INFLUENCE OF EXTRACELLULAR MATRIX OVERLAY AND MEDIUM FORMULATION ON THE INDUCTION OF CYTOCHROME P-450 2B ENZYMES IN PRIMARY CULTURES OF RAT HEPATOCYTES

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ABSTRACT:

The effect of medium formulation, composition of extracellular matrix overlay, and culture dish material on liver microsomal cytochrome P-450 (CYP) 2B induction by phenobarbital (PB) was investigated in primary cultures of rat hepatocytes. When hepatocytes were maintained on Permanox dishes with an overlay of either collagen (type I) or Matrigel, Williams’ E medium was superior to other medium formulations in terms of the magnitude of induction of CYP2B on a per milligram microsomal protein basis. Modified Chee’s medium (MCM) and hepatocyte culture medium were intermediate in their capacity to sustain induction of CYP2B by PB, and Dulbecco’s modified Eagle’s medium was slightly less effective. The overall induction of CYP2B activity by PB was, on average, 50% lower in hepatocytes cultured on polystyrene dishes (LUX). Little or no difference was observed between hepatocytes overlaid with collagen and those overlaid with Matrigel. MCM was superior to Williams’ E medium in terms of the yield of microsomal protein and the ultrastructural features of the hepatocyte monolayers. CYP2B induction by PB was optimal after 3 days of treatment in either medium. CYP1A, CYP3A, and CYP4A activities could be induced in vitro by prototypical inducing agents in hepatocytes cultured on Permanox dishes with MCM and a Matrigel overlay to comparable levels observed in vivo. The results of these studies show that medium formulation and culture vessel material, but not the type of extracellular matrix overlay, have significant effects on the induction of CYP enzymes in cultured rat hepatocytes maintained in a sandwich configuration.

Over the past two decades significant efforts have been made to establish primary cultures of rat hepatocytes in which microsomal cytochrome P-450 (CYP) enzymes belonging to the CYP2B subfamily are maintained and/or induced as they are in vivo (LeCluyse et al., 1996b). In vivo liver microsomes from untreated rats contain undetectable levels of immunoreactive CYP2B1 and low levels (10 – 40 pmol/mg) of CYP2B2 (Thomas et al., 1987; Ryan and Levin, 1990). The levels of CYP2B1 and CYP2B2 in rat liver microsomes are coordinately regulated in a dose-dependent manner by phenobarbital (PB) and PB-type inducers (Adesnik et al., 1981; Waxman et al., 1990). The marked induction (~50-fold) of CYP2B1 and CYP2B2 enzymes involves transcriptional activation of their respective genes by mechanisms that have yet to be fully elucidated.

Three factors have been consistently shown to have significant effects on CYP enzyme expression and induction in vitro. These are: 1) the biophysical nature and composition of the extracellular matrix (Ben-Ze’ev et al., 1988; Lindblad et al., 1991), 2) the formulation of culture medium (Jauregui et al., 1986; Turner and Pitot, 1989), and 3) the combination and concentration of hormones (Dich et al., 1988; Dahn et al., 1993). Unfortunately, direct comparisons of rat hepatocyte culture protocols designed to study CYP expression are complicated by the number of culture variables and the interdependence of substratum, extracellular matrix overlay, medium formulation, and hormone supplementation. Comparisons between various studies are also complicated by the endpoints used to monitor CYP expression. For example, Sidhu et al. (1993) have shown that modified Williams’ E medium (WEM) and modified Chee’s medium (MCM) are equally effective in supporting induction of CYP2B mRNA by PB, but the latter is superior in supporting induction of CYP2B protein and activity. The age of the cultures before inducers are introduced, the relative potency of inducers within each class, the final concentration of inducers in culture medium, and the duration of exposure to inducers also have profound effects on the induction of CYP enzymes in cultured hepatocytes. Therefore, the purpose of this study was to determine the effects of a number of culture conditions on the overall cell morphology and the induction of CYP2B enzymes by PB in long-term cultures of rat hepatocytes.

