Strain differences in the maintenance of cytochrome *P*-450 and mixed-function-oxidase activities in cultured rat hepatocytes

Effect of prostaglandins

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The mixed-function-oxidase (MFO) activities, ethoxyresorufin and pentoxyphenoxazone *O*-dealkylase, of cultured Hooded-Lister(HL)-rat hepatocytes declined rapidly during 72 h of culture, whereas in Sprague-Dawley(SD)-rat hepatocytes the MFO activities increased between 24 and 72 h in culture. Cytochrome *P*-450 content declined at the same rate in both HL- and SD-rat hepatocyte cultures. NADPH:cytochrome *c* reductase and NADH:cytochrome *b*₅ reductase were more stable in SD- than in HL-rat hepatocyte cultures. 16,16-Dimethylprostaglandins E₂ and F_{2α} improved the maintenance of cytochrome *P*-450 content, MFO activity and NADPH:cytochrome *c* reductase in the HL-rat hepatocyte cultures. In SD-rat hepatocytes, the prostaglandins had no effect on cytochrome *P*-450 content or NADPH:cytochrome *c* reductase activity, whereas they prevented the increase observed in MFO activities between 24 and 72 h after culture.

INTRODUCTION

Primary culture of hepatic parenchymal cells has become an established technique for studying xenobiotic metabolism, toxicity and carcinogenesis under defined conditions *in vitro* (Sirica & Pitot, 1980; Williams *et al.*, 1982). However, this application is severely limited by phenotypic changes with respect to a number of specific functions (Guillouzo *et al.*, 1981). For example, several studies have shown that the concentration of cytochrome *P*-450 and cytochrome *P*-450-dependent mixed-function-oxidase (MFO) activities decline substantially during the first 24–48 h of culture (Dickens & Peterson, 1980; Lake & Paine, 1982).

Many laboratories are working to devise a chemically defined culture medium which effectively maintains the concentration of cytochrome *P*-450 and the activity of MFO in primary cultures of hepatocytes at levels comparable with those in the liver *in vivo*. Progress has been slow, and successes reported by one laboratory have not been reproduced by others. This paper demonstrates that the problem of inter-laboratory variation in results is exacerbated by strain differences in the maintenance of MFO activity during culture of rat hepatocytes.

EXPERIMENTAL

Materials

Flow Laboratories supplied the foetal-calf serum, BME vitamin solution and Fungizone. Bovine serum albumin (fraction V), NADH, NADPH, isocitric acid, isocitrate dehydrogenase, cytochrome *c* and Lubrol PX were from Sigma. Collagenase was from Boehringer Mannheim. Resorufin, ethoxyresorufin and pentoxyphenoxazone were synthesized as described previously (Burke & Mayer, 1983). DMPGE₂ and DMPGF_{2α} were generously

given by Dr. J. E. Pike, Upjohn Co., Kalamazoo, MI, U.S.A.

Primary culture of hepatocytes

Hepatocytes from male HL rats (180–220 g) and male SD rats (180–220 g) were prepared (≥ 90% viable by Trypan Blue exclusion) and cultured on collagen-coated Petri dishes as described previously (Moldeus *et al.*, 1978; Morrison *et al.*, 1983). The medium used was modified Earle's medium as described previously (Morrison *et al.*, 1983), supplemented with 100 μM-hydrocortisone 21-succinate, 100 μM-5-aminolaevulinic acid, 5% (v/v) foetal-calf serum and insulin (1 i.u./litre) (Pork Insulatard; Wellcome). DMPGE₂ and DMPGF_{2α} were added daily to the medium (1 μM) in dimethyl sulphoxide (0.1%, v/v) starting from the initiation of cultures.

Preparation of whole-cell homogenate

All enzymic measurements on fresh and cultured cells were performed with cell homogenates prepared as described previously (Grant *et al.*, 1985). For cytochrome *P*-450 measurements, homogenates were prepared in 0.1 M-sodium phosphate buffer, pH 7.6, containing 1 mM-dithiothreitol, 1 mM-EDTA, 15% (v/v) glycerol and 0.2% (v/v) Lubrol PX. Cells used for all determinations other than those of cytochrome *P*-450 were homogenized in 0.1 M-sodium phosphate buffer, pH 7.6. Homogenates were stored at –80 °C for up to 1 month until analysis.

