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The influence of the choice of digestion enzyme used to prepare rat hepatocytes on xenobiotic uptake and efflux

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ABSTRACT

Isolated rat hepatocytes are widely used to assess the metabolism and toxicity of xenobiotics. The choice of digestion enzyme used to prepare the cells has been shown previously to influence their metabolic capability. This study investigates the effect of the digestion enzyme (collagenase II, collagenase A/trypsin inhibitor, or collagenase plus dispase) on the uptake of xenobiotics into, and efflux from, hepatocytes. The choice of digestion enzymes used in this study does not affect uptake of either pravastatin (an organic anion probe substrate for Oatp transporter) or metformin (an organic cation probe substrate for Oct transporter). With regard to efflux transporters, hepatocyte differentiation was better maintained when cells were isolated using collagenase II alone.

1. Introduction

Isolated hepatocytes provide a useful in vitro system for the evaluation of xenobiotic metabolism and toxicity. Since its introduction, the two stage perfusion method for preparation of hepatocytes has undergone several modifications and considerable variations now exist in the composition of physiological buffers, digestion enzymes and post-isolation treatment of cells. We recently compared the activities of both phase I and II enzymes in hepatocytes prepared by three different digestion enzymes: type II collagenase, collagenase type A with trypsin inhibitor, and a preparation containing collagenase and dispase (Sinclair et al., 2009). The latter two digestion methods produced cells with greater cytochrome activity towards testosterone, and glutathione conjugation of the hepatotoxin, troglitazone, than cells prepared by type II collagenase alone. It is clear that the method of preparation of the hepatocytes has significant implications for the generation of xenobiotic metabolism and toxicity data. In order to choose the most appropriate method for such studies in the present manuscript we investigated whether the composition of the digestive enzyme cocktail influenced the uptake of xenobiotics into, and efflux of xenobiotics from hepatocytes.

Uptake represents the first stage in the hepatic processing of xenobiotics. The function and expression of the uptake transporters is unstable after isolation of hepatocytes (Rippin et al., 2001; Liu et al., 1998; Jigorel et al., 2005), and rapid decreases in the mRNA and function of hepatic bile acid uptake transporter (Ntcp), the organic anion transporting polypeptide (Oatp1,Oatp2 and Oatp4), organic cation transporter,(Oct) and the organic anion transporter (Oat2) have been measured in cultured cells, but it is unclear if the isolation process itself has any effect. To investigate the effect of the isolation procedure without the confounding effect of the instability of uptake transporters reported in culture, this study used freshly isolated cell suspensions to measure uptake. The procedure has been widely used to measure the contribution of drug uptake rates to clearance (Soars et al., 2007). Oatp plays an important role in the hepatic clearance of many pharmaceutically relevant drugs, and the uptake of the drug pravastatin was measured to assess its function post-isolation. At physiological pH pravastatin is a monovalent anion. It has previously been used as a probe substrate for Oatp in isolated hepatocytes (Soars et al., 2007), and its uptake involves Oatp1a1, Oatp1a4 and Oatp1b2 (Hsiang et al., 1999; Tokui et al., 1999; Sasaki et al., 2004). At low concentrations of pravastatin over 90% of its uptake into hepatocytes is carrier mediated, with little contribution from passive uptake (Nezzasa et al., 2003). Metformin, a biguanide antidiabetic drug, was used as a substrate for the polyspecific Oct. Metformin is a hydrophilic monovalent cation at physiological pH and Oct1 plays an important role in its hepatic uptake (Wang et al., 2002; Umehara et al., 2007).

The multi-drug resistance associated proteins (Mrps) play a major role in the hepatic excretion of organic anions. Mrp2 in particular mediates biliary excretion of numerous drugs and their metabolites and thus has an important protective function.

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Canalicular efflux via Mrp2 decreases after isolation of hepatocytes, and this is thought to be due to an internalisation of the protein (Bow et al., 2008). Over time in culture, internalised Mrp2 is re-inserted into the canalicular membrane and its function is restored (Zhang et al., 2005; Liu et al., 1999). During de-differentiation of hepatocytes, sinusoidal efflux via Mrp3 may also be upregulated (Luttringer et al., 2002). The function of the efflux transporters can be measured using 5 (and 6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA) using the method of Zhang et al. (2005) to distinguish canalicular from sinusoidal efflux from the cells. Briefly, the CDFDA diffuses into cells and is instantaneously hydrolysed by intracellular esterases to membrane impermeable fluorescent CDF and the diacetate. Cultured hepatocytes were pre-incubated in the presence of calcium ions, followed by incubation with CDFDA. After a defined period extracellular substrate was removed and the accumulation of CDF measured in cell lysates. In the presence of calcium ions, bile canaliculi remain intact thus total (intracellular plus canalicular) accumulation of CDF is measured. In the absence of calcium ions, bile canaliculi are disrupted and only intracellular CDF accumulation is measured. The overall efflux of CDF (sinusoidal plus canalicular) can be expressed as the biliary excretion index (BEI):

BEI = (CDF accumulation in cells plus bile – CDF accumulation in cells)/CDF accumulation in cells plus bile.

In the present study we have applied this method to monolayers of hepatocytes cultured on collagen-coated polystyrene dishes. In preliminary studies we found that the accumulation of CDF was not significantly different in monolayers and in sandwich cultures at 72 h in culture, and since our interest was predominantly in the influence of digestion enzymes on the rate of de-differentiation of hepatocytes we concentrated our studies on the simpler widely used monolayer model of hepatocyte culture.

