Bilirubin inhibits bile acid induced apoptosis in rat hepatocytes

A Granato, G Gores, M T Vilei, R Tolando, C Ferreira, M Muraca

Background and aims: Hydrophobic bile acids contribute to hepatocellular injury in cholestasis and rapidly induce apoptosis in vitro; however, unlike Fas agonists, cholestasis does not cause extensive hepatocyte apoptosis. As antioxidants provide protection against bile acid induced liver injury, our premise was that bilirubin, a free radical scavenger with increased plasma levels in the presence of liver disease, could protect hepatocytes against bile acid induced apoptosis.

Methods: Freshly isolated rat hepatocytes were incubated for four hours with 100 μmol/l glycochenodeoxycholate (GCDC) alone or with increasing concentrations of unconjugated (UCB) or conjugated (CB) bilirubin.

Results: Both UCB and CB inhibited GCDC induced apoptosis in a dose dependent fashion and suppressed the generation of reactive oxygen species by hepatocytes.

Conclusions: The antiapoptotic effect of bilirubin associated with its antioxidant properties indicates that hyperbilirubinemia may have a protective role in liver disease.

In patients with cholestasis, accumulation of bile acids within the liver contributes to hepatocellular damage. While high bile acid concentrations can induce hepatocyte necrosis, lower concentrations of these compounds are associated with apoptosis which can be triggered by specific cell surface “death” receptors (extrinsic pathway) or by mitochondrial dysfunction induced by internal stress (intrinsic pathway), such as the generation of reactive oxygen species (ROS).”

Despite an increasing body of evidence demonstrating that bile acid mediated hepatocyte apoptosis plays an important role in hepatobiliary diseases, the incidence of parenchymal liver cell apoptosis in cholestatic disorders is lower than expected on the basis of in vitro studies. Possible explanations for this discrepancy include activation of antiapoptotic pathways and the presence of protective humoral factors. Free radical scavengers such as lazaroïds, α-tocopherol, and ebselen inhibit bile acid induced hepatocyte apoptosis in vitro. Bilirubin, a yellow tetrapyrrole derived from the enzymatic degradation of haeme, also accumulates in the plasma of patients with cholestasis due to impaired biliary excretion. This lipid soluble pigment is commonly considered merely a toxic waste compound because neonatal hyperbilirubinemia can lead to brain damage (“kernicterus”). However, the pigment acts as an efficient free radical scavenger in vitro at micromolar concentrations. Prior to biliary excretion, bilirubin is conjugated with one or two molecules of glucuronic acid, yielding a water soluble compound. Unconjugated (UCB) and conjugated (CB) bilirubin accumulate in tissues and plasma during cholestasis.

In the present work, we demonstrate that both UCB and CB effectively inhibit bile acid induced apoptosis in freshly isolated hepatocytes, an effect associated with suppression of ROS generation, suggesting that these pigments may play a protective role in cholestatic disorders.

MATERIALS AND METHODS

Materials

CD rats were purchased from Charles River (Kislegg, Germany). William’s E medium, fetal bovine serum, penicillin/streptomycin, glutamine, and collagenase were from Life Technologies Inc. (Grand Island, New York, USA). Trypan blue, dexamethasone, glucagon, glycochenodeoxycholate (GCDC), diamidino-2-phenylindole dihydrochloride (DAPI), and propidium iodide were supplied by Sigma Chemical Co. (St. Louis, Missouri, USA). UCB (Sigma Chemical Co) was recrystallised before use. CB ditaurate (a commercially available surrogate for bilirubin glucuronate) was from Porphyrin Products Inc. (Logan, Utah, USA). According to the manufacturer, the pigment contains approximately 10–12% monomurate and less than 2% of UCB.

