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Mitochondrially mediated synergistic cell killing by bile acids

Anabela P. Rolo[a], Carlos M. Palmeira[a], Kendall B. Wallace[b,*]

[a] *Center for Neurosciences and Cell Biology of Coimbra, Department of Zoology, University of Coimbra, 3004-517 Coimbra, Portugal*
[b] *Department of Biochemistry and Molecular Biology, University of Minnesota School of Medicine, 10 University Drive, Duluth, MN 55812-2487, USA*

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Abstract

The accumulation of endogenous bile acids contributes to hepatocellular damage during cholestatic liver disease. To examine the controversy regarding the therapeutic use of ursodeoxycholate (UDCA) in cholestatic patients, we investigated the possible cytoprotection or synergistic effects of UDCA against chenodeoxycholate (CDCA)-induced injury to isolated rat hepatocytes. Our aim was to investigate the role of the mitochondrial permeability transition (MPT) in the mechanism of cytotoxicity caused by UDCA plus CDCA. Although not toxic by itself, UDCA potentiated the mitochondrial depolarization, ATP depletion and cell killing caused by CDCA. Fructose maintained ATP levels and prevented bile acid-induced cell killing. Cyclosporine A (CyA), a potent inhibitor of the MPT, substantially reduced mitochondrial depolarization, ATP depletion and cell killing caused by CDCA. Our results demonstrate that the synergistic cytotoxicity by UDCA plus CDCA is mediated by impairment of mitochondrial function, an event that is expressed via induction of the MPT.

Keywords: Hepatocyte; Bile acid; Ursodeoxycholate; Mitochondrial permeability transition; Cholestasis

1. Introduction

Bile formation is a vital function of the liver and its impairment is central in the pathogenesis of cholestasis. In cholestatic disease, intrahepatic accumulation of toxic bile acids promotes hepatic injury [1]. Although the cellular mechanisms involved in bile acid-induced injury are still under investigation, it is known that hydrophobic bile acids are particularly hepatotoxic. Due to their membrane-active, detergent-like properties, the cytotoxicity of bile acids has been correlated to the degree of lipophilicity of the different molecular species [2]. Mechanisms implicated in the toxicity of bile acids include stimulation of lipid peroxidation [3] and induction of mitochondrial dysfunction [4–7].

Ursodeoxycholic acid (UDCA) has been used successfully in the treatment for primary biliary cirrhosis and other cholestatic liver diseases in humans [8]. Serum liver tests and histopathological analyses suggest improvement of liver function in patients treated with UDCA. In most of the cases, UDCA shows a favourable effect on biochemical indices [9] but not on symptoms or the progression of histological damage [10]. Recently we have shown that UDCA causes synergistic cell killing by toxic bile acids in rat hepatocyte primary cultures [11]. Likewise, Krahenbuhl et al. [12] described that UDCA increases the toxicity of lipophilic bile acids at the level of the mitochondrial electron transport chain. Conversely, studies made by others described a putative beneficial effect of UDCA exerted at the level of mitochondrial function [6,13]. The aim of the present study was to clarify the mechanisms involved in the synergistic cell killing by UDCA plus chenodeoxycholate (CDCA, a lipophilic toxic bile acid) [1], especially at the level of mitochondrial function. The results demonstrate that impairment of cellular ATP generation is a final pathway leading to cell death after exposure to CDCA, and that the triggering event is induction of the mitochondrial permeability transition (MPT).

Abbreviations: CDCA, Chenodeoxycholate; CyA, cyclosporine A; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; KHH buffer, Krebs–Henseleit–HEPES buffer; LDH, lactate dehydrogenase; MPT, mitochondrial permeability transition; RPMI, Rosewell Park Memorial Institute; TMRM, tetramethylrhodamine methyl ester; UDCA, Ursodeoxycholate

* Corresponding author. Tel.: +1-218-726-8899; fax: +1-218-726-8014.
E-mail address: kwallace@d.umn.edu (K.B. Wallace).

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2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Harlan Labs, Madison, WI) weighing 186 ± 6 g were maintained in AAALAC-accredited, climate-controlled facilities and allowed free access to food (Purina Chow) and water.

