



Drug Metabolism in Hepatocyte Sandwich Cultures of Rats and Humans

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ABSTRACT. Adult hepatocytes from rat and man were maintained for 2 weeks between two gel layers in a sandwich configuration to study the influence of this culture technique on the preservation of basal activities of xenobiotic-metabolizing phase I and phase II enzymes. The response of these enzyme activities to an enzyme inducer was investigated using rifampicin (RIF). Basal levels of cytochrome P-450 (CYP) isozymes were characterized by measuring ethoxyresorufin O-deethylation (EROD), ethoxycoumarin O-deethylation (ECOD), and the specific oxidation of testosterone (T). In hepatocytes from untreated rats, CYP isozyme levels, including the major form CYP 2C11, increased during the first 3 days in culture. After this period of recovery, the levels of CYP 2C11, CYP 2A1, and CYP 2B1 decreased, whereas CYP 3A1 increased. In contrast to these dynamic changes, CYP activities such as CYP 1A2 and the major isozyme CYP 3A4 were largely preserved until day 9 in cultures of human hepatocytes. In measuring phase II activities, a distinct increase in glucuronosyltransferase (UDP-GT) activity toward *p*-nitrophenol (PNP) was found for rat and human hepatocytes over 2 weeks in culture. Sulfotransferase (ST) activity toward PNP showed an initial increase, with a maximum at day 7 and day 9 in culture, respectively, and then decreased until day 14. Glutathione S-transferase (GST) activity decreased constantly during the time of culture. Effects of the enzyme-inducing drug rifampicin on phase I and phase II enzymes were investigated using cultured human hepatocytes. Rifampicin treatment (50 μ mol/L) for 7 days resulted in a 3.7-fold induction of CYP 3A4 at day 9 in culture. ECOD activity was increased sixfold and phase II ST activity increased twofold compared to the initial value at day 3. No effect of rifampicin on CYP 3A was found in cultures of rat hepatocytes. These results demonstrate that rat and human hepatocytes preserve the major forms of CYP isozymes and phase II activities and respond to inducing drugs such as rifampicin. The novel hepatocyte sandwich culture is suitable for investigating drug metabolism, drug-drug interactions and enzyme induction. *BIOCHEM PHARMACOL* 54;7:761-772, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. primary culture; double gel; cytochrome P-450; phase II transferases; induction; rifampicin

Intact hepatocytes are well suited for the study of biotransformation reactions. Advantages are the existence of interacting enzyme systems, physiological cofactor concentrations, and three to four times higher enzyme activities than in microsomal preparations [1, 2]. Although phase I and phase II reactions are not tightly coupled, the balance between oxidation and subsequent conjugation reactions, which determines the metabolic pattern *in vivo*, is also retained in hepatocytes *in vitro*.

The major limitation to using hepatocyte cultures to study drug metabolism is the rapid loss of cytochrome P-450 (CYP)[#] and phase II catalyzing enzymes after isolation [3].

It has been shown that transcription of liver-specific genes decreases to less than 20% during isolation and culturing up to 24 hr [4]. For example, CYP 2C11, which constitutes ca. 50% of total CYP of male rat *in vivo*, decreases during the isolation procedure to 40% of the initial level and to 2% after 6 hr in culture [4]. Thus, interpretation of results is difficult with hepatocytes dedifferentiating to a fetal-like state. To stabilize a differentiated phenotype in cultured hepatocytes, a precondition for studying metabolism *in vitro*, several strategies have been developed. The most promising approach is the cultivation of hepatocytes on different extracellular matrices [3, 5] and in different culture systems [6]. In this report, a culture system of hepatocytes between two layers of collagen gel [7, 8] is shown to be a well-suited model for the study of phase I and phase II drug metabolism in different species.

Current research is focused on developing a primary hepatocyte culture in which enzyme induction can be studied *in vitro*. Due to the functional instability of the cells in culture, the response to inducers *in vitro* is much too low, compared to the situation *in vivo*. To study the regulation of

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[#] Abbreviations: CYP, cytochrome P-450; DMEM, Dulbecco's Modified Eagle's medium; WE, William's Medium E; BSP, sulfobromophthalein; RIF, rifampicin; EROD, ethoxyresorufin O-deethylation; ECOD, ethoxycoumarin O-deethylation; UDP-GT, glucuronosyltransferase; ST, sulfotransferase; PNP, *p*-nitrophenol; GST, glutathione S-transferase; FCS, fetal calf serum; T, testosterone; A, androstenedione.

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drug-metabolizing enzymes, hepatocytes must be stabilized to allow pretreatment with the inducer over an extended period of time. Due to species differences in liver response, substrate specificity, and inducibility, the use of human hepatocytes in culture is the only system making it possible to predict enzyme induction in humans [9].

MATERIALS AND METHODS

Chemicals and Reagents

William's Medium E (WE) and Dulbecco's Modified Eagle's medium (DMEM) without phenol red were purchased from Biochrom (Berlin, Germany), bovine serum albumin (BSA), Pentex® Bovine Albumin (fraction V) from Miles Diagnostica (Kankakee, IL), fetal calf serum (FCS) from Bio Whittaker Bioproducts (Verviers, Belgium), H-insulin from Hoechst AG (Frankfurt, Germany), and pentobarbital, Nembutal® from Sanofi CEVA (Hannover, Germany). Collagenase H (from *Clostridium histolyticum*) and rifampicin were obtained from Boehringer Mannheim (Mannheim, Germany), prednisolone, collagenase type IV, testosterone, testosterone- β -D-glucuronide, 7-ethoxycoumarin and umbelliferone (7-hydroxycoumarin) from Sigma (Deisenhofen, Germany), glucagon from Merck (Frankfurt, Germany), penicillin, streptomycin and L-glutamine from GIBCO (Eggenstein, Germany), glucagon from Novo (Mainz, Germany), β -glucuronidase/sulfatase (from *Helix pomatia*), ethyleneglycol-bis (β -aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), sulfobromophthalein (BSP) and heparin from Serva GmbH (Heidelberg, Germany), and resorufin and dicoumarol from Aldrich (Steinheim, Germany). [14 C]testosterone was purchased from Amersham Buchler GmbH (Braunschweig, Germany) with a specific activity of 59 mCi/mmol (7.51 MBq/mg). Steroid standards of the major monohydroxylated testosterone metabolites (OH-T) were purchased from Steraloids Inc. (Wilton, NH). [14 C]nitrophenol (*p*-nitrophenol, PNP, 65 mCi/mmol) was purchased from Biotrend Chemikalien GmbH (Cologne, Germany). All other chemicals were of either analytical, reagent or HPLC grade. Type I collagen was prepared from rat tail tendons according to the method described by Elsdale and Bard [10]. A final concentration of 1.11 mg/mL collagen was used.

