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Progressive stages of mitochondrial destruction caused by cell toxic bile salts

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ABSTRACT

The cell-toxic bile salt glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA) are responsible for hepatocyte demise in cholestatic liver diseases, while tauroursodeoxycholic acid (TUDCA) is regarded hepatoprotective. We demonstrate the direct mitochondrio-toxicity of bile salts which deplete the mitochondrial membrane potential and induce the mitochondrial permeability transition (MPT). The bile salt mediated mechanistic mode of destruction significantly differs from that of calcium, the prototype MPT inducer. Cell-toxic bile salts initially bind to the mitochondrial outer membrane. Subsequently, the structure of the inner boundary membrane disintegrates. And it is only thereafter that the MPT is induced. This progressive destruction occurs in a dose- and time-dependent way. We demonstrate that GCDCA and TCDCA, but not TUDCA, preferentially permeabilize liposomes containing the mitochondrial membrane protein ANT, a process resembling the MPT induction in whole mitochondria. This suggests that ANT is one decisive target for toxic bile salts. To our knowledge this is the first report unraveling the consecutive steps leading to mitochondrial destruction by cell-toxic bile salts.

1. Introduction

Mitochondria are critically involved in the induction of cell death. A decisive event is the induction of the mitochondrial permeability transition (MPT), an unspecific increase of the inner mitochondrial membrane permeability up to 1.5 kDa. Upon MPT induction, a colloid-osmotically driven influx of water into the mitochondrial matrix ("swelling") occurs, causing mitochondrial structure changes [1], and culminating in the distension and rupture of the outer membrane (MOMP) [2–4]. MOMP liberates Cyt C into the cytosol, the hallmark signal towards cell death [5–7]. In cell free systems, the MPT is typically assessed by an optical density (OD) decrease in mitochondrial suspensions due to mitochondrial swelling, a direct consequence of MPT induction.

Mechanistically the MPT is still a conundrum. Two major mechanistic MPT models, termed "classical" and "alternative" have been suggested. The classical model claims a non-selective pore formed by proteins from either the inner or both mitochondrial membranes [8,9]. However, the molecular constituents of the MPT pore are still under debate [5,6,9–11]. In contrast, the alternative model proposes denaturations/rearrangements of membrane proteins to cause MPT [12]. At present, it is also unclear whether the MPT, elicited by diverse stimuli, is mechanistically a uniform process resulting in equally damaged mitochondria or whether different stages of damage result from these stimuli. This question is of particular importance with respect to the ongoing discussion about the implication of the MPT in different cell death scenarios like apoptosis or necrosis [5–7,13–17].

Abbreviations: ANT, adenine nucleotide translocator; Cyt C, cytochrome C; Cys A, cyclosporine A; FCCP, carbonylcyanide-p-(trifluoromethoxy) phenyl-hydrazone; GCDCA, glycochenodeoxycholic acid; IM, inner membrane; MβCD, methyl-β-cyclodextrin; MMP, mitochondrial membrane potential; MOMP, mitochondrial outer membrane rupture; MPT, mitochondrial permeability transition; 4-MUP, 4-methylumbelliferyl phosphate; OD, optical density; OM, outer membrane; PC, phosphatidylcholine; Rh123, rhodamine 123; ROS, reactive oxygen species; TCDCA, taurochenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; VDAC, voltage-dependent anion channel; ZE-FFE, zone electrophoresis in a free flow electrophoresis device

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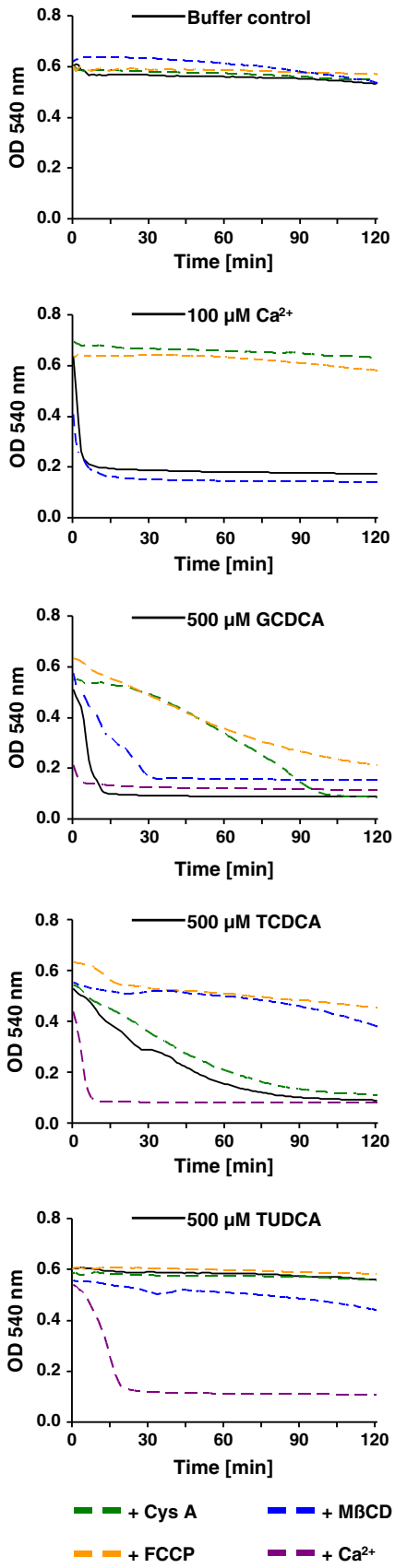
Glycine- and taurine-chenodeoxycholate (GCDCA and TCDCA) are the predominant hydrophobic bile salts accumulating in cholestatic patients, and account for liver cell death [18–22]. Bile salt-dependent mitochondrio-toxicity has been firmly established by several studies [23], which have demonstrated that bile salts impair the inner mitochondrial transmembrane potential (MMP) [24] and induce the MPT [24–26]. It remained, however, unclear how this mitochondrial destruction proceeds in detail, as such deleterious effects were especially detected in the presence of markedly elevated calcium (e.g. 50 μM in [24]), or with highly toxic doses of GCDCA [25,26].

