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# REGULATION OF HEME METABOLISM AND CYTOCHROME P-450 LEVELS IN PRIMARY CULTURE OF RAT HEPATOCYTES IN A DEFINED MEDIUM

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**Abstract**—Liver cells were prepared from adult Sprague–Dawley rats and used for the determination of δ-aminolevulinic acid synthetase (ALAS) activity and cytochrome P-450 concentrations at different time intervals in tissue culture in a serum-free synthetic medium. During the first 24 hr in culture, the level of cytochrome P-450 decreased to 30–40% of the level in isolated liver cells from untreated animals. The disappearance of cytochrome P-450 was especially fast in hepatocytes obtained from female phenobarbital-treated rats where only 40% of the original cytochrome P-450 was present after 2 hr in culture and 80% had disappeared in 2 days. The activity of ALAS increased 3- to 4-fold when measured 2 hr after plating, and it reached the maximum level in 19–24 hr when its activity was about eight times the original activity. In 2–4 days in culture, the activity of ALAS was four to five times above the original level. When the amount of δ-aminolevulinic acid (ALA) in the medium was increased from 1 to 100 μM, a decrease in ALAS was obtained, but no significant increase in cytochrome P-450 level was observed. Addition of heme to the medium gave a dose-dependent decrease in the activity of ALAS. Our data indicate that during the first 24 hr in culture the increase of ALAS activity was prevented by exogenous heme. This effect may be due to inhibition of the catalytic activity, suppression of the synthesis of the enzyme, or accelerated breakdown of the enzyme by heme.

The ultimate goal of *in vitro* studies using primary monolayer cultures of hepatocytes is to be able to investigate the regulation of highly differentiated liver cell functions under strictly controlled conditions. These studies have been hampered by the drastic changes in protein metabolism [1–3], as evidenced by lack of response to inducers of drug-metabolizing enzyme systems [4, 5] and total and/or selective decrease of cytochrome P-450 species [4–12]. An understanding of the basic reasons for failure of maintenance of the normal levels and functions of the hemoproteins in cultured hepatocytes is still lacking. This is not surprising, since the complex biosynthetic pathways of hemoproteins, which play such a critical role in drug metabolism, are not well understood. It is known, however, that for the induction of drug-metabolizing enzymes synthesis of the proteins of the endoplasmic reticulum (apocytochrome P-450) and heme have to be well coordinated [13].

Much valuable information has been obtained concerning the tissue culture conditions which preserve almost normal synthesis of proteins in primary rat hepatocytes [14]. However, much less attention has been paid to the metabolism of heme in primary cultures of rat hepatocytes. This is in contrast to the many studies on heme metabolism performed with chicken embryo liver cells in culture [15–18]. The present communication reports on the relationship of heme biosynthesis and the level of cytochrome P-450 in primary cultures of rat hepatocytes in a defined medium.

## MATERIALS AND METHODS

*Isolation of hepatocytes.* Adult female and male Sprague–Dawley rats (250–300 g) from Harlan Sprague–Dawley Inc. (Walkersville, MD) were used. Animals were anesthetized with halothane. Hepatocytes were isolated by a two-step liver perfusion technique as described by Seglen [19] and modified by Williams *et al.* [20]. During the perfusion, the collagenase solution was saturated with oxygen and recirculated 10–15 min through the liver. After the perfusion the liver was slit with several cuts, and the cells were suspended in Waymouth medium. Dispersed cells were filtered through nylon cloth of 74 μmesh and were further purified with three low-speed centrifugations to remove cell debris.

*Serum-free synthetic medium.* The following ingredients were added to Waymouth's MB 752/1 formula: $NaHCO_3$ (2.24 g/l), Hepes† (3.57 g/l), L-alanine (11.2 mg/l), L-aspargine (24 mg/l), L-serine (12.8 mg/l), streptomycin (100 mg/l) and gentamycin (50 mg/l). The pH was adjusted to 7.5 with 1 N NaOH. This basic medium was used for washing the liver cells after the perfusion. For plating the cells the following hormones, insulin ($8.3 \times 10^{-4}$ M), dexamethasone ($5 \times 10^{-8}$ M), progesterone

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† Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ALAS, δ-aminolevulinic acid synthetase; ALA, δ-aminolevulinic acid; and AIA, 2-allyl-2-isopropylacetamide.