Experimental methods

Preparation of stock solutions of UCB and CB

Stock solutions of 42.75, 21.38, and 10.69 mmol/l UCB were prepared by dissolving 25, 12.5, and 6.25 mg of pigment, respectively, in 1 ml of 0.1 N NaOH. After complete dissolution, 1.75 ml of albumin solution (70 mg bovine serum albumin (BSA) in 1.75 ml of William’s E medium) were added to each UCB solution. The solutions, constantly containing 5% fetal bovine serum to the desired pigment stock, were therefore 11, 5.5, and 2.75, respectively. As bile salts are also bound to albumin, the albumin concentration in incubation medium containing 5% fetal bovine serum to the desired pigment concentrations (200, 100, and 50 μmol/l) just before use. As the albumin content of 5% fetal bovine serum corresponded to 13.44 μmol/l, the final albumin concentration in incubation medium (4.73 μmol/l from added BSA+13.44 μmol/l from fetal bovine serum) was 18.17 μmol/l. The bilirubin/albumin molar ratios in the 200, 100, and 50 μmol/l solutions were therefore 11, 5.5, and 2.75, respectively. As bile salts are also bound to albumin, the albumin solution without bilirubin was diluted with incubation medium to obtain the same final concentration of BSA in experiments not including bile pigments. The GCDC/albumin molar ratio was 5.5.

Abbreviations: GCDC, glycochenodeoxycholate; UCB, unconjugated bilirubin; CB, conjugated bilirubin; ROS, reactive oxygen species; DAPI, diamidino-2-phenylindole dihydrochloride; BSA, bovine serum albumin; DCF, dichlorofluorescein; LDH, lactate dehydrogenase; GLDH, glutamate dehydrogenase; AST, aspartate aminotransferase.
Bilirubin inhibits hepatocyte apoptosis

UCB solution was centrifuged and the bilirubin concentration assessed in duplicate by the diazo method before and after centrifugation. No change in bilirubin concentration was observed after centrifugation, nor were pigment aggregates found at microscopic examination (400x) of the solution. The pH of the culture medium containing BSA-UCB (10 ml aliquots) was adjusted to 7.4 to 10–25 µl of 1.2 M HCl. CB was dissolved directly in William’s E medium containing BSA and fetal bovine serum as described above, and no pH correction was necessary.

Hepatocyte isolation and culture

Rat primary hepatocytes were isolated from male CD rats (150–200 g) by collagenase perfusion of the liver.18 Cell viability, determined by trypan blue exclusion, was 85–93%. After isolation, hepatocytes were resuspended in William’s E medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 3 mM glutamine, 0.16 U/ml insulin, and 0.014 µg/ml glucagon. A total of 5×10^5 cells/well, seeded onto uncoated plastic tissue culture plates, were maintained at 37°C in a 5% CO_2 humidified atmosphere for three hours. Plates were then washed with the medium to remove unattached cells and incubated with a medium containing 5% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 3 mM glutamine, 0.16 U/ml insulin, and 100 µmol/l GCDCA, alone or in combination with 50, 100, or 200 µmol/l UCB or CB for four hours at 37°C in 5% CO_2 humidified atmosphere. Elsewhere, we observed that approximately 1% of UCB added to the incubation medium is converted by cultured rat hepatocytes to CB after four hours.19

Assessment of nuclear fragmentation (apoptosis)

Isolated rat hepatocytes were plated onto uncoated plastic tissue culture plates (5×10^5 cells/well) and incubated for four hours with 100 µmol/l GCDCA alone or in combination with UCB or CB. Nuclear changes indicating apoptosis were quantified by staining rat hepatocytes with DAPI, a membrane permeant fluorescent DNA binding dye to label the nucleus of hepatocytes. Propidium iodide (1 µM) was also added to identify necrotic cells. For morphological evaluation of apoptosis at fluorescence microscopy, cultured rat hepatocytes were incubated with DAPI (1 µg/ml) for 10 minutes at 37°C. Cells were considered apoptotic if the classic features of nuclear margination/condensation and nuclear fragmentation were present. Fluorescent stained nuclei were considered fragmented if at least three separate fragments of condensate chromatin were identified in a cell. At least 300 cells in four high power fields were counted, and nuclear fragmentation was expressed as a percentage of total cells, excluding propidium iodide.20, 21