2. Materials and methods

2.1. Materials

Collagenase type II (92 units/ml), and Liver Digest Medium® were obtained from Invitrogen Ltd, Paisley, UK. Collagenase type A (0.1125 units/ml) was obtained from Roche Diagnostics, Mannheim, Germany. All other chemicals and reagents were of analytical grade or better and were purchased from Sigma–Aldrich, Dorset, UK. Solvents were obtained from VWR International Ltd, Lutterworth, UK and were of chromatographic grade or cell culture grade.

2.2. Preparation of hepatocytes and assessment of their viability

All experiments involving animals were conducted under Home Office project licence PPL 60/255. Studies were performed using suspensions of hepatocytes that had been freshly isolated from whole livers of male Sprague Dawley rats (180–200 g) by a two-stage collagenase perfusion (Segen, 1976) following anaesthesia with 60 mg/kg intraperitoneal pentobarbitone sodium (Sagatal 60 mg/ml, Aventis, UK). Three different collagenase-containing digestion systems; collagenase type II (CII; 92 units/ml), collagenase type A/trypsin inhibitor (CA (0.1125 units/ml)/TI (0.06 mg/ml)) and collagenase/dispase (C/D, Liver Digest Medium® composition not available – proprietary product) were used separately. The viabilities of hepatocyte preparations were assessed by Trypan blue exclusion, by leakage of lactate dehydrogenase (LDH) activity (Ponsoda et al., 1991), and by intracellular reduced glutathione (GSH) (Hissin and Hilf, 1976) and ATP content (Coolen et al., 2008).

Table 1
Effect of the liver digestion enzyme on hepatocyte yield and initial cell viability, as determined by Trypan blue exclusion, LDH leakage, intracellular GSH and ATP levels.

Digestion enzyme	Yield (10^8 cells/liver)	Initial viability trypan Blue exclusion (%)	GSH content (nmol/mg protein)	LDH leakage (%)	ATP content (nmol/10^6 cells)
CII	6.0 ± 1.2	79.2 ± 2.5	11.1 ± 1.9	20.5 ± 4.1	20.2 ± 5.6
CA/TI	5.0 ± 1.1	78.2 ± 1.7	12.8 ± 3.0	17.5 ± 3.5	18.6 ± 4.9
C/D	5.6 ± 0.8	78.8 ± 1.4	11.3 ± 1.4	21.0 ± 5.4	22.7 ± 3.7

CII, collagenase type II; CA/TI, collagenase type A plus trypsin inhibitor; C/D, collagenase plus dispase. Data are mean ± SEM, $n = 5$. No statistical differences were measured by analysis of variance followed by Dunnett's test.

2.3. Incubations for uptake studies

Hepatocytes (10^6 cells/ml) were incubated in 10 ml Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM HEPES in rotating round bottomed flasks and maintained at 37 °C under an atmosphere of carbogen. The substrates (1 μM final concentration of pravastatin or metformin) were added after 5 min pre-incubation to allow equilibration of the cells in the incubation system, and samples removed to measure uptake at 0, 1, 2, 4, 6, 15, 45 and 90 min. Samples (0.6 ml) were immediately centrifuged at 7000*g* for 30 s. Supernatants were flash frozen, using liquid nitrogen before being stored at −70 °C, to await analysis for disappearance of substrates from the medium.

2.4. Preparation of primary cultures of hepatocytes and measurement of efflux

Cells were seeded at 3×10^6 cells/plate onto 60 mm Petri dishes pre-coated with 625 μg rat tail collagen prepared as described by

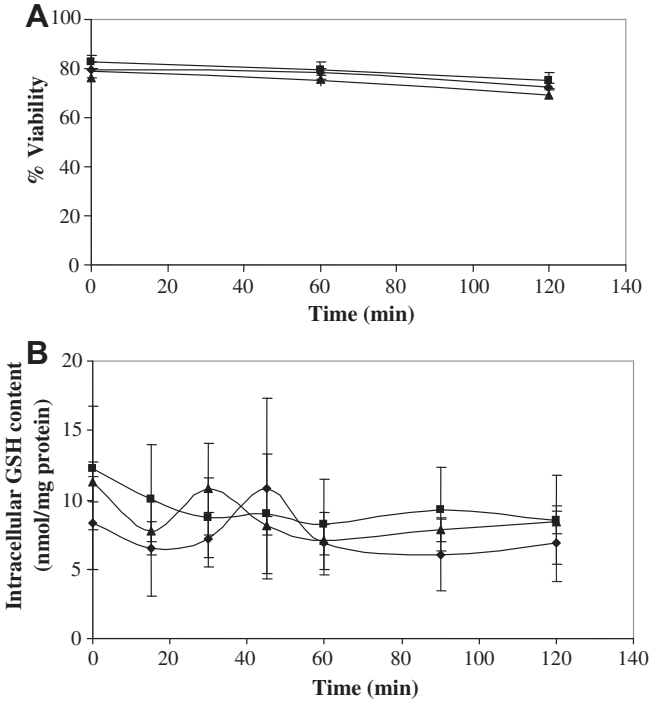

Fig. 1. Hepatocyte viability (%), determined by LDH leakage (A), and intracellular reduced glutathione (GSH) content (B), during 120 min incubation of freshly isolated suspensions of rat hepatocytes isolated using collagenase type II (CII, ♦), collagenase type A with trypsin inhibitor (CA/TI, ■) and a preparation containing collagenase and dispase (C/D, ▲). Data are mean ± SEM, $n = 5$. Statistical comparisons between CII and CA/TI or C/D were carried out by analysis of variance followed by Dunnett's multiple comparison test. No significant differences were observed.