ABSTRACT:

In the present study, we evaluated the inducibility of cytochrome P-450 (CYP) CYP1A, CYP2B, CYP3A, and CYP4A by β-naphthoflavone, phenobarbital, dexamethasone, and clofibric acid, respectively, in primary hepatocyte cultures prepared from both fresh and cryopreserved rat hepatocytes. Rat hepatocytes were successfully thawed and cultured after cryopreservation in liquid nitrogen for up to 1 month. Percentage of total recovery, viable cell recovery, and final viability of the cells were 68%, 72%, and 85%, respectively. Regardless of whether they were cryopreserved or not, cultured hepatocytes exhibited near-normal morphology. Treatment of cryopreserved hepatocytes with β-naphthoflavone caused an 8-fold increase in 7-ethoxyresorufin O-dealkylase (CYP1A1/2) activity, with an EC50 of 1.5 μM; treatment with phenobarbital caused a 26-fold increase in 7-pentoxyresorufin O-dealkylase (CYP2B1/2) activity, with an EC50 of 10 μM; treatment with dexamethasone caused a 10-fold increase in testosterone 6β-hydroxylase (CYP3A1/2) activity, with an EC50 of 1.3 μM, whereas treatment with clofibric acid caused a 3-fold increase in lauric acid 12-hydroxylase (CYP4A1-3) activity, with an EC50 of 170 μM. The induction of CYP1A, CYP2B, CYP3A, and CYP4A enzymes by these inducers was confirmed by Western immunoblotting. The patterns of P-450 induction in cryopreserved rat hepatocytes, in terms of concentration response, reproducibility, magnitude, and specificity of response, were similar to those observed in freshly isolated hepatocytes. Additionally, the magnitude and specificity of induction was similar to that observed in vivo in rats. In conclusion, under the conditions examined, cryopreserved rat hepatocytes appear to be a suitable in vitro system for evaluating xenobiotics as inducers of P-450 enzymes.

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EFFECT OF CRYOPRESERVATION ON CYTOCHROME P-450 ENZYME INDUCTION IN CULTURED RAT HEPATOCYTES

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Drugs and new molecular entities are often screened for their ability to induce cytochrome P-450 (CYP) and other phase I and II enzymes with the aim of predicting or explaining drug-drug interactions, pharmacokinetic tolerance, and/or formation of liver and thyroid tumors in rodents. Primary cultures of hepatocytes from laboratory animals and humans are reliable in vitro systems for evaluating xenobiotics as inducers of CYP enzymes. Cultured hepatocytes can potentially be used for high-throughput screening of chemical libraries to aid in the selection of drug candidates.

A major problem associated with using primary cultures of human hepatocytes for the aforementioned applications is the erratic supply of human tissue and sample-to-sample variability. Consequently, there is a need for an optimized protocol for the isolation and long-term storage of hepatocytes so that available tissue can be characterized and used more efficiently and conveniently. Cryopreservation is presently the only method for long-term storage of hepatocytes. Several groups have successfully cryopreserved hepatocytes obtained from laboratory animals and humans (Jackson et al., 1985; Chesne and Guillouzo, 1988; Loretz et al., 1989; Sun et al., 1990; de-Sousa et al., 1996; Swales et al., 1996), although their effectiveness, in terms of viable cell recovery and attachment efficiency, vary based on differences in the method used for cryopreservation. These differences include, but are not limited to, cryopreservation medium, hepatocyte density, and the rate at which hepatocytes are frozen. Hepatocytes have been successfully frozen in various media containing 20 to 90% FBS (Chesne et al., 1993) and 10 to 20% DMSO as a cryoprotectant (Loretz et al., 1989). Density of hepatocytes in the freezing media has varied from 10^6 to 10^7 cells/ml (Jackson et al., 1985; Chesne and Guillouzo, 1988; Loretz et al., 1989; Chesne et al., 1993; Swales et al., 1996) and, finally, some laboratories use a rapid rate of freezing (Gomez-Lechon et al., 1984), whereas others recommend a slow rate (Loretz et al., 1989). It has been hypothesized that a slower rate of freezing minimizes the formation of intracellular ice crystals, which may be largely responsible for diminished recovery of viable cells from preparations of cryopreserved hepatocytes (Harris et al., 1991). Additionally, rapid thawing of frozen hepatocytes at 37°C is preferred to slow thawing (Chesne and Guillouzo, 1988; Loretz et al., 1989). Depending on the cryopreservation method, viable-cell recoveries from cryopreserved hepatocytes have been reported to range from 40 to 60% (Jackson et al., 1985; Chesne and Guillouzo, 1988; Zaleski et al., 1993; Swales et al., 1996).

