Effects of Hormones on Changes in Cytochrome P-450, Prolyl Hydroxylase, and Glycerol Phosphate Acyltransferase in Primary Monolayer Cultures of Parenchymal Cells from Adult Rat Liver

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Previous studies have shown that isolation and primary culture of rat hepatocytes in a standard, chemically defined medium is associated with selective changes in microsomal function. These changes were found to be selectively sensitive to addition of hormones to the culture medium. The concentration of cytochrome P-450 declined dramatically during the first 24 hours of incubation. However, cytochrome P-450, a form of the hemoprotein induced by polycyclic aromatic hydrocarbons, was resistant to this change. Cytochrome P-450 levels selectively rose during the first ten hours in culture and, thereafter, declined at a less rapid rate than did the cytochrome P-450 in normal hepatocytes or in cells prepared from phenobarbital pretreated animals. Addition of dexamethasone to the medium at the time of cell plating partially prevented the fall of cytochrome P-450 and of 4\(^5\)C-heme in microsomes prepared from hepatocytes derived from rats given 5\(^4\)C-\(\alpha\)-aminolevulinic acid. This suggests that the steroid decreases degradation of the hemoprotein. As compared to the loss of cytochrome P-450 in cultures of normal hepatocytes, the hemoprotein fell to lower levels in hepatocytes prepared from regenerated liver four days after partial hepatectomy. This result may be related to the accelerated formation of the monolayer in the cultures of regenerated hepatocytes. Both sn-glycerol-3-phosphate acyltransferase activity and glycerol kinase activity declined in the first 24 hours of culture. The fall in the latter enzyme was partially prevented by addition of estradiol. Collagen prolyl hydroxylase, a newly discovered microsomal constituent of the hepatocyte, rose slightly during the first 24 hours in culture. This change was augmented threefold by addition of insulin to the medium. We conclude that the present hepatocyte culture system with its attendant changes in functional phenotype may be useful in better defining the role of hormones in modulating metabolic processes in the liver.

INTRODUCTION

Techniques have been developed recently which permit preparation of high yields of viable hepatocytes from adult rat [1] and the establishment of these cells in primary monolayer cultures [2]. Preparations of freshly isolated hepatocytes have

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been universally adopted for many studies of liver metabolism in vitro. However, the cells are viable for only a few hours and, therefore, this system is unsuitable for examination of the regulation of some hepatic functions in which detectable changes occur in a time frame of many hours or days. For this reason, monolayer cultures of non-proliferating hepatocytes represent a potentially attractive system for investigation of differentiated liver functions for extended periods of time in vitro. Unlike proliferating liver "cell lines" derived from hepatoma or normal liver which may exhibit only a few of the functions present in liver in vivo [3] primary cultures of rat hepatocytes display an impressive array of adult liver functions [3,4]. However, the levels of some microsomal functions in cultured cells are strikingly altered by comparison with the level in the parent liver. For example, the level of cytochrome P-450, a hemoprotein involved in drug metabolism, and the activity of sn-glycerol-3-phosphate acyltransferase, an enzyme which may control glycerol lipid formation, are lower in cultured hepatocytes than in the liver in vivo [5–7]. In contrast, the activity of collagen prolyl hydroxylase, an enzyme which we have recently discovered to be a constituent of the hepatocyte [8] rises in monolayer cultures. These changes occur largely in the first few hours following incubation of isolated hepatocytes in culture medium while the cells are adapting to the culture conditions in vitro. In attempting to understand the mechanism of these phenotypic changes in cultured cells, we are systematically examining various constituents of culture media for their ability to modulate changes in hepatocyte functions. In the present report, we have examined the effect of some hormones on the levels of these three microsomal enzymes.

MATERIALS AND METHODS

Materials

Adult male Sprague-Dawley rats (200–250 g) obtained from Flow Laboratories (Dublin, Virginia) and housed individually with free access to food and water were used for all studies. Collagenase (Type I), pronase, and amino acids were purchased from Sigma Chemical Company (St. Louis, Missouri); Eagle's Minimal Essential Vitamin Mixture from Grand Island Biological Company (Grand Island, New York); and 5-[14C]-8-aminolevulinic acid from New England Nuclear Corporation (Boston, Massachusetts). Other chemicals were of the highest purity available commercially.