Cell Culture

RAT HEPATOCYTE ISOLATION AND CULTURE. Primary rat hepatocytes were isolated [11] and cultured between two layers of collagen in a modification of the method of Dunn *et al.* [7] and Bader *et al.* [8]. Male Wistar rats (HSD Win: WV; 180–210 g) were obtained from Harlan Winkelmann (Borchen, Germany). Before use, free access to tap water and food was provided. The rats were anesthetized with pentobarbital (Nembutal®, 150 mg/kg) and heparinized (500 U/kg). The liver was perfused *in situ* with calcium-free Hanks' buffer substituted with EGTA (0.5 mmol/L) for 10 min at 37°C followed by perfusion of Hanks' buffer con-

taining collagenase type IV (0.7 U/mL) and calcium chloride (4 mmol/L) for 20 min. The hepatocytes were released from the liver, the suspension filtered through a nylon mesh with 100 μ m pore size, and then centrifuged. The resulting cell pellet was resuspended in the incubation medium [William's Medium E supplemented with 10% (v/v) FCS, prednisolone (9.6 μ g/mL), insulin (0.16 U/mL), glucagon (0.014 μ g/mL), penicillin (200 U/mL), and streptomycin (200 μ g/mL)], and the washing procedure was repeated three times. Hepatocytes were counted in a hemocytometer in the presence of 0.04% trypan blue in protein-free medium. Yields of 30–50 $\cdot 10^7$ cells were commonly achieved from one liver, with a viability of 78 to 85%. The minimum yield of hepatocytes was at least 5 $\cdot 10^7$ cells/g liver during all isolation procedures.

For surface coating of the culture dishes, collagen gels were prepared by distributing 1 mL of collagen gel solution (1 part of supplemented DMEM (10 \times), pH 7.4 and 9 parts collagen solution at 1.11 mg/mL, chilled on ice, mixed just before use) over a 60 mm glass tissue culture dish and incubated at 37°C for at least 1 hr. Hepatocytes were seeded at a density of 2 $\cdot 10^6$ cells. Four hours following seeding and attachment of the cells, culture medium was removed, and a 1 mL collagen gel solution was distributed as a second matrix layer over the cells. Three hours of incubation at 37°C were allowed for gelation and attachment of the second gel layer. Culture medium was added and changed daily.

HUMAN HEPATOCYTE ISOLATION. Human hepatocytes were isolated from specimens obtained from patients, four male and one female (aged between 35 and 83 years), undergoing partial liver resections for therapy of hepatic tumors. Small pieces of 10–20 g were removed from the safety margin of healthy tissue resected together with the nodular tumor to prevent intraoperative tumor spreading and ensure total removal of the tumor. Patients consented to the use of this tissue for research purposes. Resections were performed at the Department of Abdominal and Transplant Surgery, Medical School of Hannover, Hannover, Germany. Specimens were transferred to ice-cold physiologic saline solution. Four major vessels visible on the cut surface were cannulated. Perfusion was initiated with 200 mL buffer A containing NaCl (200 mmol/L), KCl (9 mmol/L), HEPES (10 mmol/L), and EGTA (0.5 mmol/L) at pH 7.4. This was followed by an 8-min perfusion of 200 mL of buffer B consisting of NaCl (200 mmol/L), KCl (9 mmol/L), and HEPES (10 mmol/L). Thereafter, collagenase perfusion was started with 200 mL of buffer C containing 100 mg collagenase type IV until softening of the liver specimen was obtained. Buffer C consisted of NaCl (100 mmol/L), KCl (9 mmol/L), HEPES (10 mmol/L), and CaCl₂ \cdot 2H₂O (5 mmol/L). The collagenase perfusate was recirculated with rates adapted to the size of the specimen, ranging from 10 to 50 mL/min. All perfusates were warmed to 37°C and preequilibrated with room air. Thereafter, the specimen was transferred to ice-cold washing buffer. Non-

perfused areas were removed with a scalpel blade. Cells were shaken gently until a homogeneous-appearing suspension was obtained. This suspension was filtered through a nylon mesh with 100 μm pore size and washed three times thereafter with cold buffer D. Buffer D contained 9.91 g/L Hanks buffered salts without calcium and magnesium, 2.4 g/L HEPES and 2.0 g bovine serum albumin. Hepatocyte viability ranged from 88–95%. On average, $100\text{--}400 \cdot 10^7$ cells were obtained from one isolation procedure. This protocol represented a significant improvement in yield in contrast to protocols previously reported by the same group [8, 12].

TREATMENT OF CULTURES. After 48 hr in culture, rat and human hepatocytes were exposed to rifampicin (RIF) dissolved in the incubation medium at a final concentration of 50 $\mu\text{mol/L}$. Enzyme activities were measured 7 and 12 days later. Rifampicin-containing medium was changed daily.

Enzyme Assays

Hepatocyte cultures containing $2 \cdot 10^6$ hepatocytes per dish were washed with medium and preincubated for 30 min before enzyme activity assays. Measurements of enzyme activities were performed with at least three different preparations of hepatocyte cultures; the values were expressed as means \pm SD. Preliminary experiments established that under these conditions the enzymatic reaction proceeded linearly with respect to time. Samples could be stored frozen at -20°C until analysis.

The concentration of substrates and their metabolites was monitored separately in medium and gel layer. No difference was observed, which implies that a collagen matrix has no effect on substrate penetration and metabolite release.

7-ETHOXYRESORUFIN O-DEETHYLATION (EROD) ASSAY. The CYP 1A-associated deethylation of ethoxyresorufin was measured by a modification [13] of assays developed by Burke and Mayer [14], Mayer *et al.* [15], and Klotz *et al.* [16]. Hepatocyte cultures were incubated with 3 $\mu\text{mol/L}$ 7-ethoxyresorufin and 10 $\mu\text{mol/L}$ dicoumarol. Dicoumarol was added to the assay media to prevent further biotransformation of resorufin by cytosolic diaphorase [17]. Aliquots of the supernatant medium were withdrawn after 1 hr and frozen immediately in liquid nitrogen. Formation of resorufin was quantified by HPLC analysis using an HPLC 1090 M (Hewlett-Packard, Waldbronn, Germany) equipped with a spectrofluorimeter (HP 1046 A, Hewlett Packard) with an excitation wavelength of 540 nm and emission wavelength of 586 nm. The spectrofluorimeter was calibrated using resorufin standards. Resorufin conjugates were cleaved using β -glucuronidase/aryl sulfatase from *Helix pomatia* in 100 mmol/L acetate buffer (pH 4.66) for 12 hr at 37°C .

HPLC was performed with a LiChrospher[®] 100 RP 18 column (250 \times 4 mm i.d., particle size 5 μm endcapped, E. Merck, Darmstadt, Germany) and a LiChrospher[®] 100 RP

18 guard column (4 \times 4 mm i.d., particle size 5 μm). Gradient elution was carried out at a flow rate of 1.3 mL/min. The mobile phase consisted of a 50 mmol/L ammonium acetate buffer, pH 7 (solvent A), and acetonitrile. The operating conditions were started with 100% A for 6 min, changed to 60% A after 15 min, followed by isocratic elution for 2 min, then changed to 50% A after 20 min and 0% A after 30 min.

