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SHORT COMMUNICATIONS

Effect of serum-free medium on cytochrome P450-dependent metabolism and toxicity in rat cultured hepatocytes

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Abstract—Cytochrome P450 (P450)-dependent activities in homogenates of rat hepatocytes cultured for 96 hr in serum-free and serum-containing medium were compared. Benzphetamine and erythromycin *N*-demethylases, 7-methoxy- and propoxy-coumarin *O*-dealkylases, *p*-nitrophenol hydroxylase and 7-ethoxy- and pentoxy-resorufin *O*-dealkylases were all maintained at higher levels in hepatocytes cultured in serum-free medium, although there was some selectivity with respect to the extent of the maintenance relative to the activities in fresh cells. The toxicities of coumarin, precocene I and precocene II to 24 hr hepatocyte cultures, determined as decreased survival, were also shown to be increased in serum-free medium. However, the magnitude of the difference between media with respect to the toxicity of precocene II was decreased in hepatocytes cultured for 72 hr. The observed increase in toxicity is consistent with the improved maintenance of P450 in hepatocytes cultured in serum-free medium, although there is still a selective decline in P450 activities and toxicity with increased time in culture. The activity of alcohol dehydrogenase and the toxicity of allyl alcohol were similar in hepatocytes cultured in serum-free and serum-containing medium for 96 hr. The absence of serum did not affect the non-protein sulphydryl content of the cultures.

Marked alterations in the expression and regulation of the cytochrome P450 (P450*) isozymes occur in rat hepatocytes in primary culture limiting the use of these cultures for studies of xenobiotic metabolism and toxicity. Although a range of P450-dependent activities can be maintained in serum-containing medium supplemented with dexamethasone [1, 2], isozyme-selective loss of activity occurs nonetheless, even over a 24 hr culture period. In addition to dexamethasone, the presence of other hormones has been shown to affect P450-dependent activities in freshly isolated and cultured hepatocytes [3, 4]. Therefore, the inclusion of calf serum in the medium, with its batch-to-batch variability in hormone content, may influence the expression of P450s in this culture system. Accordingly, the maintenance of a range of P450-dependent activities was determined in hepatocytes cultured in serum-free and serum-containing medium. The effect of serum-free medium on the toxicity of precocenes I and II and coumarin was also determined. The P450-dependent toxicity of these compounds to hepatocyte cultures has been reported previously [5, 6]. The non-P450 activity, alcohol dehydrogenase (ADH) and ADH-dependent allyl alcohol toxicity [7], were also determined, as were glutathione levels.

Materials and Methods

The supplies of most of the plasticware, chemicals, culture media and sera have been reported previously [2, 5]; allyl alcohol was obtained from Aldrich Chemical Co. (Gillingham, U.K.). The source and maintenance of the Wistar rats used in these studies have both been described previously [8]. Hepatocytes were isolated by lobe perfusion from adult male rats and cultured in modified Williams' Medium E containing 10% (v/v) foetal calf serum (WEC) [2]. Viability of all preparations was greater than 90%, as assessed by Trypan blue exclusion. Seeding density was 0.10–0.12 million cells/cm^2. A culture is defined as one 24-well plate or one 90-mm diameter plate. After allowing 2–4 hr for attachment, medium was replaced with either fresh WEC or the same Williams' E medium but without the serum (WEX). Medium was changed every 24 hr. Homogenates of fresh cell suspensions and 96 hr cultures were prepared by sonication [2]. The protein content of the homogenates was determined using the Bradford reagent [9], and the following enzyme activities determined as described elsewhere: benzphetamine and erythromycin *N*-demethylases (BZDM, EMDM), *p*-nitrophenol hydroxylase (PNPH), 7-ethoxyresorufin *O*-deethylase (EROD) and 7-pentoxyresorufin *O*-depentylase (PROD) [2], 7-methoxycoumarin *O*-demethylase and 7-propoxycoumarin *O*-depropylase (MCOD, PCOD; at a substrate concentration of 0.5 mM) [10], ADH [11]. Enzyme activities were expressed per mg protein. For glutathione (GSH) measurement: cultures were washed with saline, 0.3 mL 10% (w/v) sulphosalicylic acid was then added to each well and the non-protein sulphydryl (NPS) content (mainly GSH) in the supernatant was determined using the Saville assay [12]. Hepatocytes were cultured for 24 or 72 hr in WEC or WEX prior to exposure to the test compounds. Each culture was exposed to medium containing these compounds, at various concentrations, for 24 hr, after which cell survival was determined using a modification of the MTT reduction method [13]. The percentage survival at each dose was calculated relative to the appropriate solvent control for each culture (solvent concentration did not exceed 1%). A TC_{50} value was determined by interpolation from the survival curve for each culture. The effect of WEC and WEX on enzyme activities was analysed by Anova/Dunnett's test or Kruskal–Wallis/Steel's test, as appropriate, following an analysis of the homogeneity of variance. Effects on toxicity and NPS contents were analysed by paired *t*-tests. For all data presented, N refers to the number of cultures in each experiment, each culture being derived from a different animal.

