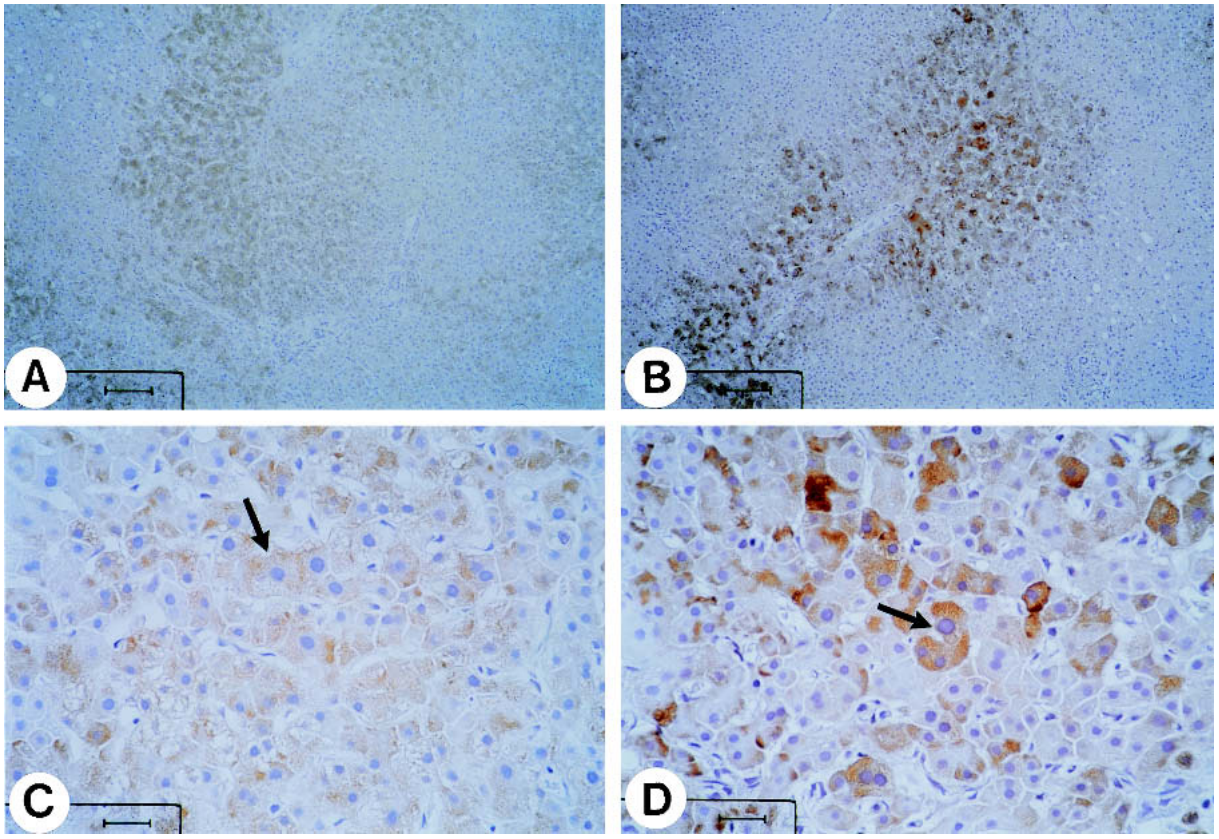


Fig. 6. Effects of phenobarbital on CYP2B6 (A,C) and 3A4 (B,D) immunoreactivities in liver slices (HL30) incubated for 72 h with 2 mM PB. *Bars* represent 100 μm (A,B) or 25 μm (C,D). Note that the distribution of CYP2B6 and 3A4 is preserved in liver slices. *Arrows* indicate positive hypertrophic hepatocytes, a cellular phenotype characteristic of enzyme induction.

lower than in cultured hepatocytes at the mRNA (2.2- to 9.8-fold vs. 0.8- to 64.2-fold over control), apoprotein (0.6- to 3.4-fold vs. 1.2- to 11.7-fold over control), and activity (0.3- to 6.3-fold vs. 0.6- to 133-fold over control) levels. However, our observations have to be confirmed by using the same donor livers for the preparation of hepatocytes and slices. CYP2C9 apoprotein content and immunoreactivity remained unchanged after treatment with PB and CPA in both cultured liver slices and hepatocytes (data not shown).