A number of studies have evaluated the metabolic capacity of
cryopreserved hepatocytes (Utesch et al., 1992; Salmon and Kohl, 1996; Swales et al., 1996). However, little is available on the inducibility of P-450 enzymes in cryopreserved hepatocytes (de-Sousa et al., 1996). In the present study, we evaluated the inducibility of CYP1A, CYP2B, CYP3A, and CYP4A by β-naphthoflavone, phenobarbital, dexamethasone, and clofibric acid, respectively, in primary hepatocyte cultures prepared from both fresh and cryopreserved rat hepatocytes.

Materials and Methods

Chemicals and Reagents. Insulin, Dulbecco’s modified Eagle’s medium (DMEM), GlutaMAX-1, modified Chee’s medium (MCM), modified Eagle’s medium (MEM) nonessential amino acids, and penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY). Matrigel and insulin, transferrin, and selenium were purchased from Collaborative Biomedical Products (Bedford, MA). Collagenase was purchased from Worthington Biochemical Corp. (Freehold, NJ). Vitrogen 100 was purchased from CellTix (Santa Clara, CA). Androstenedione, 1-arginine, bovine albumin, clofibric acid, dexamethasone, DMSO, fetal bovine serum (FBS), EGTA, d- (+)-glucose, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, 1-glutamine, 11β-hydroxytestosterone, lauric acid, NADP, β-naphthoflavone, Percoll, phenobarbital, testosterone, thymidine, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid protein assay reagents were purchased as a kit from Pierce Chemical Co. (Rockford, IL). NuPage gels and related electrophoresis reagents were purchased from Novex (San Diego, CA). Polyvinylidene difluoride membranes were purchased from Bio-Rad (Hercules, CA). 5-Bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). 7-Ethoxyresorufin, 7-pentoxyresorufin, and resorufin were purchased from Molecular Probes Inc. (Junction City, OR). [14C]Lauric acid (58 Ci/mol) was purchased from ICN Radiochemicals (Irvine, CA). 6β-Hydroxytestosterone and 16β-hydroxytestosterone were purchased from Steraloids, Inc. (Wilton, NH). Solvents were either purchased from Fisher Scientific (Pittsburgh, PA) or Aldrich Chemical Co. (Milwaukee, WI).

Hepatocyte Isolation and Culture. Hepatocytes were isolated from male Sprague-Dawley rats by a modification of the previously described two-step collagenase digestion method (Seglen, 1976; Seglen et al., 1980; Quistorff et al., 1989; LeClayse et al., 1996). Rat livers were first perfused with calcium-free buffer containing 5.5 mM glucose and 0.5 mM EGTA for 10 min at a flow rate of 35 to 40 ml/min and then 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 supplemented with 5% FBS, 2.5 μg/ml insulin, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 μM dexamethasone and washed by low-speed centrifugation (50g for 2–3 min at room temperature). The cell pellet was resuspended in 1:1 (v/v) mixture of supplemented DMEM and 90% isotonic Percoll and centrifuged (70g for 5 min at room temperature). The cell pellet was resuspended in fresh supplemented DMEM and washed once by low-speed centrifugation. Hepatocytes were resuspended in supplemented DMEM and viability was determined by trypan blue exclusion. Hepatocytes were cultured according to the method described by LeClayse et al. (1996). Briefly, 3 × 10^6 hepatocytes were added in 3 ml of supplemented DMEM to 60-mm Permanox plastic culture dishes (Nalge Nunc International, Naperville, IL) coated with a collagen substratum, and allowed to attach for 2 to 3 h at 37°C in a humidified chamber with a 95%/5% mixture of air/CO2. After 2 to 3 h, the culture dishes were gently swirled and medium containing unattached cells was aspirated and discarded. Fresh, ice-cold, serum-free MCM supplemented with 0.1 μM dexamethasone, ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 5.35 μg/ml linoleic acid, and 1.25 mg/ml albumin), 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.25 mg/ml Matrigel was added to each dish and cultures were returned to the humidified chamber. Medium was changed on a daily basis thereafter. Hepatocytes were maintained in culture for 3 days before treatment with P-450 inducers. On day 4, medium was aspirated and replaced with 3 ml of supplemented MCM containing 0.1% DMSO (negative controls) or one of four P-450 inducers (positive controls). Medium was changed once a day for 3 days before the preparation of microsomes.

