Biochemical Pharmacology, Vol. 38, No. 14, pp. 2247–2251, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

DEPENDENCE OF THE INDUCTION OF CYTOCHROME P-450 BY PHENOBARBITAL IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES ON THE COMPOSITION OF THE CULTURE MEDIUM

NANCY A. TURNER* and HENRY C. PITOT†
McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706, U.S.A.

(*Received* 5 *July* 1988; *accepted* 22 *November* 1988)

Abstract—A chemically defined medium developed for the maintenance of differentiated adult rat hepatocytes (T1) was compared with two commercially available media (Waymouth 752/1 and Leibovitz L-15) for maintenance of cytochrome P-450 metabolic activity in cultured hepatocytes. Specific metabolic activities of initially isolated cells and 72-hr control and phenobarbital-treated cultures were determined with 7-ethoxycoumarin, 7-ethoxyresorufin, and 7-pentoxyresorufin as substrates. Control and phenobarbital-treated cultures in T1 medium had a higher metabolic activity towards each of the three substrates than comparable cultures in the other media. These studies indicated that the metabolic activity and the response to phenobarbital of the major isozyme of the phenobarbital-inducible family of cytochrome P-450 were maintained in hepatocytes in T1 medium. However, there was anomalous expression and induction by phenobarbital of the major 3-methylcholanthrene-inducible isozyme, cytochrome P-450c, in cultured hepatocytes in each of the three media tested, but this response was more pronounced in T1 medium. In conclusion, the regulation of cytochrome P-450 metabolic activity in cultured hepatocytes was shown to be dependent on the composition of the culture medium.

Primary adult hepatocytes in culture rapidly lose the xenobiotic metabolizing capability of many isozymes of the cytochrome P-450 family [1–3]. Maintenance of cytochrome P-450 protein and of metabolic activities at levels initially present in isolated adult rat hepatocytes has been shown previously to be dependent both on specific factors that apparently stabilize the proteins [4] and on components within the culture medium itself [5]. On the other hand, inducers of isozymes of the cytochrome P-450 family can increase the level of activity in adult hepatocytes in culture [6, 7].

The metabolic activity of cytochrome P-450 towards several xenobiotic substrates can be determined in cultured hepatocytes. Substrates that are selectively metabolized by only one isozyme of cytochrome P-450 can be used to elucidate which of the forms contribute to the metabolism of cultured cells. Several metabolic assays are sufficiently sensitive to detect the reduced level of metabolic activity in cultured hepatocytes. 7-Ethoxycoumarin (EC)‡, a commonly used substrate both *in vivo* and *in vitro*, is deethylated by several isozymes of cytochrome P-450 [8]. Edwards *et al.* [9] have shown that 7-ethoxycoumarin-*O*-deethylase (ECOD) activity declines steadily over 96 hr in culture, but that this decline can be modulated by additions to the culture medium. Burke *et al.* [10] have shown that the alkyl derivatives of phenoxazone, 7-ethoxyresorufin (ER) and 7-pentoxyresorufin (PR), are selectively dealkylated by specific cytochrome P-450 isozymes. These studies have shown that 7-ethoxyresorufin-*O*-deethylase (EROD) activity is specific to the major MC-inducible isozyme, cytochrome P-450c, and that 7-pentoxyresorufin-*O*-dealkylase (PROD) activity is specific to the major PB-inducible isozyme, cytochrome P-450b.

In the studies reported here, we have compared the metabolic activities of the three substrates indicated above (EC, ER, and PR) as a reflection of the levels of several isozymes of cytochrome P-450, specifically P-450c and P-450b for the latter two substrates in control and PB-treated cultures of adult hepatocytes. These comparisons were made in three different media, T1 (developed in our laboratory [11]), and two commercially available media.

METHODS

Adult male Sprague–Dawley rats (150 g) were obtained from Harlan Sprague–Dawley, Inc. (Madison, WI). Unless otherwise noted, all chemicals were obtained from the Sigma Chemical Co. (St Louis, MO) and, where appropriate, were of tissue culture grade. Leibovitz L-15 and Waymouth 752/1 media were obtained from KC Biological (Lenexa, KS); Nu-Serum was purchased from Collaborative

* Present address: Smith Kline & French Laboratories, Department of Cell Biology L109, 709 Swedeland Road, Swedeland, PA 19479.

† Correspondence: Dr Henry C. Pitot, McArdle Laboratory for Cancer Research, 450 North Randall Ave., Madison, WI 53706, U.S.A.

