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Maintenance of total cytochrome *P*-450 content in rat hepatocyte culture and the abundance of CYP1A2 and CYP2B1/2 mRNAs

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mRNAs encoding cytochrome *P*-450s CYP1A2 and CYP2B1/2 have been quantified in rat hepatocytes cultured for periods up to 72 h under several different culture conditions that maintain total cytochrome *P*-450 content. When hepatocytes were cultured at either 37 or 30 °C in Williams E media, both CYP1A2 and CYP2B1/2 mRNAs declined dramatically. However, when cultured at 30 °C for 24 h, the decline in these mRNAs was not as great as that observed in cells grown at 37 °C. The addition of dimethyl sulphoxide to cells grown at 37 °C did not affect the rate of disappearance of the CYP1A2 or CYP2B1/2 mRNAs. These mRNAs also declined rapidly in cells grown in '*P*-450 medium' i.e. RPMI 1640 medium without cyst(e)ine but supplemented with 0.1 mM-δ-aminolaevulinic acid. However, the levels of CYP2B1/2 mRNAs were maintained when hepatocytes were cultured in Williams E medium supplemented with 0.5 mM-metyrapone. These conditions did not, however, maintain the levels of CYP1A2 mRNA.

INTRODUCTION

Cytochrome *P*-450 is the collective term for a gene superfamily of proteins that are important determinants of the pharmacological and toxicological activity of numerous endogenous and foreign chemicals [1]. To date, some 28 isoenzymes comprise the *P*-450 content of rat liver [2], and their relative proportions vary depending on physiological status as well as exposure to inducing chemicals [3]. In view of the diversity of mechanisms regulating cytochromes *P*-450, the defined conditions of liver cell culture are a popular model system (reviewed in [4]). However, when rat hepatocytes are cultured there is a rapid loss of many cytochromes *P*-450 [5]. Collectively these losses may involve enhanced cytochrome *P*-450 degradation and a reduced ability of cultured hepatocytes to synthesize these haemoproteins [6]. Culture systems have therefore been devised that retard the loss of total cytochrome *P*-450 content in rat hepatocyte culture. These include the culture of hepatocytes at 30 °C rather than 37 °C [7], the inclusion in the culture medium of metyrapone or dimethyl sulphoxide (DM50) at final concentrations of 0.5 mM and 2% (v/v) respectively [8,9], or culture in a medium lacking cyst(e)ine but supplemented with δ-aminolaevulinic acid (known as '*P*-450 medium') [10].

However, as cytochrome *P*-450 is a collective term for a superfamily of haemoproteins, the current work investigates the mechanisms underlying the ability of diverse culture conditions to prevent the loss of the total content of cytochromes *P*-450 by examining the effects on the expression of specific cytochromes *P*-450, namely CYP1A2 and members of the CYP2B subfamily. These cytochromes *P*-450 were chosen because previous work had shown that culture of hepatocytes with metyrapone, or in '*P*-450 medium', markedly affected the enzyme activities associated with these proteins [11,12]. Therefore, the present work describes the use of cloned cDNAs to determine the effect of various culture conditions on the expression of the mRNAs encoding CYP1A2 and CYP2B1/2 in hepatocytes.

MATERIALS AND METHODS

Isolation and culture of hepatocytes

Hepatocytes were isolated from adult male CD rats (250–290 g) (supplied by Charles River Ltd., Margate, Kent, U.K.) as described by Paine *et al.* [8], and were cultured at a density of 18×10^6 cells/20 ml of medium in 150 mm-diam. Lux Petri dishes (Flow Laboratories, Irvine, Scotland, U.K.). Hepatocytes were cultured in Williams medium E (WME) supplemented with 5% foetal calf serum, gentamicin (60 μg/ml) (all from Flow), 1 μM-insulin and 0.1 mM-cortisol 21-hemisuccinate (both from Sigma Chemical Co., Poole, Dorset, U.K.) unless stated otherwise. Where indicated, this medium was supplemented with either metyrapone (0.5 mM) or 2% (v/v) DMSO (both from Sigma). Cells were cultured at 30 or 37 °C in a humidified atmosphere of 5% CO_2 in air for 24–72 h. Another culture condition involved the incubation of cells at 37 °C in *P*-450 medium (RPMI 1640 medium prepared in this laboratory [10]), which is cyst(e)ine-free and supplemented with 0.1 mM-δ-aminolaevulinic acid (Sigma).

