RESEARCH COMMUNICATION

Altered expression of cytochrome *P*-450 mRNAs, and potentially of other transcripts encoding key hepatic functions, are triggered during the isolation of rat hepatocytes

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The abundance of 12 cytochrome *P*-450 (CYP) mRNAs was quantified in the caudate lobe of rat livers before dissociation of the organ into single cells by perfusion with 0.025% (w/v) collagenase. Comparison of the initial abundance of CYP-1A1, -1A2, -2A subfamily, -2B1/2, -2C7, -2C11, -2D subfamily, -2E1, -3A1/2 and -4A1 transcripts in the caudate lobe of the intact liver with the values found in freshly isolated hepatocytes demonstrated that the relatively brief (1 h) cell isolation and washing procedures routinely caused 2–3-fold increases in the mRNAs encoding CYP-1A2, -2B1/2, -3A1/2 and -4A1, concomitant with a 50% decline in CYP2C11 mRNA. Further changes in the expression of CYP mRNAs occurred when the hepatocytes were cultured. Thus CYP1A1 mRNA, which is not constitutively expressed in rat liver, became detectable in hepatocytes cultured for 1 h, and after 6 h CYP3A1/2 mRNA levels began to increase. In contrast, levels of all other CYP mRNAs

studied had declined after 24 h of culture concomitant with the loss of total cytochrome *P*-450 content. Culture of hepatocytes with 0.5 mM metyrapone (which prevents the loss of total *P*-450 content) increased CYP1A1 and CYP3A1/2 mRNA levels still further, such that after 72 h of culture these transcripts were conservatively 10–18-fold higher than in hepatocytes prior to culture. This suggests that these two isoenzymes comprise the bulk of the total cytochrome *P*-450 content maintained by metyrapone. Collectively, these results demonstrate that the technique commonly used to isolate rat hepatocytes alters hepatic gene expression, as illustrated by the elevation of the mRNAs encoding CYP-1A2, -2B1/2, -3A1/2 and -4A1, and that such perturbations are exacerbated during culture under standard conditions by the loss of the constitutive CYP2C11 and the precocious induction of CYP1A1 and CYP3A1/2 mRNAs.

INTRODUCTION

Liver cell culture is a popular model system for the study of hepatic functions under defined conditions. However, many hepatic functions spontaneously decline in hepatocyte culture [1]. In this respect our laboratory has been concerned with the mechanism(s) underlying the loss of 70% of the hepatocytes' cytochrome *P*-450 content during the first 24 h of culture and the ability of culture medium containing 0.5 mM metyrapone to prevent the loss of total cytochrome *P*-450 content [2]. However, the term 'cytochrome *P*-450' encompasses a gene superfamily of haemoproteins that are important determinants of normal, homeostatic body mechanisms including cholesterol, steroid, fatty acid, prostaglandin and vitamin D₃ metabolism. Furthermore, individual cytochrome *P*-450 isoenzymes play a pivotal role in determining the delicate balance between intoxication/detoxication pathways of numerous foreign chemicals including drugs [3,4]. Accordingly, in order to begin to determine which isoenzymes of cytochrome *P*-450 underlie the well-established loss of total cytochrome *P*-450 content in rat hepatocyte culture and the ability of metyrapone to prevent this phenomenon, the present work employing cDNA and oligonucleotide probes examines the effects of the hepatocyte isolation procedure and culture with and without metyrapone on the abundance of individual mRNAs that encode hepatic cytochrome *P*-450 content.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Hepatocytes were isolated from adult male CD rats (250–290 g), supplied by Charles River Ltd., Margate, Kent, U.K., following anaesthesia resulting from intraperitoneal injection of 60 mg of sodium pentobarbitone/kg body weight (Sagatal; May & Baker, Dagenham, Essex, U.K.) as previously described [5]. Before perfusion of the liver with collagenase the caudate lobe was ligated and removed for RNA extraction. Then hepatocytes were isolated by perfusion for 30 min with Hanks balanced salt solution (Flow Laboratories, Irvine, Scotland, U.K.) containing 0.025% (w/v) collagenase H (Boehringer, Lewes, E. Sussex, U.K.). After 30 min of perfusion the liver was dissociated into a cell suspension by gentle agitation in Hanks balanced salt solution containing 2% (w/v) BSA (Sigma Chemical Co., Poole, Dorset, U.K.) followed by filtration through a 125 µm pore size Nylon mesh (Nybolt no. 10.5; John Stanair & Co., Whitefield, Manchester, U.K.) and centrifugation at 50 g for 3 min. The pellet was resuspended in approx. 75 ml of culture medium comprising Williams Medium E containing 5% (v/v) foetal calf serum, 60 µg of gentamicin/ml (all from Flow Laboratories) and supplemented with 1 × 10⁻⁶ M insulin and 1 × 10⁻⁴ M hydrocortisone 21-sodium hemisuccinate (both from Sigma). A further three centrifugation steps were performed before the cells were resuspended in 35 ml of the culture medium. An aliquot was