‡ Abbreviations: DMSO, dimethyl sulfoxide; EC, 7-ethoxycoumarin; ECOD, 7-ethoxycoumarin-*O*-deethylase; ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin-*O*-deethylase; L-15, Leibovitz L-15 medium; MC, 3-methylcholanthrene; PB, sodium phenobarbital; PR, 7-pentoxyresorufin; PROD, 7-pentoxyresorufin-*O*-dealkylase; cytochrome P-450b, major PB-inducible cytochrome P-450; cytochrome P-450c, major MC-inducible cytochrome P-450; and Way, Waymouth 752/1 medium.

Research. Inc. (Waltham, MA); and sodium phenobarbital from Mallinckrodt (St Louis, MO). 7-Ethoxy- and 7-pentoxyresorufin were obtained from Molecular Probes Inc. (Junction City, OR); and resorufin, 7-hydroxycoumarin, and 7-ethoxycoumarin from the Aldrich Chemical Co. (Milwaukee, WI).

Culture of hepatocytes. Three media were chosen for comparison in this study. These were T1, a chemically defined medium developed in this laboratory for the maintenance of differentiated rat hepatocytes [11], a modification of Waymouth 752/1 (Way), and Leibovitz L-15+5% NuSerum (L-15). Hepatocytes were isolated and cultured on collagen-coated dishes in the three media as described previously [11]. Six hours after plating, PB was added in 100 μl of sterile H_2O to a final concentration of 2 mM. Subsequent media changes and addition of PB were performed daily. Microsomes were prepared in SET buffer (250 mM sucrose, 5.3 mM EDTA, 20 mM Tris, pH 7.4) from cultured hepatocytes [11].

Assay procedures. Cytochrome P-450 was measured by the method of Omura and Sato [12]. The three metabolic assays were performed on an Aminco Bowman spectrophotofluorimeter at room temperature with 20–500 mg of microsomal protein in a final volume of 1 ml. ECOD was assayed according to a modification of the method of Ullrich and Weber [13], and EROD and PROD by a modification of the procedure of Burke and Mayer [14]. The ECOD reaction was run in 66 mM Tris buffer (pH 7.4) and contained 500 μM EC (5 μl of 100 mM stock in DMSO) and 200 μM NADPH to start the reaction. The spectrophotofluorimeter was set at an excitation wavelength of 380 nm, an emission wavelength of 460 nm, and a photomultiplier slit of 1 mm. During the linear portion of the reaction, 7-hydroxycoumarin was added as an internal standard at a concentration range of 0.1 to 0.5 μM (1–5 μl of 100 μM in EtOH). EROD and PROD were assayed in 0.1 M sodium/potassium phosphate buffer containing 60 μM EDTA and 5 mM $MgSO_4$ (pH 7.6) with 1 mM NADPH. The excitation wavelength was set at 530 nm, emission at 585 nm, and the slit at 0.5 mm. The concentration of ER was 5 μM (5 μl of 1 mM ER in 1:10 DMSO to EtOH), and the resorufin standard was added at concentrations of 10–50 nM in EtOH. Protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as standard.

RESULTS

The total amount of heme-bound cytochrome P-450, as determined by the reduced CO-binding spectrum of the heme, declined in 72-hr cultures in each of the three media to approximately 15–30% of the level in initially isolated hepatocytes (Fig. 1). There was no significant difference between the levels obtained in each of the culture media. Two time–course experiments over the first 72 hr comparing T1 and L-15 showed the rate of decline to be similar in both media (data not shown). The amount of cytochrome P-450 and the metabolism of each substrate in initially isolated cells from different animals

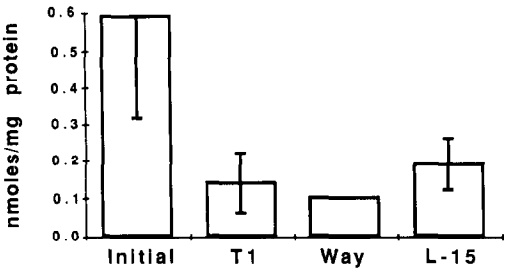

Fig. 1. Total cytochrome P-450 in initially isolated cells and control cultures after 72 hr. Hepatocytes were isolated and cultured as described in Methods. The amount of total heme-bound cytochrome P-450 was determined based on its reduced CO-binding spectrum in initially isolated cells and 72-hr control cultures in each of the three media. The data are expressed as nanomoles per milligram of microsomal protein, the mean ± SD from N = seven determinations for initially isolated cells, six for T1, and five for L-15; the value for Way was an average of two.

varied considerably; however, the levels in the cultured cells were consistent with the initial levels and between experimental groups.