Cytochrome *P*-450 and protein determination

These were determined in cell homogenates as previously described [8].

RNA isolation

The cells from four 150 mm-diam. dishes per treatment regime were harvested into 12 ml of sterile Dulbecco's phosphate-buffered saline, pH 4.5 (Flow), and the total RNA was isolated by the guanidinium/hot phenol method described by Maniatis *et al.* [13], except that the concentration of guanidinium thiocyanate (Fluka Chemicals, Glossop, Derbyshire, U.K.) was 6 M and the proteinase K step was omitted. Redistilled phenol was purchased from Gibco BRL Ltd. (Uxbridge, Middx., U.K.). The RNA obtained was dissolved in sterile diethyl pyrocarbonate-treated water, quantified by its absorbance at 260 nm and stored at −70 °C in small aliquots for future use.

Abbreviations used: WME, Williams medium E, *P*450 medium, RPMI 1640 without cyst(e)ine supplemented with 0.1 mM-δ-aminolaevulinic acid; DMSO, dimethyl sulphoxide; HBSS, Hanks balanced salt solution.

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Northern blotting and hybridization

RNA (20 μg) was electrophoresed through a 1.5% (w/v) agarose gel containing formaldehyde (1.9%, v/v), and blotted on to a nylon membrane (Hybond-N; Amersham International, Aylesbury, Bucks., U.K.) as described by Fourney *et al.* [14]. The RNA was fixed to the membrane by exposure to u.v. light at 306 nm, and the filter was stored at −70 °C until hybridization.

Cloned cDNAs coding for rat cytochromes *P*-450 CYP1A2 [15] and CYP2B2 [16] were excised from their vectors and purified from low-melting-point agarose gels as described by Maniatis *et al.* [13]. cDNA inserts were labelled with [α-^{32}P]dCTP (800 Ci/mmol; Amersham International) to a specific radioactivity of 10^8 d.p.m./μg by random priming (Multiprime Kit; Amersham International).

The membranes were pre-hybridized and then hybridized at 42 °C for 44 h as described by Pike *et al.* [17], except that the hybridization buffer contained 5 × Denhardt's solution and did not contain dextran sulphate. After hybridization, the membranes were washed as described by Pike *et al.* [17] to a final stringency of 0.1 × SSPE containing 0.1% SDS at 50 °C, and autoradiographed at −70 °C for 72 h (1 × SSPE = 0.18 M-NaCl, 10 mM-NaH_2PO_4 and 1 mM-EDTA, pH 7.7).

The content of poly(A)$^+$ RNA in each track of a Northern blot was determined by hybridization of the membrane to an oligo(dT)$_{18}$ probe (Pharmacia, Milton Keynes, Bucks., U.K.) as described by Harley [18], except that the membranes were pre-hybridized for 1 h at 25 °C and the hybridization buffer and wash solutions contained 2 × SSPE. The oligo(dT)$_{18}$ probe was end-labelled with [γ-^{32}P]ATP, as described by Maniatis *et al.* [13]. Autoradiography was performed overnight at −70 °C.

The hybridization signals corresponding to CYP1A2 and CYP2B1/2 mRNAs and poly(A)$^+$ RNA were quantified by laser densitometry using an LKB Ultra-Scan XL laser densitometer employing LKB 2400 Gel-Scan software. The hybridization signals corresponding to the *P*-450 mRNAs were normalized with respect to the poly(A)$^+$ RNA signal.

Statistics

Results are expressed as the means ± S.D. of values found in individual hepatocyte preparations ($n = 3$). Student's t test was used to determine the significance of the results. Where results are quoted without S.D. values, these are the average of two separate cell preparations and the Bonferroni correction was applied to test the significance of these values.

RESULTS

The effects of five different sets of culture conditions on the relative abundance of CYP1A2 and CYP2B1/2 mRNAs were investigated by Northern blot hybridization with cloned cDNAs (Figs. 1 and 2; Table 1). In all instances the hybridization signal corresponded to RNA species of 2100 nucleotides for CYP1A2 and 1800 nucleotides for the CYP2B subfamily, which are in accord with published data [19,20]. The apparent difference in size of the mRNA in isolated and cultured hepatocytes relative to the fresh liver sample (Figs. 1 and 2) is believed to be an artefact, as the 28 S and 18 S rRNA bands, as seen on ethidium bromide-stained gels, followed the same arched pattern.

