Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 672-679

APPARENT LACK OF CORRELATION BETWEEN THE LOSS OF CYTOCHROME P-450 IN HEPATIC PARENCHYMAL CELL CULTURE AND THE STIMULATION OF HAEM OXYGENASE ACTIVITY

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Received February 14,1978

SUMMARY The hypothesis that the stimulation of haem oxygenase activity in cultured adult rat liver parenchymal cells is intimately associated with the accelerated breakdown of the haemoprotein cytochrome P-450 was examined. Even though the time course of the loss of cytochrome P-450 and the stimulation of haem oxygenase activity were found to be compatible with this hypothesis, further work however showed that high levels of cytochrome P-450 could be maintained in liver cell culture in the face of high haem oxygenase activities.

INTRODUCTION

The activity of the hepatic microsomal cytochrome P-450 linked drug metabolising system frequently parallels the toxicity and carcinogenicity of many natural and synthetic chemicals (1,2). Since the activity of the P-450 system is affected by nutrition (3), hormonal status (4) as well as exposure to environmental inducing agents (4) many investigators have utilised the defined conditions of cell culture in order to study the factors which regulate cytochrome P-450 and hence determine toxicity and carcinogenesis. However the rapid loss of cytochrome P-450 and associated oxidations dependent on this haemoprotein during the first 24 hours of primary hepatocyte culture (5-7) have limited the kinds of questions that can be answered. The knowledge that haem, an essential component of cytochrome P-450, is degraded by

Abbreviation HBSS = Hank's balanced Salt Solution without Calcium and Magnesium.

0006-291X/78/0812-0672$01.00/0

an inducible microsomal enzyme system called haem oxygenase (8,9) coupled with the finding that the loss of cytochrome P-450 in primary hepatocyte culture appears to be associated with a reciprocal increase in haem oxygenase activity has led to the proposal (6,10) that the degradation of cytochrome P-450 haem and the stimulation of haem oxygenase activity may be casually related. This hypothesis has, up to now, been difficult to test especially as it has proved difficult to maintain cytochrome P-450 in cultured hepatocytes and additionally because no inhibitors of haem oxygenase activity are known. Recently Decad et al.(11) showed that cytochrome P-450 can be maintained in hepatocyte culture at concentrations close to the level found in liver in vivo by the incorporation of numerous hormones into the culture medium. This system therefore afforded the opportunity to test the hypothesis (10) that the stimulation of haem oxygenase activity is associated with accelerated breakdown of cytochrome P-450. The work reported here is apparently incompatible with this hypothesis and shows that high levels of cytochrome P-450 can be maintained in primary hepatocyte culture in the face of high haem oxygenase activities.

MATERIALS AND METHODS

Isolation of hepatocytes Adult (180-250 gm) male rats of the Porton derived Wistar strain, fed ad libitum on MRC 41B diet (12), were anaesthetised by injection with 60 mg/kg (i.p.) pentobarbitone (Sagatal^{R}, May & Baker, Dagenham, England), and the portal vein cannulated with an 18 gauge intravenous cannula (Argyle Medicut^{R}, Southern Syringe Services, 843 Green Lane, London N.21, England). The vena cava was then cut and the liver excised whilst perfusing via the portalvein, with Hanks Balanced salt solution without calcium and magnesium (HBSS) (Gibco Biocult Ltd., Paisley, Scotland) ± the additions described by Decad et al.(11) except that albumin, oleic and linoleic acids were omitted from the perfusion medium.

After excision of the liver the hepatocytes were isolated by perfusion at 50 cm^3/min with 0.05% w/v collagenase (Worthington Type 11, supplied by Cambrian Chemicals Croydon, Surrey, England) in HBSS ± the additions referred to above using the technique described by Judah et al.(13). This process yielded approximately 3×10^8 parenchymal cells per liver depending on the size of rat

used with a viability of 88 ± 6 (mean ± S.D. 18 perfusions) as assessed by trypan blue exclusion.