In contrast to the amount of heme-bound cytochrome P-450, the ability to metabolize specific substrates varied considerably in hepatocytes cultured in the different media. ECOD activity, a general measure of cytochrome P-450 activity, was easily detected in initially isolated cells (Table 1). After 72 hr in culture, the level of ECOD activity in Way or L-15 medium had declined to a mean value of 10 and 14%, respectively, of the level in initially isolated cells. In T1, however, the mean level had declined to only 39%. Two time–course experiments over the first 72 hr of culture showed the decline in levels to be gradual, with the level in T1 exceeding the level in equivalent cultures in L-15 or Way (data not shown). The addition of PB to the culture medium increased the level of ECOD activity in hepatocytes in each of the three media (Table 1). The addition of PB to the culture media could have been acting to maintain or stabilize the cytochrome P-450 proteins, but in T1 there was an increase in activity above the level in initially isolated cells.

EROD activity specific to cytochrome P-450c was detected at low levels in initially isolated cells (Table 1). The activity declined in 72-hr cultures of Way and L-15 to 20 and 17%, respectively, of the initial level. In T1, however, the level of EROD activity increased 2-fold over the level in initially isolated cells. Preliminary time–course experiments over the first 72 hr of culture showed a gradual decline in activity in Way and L-15, but in T1, after an initial decline in the first 24 hr, the levels gradually increased (data not shown). The addition of PB to the culture medium increased the EROD activity in each of the three media, but because of the high basal level in T1, the level in T1 + PB was significantly higher than in the other media (Table 1). In L-15 and Way, PB treatment increased the EROD activity above the level in initially isolated cells in 2 of 2 and 4 of 5 experiments, respectively. This increase above the initial levels may indicate that induction

Table 1. Metabolic activity of cultured hepatocytes

Sample*	Activity (pmol/min/mg microsomal protein) ECOD	EROD	PROD
Initial	445 ± 50 (14)	12.0 ± 3.2 (15)	2.0 ± 0.4 (5)
T1	175 ± 25† (14)	26.4 ± 5.0† (14)	0.6 ± 0.3 (4)
T1 + PB	580 ± 174‡ (7)	222.0 ± 71‡ (8)	4.1 ± 0.9§ (5)
L-15	62 ± 16 (10)	2.0 ± 1.0 (10)	0 (3)
L-15 + PB	148 ± 81 (4)	12.8 ± 4.6 (4)	0 (3)
Way	44 ± 20 (6)	2.4 ± 0.9 (6)	0 (4)
Way + PB	115 ± 62 (4)	41.7 ± 14.9 (5)	3.9 ± 1.7 (5)

* The assays were performed, as described in Methods, on microsomal preparations from initially isolated cells and 72-hr control and PB-treated cultures in each of the three media. The data are means ± SE. The numbers in parentheses are the numbers of separate microsomal preparations tested.

†–§ A one-tailed sign test indicated the following: †Activity greater than in L-15 or Way ($P < 0.02$); ‡Activity greater than in T1 alone ($P < 0.01$); §Activity greater than initial ($P < 0.03$).

of activity may be occurring as well as maintenance or stabilization of activity by PB.

Table 1 also shows the levels of PROD activity in hepatocytes cultured in each of the three media. Only in control cultures in T1 medium was the expression of PROD activity maintained, albeit at a reduced level. The addition of PB to T1 medium increased the PROD activity above the levels in initially isolated cells. In L-15 medium PB treatment did not result in detectable levels of activity. In three of five experiments, the addition of PB to Way medium resulted in an increased activity above the initial level.

The data from the three assays described above were consistent with previously reported data on the immuno-quantitation of these proteins in cultured hepatocytes [11], e.g. protein levels correlated with metabolic activity towards the appropriate substrate. Consistent with the immuno-quantitation data, the metabolic activity varied between experiments, but the relative levels in each of the three media compared with the initially isolated cells were similar in all experiments. Thus, the levels of metabolic activity in T1 medium were always higher than in L-15 or Way medium. To determine whether T1 medium allowed for significantly improved maintenance of cytochrome P-450-linked metabolic activity of cultured hepatocytes, a one-tailed sign test [16] was used (Table 1). The results of this test demonstrated that this metabolic activity of hepatocytes was indeed significantly higher in T1 medium and was further increased by PB.

DISCUSSION

In this study we compared T1 medium with two commercially available media—L-15, which is supplemented with serum, and Way, which is chemically defined—for the maintenance of cytochrome P-450 metabolic activity in cultured rat hepatocytes. In hepatocytes in T1 medium there was always a higher metabolic activity towards the three substrates than in control cultures in the other media, and a greater response to PB (Table 1). These results correlated well with an earlier study in which we used Western blotting to quantitate specific cytochrome P-450 isozymes [11]. We showed that the PB-inducible isozymes could respond to PB in cultures in T1 and to a lesser extent in Way. We also showed that there was an anomalous increase in the MC-inducible form, cytochrome P-450c, in cultured hepatocytes, particularly in T1 medium, and that PB further increased cytochrome P-450c in all three media.

