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# LIGANDS MAINTAIN CYTOCHROME P-450 IN LIVER CELL CULTURE BY AFFECTING ITS SYNTHESIS AND DEGRADATION

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SUMMARY Rat hepatocytes cultured for 24 h lose 68% of their cytochrome P-450. It is shown that this loss is due to the failure of cultured hepatocytes to synthesize cytochrome P-450 as well as enhanced degradation. Compounds that form ligands with cytochrome P-450, eg metyrapone, prevent the loss of cytochrome P-450. Ligands are generally considered to protect proteins from degradation but the present work suggests that the effect of metyrapone on cytochrome P-450 synthesis is of equal importance to its effect on degradation in preventing the loss of cytochrome P-450 in hepatocyte culture.

## INTRODUCTION

The primary culture of rat hepatocytes for 24 h results in the loss of 70% of their cytochrome P-450 concentration (1,2). This loss can be prevented by the culture of hepatocytes in a medium containing any one of a number of pyridines that form ligands with cytochrome P-450. The most potent of these are metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) and 3-hydroxypyridine (3). This finding offers the opportunity to determine the mechanism(s) underlying the loss of cytochrome P-450 in liver cell culture. Since this loss is greater than would be predicted from the half-life of cytochrome P-450 in intact liver (4) it seemed reasonable to examine the effect of metyrapone and 3-hydroxypyridine on the degradation of cytochrome P-450; especially as there are numerous examples where ligands protect proteins from degradation (5).

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In view of the difficulties involved in purifying cytochrome P-450 from hepatocyte microsomes its degradation is measured by determining the loss of radioactivity associated with microsomal haem after preferentially pre-labelling the haem prosthetic group of cytochrome P-450 with a tracer pulse of [$^{14}$C]-5-aminolaevulinic acid (4,6-9). We have also preferentially labelled cytochrome P-450 in intact liver prior to the isolation and culture of hepatocytes and have used this method to examine P-450 degradation during 24 h of culture. In addition this approach permits the effect of hepatocyte culture conditions on the synthesis of cytochrome P-450 to be determined. This is because the haem of cytochrome P-450 is not re-utilized in the synthesis of cytochrome P-450 but is degraded (10) and the only factor that can alter (eg lower) the initial specific radioactivity of cytochrome P-450 is the synthesis of new P-450 molecules containing unlabelled haem. Based on these considerations the present work demonstrates:

1) that enhanced degradation and impaired synthesis are the mechanism(s) underlying the loss of cytochrome P-450 in hepatocyte culture.

2) ligands, such as metyrapone and 3-hydroxypyridine, that maintain cytochrome P-450 in hepatocyte culture at the same concentration as found in intact liver do so by affecting both synthesis and degradation.

## MATERIALS AND METHODS

These are as previously described (3) for the isolation and culture of hepatocytes, preparation of microsomal fraction from intact liver, cytochrome P-450 and protein determinations.

### Preparation of microsomal fraction from isolated and cultured hepatocytes

Hepatocytes cultured for 24 h were scraped from 6 x 150 mm diameter petri dishes and suspended in 12 ml of 0.15 M KCl-50 mM phosphate buffer pH 7.4 (approximately 7 x $10^6$ cells/ml). Freshly isolated hepatocytes were resuspended in 0.15 M KCl-50 mM phosphate buffer at the same concentration of cells. Both isolated and cultured hepatocytes were homogenised using an Ultra-Turrax TP 18/10 blender for 30 secs. The microsomal fraction from the resultant homogenate was prepared by the same method as described for intact liver.

### Labelling of cytochrome P-450 with 5-amino[4-$^{14}$C]-laevulinic acid

200 gm male rats of the Porton derived Wistar strain fed *ad libitum* on MRC 41B diet were injected intravenously via a tail vein with 0.25 ml

0.15 M NaCl containing 5 μCi 5-amino[4-$^{14}$C]-laevulinic acid (specific activity = 42 mCi/mmol) (Radiochemical Centre, Amersham, Bucks, U.K.). Injections were carried out between 09.30 and 10.00 hrs and 2 h later the isolation and plating of hepatocytes was commenced. The procedures took approximately 2 h.

