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A cellular model to study drug-induced liver injury in nonalcoholic fatty liver disease: Application to acetaminophen

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ABSTRACT

Obesity and nonalcoholic fatty liver disease (NAFLD) can increase susceptibility to hepatotoxicity induced by some xenobiotics including drugs, but the involved mechanisms are poorly understood. For acetaminophen (APAP), a role of hepatic cytochrome P450 2E1 (CYP2E1) is suspected since the activity of this enzyme is consistently enhanced during NAFLD. The first aim of our study was to set up a cellular model of NAFLD characterized not only by triglyceride accumulation but also by higher CYP2E1 activity. To this end, human HepaRG cells were incubated for one week with stearic acid or oleic acid, in the presence of different concentrations of insulin. Although cellular triglycerides and the expression of lipid-responsive genes were similar with both fatty acids, CYP2E1 activity was significantly increased only by stearic acid. CYP2E1 activity was reduced by insulin and this effect was reproduced in cultured primary human hepatocytes. Next, APAP cytotoxicity was assessed in HepaRG cells with or without lipid accretion and CYP2E1 induction. Experiments with a large range of APAP concentrations showed that the loss of ATP and glutathione was almost always greater in the presence of stearic acid. In cells pretreated with the CYP2E1 inhibitor chlormethiazole, recovery of ATP was significantly higher in the presence of stearate with low (2.5 mM) or high (20 mM) concentrations of APAP. Levels of APAP-glucuronide were significantly enhanced by insulin. Hence, HepaRG cells can be used as a valuable model of NAFLD to unveil important metabolic and hormonal factors which can increase susceptibility to drug-induced hepatotoxicity.

1. Introduction

Liver injury can be induced by numerous drugs, herbals and industrial toxicants (Biour et al., 2004; Seeff et al., 2015; Wahlang et al., 2013). In the most severe cases, xenobiotic-induced liver injury can require hospitalization and eventually lead to death of the patient (Björnsson, 2009). Among the different predisposing factors increasing the risk of liver injury, there is growing evidence that nonalcoholic fatty liver disease (NAFLD) could play a significant role (Fromenty, 2013; Robin et al., 2005a; Tarantino et al., 2007). NAFLD is often associated with obesity and type 2 diabetes and encompasses a large spectrum of liver lesions including fatty liver, nonalcoholic steatohepatitis (NASH) and cirrhosis (Michelotti et al., 2013).

Greater hepatotoxicity in the context of obesity and NAFLD has been documented in rodents and humans with some drugs, including acetaminophen (APAP), halothane and methotrexate, as well as other xenobiotics such as ethanol and carbon tetrachloride (Donthamsetty et al., 2007; Fromenty, 2013; Michaut et al., 2014; Robin et al., 2005a; Tarantino et al., 2007). However, the mechanisms involved in this higher susceptibility are poorly understood, although different hypotheses have been put forward (Fromenty, 2013). Furthermore, these mechanisms could be complex and different from one compound to another (Carmiel-Haggai et al., 2003; Fromenty, 2013; Robin et al., 2005a). Notably, obesity and NAFLD in rodents and humans are associated with different alterations in the activity of hepatic enzymes involved in drug metabolism including cytochromes P450 (CYPs), UDP-glucuronosyltransferases and transporters (Brill et al., 2012; Canet et al., 2015). More specifically, these dysmetabolic disorders are associated with higher CYP2E1 activity, lower CYP3A4 activity and increased capacity of glucuronide conjugation for different drugs such as APAP and lorazepam (Aubert et al., 2011; Brill et al., 2012; Chalasani et al., 2003; Emery et al., 2003; Kolwankar et al., 2007; Woolsey et al., 2015).

Deciphering the mechanisms whereby some drugs and environmental toxins are more hepatotoxic in the context of obesity and NAFLD requires appropriate experimental models. Although obese

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mice and rats can be useful (Aubert et al., 2011; Carmiel-Haggai et al., 2003; Massart et al., 2012; Robin et al., 2005a), there are numerous differences between rodents and humans regarding hepatic drug metabolism (Chu et al., 2013; Martignoni et al., 2006). In addition, investigations in animals are cumbersome and ethically problematic. Thus, a relevant human cellular model could be helpful in order to study hepatotoxicity in NAFLD.

In the past few years, the metabolically competent human hepatoma HepaRG cell line has been shown to be a valuable model to study the mechanism of hepatotoxicity induced by drugs and toxicants (Anthérieu et al., 2011; McGill et al., 2011; Savary et al., 2014; Sharanek et al., 2014; Tobwala et al., 2015). Indeed, HepaRG cells express most of the enzymes and transcription factors involved in xenobiotic biotransformation and transport (Andersson et al., 2012; Aninat et al., 2006; Anthérieu et al., 2012). In addition, HepaRG cells have been successfully used to study the effects of nutrients, hormones and drugs on the expression of various enzymes involved in carbohydrate and lipid metabolism (Anthérieu et al., 2011; Madec et al., 2011; Nagasaw et al., 2007; Samanez et al., 2012).