Measurement of reactive oxygen species (ROS) generation

Isolated hepatocytes (5×10^5 cells/well) were preloaded with 10 µmol/l 2.79-dichlorofluorescein (DCF) at 37°C for 30 minutes, washed twice, and resuspended in the incubation medium with or without 100 µmol/l GCDCA and in the presence or absence of 50, 100, and 200 µmol/l UCB or CB. Aliquots of cells, removed at 0, 60, 120, 180, and 240 minutes, were analysed for fluorescence in real time at 490 nm excitation and 520 nm emission wavelengths in a 1420 Victor 2 (EG&G Wallac, Turku, Finland) fluorescence spectrophotometer.

Enzyme release

During the four hour incubation of hepatocytes with or without GCDCA, UCB, and CB, cellular release of lactate dehydrogenase (LDH), glutamate dehydrogenase (GLDH), and aspartateaminotransferase (AST) was measured hourly, and expressed as a percentage of enzyme released into the buffer of the total activity present in hepatocytes (sum of the activities measured after cell lysis and in the medium). Measurement of GLDH, LDH, and AST was achieved using enzymatic method kits (Randox, Crumlin, UK, Roche Diagnostica SpA, Monza, Italy, and Roche Diagnostica SpA, Monza, Italy, respectively). All measurements were performed using an automatic analyser (Hitachi 912, Tokyo, Japan).

Statistics

Values are expressed as mean (SD). A GraphPad InStat microcomputer program (GraphPad Software, Inc. San Diego, California, USA) was used to evaluate differences between groups with the Mann-Whitney rank sum test and regression analyses. A p value <0.05 was considered statistically significant.

RESULTS

Morphological features of apoptosis

After four hours of treatment with 100 µmol/l GCDCA, 63.32% (1.84%) of hepatocytes were apoptotic whereas <1% of cells were apoptotic in the absence of the bile acid, as assessed by nuclear fragmentation after DAPI staining (table 1, fig 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of unconjugated (UCB) and conjugated (CB) bilirubin on glycocholate-induced apoptosis. Rat primary hepatocytes were incubated for four hours with incubation medium alone or with 100 µmol/l GCDCA and with increasing concentrations of UCB and CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group (n = 6)</td>
<td>% Apoptosis</td>
</tr>
<tr>
<td>Incubation medium</td>
<td>0.66 (0.51)</td>
</tr>
<tr>
<td>UCB 50 µM</td>
<td>0.67 (0.52)</td>
</tr>
<tr>
<td>UCB 100 µM</td>
<td>0.83 (0.41)</td>
</tr>
<tr>
<td>UCB 200 µM</td>
<td>0.66 (0.51)</td>
</tr>
<tr>
<td>CB 50 µM</td>
<td>0.83 (0.41)</td>
</tr>
<tr>
<td>CB 100 µM</td>
<td>0.70 (0.54)</td>
</tr>
<tr>
<td>CB 200 µM</td>
<td>0.70 (0.55)</td>
</tr>
<tr>
<td>GCDCA 100 µM</td>
<td>63.32 (1.84%)*</td>
</tr>
<tr>
<td>GCDCA 100 µM+UCB 50 µM</td>
<td>55.97 (3.28)**</td>
</tr>
<tr>
<td>GCDCA 100 µM+UCB 100 µM</td>
<td>42.92 (2.91)**</td>
</tr>
<tr>
<td>GCDCA 100 µM+UCB 200 µM</td>
<td>38.21 (2.15)**</td>
</tr>
<tr>
<td>GCDCA 100 µM+CB 50 µM</td>
<td>52.50 (2.43)**</td>
</tr>
<tr>
<td>GCDCA 100 µM+CB 100 µM</td>
<td>35.50 (3.08)</td>
</tr>
<tr>
<td>GCDCA 100 µM+CB 200 µM</td>
<td>30.33 (4.27)</td>
</tr>
</tbody>
</table>

Results are expressed as mean (SD) per cent apoptosis occurring in GCDCA treated cells.

p<0.005 compared with GCDCA.