Analytical methods

The concentration of cytochromes *P*-450 and *P*-420 were measured as described by Omura & Sato (1964). NADPH: cytochrome *c* reductase and NADH:cytochrome *b*₅ reductase activities were measured as

Abbreviations used: MFO, mixed-function oxidase; EROD, *O*-de-ethylation of ethoxyresorufin; PPOD, *O*-dealkylation of pentoxyphenoxazone; DMPGE₂, 16,16-dimethylprostaglandin E₂; DMPGF_{2α}, 16,16-dimethylprostaglandin F_{2α}; SD, Sprague-Dawley; HL, Hooded Lister.

Fig. 1. Maintenance of EROD, PPOD, cytochrome (Cyt.) *P*-450 content and NADPH:cytochrome *c* reductase and NADH:cytochrome *b*₅ reductase activities in hepatocytes cultured from HL (▨) (*n* = 3–6) and SD (□) (*n* = 3) rats

The variation between experiments was less than 10%. All data are presented as percentages of initial values in fresh cells. Fresh-cell values (expressed per mg of cell protein) were as follows. For HL rats: cytochrome *P*-450 content, 0.38 nmol/mg; NADH:cytochrome *b*₅ reductase, 0.68 mol/min per mg; NADPH:cytochrome *c* reductase, 24.9 nmol/min per mg; EROD, 380 pmol/min per mg; and PPOD, 109 pmol/min per mg. For SD rats: cytochrome *P*-450 content, 0.41 nmol/mg; NADH:cytochrome *b*₅ reductase, 0.59 mol/min per mg; NADPH:cytochrome *c* reductase, 30.1 nmol/min per mg; EROD, 345 pmol/min per mg; and PPOD, 89 pmol/min per mg.

Fig. 2. Effect of DMPGE₂ and DMPGF_{2α} on the maintenance of cytochrome *P*-450 during culture of HL- (*n* = 3) and SD- (*n* = 2) rat hepatocytes

■, Control; □, DMPGE₂; ▨, DMPGF_{2α}. Variation between experiments was less than 10%. The data are expressed as percentages of initial values in fresh cells. Fresh-cell values were as indicated for Fig. 1.

described by Falzon *et al.* (1984). MFO activities were measured by the *O*-de-ethylation of 5 μ M-ethoxyresorufin (EROD reaction) and *O*-dealkylation of 5 μ M-pentoxyphenoxazone (PPOD reaction), which are catalysed by different isoenzymes of cytochrome *P*-450 in the rat (Burke *et al.*, 1985). Reaction mixtures contained 0.5 ml of cell homogenate and an NADPH-generating system consisting of 0.25 mM-NADP, 2.5 mM-DL-isocitric acid, 0.6 unit of isocitrate dehydrogenase and 5 mM-MgSO₄. Incubations were carried out in air in round-bottomed tubes in a shaking water bath at 37 °C, and the MFO activity quantified by direct fluorimetric detection of the resorufin formed (Grant *et al.*, 1985).

Protein was measured by the method of Lowry *et al.*

(1951), with bovine serum albumin as standard. Results are expressed in terms of total cell protein.

RESULTS

Fig. 1 shows changes in cytochrome *P*-450 concentration and in MFO and cytochrome reductase activities during the culture of hepatocytes from SD and HL rats. The major difference between the two strains of rats lies in the stability of the two MFO activities, EROD and PPOD. The activities of both EROD and PPOD declined throughout the 72 h culture period in HL-rat hepatocytes, whereas cells from SD rats showed an increase in both EROD and PPOD activity between 24

Fig. 3. Effect of DMPGE₂ and DMPGF_{2α} on the maintenance of EROD activity during culture of HL ($n = 3$)- and SD ($n = 2$)-rat hepatocytes

■, Control; □, DMPGE₂; ▨, DMPGF_{2α}. Variation between experiments was less than 10%. Data are expressed as percentages of initial values in fresh cells. Fresh-cell values were as indicated for Fig. 1.

Fig. 4. Effect of DMPGE₂ on the maintenance of NADPH:cytochrome *c* reductase activity during culture of HL ($n = 3$)- and SD ($n = 2$)-rat hepatocytes

■, Control; □, DMPGE₂. Variation between experiments was less than 5%. Data are expressed as percentages of initial values in fresh cells. Fresh-cell values were as indicated in Fig. 1.

and 72 h in culture. EROD was maintained in SD-rat hepatocytes to a greater extent than was PPOD, and showed an increase to over 160% of fresh cell levels after 48 h in culture. It is important to note that, although the catalytic function of cytochrome P-450 showed differential stability in two strains of the rat, the concentration of the cytochrome holoprotein, as measured spectrophotometrically, declined at approximately the same rate during culture of hepatocytes from both strains of rat. Both cytochrome reductase enzyme activities were more stable in SD- than in HL-rat hepatocytes.