Four medium formulations, namely, Dulbecco’s modified Eagle’s medium (DMEM), WEM, MCM, and hepatocyte culture medium (HCM), were chosen to represent both conventional formulations,
which are easily obtained commercially (WEM, DMEM) and “en-
riched” formulations, which contain superphysiological concentra-
tions of certain components, such as amino acids (MCM, HCM).
Collagen and Matrigel overlay were compared because both represent matrix components found in the space of Disse, yet are expressed
during different stages of hepatocyte differentiation (laminin = fetal;
collagen = mature; Reid et al., 1992). However, Matrigel is thought to be superior to collagen in its ability to restore the differentiated
phenotype to cultured hepatocytes, presumably because of its complex
nature (laminin, collagen type IV, entactin, etc.; Berry et al., 1991).
Finally, two commonly used, commercially available types of culture
dishes [polystyrene (LUX) and Permanox] were compared for their
influence on PB-induced CYP2B enzyme expression.

Experimental Procedures

Materials. All culture media, sera, glutaMAX media supplement, and
nonessential amino acids were obtained from Gibco BRL (Grand Island, NY).
Insulin, transferrin, selenium, linoelic acid, and BSA supplement (ITS+) and Matrigel were purchased from Collaborative Biomedical Research (Bedford, MA). Collagenase (CLS 2) was obtained from Worthington Biochemical
Corporation (Lakewood, NJ). Collagen, type I (Vitrogen) was obtained from
CelTrix (Santa Clara, CA). Petri dishes (60 mm) (LUX, Permanox) were
purchased from NUNC (Naperville, IL). The sources of the various steroids
and other reagents used in this study have been reported previously (Sonderfan
et al., 1987; Pearce et al., 1996).

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated
from rat liver by a modification of the collagenase digestion method described
by LeCluyse et al. (1996a), which was adapted from the method of Quistorff
et al. (1989). Briefly, rat livers were perfused with calcium-free buffer con-
taining 5.5 mM glucose and 0.5 mM EGTA for 10 min at a flow rate of 35 to
40 ml/min followed by perfusion with buffer containing 1.5 mM calcium and
collagenase (0.3–0.4 mg/ml) for 10 to 15 min at a flow rate of 30 to 35 ml/min.
Hepatocytes were dispersed from the digested liver in DMEM containing 5%
fetal calf serum, insulin (4 μg/ml), and dexamethasone (1 μM) and washed by
low-speed centrifugation (50g, 2 min). Cell pellets from rat liver were resus-
pended in equal volumes of medium and 90% isotonic Percoll and centrifuged
at 70g for 5 min. The resultant pellets were resuspended in fresh medium and
washed once by low-speed centrifugation. Hepatocytes were resuspended in
supplemented DMEM and viability was determined by trypan blue exclusion.
Cell preparations were not used for experimental studies unless the viability
was ≥85%.

Unless otherwise specified, approximately 3 × 10^7 hepatocytes were added
to 60-mm culture dishes in 3 ml of serum-free medium containing 0.1 μM
dexamethasone, 6.25 pg/μl insulin, 6.25 μg/ml transferrin, and 6.25 μg/ml
selenium and allowed to attach for 2 to 3 h at 37°C in a humidified chamber
with 95%/5% air/CO₂. Culture dishes were gently swirled and medium con-
sumed in equal volumes of medium and 90% isotonic Percoll and centrifuged
at 70g for 5 min. The resultant pellets were resuspended in fresh medium and
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selenium and allowed to attach for 2 to 3 h at 37°C in a humidified chamber
with 95%/5% air/CO₂. Culture dishes were gently swirled and medium con-
taining unattached cells and debris was aspirated. Cultures were overlaid with
either collagen or Matrigel as described elsewhere (LeCluyse et al., 1996a). In
addition, groups of hepatocyte cultures (n = 5–6 dishes per treatment group) were then treated for 3 consecutive days with either saline (control) or 100 μM
PB. At the end of the study period, cells were harvested for the preparation of
microsomes.