2.2. Materials

UDCA and CDCA were purchased from Sigma Chemical Co. (St. Louis, MO). UDCA was dissolved in ethanol, CDCA in water. Lactate dehydrogenase (LDH), commercial kit, was purchased from Sigma and Collagenase type 2 was from Worthington (Freehold, NJ). All other chemicals were of analytical grade and obtained from standard commercial sources. A Labsystems type 374 plate-reader was used for all measurements of fluorescence intensity.

2.3. Rat hepatocyte isolation and culture

Hepatocytes were isolated according to a modified procedure of Seglen [14]. Cells were plated on 12-well plates at a density of 0.6×10^6 cells per well. Culture medium was RPMI supplemented with 5% fetal bovine serum and 15 mM HEPES (pH 7.4). Cells were maintained in an incubator at 37 °C, 5% CO_2 and humidified atmosphere. After allowing 2–3 h for the cells to attach, the medium was replaced with Krebs–Henseleit–HEPES Buffer (KHH buffer, 119 mM, 4.9 mM KCl, 1.2 mM KH_2PO_4, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 12.5 mM HEPES, pH 7.4) and bile acids (150 μM CDCA and/or 250 μM UDCA) were added. Cells were incubated at 37 °C for 1, 2 or 4 h. In some experiments, cells were supplemented with 20 mM fructose or 1 μM CyA.

2.4. Cell viability assay

Cell viability was determined fluorometrically by estimating release of LDH into medium [15]. The determination of LDH activity is based on the reduction of pyruvate to lactate as monitored by the decrease in NADH fluorescence at 450 nm with excitation wavelength 355 nm. Fresh reaction solution was made by mixing 0.4 ml of 16.2 mM pyruvate with 10 ml of 0.2 mM NADH in phosphate buffer (pH 7.5). After incubation of hepatocytes with bile acids for 1, 2 or 4 h, 5 μl of the cell-free supernatant was added to 200 μl of fresh assay solution to initiate the reaction. Total cellular LDH was determined by lysing the cells after freezing in a solution of 0.1 M sodium phosphate buffer (pH 7.0). The amount of LDH released into the media was expressed as percent of total LDH.

2.5. Measurement of mitochondrial membrane potential ($\Delta\psi$) in hepatocytes

To monitor mitochondrial $\Delta\psi$, hepatocytes were loaded with 6.6 μM tetramethylrhodamine methyl ester (TMRM) in KHH buffer at room temperature for 10 min. The supernatant was then aspirated, and the cells returned to the original volume with KHH. TMRM is a membrane-permeable cationic fluorophore that accumulates electrophoretically in mitochondria in proportion to their $\Delta\psi$ [16]. Cell suspensions (200 μl containing 10^5 cells) were loaded into 96-well plates and fluorescence measured using excitation and emission wavelengths of 485 and 590 nm, respectively. After 5 min of recording basal fluorescence, cells were incubated with 150 μM CDCA, 250 μM UDCA and/or 1 μM CyA. Mitochondrial $\Delta\psi$ was estimated, taking into account the complete depolarization caused by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). The change in $\Delta\psi$ induced by the test agents was expressed as percent of total $\Delta\psi$.

2.6. Determination of adenine nucleotide content

After incubation of hepatocytes in culture with the compounds for 1, 2 or 4 h, adenine nucleotide content was determined by HPLC according to the method of Jones [17]. The extraction of adenylate nucleotides from cells was performed as described previously [18]. Quantification was achieved by employing a standard curve.

2.7. Statistical analysis

Solvent controls (water or ethanol) were included within each experimental determination and the data is expressed as a percentage of the respective control (mean ± S.E., $n = 4$). Ethanol itself had no effect on any of the three parameters measured. Statistical significance was determined by the one-way ANOVA Student–Newman–Keuls post *t* test for multiple comparisons. A P value of < 0.05 was considered statistically significant.

