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Xenobiotic Metabolism by Cultured Primary Porcine Hepatocytes

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ABSTRACT

Considering the large yield of viable cells comparable to human liver, primary porcine hepatocytes offer a valuable resource for constructing a bioartificial liver device. In this study, the ability of cultured primary porcine hepatocytes to detoxify xenobiotics has been examined using various known substrates of cytochrome P450 isoenzymes and UDP-glucuronosyltransferases. Present investigation demonstrated the stability of the isoenzymes responsible for the metabolism of diazepam in native state and stabilization of other isoenzymes, as judged by ethoxycoumarin *o*-dealkylase (ECOD), ethoxyresorufin *o*-dealkylase (EROD), benzyloxyresorufin *o*-dealkylase (BROD), and pentoxyresorufin *o*-dealkylase (PROD) activities following induction in culture environment, for a period of 8 days. Resorufin *O*-dealkylase activities were found to be the most unstable and deteriorated within first 5 days in culture. These activities were restored following induction with 3-methylcholanthrene (3-MC) or sodium phenobarbital (PB) to 20-fold of 1 activity for EROD, and 60 and 174% of day 1 activity for PROD and BROD on day 8, respectively. Metabolism of methoxyresorufin was most strikingly increased following induction with 3-MC to approximately 60-fold of day 1 activity, on day 8. UDP-glucuronosyltransferase-dependent glucuronidation of phenol red, however, stayed intact during the course of our study without induction. Our study indicated that porcine hepatocytes *in vitro* maintain many important liver-specific functions including detoxification (steady state and inducibility).

INTRODUCTION

LIVER FAILURE, among other diseases, remains as one of the major causes of mortality in the United States.[1] Although various approaches have been attempted to reduce mortality, orthotopic liver transplantation remains the mainstay of treatment.[2] Hepatocyte-based bioartificial liver support systems repre-

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sent a promising approach for temporary replacement of normal liver function. This extracorporeal device can be used in the treatment of acute fulminant hepatic failure, to support liver function while the liver regenerates, or, alternatively, to stabilize a critically ill patients awaiting transplantation surgery. In addition, it could provide support in chronic liver failure during acute exacerbations.

The availability of human liver cells has been and likely will remain a serious limitation to the use of primary human cells in bioartificial liver devices. Due to the large yield of viable and functional hepatocytes, porcine hepatocytes represent an attractive source of cells, which can be incorporated in a bioartificial liver device. Hence, maintenance of the hepatocellular functions and, in particular, detoxification enzyme activities in culture environments is crucial.

In spite of fairly efficient current techniques to replace some essential functions of the liver, detoxification remains among one of the most important function in liver failure.[3] Furthermore, lack of detoxification pathways and accumulation of endotoxins have been implicated in the neurological disorders occurring as part of the constellation of disorders known as hepatic encephalopathy.[4] Although the mechanism of these mental status changes is not well understood, the absence of detoxification reactions is thought to be playing a role in this complication.[5]

Presently, only limited information on the long-term functionality and detoxification ability of the primary porcine hepatocytes in culture is available. Recent studies have shown lidocain and ethoxycoumarin metabolism as indicators for cytochrome P450IA1-dependent activities with cultured porcine hepatocytes.[6] In addition, cytochrome P450 isozyme activity has also been assessed using alkyoxy resorufin substrates.[11] However, there is no complete study to demonstrate the activity of multiple isoforms of cytochrome P450 as well as the phase II conjugating enzyme activity in native and induced state in culture environment. Our investigation is an attempt to demonstrate usefulness of primary porcine hepatocyte culture as functional units for detoxification of various xenobiotic.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle medium (DMEM) with 4.5 g/L glucose, fetal bovine serum (FBS), penicillin, streptomycin, Earl's balanced salt solution (EBSS), and collagenase were purchased from GIBCO (Gaithersburg, MD), hydrocortisone from Upjohn (Kalamazoo, MI), epidermal growth factor (EGF) from Collaborative Research (Bedford, CA), and insulin from Squibb (Princeton, NJ). Goat anti-pig albumin antibody (horse radish peroxidase-conjugated) was purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Resorufin sampler kit and Hoechst dye were purchased from Molecular Probes (Eugene, OR). All other chemicals, including diazepam and its major metabolites (temazepam, nordiazepam, and oxazepam), β-glucuronidase, ethoxy-coumarin, and hydroxycoumarin were purchased from Sigma Chemical Company (St. Louis, MO). Biotin Blocking Sytem x590, and rabbit primary universal peroxidase (Kit# K684) were purchased from DAKO.

Isolation of porcine hepatocytes

Porcine hepatocytes were obtained from Organogenesis, Inc. (Canton, MA) and consisted of hepatocytes isolated from swine weighing 8–10 kg (Yorkshire/Hampshire hybrid, Parsons Inc., Hadley, MA). Briefly, pigs were systemically anesthetized (using sodium pentobarbital) and heparinized, and the portal vein and inferior vena cava were exposed and cannulated. The liver was perfused with lactated Ringer buffer (4°C, 3 liters, 300 mL/min) followed by EDTA solution (0.2% in Ringer solution, 1 liter, 300 mL/min) at 37°C. The liver was further digested with collagenase perfusion (1 mg/mL in HEPES buffer, 10 m*M*, 1.5 liter, 150 mL/min) at 37°C. Following enzymatic digestion, the tissue was sequentially processed through 200- and 100-micron stainless steel sieves and washed with cold medium. The typical yield was between 3.0×10^9 and 1.4×10^{10} cells with viability ranging from 80% to 95%, as determined by trypan blue exclusion.