7-ETHOXYCOUMARIN O-DEETHYLATION (ECOD) ASSAY. Umbelliferone (7-hydroxycoumarin) and its conjugates with D-glucuronic acid and sulfate were measured as described by Ullrich and Weber [18] and Fry and Bridges [19], using a substrate concentration of 130 $\mu\text{mol/L}$ and 170 $\mu\text{mol/L}$ for rat and human hepatocyte cultures, respectively. ECOD activity exhibits biphasic kinetics in rat liver [20]. Only the low substrate concentration was used to measure high-affinity ECOD activity. The incubation was started by adding 7-ethoxycoumarin to the supernatant medium of the cultures. Aliquots of the hepatocyte incubates were withdrawn after 2 hr and the incubation was stopped by placing the samples in a glass tube and immediately freezing the contents in liquid nitrogen. Formation of umbelliferone and its conjugates was determined by HPLC analysis. Umbelliferone concentrations were determined based on the published procedures using a spectrofluorimeter with an excitation wavelength of 320 nm and an emission wavelength of 456 nm. The spectrofluorimeter was calibrated using umbelliferone standards.

To determine the amount of the nonfluorescent glucuronide and sulfate conjugates of umbelliferone, samples were analyzed by HPLC (HP 1090 M equipped with a diode array detector and a work station, Hewlett Packard) with UV detection at 336 nm, and quantified with umbelliferone glucuronide and sulfate standards as reported [21]. HPLC was carried out on a LiChrospher[®] 100 RP 18 column (250 \times 4 mm i.d., particle size 5 μm , endcapped) with a LiChrospher[®] 100 RP 18 guard column (4 \times 4 mm i.d., particle size 5 μm) at a flow rate of 1.3 mL/min. The mobile phase consisted of a 50 mmol/L ammonium acetate buffer, pH 7 (solvent A) and acetonitrile. The operating conditions were the same as for the EROD assay.

TESTOSTERONE MONOHYDROXYLASE ASSAY. The solution of radiolabelled testosterone in toluene was evaporated to dryness and dissolved in a stock methanol solution of nonradioactive testosterone to give a specific activity of 24 mCi/mmol (3 MBq/mg). Aliquots of [¹⁴C]testosterone solution were added to give the initial concentrations of 170 $\mu\text{mol/L}$ and 220 $\mu\text{mol/L}$ testosterone in rat and human hepatocyte cultures, respectively, and incubated for 1 hr at 37°C . At this time point, 1 mL of the supernatant medium was removed and the enzymatic reaction stopped by adding 100 μL of 5% phosphoric acid. Testosterone and its metabolites were analyzed by a modification of the procedure described by Sonderfan *et al.* [22] using a Nucleosil[®] 5 C18-AB column, (250 \times 4 mm i.d., particle size 5 μm ,

Macherey and Nagel, Düren, Germany) with a LiChrospher® 100 RP 18 guard column (20 × 4 mm i.d., particle size 5 µm). Analytical HPLC was performed on an HP 1090 M using a ternary gradient with a flow rate of 1 mL/min to separate testosterone (T) and 15 metabolites (2α OH-T, 2β OH-T, 6α OH-T, 6β OH-T, 7α OH-T, 11α OH-T, 11β OH-T, 14α OH-T, 15α OH-T, 16α OH-T, 16β OH-T, 19 OH-T, androstenedione (A), T-glucuronide, and 5α-dihydrotestosterone (DHT)), which were monitored with a UV detector at 254 nm and a radioactivity detector (Ramona® 5, Raytest GmbH, Straubenhardt, Germany).

The elution conditions using 0.2% aqueous phosphoric acid (solvent A), acetonitrile (solvent B), and methanol (solvent C) were started with an isocratic elution for 20 min with 68% mobile phase A, 0% B and 32% C, then changed to 63% A, 5% B, and 32% C after 45 min, followed by isocratic elution for 5 min, then changed to 61% A, 7% B, and 32% C after 60 min, a subsequent isocratic elution for 5 min, then changed to 58% A, 10% B, and 32% C after 75 min, isocratic elution for 10 min, changed to 33% A, 35% B, and 32% C after 105 min and to 5% A, 95% B, and 0% C after 110 min.

ASSAY FOR GLUCURONOSYLTRANSFERASE (UDP-GT) AND SULFOTRANSFERASE (ST) ACTIVITY. UDP-GT and ST activity were determined using 10 µmol/L [¹⁴C]p-nitrophenol (PNP, 65 mCi/mmol). PNP glucuronide (*m/z* 315) and PNP sulfate (*m/z* 219) were identified by LC/MS (mass spectrometer comprising: PE/SCIEX/API III with MacIntosh Quadra®, Perkin-Elmer Sciex Instruments; Thornhill, Ontario, Canada, with HP 1090 M). PNP was incubated with cultured hepatocytes at 37°C for 1 hr and the reaction was terminated by the addition of 10% trichloroacetic acid. The samples were centrifuged and aliquots of the supernatant assayed for PNP glucuronide and PNP sulfate. The metabolites were determined by analytical HPLC, performed on an HP 1090 M equipped with work station and Ramona® 5 radioactivity monitor. HPLC was performed on a LiChrospher® 100 RP 8 column (250 × 4 mm i.d., particle size 5 µm) with a LiChrospher® 100 RP 8 guard column (4 × 4 mm i.d., particle size 5 µm) at a flow rate of 1.3 mL/min. The mobile phase consisted of a 50 mmol/L aqueous ammonium acetate solution (solvent A) and acetonitrile. For elution the gradient started with 100% A, changed to 80% A after 15 min, followed by isocratic elution for 5 min, changed to 20% A after 25 min and isocratic elution for 5 min with 20% A.

GLUTATHIONE S-TRANSFERASE (GST) ASSAY. GST activity was determined by measuring the disappearance of BSP from the medium. BSP content and the formation rate of BSP conjugate were monitored simultaneously by HPLC [23]. HPLC was performed on a LiChrospher® 100 RP 18 column (250 × 4 mm i.d., particle size 5 µm) and a LiChrospher® 100 RP 18 guard column (4 × 4 mm i.d., particle size 5 µm). Gradient elution was carried out at a flow rate of 1.3 mL/min. The mobile phase consisted of a 50

mmol/L ammonium acetate buffer, adjusted to pH 5.8 with formic acid (solvent A) and acetonitrile. For elution the gradient started with 100% A, changed to 60% A after 24 min, 20% A after 28 min, and isocratic elution for 6 min with 20% A. BSP (30 µmol/L and 40 µmol/L) was incubated with rat and human hepatocyte cultures, respectively, for 1 and 2 days. Samples of the supernatant medium were centrifuged, and 100 µL of the supernatant was analyzed by HPLC and quantified with BSP standards.

Cell Morphology and Quantitation

Cells were examined with an image-analysis system comprising an inverted microscope (Nikon TMS, Düsseldorf, Germany) fitted with phase contrast optics and equipped with a CCD color video camera (3 CCD camera DXC-930, Sony, Fellbach, Germany) and an adapter (CMA-02, Sony) as interface to a digital image-processing computer system (HP Vectra XM2, Waldbronn, Germany). CCD images of the microscope field of view were digitized on-line by a frame grabber (8-Bit Color frame grabber, Vidi Sys GmbH & Co. KG, Sauerlach, Germany) and visualized on a video monitor (Trinitron® PVA-1443 MD, Sony). In addition to morphological analysis, cell quantification was carried out using a cell counting program (Bayer AG, Wuppertal, Germany). An average of 100 to 200 cells were counted in a defined video frame at ×200 magnification. There were usually at least four representative areas to be counted.