Determination of the radioactivity of the microsomal fraction

The radioactivity of microsomal cytochrome P-450 was routinely determined by liquid scintillation counting after adding aliquots of a microsomal suspension to Instagel (Packard Instrument Co., Downers Grove, Illinois, U.S.A.) since 5-amino[4-$^{14}$C]-laevulinic acid is a specific haem precursor. The d.p.m. values were computed by automatic external standardization. To check the validity of measuring the radioactivity associated with microsomal haem in microsomal suspensions, microsomal haem was extracted (11) and the radioactivity determined in this extract. Both methods gave the same result.

Statistics

Results are quoted as means ± S.D. and were analysed by Student's t test.

RESULTS AND DISCUSSION

Treatment of rats with 5-amino[4-$^{14}$C]-laevulinic acid can result in an over production of hepatic haem which makes it difficult to dissociate the turnover of microsomal haemoproteins from the turnover of haem bound to microsomes (6). However the tracer pulse of 5-amino[4-$^{14}$C]-laevulinic acid used in the present study did not result in an over production of haem as the haem extracted from microsomes prepared from intact liver, isolated or cultured hepatocytes could always be accounted for by the sum of the concentrations of the two microsomal haemoproteins, cytochrome P-450 and cytochrome $b_5$. Since the half-life of cytochrome $b_5$ is considerably longer than that of cytochrome P-450 the labelling procedure used, in the present work, results in the preferential labelling of cytochrome P-450 with less than 10% of the microsomal radioactivity associated with cytochrome $b_5$ (9). Furthermore we found that the concentration of cytochrome $b_5$ only declines by 20% in hepatocyte culture and that this change is not affected by the treatments used in the present work. Accordingly we are confident that changes in the radioactivity of the microsomal fraction reflect the metabolism of the haem

prosthetic group of cytochrome P-450 and hence the metabolism of the holocytochrome.

Effect of hepatocyte culture on the degradation and synthesis of cytochrome P-450

Hepatocytes cultured for 24 h without treatment lose 68% of their cytochrome P-450 content compared to freshly isolated cells (Table 1). This loss of cytochrome P-450 is paralleled by a loss of radioactivity associated with microsomes (Table 1) and also with a loss of total microsomal haem (data not shown). The loss of microsomal radioactivity in hepatocytes cultured without treatment for 24 h was significantly ($p < 0.05$) greater than the loss found *in vivo* 24 h after the same labelling procedure had been used (Table 1). This indicates that liver cell culture results in an enhanced degradation of cytochrome P-450. However the specific radioactivity of cytochrome P-450 found in hepatocytes cultured for 24 h without treatment is not significantly different ($p > 0.05$) from that present in isolated hepatocytes prior to culture (Table 1). As the haem of hepatic cytochrome P-450 is not re-utilized *in vivo* in the synthesis of cytochrome P-450 but is degraded (10) the simplest interpretation to account for the same specific radioactivity of cytochrome P-450 in cultured and freshly isolated hepatocytes is that the synthesis of cytochrome P-450 does not occur during the first 24 h of hepatocyte culture. If the synthesis of P-450 molecules had occurred in hepatocyte culture then these would contain unlabelled haem and the specific radioactivity of cytochrome P-450 would be lower than found in freshly isolated hepatocytes. This conclusion is compatible with the finding that the specific radioactivity of cytochrome P-450 decreases in the intact liver of rats over the same period of time (Table 1). Accordingly both the impaired synthesis and enhanced degradation of cytochrome P-450 underlie its loss in hepatocyte culture.

Table 1. Changes in cytochrome P-450 concentration, microsomal radioactivity (P-450 degradation), and the specific radioactivity of cytochrome P-450 (P-450 synthesis) in intact rat liver and hepatocyte culture.