Culture of porcine hepatocytes

Type I collagen was prepared from Lewis rat tail tendons as previously described.[12] A collagen gelling solution was prepared by mixing 9 parts of a 1.1 mg/mL collagen solution with 1 part of concentrated (10×) DMEM and kept at 4°C. Culture dishes (60-mm petri dishes) were coated with 1.0 mL of collagen gelling solution and the coated dishes were incubated at 37°C for 1 h to allow for gelation. The hepatocytes were suspended in culture medium (5×10^5 cells/mL) and seeded into the dishes at density of 1 million cells/dish in 4 mL of medium. The culture medium consisted of DMEM supplemented with 10% FBS, 0.5 U/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 ug/mL hydrocortisone, 200 U/mL penicillin, and 200 ug/mL streptomycin. The cultured hepatocytes were maintained in an incubator set at 37°C under a humidified gas mixture of 90% air/10% CO_2. The medium was collected every other day and replaced with 4 mL of fresh medium. The collected samples were kept at 4°C until quantitative analysis of albumin and urea production.

Hepatocytes albumin staining and image acquisition

Cultures were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min, and permeabilized for 10 min with 0.1% Triton in PBS. Endogenous avidin binding activity of hepatic tissue was blocked by 20-min incubations with 0.1% avidin and 0.01% biotin in 50 mM Tris-HCl, respectively. Endogenous peroxidase activity was blocked by 30 min of incubation with a hydrogen peroxide (H_2O_2) and sodium azide solution. Rabbit anti-pig albumin antibodies were used with H_2O_2 detection by use of a biotinylated secondary antibody to rabbit (IgG), streptavidin-labeled horse radish peroxidase and hydrogen peroxide in the presence of 3-amino-9-ethylcarbazole as a substrate. Specimens were observed and recorded using a Nikon Diaphot microscope equipped with a CCD camera (Optronics CCD V1470), and MetaMorph Image Analysis System (Universal Imaging, Westchester, PA).

Measurements of albumin and urea production

Albumin produced by porcine hepatocytes in culture was measured in medium samples collected every other day using previously described enzyme-linked immunosorbent assay (ELISA).[12] Urea concentrations in the collected medium were assayed using a commercially available kit from Sigma (kit No.535-A).

Measurement of cytochrome P450 activity

Resorufin O-*dealkylase activity:* Cytochrome P450-dependent resorufin *o*-dealkylase activity (BROD, PROD, EROD, and MROD) was measured using resorufin substrates namely pentoxy-, benzyloxy-, ethoxy-, and methoxyresorufin. The incubation mixture contained resorufin substrates (pentoxy-, ethoxy-, or methoxyresorufin, final concentration 5 μM) and dicumarol (80 μM) in EBSS. Dicumarol was added to incubation mixture on day 1 to prevent the disappearance of resorufin fluorescence due to further metabolism by cytosolic oxidoreductases.[13] An 80 μM concentration of dicumarol was chosen on the basis of a dose–response study using various concentrations of dicumarol (0–200 μM, $n = 4$ dishes per concentration, 1×10^6 hepatocytes/dish with pentoxyresorufin (5 μM) as the substrate.

The prepared solutions were preheated to 37°C, prior to incubation with hepatocytes. The petri dishes (1×10^6 hepatocytes/petri dish, $n = 4$ per substrate) were aspirated and washed with 2 mL of EBSS (37°C), and further incubated with 2 mL of EBSS at 37°C for 5–7 min, to remove the residual medium. Following removal of EBSS, the incubation mixture was added (2 mL per dish), and the dishes were incubated at 37°C in a 10% CO_2 incubator. At various time points (5, 15, 25, and 35 min) following incubation, 100 μL of the mixture was transferred into a 96-well plate. The fluorescence of the plate was measured using a fluorescence plate reader (Cytocalc, Molecular Devices, Sunnyvale, CA, ext. 530 nm and emis. 590 nm) at the end of 35 min of incubation. A standard curve of resorufin fluorescence was constructed using concentrations ranging from 1 to 1,000 nmol in EBSS. A linear curve was obtained with an r^2 of 0.99. The constructed standard curve was used to convert the fluorescence values obtained from the plate reader to nanomoles of resorufin. Rate of formation of resorufin, as calculated from the early linear increase in the fluorescence curve, was defined as cytochrome P450 activity and expressed as nmol/min.