3. Results

Addition of CDCA to rat hepatocytes in primary culture was previously shown to cause a dose-dependent decrease in cell viability [11]. Furthermore, CDCA's toxicity was increased in the presence of UDCA. In the present work, we have also observed that although nontoxic per se, UDCA causes synergistic cell killing by CDCA as measured by LDH leakage (Fig. 1). The control samples contained the bile acid solvent at adequate concentrations. Cytotoxicity of CDCA and UDCA plus CDCA was time-dependent, with cell viability decreasing progressively throughout the incubation period. After 4 h, cell viability was only 41 ± 2% for UDCA plus CDCA compared to a

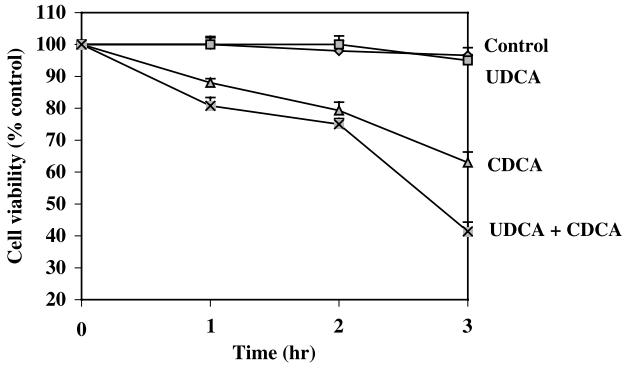

Fig. 1. Changes in cell viability following incubation of hepatocytes in KHH buffer with bile acids (150 μM CDCA and/or 250 μM UDCA) for 1, 2 or 4 h. Cell viability was determined fluorometrically by estimating the release of LDH into media. Total LDH was determined after lysing the cells; the amount of LDH released into media at any given time during the incubation was expressed as the percentage of total LDH. Percent viability was obtained by comparing with control cell viability, considered as 100% at time 0. Using this method, control cell viability was 89 ± 6% after 4 h of incubation. Data represent the mean ± S.E. of measurements from four separate animals.

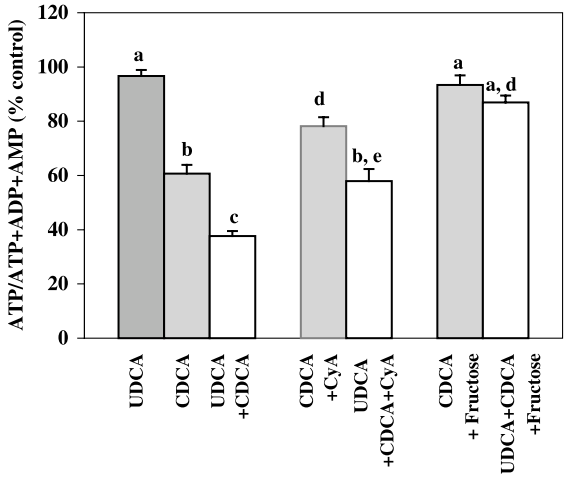

Fig. 2. Adenine nucleotide content of hepatocytes following 4-h incubation in KHH buffer with bile acids (150 μM CDCA and/or 250 μM UDCA). In some experiments, cells were supplemented with 20 mM fructose or 1 μM CyA. Adenine nucleotides were determined by HPLC according to the method of Jones [17]. Using this method, content for control cells was: AMP, 17 ± 4 nmol/million cell; ADP, 46 ± 3 nmol/million cell; ATP, 75 ± 3 nmol/million cell. Data are expressed as percent content compared to untreated cells. Data represent the mean ± S.E. of measurements from four separate animals. Values that share the same letter are not statistically different ($P < 0.05$).

63 ± 2% for CDCA alone. UDCA by itself had no significant effect on LDH release. The concentrations used were determined by taking into account previous data [11], where we describe the cytotoxicity of bile acids in terms of dose and time dependence. The concentrations used are below those that cause detergent-like nonspecific cell killing.