($1 \times 10^{-6}$ M), testosterone ($1 \times 10^{-6}$ M), estradiol ($1 \times 10^{-6}$ M) glucagon ($1 \times 10^{-6}$ M), and thyroxin ($1.2 \times 10^{-5}$ M), and the following vitamins, α-tocopherol ($1.2 \times 10^{-5}$ M) and L-ascorbic acid ($3 \times 10^{-4}$ M), were added. In addition, transferrin ($6 \times 10^{-8}$ M), linoleic acid ($1.8 \times 10^{-5}$ M), and δ-aminolevulinic acid (ALA) ($1 \times 10^{-6}$ M) were added to the medium.

*Hepatocyte cultures.* Waymouth's medium (1.5 ml), supplemented with vitamins and hormones as described earlier, and containing hepatocytes ($0.75 \times 10^6$ cells/ml), was pipetted onto collagen-coated wells of 40 mm in diameter. The cells were incubated at 37° in a humidified incubator in a 5% $CO_2$, 95% air atmosphere. The medium was changed after 4 hr of incubation to remove the unattached cells and afterwards every 24 hr. In some experiments, leupeptin and pepstatin (1 μg/ml) were added to the medium to inhibit the effect of lysosomal proteases.

*Addition of chemicals.* 2-Allyl-2-isopropylacetamide (AIA) was dissolved in phosphate-buffered saline and added to the defined medium at a $3.5 \times 10^{-5}$ M concentration. Heme was dissolved in 0.25% sodium carbonate solution, and pH was adjusted to 8 with HCl.

*Assays for δ-aminolevulinic acid synthetase and cytochrome P-450.* The determination of δ-aminolevulinic acid synthetase (ALAS) activity in hepatocytes was performed by scraping off the cells from two culture dishes and suspending in 1 ml of 10 mM Tris, 0.154 M NaCl buffer, pH 7.4. Homogenates were prepared with a Potter–Elvehjem homogenizer with 0.05 to 0.10 mm clearance. Homogenates were kept frozen overnight at −80°. ALAS activity was determined by the radiochemical method of Ebert *et al.* [21] using a succinyl-CoA generating system as described by Condie and Tephly [22]. The modifications as described by Scotto *et al.* [23] were necessary to determine the enzyme activity in tissue culture in the presence of succinyl-CoA generating system. For preparation of microsomes, hepatocytes from six tissue culture wells were pooled. The wells were washed twice with cold phosphate-buffered saline. Cells were homogenized in 0.25 M cold sucrose solution, sonicated for 10 sec while kept on ice, and centrifuged at 8000 *g* for 15 min. The supernatant fraction thus obtained was centrifuged at 150,000 *g* for 1 hr. The microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. Cytochrome P-450 concentration was determined by the method of Omura and Sato [24] using an Aminco DW-2 spectrophotometer equipped with a beam condensing system for micro cells. The method of Lowry *et al.* [25] was used for protein determination.

## RESULTS

Figure 1 shows that 60–70% of the original cytochrome P-450 disappeared during the first 24 hr in culture. However, the pattern of disappearance was much more gradual in cells from male rats treated with phenobarbital than from female phenobarbital-treated animals, where only 40% of the original cytochrome P-450 was present after 2 hr in culture. In addition, a prominent destruction of cytochrome P-450 was evidenced in phenobarbital-treated female rats during the preparation of the cells, because the amount of cytochrome P-450 present was only 80% of the amount of untreated female rats. After 48 hr in culture the level of cytochrome P-450 remained the same as during the 24-hr period except in cells obtained from female phenobarbital-treated rats where a further 50% decrease was recorded. When leupeptin and pepstatin were added to the tissue culture medium of phenobarbital-treated female rats, no differences in the level of cytochrome P-450 were observed (Table 1). The presence or absence of ALA in the tissue culture medium did not affect the level of cytochrome P-450 after 24 hr in culture.