Cryopreservation and Thawing. Hepatocytes were cryopreserved and stored for up to 1 month in liquid nitrogen according to a method based on the method of Ulrich et al. (1998). Briefly, cells were suspended in FBS (containing 10% DMSO) at a density of 1 × 10^7 cells/ml and were aliquoted (4.5 ml) into Cryotubes (NUNC, Roskilde, Denmark). These vials were placed in a microprocessor-controlled liquid nitrogen freezing chamber (model 1010, Forma, Marietta, OH) and frozen as follows. From 4°C the chamber was cooled at −1°C/min until the sample reached −4°C. The chamber was then cooled at −25°C/min until the chamber temperature reached −40°C (sample temperature of −8 to −10°C). The chamber was then warmed at 15°C/min to −12°C and cooled again at the rate of −1°C/min until the chamber reached −40°C. Finally, the chamber was cooled at −10°C/min until the sample and freezing chamber both reached −90°C at which time the frozen cell suspensions were immediately placed in liquid nitrogen.

After 5 to 30 days in liquid nitrogen, suspensions were thawed in a 37°C water bath. The contents of each tube were mixed with excess supplemented DMEM and washed by low-speed centrifugation. Cell pellets were resuspended in fresh supplemented DMEM and post-thaw viability was determined by trypan blue exclusion. Viable cells were isolated by Percoll centrifugation and cultured as described above.

Cell Harvest and Preparation of Microsomes. At the end of the culture period, hepatocyte cultures were rinsed twice with ice-cold PBS. An aliquot (0.5 ml) of ice-cold homogenizing buffer (50 mM Tris-HCl, 150 mM KCl, 2.0 mM MgCl2) was added to each 107 hepatocytes and allowed to attach for 2 to 3 h at 37°C in a humidified chamber with a 95%/5% mixture of air/CO2. After 2 to 3 h, the culture dishes were gently swirled and medium containing unattached cells was aspirated and discarded. Fresh, ice-cold, serum-free MCM supplemented with 0.1 μM dexamethasone, ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 5.35 μg/ml linoleic acid, and 1.25 mg/ml albumin), 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.25 mg/ml Matrigel was added to each dish and cultures were returned to the humidified chamber. Medium was changed on a daily basis thereafter. Hepatocytes were maintained in culture for 3 days before treatment with P-450 inducers. On day 4, medium was aspirated and replaced with 3 ml of supplemented MCM containing 0.1% DMSO (negative controls) or one of four P-450 inducers (positive controls). Medium was changed once a day for 3 days before the preparation of microsomes.

Fig. 1. Photomicrographs of primary cultures of rat hepatocytes prepared from freshly isolated (A) and cryopreserved (B) rat hepatocytes. Cryopreserved or freshly isolated rat hepatocytes were isolated and cultured for up to 6 days as described in Materials and Methods. On day 6, cultures were observed under a phase-contrast microscope and pictures were taken with a 35 mm Nikon camera. Magnification, 61.25×.
mM EDTA) was added to each dish. Cells were scraped from the dishes with a rubber policeman, collected, and homogenized by sonication with a Vibra-cell probe sonicator (Sonics & Materials, Danbury, CT) at approximately 40 W for 15 s. Cell lysates were centrifuged at 9,000 g for 20 min at 4°C. Supernatant fractions were collected and centrifuged at 100,000 g for 60 min at 4°C. The final microsomal pellets were resuspended in 250 mM sucrose (0.1–0.5 ml) with the aid of a Potter-Elvehjem tissue grinder fitted with a Teflon pestle. A 20-μl aliquot was removed to determine the protein concentration with a bicinchoninic acid protein assay kit, according to Technical Bulletin 23225X from Pierce Chemical Company (Smith et al., 1985; Wiechelman et al., 1988). Microsomal samples were subsequently stored at −20°C.