* Abbreviations: P450, cytochrome P450; ADH, alcohol dehydrogenase; BZDM, benzphetamine *N*-demethylase; COU, coumarin; EMDM, erythromycin *N*-demethylase; EROD, 7-ethoxyresorufin *O*-deethylase; GSH, glutathione; MCOD, 7-methoxycoumarin *O*-demethylase; MTT, 3 - (4 - dimethylthiazol) - 2 - yl) - 2,5 - diphenyltetrazolium bromide; NPS, non-protein sulphydryls; PCOD, 7-propoxycoumarin *O*-depropylase; PII, precocene II; PNPH, *p*-nitrophenol hydroxylase; PROD, 7-pentoxyresorufin *O*-depentylase; TC_{50} toxic concentration causing 50% loss of cell survival; WEC, serum-containing Williams' E; WEX, serum-free Williams' E.

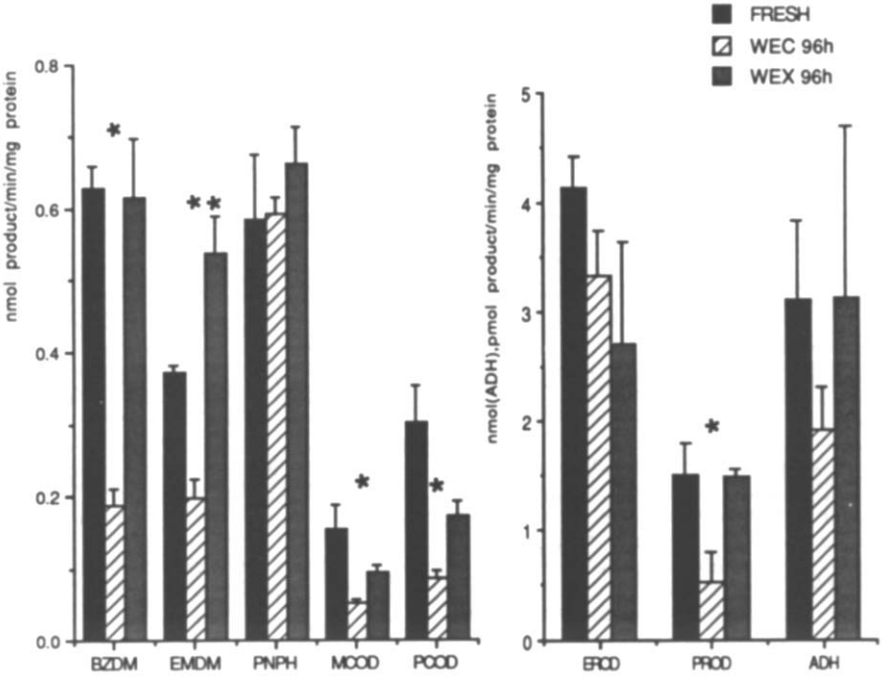

Fig. 1. Activities determined in homogenates of freshly isolated hepatocytes and 96 hr cultures of hepatocytes. Activities in homogenates were determined as described in Materials and Methods. Values shown are mean ± SEM, N = 4 (ADH N = 3); *Activities significantly different to fresh, $P < 0.05$ (Anova/Dunnett's test; Kruskal–Wallis/Steel: MCOD, PCOD and ADH).

Results and Discussion

Survival of hepatocytes in WEX. Overall, survival of hepatocytes cultured in WEX was 80–81% of the survival of cells cultured in WEC, both at 24 and 72 hr (paired *t*-test, $P < 0.05$). Although the survival was lower in WEX, all toxicity data were calculated relative to the control wells on each plate.

Maintenance of enzyme activities in WEX. The data are presented in Fig. 1. ADH, EROD and PNPH activities did not significantly decline over the 96 hr culture period in either medium, whereas the other activities measured were maintained in WEX but significantly declined in WEC. These results are consistent with previous experiments in which maintenance of several activities for 24 hr in WEC was followed by a general decline in most of the activities measured by 72 hr in culture, although selective maintenance of some activities was observed even at this time-point [2, 14]. It is possible that those activities that are markedly better maintained in WEX, e.g. BZDM and PROD, are mediated by P450s whose expression is suppressed by hormones present in serum. It has been reported that both of these activities are mediated by the testosterone 16α-hydroxylase, P4502C11, in untreated male rats [15] and in hepatocyte cultures [16], and that expression of this male-specific P450 is developmentally regulated [17, 18], this latter observation providing strong evidence for hormonal regulation of P4502C11. MCOD and PCOD activities were the least well maintained in WEX (50–60% of the fresh activity; not statistically significant) and have been shown to be influenced by the age and sex of the hepatocyte donor [14]; PCOD activity has also been associated with the steroid-inducible/sex-specific P4503A family [19], as has EMDM, which was significantly better maintained in WEX. All these observations provide strong evidence for hormonal regulation of P4503A. As a whole, these results indicate that the maintenance of at least some P450 activities over a 96 hr culture period is superior in serum-free medium.