Statistics

Each enzyme assay was carried out with at least three different cell preparations. The values are expressed as arithmetic means ± SD. Statistical significance was analyzed for enzyme activity data, representing five separate hepatocyte preparations. Prior to the statistical analysis, a logarithmic transformation was performed on raw data to take into account variance heterogeneity and distributional irregularities. Thereafter, Student's *t*-tests were used to assess the statistical relevance of the findings.

RESULTS

Phase I Enzyme Activities

Dealkylation of 7-ethoxyresorufin by rat and human hepatocytes is shown in Fig. 1. In rat hepatocytes, the formation of total resorufin over the 14 days in culture was maintained at the day 0 level, whereas resorufin conjugates increased, with a maximum at day 7. EROD activity in human cells increased, but the same proportion of free resorufin to its phase II conjugates was observed throughout the 14 days in culture (Fig. 1). ECOD enzyme activity decreased in both species after 3 days in culture (Figs. 2 and 3). The lowest activity was observed for rat cells at day 10, increasing to 74% after 2 weeks. The ECOD activity of human hepatocytes declined to 66% of the value at day 3 and remained at

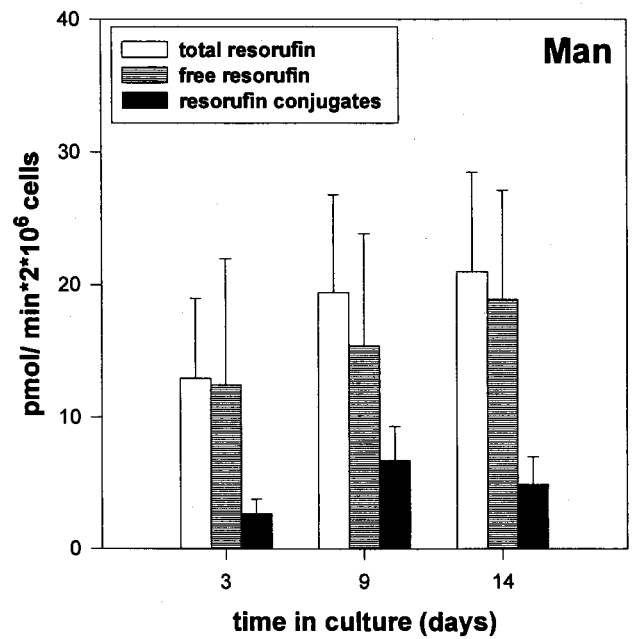
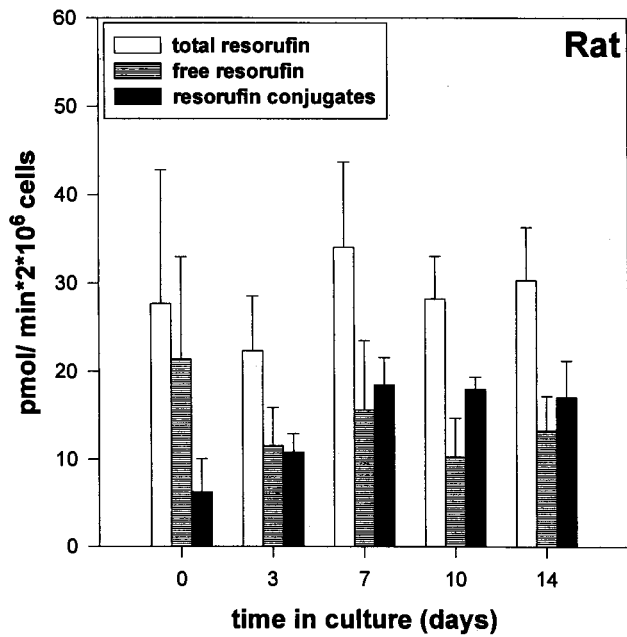


FIG. 1. EROD activity and conjugation of resorufin by rat and human hepatocytes in sandwich culture (values are expressed as means \pm SD, $n = 4$).

this level until day 14 (Fig. 3). In rat and human hepatocytes, deethylation of 7-ethoxycoumarin to umbelliferone was followed by an extensive conjugation with glucuronic acid and sulfate.

Testosterone was oxidized by freshly isolated rat hepatocytes to hydroxytestosterone metabolites and androstenedione (Table 1). The time courses of CYP-specific testosterone hydroxylase activities in rat hepatocytes are shown in Fig. 4. The male-specific form CYP 2C11, measured by the activity of testosterone 2α -hydroxylase, reached a maxi-

mum after 3 days in culture (Fig. 4). A similar time course was found for 16β -hydroxylase activity (CYP 2B1) (Fig. 4). Testosterone oxidation in human hepatocytes (Fig. 5) showed 6β -hydroxytestosterone, a functional marker of CYP 3A4, as the main metabolite with 2β -hydroxylase having the second highest activity, followed by androstenedione formation.

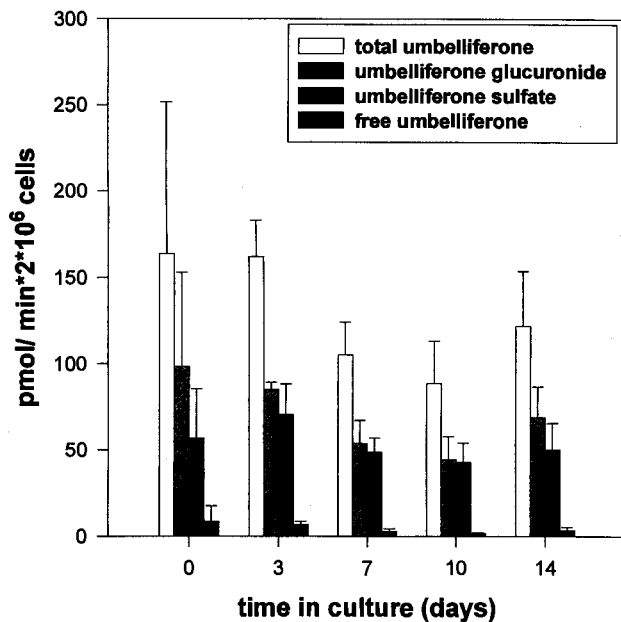


FIG. 2. ECOD activity and conjugation of umbelliferone by rat hepatocytes in sandwich culture (values are expressed as means \pm SD, $n = 4$).