Experimental Procedures

Primary culture of adult rat liver parenchymal cells in monolayer. Hepatocyte cultures were prepared by a previously described method with minor modifications [6]. In brief, either normal or regenerated liver (four days following a two-thirds heptectomy) was perfused without circulation in situ with calcium-free salt solution followed by culture medium containing 0.03% collagenase. Hepatocytes were separated cleanly from other cellular components of the liver by repeated centrifugation and resuspension in culture medium. The cells were placed in 60 mm plastic Petri dishes precoated with rat tail collagen [9] in a final volume of 3.0 ml of chemically defined culture medium. Inoculation of plates with 2.5 × 10⁶ parenchymal cells leads to formation of a viable confluent monolayer firmly adherent to the bottom of the dish after four to eight hours of incubation at 35°C in humidified air with 5% CO₂. Approximately 90% of the plated cells remained attached to the culture dish after 24 hours of incubation as judged by recovery of DNA. Viability of the attached cells was greater than 95% as judged by exclusion of Trypan Blue. The control medium was
changed every 24 hours in these experiments and consisted of a modification of Leibowitz's L-15 formulation from Microbiological Associates (Bethesda, Maryland) in that phenol red, pyruvate, galactose, and amphotericin B were omitted and penicillin (100 U per liter) and glucose (10 mM) were added [6].

**Analytical Procedures**

Cytochrome P-450 was measured in 10,000 × g supernatant of cell homogenates as described previously [6]. Following reduction of the contents of the sample and reference cuvettes with dithionite, the carbon monoxide difference spectrum was recorded using an Aminco DW-2A (American Instrument Company, Silver Spring, Maryland) split beam recording spectrophotometer. Extraction and crystallization of labeled heme was carried out by the method of Labbe and Nishida [10] with rat erythrocytes as heme carrier. Published procedures were used for measurement of the activities of glycerol kinase and sn-glycerol-3-phosphate acyltransferase [7], prolyl hydroxylase [11], and protein [12].

**RESULTS**

We have previously shown that the level of cytochrome P-450 in hepatocytes incubated in control culture medium for 24 hours is less than 20% of the initial level in the freshly isolated hepatocytes [5,6] (Table 1). This change occurs selectively by contrast with the levels of other microsomal enzymes such as glucose-6-phosphatase [2] or gamma-glutamyl-transpeptidase which remain unchanged for several days in culture (unpublished observations). To obtain starting material for cultures in these experiments, we have routinely prepared hepatocytes from the regenerated liver, four days following a two-thirds hepatectomy [2], because we have observed that omission of this step results in less reliable formation of successful monolayer cultures in serum-free culture medium. As shown in Fig. 1, the extent of the fall in cytochrome P-450 appears to be related in part to the use of regenerated hepatocytes since levels of the hemoprotein are lower in cultures of regenerated hepatocytes after 24 hours of incubation than in cultures of cells prepared from normal liver. This difference is due largely to the fact that in cultures of normal hepatocytes, the concentration of cytochrome P-450 rose above in vivo levels during the first six to ten hours of incubation, whereas in regenerated cell cultures, cytochrome P-450 concentration was only maintained at in vivo levels for eight hours of incubation. After the first 12 hours of incubation in both types of cultures, cytochrome P-450 fell at similar rates (Fig. 1).

It is known that there exist at least two forms of cytochrome P-450 which differ in their catalytic specificities and which are selectively induced either by such lipophilic drugs as phenobarbital (cytochrome P-450) or by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (cytochrome P1-450) [13,14]. In cultured cells derived from phenobarbital pretreated rats, the concentration of cytochrome P-450 rose briefly six hours after incubation and thereafter declined. In contrast, cytochrome P1-450 in cultured cells derived from 3-methylcholanthrene pretreated rats rose steadily during the first ten hours of incubation. Cytochrome P-450 declined in cultured cells from all experimental groups, but levels in methylcholanthrene treated rats were higher at 48 hours.

Although the decline in cytochrome P-450 was unaffected by addition of either insulin or estradiol to the medium, the concentration of the hemoprotein in cells incubated in the presence of dexamethasone (10⁻⁶M) was nearly twofold higher than
FIG. 1. Effect of pretreatment of rats on the fall in cytochrome P-450 in cultured hepatocytes. Four days prior to perfusion of the liver with collagenase, rats were given phenobarbital (75 mg/kg, intraperitoneally, each day for three days), 3-methylcholanthrene (20 mg/kg in corn oil, each day for two days), or saline or were subjected to a two-thirds hepatectomy. The hepatocytes were isolated as usual and were placed in the standard culture medium supplemented with 2% dialyzed fetal calf serum and ascorbate (50 mg/l). At the indicated times, 10,000 g supernatants of cell homogenates were prepared and cytochrome P-450 or cytochrome P-450 was measured as mentioned in Materials and Methods. Results were calculated as n moles of cytochrome P-450 per mg protein in the 10,000 g supernatant and the data are expressed as percentages of the initial value in the freshly isolated hepatocytes. Each point represents the average of duplicate determinations in a single batch of cells.