The total heme-bound cytochrome P-450 declined in each of the three media to similar levels (Fig. 1). The determination of total cytochrome P-450 based on the reduced binding spectrum of the CO-bound heme is not a sensitive assay and does not necessarily measure metabolically active enzyme. Lake and Paine [17] have demonstrated that hepatocytes in a "P-450 medium" maintain the total heme-bound cytochrome P-450 at much higher levels than their control medium. However, hepatocytes in this medium have decreased metabolic activity towards several substrates (including EC and ER) compared with the control medium.

We compared the metabolic activity of hepatocytes cultured in each of the three media towards three substrates that are commonly used as a measure of the xenobiotic metabolic activity of hepatocytes. ECOD activity (metabolically linked to several cytochrome P-450s) declined in all three media in 72-hr cultures, but was always higher in T1 medium than in equivalent cultures in Way or L-15. Our results concurred with the studies of Edwards *et al.* [9], which demonstrated that ECOD activity declines in a modification of Way medium after 72 hr in culture to levels similar to what we detected, and with the results of Lake and Paine [17], which also demonstrated a decline in activity in Williams' E medium to 22% of the initial level after 72 hr in culture. We also showed that PB increased the ECOD activity in each of the three media and that this increase in T1 medium was 2-fold higher than the levels in initially isolated cells.

The level of EROD activity (metabolically linked to cytochrome P-450c) decreased in cultures in Way and L-15, but increased over the level in initially

isolated hepatocytes in T1 medium. These results also concur with the study of Lake and Paine [17]. In their culture system, after an initial drop in the first 24 hr, the level of EROD activity increased to levels above the initially isolated cells. Dickins and Peterson [4] used a modification of Waymouth MB 752/1 designated as WOBA-M_2 and compared it with the medium supplemented with the hormonal mixture of Decad *et al.* [18], designated AB medium, for maintenance of metabolic activity. In the WOBA-M_2 medium, ECOD and EROD activities declined to levels similar to our data for Way medium. In the hormonally supplemented AB medium, however, there was maintenance of ECOD activity similar to T1 and increased EROD activity abvoe the levels of initially isolated cells.

In contrast to the situation *in vivo*, PB treatment increased the EROD activity from hepatocytes in each of the three media. The increase in cytochrome P-450c-associated activity in culture and an anomalous inductive response to PB treatment have been shown by other researchers under various culture conditions. In a different culture system the level of ECOD activity declined in the first 24 hr, then gradually increased in culture [8]. In those studies inhibitors of cytochrome P-450 metabolism were used to determine the contribution of the types of cytochrome P-450 on metabolism in isolated hepatocytes. Their results indicated that cytochrome P-450c type activity increases in culture and is further increased by treatment with PB. In addition, PB increased cytochrome P-450b-associated ECOD activity, although to a lesser extent than P-450c. These results are similar to those reported here in that we detected an increase in basal cytochrome P-450c type metabolism in cultures in T1 and increased metabolic activity associated with cytochrome P-450c in response to PB in all three media. Consistent with our data, Owens and Nebert [19] reported that, in cultures of rat hepatocytes, cytochrome P-450c increases from the initial value in isolated cells and is further induced by PB. Fahl *et al.* [3] also demonstrated that PB treatment of hepatocytes on collagen gels results in an altered metabolism of benzo[*a*]pyrene that resembled cytochrome P-450c-associated metabolism. The mechanism for the *in vitro* induction of the basal level of cytochrome P-450c-associated metabolism and the further increase by PB treatment is not known.

We observed an increase in the metabolism of PR associated with cytochrome P-450b in cultured hepatocytes treated with PB. In T1, the level of PROD activity declined slightly from the level in initially isolated cells, but no activity was detected in control cultures in Way or L-15. The maintenance of cytochrome P-450b-associated activity in T1 medium may provide a unique experimental system for studying the regulation of this cytochrome P-450 family. Treatment with PB increased the PROD activity in T1 and to a variable extent in Way, but had no effect in L-15.