Time course of CYP enzyme induction. Hepatocytes were plated onto
Permanox culture dishes and overlaid with Matrigel as described above.
Hepatocytes were maintained in MCM supplemented with ITS+ and 0.1 μM
dexamethasone before initiating treatment with inducers. Groups of hepatocyte
cultures (n = 5–6 dishes per treatment group) were then treated for 1 to 4
consecutive days with 0.1% dimethyl sulfoxide (control) or 100 μM PB. At the
appropriate time point, cells from the corresponding groups were harvested for
the preparation of microsomes.

Dose response of P-450 enzyme induction. Hepatocytes were plated onto
Permanox culture dishes and overlaid with Matrigel as described above.
Hepatocytes were maintained in either WEM or MCM supplemented with
ITS+ and 0.1 μM dexamethasone before initiating treatment with PB. Groups
of hepatocyte cultures (n = 5–6 dishes per treatment group) were then treated
for 3 consecutive days with either 10, 25, 100, 250, 750, or 1500 μM PB.
Control cultures were treated with saline vehicle alone. At the end of the study
period, cells were harvested for the preparation of microsomes.

Cell harvest and microsome preparation. At the appropriate time points,
cultures of hepatocytes from each treatment group were rinsed twice with
ice-cold phosphate-buffered saline. Homogenization buffer (50 mM Tris-HCl,
ph 7.0, 150 mM KCl, 2 mM EDTA; 0.5 ml/dish) was added to each dish and
cells were scraped with a rubber policeman. Harvested cells from each treat-
ment were then pooled and sonicated with a Vibra-Cell probe sonicator (Sonics
& Materials, Danbury, CT) at 40 W for 15 to 20 s (Wortelboer et al., 1990).
Cell lysates were centrifuged at 9000g for 20 min at 4°C. Supernatant fractions
were collected and centrifuged at 100,000g for 60 min at 4°C. The final
microsomal pellets were resuspended in 0.25 to 0.5 ml of 0.25 M sucrose with
the aid of a Dounce homogenizer fitted with a Teflon pestle. A small aliquot
was taken for protein determination and all samples were subsequently stored
at −80°C.

Protein determination. The concentration of protein was determined with a
commercially available kit (BCA Protein Assay kit; Pierce Chemical Co.,
Rockford, IL) with bovine serum albumin as a standard (supplied with the
BCA Protein Assay kit).

P-450 enzyme activities. 7-Ethoxyresorufin and 7-pentoxyresorufin O-deal-
kyase (PROD), testosterone, and lauric acid hydroxylase activities were
determined as described by Pearce et al. (1996).

SDS-polyacrylamide gel electrophoresis and Western immunoblotting. The
levels of CYP2B in rat hepatocyte microsomes were determined by Western
immunoblot analysis with polyclonal antibodies against purified rat liver
microsomal enzymes as described previously (Parkinson and Gemzik, 1991).
Electron microscopy. Hepatocytes were fixed with 2.5% glutaral-
dehyde in 0.1 M phosphate buffer, pH 7.2, for 20 to 30 min. Cultures were then
postfixed with 1% OsO₄ for 30 min, stained en bloc with 1% uranyl acetate,
derhydrated in an acetone series, and embedded in Epon resin. Thin sections
were cut perpendicular to the plane of the monolayer, poststained with uranyl
acetate and lead citrate, and viewed with a JEOL 1200EXII electron micro-
scope.

Statistical analysis. Data were analyzed for statistically significant differ-
ences between control and treated rat hepatocyte cultures by Dunnett’s two-
way ANOVA at the 5% level of significance (Dunnett, 1964).

Results

Media and Overlay Effects on P-450 Enzyme Induction. Hepa-
tocytes were cultured on a simple substratum of collagen, type I, with
one of four different medium formulations. In addition, cultures were overlaid with a top layer of either collagen (type I) or Matrigel.
Hepatocyte cultures were then examined for the induction of CYP2B
by the prototypical inducer PB. The effects of medium formulation
and matrix overlay on microsomal PROD activity in primary cultures
of rat hepatocytes treated with 100 μM PB are shown in Fig. 1A.
WEM was apparently superior to other medium formulations in terms
of the magnitude of CYP2B induction (on a per mg microsomal
protein basis). MCM and HCM were similar in their capacity to
sustain induction of CYP2B by PB, and DMEM was only slightly less
effective when hepatocytes were maintained on Permanox dishes. The
changes in PROD activity observed in cultures maintained in different
media were also reflected qualitatively by marked increases in the immunoreactive protein on Western blots (Fig. 1B).