After treatment of liver slices with 2 mM PB for 72 h, immunohistochemical study of CYP2B6 and 3A4 showed increases in staining intensities that preserved their distribution, i.e., random and centrilobular, respectively (Fig. 6A,B). Some positive hepatocytes for CYP 2B6 and 3A4 immunoreactivities were also hypertrophic with a ground glass appearance (Fig. 6C,D). This phenotype is related to smooth endoplasmic reticulum proliferation and associated with enzyme induction. No immunoreactivity was observed in any other cell types in response to PB treatment. Similar results have been obtained with 500 μM CPA (data not shown). By contrast, in cultured

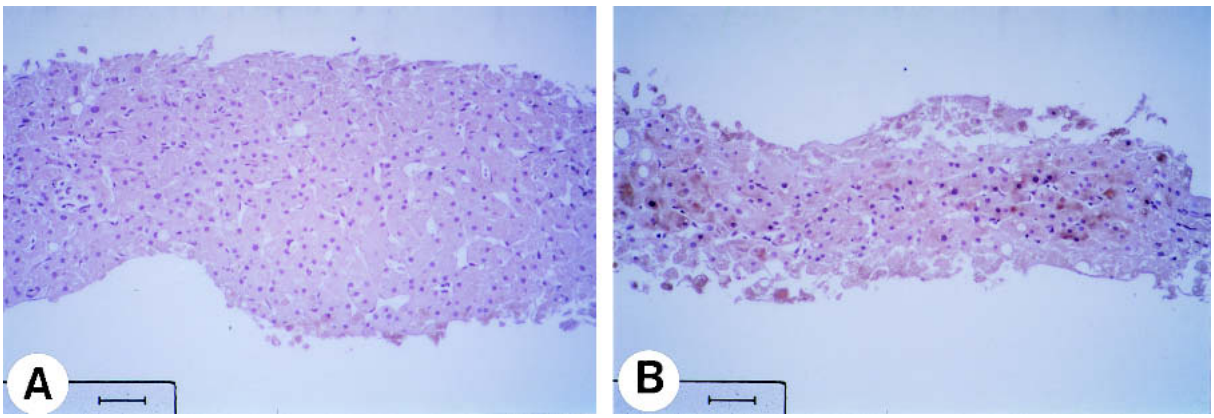


Fig. 7. CYP3A4 immunoreactivity in cross sections of human liver slices (HL26) after 72 h of culture. Control slice (A) and slice treated with 2 mM PB (B). *Bars* represent 50 μm. Note that immunoreactivity following induction can be detected throughout the entire slice including central cell layers.

hepatocytes, marked PB- and CPA-related increases in CYP2B6 and 3A4 immunoreactivities affected all the hepatocytes (data not shown). Moreover, there was no cellular hypertrophy associated with the induction of CYP2B6 and 3A4. In histologic cross sections after exposure of the liver slices to PB, CYP 3A4 immunoreactivity was clearly observed even in the middle of the slices, whereas little or no immunoreactivity was present in controls (Fig. 7A,B). Similar results have been obtained with 500 μM CPA (data not shown).

DISCUSSION

The morphologic integrity of human liver slices after 72 h of culture was documented for the first time by histologic examination. The histologic pattern of slices was well preserved after 72 h of culture, with at most 20% of hepatocellular necrosis, indicating that our culture conditions (particularly the use of 40% oxygen) are suitable. Histologic examination of slices treated with 2 mM PB or with 500 μM CPA for 72 h showed hypertrophic hepatocytes with a ground glass appearance, which is characteristic of proliferation of smooth endoplasmic reticulum (24) and consistent with the induction of microsomal enzymes.

CYP2B6, 3A4, and 2C9 apoprotein levels at T_0 were in the same ranges in liver slices and in hepatocytes (see the legend to Fig. 2). Our attempt to compare the relative CYP stability data in cultured hepatocytes and liver slices has been complicated by our experimental design: (a) different individual liver donors have been used to prepare either hepatocyte or liver slice cultures because the amount of human material was very limited; (b) different preculture times have been used for hepatocytes or slices, to assess the responsiveness of each culture system, when cellular organization was the best to mimic the *in vivo* situation (e.g., cell–cell interactions, differentiated functions); and (c) the relatively small number of samples processed has complicated the identification of the interindividual variability contribution. Figure 2 showed that the CYPs' apoprotein levels were reduced over the 72-h culture period in both models. The rapid decline of CYP2B6, 3A4, and 2C9 apoproteins with time in culture in hepatocyte is consistent with the literature (19,25). In cultured human liver slices, the stability of the CYP2B6, 3A4, and 2C9 apoprotein levels throughout culture and the high interindividual variability obtained in our study are in agreement with data from Renwick *et al.* (7), although illustrated by only one sample for CYP2B6 apoprotein. Other authors have shown that the levels of CYP2B6, 3A4, and 2C9 activities declined rapidly in human liver slices from the beginning of the culture (6). These discrepancies could be explained by differences in human sample procurement (time of warm and cold ischemia) and/or liver slice culture conditions (media used, oxygen tension, etc.). The maintenance of an original tissue matrix in liver slices has been for the first time correlated by the maintenance of the region specificity of CYP expression. We have shown that human liver slices display the same region specificity of CYP2B6, 2C9, and 3A4 expression as intact liver (26,27). Indeed, in human liver slices, CYP3A4 was located predominantly in the centrilobular region, while CYP2B6 and 2C9 expression was randomly distributed throughout the parenchyma.

