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Modulation of CYP1A1 and CYP2B1 expression upon cell cycle progression in cultures of primary rat hepatocytes

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Abstract

Primary cultures of epidermal growth factor (EGF)-stimulated hepatocytes are a valuable tool to study the regulation of hepatocyte proliferation. As progression through the cell cycle is generally associated with a reduction in liver-specific functions, we studied the effects of a proliferative response triggered by EGF on the albumin secretion and urea production, and on cytochrome P450 (CYP) 1A1 and CYP2B1 expression and their corresponding 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-pentoxyresorufin-*O*-dealkylase (PROD) activities. It was found that cell cycle entry is associated with decreased albumin secretion and urea production. Furthermore, western blot analysis revealed that in hepatocytes cultured under proliferative conditions, the protein expression of CYP1A1 and CYP2B1 was substantially decreased, as well as the CYP2B-mediated PROD activity. In contrast, EROD activity was not altered. In addition, the expression levels of the liver enriched transcription factors (LETFs) hepatic nuclear factor (HNF) 3β and HNF4α were downregulated under proliferative conditions, whereas the expression of HNF1α remained constant. In conclusion, we show that in cultured primary hepatocytes, cell cycle progression significantly modulates albumin secretion, urea production and CYP-mediated biotransformation, probably involving transcriptional regulation by hepatic nuclear factors. Therefore, in order to maintain primary hepatocytes functional in culture, cell cycle inhibition must be achieved.

Keywords: EGF; Rat hepatocytes; Cytochrome P450; Proliferation; Cell cycle; Hepatic nuclear factors

1. Introduction

The use of primary hepatocytes in culture has gained interest in the pharmaceutical industry, as a promising alternative to *in vivo* experimentation for early lead selection, elucidation of biotransformation patterns, detection of drug–drug interactions and hepatotoxicity (Ulrich et al., 1995; Groneberg et al., 2002). The loss of liver-specific functions, such as biotransformation capacity, upon isolation and subsequent culture of the hepatocytes, however, remains a major shortcoming (Vanhaecke and Rogiers, 2005). The molecular mechanisms underlying this dedifferentiation seem to be rather complex and are triggered by a number of factors. In liver, mature hepatocytes are fully differentiated and remain quiescent (G_0). Upon isolation, however, they enter the cell cycle and progress to mid-late G_1 phase, where they are arrested at the mitogen-dependent restriction point (Loyer et al., 1996). Upon stimulation by mitogens, such as transforming growth factor alpha (TGF-α) and epidermal growth factor (EGF), hepatocytes pass beyond this restriction point, DNA is synthesised and mitosis is completed (Etienne et al., 1988; Loyer et al., 1996). Transcriptional regulation by several

Abbreviations: C/EBP, CCAAT/enhancer binding protein; CDK1, cyclin-dependent kinase 1; CYP, cytochrome P450; BSA, bovine serum albumin; DSM, differentiation stimulating medium; EGF, epidermal growth factor; EROD, 7-ethoxyresorufin-*O*-deethylase; FBS, fetal bovine serum; FIH, freshly isolated hepatocytes; HNF, hepatic nuclear factor; LETFs, liver enriched transcription factors; M199, medium 199; MEM, minimum essential medium; PROD, 7-pentoxyresorufin-*O*-dealkylase; PSM, proliferation stimulating medium; TGF-α, transforming growth factor alpha; WEM, Williams E medium.

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liver enriched transcription factors (LETFs), including members of the CCAAT/enhancer binding protein (C/EBP), hepatic nuclear factor 1 (HNF1), HNF3 and HNF4 families, are indispensable for liver development, hepatocyte homeostasis and differentiation (Cereghini, 1996). As it was previously suggested that alterations in mRNAs of LETFs potentially underlie the loss of the hepatic phenotype during isolation and subsequent cultivation, and are closely related to the rapid and substantial decline in cytochrome P450-mediated phase I biotransformation (Padgham et al., 1993), we used hepatocytes kept either under differentiation or proliferating conditions, to investigate the modulation of basic metabolic competence (urea production and albumin secretion) and the phase I biotransformation enzymes CYP1A1 and CYP2B1, as well as the potential involvement of several hepatic nuclear factors. Proliferating conditions were obtained by using EFG-stimulated hepatocytes, an *in vitro* model successfully used by our group to study the regulation of hepatocyte proliferation (Papeleu et al., 2003, 2004).