The nomenclature used for the cytochrome *P*-450 gene superfamily in this paper is that recommended in [23].

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Table 1 Sources of cDNAs employed and size of hybridization signal detected

Abbreviation: n.d., not detected.

CYP cDNA	Size of hybridization signal detected (kb)	Reference
1A1	2.7	[6]
1A2	2.1	[7]
2A6	2.0	[8]
2B1/2	1.8	[9]
2C7	2.0	[10]
2C11	2.1	[11]
2D6	n.d.	[12]
2E1	2.0	[13]
3A1	2.5	[14]
4A1	2.2	[15]

diluted 1:10 in PBS (pH 7.4) containing 0.1% (w/v) Trypan Blue and the cell number and viability enumerated using a haemocytometer. The viability of hepatocytes, as assessed by Trypan Blue exclusion, was routinely greater than 85%. Cells (1.8×10^7) were suspended in 20 ml of culture medium $\pm$ 0.5 mM metyrapone and added to 150 mm-diam. Lux Scientific plastic Petri dishes purchased from Flow Laboratories. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air.

RNA isolation and hybridizations

Total RNA was isolated from the intact liver, freshly isolated hepatocytes and hepatocyte cultures by a guanidinium/hot phenol method and Northern blots performed as previously described [2]. Cloned cDNAs coding for rat and human cytochromes *P*-450 (see Table 1) were labelled with [α -³²P]dCTP (3000 Ci/mmol) to a specific radioactivity of 1×10^9 d.p.m./ μ g using a Multiprime oligonucleotide-labelling kit (Amersham International, Aylesbury, Bucks., U.K.). The CYP2C11 oligonucleotide, as described by Waxman [11], was 5'-end-labelled with [γ -³²P]ATP (3000 Ci/mmol) to a specific radioactivity of 1×10^8 d.p.m./ μ g using a 5'-end-labelling kit (Promega, Southampton, Hants., U.K.). All hybridizations were carried out at 42 °C as previously described [2] except those with the CYP2C11 oligonucleotide. Prehybridization and hybridization with the labelled CYP2C11 oligonucleotide were performed at 65 °C using Quickhyb solution (Stratagene, Cambridge, U.K.) as described in the Quickhyb protocol. When hybridization was complete the blots with cDNAs were washed twice for 10 min in double-strength SSPE (SSPE = 0.15 M NaCl/9 mM NaH₂PO₄/1.25 mM EDTA, pH 7.4), containing 0.1% SDS at 25 °C, followed by two 10 min washes in 0.1 $\times$ SSPE/0.1% SDS at 25 °C and finally two washes in 0.1 $\times$ SSPE/0.1% SDS at 55 °C. Blots probed with the CYP2C11 oligonucleotide were washed twice in double-strength SSPE/0.1% SDS at 25 °C for 15 min, then for 30 min in 0.1 $\times$ SSPE/0.1% SDS at 25 °C. The hybridization signals corresponding to cytochrome *P*-450 mRNAs (Table 1) were detected by autoradiography, quantified by laser densitometry, and normalized with respect to the amount of poly(A)⁺ RNA loaded on to each track, as described previously [2], except for the experiment with α -amanitin (Sigma). Normalization of the individual CYP mRNA signals to poly(A)⁺ mRNA was

preferred over normalization to a common 'housekeeping' gene since hepatocyte culture conditions can, for example, affect the expression of the β -actin gene [16]. In the experiment with α -amanitin the synthesis of poly(A)⁺ mRNA is inhibited [17] and so the amount of RNA (predominately 28 S and 18 S rRNA) loaded in each track of the Northern blot was normalized to the individual cytochrome *P*-450 mRNA signal by reference to the ethidium bromide-stained gel, as previous studies [2] have shown quantification of RNA by its absorbance at 260 nm to be unreliable. This correction for the amount of RNA loaded on to the gel in the experiments with α -amanitin was achieved by laser densitometry of the negative of the photographed ethidium bromide-stained gel.