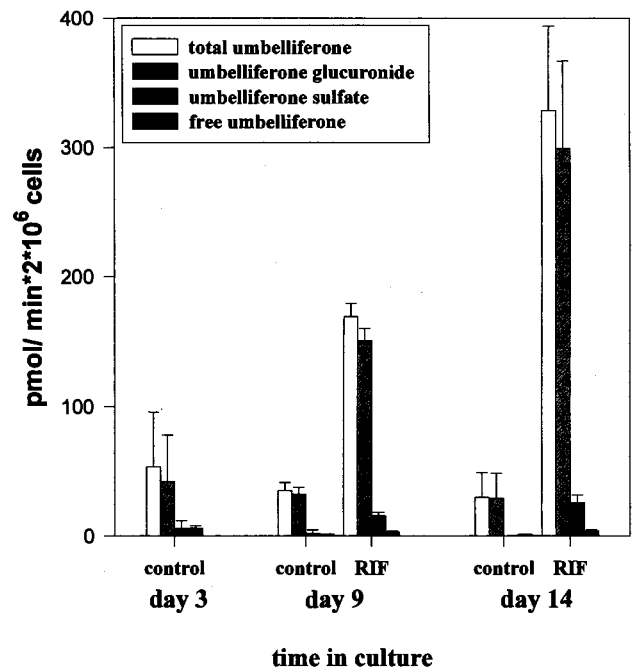


FIG. 3. ECOD activity and conjugation of umbelliferone in control and rifampicin-treated ($50 \mu\text{mol/L}$) human hepatocytes in sandwich culture (values are expressed as means \pm SD, $n = 3$).

TABLE 1. Testosterone hydroxylation and formation of androstendione (A) (pmol/min · 2 · 10⁶ cells) in primary cultures of hepatocytes from male rats (values are expressed as means ± SD, n = 3)

Time in culture (days)	Testosterone oxidation										
	2α	2β	6α	6β	7α	11α	15α	16α	16β	A	
0	221.0 ± 74.9	25.7 ± 20.7	47.5 ± 1.0	164.8 ± 37.8	57.9 ± 4.3	43.9 ± 31.1	7.1 ± 4.1	217.9 ± 69.5	13.9 ± 19.6	112.3 ± 64.3	
3	324.7 ± 93.3	78.7 ± 32.0	38.1 ± 7.2	89.9 ± 19.7	21.3 ± 15.1	63.2 ± 36.3	7.5 ± 1.4	362.3 ± 147.5	40.4 ± 5.3	195.7 ± 94.9	
7	36.0 ± 3.6	101.1 ± 29.3	54.9 ± 7.5	193.9 ± 87.1	21.5 ± 15.5	10.2 ± 14.4	4.5 ± 3.2	71.0 ± 24.9	27.9 ± 5.6	94.7 ± 31.2	
10	11.7 ± 16.6	106.5 ± 17.3	62.0 ± 15.0	256.7 ± 59.4	19.5 ± 14.2	8.7 ± 12.2	4.6 ± 3.3	44.3 ± 18.4	30.2 ± 2.9	44.2 ± 6.8	
14	8.7 ± 12.3	149.1 ± 42.7	76.0 ± 6.2	329.1 ± 26.1	12.2 ± 17.3	8.7 ± 12.3	6.3 ± 4.6	41.5 ± 5.9	34.2 ± 2.8	37.3 ± 10.7	

Human hepatocytes also contained testosterone 7α-, 6α-, 16α-, 15α-, 11α-, and 16β-hydroxylase activities which were completely preserved for 9 days in culture, as shown in Table 2. Of these minor metabolites, only 6α- and 7α-hydroxytestosterone were detectable at day 14.

Phase II Enzyme Activities

In rat hepatocytes, an identical time course was found for both GT forms (Table 3). Starting from the enzyme activity in freshly isolated hepatocytes, PNP UDP-GT and testosterone UDP-GT showed a marked increase in activity, reaching an initial maximum at day 7 (Table 3). Glucuronidation of PNP in human hepatocytes showed an increase to 355% at day 9 compared to the enzyme activity at day 3 (Table 4). This phase II activity was not maintained, declining to 181% at day 14. The time course of sulfotransferase activity (ST) toward PNP was quite similar in rat and human hepatocyte cultures (Tables 3 and 4). After an initial increase with a maximum at day 7 for rat hepatocytes and at day 9 for human hepatocytes, enzyme activity decreased over the 14 days in rat hepatocyte cultures to 151% and in human hepatocytes to 40% of the initial value. GST activity showed a similar time course in rat and human hepatocytes (Tables 3 and 4). As shown in Table 4, a decline to 21% of the value at day 3 was observed for GST activity in human hepatocytes.

Effects of Rifampicin on Phase I and Phase II Enzyme Activities in Human Hepatocytes

After 24 hr in culture, human hepatocytes were exposed to 50 μmol/L RIF in the culture medium for 12 days. The

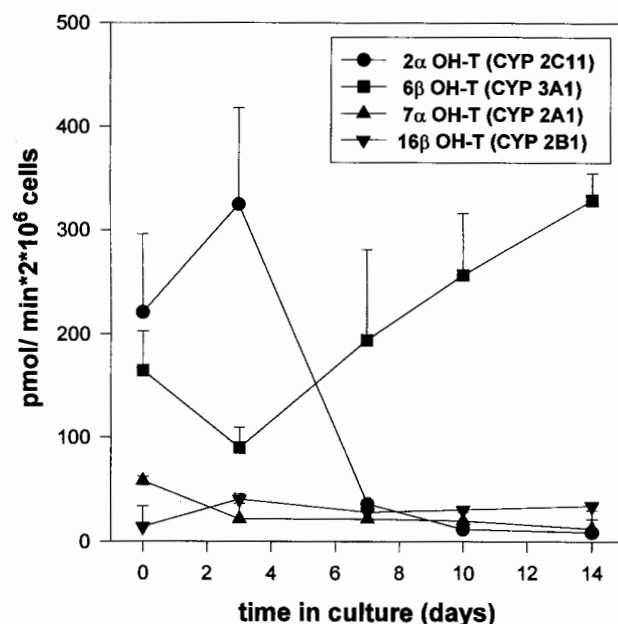


FIG. 4. Testosterone hydroxylase activity of rat hepatocytes in sandwich culture representing single CYP isozymes (values are expressed as means ± SD, n = 3).