Culture of cells The resultant cell suspension containing approximately 90% parenchymal cells contaminated with 10% reticuloendothelial cells as assessed by their size distribution (r.e. cells 5-15 μm, parenchymal cells > 16 μm) on a Coulter counter model ZB_I was added to the required volume of culture medium and seeded at a density of 7 x 10^6 cells/10 cm^3 culture medium into a 100 mm diameter petri dish (Corning ™ obtained from Fisons, Loughborough, England) which were either untreated or coated in the laboratory with rat tail collagen exactly as described by Michalopoulos and Pitot (14). The time taken from the start of the perfusion to seeding the cells in petri dishes was approximately 60 minutes.

Culture medium was either Williams medium E containing 5% (v/v) foetal calf serum and 5 mg% gentamicin or Waymouths MB 752/1 medium without serum but ± the additions described by Decad et al. (11). All media, serum and gentamicin were purchased from Flow Laboratories Irvine, Scotland.

Biochemical determinations The cytochrome P-450 content and haem oxygenase activity of the intact liver was determined by ligating and excising a liver lobe perfused with HBSS prior to contact with collagenase. The P-450 content and haem oxygenase activity of the lobe and isolated hepatocytes were determined immediately after isolation by homogenisation in a volume of 0.15 M KCl in 50 mM tris buffer pH 7.4 calculated to give a 10% w/v homogenate. Biochemical determinations were performed on cultured hepatocytes after each 100 mm petri dish had been washed with 20 cm^3 ice cold 0.15 M NaCl and stored overnight as a monolayer at -70°C. The cells were then scrapped from 2 x 100 mm petri dishes and pooled into 5 cm^3 ice cold - 0.15 M KCl in 50 mM tris buffer pH 7.4 for the cytochrome P-450 determination or into 1.5 cm^3 ice cold 0.15 M KCl in 50 mM tris buffer pH 7.4 for the haem oxygenase assay. The cells destined for the P-450 determination were then sonicated for 10 sec using a Dawe Soniprobe sonicator (Dawe Instrument Ltd., London, W.3. England) at an amplitude of 2. No loss or gain of cytochrome P-450 was found when liver homogenates were diluted to the same protein concentration as the cultured cells and sonicated for 10-20 seconds. However sonication of liver homogenates under these conditions resulted in a marked loss of haem oxygenase activity. Accordingly the cells destined for assay of haem oxygenase activity were homogenised by 20 up and down strokes of glass homogeniser (Jencons Scientific Ltd., Hemel Hempstead, England).

Cytochrome P-450 determination Since the amount of cytochrome P-450 recovered in 10,000 g supernatant varies with the amount initially present (15) cytochrome P-450 was measured in homogenates rather than 10,000 g supernatants in order to avoid any losses during preparation exaggerating the effects of the culture conditions employed. Accordingly cytochrome P-450 was determined in homogenates of perfused liver, isolated cells and cultured cells by recording the difference spectrum of reduced homogenate + CO vs homogenate + CO between 490 nm - 420 nm exactly as described by McLean and Day (15). In addition P-450 was also determined by the standard technique of Omura and Sato (16) by recording the difference spectrum of reduced homogenate + CO vs reduced homogenate. Both techniques gave values of P-450 that were within 10% of each other with homogenates of perfused liver or isolated cells as well

TABLE 1 Time course of the loss of cytochrome P-450 and increase in haem oxygenase activity in adult rat liver parenchymal cells cultured in William's medium E containing 5% foetal calf serum.