Diazepam metabolism: To assess P450-dependent diazepam metabolism, porcine hepatocytes (1×10^6 hepatocytes/petri dish, $n = 4$) were incubated with medium containing diazepam (50 μg/mL) for 24 h at 37°C in a 10% CO_2 incubator. Preliminary experiments comparing diazepam concentrations (10–50 μg/mL) and incubation times ranging from 4 to 24 h established that 50 μg/ml diazepam provided sufficient drug and metabolite for analytical detection by HPLC without significant substrate depletion. After incubation, medium samples were harvested and 200 μL of the collected medium was diluted with 200 μL of a 50:50 mixture of acetonitrile and methanol to precipitate the proteins. The mixture was centrifuged at 10,000 rpm for 10 min and supernatant was injected to high-performance liquid chromatography (HPLC) (100 μL). The HPLC system for analysis of diazepam and metabolites was as previously described.[14] The retention time of diazepam and major metabolites (temazepam, nordiazepam, and oxazepam) in the medium samples were confirmed using authentic diazepam and metabolite standards. The quantitative analysis was carried out using the standard curves of diazepam and metabolites prepared in medium.

Ethoxycoumarin o-*dealkylase activity:* Cytochrome P450-dependent ethoxycoumarin *o*-dealkylase (ECOD) activity of cultured porcine hepatocytes was monitored fluorometrically using a multiwell plate scanner fluorometer. The fluorescence of hydroxycoumarin, formed from ethoxycoumarin by action of cytochrome P450 isoenzymes, was measured at excitation wavelength of 355 nm and emission of 460 nm. Briefly, ethoxycoumarin (4 μM, in EBSS) was incubated with porcine hepatocytes (1×10^6 hepatocytes/petri dish, $n = 4$) for 4 h at 37°C in 10% CO_2 incubator. At the end of incubation, the supernatant was removed and stored at −80°C. Hepatocytes were isolated from collagen gel following collagenase treatment (4 mg/mL, 1 mL, 10 min incubation). The isolated cells were combined with the previously collected supernatants and stored at −80°C until further analysis. At the time of analysis, the supernatant and cell mixtures were sonicated to disrupt the cell membrane and homogenized. Hundred microliter samples of homogenate were transferred to a 96-well plate and the fluorescence of the plate was recorded. A previously constructed standard curve of hydroxycoumarin was used to convert the fluorescence values to the concentrations of hydroxycoumarin in the samples.

Phenol red glucuronidation

Phenol red glucuronidation assay was carried out as described elsewhere.[13] Briefly, the phenol red-containing medium was collected from cultured hepatocytes (1×10^6 hepatocytes/petri dish, $n = 4$) and the pH was adjusted to 5 with 50% glacial acetic acid. Two aliquots of the adjusted medium were dispensed into two test tubes. To one of the tubes 0.1 mL of 10mg/mL β-glucuronidase solution in 0.2 *M* acetate buffer was added. The second tube was used as a blank with culture medium incubated with 0.2 M acetate buffer without enzyme. Compared to control medium (without cells), the phenol red metabolism was calculated after 3 h incubation at 37°C following addition of glycine buffer to each sample and determining the absorbance at 546 nm.

Cytochrome P450 induction

3-Methylcholanthrene (3-MC) (2 μM) or sodium phenobarbital (1 mM) was used as inducer of cytochrome P450 activities. Various concentrations of 3-MC (0.5–4 μM) or sodium phenobarbital (0.5–4 mM) were tested in the medium for 10 days, and the albumin production of porcine hepatocytes was monitored. Presence of 3-MC (up to 4 μM) and PB (up to 2 mM) did not interfere with albumin production of porcine hepatocytes (data not shown). Therefore, induced hepatocyte cultures received 4 mL of medium containing the inducer (PB, 1 mM, or 3-MC, 2 μM), starting on day 1 of culture and replaced every other day.

RESULTS

The present study was designed to investigate the functionality of detoxification enzymes in cultured primary porcine hepatocytes.

Hepatocyte morphology

The purity of the initial cell population was determined using a porcine albumin-specific antibody and *in situ* immunostaining. Ninety eight (98%) percent of the areas were stained for albumin, indicating 98% purity of the initial hepatocyte population. Porcine hepatocytes retained their cuboidal morphology and distinct intercellular boundaries for 8 days in culture.

Dicumarol dose–response study

It is known that the metabolism of alkoxyresorufin derivatives involves dealkylation by microsomal cytochrome P450 to form resorufin followed by reduction of the resorufin to nonfluorescent products by cytosolic oxidoreductases. In our study, a dose–response study was carried out to look at the effect of various concentrations of dicumarol, a substrate and competitive inhibitor of cytosolic oxidoreductases, on stabilization of resorufin fluorescence formed by microsomal enzymes. The results are shown in Fig. 1 with PROD activity. The presence of dicumarol up to 80 μM effectively inhibited the metabolism of resorufin by cytosolic quinone oxidoreductases within the time period of assay (35 min). However, higher concentrations of dicumarol (above 100 μM up to 200 μM) seemed to have an adverse effect and resulted in lower fluorescence values, possibly due to cellular damage. Eighty micromolar concentration of dicumarol was used in subsequent studies with one million porcine hepatocytes.