When hepatocytes were cultured on polystyrene dishes (LUX), WEM again supported the highest degree of CYP2B induction, and MCM and HCM were only slightly less effective (Fig. 2). However, induction of CYP2B by PB was ~50% lower in cultures maintained on polystyrene dishes than in those on Permanox dishes. Moreover, hepatocytes maintained on polystyrene dishes in DMEM responded only poorly to PB treatment compared with those maintained on Permanox dishes. Regardless of the type of Petri dish used for the experiments, no obvious improvement could be discerned in the induction of CYP2B activity by cultures maintained with a Matrigel versus a collagen overlay. However, all conditions were superior to the induction of CYP2B by PB observed in cultures of hepatocytes without an overlay (data not shown; Sidhu et al., 1993; LeCluyse et al., 1996a).

Although the data suggest that WEM facilitates the highest overall induction of CYP2B by PB, other cellular functions were expressed poorly in cultures maintained in WEM compared with those maintained in MCM. For example, medium formulation affected the yield of microsomal protein per dish as shown in Fig. 3. Protein yields were often 1.5 to 2.0 times greater from cultures of hepatocytes without an overlay (data not shown; Sidhu et al., 1993; LeCluyse et al., 1996a).

Concentration-Response Relation for P-450 Induction by PB. The effects of PB concentration on the induction of PROD activity in cultures of hepatocytes maintained in either WEM or MCM are shown in Fig. 6A. A concentration-dependent increase in microsomal PROD activity was observed between 10 and 250 μM PB in cultures of hepatocytes maintained in either medium. At higher concentrations, PB caused a marked reduction in PROD activity. In contrast to CYP2B activity, CYP1A (7-ethoxyresorufin O-dealkylase) and CYP3A (testosterone 6β-hydroxylase) activities did not increase significantly over control levels at concentrations of PB below 250 μM, but increased in a concentration-dependent fashion at higher doses of PB (data not shown).

Time Course of P-450 Induction Response. The effects of treatment time on CYP2B enzyme induction by PB (100 μM) are shown
in Fig. 7. PB caused a time-dependent increase in the rate of PROD over the first 72 h of treatment. However, PROD activity did not appear to be increased further by an additional 24 h of treatment with the inducer. The increase in PROD activity observed over the first 72 h was paralleled by a corresponding increase in immunoreactive CYP2B protein (Fig. 7, top).

Induction of Other Major P-450 Subfamilies In Vitro. The culture conditions found to be both optimal and the most time-effective for assessing the induction of CYP2B enzymes, namely MCM, collagen-coated Permanox dishes, and a Matrigel overlay, were utilized to examine the induction of other P-450 enzymes. Figure 8 shows the results of three separate experiments designed to examine the induction of CYP1A, CYP2B, CYP3A, and CYP4A by β-naphthoflavone, PB, dexamethasone, and clofibric acid in primary cultures of rat hepatocytes. Induction of rat CYP1A, CYP2B, CYP3A, and CYP4A enzymes was determined in microsomes prepared from three separate groups of hepatocytes, based on measurements of 7-ethoxyresorufin O-dealkylase, PROD, testosterone 6β-hydroxylation, and lauric acid 12-hydroxylation, respectively (Pearce et al., 1996). The corresponding means ± S.E. for each group are compared with representative values obtained from liver microsomes from male rats treated with P-450 enzyme inducers in Table 1. The results showed that the culture conditions found to be optimal for CYP2B induction supported the induction of CYP1A, CYP3A, and CYP4A as well. In addition, induction of these P-450 enzymes in cultured rat hepatocytes was reproducible from one experiment to the next and, generally, within a factor of 2 to 4 of induced activities observed in vivo, with the exception of the induction of CYP1A by β-naphthoflavone (Table 1).