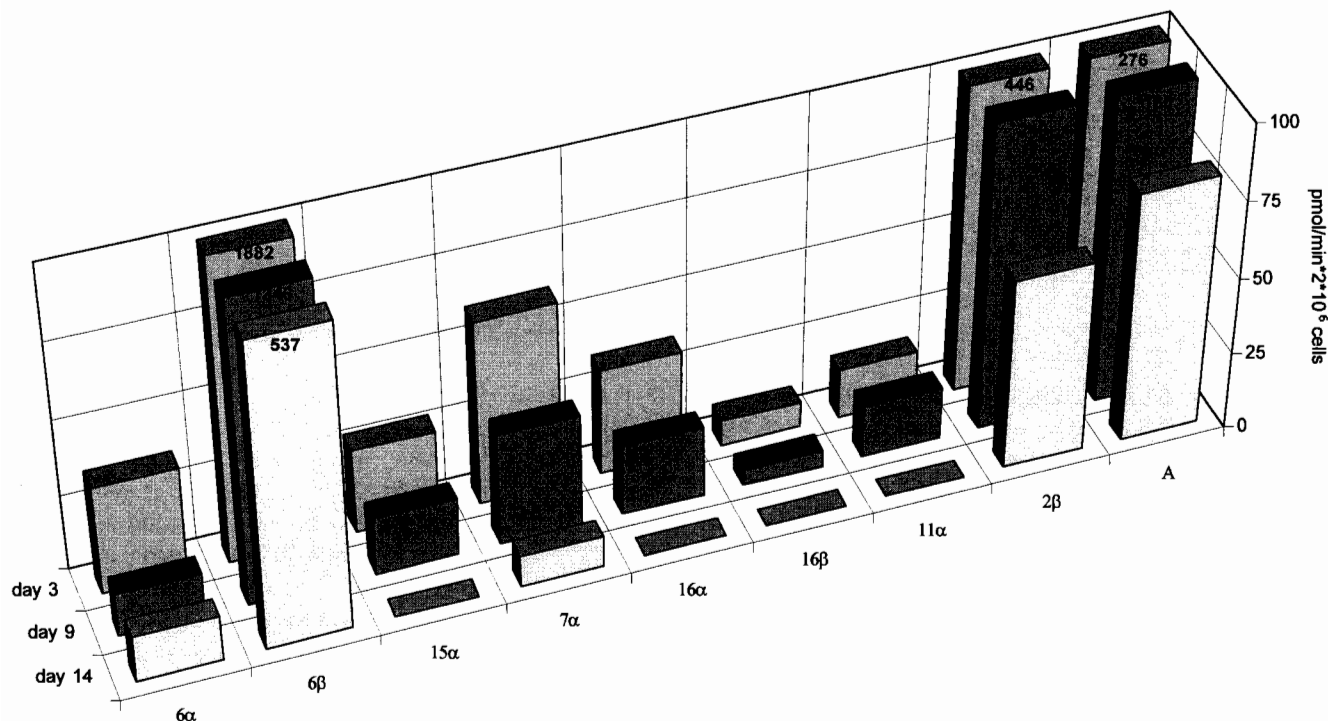


FIG. 5. Testosterone hydroxylation and formation of androstenedione (A) in primary cultures of human hepatocytes (values are expressed as means \pm SD, $n = 5$).

effects of rifampicin on phase I and phase II enzymes were studied using 7-ethoxyresorufin (CYP 1A2), 7-ethoxycoumarin (mainly CYP 1A2), testosterone (CYP 3A4), PNP (UDP-GT and ST activity), and BSP (GST activity) as substrates. Prior to the statistical analysis of CYP 3A4

induction, a logarithmic transformation was performed on raw enzyme data to take into account variance heterogeneity and distributional irregularities. Thereafter, Student's *t*-tests were used to assess the statistical relevance of the findings. Statements of statistical interference are therefore

TABLE 2. Testosterone hydroxylation and formation of androstenedione (A) ($\text{pmol}/\text{min} \cdot 2 \cdot 10^6$ cells) in primary cultures of human hepatocytes with and without rifampicin (RIF) treatment (values are expressed as $\text{pmol}/\text{min} \cdot 2 \cdot 10^6$ cells and are means \pm SD, $n = 5$).

Testosterone oxidation	Day 3		Day 9		Day 14	
	Control	Control	RIF	Control	RIF	
2α	0.0 \pm 0.0 (100%)	0.0 \pm 0.0 —	34.5 \pm 27.5 —	0.0 \pm 0.0 —	7.4 \pm 14.8 —	
2β	446.3 \pm 359.1 (100%)	240.0 \pm 182.6 (54%)	624.6 \pm 220.2 (140%)	62.3 \pm 47.8 (14%)	475.5 \pm 180.0 (107%)	
6α	35.6 \pm 58.0 (100%)	14.2 \pm 17.8 (40%)	209.7 \pm 313.4 (589%)	14.5 \pm 12.5 (41%)	176.9 \pm 274.2 (497%)	
6β	1882 \pm 1061 (100%)	1346 \pm 890.6 (72%)	5930 \pm 904 (315%)	536.6 \pm 365.7 (29%)	6306 \pm 1450 (335%)	
7α	60.5 \pm 51.0 (100%)	36.7 \pm 39.5 (61%)	547.5 \pm 217.7 (905%)	9.7 \pm 13.7 (16%)	644.8 \pm 270.3 (1066%)	
11α	16.0 \pm 21.7 (100%)	17.1 \pm 21.5 (107%)	256.6 \pm 161.5 (1608%)	— (0%)	210.8 \pm 109.7 (1321%)	
15α	27.7 \pm 34.3 (100%)	18.6 \pm 30.3 (67%)	47.5 \pm 46.2 (171%)	— (0%)	42.7 \pm 39.2 (154%)	
16α	34.9 \pm 20.9 (100%)	22.9 \pm 21.3 (66%)	60.0 \pm 49.0 (172%)	— (0%)	24.5 \pm 25.7 (70%)	
16β	8.5 \pm 10.6 (100%)	5.2 \pm 10.4 (62%)	31.1 \pm 26.8 (368%)	— (0%)	24.5 \pm 22.6 (290%)	
A	276.1 \pm 34.6 (100%)	185.2 \pm 109.6 (67%)	289.0 \pm 242.5 (105%)	81.2 \pm 71.8 (29%)	205.9 \pm 126.1 (75%)	

Data in parentheses are given in percent of the initial value on day 3 in culture (100%).

TABLE 3. UDP-GT, ST, and GST activities (pmol/min · 2 · 10⁶ cells) in primary cultures of hepatocytes from male rats (values are expressed as means ± SD, n = 4–5)

Time in culture (days)	PNP UDP-GT	Testosterone UDP-GT	ST	GST
0	63.7 ± 23.7	52.1 ± 37.7	48.0 ± 33.9	52.6 ± 2.6
3	263.7 ± 54.9	728.9 ± 199.7	105.1 ± 40.0	28.6 ± 5.7
7	456.8 ± 97.6	1260.5 ± 408.9	121.6 ± 57.3	13.8 ± 3.4
10	408.8 ± 67.2	917.9 ± 217.3	70.4 ± 37.6	8.8 ± 1.5
14	468.0 ± 112.8	1221.9 ± 293.1	72.5 ± 37.1	7.0 ± 2.1

based on logarithmic transformed data. To calculate ratios and confidence intervals, geometric means were used for CYP 3A4-mediated testosterone 6 β -hydroxylase activity.