Resorufin o*-dealkylase activity*

Cytochrome P450-dependent BROD, PROD, EROD, and MROD activities were measured on various days following culture (Fig. 2). All activities were detectable on day 1 following initiation of culture. The

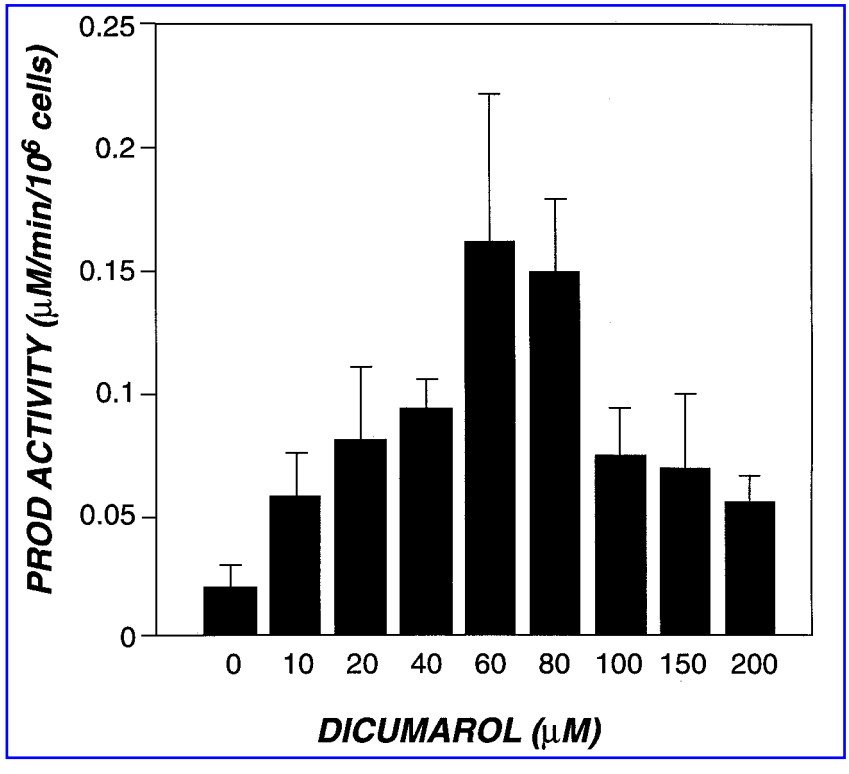

FIG. 1. Effect of dicumarol concentrations on the stabilization of resorufin fluorescence as depicted by PROD activity (1×10^6 porcine hepatocytes/dish, $n = 4$ per concentration of dicumarol).

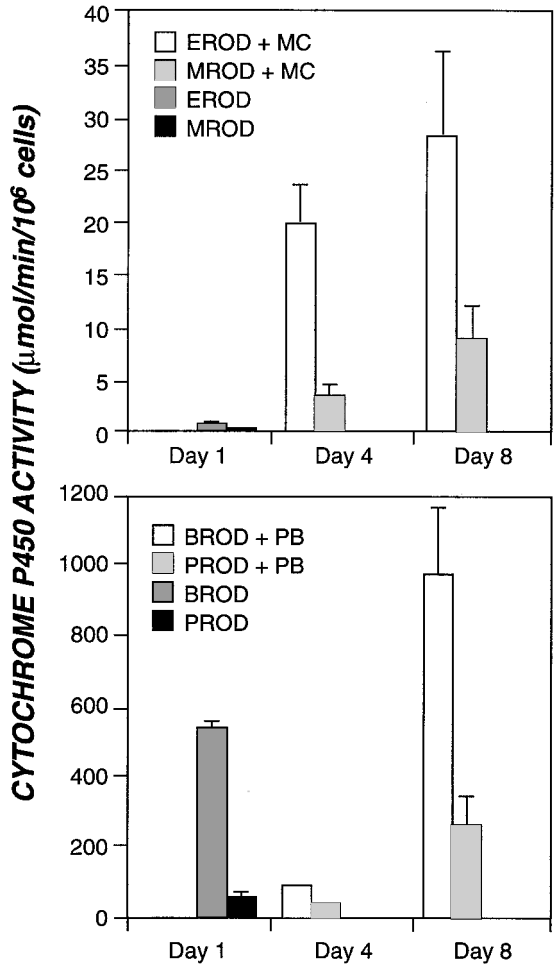

FIG. 2. Activity of various cytochrome P450 isoenzymes in porcine hepatocytes on days post culture (1×10^6 porcine hepatocytes/dish, $n = 3$ per condition from two separate isolations).

EROD activity, showed the highest levels and continued to be detectable up to day 5. Low levels of PROD and MROD activity were detected on day 1, which declined rapidly (by day 4) in culture. In spite of reasonable levels of BROD activity on day 1 it deteriorated within 24 h. There was no measurable activity for any forms of isoenzymes on day 8 of culture (Fig. 2).

Diazepam metabolism

The ability of cultured hepatocyte to metabolize diazepam was investigated by incubating diazepam (50 μg/mL) with porcine hepatocytes on various days post culture. The amounts of diazepam metabolized over 24 h by one million hepatocytes are presented in Fig. 3. Approximately 70% of the diazepam present in the medium was metabolized by cultured hepatocytes. These levels were stable with the following days, and approximately 10% reduction was observed in the following days in culture. The amount of major metabolites of diazepam formed by cultured porcine hepatocytes is presented in Fig. 4. Nordiazepam was the primary metabolite detected in the medium followed by temazepam. A small amount of oxazepam was detected on day 1, hence the levels were below our detection limits on subsequent days. While the total amounts of temazepam and nordiazepam formed by hepatocytes were reduced by 50% by day 4, these levels showed little or no change on the following days.