Statistics

The change in abundance (fold) of individual CYP mRNAs is expressed as the mean $\pm$ S.D. of the number (*n*) of individual observations with hepatocyte preparations derived from different rats. A two-tailed unpaired Student's *t*-test was used to determine statistical significance.

RESULTS AND DISCUSSION

In order to determine the effect of perfusing rat liver with 0.025% (w/v) collagenase and its subsequent dissociation into single hepatocytes, the smallest (caudate) lobe of the liver was ligated and removed before addition of collagenase to the perfusate. The results presented in Table 2 show that during the isolation of hepatic parenchymal cells the abundances of CYP-1A2, -2B1/2, -2E1, -3A1/2 and -4A1 mRNAs increase, whereas the level of mRNA encoding CYP2C11 decreases. Although the liver comprises parenchymal and non-parenchymal cells, the former population expresses the higher levels of cytochrome *P*-450 isoenzymes [18], making it unlikely that the loss of RNA associated with discarding the non-parenchymal cells is responsible for the elevation in CYP mRNA levels observed,

Table 2 Alteration of CYP mRNA levels during the hepatocyte isolation procedure

Rat hepatocytes were isolated as previously described [5]. Prior to the addition of collagenase to the perfusion medium the caudate liver lobe was removed. Total RNA was prepared from both the lobe and the freshly isolated cells. The results are expressed as the change (fold) compared with the value determined in the respective caudate lobe. Where mean and S.D. are quoted the number of individual rats studied (*n*) is given in parentheses. * denotes significantly different (*P* < 0.01) from the value found in caudate lobe before perfusion by two-tailed, unpaired Students' *t*-test. Abbreviation: n.d., not detectable.

CYP	Change in mRNA abundance in freshly isolated hepatocytes relative to the value determined in the intact caudate lobe (fold)
1A1	n.d.
1A2	2.4 $\pm$ 0.9* (9)
2A subfamily	1.1
2B1/2	2.5 $\pm$ 0.9* (10)
2C7	0.8
2C11	0.4 $\pm$ 0.1* (4)
2D subfamily	n.d.
2E1	1.8
3A1/2	3.5 $\pm$ 1.1* (10)
4A1	3.3 $\pm$ 0.2* (3)

Table 3 Abundance of CYP mRNAs in different liver lobes of the rat

Five rats were anaesthetized as described in the Materials and methods section and blood cleared from their livers by perfusion with Hanks balanced salt solution for approx. 2 min. Then the individual lobes [22] were removed, RNA extracted, and the abundance of the individual CYP mRNAs quantified. The results are presented as the means $\pm$ S.D. ($n = 5$). A two-tailed, unpaired Student's *t*-test was used to determine statistical significance, but none of the values were statistically different ($P > 0.05$) from the value determined in the caudate lobe. Abbreviation: n.d., not detectable.

CYP	Change of abundance of CYP mRNA in individual liver lobes compared with the caudate lobe (fold)		
	Median	Left lateral	Right lateral
1A1	n.d.	n.d.	n.d.
1A2	1.0 ± 0.2	0.9 ± 0.4	1.0 ± 0.3
2B1/2	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.3
2C11	1.5 ± 0.8	1.1 ± 0.5	0.9 ± 0.5
3A1/2	1.6 ± 0.6	1.4 ± 0.5	1.0 ± 0.5
4A1	1.4 ± 0.8	1.3 ± 0.8	1.3 ± 0.6

Table 4 The effect of culturing hepatocytes in the presence or absence of metyrapone upon the relative abundance of various *P*-450 mRNAs