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Both UCB and CB inhibited GCDC induced apoptosis in a concentration dependent fashion. The inhibitory effect of CB on GCDC induced apoptosis was significantly stronger compared with UCB (fig 2).

GCDC induced ROS generation
In order to investigate any relationship between bilirubin inhibition of GCDC induced apoptosis and the antioxidant properties of the pigment, ROS generation was assessed in rat hepatocytes treated with 100 μmol/l GCDC in the presence or absence of 50, 100, and 200 μmol/l UCB or CB, as described above. Addition of both pigments strongly suppressed the increase in GCDC-stimulated DCF fluorescence, thus indicating strong suppression of ROS generation (figs 3, 4; only data obtained at a concentration of 100 μmol/l are shown). Inhibition of ROS generation was not significantly different with UCB or CB at any concentration tested.

Enzyme release
Incubation with 100 μmol/l GCDC was followed by increased release of LDH, GLDH, and AST with respect to control samples. Coincubation with 100 μmol/l UCB or CB strongly reduced release of all enzymes tested (figs 5, 6).

DISCUSSION
Although our understanding of the pathogenesis of cholestatic liver disease is incomplete, it is generally believed that accumulation of toxic hydrophobic bile acids, such as deoxycholic acid conjugates, within the hepatocyte can contribute to liver injury by inducing hepatocyte apoptosis. Antioxidants, such as α-tocopherol or lazaroid, reduce both the generation of ROS and cell injury in freshly isolated hepatocytes treated with GCDC as well as in the intact rat infused with taurochenodeoxycholic acid. Bilirubin, the yellow pigment which accumulates in the plasma of patients with cholestasis, was recognised as an antioxidant of possible physiological importance approximately 15 years ago, and its activity as a free radical scavenger was demonstrated in model membrane systems, being equal to or even surpassing...
Bilirubin inhibits hepatocyte apoptosis. Enzyme activity released in the incubation medium was expressed as percent of total enzyme activity measured on cell lysates at the end of each time point. Release of lactate dehydrogenase (LDH) (A), glutamate dehydrogenase (GLDH) (B), and aspartate aminotransferase (AST) (C) to control medium and after incubation with UCB, GCDC, and GCDC + UCB for four hours (mean (SD), n = 6).

**Figure 5** Bile acid stimulated enzyme release from hepatocytes was inhibited by unconjugated bilirubin (UCB). Hepatocytes were incubated with 100 μmol/l glycochenodeoxycholate (GCDC) with or without 100 μmol/l UCB. Enzyme activity released in the incubation medium was expressed as percent of total enzyme activity measured on cell lysates at the end of each time point. Release of lactate dehydrogenase (LDH) (A), glutamate dehydrogenase (GLDH) (B), and aspartate aminotransferase (AST) (C) to control medium and after incubation with UCB, GCDC, and GCDC+UCB for four hours (mean (SD), n = 6).

**Figure 6** Bile acid stimulated enzyme release from hepatocytes was inhibited by conjugated bilirubin (CB). Hepatocytes were incubated with 100 μmol/l glycochenodeoxycholate (GCDC) with or without 100 μmol/l CB. Enzyme activity released in the incubation medium was expressed as percent of total enzyme activity measured on cell lysates at the end of each time point. Release of lactate dehydrogenase (LDH) (A), glutamate dehydrogenase (GLDH) (B), and aspartate aminotransferase (AST) (C) to control medium and after incubation with CB, GCDC, and GCDC+CB for four hours (mean (SD), n = 6).

that of α-tocopherol. As bilirubin interacts with biomembranes, it was postulated that this pigment could prevent lipid peroxidation associated with alterations in the physicochemical properties of the membranes leading to cell dysfunction and death. The protection against oxidative stress provided by the pigment has been demonstrated in several in vitro and in vivo studies.