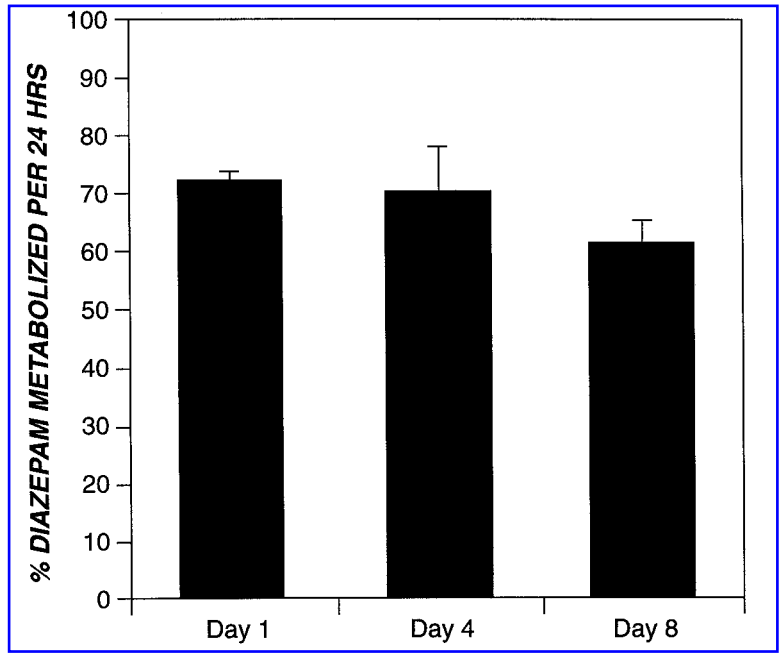

FIG. 3. Diazepam metabolism by porcine hepatocytes on days post culture (1×10^6 porcine hepatocytes/dish, $n = 3$ dishes from two separate isolations).

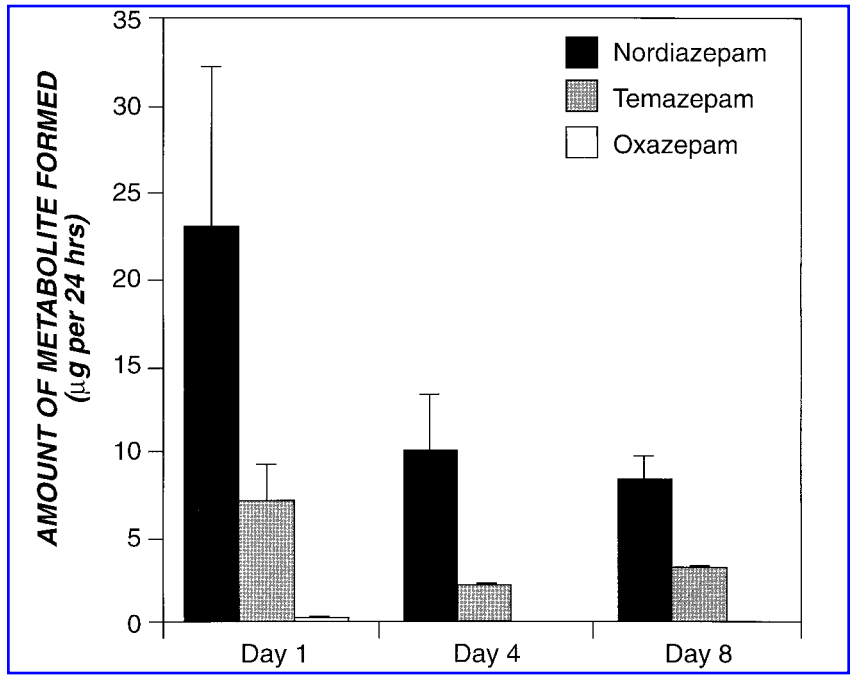

FIG. 4. Major metabolites of diazepam formed within 24 h by porcine hepatocytes (1×10^6 porcine hepatocytes/dish, $n = 3$ dishes from two separate isolations).