Discussion

Media and Overlay Effects on P-450 Enzyme Induction. The aim of this study was to examine in vitro conditions for evaluating drugs and other xenobiotics as CYP inducers in primary cultures of rat hepatocytes. Effects of media formulation and extracellular matrix overlay were assessed on CYP induction by prototypical inducers in rat hepatocyte cultures to determine the optimum response in vitro. CYP2B induction was chosen as the target response because it is one of the most liver-selective in its regulation and has historically been one of the most difficult responses to restore in primary cultures of rat hepatocytes (LeCluyse et al., 1996b). Four medium formulations, namely, DMEM, WEM, MCM, and HCM, were chosen to represent both standard formulations, which are easily obtained commercially (WEM, DMEM) and enriched formulations, which contain superphysiological concentrations of certain components, especially amino acids (MCM, HCM; LeCluyse et al., 1996b).

The results of this study showed that WEM was superior to other medium formulations in terms of the magnitude of CYP2B induction and specific content of CYP2B in microsomes. MCM and HCM were intermediate in their capacity to sustain induction of CYP2B by PB, and DMEM was slightly less effective when hepatocytes were main-
Overall induction of CYP2B by PB was, on average, 50% lower in cultures maintained on LUX dishes compared with those on Permanox dishes. In addition, hepatocytes maintained on polystyrene dishes in DMEM responded poorly to PB treatment in comparison to those maintained on Permanox dishes. The difference in CYP2B induction response between hepatocytes cultured on different types of Petri dishes is not understood at this point but does not appear to be related to differences in the viability and/or integrity of the cells based on trypan blue exclusion and lactate dehydrogenase release (results not shown).

Throughout this study, a matrix overlay was used because it has been reported to enhance P-450 enzyme induction by prototypical inducers, such as PB, and to facilitate a more normal cellular architecture (Musat et al., 1993; Sidhu et al., 1993, 1994; LeCluyse et al., 1994). Matrigel was compared with collagen (type I) as the overlay matrix because both extracellular matrices have been shown to enhance the performance of hepatocyte cultures and to prolong viability, yet no side-by-side comparisons have been reported. Matrigel is more expensive than collagen, type I (per milligram basis), and its composition is complex and variable. However, Matrigel is believed to be superior to collagen in its ability to restore the differentiated phenotype to cultured hepatocytes, presumably due to its complex nature and the apparent requirement for laminin to maintain the PB-mediated CYP2B induction response (Kleinman et al., 1982, 1986; Brown et al., 1995).

Although previous reports have shown the importance of the extracellular matrix environment for restoring the PB-mediated induction of CYP2B enzymes in vitro (Sidhu et al., 1993, 1994), our results showed that little or no difference in the induction of CYP2B by PB was observed between hepatocytes overlaid with collagen and those overlaid with Matrigel. These data suggest that the type of extracellular matrix material used for overlaying hepatocyte monolayers is not crucial, but merely the presence of an overlay is sufficient for restoring P-450 enzyme induction response. These results are in good agreement with those of Dunn et al. (1991), who showed that albumin production and secretion were not dependent on the composition of the overlay matrix. However, Brown et al. (1995) showed that purified laminin (the major component of Matrigel) alone or peptides known to mimic the activities of laminin were sufficient to restore the PB responsiveness to cultured rat hepatocytes maintained on collagen-coated dishes. Aside from the specific mechanism of action, overlaying monolayers with Matrigel holds the distinct advantage of saving the overlay matrix material used for overlaying hepatocyte monolayers is not understood at this point but does not appear to be related to differences in the viability and/or integrity of the cells based on trypan blue exclusion and lactate dehydrogenase release (results not shown).