**Enzyme Assays.** The O-dealkylation of 7-ethoxyresorufin and 7-pentoxyresorufin, the 6β- and 16β-hydroxylation of testosterone, and the 12-hydroxylation of lauric acid were determined by methods described previously (Burke and Mayer, 1974; Wood et al., 1983; Sonderfan et al., 1987; Sonderfan and Parkinson, 1988; Romano et al., 1988; Giera and Van Lier, 1991; Burke et al., 1994; Pearce et al., 1996).

**Western Immunoblotting.** Microsomal samples were analyzed by Western immunoblotting to determine levels of immunoreactive CYP1A, -2B, -3A, and -4A. Microsomes were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, based on the method originally described by Laemmli (1970). Briefly, microsomes were mixed in a 1:1 ratio with NuPage sample dilution buffer (pH = 8.5) containing 580 mM sucrose, 280 mM Tris-Base, 211 mM Tris-HCl, 2% sodium dodecylsulfate, 1.0 mM EDTA, 50 mM dithiothreitol, and 440 μM Serva Blue G250, and heated at 100°C for 2 to 5 min. The denatured proteins were subjected to electrophoresis on precast 4 to 12% NuPage bis-Tris gels (pH = 5.6 gels; constant voltage of 200 V; electrophoresis time ~55 min) (Novex, San Diego, CA). Proteins were transferred electrophoretically to polyvinylidene difluoride membranes and subjected to immunoblotting, based on the method by Towbin et al. (1979), with a Blot Module from Novex. Membranes were incubated in blocking buffer containing 10% (w/v) Carnation nonfat dry milk and 0.05% (v/v) Tween 20 in Tris buffered saline (10 mM Tris-HCl and 150 mM NaCl, pH = 7.4) and then probed with polyclonal antibodies raised against purified rat liver microsomal CYP1A1, CYP2B1, CYP3A1 (Parkinson and Gemzik, 1991), or CYP4A1/2 (a gift from Dr. James Hardwick, Northeastern Ohio University, Roostown, OH) at final concentrations ranging from 0.5 to 5 μg/ml. The secondary antibody was affinity purified goat-anti-rabbit IgG (H + L) conjugated with alkaline phosphatase from Kirkegaard & Perry and was diluted in blocking buffer to a final concentration of 0.3 μg/ml. Membranes were washed three times with Tris buffered saline and the proteins visualized by incubation with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphate substrate.

**Fig. 2.** Effect of cryopreserving rat hepatocytes on the induction of 7-ethoxyresorufin O-dealkylase (CYP1A) activity by β-naphthoflavone.

Cryopreserved or freshly isolated rat hepatocytes were isolated and cultured for up to 6 days as described in Materials and Methods. A, cultures were treated daily for 3 consecutive days with either vehicle (0.1% DMSO) or various concentrations of β-naphthoflavone (0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, and 50 μM). B, C, and D, cultures were treated daily for 3 consecutive days with β-naphthoflavone (50 μM), phenobarbital (100 μM), dexamethasone (10 μM), or clofibric acid (100 μM). At the end of the treatment period, hepatocytes were harvested and microsomes were prepared and analyzed for CYP1A activity as described in Materials and Methods. Where error bars are shown, data are mean ± S.D. of three preparations. *p < .05 as determined by ANOVA followed by a Dunnett’s post hoc test.
Results

Evaluation of Cryopreserved Hepatocytes. Percentage of total recovery and percentage of viable recovery were estimated in several preparations of cryopreserved hepatocytes. Percentage of total recovery, defined as the total number of cells (dead plus viable) recovered post-thaw compared with the total number of cells frozen, was approximately 68%. Percentage of viable recovery, defined as the number of viable cells recovered post-thaw compared with the number of viable cells frozen, was approximately 72%. Viability of the final preparation of hepatocytes (after Percoll gradient centrifugation) was greater than 85% for each preparation of hepatocytes. Cryopreserved hepatocytes appeared to attach to collagen-coated culture dishes with a slightly lower efficiency than freshly isolated hepatocytes. After 6 days in culture, representative sections of culture dishes seeded with cryopreserved or not, hepatocytes exhibited near-normal morphology; the cells were cuboidal and contained granular cytoplasm with one or two centrally located nuclei (Fig. 1). In all cases, the cultured hepatocytes were free of detectable autophagic and lipid vesicles. Even though the collagen substrate and Matrigel overlay caused cells to spread and flatten to a certain degree, the hepatocytes remained in chords or trabeculae.