Upon Northern blot hybridization with a CYP1A2 cDNA, a less intense hybridization signal was obtained from RNA extracted from freshly isolated hepatocytes than from RNA extracted from intact liver (Fig. 1, lanes 1 and 9), despite the fact that equal amounts of each RNA sample (as judged by A_{260} measurements) were loaded on to the gel. A similar discrepancy was observed when blots were hybridized with a CYP2B1/2 cDNA (Fig. 2, lanes 1 and 9). However, when the hybridization signals were normalized for the amount of poly(A)$^+$ RNA on the membrane, it was found that the abundances of CYP1A2 and CYP2B1/2 mRNAs were the same in freshly isolated hepatocytes as in intact liver. The effect of the culture conditions upon the abundance of these mRNAs is described below.

(*a*) Standard conditions (WME) at 37 °C

When hepatocytes were cultured at 37 °C in WME, their total *P*-450 content decreased to 49 ± 15% of the initial value after 24 h (Table 2). Concomitant with this decrease, the abundance of the mRNAs encoding CYP1A2 and the CYP2B subfamily declined to 27% and 28% of their initial values respectively

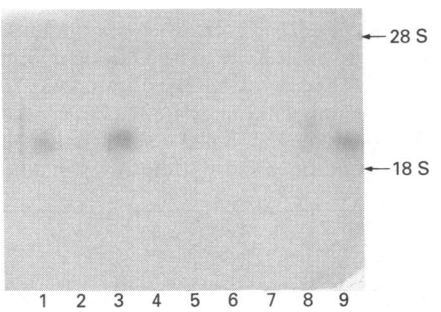

Fig. 1. Northern blot of purified rat hepatocyte RNA probed with a ^{32}P-labelled cDNA probe to CYP1A2 mRNA

CYP1A2 was visualized on a Northern blot probed with a specific CYP1A2 cDNA as described in the Materials and methods section. Lane 1, freshly isolated hepatocytes; lane 2, control hepatocytes cultured for 24 h; lane 3, metyrapone-treated hepatocytes cultured for 24 h; lane 4, control hepatocytes cultured for 48 h; lane 5, metyrapone-treated hepatocytes cultured for 48 h; lane 6, control hepatocytes cultured for 72 h; lane 7, metyrapone-treated hepatocytes cultured for 72 h; lane 8, freshly isolated hepatocytes; lane 9, intact liver lobe from the same animal as the cultured hepatocytes prior to isolation. The apparent difference in size of the mRNA in isolated and cultured hepatocytes relative to that from the fresh liver sample is believed to be an artefact of the method of data presentation.

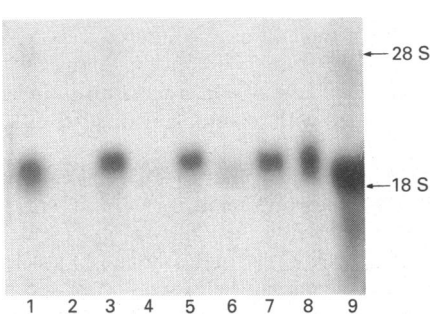

Fig. 2. Northern blot of purified rat hepatocyte RNA probed with a ^{32}P-labelled cDNA probe to CYP2B1/2 mRNAs

CYP2B1/2 mRNAs were visualized on a Northern blot probed with a specific CYP2B2 cDNA which, because of 97% sequence identity, will cross-hybridize to CYP2B1 [16]. Lane 1, freshly isolated hepatocytes; lane 2, control hepatocytes cultured for 24 h; lane 3, metyrapone-treated hepatocytes cultured for 24 h; lane 4, control hepatocytes cultured for 48 h; lane 5, metyrapone-treated hepatocytes cultured for 48 h; lane 6, control hepatocytes cultured for 72 h; lane 7, metyrapone-treated hepatocytes cultured for 72 h; lane 8, freshly isolated hepatocytes; lane 9, intact liver lobe from the same animal as the cultured hepatocytes prior to isolation. The apparent difference in size of the mRNAs in isolated and cultured hepatocytes relative to the fresh liver sample is believed to be an artefact of the method of data presentation.

Table 1. Effect of hepatocyte culture conditions on the relative abundance of CYP1A2 and CYP2B1/2 mRNAs

Rat hepatocytes were cultured under the various regimes for 0–72 h, and the abundances of cytochrome CYP1A2 and CYP2B1/2 mRNAs were determined as described in the Materials and methods section. The results are presented as the means ± S.D. of at least three individual cell preparations, unless quoted without S.D., in which case these are the averages of two separate cell preparations and the Bonferroni correction was used to test the statistical significance of these results. * Denotes significantly different ($P < 0.05$) from isolated cell value. N.D., not detectable.

