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Toxicity of peroxisomal C_{27}-bile acid intermediates

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ABSTRACT

Peroxisomes play an important role in bile acid biosynthesis because the last steps of the synthesis pathway are performed by the β-oxidation system located inside peroxisomes. As a consequence, C_{27}-bile acid intermediates accumulate in several peroxisomal disorders. It has been suggested that C_{27}-bile acids are especially toxic and contribute to the liver disease associated with peroxisomal disorders. For this reason, we investigated the toxicity of C_{27}-bile acids and the underlying mechanisms. We studied the effects of conjugated and unconjugated C_{27}-bile acids on cell viability, mitochondrial respiratory chain function and production of oxygen radicals in the rat hepatoma cell line McA-RH7777. Cell viability decreased progressively after incubation with increasing concentrations of different bile acids with dihydroxycholestanoic acid (DHCA) being clearly the most cytotoxic bile acid. In addition, the different bile acids caused a dose-dependent decrease in ATP synthesis by isolated mitochondria oxidizing malate and glutamate. Finally, there was a dose-dependent stimulation of ROS generation in the presence of C_{27}-bile acids. In conclusion, our studies showed that C_{27}-bile acids are more cytotoxic than mature C_{24}-bile acids. In addition, C_{27}-bile acids are potent inhibitors of oxidative phosphorylation and enhance mitochondrial ROS production by inhibiting the respiratory chain.

Introduction

Bile acids are formed in the liver from cholesterol via multiple pathways involving several enzymes localized in different subcellular compartments [1]. Bile acid biosynthesis consists of modification of the ring structure of cholesterol, oxidation and shortening of the side chain, and finally conjugation of the bile acid with an amino acid. Peroxisomes play an important role in the synthesis of bile acids because the last steps of the biosynthesis pathway, the shortening of the side chain, are performed by the β-oxidation system located inside the peroxisome [2]. In addition, the enzyme responsible for conjugation, bile acyl-CoA: amino acid *N*-acyltransferase (BAAT), is present inside peroxisomes [3,4].

In the classic bile acid biosynthesis pathway (reviewed in [1]), the ring structure of cholesterol is modified first, and subsequently the sterol side chain is converted into a carboxylic acid by the mitochondrial sterol 27-hydroxylase (CYP27A1). The products of this reaction are the C_{27}-bile acid intermediates 3α,7α-dihydroxycholestanoic acid (DHCA) and 3α,7α,12α-trihydroxycholestanoic acid (THCA). These C_{27}-bile acid intermediates have to be activated to their CoA-ester, which occurs predominantly at the endoplasmic reticulum [5], and are then transported into the peroxisome. Here, the (25*R*)-isomer of DHC- and THC-CoA, which are exclusively formed by CYP27A1, are first converted into the (25*S*)-isomer by α-methylacyl-CoA racemase (AMACR). This conversion is necessary because the peroxisomal β-oxidation system can only handle *S*-isomers. (25*S*)-DHC- and THC-CoA are then oxidized by the first enzyme of the β-oxidation system, i.e. branched-chain acyl-CoA oxidase (BCOX). The resulting enoyl-CoA esters are subsequently hydrated into a hydroxyacyl-CoA and then dehydrogenated into a β-ketoacyl-CoA. Both these steps are catalyzed by D-bifunctional protein (DBP), alternatively named multifunctional protein 2 or multifunctional enzyme 2. Finally, sterol carrier protein X (SCPx) is responsible for the thiolytic cleavage of the β-ketoacyl-CoA esters of DHCA and THCA. Cleavage of the side chain of DHC-CoA by β-oxidation results in the formation of chenodeoxycholic acid (CDCA), whereas cleavage of the side chain of THC-CoA results in cholic acid (CA).

Patients with Zellweger syndrome, who lack functional peroxisomes, and patients with a defect of one of the peroxisomal enzymes involved in bile acid biosynthesis, i.e. AMACR-, DBP- and SCPx-deficient patients, accumulate C_{27}-bile acid intermediates [2]. These C_{27}-bile acid intermediates have altered physical properties when compared to mature C_{24}-bile acids and are only partly conjugated in patients, most likely due to low affinity of the conjugating enzyme BAAT towards C_{27}-bile acyl-CoAs [6,7]. They are excreted in bile, but studies in DBP- and AMACR-deficient knockout mice have shown that they are not excreted as efficiently as C_{24}-bile acids [8,9]. It has been suggested that the C_{27}-bile acid inter-

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mediates are especially toxic and contribute to the liver disease associated with peroxisomal disorders [10,11]. Patients with Zellweger syndrome have cholestasis, hepatomegaly, elevated serum liver enzymes, excessive hepatocyte iron stores and steatorrhea. Lesions of cholangioles, inflammation and bile duct proliferation have also been reported [10]. In two siblings with AMACR deficiency, who had cholestasis and a deficiency of fat-soluble vitamins, histological examination of the liver revealed giant cell transformation, the prevalence of necrotic hepatocytes and periportal hemosiderosis [12].

There are a number of reports in literature describing treatment of patients with a peroxisomal disorder with C_{24}-bile acids [12–14]. In addition, bile acid treatment was studied in peroxisome-deficient PEX2 Zellweger mice [11]. The rationale behind this treatment is to lower the levels of the potentially hepatotoxic C_{27}-bile acid intermediates by downregulation of the bile acid biosynthesis pathway and by increasing the bile flow. The treatment has been shown to reduce C_{27}-bile acid accumulation and to improve liver histology in the treated patients. These studies clearly demonstrate that the bile acid abnormalities contribute to the liver pathology, however, the direct effects of the C_{27}-bile acid intermediates cannot be deduced from these studies especially since multiple peroxisomal functions are disturbed in these patients and this mouse model for Zellweger syndrome. For this reason, we have studied the toxicity of conjugated and unconjugated C_{27}-bile acid intermediates and the underlying mechanisms. We have examined their effects on cell viability, mitochondrial respiratory chain function and the production of oxygen radicals in a rat hepatoma cell line and compared the results with the effects of the mature C_{24}-bile acids.