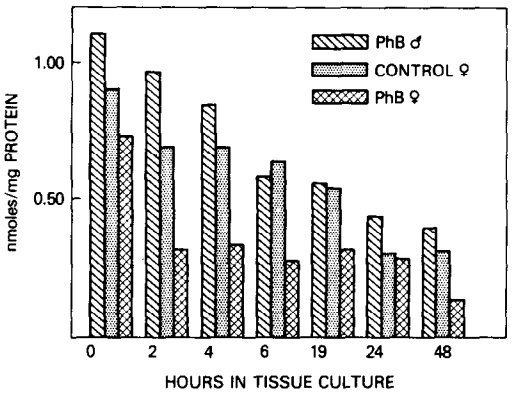


Fig. 1. Cytochrome P-450 levels at different time intervals from control and phenobarbital-treated female and phenobarbital-treated male rats. The original pre-culture levels of cytochrome P-450 in cell preparations obtained from PhB treated ♂, control ♀ and PhB ♀ were 1.11, 0.90 and 0.73 nmoles/mg microsomal protein. Values are means of three experiments.

The activity of ALAS was increased three to four times when measured 2 hr after the plating of the cells, and it reached the maximum in 19–24 hr (Fig. 2). In 48 hr the activity of ALAS decreased but still remained about four to five times higher than the original level. In 4 days in tissue culture the value for ALAS for phenobarbital-treated females was four times above the original value.

Table 1. Effect of leupeptin and pepstatin on cytochrome P-450 level in liver cells obtained from phenobarbital-treated rats

| Time in tissue culture (hr) | Cytochrome P-450* (pmoles/mg microsomal protein) | |
|---|---|---|
| | − Leup. − Pepst. | + Leup. + Pepst. |
| 0 | 747 | 718 |
| 2 | 303 | 318 |
| 4 | 331 | 308 |
| 6 | 284 | 295 |
| 19 | 322 | 356 |
| 24 | 233 | 250 |

* Each value is the mean of two experiments.

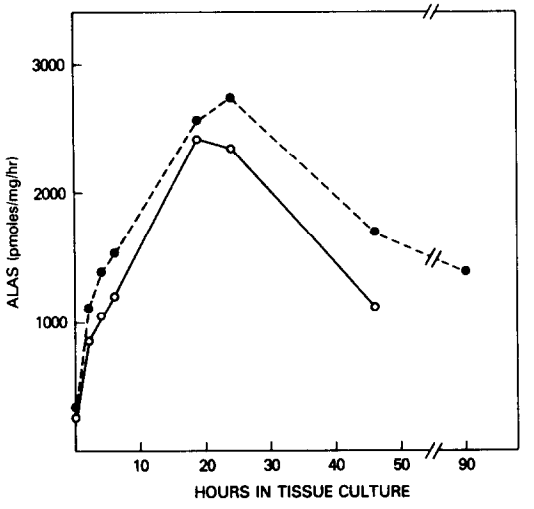


Fig. 2. δ-Aminolevulinic acid synthetase levels at different time intervals from control (———) and phenobarbital (– – –) -treated female rats. Values are means of three experiments.

Table 2. δ-Aminolevulinic acid synthetase in liver cells after 19 hr in culture

| Medium | ALAS* (pmoles/mg/hr) | % |
|---|---|---|
| Control† | 2012 ± 51 | 100 |
| − ALA | 2637 ± 110 | 131 |
| + ALA (10 μM) | 1480 ± 56 | 74 |
| + ALA (100 μM) | 1198 ± 187 | 60 |
| − Steroids‡ | 1881 ± 140 | 93 |
| + Heme (1.5 μM)§ | 1815 ± 7 | 90 |
| + Heme (3 μM)§ | 1500 ± 81 | 75 |
| + Heme (6 μM)§ | 1146 ± 27 | 57 |
| + AIA (0.35 mM)§¶ | 2016 ± 55 | 105 |

* Each value is the mean ± S.E. of three to five experiments.

† Control medium contained 1 μM ALA.