Induction of 7-Ethoxyresorufin O-dealkylase (EROD) (CYP1A1/2) Activity. The effects of treating freshly isolated or cryopreserved rat hepatocyte cultures with the CYP1A enzyme inducer β-naphthoflavone were reproducible between three preparations of cryopreserved rat hepatocytes (Fig. 2B). The magnitude of CYP1A induction in freshly isolated and cryopreserved hepatocytes, on a picomole per minute per milligram basis, was comparable with the response observed in vivo (Table 1).

Induction of 7-pentoxyresorufin O-dealkylation (PROD) (CYP2B1/2) Activity. The effects of treating freshly isolated or cryopreserved rat hepatocyte cultures with the CYP2B enzyme inducer phenobarbital are shown in Fig. 4, A and B. The induction of PROD (CYP2B1/2) activity by phenobarbital was concentration dependent, with an estimated EC_{50} of ~10 μM (Fig. 4A). The concentration-response curves for induction of CYP2B1/2 by phenobarbital were similar for the cultures prepared from freshly isolated or cryopreserved cells. Furthermore, both the absolute “rates” (expressed as picomole per minute per milligram microsomal protein) and fold induction of CYP1A1/2 by β-naphthoflavone were reproducible between three preparations of cryopreserved rat hepatocytes (Fig. 2B). The magnitude of CYP1A induction in freshly isolated and cryopreserved hepatocytes, on a picomole per minute per milligram basis, was comparable with the response observed in vivo (Table 1).

The effects of treating cultured rat hepatocytes with β-naphthoflavone, phenobarbital, dexamethasone, or clofibric acid, prototypical inducers of CYP1A, CYP2B, CYP3A, and CYP4A enzymes, respectively, on EROD activity are shown in Fig. 2, C and D. In cryopreserved hepatocytes β-naphthoflavone (50 μM) caused an 8-fold increase in EROD activity, whereas phenobarbital (100 μM) caused a 3-fold increase. Similar results were obtained with freshly isolated hepatocytes (Fig. 2D). Western immunoblotting established that treatment of cryopreserved hepatocytes with β-naphthoflavone, but not phenobarbital, dexamethasone, or clofibric acid, caused a marked increase in immunoreactive CYP1A1 and CYP1A2 (Fig. 3A).

### Table 1

<table>
<thead>
<tr>
<th>P-450 Enzyme</th>
<th>In Vivo Induction in Male Rats</th>
<th>In Vitro Induction</th>
<th>Comparison of In Vivo with In Vitro</th>
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<tr>
<td></td>
<td>Enzyme activity (pmol/min/mg protein)</td>
<td>Ratio of enzyme activity</td>
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<tr>
<td>Clofibric acid</td>
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<td>3,750</td>
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</table>

*Enzyme activity of CYP1A, CYP2B, CYP3A, and CYP4A enzymes was determined as EROD, PROD, testosterone 6β-hydroxylation, and lauric acid 12-hydroxylation respectively, as described in Materials and Methods.

1Liver 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 clofibric acid at a dosage of 5 mg/kg, 100 mg/kg, 50 mg/kg, and 200 mg/kg, respectively. Microsomes were provided by Xenotech, LLC, which also provided data on activity of P-450 enzymes. Data shown are averages of duplicate determinations from three to five experiments.

2Liver microsomes from cryopreserved rat hepatocytes (prepared from freshly isolated rat hepatocytes) were treated with P-450 enzyme inducers and activity of P-450 enzymes was determined as described in Materials and Methods. Data shown are averages of duplicate determination from a single experiment (Fig. 2A, 4A, 5A, and 6A). Data for induced samples are maximum response observed in vivo (Table 1).

3Liver microsomes from cultured rat hepatocytes (prepared from freshly isolated or cryopreserved cells) were treated with P-450 enzyme inducers and activity of P-450 enzymes was determined as described in Materials and Methods. Data shown are averages of duplicate determination from a single experiment (Fig. 2A, 4A, 5A, and 6A). Data for induced samples are maximum response observed in vivo (Table 1).