| Preparation | No.of separate preparations | Cytochrome P-450/ mg microsomal protein (% isolated hepato-cytes) | $^{14}C$ radioactivity/ mg microsomal protein (% isolated hepato-cytes) | $^{14}C$ radioactivity/ pmole P-450 (% isolated hepato-cytes) |
|---|---|---|---|---|
| Isolated hepatocytes | 6 | 100 | 100 | 100 |
| Liver *in vivo* 24 h after injection of $^{14}C$ | 3 | 100 | 50 ± 5 | 45 ± 9 |
| Hepatocytes cultured for 24 h with: | | | | |
| a) no treatment | 6 | 32 ± 6 | 34 ± 5 | 105 ± 6 |
| b) 5mM 3-hydroxypyridine | 3 | 76 ± 3 | 43 ± 3 | 57 ± 6 |
| c) 0.5mM metyrapone | 6 | 85 ± 6 | 47 ± 4 | 56 ± 3 |
| d) 0.5mM metyrapone + 5μM cycloheximide | 3 | 84 ± 3 | 65 ± 1 | 81 ± 5 |
| e) 5μM cycloheximide | 3 | 53 ± 6 | 50 ± 1 | 97 ± 13 |

200 gm male rats were injected with 5μCi 5-amino[4-$^{14}C$]-laevulinic acid, 2 h later the isolation and culture of hepatocytes was commenced. Hepatocytes were cultured for 24 h prior to harvesting and assay of cytochrome P-450 and microsomal radioactivity as described in the Materials & Methods. The loss of microsomal radioactivity in intact liver is compared to isolated hepatocytes and was determined 24 h after injecting [$^{14}C$]-5-aminolaevulinic acid during which period the rats were allowed food and water *ad libitum*. The results are expressed as the mean ± S.D. of the percentage of the initial concentration of cytochrome P-450 and the initial microsomal radioactivity remaining after 24 h.

The initial concentration of cytochrome P-450 (pmoles/mg microsomal protein) in isolated cells was 562 ± 32. The microsomal radioactivity (d.p.m./mg microsomal protein)present in isolated cells was 4200 ± 200. Accordingly the initial specific radioactivity of cytochrome P-450 (d.p.m./pmol P-450) in isolated cells was 7.4 ± 0.2.

Effect of treatments that maintain cytochrome P-450 in hepatocyte culture on its synthesis and degradation

The culture of hepatocytes for 24 h in a medium containing 0.5 mM metyrapone or 5 mM 3-hydroxypyridine maintains cytochrome P-450 at a concentration that is not significantly different ($p > 0.05$) from that found in freshly isolated cells (Table 1). Both compounds form high affinity ligands with cytochrome P-450 (3) and since there are numerous examples whereby ligands can protect proteins from degradation (5) one might expect these compounds to maintain cytochrome P-450 in hepatocyte culture solely by inhibiting its degradation. However the effect of metyrapone or 3-hydroxypyridine on the degradation of the cytochrome is insufficient to account for the maintained amount of cytochrome P-450 (Table 1). In addition these compounds significantly ($p < 0.05$) lower the specific radioactivity of cytochrome P-450 demonstrating that they have an important effect on the synthesis of the cytochrome, which does not occur in untreated cells. It can be estimated from the specific radioactivity of cytochrome P-450 in hepatocytes cultured with metyrapone or 3-hydroxypyridine that 40-50% of the maintained level of cytochrome P-450 is due to newly synthesized cytochrome. In contrast the results in Table 1 show that the ability of metyrapone to maintain high concentrations of cytochrome P-450 in cultured hepatocytes is apparently independent of protein synthesis, as cells cultured with metyrapone and cycloheximide contain a cytochrome P-450 concentration that is not significantly different ($p > 0.05$) from that found in cells cultured with metyrapone alone. This results from the inability of medium containing 5 μM cycloheximide, which was found to inhibit general protein synthesis by 95%, to completely inhibit the effect of metyrapone on the synthesis of the cytochrome and from the additive effects of cycloheximide and metyrapone on cytochrome P-450 degradation (Table 1). The failure of cycloheximide to completely inhibit the effect of metyrapone on cyto-

chrome P-450 synthesis is demonstrated by the finding that the specific radioactivity of cytochrome P-450 in hepatocytes cultured with cycloheximide and metyrapone is significantly different ($p < 0.05$) from untreated cells (Table 1).

In conclusion the results presented clearly show that the mechanisms underlying the loss of cytochrome P-450 in cultured hepatocytes are impaired synthesis and enhanced degradation. Metyrapone and 3-hydroxypyridine maintain cytochrome P-450 by affecting both processes.

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