‡ Progesterone, testosterone, β-estradiol and dexamethasone were omitted; 1 μM ALA was present.

§ A 1 μM concentration of ALA was present.

¶ 2-Allyl-2-isopropylacetamide (AIA) was added to the medium after 4 hr in culture.

Medium containing no aminolevulinic acid (ALA) gave somewhat higher values for ALAS than our control medium which contained 1 μM ALA (Table 2). When this amount was increased to 10 or 100 μM, a decrease in ALAS activity was obtained. When progesterone, testosterone, estradiol and dexamethasone were omitted from the culture, no significant difference in activity of ALAS was observed as compared to the control medium. Addition of heme to the medium gave a dose-dependent decrease in the activity of ALAS (Fig. 3). No induction of ALAS was obtained when AIA was added to the tissue culture medium at 4 hr after plating the cells.

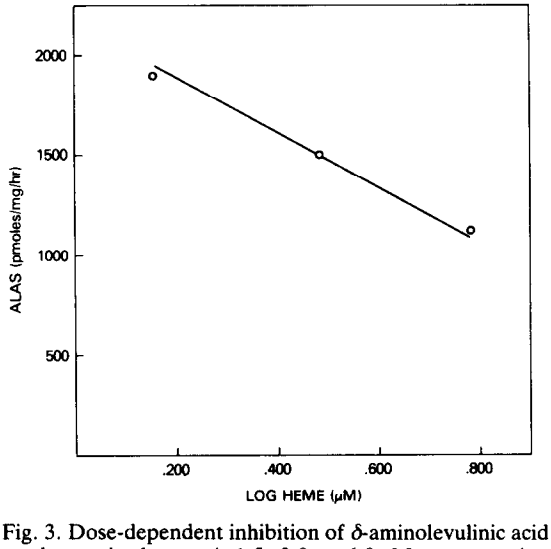


Fig. 3. Dose-dependent inhibition of δ-aminolevulinic acid synthetase by heme. A 1.5, 3.0 or 6.0 μM concentration of heme was added to the tissue culture medium. Cells were harvested after 24 hr in culture and analyzed for δ-aminolevulinic acid synthetase in triplicate. Values are averages of three experiments.

## DISCUSSION

Adult liver cells maintained in culture under defined conditions should be most valuable for studies on metabolic processing of drugs and carcinogens *in vitro*. However, the usefulness of such cells has been restricted by the lack of similarities in biological responses under the culture conditions and *in vivo* [4, 7, 12]. The most commonly used criteria for optimal tissue culture conditions are the maintenance and inductibility of cytochrome P-450. It is known that hepatocytes in primary cultures undergo both morphological and biochemical changes, and this is especially manifested in the rapid and selective loss of cytochrome P-450 [5–12]. Partial inhibition of the rapid decline of cytochrome P-450 has been obtained by adding different hormones [26], ALA [6, 26, 27], ascorbic acid [6, 9], or omitting cystine and/or cysteine [10]. Fry *et al.* [28] reported that, in rat hepatocytes cultured for up to 4 days in the absence of any known exogenous inducer, almost all phenobarbital-type cytochrome P-450 had disappeared, and only P-448 was present. Similarly, Guzelian *et al.* [5] observed that the level of cytochrome P-450 declines in hepatocytes under a variety of tissue culture conditions.

In our hands, Waymouth's medium supplemented with several vitamins, hormones and ALA did not maintain the level of cytochrome P-450 during the 24-hr tissue culture period. There was a 60–70% loss of cytochrome P-450 during day 1 in culture whereas during day 2 in culture the loss was insignificant, except for cells obtained from phenobarbital-treated female rats, which lost 80% of their original cytochrome P-450 in 2 days in culture. In addition, there seemed to be a significant loss of cytochrome P-450 from hepatocytes obtained from phenobarbital-treated female rats during perfusion and handling before plating the cells. In contrast, the survival (as measured by exclusion of trypan blue) and attach-

Table 3. Effect of aminolevulinic acid on the cytochrome P-450 level in liver cells obtained from control rats

| ALA added ($\mu$M) | Cytochrome P-450* (pmoles/mg microsomal protein) |
|---|---|
| None | 284 |
| 1 | 212 |
| 10 | 222 |
| 100 | 289 |

* Each value is the mean of two experiments. Cells were harvested 24 hr after plating.

ment to the collagen-coated plates were similar in all preparations. When leupeptin and pepstatin were added to the medium, no improvement in the level of cytochrome P-450 was observed (Table 1).