4Values in parentheses show fold induction compared with controls.
tively, on PROD (CYP2B) activity are shown in Fig. 4, C and D. In cryopreserved hepatocytes, phenobarbital (100 μM) caused a 26-fold induction of PROD activity, whereas the other inducers had little or no effect. Similar results were obtained with freshly isolated hepatocytes (Fig. 4D). Western immunoblotting established that treatment of cryopreserved hepatocytes with phenobarbital, but not β-naphthoflavone, dexamethasone, or clofibric acid, caused a marked increase in immunoreactive CYP2B1 and CYP2B2 (Fig. 3B).

Induction of CYP2B1/2 in cultured hepatocytes was measured by testosterone 16β-hydroxylase activity. The pattern of induction of testosterone 16β-hydroxylase activity (data not shown) was similar to that shown in Fig. 4D for PROD activity.

Induction of Testosterone 6β-Hydroxylase (CYP3A1/2) Activity. The effects of treating freshly isolated or cryopreserved rat hepatocyte cultures with the known CYP3A1/2 enzyme inducer dexamethasone are shown in Fig. 5, A and B. The induction of testoste

2 The major dexamethasone-inducible P-450 enzyme in rats was originally named cytochrome P-450p or P-450PCN, and was subsequently renamed CYP3A1 when the cDNA thought to encode this enzyme was isolated and sequenced (Gonzalez et al., 1985). However, it now appears that cytochrome P-450p is encoded by CYP3A23, and that CYP3A1 may be an allelic variant of this gene (Gonzalez et al., 1986; Ribeiro and Lechner, 1992; Kirata and Matsubara, 1993; Komori and Oda, 1994; Nagata et al., 1996; Mahnke et al., 1997; Wang and Strobel, 1997). For simplicity, the old nomenclature has been retained and the 6β-hydroxylase (CYP3A3) activity by dexamethasone was concentration dependent, with an estimated EC50 of ~1.3 μM (Fig. 5A). The concentration-response curves for induction of CYP3A1/2 by dexamethasone were similar for the cultures prepared from freshly isolated or cryopreserved cells. Furthermore, both the absolute "rates" (expressed as picomole per minute per milligram microsomal protein) and fold induction of CYP3A by dexamethasone were comparable between three preparations of cryopreserved rat hepatocytes (Fig. 5B). The magnitude of CYP3A induction in freshly isolated and cryopreserved hepatocytes, on a picomole per minute per milligram, was comparable with the response observed in vivo (Table 1).

The effects of treating cultured rat hepatocytes with β-naphthoflavone, phenobarbital, dexamethasone, or clofibric acid, prototypical inducers of CYP1A, CYP2B, CYP3A, and CYP4A enzymes, respectively, on testosterone 6β-hydroxylase activity are shown in Fig. 5, C and D. In cryopreserved hepatocytes, dexamethasone (10 μM) caused a 10-fold induction of testosterone 6β-hydroxylase activity, whereas phenobarbital (100 μM) caused a 2-fold increase (Fig. 5C). Similar results were obtained with freshly isolated hepatocytes (Fig. 5D). Western immunoblotting established that treatment of cryopreserved hepatocytes with dexamethasone and phenobarbital, but not β-naph-
thoflavone or clofibric acid, caused a corresponding increase in immunoreactive CYP3A1 and CYP3A2 (Fig. 3C).

**Induction of Lauric Acid 12-Hydroxylase (CYP4A) Activity.** The effects of treating freshly isolated or cryopreserved rat hepatocyte cultures with the CYP4A enzyme inducer, clofibric acid, are shown in Fig. 6, A and B. The induction of lauric acid 12-hydroxylase (CYP4A1-3) by clofibric acid was concentration dependent, with an EC50 of \(170 \mu M\) (Fig. 6A). The concentration-response curves for induction of CYP4A by clofibric acid were similar for cultures prepared from freshly isolated or cryopreserved cells. Furthermore, both the absolute “rates” (expressed as picomole per minute per milligram microsomal protein) and fold induction of CYP4A by clofibric acid were reproducible between three preparations of cryopreserved rat hepatocytes (Fig. 6B). The magnitude of CYP4A induction in freshly isolated and cryopreserved hepatocytes, on a picomole per minute, was comparable with the response observed in vivo (Table 1).