Culture medium	Temp. (°C)	Culture period (h)...	Abundance of CYP1A2 mRNA (% of initial value) 24	48	72	Abundance of CYP2B1/2 mRNAs (% of initial value) 24	48	72
WME	37		27±15*	9±6*	N.D.	28±18*	36±8*	27±11*
WME	30		88	27	15	71	23	10
WME+DMSO	37		36±22*	9±4*	7±4*	29±12*	14±6*	18
P-450 medium	37		46±24*	12±9*	15±5*	22±5*	25±7*	34±10*
WME+metyrapone	37		58±19*	26±8*	12±2*	81±45	126±12*	167±22*

Table 2. Effect of hepatocyte culture conditions on the loss of cytochrome *P*-450 content

Rat hepatocytes were cultured under the various regimes for 0–72 h and their cytochrome *P*-450 content was determined in cell homogenates as described in the Materials and methods section. The results are presented as the means ± S.D. of at least three individual determinations. The initial cytochrome *P*-450 concentration of freshly isolated hepatocytes before culture was 202±42 pmol/mg of protein. In previous studies [23] the *P*-450 content of hepatocytes isolated by perfusion of the liver with collagenase were not found to be different from the *P*-450 content of the donor liver. * Denotes significantly different ($P < 0.05$) from untreated value (WME at 37 °C) at same time point.

Culture medium	Temp. (°C)	Culture period (h)...	Abundance of cytochrome *P*-450 (% of initial value) 24	48	72
WME	37		49±15	41±10	31±6
WME	30		89±11*	50±14	39±5
WME+DMSO	37		79±8*	76±6*	31±3
P-450 medium	37		80±9*	59±6*	56±13*
WME+ metyrapone	37		92±17*	111±28*	113±30*

(Table 1; lane 2 in Figs. 1 and 2). Longer periods of culture in this medium resulted in a further decline in CYP1A2 mRNA abundance, and by 72 h of culture this was below the level of detection (Table 1). In contrast, the abundance of CYP2B1/2 mRNA after 48 and 72 h of culture remained similar to that found after 24 h of culture (Table 1).

(*b*) Standard conditions (WME) at 30 °C

Reducing the temperature at which hepatocytes were cultured in WME retarded the decline in the total cytochrome *P*-450 content during the first 24 h of culture ($P < 0.05$) (Table 2), and also retarded the decline of CYP1A2 and CYP2B mRNAs; these values were not found to be statistically different using the Bonferroni correction compared with the isolated cell value.

(*c*) Standard (WME) medium containing 2% (v/v) DMSO

The culture of hepatocytes at 37 °C in WME supplemented with 2% (v/v) DMSO also retarded decline in the total *P*-450 content ($P < 0.05$) (Table 2), but had little effect on the decline of CYP1A2 and CYP2B1/2 mRNAs compared with culture at 37 °C in medium without DMSO (Table 1).

(*d*) *P*-450 medium

The culture of hepatocytes at 37 °C in this medium, which is based on RPMI 1640 lacking cyst(e)ine and supplemented with 0.1 mM-aminolaevulinic acid, 5% foetal calf serum, gentamicin (60 μg/ml), 1 μM-insulin and 0.1 mM-cortisol 21-hemisuccinate, had little effect on the decline in the CYP1A2 and CYP2B1/2 mRNAs compared with culturing hepatocytes at 37 °C in WME (Table 1).

(*e*) Standard medium containing 0.5 mM-metyrapone

The treatment found to be most effective in preventing the loss of the total amount of cytochrome *P*-450 throughout the 72 h period of study was the culture of hepatocytes at 37 °C in WME containing 0.5 mM-metyrapone (Table 2). These culture conditions also maintained the abundance of CYP2B1/2 mRNAs at initial or higher levels throughout the 72 h period of study (Table 1; lanes 3, 5 and 7 in Fig. 2). However, CYP1A2 mRNA levels were not maintained in the presence of metyrapone, and declined throughout the culture period (Table 1; lanes 3, 5 and 7 in Fig. 1).