Condition	Cytochrome P-450 concentration p moles/mg protein	Haem oxygenase activity p moles bilirubin formed /min/mg protein
Liver lobe	168 ± 28 (n=6)	16 ± 5 (n=4)
Isolated cells	150 ± 37 (n=8)	17 ± 5 (n=4)
Cells after 4 hr culture	127 ± 23 (n=8)	33 ± 3 (n=4)*
Cells after 8 hr culture	106 ± 11 (n=4)	61 ± 10 (n=4)*
Cells after 16 hr culture	35 ± 4 (n=4)*	115 ± 26 (n=4)*
Cells after 24 hr culture	31 ± 12 (n=8)*	89 ± 28 (n=4)*

Rat liver parenchymal cells were isolated by perfusing the liver with 0.05% (w/v) collagenase in HBSS -additions as described in the Methods Section. The isolated cells were then seeded in Williams medium E containing 5% (v/v) foetal calf serum at a density of 7 x 10^6 cells/10 cm^3 medium in 100 mm diameter plastic petri dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were harvested after the respective time in culture and the level of cytochrome P-450 and haem oxygenase activity assayed in cell homogenates as described in the Methods Section. Similarly cytochrome P-450 was determined in homogenates of liver and isolated cells but due to the low rate of haem oxygenase activity in homogenates as well as their turbidity the haem oxygenase activity of intact liver and isolated cells was measured in a post mitochondrial supernatant which was prepared by centrifuging homogenates at 10,000 g for 20 minutes. The haem oxygenase activity of the post mitochondrial supernatant was then corrected for the recovery (74%) of microsomes using cytochrome P-450 as the microsomal marker and is expressed above per mg homogenate protein. All results are expressed as the mean ± S.D. of individual values and n represents the number of separate experiments.

* indicates significantly different from isolated cell value ($P < 0.05$).

as in cultured cells which contained more than 60 p moles cytochrome P-450 per mg protein. Below this concentration interference from reduced mitochondrial cytochromes in the reduced + CO vs CO procedure resulted in the loss of the distinct P-450 peak. Accordingly the P-450 concentration of cells containing less than 60 p moles/mg protein had to be determined by the reduced + CO vs reduced difference spectrum using the correction described by Kowal *et al.*(17) for the interference produced by the interaction of cytochrome a_3 + CO. Preliminary experiments (Paine, Williams and Legg unpublished) have shown that the P-450 concentrations found under the conditions shown in Tables 1 and 2 are in good agreement with the level of pyramidon demethylase, a P-450 linked enzyme whose activity has been shown to parallel the concentration of cytochrome P-450 (7).

Haem Oxygenase activity was assayed on Perkin-Elmer 356 spectrophotometer in the dual beam mode exactly as described by De Matteis & Gibbs (18).

Protein was assayed by the method of Lowry *et al.*(19) using crystalline bovine serum albumin (Sigma) as the standard.

Statistics The results are expressed as the mean ± S.D. of individual values of cytochrome P-450/mg protein and haem oxygenase activity (expressed as p moles bilirubin formed/minute/mg protein) averaged from duplicate determinations, which were found to agree within 10% using cells from the same experiment; n represents the number of separate experiments. Student's t test was used as a test of significance.

RESULTS AND DISCUSSION

Table 1 shows that there is no loss of cytochrome P-450 during the cell isolation (ie perfusion) technique or during the first 8 hours of culture. In contrast, however the haem oxygenase activity of the cultured cells rises rapidly during the first 8 hours of culture and after 16 hours reaches maximum activity, which is 6.8 fold higher than found in the intact liver or freshly isolated cells. Concomittant with this rise in haem oxygenase activity the level of cytochrome P-450 falls such that after 16 hours in culture the P-450 concentration is only 20% of that found in freshly isolated cells. Such a similar time course in the loss of cytochrome P-450 and rise in haem oxygenase activity has undoubtably encouraged the proposal that the stimulation of haem oxygenase activity is intimately associated with the turnover of cytochrome P-450 in hepatic parenchymal cells in culture (6,10).