Incubation of hepatocytes with a toxic dose of CDCA and UDCA plus CDCA resulted in a decrease of intracellular adenine nucleotide content (Fig. 2), which appeared to parallel cell death. UDCA at 250 μM had no effect on cellular ATP levels. It has been shown that fructose, an alternate carbohydrate source for glycolysis, protects against cell killing induced by various toxic compounds [19] and also in hypoxic injury [20]. We investigated the hypothesis that fructose, by providing an alternate source of ATP, may prevent bile acid-induced toxicity. In agreement with previous findings, 20 mM fructose afforded almost complete protection against ATP depletion after 4 h of exposure to CDCA or UDCA plus CDCA (Fig. 2). In addition to preventing ATP depletion, fructose also protected against UDCA plus CDCA synergistic cytotoxicity (Fig. 3). These results suggest that inhibition of mitochondrial ATP formation by oxidative phosphorylation is a key event in the cytotoxicity of bile acids.

Impairment of mitochondrial function by bile acids may have drastic consequences on cellular function through the perturbation of bioenergetic charge and metabolism of the cell. We next assessed mitochondrial function during exposure of isolated cells to bile acids. Changes of mitochondrial membrane potential were monitored by the cellular retention of the fluorescent probe, TMRM.

Similar to the results reported with isolated mitochondria [7], CDCA addition resulted in a marked, almost complete decrease of TMRM retention in isolated hepatocytes (Fig. 4). UDCA induced 30% depolarization. CDCA plus UDCA caused more substantial mitochondrial depolarization when compared with CDCA alone. CyA inhibited both CDCA- and UDCA plus CDCA-induced depolarization of mitochondrial membrane potential (Fig. 4). Additionally, CyA

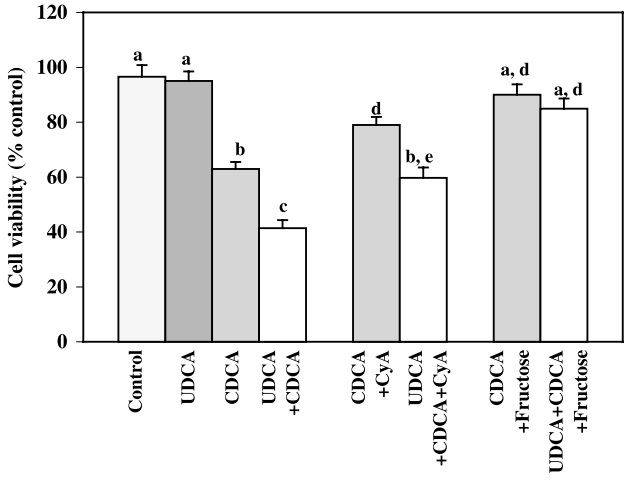

Fig. 3. Changes in cell viability following 4-h incubation of hepatocytes in KHH buffer with bile acids (150 μM CDCA and/or 250 μM UDCA). In some experiments, cells were supplemented with 20 mM fructose or 1 μM CyA. Cell viability was determined as described in Fig. 1. Data represent the mean ± S.E. of measurements from four separate animals. Values that share the same letter are not statistically different ($P < 0.05$).

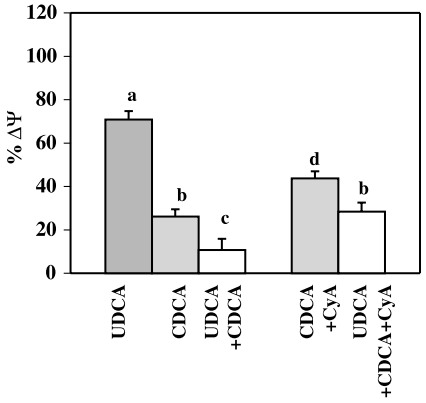

Fig. 4. Mitochondrial depolarization induced by bile acids (150 μM CDCA and/or 250 μM UDCA). In some experiments, cells were supplemented with 1 μM CyA. Mitochondrial $\Delta\psi$ was measured in cell suspensions as described in Materials and methods. The change in $\Delta\psi$ induced by the test agents was expressed as percent $\Delta\psi$ in the absence of bile acids, taking into account the complete depolarization of mitochondrial membrane potential by FCCP. Data represent the mean ± S.E. of measurements from four separate animals. Values that share the same letter are not statistically different ($P < 0.05$).

also prevented both ATP depletion (Fig. 2) and the associated synergistic cell killing (Fig. 3), indicating that bile acids cytotoxicity is a consequence of impairment of mitochondrial function. CyA by itself did not affect mitochondrial membrane potential, ATP levels or cell killing (data not shown).

