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Differential sensitivity of metabolically competent and non-competent HepaRG cells to apoptosis induced by diclofenac combined or not with TNF-α☆

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HIGHLIGHTS

- HepaRG hepatocytes are less sensitive to DCF than HepG2 cells and undifferentiated HepaRG cells.
- The lower sensitivity of HepaRG hepatocytes is related to their high detoxifying capacity.
- Inhibition of glutathione transferases results in increased DCF cytotoxicity.
- TNF-α potentiation of DCF cytotoxicity is not observed in undifferentiated HepaRG cells.
- DCF-induced cholestasis typified by bile canaliculi dilatation is not aggravated by TNF-α.

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ABSTRACT

The role of reactive metabolites and inflammatory stress has been largely evoked in idiosyncratic hepatotoxicity of diclofenac (DCF); however mechanisms remain poorly understood. We aimed to evaluate the influence of liver cell phenotype on the hepatotoxicity of DCF combined or not with TNF-α using differentiated and undifferentiated HepaRG cells, and for comparison, HepG2 cells. Our results demonstrate that after a 24 h-treatment metabolizing HepaRG cells were less sensitive to DCF than their undifferentiated non-metabolizing counterparts as shown by lower oxidative and endoplasmic reticulum stress responses and lower activation of caspase 9. Differentiated HepaRG cells were also less sensitive than HepG2 cells. Their lower sensitivity to DCF was related to their high content in glutathione transferases. DCF-induced apoptotic effects were potentiated by TNF-α only in death receptor-expressing differentiated HepaRG and HepG2 cells and were associated with marked activation of caspase 8. TNF-α co-treatment did not aggravate DCF-induced cholestatic features. Altogether, our results demonstrate that (i) lower sensitivity to DCF of differentiated HepaRG cells compared to their non-metabolically active counterparts was related to their high detoxifying capacity, giving support to the higher

Abbreviations: ABT, 1-aminobenzotriazole; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; AC-IETD-AMC, Ac-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin; AC-LEHD-AMC, Ac-Leu-Glu-His-Asp-7-Amino-4-methylcoumarin; BSEP, bile salt export pump; CDF, 5 (and 6)-carboxy-2′,7′-dichlorofluorescein (CDF); CRP, C-reactive protein; CYP, cytochrome P450; DCF, diclofenac; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; H2-DCFDA, 2′,7′-dichlorodihydrofluorescein; HO1, heme oxygenase 1; IL-8, interleukin-8; MnSOD, manganese superoxide dismutase; MRP2, multidrug associated protein 2; MTT, methylthiazoltetrazolium; NAC, *N*-acetyl cysteine; NTCP, Na^+-dependent taurocholate cotransporting polypeptide; PBS, phosphate buffered saline; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; TNF-α, tumor necrosis factor α.

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HepG2 cells
Primary human hepatocytes

sensitivity of nonhepatic tissues than liver to this drug; (ii) TNF-α-potentiation of DCF cytotoxicity occurred only in death receptor-expressing cells.

1. Introduction

Drug-induced idiosyncratic hepatotoxicity represents 13–17% of all cases of acute liver failure (Bjornsson and Olsson, 2006). Formation of reactive metabolites, generation of oxidative stress, inflammatory stress and immune response are recognized to be potential critical determinants (Uetrecht, 2006).

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug widely used for the treatment of rheumatoid arthritis, osteoarthritis and acute injury pain. Its administration has been associated with adverse effects in various organs, especially the gastrointestinal tract and kidney. DCF has also caused rare cases of hepatocellular injury, cholestasis or mixed hepatocellular injury and cholestasis (Banks et al., 1995; Breen et al., 1986; Watanabe et al., 2007). DCF-induced liver toxicity has been related to the formation of reactive metabolites, i.e. quinone imines from 4'OH-DCF and 5'OH-DCF, and acyl glucuronides primarily catalyzed by UGT2B7 (Bort et al., 1999; Kretz-Rommel and Boelsterli, 1993; Wang et al., 2004). DCF-acyl glucuronides can bind selective proteins, including dipeptidyl peptidase IV, a canalicular membrane protein (Seitz and Boelsterli, 1998; Seitz et al., 1998). However, the role of these protein adducts in the pathogenesis of DCF-associated liver toxicity remains unclear (Aithal and Day, 2007; Banks et al., 1995).