| Experiment | Time of Incubation | Additions to the Medium | Cytochrome P-450 n mole/mg Protein | 14C-Heme DPM/mg Protein |
|------------|--------------------|-------------------------|-----------------------------------|-------------------------|
| I          | 0                  | None                    | 0.2489                            | 3440                    |
|            | 24 hr.             | None                    | 0.0458                            | 1401                    |
|            | 24 hr.             | Dexamethasone           | 0.0779                            | 1994                    |
|            |                    | (1 x 10^-6M)            |                                    |                         |
| II         | 0                  | None                    | 0.1773                            | 2570                    |
|            | 24 hr.             | None                    | 0.0154                            | 1324                    |
|            | 24 hr.             | Dexamethasone           | 0.0241                            | 1885                    |
|            |                    | (1 x 10^-6M)            |                                    |                         |

Donor animals were injected intravenously with 10 µCi of 5-[^14]C]-δ-aminolevulinic acid and operated on two hours later. The liver was perfused with collagenase solution for preparation of isolated hepatic parenchymal cells and, following three washes in culture medium, the cells were plated in complete culture medium, with or without dexamethasone. At time zero and after 24 hours in culture, cells were disrupted and microsomes were prepared for measurement of protein and of cytochrome P-450 and for quantitation of labeled heme as described in Materials and Methods. Each value represents an analysis of the cells pooled from four culture plates or the equivalent amount of freshly isolated cells.
that in cells incubated in control medium (Table 1). To elucidate whether dexamethasone increased synthesis of cytochrome P-450, or decreased degradation of the hemoprotein (or both), rats were given a pulse of 5-\(^{14}\text{C}\)-\(\delta\)-aminolevulinic acid, a specific heme precursor, two hours before the hepatocytes were isolated and placed in cultures. This protocol would be expected to label primarily the heme of cytochrome P-450 [15]. In the presence of dexamethasone in the medium at 1 \(\times\) 10\(^{-6}\) M, the minimal concentration which consistently affected the subsequent concentration of cytochrome P-450 in culture, the decay of microsomal \(^{14}\text{C}\)-heme was reduced by comparison with that in control cultures (Table 1). This suggests that the higher level of cytochrome P-450 in cultured cells exposed to dexamethasone for 24 hours is due primarily to decreased degradation of the hemoprotein.

The selectivity of the change in cytochrome P-450 is underlined by survey of other microsomal functions in cultured cells. As shown in Table 2, \(sn\)-glycerol-3-phosphate acyltransferase activity declined to 44% of its initial activity after 24 hours in culture and was only slightly reduced thereafter. By contrast, the activity of glycerol kinase, an enzyme not thought to be localized to the endoplasmic reticulum, fell to 28% of its initial level after 24 hours of culture. Neither insulin, steroids, nor estradiol, when added to the culture media, had a significant effect on the subsequent levels of \(sn\)-glycerol-3-phosphate acyltransferase activity. In contrast, estradiol partially prevented the fall in glycerol kinase activity.

Prolyl hydroxylase activity is present in freshly isolated hepatocytes at a specific activity approximately one-half that of the intact liver [8]. Because hepatocytes contain 95% of liver protein [16], it may be inferred that 50% of hepatic prolyl hydroxylase activity resides in the hepatocyte. Furthermore, when parenchymal and

| Age of Monolayer (hours) | Addition (molar) | n moles/min/mg Protein ± SEM |
|-------------------------|-----------------|-----------------------------|
|                         |                 | GK                          | GPAT                        |
| **Experiment A**        |                 |                             |                             |
| 0                       | —               | 6.41 ± 0.59                 | 0.34 ± 0.06                 |
| 12                      | —               | 2.85 ± 0.26*                | 0.17 ± 0.01**               |
| 24                      | —               | 1.81 ± 0.22*                | 0.15 ± 0.01*                |
| 48                      | —               | 1.94 ± 0.22*                | 0.12 ± 0.01*                |
| **Experiment B**        |                 |                             |                             |
| 24                      | Control         | 1.12 ± 0.07                 | 0.20 ± 0.02                 |
| 24                      | Estradiol       | 3.49 ± 0.23*                | 0.17 ± 0.01                 |

Cultures were prepared as usual from regenerated liver and were incubated in the standard culture medium. Glycerol kinase and \(sn\)-glycerol-3-phosphate acyltransferase were measured in monolayer homogenates as described under Methods. In Experiment A, the enzyme activity was measured in homogenates of monolayers that were 0, 12, 24, and 48 hours old. In Experiment B, sufficient estradiol was added two hours after the hepatocytes were plated to produce the indicated medium concentration. After 24 hours of drug exposure, monolayer homogenates were prepared and the appropriate enzyme activity measured. Values from the batches of cells in Experiment A served as 0 time levels for Experiment B.

Each point represents the mean ± SEM of at least three experiments in which each enzyme activity was measured in triplicate from two separate plates.

* \(p < 0.01\) level of significance from 0 age monolayer or 24 hour control.