Rat hepatocytes were isolated from a single liver except where results are quoted as the mean $\pm$ S.D., in which cases values were determined in hepatocyte cultures prepared from three separate rats ($n = 3$). Hepatocytes were cultured with (M) and without (C) 0.5 mM metyrapone for 0–72 h and the abundances of various cytochrome *P*-450 mRNAs were determined as described in the Materials and methods section. The control value at 24 h for CYP1A1 is quoted as 100%, as *P*-4501A1 mRNA is not constitutively expressed in untreated rat liver. All other values are compared with the value found in freshly isolated hepatocytes. * denotes significantly different value ($P < 0.05$) by two-tailed, unpaired Student's *t*-test from the 24 h untreated value (CYP1A1) or freshly isolated hepatocytes. Abbreviation: n.d., not detectable.

CYP	Change in CYP mRNA abundance compared with the initial value of freshly isolated hepatocytes after culture without (C) or with (M) 0.5 mM metyrapone (fold)			
	Culture period (h) ... 24		72	
	C	M	C	M
1A1	1.0 ± 0.0	$14.3 \pm 7.0^*$	2.3 ± 1.4	$12.4 \pm 7.5^*$
1A2	0.2	0.7	n.d.	0.2
2A subfamily	0.1	0.3	0.1	0.2
2B1/2	0.3	0.8	0.3	1.7
2C7	0.3	0.2	0.1	0.1
2C11	n.d.	n.d.	n.d.	n.d.
2D subfamily	n.d.	n.d.	n.d.	n.d.
2E1	0.2	0.1	0.1	n.d.
3A1/2	1.2 ± 0.3	$6.0 \pm 1.8^*$	$2.1 \pm 1.0^*$	$18.4 \pm 5.5^*$
4A1	0.2	0.2	n.d.	n.d.

especially as the RNA content of purified parenchymal cells before culture is not significantly different from the RNA content of whole liver [19]. In addition, a loss of non-parenchymal cell mRNA would not account for the dramatic decline of CYP2C11 mRNA during the cell isolation procedure (Table 2). Furthermore, the changes in the abundance of CYP-1A2, -2B1/2, -2C11, -3A1/2 and -4A1 observed between the intact caudate lobe of the liver and the parenchymal cell fraction before culture are not due to differences in their expression between the caudate

Figure 1 Northern blots of rat hepatocyte RNA probed with 32 P-labelled cDNAs to CYP1A1 (a) and CYP3A1/2 (b)

(a) Northern blot probed with cDNA to CYP1A1. Lane 1, freshly isolated hepatocytes; lanes 2, 4 and 6, hepatocytes cultured without treatment for 24 h, 48 h and 72 h respectively; lanes 3, 5 and 7, hepatocytes cultured with 0.5 mM metyrapone for 24 h, 48 h and 72 h respectively. (b) Northern blot probed with cDNA to CYP3A1. Lane 1, freshly isolated hepatocytes; lane 2, intact liver; lanes 3, 5 and 7, hepatocytes cultured without treatment for 24 h, 48 h and 72 h respectively; lanes 4, 6 and 8, hepatocytes cultured with 0.5 mM metyrapone for 24 h, 48 h and 72 h respectively.