In this report, we compared the mitochondrio-toxicity of GCDCA, TCDCA, TUDCA and the "classical" MPT-inducer calcium. OD measurements of mitochondrial suspensions demonstrate the MPT induction upon cell toxic bile salt exposure. However, we find that this approach is of limited use in order to reveal the detailed sequence of mitochondrial destruction elicited by GCDCA and TCDCA. We therefore concentrate on an alternative approach, using zone electrophoresis in a free-flow device (ZE-FFE), in order to separate mitochondria with progressive membrane damages, i.e., losses of outer membrane parts, upon bile salt exposure [27,28]. We find that bile salts initially bind to the mitochondrial outer membrane and mediate structural alterations of the inner boundary membrane, i.e., the inner membrane parts directly adjacent to the outer membrane. Subsequently, a significant increase of the inner membrane permeability irreversibly directs the mitochondria towards MPT and MOMP. As these findings point to a destructive progression of the bile salts via the mitochondrial membranes, we further concentrated on the participation of two major abundant mitochondrial proteins residing in the outer and inner mitochondrial membrane, VDAC and ANT respectively. To that end we employed dye-loaded liposomes reconstituted with these proteins, which especially point to the inner membrane protein ANT to mediate the process of toxic bile salt derived mitochondrial destruction. To our knowledge, this is the first report, which dissects the mitochondrial destruction into defined stages upon direct exposure to cell-toxic bile salts in the patho-physiological concentration range. This study may therefore serve as a blueprint for the elucidation of mitochondrio-toxic events elicited by other pathological situations or chemical exposures.



2. Material and methods

2.1. Mitochondrial analyses

Fresh rat liver mitochondria were essentially isolated as described elsewhere [29]. In brief, cell debris of homogenized rat liver tissue was removed by two consecutive centrifugations (800 × *g*, 10 min each). Thereafter, mitochondria were pelleted from the supernatant (9000 × *g*, 10 min), further purified by Percoll™ density gradient centrifugation (9000 × *g*, 10 min), subsequently washed and finally pelleted at 9000 × *g* [29]. Isolated mitochondria were subjected to quantification and kept on ice until use. Membrane potential (MMP) was monitored by the rhodamine 123 quenching method at Ex 485/20 nm, Em 528/20 nm [30] in 96-well plates containing 3 mg/ml freshly isolated mitochondria in a Synergy2 plate reader (BioTek, Bad Friedrichshall, Germany). FCCP (1 μM) served as internal control for MMP dissipation. MPT-induction was followed by light scattering at 540 nm in 1 min intervals for 2 h under the same conditions as for MMP. Parallel monitoring of MMP–MPT (Fig. 2) was done as above in the same wells. Measuring intervals were 1 min 13 s with 31 s delays for MPT and MMP measurements. In control experiments independence of both assessed parameters was validated. Results were directly exported into Excel to display the respective MMP/MPT curves (Fig. 1, S1, 2A–C upper panels, S2A–E upper panels).

Fig. 1. Pharmacological interventions demonstrate the mechanistic difference of calcium and bile salt mediated MPT. Optical density measurements ($OD_{540\ nm}$) of freshly isolated rat liver mitochondria incubated with buffer (control), 100 μM calcium or 500 μM bile salts (control: n = 7, Ca^{2+}: n = 6, GCDCA: n = 5, TCDCA: n = 3, TUDCA: n = 4) and co-incubated with either 5 μM Cys A (control: n = 3, Ca^{2+}: n = 6, GCDCA: n = 5, TCDCA: n = 3, TUDCA: n = 4), or 1 μM FCCP (control: n = 9, Ca^{2+}: n = 7, GCDCA: n = 9, TCDCA: n = 6, TUDCA: n = 2), or 500 μM MβCD (n = 4), or upon a mitochondrial calcium preload (20 μM; GCDCA: n = 5, TCDCA and TUDCA: n = 3).