2. Materials and methods

2.1. Chemicals and reagents

Bovine serum albumin (BSA), crude collagenase type I, L-glutamine, bovine insulin, minimum essential medium (MEM), medium 199 (M199), ethoxy/pentoxyresorufin and resorufin were purchased from Sigma–Aldrich (Belgium). Recombinant human EGF came from Promega (The Netherlands). Williams E medium (WEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Belgium) and Invitrogen (Belgium), respectively, whereas glucagon came from Novo Nordisk (Belgium). All other chemicals and reagents were commercial products of the highest available grade.

2.2. Isolation and culture of rat hepatocytes

Male outbred Sprague–Dawley rats (200–300 g) were purchased from Charles River Laboratories (Belgium). Hepatocytes were isolated and viability was tested by trypan blue exclusion as previously described (Papeleu et al., 2005). All experiments were performed in accordance with the regulations of the Animal Experiments Ethical Committee of the Vrije Universiteit Brussel. Hepatocytes (viability > 80%) were cultured for 48 h as a monolayer (Henkens et al., 2005) in a medium and at a density either stimulating proliferation or favouring differentiation. The former, referred to as "proliferation stimulating medium (PSM)", consisted of MEM/M199 (3:1, v/v), supplemented with 1 mg/ml BSA, 5 μg/ml bovine insulin, 2 mM L-glutamine, antibiotics (7.3 IU/ml benzyl penicillin, 50 μg/ml streptomycin sulphate, 50 μg/ml kanamycin monosulphate and 10 μg/ml sodium ampicillin) and 10% (v/v) FBS at a density of 0.4×10^5 cells/cm^2. The latter, referred to as "differentiation stimulating medium (DSM)", was composed of WEM, supplemented with 2 mM L-glutamine, 7 ng/ml glucagon, antibiotics and 10% (v/v) FBS at a density of 0.57×10^5 cells/cm^2. Consequently, cells were placed at 37 °C in an atmosphere of 5% CO_2 and 95% air at 100% relative humidity. After 4 h, medium was removed and MEM/M199 was renewed daily with serum-free MEM/M199 containing 0.5 μg/ml hydrocortisone and 50 ng/ml EGF, whereas WEM was supplemented with 25 μg/ml hydrocortisone hemisuccinate and 5 μg/ml bovine insulin. Twenty four hours after seeding WEM was renewed daily with serum-free culture medium.

2.3. Secretion of albumin and urea production

For measuring albumin secretion, medium samples were collected, followed by harvesting of the cells (cultured in 35 mm ∅ dishes). Medium was analyzed for its albumin content by an ELISA technique according to Dunn et al. (1991). For the determination of urea secretion, the hepatocyte monolayers (35 mm ∅ dishes) were incubated with 10 mM NH_4Cl, 6 h prior to medium collection and cell harvesting. Urea concentration in the medium was analyzed spectrophotometrically using a Quantichrom Urea Assay kit (BioAssay Systems, CA, USA). Total cellular protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Germany), using BSA as a standard.

2.4. EROD and PROD activity measurements

Microsomal fractions from both freshly isolated and cultured hepatocytes (15 cm ∅ dishes) were prepared (Hales and Neims, 1977), and stored at −80 °C. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Germany), using BSA as a standard. Microsomes were incubated with 5 μM ethoxy- or pentoxyresorufin for EROD and PROD determinations, respectively. Resorufin formed was measured fluorimetrically (Burke and Mayer, 1974).

2.5. Preparation of cell lysates and immunoblot analysis

Cells (10 cm ∅ dishes) were harvested by scraping, washed twice with ice-cold PBS, and lyzed in modified Tween-20 buffer (Albrecht and Hansen, 1999). Total cellular protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Germany), using BSA as a standard. Proteins (25 or 50 μg) were resolved on SDS–PAGE (7.5 or 10% w/v) and blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, UK). Equal protein loading was examined by reversible 0.1% (w/v) Ponceau Red staining of the membranes. After blocking the membranes with 5% (w/v) non-fat milk in 0.1% (v/v) Tween-20 in Tris buffered saline (pH 7.6), the membranes were probed overnight at 4 °C with anti-CYP1A1, anti-CYP2B1 (BD Gentest, Belgium), anti-HNF1α, anti-HNF3β, anti-HNF4α, anti-CDK1 (Santa Cruz Biotechnology, CA, USA) and anti-cyclin D1 (Neomarkers, CA,