The effects of treating cultured rat hepatocytes with \(\beta\)-naphthoflavone, phenobarbital, dexamethasone, or clofibric acid, prototypical inducers of CYP1A, CYP2B, CYP3A and CYP4A enzymes, respectively, on lauric acid 12-hydroxylase activity are shown in Fig. 6, C and D. In cryopreserved hepatocytes, clofibric acid (100 \(\mu M\)) caused a 3-fold induction of lauric acid 12-hydroxylase activity, whereas the other inducers had little or no effect (Fig. 6C). It should be noted that these experiments were carried out with low concentrations of clofibric acid that were not sufficient to cause maximal induction of CYP4A (Fig. 6A). Similar results were obtained with freshly isolated hepatocytes (Fig. 6D). Western immunoblotting established that treatment of cryopreserved hepatocytes with clofibric acid, but not \(\beta\)-naphthoflavone, phenobarbital, or dexamethasone, caused a marked increase in immunoreactive CYP4A proteins (Fig. 3D).

**Discussion**

Several studies have shown that cryopreserved rat hepatocytes retain adequate viability (Chesne and Guillouzo, 1988; Chesne et al., 1993; de-Sousa et al., 1996; Swales et al., 1996), metabolic compe-
tency (Jackson et al., 1985; Utesch et al., 1992; Salmon and Kohl, 1996; Swales et al., 1996), and ability to attach to culture dishes coated with extracellular matrix such as collagen (Utesch et al., 1992; de-Sousa et al., 1996; Salmon and Kohl, 1996; Swales et al., 1996). However, little information is available regarding the inducibility of P-450 enzymes in cryopreserved hepatocytes. Several factors must be considered when evaluating hepatocytes as an in vitro system for induction studies. These include percentage of recovery of hepatocytes, attachment efficiency to cell culture dishes, morphology of cultured hepatocytes, cell density (confluency), and responsiveness to various P-450 enzyme inducers. These factors have been studied previously with both fresh and cryopreserved hepatocytes (Jackson et al., 1985; Powis et al., 1987; Chesne and Guillouzo, 1988; Loretz et al., 1989; Chesne et al., 1993; de-Sousa et al., 1996), with the exception that cryopreserved hepatocytes have not been evaluated for their responsiveness to inducers of all families of P-450 enzymes. In the present study, we demonstrate that cryopreserved rat hepatocytes, much like freshly isolated rat hepatocytes, respond to P-450 enzyme inducers when cultured under conditions that restore near-normal cellular morphology. The magnitude of P-450 enzyme induction response in cryopreserved hepatocytes was generally reproducible and was comparable with the response observed in vivo (Table 1).

Data presented in this article show that rat hepatocytes were successfully thawed and cultured after cryopreservation in liquid nitrogen for up to 1 month. Other studies have suggested that cryopreserved hepatocytes, when frozen appropriately, are stable for up to 4 years in liquid nitrogen (Chesne et al., 1993). We have thus far determined that they are stable in liquid nitrogen for up to 3 months (data not shown). Studies are underway to evaluate the effects of long-term storage of cryopreserved hepatocytes. In the current study, the recovery of viable cells was ~72% (ranging from 53% to 96%) suggesting that, on average, 28% of the viable hepatocytes were lost as a result of freezing, storage, and thawing. Furthermore, attachment efficiency of cryopreserved hepatocytes in culture appeared to be slightly lower than that observed with freshly isolated hepatocytes (data not shown). Both a reduced viable cell recovery and attachment efficiency have been reported previously (Chesne and Guillouzo, 1988; Chesne et al., 1993; Swales et al., 1996). Despite these minor concerns, cryopreserved rat hepatocytes can be cultured and maintained as reliably as freshly isolated hepatocytes.