The regulation of cytochrome P-450 levels, and that of other cellular hemoproteins, is dependent to a great extent on the metabolism of heme. When hemoproteins are being destroyed there is a release of heme, which is thought to enter the "free-heme pool" [13]. ALAS is the rate-limiting enzyme in the biosynthetic pathway of heme [29], and its synthesis is under negative feedback regulation by heme [30]. Heme is an inducer of heme oxygenase [31] which is known to increase up to 6-fold under the tissue culture conditions [32, 33], indicating the presence of an excess of free heme [5]. However, the present data show a prominent increase in the activity of ALAS after 2 hr in tissue culture, and in 19–24 hr its activity was about eight times the original activity and remained three to four times higher than the original value even after 4 days in culture. The induction of ALAS seems to indicate the lack of heme or severe disturbances somewhere along the heme biosynthetic pathway. This interpretation is further supported by the dose-dependent inhibition of ALAS activity when exogenous heme was included (Fig. 3). Inclusion of ALA in the culture medium has been reported to promote the maintenance of cytochrome P-450 in tissue culture [6, 26, 27]. ALA (1 $\mu$M) was included routinely in a culture medium. When it was omitted entirely, a 30% increase in ALA synthetase activity was obtained after 19 hr in culture. On the contrary, when the amount of ALA was increased from 1 to 100 $\mu$M, activity of ALAS dropped (Table 2). No significant increase of cytochrome P-450 levels was observed after the ALA treatment (Table 3). Similar effects of high ALA concentration on ALAS activity have been observed in rats *in vivo* [34].

Edwards and Elliott [35, 36] reported that steroid hormones (progesterone, hydrocortisone, testosterone and 17$\beta$-estradiol) are inducers of ALAS in rat liver cell suspensions in the presence of cyclic AMP. When these hormones were excluded from the culture medium in our system, no decrease in ALAS activity was observed as compared to the control medium with the steroids included (Table 2).

AIA is a porphyrogenic chemical which, when administered to animals or added to chicken embryo liver cells in culture, produces a prominent induction of ALAS [18]. Edwards and Elliott [35] obtained 4- to 5-fold induction of ALAS in rat liver cell suspensions after treatment with AIA in Waymouth's medium in the presence of calf serum and dibutyrul cyclic AMP [35]. AIA is metabolized by a phenobarbital-type P-450 species and binds covalently to the prosthetic heme causing its destruction [37, 38]. No induction of ALAS activity was observed when AIA was applied to the cells after 4 hr in culture. This lack of ALAS induction is most likely due to absence of the form(s) of cytochrome P-450 responsible for the metabolism of AIA, or the regulation of ALAS may be altered in cultured rat hepatocytes.

Our data indicate that during the first 24 hr in culture there may be a shortage of heme as evidenced by an increase of ALAS activity. However, the observed inhibition of the activity of ALAS by exogenous heme could be due to the inhibition of the catalytic activity, suppression of the synthesis of the enzyme, or accelerated breakdown of the enzyme by heme. It is of interest to note that during this period the cytochrome P-450 system was not induced in liver cells by phenobarbital, whereas if the cells are treated after 24 hr in culture and analyzed at 96 hr, an induction is obtained [39]. In addition, selenium seems to increase the induction of phenobarbital-type cytochrome P-450 [40]. The present study shows that after 24 hr in culture the amount of heme in the free heme pool had increased as evidenced by the decrease of ALAS activity. The availability of heme for the synthesis of hemoproteins might explain the importance of timing for induction of cytochrome P-450 by phenobarbital. However, the inability of ALA to increase cytochrome P-450 indicates that synthesis and/or availability of apocytochrome P-450, in addition to that of heme, may also determine the induction of cytochrome P-450.

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