RIF increased testosterone 6 β -hydroxylase activity in hepatocytes of five donors (Table 2). This enzyme activity associated with CYP 3A4 was induced 3.7-fold ($P = 0.020$) at day 9 compared to activity at day 3 (Fig. 6). RIF induction was 5.8-fold that of the control value at day 9 ($P = 0.017$). No effect of RIF (50 μ mol/L) on testosterone 6 β -hydroxylase activity was found in rat hepatocytes (data not shown). After 7 and 12 days of RIF treatment, ECOD activity was induced 3.2- and 6.2-fold, respectively, compared to the activity at day 3 (Fig. 3), while EROD activity was 1.4-fold compared to the control value at day 14 (14.4 pmol/min · 2 · 10⁶ cells, n = 3). In addition, two testosterone hydroxylase activities, 7 α - and 11 α -hydroxylase, were significantly induced 9.1- and 16.1-fold at day 9 (Table 2). As summarized in Table 5, RIF also influenced phase II enzyme activities. At day 9, UDP-GT and ST activity toward PNP increased 1.5- and 2.1-fold compared to the control value of day 9. During RIF treatment, these phase II enzyme activities were maintained at 159 and 179% of the value at day 9 until day 14, whereas in control cultures a loss of the phase II activities to 47 and 25% was observed. RIF treatment had nearly no stabilization effect on GST activity (Table 5).

DISCUSSION

Preservation of Drug-Metabolizing Enzyme Activities

The substrates selected for the qualitative and quantitative assessment of phase I and phase II enzyme activities fulfill several important criteria. All easily penetrate liver cells to form metabolites, which are released into the media without any enrichment in the cellular layer or binding to the

TABLE 4. UDP-GT, ST, and GST activities (pmol/min · 2 · 10⁶ cells) in primary cultures of hepatocytes (values are expressed as means ± SD, n = 3)

Time in culture (days)	PNP UDP-GT	ST	GST
3	108.0 ± 90.5	55.5 ± 20.5	53.9 ± 15.5
9	427.0 ± 54.0	115.0 ± 35.0	22.4 ± 3.8
14	195.5 ± 51.0	22.0 ± 5.5	11.2 ± 6.0

extracellular matrix (data not shown). CYP isozymes were determined by measuring their activity in forming specific metabolites. Enzymatic reactions are performed directly within the intact cells, therefore making preparation of microsomal fractions unnecessary. To delay the dedifferentiation of hepatocytes in culture and the accompanying loss of CYP activity, we used rat and human hepatocytes in culture overlaid with matrix. Hepatocytes in this sandwich culture reconstitute a physiological polyhedral cell shape, respond to stimulation [12], and have been successfully used to predict drug metabolism [8].

Enzyme activities such as 7-ethoxyresorufin deethylase and certain testosterone hydroxylases, e.g., 6 β -hydroxylase, are associated with single CYP isozymes [14, 24]. On the other hand, 7-ethoxycoumarin deethylase, other testosterone hydroxylation reactions, and *p*-nitrophenol and BSP conjugation reactions are performed by more than one form of CYP and UDP-GT, ST and GST, respectively. In sandwich culture, the formation rate of resorufin in rat (CYP 1A1) was maintained over 14 days at the initial enzyme level, in contrast to conventional culture systems

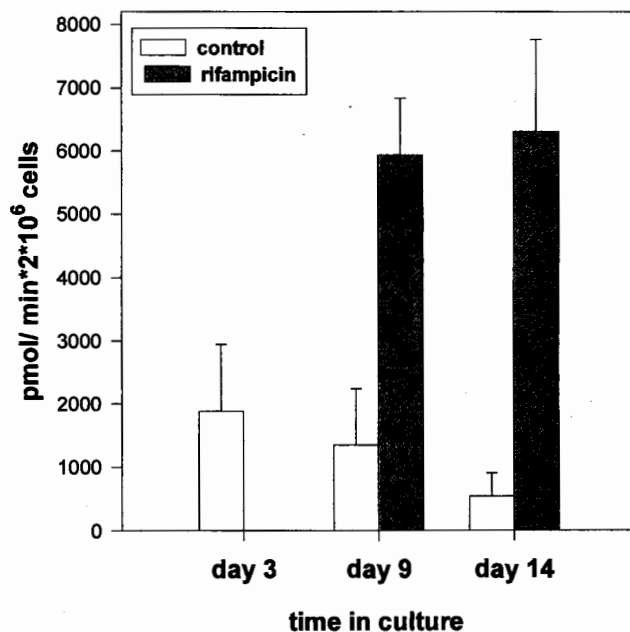
**FIG. 6.** Effect of rifampicin treatment (50 μ mol/L) on testosterone 6 β -hydroxylase activity in cultured human hepatocytes (values are expressed as means ± SD, n = 5).

TABLE 5. UDP-GT, ST, and GST activities in primary cultures of human hepatocytes treated with and without rifampicin (RIF) (values are expressed as pmol/min · 2 · 10⁶ cells and are means ± SD, n = 3)

	Day 3 control	Day 9		Day 14	
		Control	RIF	Control	RIF
UDP-GT	136.9 ± 71.8 (100%)	349.2 ± 74.8 (255%)	521.0 ± 101.0 (381%)	162.7 ± 79.0 (119%)	554.5 ± 140.0 (405%)
ST	52.3 ± 23.3 (100%)	72.6 ± 39.0 (139%)	149.0 ± 30.3 (285%)	18.4 ± 6.5 (35%)	129.7 ± 42.0 (248%)
GST	53.9 ± 15.5 (100%)	22.4 ± 3.8 (41%)	28.9 ± 9.9 (54%)	11.2 ± 6.0 (21%)	23.2 ± 7.5 (43%)

Data in parentheses are given in percent of the initial value on day 3 in culture (100%).

[25]. In human hepatocytes, EROD activity (CYP 1A2) increased over 14 days. On fibronectin-coated plates [22, 26] or without coating cell dishes [1, 27], loss of EROD activity was observed during this time. 7-Ethoxycoumarin is O-deethylated by at least 10 CYP isozymes present in male rat liver (CYP 1A1/2, CYP 2A1, CYP 2B1/2, CYP 2C6/7, CYP 2C11, CYP 2C13, and CYP 2E1), of which the 1A and 2B subfamilies are the most effective forms [28]. In humans, 7-ethoxycoumarin is deethylated primarily by CYP 1A2, CYP 2B6, and CYP 2E1 [29, 30]. ECOD activity of human hepatocytes in sandwich culture showed a moderate decrease until day 9, but remained stable at least until day 14. Similar values are reported for freshly isolated human hepatocytes [31]. In rat hepatocytes, ECOD activity was constant for 3 days, then decreased to approximately half of this value before increasing again. Thus, the continuous decline in ECOD activity observed within a few days for rat and human hepatocytes cultured on fibronectin [32, 33] or collagen [34] was prevented by using the sandwich culture system. In addition, the low amount of free umbelliferone in rat and human hepatocyte cultures during the culture time indicates that coupling of phase I and phase II reactions was completely maintained.