** \(p < 0.05\) level of significance from 0 age monolayer or 24 hour control.

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nonparenchymal cells were prepared from the same liver, greater than 90% of total prolyl hydroxylase recovered was associated with the parenchymal cells (unpublished observation). This excludes the possibility that contamination of the cultures with nonparenchymal cells accounts for the presence of the enzyme in hepatocyte cultures. Confirming this biochemical evidence, the enzyme has been localized to the hepatocyte by immunofluorescence using antibody directed against purified rat skin prolyl hydroxylase (unpublished observations). The level of this enzyme rose by 50% during the first 24 hours of incubation in control medium (Fig. 2). This increase was marked (250%) when insulin was present in the culture medium. In contrast, dexamethasone had no stimulatory effect on hepatocyte prolyl hydroxylase activity (data not shown). The insulin stimulated rise in prolyl hydroxylase activity was blocked by cycloheximide [8], and thus, this change appears to represent de novo protein synthesis rather than enzyme activation.

**DISCUSSION**

Cytochrome P-450 concentration declines dramatically during the first 24 hours in culture, at a rate suggesting accelerated degradation of the hemoprotein by comparison with its overall half-life of 24–48 hours in vivo [17,18]. Dexamethasone partially prevented the fall in cytochrome P-450 in culture apparently by decreasing the degradation of the hemoprotein. This finding is consistent with the observation that cytochrome P-450 falls in adrenalectomized rats [19] and supports the view that steroid hormones regulate cytochrome P-450 by a direct effect on the liver. We have previously reported that the rates of microsomal metabolism of aminopyrine or aniline declined in cultured hepatocytes, in parallel with the concentration of cytochrome P-450 [6]. In contrast, p-nitroanisole-O-demethylase activity remained unchanged in monolayer cultures [2,6]. Furthermore, only the latter enzyme activity was inducible in culture by polycyclic aromatic hydrocarbons, whereas none of the drug metabolizing enzymes was consistently induced in culture by phenobarbital [6].
A postulated explanation for these findings is that p-nitroanisole-O-demethylase activity is supported by a subspecies of cytochrome P-450, one which resists the conditions of cell culture. The present results are consistent with this idea in that cytochrome P-450, which mediates O-demethylation of p-nitroanisole [20], selectively increases in the first hours of culture and declines more slowly than does the form of cytochrome P-450 induced by phenobarbital.

During the first 24 hours of incubation, cytochrome P-450 declined to lower levels in cells derived from regenerated liver than in those from normal rats. This result is surprising because general protein turnover is significantly retarded in regenerated liver [21]. We have observed that the process of monolayer formation which involves attachment of cells to the collagen substratum followed by flattening and intercellular contact, occurs more rapidly (4–6 hours) with cells derived from regenerated liver than in cultures prepared with normal hepatocytes (18–24 hours). It is possible that upon isolation and incubation of the hepatocyte, mechanisms which degrade cytochrome P-450 may be activated as part of a coordinated redirection of cell energies toward the process of monolayer formation. In line with this idea is the fact that addition of cycloheximide or other inhibitors of protein synthesis to the medium at the time of cell plating blocks monolayer formation and, paradoxically, prevents partially the fall in cytochrome P-450 levels [22].

We have recently discovered that collagen prolyl hydroxylase is a constituent of the endoplasmic reticulum of the hepatocyte. In proliferating cell systems, prolyl hydroxylase activity is increased by lactate or ascorbate through activation of the enzyme [23]. Although addition of lactate or ascorbate to the medium has no effect on prolyl hydroxylase activity in cultured hepatocytes (unpublished observations), insulin increased prolyl hydroxylase activity (Fig. 2), apparently by increasing synthesis of the enzyme. This effect of insulin is selective in that the levels of cytochrome P-450 and sn-glycerol-3-phosphate acyltransferase are unchanged by the presence of insulin in the medium. Insulin has not been identified previously as a regulatory factor in collagen synthesis. Nevertheless, localization of prolyl hydroxylase to the hepatocyte raises the possibility that the parenchymal cell may participate in the formation of hepatic fibrogenesis and that insulin may influence this process.

The absence of most differentiated hepatic functions is characteristic of proliferating liver cell culture systems. Although it has been commonly assumed that this "dedifferentiation" is linked to the process of cell replication, the present data suggest that some loss of function may occur early as the cells adapt to culture conditions and in the absence of cell division. It is important to understand the reasons for these changes in order to design culture systems in which differentiated functions are maintained. The present data suggest that hormones are important modulators of hepatic functions in culture and the absence of these substances from the culture medium contributes to culture-related phenotypic changes. This culture system should prove useful for determining how hormones act in direct contact with cells of the liver to selectively alter enzyme functions within a given organelle.

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