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PRELIMINARY COMMUNICATION

LONG-TERM MAINTENANCE AND INDUCTION OF CYTOCHROME P-450 IN PRIMARY CULTURES OF RAT HEPATOCYTES*

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The cytochrome P-450 system is known to be a necessary catalytic component of the liver in the metabolism of a number of xenobiotics. Primary cultures of rat hepatocytes have been advocated as experimental systems to study the metabolic activation and toxicity of carcinogens and other xenobiotics. Unfortunately, levels of cytochrome P-450 drop precipitously after 24 hr in culture (1-3); however, some investigators have reportedly reduced the loss of P-450 in 24-hr cultures by supplementation of the medium with hormones and other substances (4-7) or by use of special substrata for cell attachment (2, 3). Recently we showed longer term maintenance of P-450 in hepatocyte cultures grown in medium containing nicotinamide‡. The present report provides additional information on the value of cystine-free medium (8) in maintaining high levels of P-450 in culture for extended periods and on the induction of cytochrome P-450 in vitro by phenobarbital.

MATERIALS AND METHODS

Culture medium and fetal bovine serum were purchased from the Grand Island Biological Co., Grand Island, NY. The other chemicals and hormones were obtained from the Sigma Chemical Co., St. Louis, MO.

Culture medium. The dissociation medium was Hanks' Ca^{2+}-free balanced salt solution (BSS) modified to give an osmolarity value of 307 mOsm (9). The culture medium used was Dulbecco-Vogt's modification of Eagle's minimum essential medium deficient in arginine but supplemented with 1×10^{-4}M ornithine as previously described (9). A second medium was prepared by eliminating cystine. Both types of media were supplemented with hydrocortisone sodium succinate (0.05 g/l), insulin (0.01 g/l), nicotinamide (0.3 g/l), 10% (v/v) newborn bovine serum, 1% bovine albumin fraction V, potassium penicillin G (200 units/ml), streptomycin (200 μg/ml) and amphotericin (4 μg/ml). The media were termed complex medium and complex medium less cystine.

Isolation of hepatocytes. The isolation and cultivation of parenchymal hepatocytes from livers of 7- to 10- day-old Sprague-Dawley rats have been described in detail elsewhere (9). The procedure utilized 0.05%

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collagenase (w/v) to dissociate the tissue into single cells, which were plated into 60 x 15 mm tissue culture dishes (4-5 x 10^6 cells per dish, Falcon).

Assays. The hepatocytes were cultured in complex medium or complex medium less cystine for periods of 1 to 7 days. In some experiments, phenobarbital sodium was dissolved in the nutrient medium, and the solutions were titrated to pH 7.4 with hydrochloric acid. For analysis of cytochrome P-450 it was necessary to pool the cells from approximately fifteen culture dishes. The microsomal fraction of the cells was obtained by differential centrifugation (10), and cytochrome P-450 levels were determined by the method of Omura and Sato (11). Microsomal protein content was measured by the method of Lowry et al. (12).

The viability and integrity of the cultured cells were assessed by the trypan blue-dye exclusion method (9) and by measurement of leakage of lactate dehydrogenase (LDH) from the cells into the culture medium (9).

Student's t-test was used as a test of statistical significance ($P < 0.05$) between group values.

RESULTS AND DISCUSSION

The integrity and viability of hepatocytes were comparable for cultures grown in complex medium, complex medium less cystine, and phenobarbital-supplemented medium. Ninety percent or more of the cells in each set of cultures excluded trypan blue. LDH leakage was minimal in all cultures (data not shown).

Hepatocytes grown for 3 days or longer in complex medium lacking cystine had substantially higher levels of cytochrome P-450 than hepatocytes grown in complex medium containing cystine (Table 1). In fact there appeared to be an induction of P-450 after 3 days in the cells cultured in the cystine-free medium, in that their P-450 levels were nearly twice those of freshly isolated cells or cells grown for 3 days in medium containing cystine. After as long as 7 days in culture, levels of P-450 in the cells grown in medium without cystine were still comparable to P-450 levels in freshly isolated hepatocytes. This is the first report, to our knowledge, of a primary culture system in which stable P-450 levels are maintained for longer than 24-48 hr.

It has been shown previously by Paine and Hockin (8) that removal of cystine or cysteine from the culture medium reduces the loss of cytochrome P-450 in 24-hr-old primary cultures of adult rat hepatocytes. We have confirmed and extended these results in primary cultures of postnatal rat hepatocytes by demonstrating the long-term maintenance of P-450 in culture. The mechanism by which sulfur-containing amino acids may be responsible for the loss of P-450 in cultured hepatocytes is not yet known. However, it is interesting in the present study to note that removal of cystine from the medium and addition of phenobarbital to complex medium influence P-450 levels similarly (i.e. both produce a doubling of P-450 content). It can be seen in Table 2 that phenobarbital treatment in vitro on days 2-4 produced a greater than 2-fold increase over controls in P-450 levels after 4 days in culture.

In summary, we have shown that the usual rapid and pronounced decline in cytochrome P-450 levels in cultured hepatocytes can be prevented for as long as 7 days by utilization of cystine-free medium containing nicotinamide and certain other supplements. Retention of P-450 is of critical importance if a cell culture system is to be used to investigate the metabolism and toxicity of xenobiotics. We have also demonstrated the apparent induction of cytochrome P-450 in the cultured hepatocytes by phenobarbital.

Table 1. Levels of microsomal cytochrome P-450 in isolated hepatocytes from postnatal rats and in primary cultures of postnatal rat hepatocytes*

Liver microsomal preparations	Cytochrome P-450 content (nmoles/mg protein)
Freshly isolated hepatocytes	0.490 ± 0.07 (4)
One-day cultures	
Complex medium	0.490 ± 0.03 (7)
Complex medium less cystine	0.423 ± 0.01 (7)
Three-day cultures	
Complex medium	0.580 ± 0.03 (9)
Complex medium less cystine	0.975 ± 0.06† (4)
Five-day cultures	
Complex medium	0.330 ± 0.07 (5)
Complex medium less cystine	0.522 ± 0.11 (4)
Seven-day cultures	
Complex medium	0.240 ± 0.05 (3)
Complex medium less cystine	0.559 ± 0.02† (3)

*Results are means ± S.E.; number of experiments is in parentheses. Cytochrome P-450 was measured immediately after isolation of the hepatocytes prior to plating and after 1, 3, 5, and 7 days in culture. Microsomal yield was approximately 0.0124 mg per 10^6 cells.
†Significantly higher than levels in cells grown in complex medium ($P < 0.05$).

Table 2. Levels of microsomal cytochrome P-450 in primary cultures of postnatal rat hepatocytes treated with phenobarbital*

Liver microsomal preparation	Cytochrome P-450 content (nmoles/mg protein)
Controls	0.66 ± 0.062 (5)
1×10^{-3} M Phenobarbital	1.05 ± 0.114† (5)
2×10^{-3} M Phenobarbital	1.53 ± 0.175† (5)

*Results are means ± S.E.; number of experiments is in parentheses. Cytochrome P-450 was measured in hepatocytes grown in complex medium after 4 days in culture. Phenobarbital was added to the cultures on days 2-4. Microsomal yield was approximately 0.0124 mg per 10^6 cells.
†Significantly higher than controls ($P < 0.05$).

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