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Cytochrome P-450 activity in hepatocytes following cryopreservation and monolayer culture

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Hepatocytes in suspension or in primary culture are useful for the *in vitro* study of drug and carcinogen metabolism [1]. One of the biggest practical requirements is the ability to prolong the useful lifetime of hepatocyte preparations. Effective techniques for storing hepatocytes would be particularly useful when applied to human liver, for which availability is limited. Frozen hepatocytes may also be valuable for transplantation in the treatment of liver injury [2]. Certain studies have indicated that the viability and efficiency of drug metabolism of frozen and thawed rat hepatocytes was reasonably comparable with that of freshly prepared cells [3, 4]. Other studies, however, indicate marked abnormal morphology and metabolism following a similar cryopreservation protocol [5]. We have further investigated cell viability and cytochrome P-450-catalysed drug metabolism in post-mitochondrial supernatants derived from hepatocytes, in suspension and in primary monolayer culture, following cryopreservation. The effect of urea as a possible neutralizer of cryoprotectant toxicity [6] was also investigated.

The animals used were adult male Wistar rats (approximately 250 g) which were provided with food (422-modified diet, Heygates and Sons Ltd., Northampton, U.K.) and water *ad lib*. The procedure for isolation of hepatocytes was based on that of Seglen [7] with the following modifications. The rat was anaesthetized with an intraperitoneal injection of sodium pentobarbitone (60 mg/kg). The liver was preperfused at 37° with 100 ml of Krebs–Henseleit (KH) buffer pH 7.4 which was Ca^{2+}-free and contained *N*-tris-[hydroxymethyl]-methyl-2-amino ethane sulfonic acid (TES; 32 mM). The liver was then perfused with re-circulating KH buffer containing Ca^{2+} (2 mM), TES (32 mM) and 0.075% (w/v) collagenase (type IV Sigma or *Clostridium histolyticum*; BCL). The perfusion flow rate was 20 ml/min throughout. The cell suspension so prepared was kept on ice in KH and TES buffer prior to use and cells were counted using a Neubauer haemocytometer. This method provided freshly isolated hepatocytes which were approximately 85% viable (by trypan blue exclusion tests [8]) and approximately 90% viable (by lactate dehydrogenase (LDH) leakage tests [8]). Attachment of these cells to culture dishes was 86 ± 8%.

Isolated hepatocytes were suspended in culture medium to allow distribution of 5 ml of cell suspension (containing 2.5×10^6 hepatocytes) to each sterile 60 mm × 15 mm culture dish (Nunc). The culture medium consisted of modified Eagles medium (Gibco) with the following alterations: methionine (1 mM), δ-aminolaevulinic acid (77 μM), dexamethasone (260 nM), foetal calf serum (2.5%), horse serum (10%), insulin (13.25 IU/l), total glutamine (2.0 mM) and gentamycin (50 μg/ml). Cysteine was omitted. The culture dishes were then incubated for 20 hr in a 5% CO_2, humidified incubator at 37°. At 4 hr, the culture medium and non-attached cells were replaced with fresh medium (5 ml) and the number of cells removed were counted in a haemocytometer.

For freezing of cells in suspension, the washed hepatocyte preparation was centrifuged at 50 g for 1 min and the cells were resuspended in modified Eagles medium (MEM, Gibco) containing 1% bovine serum albumin and kept on ice in 2 ml polypropylene cryotubes (Nunc). Addition of cryoprotectant was based on the method of Van der Meulen *et al.* [9]. Thus 0.9 ml of cell suspension (approximately 1.0×10^7 cells/ml) was diluted over a period of 10 min with an equal volume of ice-cold MEM containing 20% dimethylsulphoxide (DMSO) with or without urea (1.1 M). After a further 10 min, the samples were suspended, via an insulating platform, in the vapour phase of a flask containing liquid nitrogen. The rate of cooling was between 1° and 2° per min. After 3 hr, the tubes were transferred into the liquid phase of liquid nitrogen for storage. The samples were removed from liquid nitrogen after 7–10 days and rapidly thawed in a water bath at 37°. The cells were then washed three times in KH buffer containing TES (32 mM).

To prepare post-mitochondrial supernatants (S9), hepatocytes were homogenized in 0.15 M KCl, 3 mM Tris/HCl buffer (pH 7.2 at 4°) until >90% of the cells were disrupted. Cultured cells were rinsed twice with buffer and the cells were scraped off the plates using a plastic wedge prior to homogenization in a Potter–Elvehjem apparatus with a close-fitting PTFE plunger. The homogenates were then centrifuged at 9000 *g* for 20 min and the supernatant (S9) was used for determination of cytochrome P-450 activity. 7-Ethoxycoumarin *O*-deethylase activity was measured by the method of Greenlee and Poland [10] and aminopyrine *N*-demethylase activity was assessed by the method of Lake *et al.* [11] but using a total incubation volume of 1.5 ml. S9 protein was estimated by the Lowry method [12].