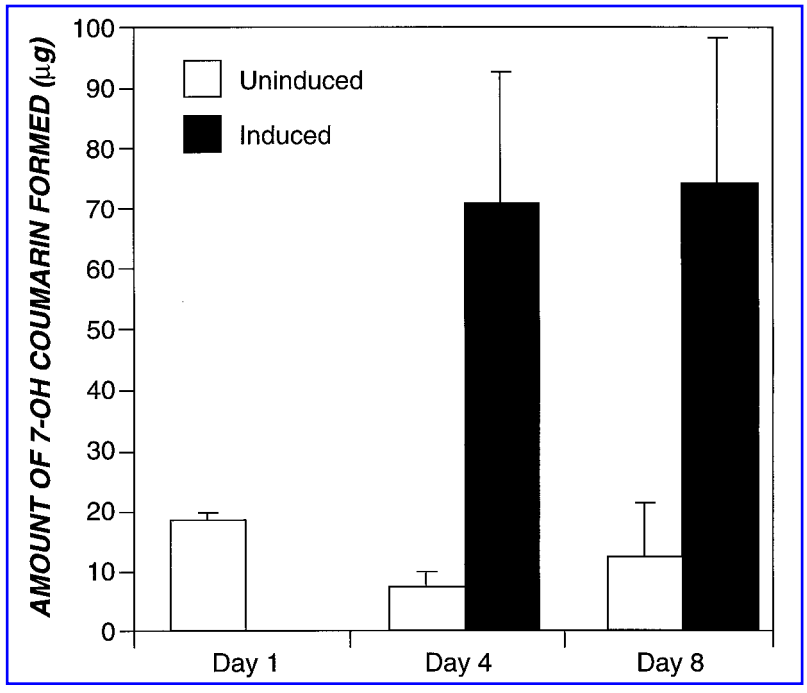

FIG. 5. ECOD activity of porcine hepatocytes in native and induced cultures (1×10^6 porcine hepatocytes/dish, $n = 3$ per condition from two separate isolations).

Ethoxycoumarin o-*dealkylase activity*

Assay of ECOD activity in cultured porcine hepatocytes was used as another measure of drug-metabolizing activity in these hepatocytes. The ECOD activity presented as amount of hydroxycoumarin formed following incubation with ethoxycoumarin is shown in Fig. 5. Hydroxycoumarin formation was reduced approximately by 50% by day 4 yet stayed relatively constant thereafter.

Glucuronidation of phenol red

The activity of phase II metabolic enzyme, UDP-glucuronosyl transferase, was measured by glucuronidation of phenol red (Fig. 6). Porcine hepatocytes maintained their glucuronidation activity over the course of our study. Roughly about 15–20% of the phenol red present in the medium was glucuronide conjugated by porcine hepatocytes.

Induction of cytochrome P450

Our studies with alkoxyresorufin and ethoxycoumarin as substrates for various cytochrome P450 isoenzymes indicated a loss of activity of the involved isozymes after day 1 in culture. Therefore, various inducers, such as phenobarbital (PB) and 3-MC, were used to examine the inducibility of these isoforms. Use of two different inducers produced different patterns and kinetics of induction. Sodium PB was used as an inducer of BROD and PROD, and 3-MC was used for induction of EROD and MROD as well as ECOD activities. Continuous induction by 3-MC resulted in a 22-fold increase in the EROD activity on day 4 and an almost 16-fold increase by day 8, as compared to day 1 activity (Fig. 7). MROD activity was induced most strikingly with 3-MC and reached almost 40-fold of day one activity on day 4 and 60-fold on day 8 (Fig. 7).

Continuous induction with PB on the other hand, resulted in an increased activity of BROD and PROD on days 4 and 8 of culture (Fig. 7). This increase was relatively low on day 4 but reached to almost 59% of the day 1 activity for PROD and 174% of the day 1 activity for BROD, on day 8 (Fig. 7).

Use of 3-MC as an inducer of ethoxycoumarin-dependent cytochrome P450 activity resulted in 4-fold

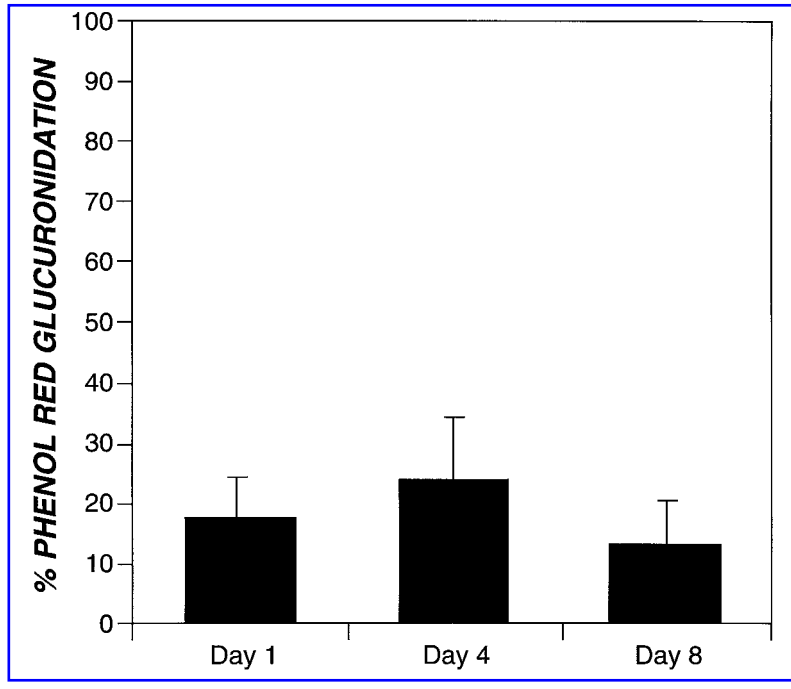

FIG. 6. Glucuronidation of phenol red by porcine hepatocytes on days post culture (1×10^6 porcine hepatocytes/dish, $n = 3$ per condition from two separate isolations).

increase in the activity on day 4 compared to day 1. Continuous exposure to inducer on the following days did not further enhance the ECOD activity and the levels remained unchanged on day 8 (Fig. 5).

