Bilirubin inhibits bile acid induced apoptosis in rat hepatocytes

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

See end of article for authors’ affiliations

Correspondence to:
Dr M Muraca, Department of Medical and Surgical Sciences, University of Padova, Via Giustiniani, 2, I-35128 Padova, Italy; muraca@unipd.it

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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 necrosis, lower concentrations of these compounds are associated with apoptosis in vitro. 8 Possible explanations for this discrepancy include activation of antiapoptotic pathways and the presence of protective humoral factors. Free radical scavengers such as lazaroids, 10 α-tocopherol, and 4-ethylselen 11 inhibit bile acid induced apoptosis in vitro. Bilirubin, a yellow tetapyrrole derived from the enzymatic degradation of haeme, also accumulates in the plasma of patients with cholestasis due to impaired biliary excretion. 12 This lipid soluble pigment is commonly considered merely a toxic waste compound because neonatal hyperbilirubinaemia may have a protective role in liver disease.

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. 17 CB ditaurate (a commercially available surrogate for bilirubin glucurate) was from Porphyrin Products Inc. (Logan, Utah, USA). According to the manufacturer, the pigment contains approximately 10–12% monotaurate 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, 1.750 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 protected from light, were diluted with 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 was 1.594 μmol/l from added BSA +13.44 μmol/l from fetal bovine serum. The bilirubin/albumin molar ratio in the 200, 100, and 50 μmol/l solutions were therefore 11.55, 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 (400×) of the solution. The pH of the culture medium containing BSA-UCB (10 ml aliquots) was adjusted to 7.4 by adding 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. 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 100 μg/ml glucagon. A total of 5×10⁵ cells/well, seeded onto uncoated plastic tissue culture plates, were maintained at 37°C in a 5% CO₂ 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 μg/l GCDC, alone or in combination with 50, 100, or 200 μg/l UCB or CB for four hours at 37°C in 5% CO₂ 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.⁹

Assessment of nuclear fragmentation (apoptosis)

Isolated rat hepatocytes were plated onto uncoated plastic tissue culture plates (5×10⁵ cells/well) and incubated for four hours with 100 μg/l GCDC 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.⁹ ¹⁰

Measurement of reactive oxygen species (ROS) generation

Isolated hepatocytes (5×10⁵ cells/well) were preloaded with 10 μM 2.79-dichlorofluorescein (DCF) at 37°C for 30 minutes, washed twice, and resuspended in the incubation medium with or without 100 μl/l GCDC and in the presence or absence of 50, 100, and 200 μl/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 GCDC, UCB, and CB, cellular release of lactate dehydrogenase (LDH), glutamate dehydrogenase (GLDH), and aspartate aminotransferase (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 μg/l GCDC, 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>Experimental group (n = 6)</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<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>GCDDCA 100 μM</td>
<td>63.32 (1.84)</td>
</tr>
<tr>
<td>GCDDCA 100 μM+UCB 50 μM</td>
<td>55.97 (3.28)**</td>
</tr>
<tr>
<td>GCDDCA 100 μM+UCB 100 μM</td>
<td>42.92 (2.91)*</td>
</tr>
<tr>
<td>GCDDCA 100 μM+UCB 200 μM</td>
<td>38.21 (2.15)**</td>
</tr>
<tr>
<td>GCDDCA 100 μM+CB 50 μM</td>
<td>35.16 (2.43)**</td>
</tr>
<tr>
<td>GCDDCA 100 μM+CB 100 μM</td>
<td>35.50 (3.08)</td>
</tr>
<tr>
<td>GCDDCA 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 GCDDCA treated cells. p<0.005 compared with GCDDCA.
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 the 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.\(^7\)\(^,\)\(^8\) Antioxidants, such as vitamin E (alpha-tocopherol or lazaroid), reduce both the generation of ROS and cell injury in freshly isolated hepatocytes treated with GCDC\(^4\)\(^,\)\(^9\) as well as in the intact rat infused with taurochenodeoxycholic acid.\(^10\) Bilirubin, the yellow pigment which accumulates in the plasma of patients with cholestasis, was recognized 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...
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 bilirubin 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 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, 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 neurons it has not been shown to have any adverse effects on hepatocytes. Moreover, ursodeoxycholic acid is an antiapoptotic hydrophilic bile acid² whereas GCDC is a detergent bile 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 devices 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, these findings suggest that physiological jaundice may also have antioxidant properties useful in the newborn. Further studies are needed to assess the clinical relevance of these findings.

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Authors’ affiliations
A Granato, M T Vilei, C Ferraresso, M Muraca, Clinica Medica 1, University of Padova, Italy
G Gores, Division of Gastroenterology and Hepatology, Mayo Medical School, Clinic and Foundation, Rochester, Minnesota, USA
R Tolando, GlaxoSmithKline Research Centre, Verona, Italy

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