and the other three liver lobes, all of which contribute to the average value obtained for the isolated hepatocytes, as no significant differences in the abundance of these CYP mRNAs were found between the caudate, left lateral, right lateral or median liver lobes of five individual rats (Table 3). Therefore the brief 30 min perfusion with collagenase followed by four 3-min-long washes at 50 g are sufficient to trigger 2–3-fold changes in the expression of CYPs 1A2, 2B1/2, 2C11, 3A1/2 and 4A1. This effect of the cell isolation and washing procedure on CYP mRNA abundance could be due to the loss of specific regulatory factors present in either the intact liver or blood (e.g. glucocorticoids, growth hormone, liver-specific differentiation factors), or may be due to induction by the anaesthetic commonly used for surgery, namely pentobarbitone. Pentobarbitone, being a barbiturate like phenobarbitone, may well induce certain forms of cytochrome *P*-450, e.g. CYP2Bs and CYP3A1/2 [3]. However, the *P*-450 mRNAs elevated during the cell isolation procedure have also been shown to be down-regulated by growth hormone and thyroid hormone [20] while three of them CYP1A2, CYP2Bs and CYP3A1, are induced by glucocorticoids [3]. It should therefore be noted that the medium used by us and many others to wash the cells before culture contains 1×10^{-4} M hydrocortisone and 1×10^{-6} M insulin. Furthermore, it also contains 5% (v/v) foetal calf serum and this will contribute growth and thyroid hormones. Therefore the elevation of levels of CYP2B1/2 and CYP3A1/2 mRNAs seem, at present, most likely to result from induction by the anaesthetic routinely used by us and many other workers for the preparation of rat hepatocytes, rather than from a lack of growth or thyroid hormones. Whatever the mechanisms underlying the elevated abundance of specific *P*-450 mRNAs during the hepatocyte isolation procedure, the results presented in Table 4 show that these effects are not sustained, except for CYP3A1/2, when the hepatocytes are placed in culture, as a general decline in constitutively expressed cytochrome *P*-450s occurs over the next 24–72 h. In contrast, CYP1A1 mRNA, which is not constitutively expressed in normal rat liver (Table 2 and Figure 1a), spontaneously increases in 'control/untreated' rat hepatocytes in culture, as does the expression of CYP3A1/2 (Table 4). The results presented in Table 4 and Figure 1 also show that culture of rat hepatocytes with 0.5 mM metyrapone increases the abundance of CYP1A1 and CYP3A1/2

Figure 2 Effect of α -amanitin on the abundance of CYP1A1 and CYP3A1/2 mRNAs

Hepatocytes were cultured for 18 h with 0.5 mM metyrapone and then the medium was changed to medium $\pm$ 0.5 mM metyrapone $\pm$ 1 μ g of α -amanitin/ml and the cells were cultured for a further 6 h before extraction and analysis of CYP1A1 and CYP3A1/2 mRNAs as described in the Materials and methods section.

mRNAs still further, such that these are, conservatively, 12–18-fold higher after 72 h of culture than the level found in the intact liver. A more detailed, albeit preliminary, analysis of the time course of these early changes in CYP1A1 and CYP3A1/2 expression produced by metyrapone demonstrated that the mRNA-encoding CYP1A1 becomes detectable after only 1 h of placing the isolated hepatocytes into culture, while CYP3A1/2 mRNAs increase after between 6 and 24 h of culture. In order to determine if these changes in expression of CYP1A1 and CYP3A1/2 during the first 24 h of culture were transcriptional in origin, the hepatocytes were allowed to attach to the Petri dishes for 18 h (overnight) and were then treated with α -amanitin, an inhibitor of RNA polymerase II [17]. Treatment of hepatocyte cultures with 1 μ g of α -amanitin/ml of medium between 18 and 24 h of culture inhibited the increase in CYP1A1 mRNA (Figure 2a) over this time course by 80–90%, while in the same experiment treatment with α -amanitin was without effect on the accumulation of CYP3A1/2 mRNA (Figure 2b). These preliminary results, which need to be confirmed by nuclear 'run-on' experiments, indicate that during the first 24 h of culture CYP1A1 is activated by transcriptional mechanisms while the accumulation of CYP3A1/2 mRNAs may occur by post-transcriptional mechanisms. Nevertheless, the purpose of this paper is to draw attention to the fact that the previously assumed, somewhat innocuous, dissociation of an intact liver into single hepatocytes before culture by an established, routine method has reproducible effects on one aspect of differentiated hepatic function which is immediately observable as the altered abundance of CYP-1A2, -2B1/2, -2C11, -3A1/2 and -4A1 mRNAs. Furthermore, within 1 h of culture CYP1A1 mRNA, which is not constitutively

expressed in the liver, becomes detectable, while 6–24 h later the mRNAs encoding CYP3A1/2 rise by an apparently different mechanism in the face of other constitutively expressed *P*-450s declining over the same time period. In conclusion, the changes in the expression of cytochrome *P*-450 isoenzymes and potentially other key hepatic functions during the isolation of liver cells and their subsequent culture may provide a unique opportunity to study the signals involved in regulating hepatocyte differentiation [21].

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