By using HepaRG cells, the aim of the present study was two-fold. First, we sought to set up a cell model of NAFLD, in particular regarding the alterations of CYP2E1 and CYP3A4 activity that are observed in this hepatic disease. To this end, differentiated HepaRG cells were treated for one week with stearic acid (C18:0) or oleic acid (C18:1) in the presence of different concentrations of insulin. The insulin effect was investigated because previous studies suggested that this hormone could modulate CYP2E1 expression and activity (Ioannides et al., 1988; Moncion et al., 2002; Woodcroft and Novak, 1999). Second, we used this cell model of NAFLD in order to determine the cytotoxic effects of the painkiller APAP. Indeed, some data in rodents and humans suggested that NALFD could be associated with more severe APAP-induced hepatotoxicity, especially after an overdose (Aubert et al., 2012; Kon et al., 2010; Michaut et al., 2014; Myers and Shaheen, 2009; Nguyen et al., 2008). One of these rodent studies proposed that higher APAP hepatotoxicity in NAFLD could be attributed to greater activity of CYP2E1 (Aubert et al., 2012), the primary enzyme responsible for the biotransformation of APAP to the highly toxic metabolite N-acetyl-*p*-benzoquinone imine (NAPQI) (Hinson et al., 2010; McGill and Jaeschke, 2013). Overall, our results indicate that HepaRG cells loaded with stearic acid can be used as a valuable model to study the mechanisms whereby APAP is more toxic in the context of NAFLD.

2. Materials and methods

2.1. Chemicals

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), APAP, APAP-β-D-glucuronide, APAP-sulfate, testosterone, 6β-hydroxytestosterone, chlormethiazole (CMZ), chlorzoxazone (CZX), metformin, dimethyl sulfoxide (DMSO), oleic acid, stearic acid, insulin and oil Red O were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). William's E medium was obtained from Eurobio laboratories (Les Ulis, France). Fetal Bovine Serum (FBS) was supplied by Lonza (Levallois-Perret, France). Glutamine, penicillin and streptomycin were obtained from Invitrogen (Cergy Pontoise, France). Hydrocortisone hemisuccinate was purchased from Upjohn Pharmacia (Guancourt, France). Protease and phosphatase inhibitors were purchased from Roche Diagnostics (Indianapolis, IN).

2.2. Cell cultures and treatments

Native HepaRG cells were cultured as previously described (Aninat et al., 2006). Briefly, HepaRG cells were seeded at a density of 2.6×10^4 cells/cm^2 and were first incubated in a William's E medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 5 μg/ml insulin and 50 μM hydrocortisone hemisuccinate. After 2 weeks, cell differentiation was induced by the same culture medium supplemented with 2% DMSO (differentiation medium) for 2 additional weeks. Cells were subsequently treated for 1 week with different concentrations of insulin (0, 0.01 or 5 μg/ml), stearic acid (0 or 100 μM), or oleic acid (0 or 100 μM). In some experiments, lower or higher concentrations of fatty acids were used. For the APAP experiments, cells were incubated with different concentrations of this drug (0 to 20 mM) during the last 6, 24 or 48 h of the 7-day treatment, so that untreated and APAP-treated cells were investigated at the same time point (i.e. at day 7). Stearic acid, oleic acid and APAP were dissolved in DMSO whose final concentration in cultures was always set at 2%. For the investigations performed with the prototypical CYP2E1 inhibitor CMZ, cells were incubated 1 week with 150 μM of CMZ after the end of differentiation. Whatever the treatments, the culture medium was renewed every 2 or 3 days during the 7-day experiments. For the experiments carried out with the AMP-activated protein kinase (AMPK) activators AICAR and metformin, cells were first preincubated for 6 h with or without 500 μM of each activator. Cells were then treated for 24 h with 20 mM of APAP, with or without each AMPK activator (500 μM). HepaRG cells were used at passages 11 to 15.

Primary human hepatocytes (PHH) from adult donors were prepared at Biopredic International (Saint-Grégoire, France) by perfusion of liver fragments provided by the Centre de Ressources Biologiques (CRB) Santé de Rennes. Information about the liver donors is provided in the Supplementary Table 1. Hepatocytes were then immediately seeded at a density of 11×10^4 cells/cm^2 in a William's E medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 5 μg/ml insulin and 5 μM hydrocortisone hemisuccinate. The medium was discarded 12 h after seeding and cells were then maintained in the same differentiation medium used for HepaRG cells, which was renewed every other day. Two days after seeding, PHH were then treated or not with oleic acid, stearic acid and insulin for one week.

2.3. Oil red O staining, cellular triglycerides and apolipoprotein B in culture medium

For staining of neutral lipids, live cells were washed with phosphate-buffered saline, incubated for 45 min at room temperature with an oil red O-saturated solution, washed again, and observed under a phase-contrast microscope. Triglyceride quantification (nmol/mg of proteins) was measured with a colorimetric kit purchased from Biovision (Milpitas, CA), using the manufacturer's recommendations. Apolipoprotein B (apoB) levels (μg/ml) in culture medium were determined with the human ELISA kit purchased from Abcam (Cambridge, UK), according to the manufacturer's instructions.

2.4. Determination of CYP2E1 and CYP3A4 activities

In order to measure CYP2E1 and CYP3A4 activities, HepaRG cells or PHH were incubated for 6 and 2 h in phenol red-free and DMSO-free William's E medium containing 300 μM CZX or 200 μM testosterone, respectively. At the end of the incubation, aliquots of culture media were collected and stored at −80 °C until analysis. 6-hydroxychlorzoxazone was then quantified by high-performance liquid chromatography (HPLC)-tandem mass spectrometry (Xenoblis, Rennes, France), whereas 6β-hydroxytestosterone was measured by HPLC analysis, as previously described (Aninat et al., 2006).

2.5. Cellular ATP and GSH

Cellular ATP was measured with the CellTiter-Glo® assay purchased from Promega (Charbonnieres, France), using the manufacturer's recommendations. Briefly, HepaRG cells were incubated with the reagent 10 min at 37 °C and the luminescent signal was quantified using a