PB Concentration Response. The importance of drug concentration is illustrated in Fig. 6, which shows the enzyme-inducing effects of a wide range of concentrations of PB in cultured rat hepatocytes. Over the range of 10 to 100 μM, PB caused a concentration-dependent induction of PROD activity. A plateau was observed between 100 and 300 μM (a range that compares favorably with plasma concentrations in rats administered PB at sedating dosages of 80–100 mg/kg, which is the dosage range typically used to achieve maximal CYP2B induction in vivo; Madan et al., 1999). At concentrations greater than 300 μM, the ability of PB to induce CYP2B activity steadily declined (as did the levels of immunoreactive protein), such that millimolar concentrations of PB were no more effective than 10 μM PB at inducing CYP2B enzymes. The apparent loss of CYP2B induction at high dosages of PB was not a consequence of cell toxicity, and, indeed, millimolar concentrations of PB were extremely effective at inducing CYP1A1, a phenomenon that is not observed in vivo (arguably because millimolar concentrations of PB cannot be achieved in vivo), but that has been reported as an in vitro phenomenon by other investigators (Sidhu et al., 1993). A practical conse-

![Fig. 6. Concentration-response relationship for induction of microsomal PROD activity (CYP2B) by PB in primary cultures of rat hepatocytes maintained in either WEM or MCM.](image_url)

Hepatocytes were maintained for 3 days in culture without treatment followed by 3 consecutive days of treatment with 10, 25, 100, 250, 750, or 1500 μM PB. Hepatocytes from each treatment group were then harvested and microsomes were prepared as outlined in Experimental Procedures.

![Fig. 7. Time course of induction of microsomal PROD activity and immunoreactive CYP2B protein by PB (100 μM) in rat hepatocytes cultured on collagen-coated Permanox dishes with a Matrigel overlay in modified MCM.](image_url)

Hepatocytes were maintained for 3 days in culture without treatment followed by treatment with 100 μM PB for 24, 48, 72, or 96 h. The data represent the mean ± S.E. from three separate experiments. *Significantly different from each other according to Dunnett’s test at the 5% level of significance (p = .05). All values were significantly different from controls.
In Vitro Prediction of P-450 Induction. The results from these studies emphasize three important features of the rat hepatocyte culture technique for measuring P-450 enzyme induction. First, overall, the rat hepatocyte technique for measuring P-450 enzyme induction in vitro is suitably reproducible. Second, the in vitro technique provides a sensitive method for detecting P-450 enzyme induction because the control enzyme activities are lower than those observed in vivo, whereas the induced enzyme activities for CYP2B, CYP3A, and CYP4A are comparable (within a factor of 2–4) to those observed in vivo (Table 1; Parkinson, 1996). By contrast, the induced values for CYP1A in vitro were only within a factor of 7 to those observed in vivo, yet the fold induction observed for CYP1A was essentially the same in vivo and in vitro. It is clear from a comparison of in vitro and ex vivo data that induction of P-450 activity in cultured rat hepatocytes does not simply involve restoring activity to the original control level, an observation that was confirmed by Western immunoblotting.

In contrast, it is possible for hepatocytes to respond to CYP1A inducers (as do most extrahepatic tissues and several immortalized cell lines) and yet not respond to PB (which fails to induce in most extrahepatic tissues and immortalized cell lines, suggesting that in-vivo induction of P-450 enzyme activities are described in Experimental Procedures.)

TABLE 1

<table>
<thead>
<tr>
<th>P-450 Enzyme</th>
<th>In Vivo Induction in Male Rat</th>
<th>In Vitro Induction in Hepatocytes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control Activity</td>
<td>Induced Activity</td>
</tr>
<tr>
<td>CYP1A</td>
<td>152 ± 27</td>
<td>3,320 ± 183</td>
</tr>
<tr>
<td>CYP2B</td>
<td>23.8 ± 4.2</td>
<td>1,460 ± 180</td>
</tr>
<tr>
<td>CYP3A</td>
<td>2,460 ± 780</td>
<td>12,693 ± 2,255</td>
</tr>
<tr>
<td>CYP4A</td>
<td>489 ± 52</td>
<td>10,693 ± 620</td>
</tr>
</tbody>
</table>

The enzyme activities of CYP1A, CYP2B, CYP3A, and CYP4A enzymes were determined as 7-ethoxyresorufin O-dealkylation, 7-pentoxyresorufin O-dealkylation, testosterone 6β-hydroxylation, and lauric acid 12-hydroxylation, respectively (Pearce et al., 1996).