These observations lead to the hypothesis that CDCA and the bile acid combination induced the MPT within intact hepatocytes. We should note that bile acids caused an abrupt leakage of TMRM from mitochondria within as little as 10 min. This preceded the loss of cell viability, which was significant only after 4 h of incubation (Fig. 1). These observations indicate that mitochondrial dysfunction precedes the onset of cell death.

4. Discussion

In patients with cholestatic liver disease, hepatocellular damage may be aggravated by increased bile acid concentrations [1]. At high bile acid concentrations, hepatocellular damage may reflect the detergent effects of the compounds leading to direct disruption of cell membranes [21]. Physiological intracellular concentrations of bile acids during cholestasis have been reported to be ~ 800 μM [22] and a similar pattern of hydrophobic bile acids accumulation in liver and serum of patients has been described [23]. In most patients, however, such bile acid concentrations do not correlate with massive hepatocellular injury but with a progressive cell dysfunction and destruction or loss of intrahepatic or extrahepatic bile ducts [24]. Therefore, hepatocellular damage at cholestatic relevant concentrations is likely due to other more specific actions of the bile acids.

Hydrophobic bile acids, such as CDCA, are implicated to be major contributors to cholestatic liver diseases. Two mechanisms of cell death induced by exposure of rat hepatocytes to toxic bile acids have been described: necrosis at high concentrations and apoptosis at lower concentrations [22,25]. Since liver damage in chronic cholestasis is largely mediated by the effects of high concentrations of retained bile acids in the liver, we wanted to determine if MPT induction by bile acids would cause bioenergetic collapse and cell necrosis, and to examine the role of UDCA in the process.

At high concentrations, bile acids cause detergent-like nonspecific disruption of biological membranes. However, it is important to note that the concentrations of bile acids achieved in these incubations (150–250 μM) are well within the range observed in liver from humans and rats with cholestatic liver disease. Fischer et al. [23] reported that the levels of bile acids in humans are 215 ± 39.1 nmol/g liver (wet weight) in chronic cholestasis. Assuming 100×10^6 hepatocytes/g wet weight and 5 μl of water/10^6 hepatocytes [26], the intracellular concentration of bile acids is estimated to be ~ 430 μM. CDCA contributed 41% to total bile acids and was elevated fourfold [23]. Setchell et al. [24] reported total bile acid concentrations in human liver tissue as 61.6 ± 29.7 nmol/g (~ 123 μM), comprising mainly CDCA and cholic acid. Following UDCA or tauroursodeoxycholate administration [24], higher concentrations and an enrichment in UDCA (30%) at the expense of hydrophobic bile acids were observed. In a model of experimental cholestasis, Setchell et al. [24] reported bile acid concentrations in liver tissue of sham-operated rats as 130.8 ± 21.3 nmol/g (~ 260 μM), and increased seven- to eightfold with bile duct ligation. Therefore, we contend that the mitochondrial-specific toxicity observed in these studies is relevant to what might be observed in vivo, in either rats or humans.

Several reports suggest a beneficial effect of UDCA in treating cholestatic liver disease [8,27,28], possibly by competitive antagonism of key cytotoxic responses induced by hydrophobic bile acids. Serum liver tests and histopathological studies suggest improvement of liver function in cholestatic patients treated with UDCA. In most of the cases, UDCA shows a favourable effect on biochemical indices [9] but not on the symptoms or the progression of histological stage [10]. Indeed, the recent report by Neuberger [10] draws attention to the necessity to re-examine the therapeutic benefit of UDCA. Several reports fail to show cytoprotection by UDCA against toxic bile acids at the level of mitochondrial function or cell viability [7,11,12,29]. In fact, many reports demonstrate the opposite effect, where UDCA causes a synergistic killing by CDCA of hepatocytes in primary culture [11,12].