Materials and methods

Materials

The rat hepatoma cell lines McA-RH7777 (ATCC Crl-1601) and FaO (a gift from Dr. Latruffe, Université de Bourgogne, Dijon, France) were used in this study. Amplex Red was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA), Antimycin A and DMSO from Sigma–Aldrich (St. Louis, MO, USA), The CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega (Madison, WI, USA), horseradish peroxidase and Cu,Zn-superoxide dismutase from Boehringer Mannheim (Mannheim, Germany). CDCA, g-CDCA, CA, t-CA and g-CA were from Sigma–Aldrich, whereas DHCA and THCA were purchased from Dr. H. ten Brink (VU University, Amsterdam, The Netherlands). t-THCA and g-THCA were synthesized as described in [15] for the synthesis of conjugated C_{24}-bile acids. All other chemicals were of analytical grade.

Cell viability

Cell viability was determined by the release of lactate dehydrogenase (LDH) in the medium and with The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). McA-RH7777 and FaO hepatic cells were seeded in 48-well plates at equal density in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), and allowed to attach over night. The next day, cells were incubated with increasing concentrations of C_{24}- and C_{27}-bile acids in MEM/10% FCS in a final volume of 200 μl, and incubated for 3 h at 37 °C and 5% CO_2. For the cell proliferation assay, 33 μl of CellTiter 96® AQueous One Solution Reagent (containing MTS tetrazolium) was added to the incubation mixture and absorption was measured at 485 nm. The quantity of formazan product as measured by the amount of 485 nm absorbance is directly proportional to the number of metabolically active cells in culture. Protein content was determined before incubation with bile acids in parallel wells and was always between 50 and 100 μg protein per well. For the LDH activity measurements, the medium was collected after the 3 h-incubation, centrifuged for 5 min at 250 × g_{av} and the supernatant was used for activity measurements of LDH released in the medium. The pellet containing detached cells was added to the cell suspension obtained by lysis of the attached cells with 200 μl 0.1% Triton in PBS. The LDH activity in the medium was expressed as percentage of the total activity in medium plus cells. The LDH activity measurement is based on the reduction of pyruvate to lactate, as monitored by the decrease in NADH absorption at 340 nm in time [16]. Briefly, 25 μl of sample was incubated with 50 mM KPi pH 7.4, 0.2 mM NADH, 0.1% Triton X-100 in a final volume of 250 μl and the reaction was started by the addition of 1 mM pyruvate.

Poly ADP ribose polymerase (PARP) cleavage

Apoptosis was investigated by studying PARP cleavage after incubation of McA-RH7777 cells with 10 and 100 μM of C_{24}- and C_{27}-bile acids in DMEM/10% FCS for 3 h at 37 °C and 5% CO_2. After incubation cells were harvested, sonicated in PBS in the presence of a protease inhibitor cocktail (Complete mini, Roche), and subsequently taken up in SDS–PAGE sample buffer containing ureum. Seventy-five micrograms of protein was subjected to electrophoresis on a 6% (w/v) SDS–polyacrylamide gel essentially as described by Laemmli [17] and transferred to a nitrocellulose sheet. After blocking of non-specific binding sites with 0.2% I-Block (Tropix, Bedford, MA) in 0.1% Tween-20/PBS for 1 h, the blot was incubated for 2 h with rabbit polyclonal antibodies α-PARP (Promega, G7341) diluted 1:3000 in 0.2% I-Block. Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase and CDP-star were used for detection according to the manufacturer's instructions (Applied Biosystem).

Preparation of mitochondria

Mitochondria were isolated by differential centrifugation from cultured McA-RH7777 cells using a modification of the method of de Duve [18]. After washing the cells (two culture flasks, 225 cm^2) with PBS, they were harvested in 2 ml incubation medium consisting of 250 mM mannitol, 5 mM Tris, 0.5 mM EGTA, final pH 7.4 and lysed by passaging the cells five times through a ball-bearing cell cracker (size 8). The cell suspension was centrifuged at 4 °C (10 min, 600 × g_{av}). Subsequently, the supernatant was centrifuged for 10 min, 3600 × g_{av} at 4 °C. Finally, the pellet was resuspended in 500 μl incubation medium (250 mM mannitol, 5 mM Tris, 0.5 mM EGTA, final pH 7.4). Protein content was determined using bicinchoninic acid [19].

Citrate synthase latency

Citrate synthase latency was determined essentially as described in [20] by incubation of mitochondria (0.075 mg/ml) in incubation medium consisting of 150 mM KCl, 25 mM Tris, 2 mM EDTA, 10 mM KPi, 1 mM ADP, 1 mg/ml BSA, 1 mM oxaloacetate and 1 mM acetyl-CoA (final pH 7.4) with increasing concentrations of bile acids in a final volume of 200 μl. The reactions were allowed to proceed for 30 min at 37 °C and terminated by boiling the samples for 10 min. After cooling the samples, protein was removed by centrifugation and citrate was measured spectrophotometrically in the supernatant as described [21].

ATP and aspartate synthesis

ATP and aspartate synthesis by isolated mitochondria were measured in the presence and absence of conjugated and unconjugated C_{24}- and C_{27}-bile acids essentially as described in [22,23].