Liver microsomes from male Sprague-Dawley rats treated with P-450 enzyme inducers. Rats were dosed once per day for 4 days with isotonic saline, β-naphthoflavone, phenobarbital, dexamethasone, or clofibrate at a dosage of 5, 100, 80, 50, and 200 mg/kg, respectively. Data on the activity of P-450 enzymes were provided by XenoTech, LLC (Kansas City, KS).

Liver microsomes from cultured rat hepatocytes were treated with P-450 enzyme inducers and activity of P-450 enzymes was determined as described in Experimental Procedures. Data are the means ± S.E. from three separate experiments (Fig. 8).

Activities are reported as pmol/min/mg protein and are averages of duplicate determinations from three to five experiments.

The bell-shaped curve shown in Fig. 6 is that new chemical entities should be examined for their ability to induce CYP at multiple concentrations, preferably over a range that is clinically and/or toxicologically relevant.

In Vitro Prediction of P-450 Induction. The results from these studies emphasize three important features of the rat hepatocyte culture technique for measuring P-450 enzyme induction. First, overall, the rat hepatocyte technique for measuring P-450 enzyme induction in vitro is suitably reproducible. Second, the in vitro technique provides a sensitive method for detecting P-450 enzyme induction because the control enzyme activities are lower than those observed in vivo, whereas the induced enzyme activities for CYP2B, CYP3A, and CYP4A are comparable (within a factor of 2–4) to those observed in vivo (Table 1; Parkinson, 1996). By contrast, the induced values for CYP1A in vitro were only within a factor of 7 to those observed in vivo, yet the fold induction observed for CYP1A was essentially the same in vivo and in vitro. It is clear from a comparison of in vitro and ex vivo data that induction of P-450 activity in cultured rat hepatocytes does not simply involve restoring activity to the original control level, an observation that was confirmed by Western immunoblotting.

Third, cultured rat hepatocytes responded to all four types of P-450 inducers examined. In this context, it is noteworthy that we place considerable emphasis on the response elicited by PB. Induction of CYP2B enzymes by PB is the least robust of the P-450 induction responses depicted in Fig. 8. As a rule of thumb, hepatocytes that respond well to PB will respond to the other types of P-450 inducers. In contrast, it is possible for hepatocytes to respond to CYP1A inducers (as do most extrahepatic tissues and several immortalized cell lines) and yet not respond to PB (which fails to induce in most extrahepatic tissues and immortalized cell lines, suggesting that in-

FIG. 8. Extent and reproducibility of the induction of CYP1A (A), CYP2B (B), CYP3A (C), and CYP4A (D) enzyme activities by prototypical inducers in primary cultures of rat hepatocytes.

Hepatocytes were isolated from three separate livers and cultured on collagen-coated Permanox dishes with a Matrigel overlay in MCM. Hepatocyte cultures were treated for 3 consecutive days with solvent alone (control), or with 50 µM β-naphthoflavone, 100 µM PB, 10 µM dexamethasone, or 100 µM clofibrate, as indicated in the figure. Microsomes were subsequently prepared and analyzed for CYP1A, CYP2B, CYP3A, and CYP4A enzyme activities as described in Experimental Procedures.
duction of CYP2B enzymes is highly dependent on the expression of certain liver-specific genes).

In conclusion, when rat hepatocytes are cultured under conditions that restore near normal morphology and expression of liver-specific genes, CYP enzymes can be induced in vitro to levels that are comparable to those achieved in vivo. This in vitro technique provides a sensitive method for evaluating the ability of drugs to induce microsomal P-450 enzymes and can be used as an initial screen for P-450 induction if attention is paid to the potential for false negative and false positive results. The technique would appear appropriate for screening back-up compounds or possibly screening combinatorial libraries, where the risk of false results can be arguably offset by the benefit of high throughput. Primary cultures of hepatocytes would be particularly useful for comparing the enzyme-inducing capabilities of a racemic mixture with that of its individual enantiomers, or evaluating the enzyme-inducing potential of any compound whose supply is too limited to permit an ex vivo analysis of enzyme induction. Thus, primary cultures of rat hepatocytes maintained under appropriate culture conditions should serve as a useful tool in preclinical drug development as a screen for inducers of P-450 enzyme activity.

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References