A major difference was observed between hepatocytes from SD and HL rats in the effects of 1 μ M-DMPGE₂ or DMPGF_{2α} in culture. DMPGE₂ improved the maintenance of cytochrome P-450 content in HL- but not in SD-rat hepatocytes (Fig. 2). By comparison, DMPGF_{2α} was ineffective in this respect.

Fig. 3 shows that both prostaglandins markedly increased the maintenance of EROD activity in HL-rat

hepatocytes, with DMPGE₂ being again more effective than DMPGF_{2α}. EROD activity at 24 h in hepatocytes from SD rats was lower in cultures containing DMPGF_{2α} than in control cultures. Moreover, both prostaglandins prevented the spontaneous increase in EROD activity observed in SD-rat hepatocytes. The same effects of the prostaglandins were observed with the PPOD reaction (results not shown). Fig. 4 illustrates that DMPGE₂ prevented the rapid decline in NADPH:cytochrome *c* reductase activity that occurred over 96 h culture of HL-rat hepatocytes, but had no effect on the superior maintenance of this enzyme activity in SD-rat hepatocytes.

DISCUSSION

Considerable species differences in the optimal conditions for the primary culture of hepatocytes and in the level of maintenance of cytochrome P-450 concentrations have been demonstrated previously (Maslansky & Williams, 1982). Rat hepatocytes in culture maintain cytochrome P-450 concentrations less effectively than do those from mice, hamsters or rabbits. To our knowledge there have been no previous reports of strain differences in the maintenance of cytochrome P-450 and MFO activity during culture of rat hepatocytes.

Although there was no difference in the maintenance of the overall cytochrome P-450 content of cultured SD- and HL-rat hepatocytes, the maintenance of MFO activities varied considerably. SD-rat hepatocytes maintained MFO activity to a greater extent than those from HL rats, and, in fact, both EROD and PPOD activity increase over time in culture in SD-rat hepatocytes. The reason for this increase in enzyme activity in SD-rat hepatocyte cultures is not known. Increased UDP-glucuronyltransferase activity has also been observed during culture of SD-rat hepatocytes, and this increase is prevented by inclusion of cycloheximide, a protein-synthesis inhibitor, in the culture medium (Grant & Hawksworth, 1986). Increased UDP-glucuronyltransferase activity during culture appears to be due to protein synthesis *de novo*, but we have no evidence at present that synthesis of cytochrome P-450 isoenzymes *de novo* is taking place during culture.

The increase in EROD activity in SD-rat hepatocytes was markedly greater than that in PPOD. EROD activity is preferentially catalysed by the 3-methylcholanthrene-inducible isoenzyme(s) of cytochrome P-450 in the rat (Burke *et al.*, 1985), and the substantial increase in this reaction during culture may reflect the prominence of this type of cytochrome P-450 in cultured hepatocytes (Fry & Bridges, 1979). Steward *et al.* (1985) demonstrated immunochemically that seven different isoenzymes of cytochrome P-450 declined at different rates during rat hepatocyte culture and responded differentially to attempts to maintain the cytochrome P-450 concentration by the addition of hormones or the use of cysteine-free medium. Our results suggest that strain-specific cytochrome P-450 isoenzymes may decline at different rates in cultured HL- and SD-rat hepatocytes and respond differentially to the presence of prostaglandins in the culture medium.

HL-rat hepatocytes responded to the prostaglandins, particularly DMPGE₂, by an improved maintenance of cytochrome P-450, MFO activity and NADPH:cytochrome *c* reductase activity. In contrast, prostaglandins

prevented the spontaneous increase in EROD activity observed in SD-rat hepatocytes. Prostaglandins are known to have cytoprotective properties in the gastrointestinal tract (Robert, 1981) and the liver (Stachura *et al.*, 1981; Funck-Brentano *et al.*, 1984) and to protect hepatic cytochrome *P*-450 *in vivo* against decreases caused by endotoxins (Falzon *et al.*, 1984). The mechanism of prostaglandin hepatic cytoprotection is not understood at present, but it may involve stabilization of cell membranes (Stachura *et al.*, 1981). Prostaglandins may influence the maintenance of drug-metabolizing activities in cultured hepatocytes by the same mechanism(s) as those involved *in vivo*.

The results presented here demonstrate that the stability of drug-metabolizing enzymes during primary culture of hepatocytes and the response of these enzymes to prostaglandins during culture is markedly different in two strains of rat. Similar inter-strain, and even intra-strain, differences may account to some extent for the problems in reproducing successes in maintaining cytochrome *P*-450 and associated MFO activities during culture of rat hepatocytes in different laboratories.

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