The findings in the present study demonstrate that bilirubin inhibits GCDC induced apoptosis in rat hepatocytes, and that this effect is associated with inhibition of ROS generation in the same culture system, thus suggesting a link between the antioxidant properties of the pigment and its ability to prevent bile acid induced apoptosis. Four hour incubation of hepatocytes with GCDC led to a slight increase in LDH, GLDH, and AST, which was prevented by addition of bile pigments to the medium (figs 5, 6), further suggesting that the membrane protective activity of bilirubin could help to reduce bile acid induced cell death.

The range of bilirubin concentrations tested in the in vitro assays are commonly found in the plasma of patients with cholestatic disorders. In these patients, plasma concentrations of bile acids and bilirubin are about equimolar. The final BSA concentration in incubation medium, corresponding to 18.17 μmol/l, was well below both physiological levels (approximately 600 μmol/l) and levels observed in patients with end stage cholestatic liver disease (approximately 300 μmol/l). However, low albumin concentrations have been used in all published studies involving apoptosis induced in vitro by toxic albumin bound compounds in order to achieve, in short incubation periods, significant interaction of the test compound with cultured cells, thus mimicking the conditions of a prolonged exposure in vivo (such as in cholestasis). Moreover CB, which is not bound to albumin and is the most abundant pigment fraction in the blood of cholestatic patients, inhibited bile acid induced apoptosis to an even greater extent than UCB (fig 2). Preliminary results showed that CB also behaves as a free radical scavenger. In the present experimental setup, suppression of ROS generation was similar with the two pigments. However, further studies are needed to compare the relative antioxidant effect of UCB and CB.

The above findings indicate that bilirubin may prevent the bile acid induced liver injury associated with cholestatic disorders, thus supporting the hypothesis that the pigment plays a “beneficial” role as a powerful biological antioxidant. In our incubation system with freshly isolated hepatocytes, addition of 100 μmol/l UCB or CB almost completely prevented GCDC induced ROS formation (figs 3, 4), but the average inhibition of apoptosis at the same pigment concentration was only 25.9% and 34.5%, respectively. These data lend support to the hypothesis that
oxidative stress is not the main trigger of apoptosis but is a secondary phenomenon, amplifying the toxic effect of bile acids.¹

According to Silva and colleagues,⁶ UCB induced apoptosis in cultured neuronal cells, and this effect could be prevented by ursodeoxycholic acid. These results seem opposite to our findings. However, our experimental conditions are not comparable with those of Silva et al because while UCB is toxic to neurones it has not been shown to have any adverse effects on hepatocytes. Moreover, ursodeoxycholic acid is an antiapoptotic hydrophilic bile acid⁷ whereas GCDC is a detergent toxic compound.

In conclusion, our experimental findings indicate that the antioxidant properties of bilirubin may be relevant in liver disease, suggesting a protective role of the pigment accumulating in plasma and tissues of patients with cholestasis. The present results could have therapeutic implications as removal of bile pigments from plasma by liver support devices⁸–¹⁰ might not be necessarily advantageous in patients with liver disease. As concentrations of UCB were in a similar range as serum total bilirubin levels in physiological jaundice, with liver disease, suggesting a protective role of the pigment accumulating in plasma and tissues of patients with cholestasis. These results seem opposite to our findings. However, our experimental conditions are not comparable with those of Silva et al because while UCB is toxic to neurones it has not been shown to have any adverse effects on hepatocytes. Moreover, ursodeoxycholic acid is an antiapoptotic hydrophilic bile acid⁷ whereas GCDC is a detergent toxic compound.

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