DCF and its CYP-mediated metabolites 4'OH-DCF and 5'OH-DCF induce concentration-dependent apoptosis at equimolar concentrations, the greatest pro-apoptotic activity being produced by 5'OH-DCF in primary human hepatocytes (Bort et al., 1999). However, DCF does not appear to be more cytotoxic to human hepatocytes than to HepG2 cells which usually express low drug metabolizing enzyme activities (Fredriksson et al., 2011; Gomez-Lechon et al., 2003b). Moreover, DCF has been shown to be toxic to non-hepatic organs and cells at doses even lower than those required for liver toxicity (Ng et al., 2008). All these data make questionable the direct involvement of CYP-derived metabolites in DCF cytotoxicity *in vivo* and *in vitro*. DCF-induced apoptosis is strongly potentiated by TNF-α in HepG2 cells (Fredriksson et al., 2011; Maiuri et al., 2015) and lipopolysaccharide in rodent liver (Deng et al., 2006). The involvement of the intrinsic apoptotic pathway characterized by disruption of mitochondrial integrity has been demonstrated in various studies (Fredriksson et al., 2011; Gomez-Lechon et al., 2003a), and oxidative and endoplasmic reticulum (ER) stresses have been identified as independent cytotoxic responses to both DCF alone and the combination DCF/TNF-α (Fredriksson et al., 2014). The synergistic effect of DCF/TNF-α co-treatment appeared to occur mostly via activation of the extrinsic apoptotic pathway (Fredriksson et al., 2011).

In the present work, we further investigated mechanisms of DCF hepatotoxicity and cross-talk between hepatocyte apoptosis induced by DCF and TNF-α challenge using differentiated, metabolically competent and undifferentiated, non metabolically competent HepaRG cells and for comparison, HepG2 cells. At their undifferentiated stage HepaRG cells express markers of progenitors and do not exhibit detectable drug metabolizing enzyme activities; however, they can reach the capacity to express functions of mature hepatocytes, including activities of CYP2C9 and CYP3A4 which are the main CYPs involved in the formation of CYP-mediated metabolites of DCF (Aninat et al., 2006; Guillouzo and Guguen-Guillouzo, 2008), as well as detoxifying enzymes (Aninat et al., 2006; Gerets et al., 2012; Rogue et al., 2012). We show here that sensitivity of liver cells to DCF-induced apoptosis was related to their phenotype and that potentiation by TNF-α was observed only in differentiated HepaRG cells expressing liver-specific functions and in HepG2 cells.

2. Materials and methods

2.1. Chemicals and reagents

1-Aminobenzotriazole (ABT), diclofenac sodium salt (DCF), dithiothreitol (DTT), methylthiazoltetrazolium (MTT), *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), *N*-acetyl-cysteine (NAC), ethacrynic acid, 6β-hydroxy-testosterone and testosterone were purchased from Sigma Aldrich (St. Quentin Fallavier, France). 2′,7′-Dichlorodihydrofluorescein (H_2-DCFDA) was from Invitrogen Molecular Probe (Cergy-Pontoise, France). Ac-Ile-Glu-Thr-Asp-7-Amino-4-methylcoumarin (AC-IETD-AMC) and Ac-Leu-Glu-His-Asp-7-Amino-4-methylcoumarin (AC-LEHD-AMC) were supplied by Enzo Life Sciences (Lyon, France). TNF-α was provided by Promocell (Nuremberg, Germany). eIF2α (catalog 9722) and phospho-eIF2α (Ser51) (catalog 3597) were from Cell Signaling Technology (Danvers, MA, USA). CXCL8/IL-8 and Human C-reactive protein (CRP) DuoSet kits were from R&D (Abingdon, United Kingdom). N-benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp (OMe)-fluoromethyl ketone (z-LEHD-fmk) was purchased from BD Biosciences (Le Pont de Claix, France) and etanercept was from Amgen (Thousand Oaks, CA, USA). Glutathione transferases (GST) A1/2 and M1/2 antibodies were gifts from Dr Caroline Aninat (Rennes).

2.2. Cell cultures and treatments

2.2.1. Cell cultures

HepaRG cells were seeded at a density of 2.6×10^4 cells/cm^2 in Williams' E medium supplemented with 10% Hyclone® fetal bovine serum (Thermo scientific, San Jose, USA), 100 U/mL penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, and 50 μM hydrocortisone hemisuccinate. After 2 weeks, these undifferentiated cells were shifted to the same medium supplemented with 1.7% dimethyl sulfoxide (DMSO) for further 2 weeks in order to obtain cells expressing liver-specific functions. At that time, cultures contained hepatocyte-like and progenitors/primitive biliary-like cells in nearly equal proportions (Cerec et al., 2007).

HepG2 cells were seeded at a density of 2.6×10^4 cells/cm^2 in minimum essential medium-α supplemented with 10% Hyclone® fetal bovine serum, non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin, and were used at subconfluence.

Primary human hepatocytes were obtained from Biopredic International (St Grégoire, France). They were isolated by collagenase-perfusion of liver biopsies from adult donors (Guguen-Guillouzo et al., 1982). These cells were cultured at a density of $1.5.10^5$/cm^2 in a Williams' E medium containing 10% Hyclone® fetal bovine serum without hydrocortisone hemisuccinate for the first 24 h and in a medium deprived of serum and hydrocortisone thereafter. Cultures were used at day 4.

2.2.2. Treatments

All treatments were performed on cells maintained in a medium containing 2% Hyclone® fetal bovine serum and 0.2%