Albumin and urea production by cultured porcine hepatocytes

To monitor further the functionality of porcine hepatocytes in culture, albumin production and urea secretion by porcine hepatocytes were measured as markers of liver-specific functions (Table 1). Both albumin and urea production by porcine hepatocytes showed a steep increase during the 8 days in culture. The continuous albumin production and urea secretion of porcine hepatocytes can be accepted as an indicator for the stabilization and adaptation of these cells to the culture environment.

DISCUSSION

In the near future, hepatocyte-based bioartificial liver systems are expected to become part of the treatment as life-prolonging systems for patients with acute liver failure awaiting liver transplantation. Considering the limited supply of human hepatocytes, the alternative resources include the use of liver cells from other species. Porcine hepatocytes have been considered due to anatomical and physiological similarities between the species (pigs and human) as well as the availability of the large number of cells that are required for constructing a bioartificial liver assist device. Presently, relatively limited information is available about the activity of cytochrome P450 isoenzymes in porcine hepatocytes when compared to other species such as rat, rabbit, or guinea pig.[15–18] Our study was designed to evaluate a wide range of liver-specific functions of primary porcine hepatocytes cultured on a monolayer of collagen gel including detoxification (both phase I and phase II metabolic functions), synthesis of proteins, and secretion of urea.

Diazepam has been used as the drug of choice to assess the cytochrome P450 activity due to the commonly accepted role of benzodiazepine-like substances in the pathogenesis of hepatic encephalopathy.[4] In rats, diazepam metabolism is primarily regulated by cyp2B2,[19] whereas in humans the major forms are cyp2C18/19 and 3A4.[20,21] In porcine hepatocytes, however, the P450 isoforms that govern diazepam me-

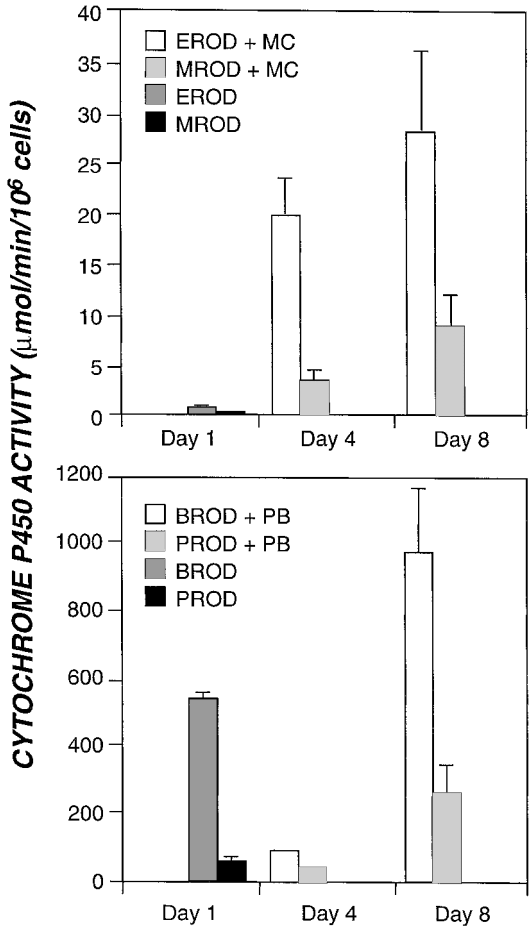

FIG. 7. Effect of inducers (PB, sodium phenobarbital 1 mM; MC, 3-methylcholanthrene, 2 μM) on the resorufin dealkylase activities (compared to day 1 uninduced cultures).

tabolism have not been identified yet. In our study, diazepam metabolism by porcine hepatocytes was shown to be stable up to 8 days, whereas the levels of the major metabolites temazepam and nordiazepam were only stabilized following an initial drop. This initial drop contributed to the decline in the amounts of oxazepam, formed by demethylation of temazepam and/or hydroxylation of nordiazepam, to undetectable levels in the following days. Our observation indicates the importance of hydroxylation (diazepam to temazepam) as the major metabolic pathway over demethylation in porcine hepatocytes. Hence, it is not

TABLE 1. ALBUMIN AND UREA PRODUCTION BY PORCINE HEPATOCYTES (1×10^6) CULTURED ON SINGLE LAYER OF COLLAGEN GEL

Days in culture	*Albumin production (μg)*	*Urea production (μg)*
2	0.9 ± 0.2	287.7 ± 13
4	6.5 ± 1.0	176.6 ± 10
6	25.9 ± 3.1	263.4 ± 17
8	46.3 ± 7.0	221.8 ± 15

known whether other metabolic pathways are responsible for the continuous metabolism of diazepam. Additional metabolic pathways such as C5 ring-hydroxylation,[22] or diazepine ring-opening and *N*-deethylation[23] to form benzophenone, or N4 oxidation,[24] all have been reported in other species, but standards were unavailable to authenticate these routes.