Toxicity in hepatocyte cultures. The toxicity of coumarin, the precocenes and allyl alcohol is mediated by a balance of activation pathways—P450 in the case of coumarin and the precocenes, ADH in the case of allyl alcohol—and inactivation via conjugation with GSH. The NPS content of hepatocytes cultured in serum-free medium was not significantly different from that of cultures maintained in WEC, at either 24 or 72 hr although the content at 72 hr was only 65–72% of the 24 hr value (Table 1).

The precocenes and coumarin produced a dose-dependent toxicity in both media and all three compounds were more toxic, as judged by TC_{50} values (Table 1) to cultures maintained in WEX. In 24 hr cultures the greatest effect of WEX was seen for precocene II (PII), where the difference in TC_{50} values was 3.4-fold; the differences for PI and coumarin were 1.7- and 1.5-fold, respectively. Increased toxicity of PII was also seen in 72 hr cultures, although the difference in TC_{50} values was not as marked. The small (20%) but significant reduction in survival in WEX relative to WEC at 24 hr noted above is unlikely to be a significant contributor to the enhanced toxicity observed in WEX at this time for the following reasons: (i) the magnitude of the differences in toxicity is much greater than the difference in survival; and (ii) the toxicity of allyl alcohol was similar in the two media, ADH activity being similar in the two culture conditions at this time-point (data not shown). However, this increased toxicity of PII in WEX-maintained cultures relative to WEC was not as marked in 72 hr cultures (1.4-fold difference in TC_{50} values).

The toxicity of PII in serum-free medium supplemented with 10% bovine serum albumin was not significantly different to that seen in WEX (data not shown). This observation coupled with the difference in TC_{50} values

Table 1. NPS content of rat hepatocyte cultures and toxicity of precocenes, coumarin and allyl alcohol

Compound	TC_{50} in μM; mean ± SEM (N)		nmol NPS/well; mean ± SEM (4)	
	WEC	WEX	WEC	WEX
24 hr cultures			7.50 ± 0.53	8.16 ± 0.42
Coumarin	670 ± 22 (4)	447 ± 35 (4)*		
Precocene I	131 ± 27 (4)	77 ± 17 (4)*		
Precocene II	127 ± 19 (4)	37 ± 4 (4)*		
Allyl alcohol	18.7 ± 0.7 (3)	19.7 ± 0.9		
72 hr cultures			5.38 ± 0.34	5.34 ± 0.25
Precocene II	533 ± 17 (4)	369 ± 57 (4)*		
Allyl alcohol	5.3 ± 0.3 (3)	6.3 ± 0.3 (3)		

Cultures were exposed to medium containing the test compound as described in the methods, after which survival relative to the solvent control was determined using MTT. TC_{50} values were calculated from dose–response curves of percentage survival.

* Mean TC_{50} in WEX significantly different to that in WEC, $P < 0.05$ (paired t-test).

NPS content of cultures was determined as described in Materials and Methods; at both time points there is no significant difference in mean NPS content between media (paired t-tests).

between media for PII at 24 hr (3.4-fold) and 72 hr (1.4 fold) indicates that a reduction in the effective concentration of PII by binding to serum albumin (the major serum binding protein) is unlikely to be a prime cause of the increased toxicity over a 24 hr exposure period in serum-free medium. Thus, the increased toxicity seen in WEX-maintained cultures is consistent with the superior maintenance of at least some forms of P450 in WEX.

In contrast to PII, the toxicity of allyl alcohol, was similar in hepatocytes maintained in WEC and in WEX, both at 24 and 72 hr, consistent with the lack of effect of serum omission on ADH activity. The enhanced toxicity at 72 hr (3-fold greater than at 24 hr) can be explained by reduced GSH conjugation of acrolein (the ultimate toxic species derived from allyl alcohol) due to the decreased GSH content of the 72 hr cultures relative to the 24 hr cultures. Paradoxically, however, the toxicity of PII was lower after 72 hr in culture when compared with 24 hr in culture (Table 1), which may be caused by either a time-dependent selective decline in P450-related activity (possibly associated with MCOD/PCOD activity, see Fig. 1) or alterations in other inactivation pathways not measured in this study. Differences between PII and allyl alcohol in terms of culture time-dependent alteration in toxicity suggest differences in the primacy of the activation/inactivation components as determinants of toxicity for these two agents.

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