Pilot studies, in accord with other findings [3], indicated that recovery of viable cells from cryopreservation was greater using DMSO as a cryoprotectant than was found using glycerol. The recovery of cells frozen in a medium containing 10% DMSO was 56.0% (Table 1). The viability of recovered cells as measured by trypan blue exclusion and LDH release was 75.4 and 74.9% respectively of the cell viability prior to freezing. Ability of hepatocytes to attach to culture dishes (also a measure of viability) was markedly reduced following cryopreservation (66% of the values derived from freshly isolated cells). Thus, in summary, the recovery of viable cells was estimated to be 42.2, 41.9 and 37% as measured by trypan blue exclusion, LDH release and attachment to culture dishes respectively.

The monooxygenase activity of S9 fractions from the recovered cells was measured towards 7-ethoxycoumarin (a relatively non-specific marker of cytochrome P-450 activity which is induced by phenobarbitone and 3-methylcholanthrene [13]) and aminopyrine (a marker more specific for cytochrome P-450 inducible by phenobarbitone [14])

Table 1. Recovery and viability of hepatocytes following cryopreservation

	Cryopreservation conditions	
	10% DMSO	10% DMSO + Urea
% Cell recovery	56.0 ± 7.3 (9)	46.6 ± 14.5 (4)
Viability of recovered hepatocytes (% of original viability)		
Trypan blue exclusion	75.4 ± 7.2 (9)	66.8 ± 8.4 (4)
Intracellular containment of LDH	74.9 ± 5.2 (3)	69.4 ± 8.5 (3)
Attachment to culture dishes	66 ± 13 (3)	52 ± 4 (3)

Values represent the mean ± S.E. Each estimation was carried out in at least duplicate and the number of experiments is given in brackets. Cell recovery was measured using a haemocytometer.
DMSO, Dimethylsulphoxide.
LDH, Lactate dehydrogenase.

Table 2. Activity of cytochrome P-450-mediated monooxygenase in hepatocytes following cryopreservation

	Freshly isolated hepatocytes		Hepatocytes recovered from cryopreservation	
	Not cultured	After monolayer culture (20 hr)	Not cultured	After monolayer culture (20 hr)
7-Ethoxycoumarin *O*-deethylase	100	49.7 ± 7.9 (3)	146.8 ± 20.9 (6)	21.5 ± 3.1 (5)
Aminopyrine *N*-demethylase	100	46.5 ± 11.6 (3)	156.1 ± 57.0 (7)	Not measured

Enzyme activity is expressed as % of activity found in S9 preparations from freshly isolated hepatocytes which were 16.7 ± 1.5 nmole/min/mg S9 protein (7-ethoxycoumarin *O*-deethylase; N = 8) and 1.05 ± 0.29 nmole/min/mg S9 protein (aminopyrine *N*-demethylase; N = 12). Values (measured in duplicate) are from the number of experiments shown in brackets.

(Table 2). Cells recovered from the procedure of freezing and thawing have a relatively high cytochrome P-450 activity per mg of S9 protein. This finding is in agreement with the increase in cytochrome P-450 per mg of microsomal protein observed by Novicki *et al.* [3] and may be due to alteration of cellular protein levels or due to selective survival of hepatocytes with relatively high cytochrome P-450 activity. The results contrast with those of Inaba *et al.* [15] where metabolism of aminopyrine in hepatocytes frozen rapidly to −70° was reduced to 10% of the original activity after 7 days.

Although cytochrome P-450 activity was preserved, the viability of the cells was markedly decreased and those cells which were able to be maintained in primary monolayer culture for 20 hr exhibited only 21.5% of the 7-ethoxycoumarin *O*-deethylase activity observed in freshly isolated hepatocytes (expressed per mg of S9 protein). Under the same conditions of culture, non-frozen hepatocytes maintained a level of 49.7% of this enzyme activity.

Urea has been used [6] to protect renal cortical slices against DMSO-induced damage to lysine residues in proteins. However, hepatocytes cryopreserved in the presence of urea (0.55 M) were not recovered in higher yields or with a greater viability (Table 1). Aminopyrine *N*-demethylase activity in S9 preparations from hepatocytes frozen with 10% DMSO plus urea was similar to that in hepatocytes frozen from 10% DMSO alone.

In summary, of hepatocytes frozen, approximately 40% were recovered as viable. Cytochrome P-450 activity was maintained following cryopreservation; however, this activity during subsequent monolayer culture was dramatically reduced compared with non-frozen cells. The integrity of hepatocytes is therefore markedly altered as a result of freezing and thawing in agreement with the extensive ultrastructural damage observed by Fuller *et al.* [5]. This cellular damage could not be inhibited by using urea to reduce DMSO-toxicity.

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Interactions with calmodulin: potential mechanism for some inhibitory actions of tetracyclines and calcium channel blockers

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A role for calcium–CaM* in the hydroosmotic action of vasopressin (VP) has been postulated based on results obtained with phenothiazines [1–3], drugs that inhibit the action of the calcium–CaM complex [4]. Other drugs that inhibit the action of VP include calcium channel blockers [5] and various tetracyclines [6].

The inhibition by tetracyclines and calcium blockers may involve interaction with cAMP-related enzymes [6–8] and correlates with their binding to specific proteins [9].