DISCUSSION

The present work shows that the loss of total *P*-450 content in rat hepatocyte cultures is paralleled by a loss of specific *P*-450 mRNAs. Retarding the loss of total *P*-450 content by culturing hepatocytes at 30 °C, particularly for 24 h, also retarded the decline of both CYP1A2 and CYP2B1/2 mRNAs. In contrast to culture at 30 °C, culture of hepatocytes in medium containing 2% (v/v) DMSO maintained total *P*-450 content, but had no significant effect on the loss of CYP1A2 and CYP2B1/2 mRNAs. Accordingly, the DMSO culture system may maintain the total *P*-450 content of cultured rat hepatocytes by affecting the expression of *P*-450 isoenzymes other than the CYP1A2 and CYP2B subfamilies. However, we specifically chose to study the effects of hepatocyte culture conditions on the expression of the CYP1A and CYP2B subfamilies because previous enzymological studies demonstrated that the culture of hepatocytes with metyrapone or in *P*-450 medium had markedly different effects on xenobiotic metabolism mediated by these cytochromes. Thus, although both media maintained total cytochrome *P*-450 content, only hepatocytes cultured with metyrapone fully maintained ethylmorphine *N*-demethylase activity, which was considered, at the time, to be associated with the phenobarbitone-inducible CYP2B subfamily. Furthermore, only culture with metyrapone markedly induced enzyme activities associated with the CYP1A subfamily [11,12]. However, subsequent immunochemical quant-

ification of these cytochromes in hepatocyte culture produced somewhat contradictory results, in that although the CYP1A apoproteins were increased by metyrapone, CYP2B apoproteins were not detectable [21]. The present work also appears, at first sight, to be at variance with these apoprotein levels, because although CYP2B1/2 mRNAs are maintained or elevated by metyrapone in culture at the same level as found in the donor liver, the apoproteins are not immunochemically detectable. This is in agreement with others [22], but it must be emphasized that neither are the apoproteins detectable in the intact liver [21,22]. The ability of metyrapone to maintain the content of CYP2B1/2 mRNAs in hepatocyte culture may, therefore, actually be in accord with its ability to maintain the enzymic activities associated with this family of *P*-450s at the same level as found in the intact liver. In contrast, culture of hepatocytes with metyrapone had little effect on the level of CYP1A2 mRNA, even though this treatment is known to markedly elevate CYP1A apoproteins and associated enzyme activities [11,12,22]. A problem here may be related to the knowledge that the CYP1A subfamily comprises two genes, namely CYP1A1 and CYP1A2, and although the antibody employed in our previous immunochemical quantification of this subfamily reacts with both isoenzymes, the cDNA probe employed in the current work is specific for CYP1A2 [15]. The lack of change in the level of CYP1A2 mRNA observed in the present work, coupled to knowledge that metyrapone elevates the amounts of the CYP1A apoproteins detected immunochemically, suggests that the effect of metyrapone in hepatocyte culture is to elevate CYP1A1. Indeed, where form-specific antibodies have been employed, metyrapone has been observed to elevate CYP1A1 apoprotein levels in hepatocyte culture [22]. However, a further problem to be addressed here is that CYP1A1 is generally considered to be expressed in normal liver at undetectable levels, while CYP1A2 comprises about 2–3% of total hepatic cytochrome *P*-450 content [5,22]. These considerations suggest that the culture of rat hepatocytes with metyrapone may be producing considerable changes in CYP1A1 gene expression to account for the finding that the culture of rat hepatocytes with metyrapone can increase CYP1A apoproteins to 40% of the total cytochrome *P*-450 [21]. Alternatively, the lack of change in CYP1A2 mRNA in the presence of metyrapone observed in the present work, but the increased amounts of immunochemically detectable CYP1A apoproteins observed by us [21] and others [5,22], may suggest that metyrapone is affecting the translatability of the mRNA encoding CYP1A or else the stability of the apoprotein. As mentioned in the Introduction, the ability of metyrapone to maintain the total cytochrome *P*-450 content of cultured rat hepatocytes is due to an effect on both the synthesis and the degradation of cytochromes *P*-450 [6]. Accordingly, the combination of immunochemical and molecular biology techniques of quantification of individual forms of cytochrome *P*-450 may prove useful in identifying the many points of their regulation. Such an approach may be particularly pertinent to studying the regulation of cytochromes *P*-450 in rat hepatocyte culture, as the present study, albeit limited, demonstrates that the expression of the isoenzymes comprising the total amount of *P*-450 may display a fascinating non-uniformity in their response to the culture conditions. This merits more extensive studies on a form-by-form basis.

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