Decad et al.(11), showed that the addition of several hormones to the culture medium maintained the cytochrome P-450 content at the same concentration found in liver, when the isolated hepatocytes were cultured in collagen coated petri dishes. However these authors gave no details as to whether the hormones were added to the medium used for cell isolation (ie perfusion) nor for that matter the cytochrome P-450 concentration of cells cultured on a plastic rather than a collagen coated surface. In the course of determining the effects of these variables the conditions that maintained cytochrome P-450 found in hepatocytes after 24 hours in culture at low, intermediate and high concentrations were found. These conditions and the cytochrome P-450 concentration found under them are shown in Table 2. The highest concentration of cytochrome P-450 was found when the several hormones included in the culture medium by Decad et al.(11) were also added to the perfusion medium and the cells cultured on a collagen substratum. The

TABLE 2 Effect of isolation and culture conditions on the levels of cytochrome P-450 and haem oxygenase activity of isolated rat hepatic parenchymal cells after 24 hours culture in MB 752/1 medium.

Treatment additions to: Perfusion Medium	Culture Medium	Substratum	Cytochrome P-450 p moles/mg protein n=4	Haem oxygenase activity p moles bilirubin formed /min/mg protein n=4
-	-	Plastic	39 ± 17	83 ± 15
+	-	Plastic	67 ± 5	82 ± 3
+	+	Plastic	83 ± 6	81 ± 20
+	+	Collagen	121 ± 20	97 ± 25

Rat liver parenchymal cells were isolated by perfusing the liver with 0.05% (w/v) collagenase in HBSS ± the additions described by Decad et al.(11) but without albumin, oleic and linoleic acids. The isolated cells were then seeded in Waymouths MB 752/1 medium ± all the additions described by Decad et al.(11) at a density of 7 x 10^6 cells/10 cm^3 medium in 100 mm diameter plastic petri dishes which were either untreated (plastic substratum) or pre-coated with rat tail collagen (collagen substratum) as described in the Methods Section. 4 Hours after seeding the cells the medium was aspirated and replaced with 10 cm^3 of the respective medium. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air throughout the experiment. 24 Hours after seeding the cells into petri dishes the cell monolayer was washed and the cells harvested for P-450 and haem oxygenase determination as described in the Methods Section. The results are expressed as the mean ± S.D. of individual values and n represents the number of separate experiments.

P-450 concentration of 121 ± 20 (n = 4) p moles/mg protein found after 24 hours culture under these conditions (Table 2) is not significantly different from the P-450 concentration of 150 ± 37 (n = 8) found in freshly isolated cells (Table 1). Cells isolated and cultured in the presence of hormones but grown on a plastic substratum had significantly lower concentration of cytochrome P-450 than cells cultured on collagen (Table 2). Omission of the hormone mixture from the culture medium altogether significantly lowered the P-450 concentration still further whilst the complete absence of hormones in the perfusion and culture medium resulted in an even lower P-450 content being found after 24 hours in culture (Table 2). The concentration of cytochrome P-450 found under these four conditions are significantly different from each other ($P < 0.05$) and therefore seem to represent distinct groups of cells containing 26%, 45%, 55% and 100% of the P-450 concentration found in liver (compare

Tables 1 and 2). Inspite of these differences in P-450 concentration, all 4 groups of cells contained highly elevated levels of haem oxygenase activity compared to the intact liver (cf Tables 1 and 2). Furthermore even though the P-450 concentrations range from 26% to 100% of the P-450 concentration found in intact liver there is no significant difference in the haem oxygenase activity found in cells containing different P-450 concentrations. Accordingly the results in Table 2 suggest that haem oxygenase activity is not the sole determinant of the cytochrome P-450 concentration of cultured cells and clearly show that high levels of cytochrome P-450 can be maintained in the face of high levels of haem oxygenase activity. Whether this is due to an increased rate of haem synthesis under the conditions employed remains to be determined.

Acknowledgements We are grateful to Dr. F. De Matteis and A.H. Gibbs for advice, encouragement and help with the haem oxygenase assay.

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