The observations and their similarities to the effects of trifluoperazine on the action of VP [1–3] led us to consider the possibility that these different drugs may all interfere with calcium–CaM-dependent steps.

Methods

Determination of CaM activity. CaM activity was determined by the activation of CaM-deficient phosphodiesterase as outlined by Cheung [10] and previously described in detail from this laboratory [1]. The standard assay contained 4 μM [^{3}H]cyclic AMP (200,000 cpm) 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.05 to 0.5 mM $CaCl_2$ (as indicated), 40 mM Tris, pH 8.0, and 2.5 μg of CaM-deficient phosphodiesterase. Various amounts of purified CaM (sp. act. 40,000 units/mg) were added as indicated in Results. Assays using heat-inactivated phosphodiesterase served as blanks and were less than 15% of the lowest activity. The various tetracyclines and most calcium antagonists were made up fresh daily in assay buffer and kept in the dark. Nifedipine was prepared daily as a concentrated solution in ethanol–PEG 4000 (1:1) and diluted in buffer prior to the experiment. Incubations were carried out in duplicate at 30° for 30 min while protected from light.

The reaction was terminated by the addition of 5 μl of a stopping solution (containing 0.2 M EDTA and adenosine, 5'-AMP and cAMP, each at 12.5 M), and the tubes were transferred on ice. The amount of [^{3}H]cyclic AMP hydrolyzed during the reaction was determined by thin-layer chromatography using the method of Rangel-Aldao *et al.* [11] as previously described [1]. Each result was calculated as the mean of the duplicate determinations which varied by less than 5%.

Results

Effects of tetracyclines on in vitro *CaM activity*. Purified CaM caused a dose-dependent activation of CaM-deficient phosphodiesterase activity that was calcium dependent (Fig. 1). Addition of calcium from 0.02 to 0.5 mM resulted in comparable and full stimulation. Addition of equal concentrations (0.05 mg/ml or 125 μM) of various tetracyclines caused the CaM dose–response curve to shift to the right to different degrees (Fig. 1). The tetracyclines showed little effect on basal phosphodiesterase activity. By far the strongest inhibitor of CaM activation was 5,6-DMC. At 0.05 mg/ml (125 μM) of 5,6-DMC, the inhibition could not be totally overcome by CaM up to 10 μg/assay (i.e. 100-fold excess over that needed for maximal activation). At a lower concentration of 5,6-DMC (0.01 mg/ml or 25 μM), its inhibitory effect could be overridden by higher concentrations of CaM (Fig. 1). The inhibitory action of these tetracyclines on CaM activation of phosphodiesterase cannot be explained by chelation of calcium, as increasing the calcium concentration did not attenuate the effect of DMC or 5,6-DMC. [Under control conditions, 92 pmoles cAMP were hydrolyzed and values with DMC or 5,6-DMC (125 μM) were inhibited to 24.5 and 18.9 at 0.05 mM Ca^{2+}, 25.0 and 20.2 at 0.5 mM Ca^{2+}, and 24.9 and 20.1 at 1 mM Ca^{2+} respectively.] Figure 2 shows the effect of increasing concentrations of tetracyclines on phosphodiesterase activity in the absence and presence of CaM (0.1 μg or 25 μM). Half-maximal inhibition for CaM-stimulated activity occurred at about 0.008 mg/ml (approximately 20 μM) for 5,6-DMC and at about 0.04 mg/ml (approximately 100 μM) for DMC and 100% inhibition at 0.025 (60 μM) and 0.05 mg/ml (125 μM) respectively. These concentrations are almost one order of magnitude lower than those required for the inhibition of VP-induced water flow in the toad bladder [6, 9]. Thus, effective inhibitory intracellular concentration could be attained with the concentrations used in the transport experiments. Also the inhibitory potency for CaM activation by the various tetracyclines was (by decreasing inhibition): 5,6-DMC > DMC ≫ DOC > TC > OTC. This sequence is identical to that described for the effects of tetracyclines on VP-induced water flow in the toad bladder [6, 9].

Effect of "calcium-blockers" on in vitro *CaM activity*. At 100 μM, diltiazem caused a small (20%) but reproducible inhibition of the CaM-activated phosphodiesterase whereas verapamil and its analogue D600 resulted in a 40–50% inhibition (Fig. 3). The inhibitory effect of 100 μM D600 could be overcome by increasing the CaM concentration 10-fold (two experiments). Nifedipine (15–30 μM) progressively inhibited phosphodiesterase activity in both the absence and presence of CaM. The inhibitory effect of nifedipine (15 μM) was unaffected by increasing CaM up to 1.0 μg/assay. This pattern may indicate an unspecific effect of nifedipine on the phosphodiesterase enzyme itself.

Discussion

The inhibition of the action of VP by tetracyclines may involve binding of the drugs to cellular proteins that have a role in VP-mediated water transport [6, 9]. Based on the

* Abbreviations: VP, vasopressin; CaM, calmodulin; TFP, trifluoperazine; 5,6-DMC, 5,6-anhydrodemeclocycline; DMC, demeclocycline; DOC, doxycycline; OTC, oxytetracycline; and TC, tetracycline.