**Fig. 5. Effect of cryopreserving rat hepatocytes on induction of testosterone 6β-hydroxylase (CYP3A) activity by dexamethasone.**

Cryopreserved or freshly isolated rat hepatocytes were isolated and cultured for up to 6 days as described in Materials and Methods. A, cultures were treated daily for 3 consecutive days with either vehicle (0.1% DMSO) or various concentrations of dexamethasone (0.5, 1.0, 5.0, and 50 μM). B, C, and D, cultures were treated daily for 3 consecutive days with β-naphthoflavone (50 μM), phenobarbital (100 μM), dexamethasone (10 μM), or clofibric acid (100 μM). At the end of treatment period, hepatocytes were harvested and microsomes were prepared and analyzed for CYP3A activity as described in Materials and Methods. Where error bars are shown, data are mean ± S.D. of three preparations. *p < .05 as determined by ANOVA followed by a Dunnett’s post hoc test.
Regardless of whether the hepatocytes were cryopreserved or not, the constitutive expression of cytochrome P-450 was markedly reduced in culture (Table 1). This is consistent with previous studies in which a decrease in both cytochrome P-450 mRNA (Kocarek et al., 1993) and protein (Woodcroft and Novak, 1998) has been reported within 1 to 2 days of culture. This decrease in P-450 levels is thought to result from the loss of those factors that maintain the constitutive expression of P-450 enzymes in vivo. These factors are poorly understood, but are known to include hormones and environmental factors such as diet and, in the case of humans, medications, smoking, and alcohol consumption. This does not present a problem when cultured hepatocytes are used as an in vitro system for the evaluation of drugs and chemicals as inducers of P-450 enzymes. However, it is for this reason that freshly isolated hepatocytes but not cultured hepatocytes are suitable for in vitro metabolism studies. Although several studies have evaluated the metabolic capacity of hepatocytes (Utesch et al., 1992; Salmon and Kohl, 1996; Swales et al., 1996), it remains to be seen whether hepatocytes cryopreserved according to the method described herein can be used for short-term metabolism studies.

Hepatocyte cultures, irrespective of whether they were seeded with cryopreserved or freshly isolated hepatocytes, were responsive to increasing concentrations of prototypical P-450 inducers. In most cases, a traditional log concentration-response curve was observed (Figs. 2A, 5A, and 6A). A notable exception was the effect of phenobarbital on the expression of CYP2B1/2, which was best characterized by a bell-shaped concentration-response curve (Fig. 4A). Over a concentration range of 10 to 100 μM, phenobarbital caused a concentration-dependent induction of CYP2B activity, as measured by 7-pentoxyresorufin O-dealkylation and testosterone 16β-hydroxylation (data not shown). A plateau was observed at ~100 μM; a concentration that compares favorably with the plasma concentration in rats administered phenobarbital at sedating dosages of 80 to 100 mg/kg, the dosage range that is typically used to achieve maximal CYP2B induction in vivo. Above 300 μM, the ability of phenobarbital to induce CYP2B activity steadily declined, such that millimolar concentrations of phenobarbital were no more effective than 10 μM phenobarbital at inducing CYP2B enzymes. Attenuation of CYP2B induction at high doses of phenobarbital is not a consequence of cell toxicity and, indeed, millimolar concentrations of phenobarbital are
extremely effective at inducing CYP1A1 (data not shown). This phenomenon is not observed in vivo (arguably because millimolar concentrations of phenobarbital cannot be achieved in vivo), but has been reported as an in vitro phenomenon by other investigators (Sidhu et al., 1993). This is substantiated by data shown in Fig. 2. C and D in which a 3-fold induction of EROD by phenobarbital was observed.

A practical consequence of the bell-shaped curve shown in Fig. 3A is that artifacts can be observed when high concentrations of a drug or new molecular entity are examined.

The magnitude and specificity of the response of cryopreserved rat hepatocytes to phenotypically P-450 enzyme inducers were reproducible from one experimental rat to another (Figs. 2B, 4B, 5B, and 6B) and were similar to those observed in cultures prepared from freshly isolated hepatocytes. This suggests that the process of cryopreservation does not affect the specific drug-cell interactions required for induction of these P-450s in rat hepatocytes.

The patterns of P-450 induction in cryopreserved rat hepatocytes, in terms of concentration response, reproducibility, magnitude of response, and specificity, were similar to those observed in freshly isolated hepatocytes. Furthermore, the magnitude and specificity of induction was similar to that observed in rats in vivo. In conclusion, under the conditions examined, cryopreserved rat hepatocytes appeared to be a suitable in vitro system for evaluating xenobiotics as inducers of P-450 enzymes.

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