Purified human CYP2A6 has been shown to catalyze the ECOD activity.[25] In other studies, the human liver microsomal levels of CYP2E1 have been correlated with the levels of ECOD activities.[26] In rats, however, CYP450 1A1 is responsible for the metabolism of ethoxycoumarin.[18] As with the formation of the metabolites of diazepam, the formation of 7-OH coumarin declined after day 1 in culture but stayed stable in the following days. Although the cytochrome P450 form responsible for the metabolism of 7-ethoxycoumarin in porcine hepatocytes is unknown, higher metabolic activity toward diazepam may well be related to the difference between the involved isoforms and/or the maintenance of these enzymes in the culture environment in this species. The P450 activities related to the BROD, PROD, and MROD were lost after the first day in culture, whereas EROD activity was maintained up to day 5 (about 7.5% of the initial value). These results were in qualitative agreement with our concurrent studies with porcine hepatocytes exposed to different protocols.[10] These results suggest that maintenance of some of the cytochrome P450 families may not be supported by the described culture conditions. In rats, EROD, BROD, PROD, and MROD activities are related to CYP450 1A1, 2B2, 2B1, and 1A2 isoenzymes, respectively. In various studies, it has been shown that activity of cyp2B1/2 in particular has been relatively difficult to maintain in culture without complex systems such as co-cultures or complex extracellular matrices.[27,28] The rapid loss of cytochrome P450-dependent enzyme activities in our porcine hepatocyte culture is consistent with earlier reports on cultured hepatocytes from species such as rat, rabbit, or hamsters.[16–18] Previous investigators have proposed several mechanisms that may contribute to the loss of cytochrome P450 activities in culture.[29,30] P450 activities that had diminished over a 1- or 5-day period (BROD, PROD, MROD, EROD, and ECOD) were all inducible. After continuous induction with MC, there was a substantial increase in the EROD activity on day 4 (22-fold higher than the day 1 activity) that was maintained at a relatively steady levels up to day 8. In contrast, MROD activities exhibited the highest response to the inducer, and the levels increased dramatically from about 40-fold on day 4 to 60-fold on day 8 relative to their day 1 activity.

Sodium PB treatment, however, resulted in a modest elevation of PROD and BROD activities. Longer exposures to the inducer resulted in much higher activities for both isoenzymes on day 8 as shown by approximately two-fold of day 1 activity for BROD and 60% of their corresponding values on day 1 for PROD. In the porcine hepatocyte culture system, the activities of enzymes belonging to the 3-MC-inducible family, namely EROD, ECOD, and MROD, were much better maintained than those of PROD and BROD, which belong to the PB-inducible family. These observations demonstrate that the changes in the induction behavior of these isoenzymes were similar in rat and porcine hepatocytes. Hence, there are certain species specific differences between cultured pig and rat hepatocytes. 3-MC is known to be a strong inducer of EROD activity and only a modest inducer of MROD in rat hepatocytes,[31] whereas porcine hepatocytes seemed to exhibit a greater response to methoxyresorufin metabolism than EROD following induction with MC. This may indicate that MROD activity is likely to be preferentially catalyzed by one or more of the family of P450 forms that are inducible by MC.

Interestingly, the activity of phase II-conjugating enzyme, UDP-glucuronosyltransferase, was stable in the cultured hepatocytes as judged by the ability of the hepatocytes to form phenol red glucuronide for at least 8 days. Other liver-specific functions of hepatocytes, as measured by their urea and albumin production, were maintained at elevated levels over the course of 8 days in culture. Albumin accounts for 80% of all hemoproteins secreted by liver into the blood.[32] Albumin secretion is a specific marker to identify hepatocytes and is used frequently as an index of viability *in vitro*.[33] Urea synthesis, however, is the pathway for the detoxification of the ammonia. Both of these functions were unaltered by the culture environment.

It should be emphasized that further refinement of the present formulation of extracellular matrix or the medium composition may improve the utility of porcine hepatocyte cultures for various studies.[10] These could include a more detailed examination of the hormonal requirements of the porcine hepatocytes as well as supplementation of essential amino acids in the formulation of the medium. There have been many attempts to maintain the levels of P450 and its monooxygenase activities in cultured rat hepatocytes. These attempts include modifying the composition of the medium (by addition of hormones, chemicals, ligands,

and/or inducers to the culture medium) or by incorporating different cell types as in a co-culture model.[34–37] From their work, it appears that the life time of the primary cultures can be extended by modifying the conditions of the culture. Perhaps one of the significant findings in our study is that porcine hepatocyte culture supports the maintenance of cyp2C19 and 3A4, important human iosforms that are involved in the metabolism of many xenobiotics such as diazepam, as well as phase II glucuronidation activities without a need for induction. Nevertheless porcine hepatocytes cultured under the conditions described in this report were shown to exhibit reasonable responsiveness to inducers, which is crucial for the clinical applications of the bioartificial liver assist devices using porcine hepatocytes. Alternatively, an induced cultured porcine hepatocytes system can be used to examine the metabolism of xenobiotics and may ultimately replace or reduce the use of experimental animals for pharmaco-toxicological studies.

ACKNOWLEDGMENT

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