The present study is the first demonstration that CYP2B6 and 3A4 mRNA, apoprotein and enzyme-related activity can be induced by PB and CPA in human liver slices, extending the short list of CYP inducers evaluated in this *in vitro* model (3–5,8,9). In most of our hepatocyte and slice cultures, PB and CPA gave the same pattern of responses, suggesting that the two compounds regulate CYP2B6 and 3A4 through similar pathways. No induction of CYP2C9 by PB or CPA was observed in hepatocytes or liver slices, in keeping with a recent report (13). Nevertheless, these results are in contrast with several published data on human hepatocytes (10,14,15). These discrepancies could be due to the specificity/sensibility of the antibodies used in the different studies and/or a basal CYP2C9 expression more or less preserved with time in culture in these studies, leading to a large magnitude of responses between cultures. By contrast, CYP2B6 and 3A4 are induced by PB and CPA in hepatocytes and liver slices. Both CYPs were regulated at the transcriptional level, as indicated by combined increases in mRNA transcripts, apoprotein contents, and activity levels. Some differences among the three parameters measured with respect to the magnitude of the induction response appeared from the limited comparisons we can make in Figs. 4 and 5. For example, the fold increase in CYP2B6 mRNA in samples HL24 and HL25 was much greater than in HL23, whereas the opposite occurred considering the apoprotein levels. We can't exclude that even if all the factors required for mRNA synthesis are available, apoprotein synthesis and/or stability of neo-synthesised protein may be impaired or not optimal, depending on the specific factors available in each human cultures. Similarly, CYP2B6 activity level was increased in HL30 and 36, but was not with respect to the apoprotein level. However, enzymatic activities we have chosen for determining CYP2B6 and 3A4 activities are not totally specific of these CYP isoforms. Indeed, testosterone 16β-hydroxylation (CYP2B6-related activity) may also be mediated to a certain degree by CYP3A4 and CYP2C (22) and testosterone 6β-hydroxylation (CYP3A4-related activity) by CYP2C (23). Considering the low levels of response (especially at the apoprotein and activity levels) in liver slices, we can't exclude that weak inducers could be difficult to identify using this system.

The inducibility of CYP2B6 and 3A4 by PB and CPA was lower in cultured liver slices than in hepatocytes. The reason for the differences in response between liver slices and hepatocytes is unclear. Several authors have postulated that substrates diffuse less efficiently in slices than in cultured hepatocytes (28). However, our analysis of histologic cross sections showed that increases in CYP3A4 immunoreactivity after treatment with PB or CPA clearly occurred in the central portion of the slice, indicating that inducing drugs diffused adequately into the slices. On the other hand, some differences in immunohistologic findings have been obtained between hepatocyte and slice cultures. In liver slices, CYP2B6 and 3A4 immunoreactivity following PB- and CPA-related induction displayed their original distribution. In addition, some positive hepatocytes were hypertrophic with the aspect of ground glass cytoplasm, typical of enzyme induction. In contrast, all cultured isolated hepatocytes were stained and did not show any morphologic feature of enzyme induction. These findings could not derive from the selection of a hepatocyte subpopulation that could predominantly express CYP because no Percoll gradient, which is known to cause the loss of cell subpopulations depending on the gradient density, was used during our hepatocyte preparation. These results are in

keeping with published data on the induction of CYP3A4 by rifampicin in cultured human hepatocytes (15). It is not surprising that hepatocytes that lost their zonal specific phenotype produce CYP, as all cells are also positive in the human fetal liver and in some pathologic situations (26). Several attempts to stabilize the original phenotype of human hepatocytes in culture have been described in the literature. Cocultures are useful *in vitro* models because other cell types in liver can modulate CYP expression. For example, Kupffer cells produce inflammatory factors (cytokines), which downregulate the expression of different CYP isoforms (29). On the other hand, other cell types in liver are implicated in the response to xenobiotics (21,30). Cocultures or the addition of extracellular matrix (matrigel or collagen) improves CYP expression and inducibility in human hepatocytes with respect to the conventional culture, but no one of these modifications appeared superior to the others (12). Factors that could influence the maintenance of CYP responsiveness in culture systems remain to be determined.

The present data illustrate the donor-related variability of hepatocyte and liver slice response. This variability affected both the basal expression and inducibility of CYP. In humans, the interindividual variability in CYP induction has been evidenced *in vivo*. The sources of this variation are interindividual nongenetic (gender, age, nutritional status, drug medication, pathology, etc.) and genetic factors. Nevertheless, the degree to which variability in CYP induction *in vitro* reflects variability *in vivo* is not known. Then it is difficult to conclude that one particular *in vitro* model better reflects the *in vivo* situation. For this purpose, it would be interesting to know the exact drug background of each patient and the genetic profile, to ascertain whether genetic differences are responsible for the variability observed *in vitro*.

In conclusion, our study demonstrates the potential of cultured human liver slices as an alternative *in vitro* system to hepatocyte cultures for studying the effects of xenobiotics on human CYP isoenzymes. Strengths of cultured human liver slices have been evidenced immunohistologically by the maintenance of region specificity of CYP expression, as found in intact liver. Moreover, histologic and immunohistologic examinations have shown that the region specificity of CYP induction obtained after treatment with PB and CPA is preserved in liver slices.

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