In conclusion, we have developed a chemically defined culture medium for the maintenance of cytochrome P-450 metabolic activities. In T1 medium the anomalous increase in cytochrome P-450c-associated metabolism in cultured hepatocytes was greater than in the other two media, but in T1 medium the basal level of cytochrome P-450b-associated activity was decreased only slightly from initially isolated cells, whereas in Way or L-15 no basal activity was detected. PB treatment increased the cytochrome P-450b-associated metabolism of PR 2-fold over the level of initially isolated cells in hepatocytes in T1. T1 medium represents a substantial improvement in the maintenance of cytochrome P-450 metabolic activity in cultured hepatocytes since we have maintained higher levels of basal cytochrome P-450 metabolic activity in the absence of serum, hormone supplementation, or complex culture substrata than in other culture media. In addition, we have demonstrated an increase in cytochrome P-450b-associated metabolic activity in response to PB.

Acknowledgements—We thank Mr Gerald L. Sattler and Ms Wendy Kennan for expert technical assistance. This work was supported by National Cancer Institute Grants CA-22484 and CA-07175, and by National Institutes of Health Grant CA-16265.

REFERENCES

1. Guzelian PS, Bissell DM and Meyer UA, Drug metabolism in adult rat hepatocytes in primary monolayer culture. *Gastroenterology* **72**: 1232–1239, 1977.
2. Paine AJ and Legg RF, Apparent lack of correlation between the loss of cytochrome P-450 in hepatic parenchymal cell culture and the stimulation of haem oxygenase activity. *Biochem Biophys Res Commun* **81**: 672–679, 1978.
3. Fahl WE, Michalopoulos G, Sattler GL, Jefcoate CR and Pitot HC, Characteristics of microsomal enzyme controls in primary cultures of rat hepatocytes. *Arch Biochem Biophys* **192**: 61–72, 1979.
4. Dickins M and Peterson RE, Effects of a hormone-supplemented medium on cytochrome P-450 content and mono-oxygenase activities of rat hepatocytes in primary culture. *Biochem Pharmacol* **29**: 1231–1238, 1980.
5. Grant MH, Melvin MAL, Shaw P, Melvin WT and Burke MD, Studies on the maintenance of cytochromes P-450 and b_5, monooxygenases and cytochrome reductases in primary cultures of rat hepatocytes. *FEBS Lett* **190**: 99–103, 1985.
6. Michalopoulos G, Sattler CA, Sattler GL and Pitot HC, Cytochrome P-450 induction by phenobarbital and 3-methylcholanthrene in primary cultures of hepatocytes. *Science* **193**: 907–909, 1976.
7. Newman S and Guzelian PS, Stimulation of *de novo* synthesis of cytochrome P-450 by phenobarbital in primary nonproliferating cultures of adult rat hepatocytes. *Proc Natl Acad Sci USA* **79**: 2922–2926, 1982.
8. Fry JR, Wiebkin P and Bridges JW, 7-Ethoxycoumarin-*O*-deethylase induction by phenobarbitone and 1,2-benzanthracene in primary maintenance cultures of adult rat hepatocytes. *Biochem Pharmacol* **29**: 577–581, 1980.
9. Edwards AM, Glistak ML, Lucas CM and Wilson PA, 7-Ethoxycoumarin deethylase activity as a convenient measure of liver drug-metabolizing enzymes: regulation in cultured rat hepatocytes. *Biochem Pharmacol* **33**: 1537–1546, 1984.
10. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
11. Turner NA, Wilson NM, Jefcoate CR and Pitot HC,

The expression and metabolic activity of cytochrome P-450 isozymes in control and phenobarbital-induced primary cultures of rat hepatocytes. *Arch Biochem Biophys* **263**: 204–215, 1988.
12. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
13. Ullrich V and Weber P, The O-dealkylation of 7-ethoxycoumarin by liver microsomes. *Hoppe-Seyler's Z Physiol Chem* **353**: 1171–1177, 1972.
14. Burke DM and Mayer RT, Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450 binding of phenoxazone and a homologous series of its *n*-alkyl ethers (alkoxyresorufins). *Chem Biol Interact* **45**: 243–258, 1983.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Dixon WS and Mood AM, The statistical sign test. *J Am Statistical Assoc* **41**: 557–566, 1946.
17. Lake BG and Paine AJ, The effect of hepatocyte culture conditions on cytochrome P-450 linked drug metabolising enzymes. *Biochem Pharmacol* **31**: 2141–2144, 1982.
18. Decad GM, Hsieh DPH and Byard JL, Maintenance of cytochrome P-450 and metabolism of aflatoxin B_1 in primary hepatocyte cultures. *Biochem Biophys Res Commun* **78**: 279–287, 1977.
19. Owens IS and Nebert DW, Aryl hydrocarbon hydroxylase induction in mammalian liver-derived cell cultures. *Mol Pharmacol* **11**: 94–104, 1975.