However, the loss and regeneration of CYP enzyme activity does not affect all CYP isozymes in the same way during the time of culture. Therefore, it is necessary to identify and monitor the levels of CYP isozymes present in the hepatocytes during cultivation and to compare them with the values found *in vivo*. The metabolism of testosterone can be used to monitor the relative concentrations of individual CYP isozymes. The different regio- and stereoselective hydroxylation reactions have been associated with single isozymes [22, 35]. With freshly isolated hepatocytes from male rat, testosterone was predominantly metabolized to 2 α -, 6 β -, 16 α -hydroxytestosterone and androstenedione (Table 1), and 2 β -, 6 α -, 7 α -, 11 α -, 15 α -, and 16 β -hydroxytestosterone were formed as minor metabolites. Similar results have been reported previously [1, 22, 36]. Most striking was the time course of CYP 2C11 activity, measured as 2 α - and 16 α -hydroxylase activity in rat hepatocytes. Testosterone 2 α -hydroxylation is a biotransformation step specific for CYP 2C11, the main CYP isozyme of male rat. CYP 2C11 in male rat liver represents approxi-

mately half of total spectrally determined CYP [37]. Typically, the expression of this male-specific form is rapidly lost in culture [38–42]. In the sandwich culture system, both CYP 2C11-dependent activities increased to 150 and 165%, respectively, compared to the value in freshly isolated cells. To our knowledge, this is the first time that regeneration of this vulnerable CYP activity, which is dramatically reduced during the hepatocyte preparation process [4], could be demonstrated in cell culture. The increase in CYP 2C11 activity in rat hepatocytes in sandwich culture is even more remarkable, as the expression of CYP 2C11 is regulated *in vivo* by gonadal and pituitary hormones [37], which are not present in the medium. The CYP 2C11 isozyme is regulated *in vivo* by growth hormone [43], but supplementation of the medium with growth hormone does not prevent the loss of CYP 2C11 [40]. The lack of growth hormone in the culture medium probably explains the increases in CYP 3A-dependent testosterone 6 β - and 2 β -hydroxylase activity [44] as seen in rat cultures after day 3. CYP 3A2 is suppressed *in vivo* by growth hormone. Culturing hepatocytes in the sandwich system preserved CYP 2A1 and CYP 2B1 as well. CYP 2A1, associated with testosterone 7 α -hydroxylase activity in rat, declined to ca. half of the initial enzyme activity but remained on this level until day 10. CYP 2B1-associated testosterone 16 β -hydroxylase activity increased until day 3 and was stably expressed thereafter until day 14. Similar results for 7 α - and 16 β -hydroxylase activity have been observed [41]. In addition, both hydroxylase activities were reported to be not stably expressed in hepatocyte culture or even undetectable [26, 38]. In contrast to the dynamic changes in the initial levels of individual CYP isozymes in rat hepatocytes, which may limit their use (for some studies) to 1 week, the situation in human hepatocytes is characterized by only a moderate decline in enzyme activities until day 9 in sandwich culture. The enzyme pattern of the testosterone hydroxylase activities was the same at day 3 and 9. The activity of testosterone 6 β -hydroxylase, marker enzyme for CYP 3A4, was reduced slightly until day 9. A lower activity was reported for human hepatocytes in a conventional culture system [45]. For all other testosterone hydroxylation reac-

tions, no unequivocal association with single human liver isozymes was described.

Compared to monooxygenase activity, the ability of isolated hepatocytes to perform phase II reactions has often been ignored, although for many compounds glucuronidation is the only biotransformation pathway. There exist multiple isoforms of UDP-GT that have been classified into two families: bilirubin/phenol UDP-GT 1 and steroid/bile acid UDP-GT 2 [46]. Steroid UDP-GT and PNP UDP-GT activities have been studied in primary cultures of rat hepatocytes. A marked decrease in steroid UDP-GT at day 5 was reported, whereas PNP UDP-GT activity increased during a 3 day culture period [47].

In contrast, we found nearly identical time courses for testosterone UDP-GT and PNP UDP-GT in rat hepatocytes. One explanation may be that PNP as well as testosterone are accepted as substrates by both isoforms, suggesting a considerable overlap of substrate specificity. Both isoforms showed an extensive increase in activity during cultivation time. The same increase in UDP-GT activity toward PNP was found for human hepatocytes. Testosterone did not appear to be a good substrate for the human steroid UDP-GT; testosterone glucuronide was only found in one liver sample in minor amounts.

There is little information available on the maintenance of ST in human hepatocyte cultures. It has been reported that ST showed instability when 1-naphthol was used as a substrate. Naphthol was converted to its sulfate conjugate only in fresh human hepatocytes, whereas no ST activity was found in cultured cells [48]. PNP is a substrate of GT and phenol ST. In the sandwich system, this ST activity was maintained over 14 days in rat and human hepatocytes. Different results have also been reported for human GST. We found GST activity to be unstable, showing a continuous decrease during culture time, as reported previously [31]. In contrast to these findings, GST activity was described to be stable in hepatocyte cultures for 3 days or even to increase with time [48].

RIFAMPICIN INDUCTION. Primary hepatocytes in culture have been extensively used to examine the response of drug-metabolizing enzymes to xenobiotic treatment. When studying the regulation of gene expression *in vitro*, the maintenance of hepatocyte function for an extended period of time is a prerequisite. As shown for the phase I and phase II drug-metabolizing enzymes, the hepatocyte sandwich culture is an improvement in prolonging these specific functions and thereby making induction studies possible.

Rifampicin shows a three- to fourfold induction of CYP 3A4 in humans *in vivo*, whereas rifampicin has no inducing effect in the rat *in vivo* [49–51].

It has recently been reported that rifampicin induction in human hepatocytes on Matrigel maintained 65 and 22% of CYP 3A4 immunoreactive protein during culture at 36 and 102 hr, respectively [52]. Treatment of these cultures with rifampicin induced CYP 3A4 protein twofold compared to cultures with standard medium.

We investigated the effects of rifampicin on phase I and phase II enzymes in human hepatocytes. The inducing agent rifampicin was added 48 hr after hepatocyte isolation. Rifampicin was demonstrated to be a potent inducer of testosterone 6 β -hydroxylase (CYP 3A4) showing a 3.7-fold (vs. control day 3) and 5.8-fold (vs. control day 9) induction in culture. ECOD activity in human hepatocytes was increased sixfold compared to the initial values at day 3. This induction is probably associated with isozyme activities other than CYP 1A2, because CYP 1A2 (EROD activity) showed a lower increase.

An increase in activity was also observed for phase II enzymes. *In vivo*, UDP-GTs can be induced by many typical microsomal enzyme inducers [53]. Because a good marker substrate to monitor phase II capacity in humans *in vivo* is not available, studies on the effect of smoking or the use of oral contraceptive steroids have reported conflicting conclusions [53].

There are no data in the literature reporting on the effect of rifampicin on phase II enzymes in cultured hepatocytes. Rifampicin treatment for 7 and 12 days in sandwich culture resulted in a twofold increase of ST and a 1.3-fold increase of UDP-GT, respectively. No effect was observed for GST.

The novel hepatocyte sandwich culture system is suitable for investigating drug metabolism, drug–drug interactions, and induction. We have demonstrated that rat and human hepatocytes maintain their liver-specific functions: (a) CYP isozymes, especially the major forms in rat (CYP 2C11, CYP 2A1, CYP 2B1, and CYP 3A1) and man (CYP 3A4 and CYP 1A2), were preserved up to 14 days; (b) the phase II enzyme activities UDP-GT, ST, and GST were maintained over 14 days in culture at adequate levels; and (c) the response of hepatocytes in sandwich culture to rifampicin induction was as sensitive as *in vivo*.

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