Because of the importance of mitochondria in cellular energy metabolism (they provide about 90% of the total ATP of liver cells), alterations in normal oxidative phosphorylation may play an important role in cell pathogenesis. Indeed, a number of observations suggest that mitochondria

are a primary target of chemical-induced injury and that their dysfunction ultimately leads to cell death [30,31]. Both morphological and biochemical observations support the concept that bile acids may be cytotoxic by causing mitochondrial dysfunction [3–7]. Long-term cholestasis caused by bile duct ligation is known to lead to impaired hepatic mitochondrial function in the rat [5], which includes decreased activities of complexes of the electron transport chain [5,32], decreased activities of enzymes involved in β-oxidation [33] and impaired antioxidative defense mechanisms [34,35]. Alterations in hepatic energy metabolism, in particular glycogen metabolism [36], are also observed. Enlarged, swollen mitochondria are apparent in histopathologic sections obtained from this model of extrahepatic cholestasis [4].

In the present work, we confirm and extend previous findings that mitochondria are an important target of bile acid toxicity [7,11]. Furthermore, our results demonstrate that synergistic cell killing by UDCA plus CDCA is manifested by inhibition of mitochondrial ATP generation. Consistent with this conclusion is the previous report that glycochenodeoxycholate, the glycine conjugate of CDCA, induces lethal hepatocellular injury dependent on ATP depletion [22]. Fructose, which is an alternate glycolytic source of cellular ATP, protects against ATP depletion and cell killing induced by CDCA alone and in combination with UDCA. Because mitochondria are the primary source of ATP in liver cells, our results indicate that impairment of mitochondrial oxidative phosphorylation is an early and critical event in the mechanism of bile acid cytotoxicity.

Induction of the MPT is widely implicated in the pathophysiology of cell death caused by a number of agents [37]. It is characterized by an increase in nonspecific permeability of the inner membrane to low-molecular-weight solutes leading to mitochondrial membrane depolarization, mitochondrial calcium release, mitochondrial swelling and inhibition of oxidative phosphorylation [38]. The MPT is suggested to reflect the opening of a pore composed of assorted elements, the formation of which is inhibited by CyA [39]. Induction of the MPT likely underlies bile acid-induced mitochondrial uncoupling as reflected by the fact that CyA protected against mitochondrial depolarization, ATP depletion and cell killing. However, protection by CyA was not complete, which may reflect the previously reported direct effect of bile acids on the respiratory chain [7]. It has been shown that reactive oxygen species are generated by isolated rat hepatocytes and rat liver mitochondria exposed to hydrophobic bile acids [40,41], the impaired electron transport being responsible for mitochondrial superoxide generation. Furthermore, oxidative stress or, in general, oxidizing conditions are inducers of the MPT [38]. Gumpricht et al. [42] presented evidence that glutathione status of isolated rat hepatocytes modulates bile acid-induced necrosis but not apoptosis, which implicates oxidative stress in the pathogenesis of bile acid-induced necrosis.

In conclusion, our results indicate that UDCA potentiates the cytotoxicity of CDCA by promoting induction of the MPT, leading to inhibition of ATP synthesis and metabolic starvation of the cell. This is consistent with previous reports describing the potentiation of CDCA-induced mitochondrial dysfunction by UDCA [7]. Furthermore, this research adds to the growing literature that indicates that UDCA potentiates photodynamic therapy [43] and induces apoptosis in hepatocellular carcinoma cells [44]. In view of the wide implication of the MPT in chemical-induced cell injury, these data draw into question the potential benefit of prescribing UDCA to treat cholestatic disease. If accumulation of hydrophobic bile acids is indeed a primary factor in the pathogenesis of cholestatic liver disease, then results from this study may provide potential new insights regarding the failure of UDCA for preventing the hepatocellular damage observed clinically in cholestatic liver injury. Induction of the MPT and subsequent cell injury may represent a primary pathway for the progressive and continued liver dysfunction associated with this disease.

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