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Cytotoxic bile acids, but not cytoprotective species, inhibit the ordering effect of cholesterol in model membranes at physiologically active concentrations

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ABSTRACT

Submillimolar concentrations of cytotoxic bile acids (BAs) induce cell death via apoptosis. On the other hand, several cytoprotective BAs were shown to prevent apoptosis in the same concentration range. Still, the mechanisms by which BAs trigger these opposite signaling effects remain unclear. This study was aimed to determine if cytotoxic and cytoprotective BAs, at physiologically active concentrations, are able to modulate the biophysical properties of lipid membranes, potentially translating into changes in the apoptotic threshold of cells. Binding of BAs to membranes was assessed through the variation of fluorescence parameters of suitable derivatized BAs. These derivatives partitioned with higher affinity to liquid disordered than to the cholesterol-enriched liquid ordered domains. Unlabeled BAs were also shown to have a superficial location upon interaction with the lipid membrane. Additionally, the interaction of cytotoxic BAs with membranes resulted in membrane expansion, as concluded from FRET data. Moreover, it was shown that cytotoxic BAs were able to significantly disrupt the ordering of the membrane by cholesterol at physiologically active concentrations of the BA, an effect not associated with cholesterol removal. On the other hand, cytoprotective bile acids had no effect on membrane properties. It was concluded that, given the observed effects on membrane rigidity, the apoptotic activity of cytotoxic BAs could be potentially associated with changes in plasma membrane organization (e.g. modulation of lipid domains) or with an increase in mitochondrial membrane affinity for apoptotic proteins.

1. Introduction

Bile acids (BAs) are naturally-occurring detergents that solubilize dietary lipids in the intestinal tract and in the bile [1]. Submillimolar concentrations of hydrophobic BAs, such as deoxycholic acid (DCA) or chenodeoxycholic (CDCA), activate cell death receptors in a ligand independent manner [2], stimulate p53, EF2-1 and Cyclin D1 expression [3], induce oxidative damage [4], promote both Bcl-2 associated protein X (Bax) translocation to mitochondria [5] and mitochondrial dysfunction [4,6]. Any of these modifications alone are sufficient for caspase activation and initiation of apoptosis [3,7]. On the other hand, more hydrophilic BAs, such as ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA), prevent the formation of reactive oxygen species [6], Bax translocation to mitochondrial membrane [8], mitochondrial dysfunction [4] and death-receptor induced apoptosis [9], ultimately inhibiting apoptosis. These hydrophilic or cytoprotective BAs are also known to activate pro-survival proteins such as the phosphatidyl inositol 3-phosphate (PI3K), the serine threonine kinase Akt and the mitogen-activated protein (MAP) Kinase Kinase Kinase [10]. Moreover, both pro-apoptotic proteins Cyclin D1 and EF2-1 have their expression levels decreased by UDCA or TUDCA incubation [11,12], even in the presence of cytotoxic DCA [3]. The molecular mechanisms associated with such different outcomes, and produced by these very similar molecules, remain elusive.

Since BAs are amphipathic molecules, biological membranes might constitute one of the targets for their biological actions. At the range of BA concentrations at which these effects are observed, 50–100 μM, a significant enrichment of BAs in cellular membranes is expected to

Abbreviations: di-4-ANEPPDHQ, 1-[2-Hydroxy-3-(N,N-di-methyl-N-hydroxyethyl) ammoniopropyl]-4-[β-[2-(di-n-butylamino)-6-napthyl] vinyl]pyridinium dibromide; BA(s), Bile acid(s); Bax, Bcl-2-associated X protein; CDCA, Chenodeoxycholic acid; Chol, Cholesterol; DCA, Deoxycholic acid; DHE, Dehydroergosterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPE-biotin, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-biotinyl; FRET, Förster resonance energy transfer; GUV, Giant unilamellar vesicles; l_d, Liquid disordered; l_o, Liquid ordered; LUV, Large unilamellar vesicles; NBD, nitro-2-1,3-benzoxadiazol-4yl; DPPE-NBD, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(nitro-2-1,3-benzoxadiazol-4yl); POPC, 1-palmitoyl-2-oleoyl-*sn*-glicero-3-phosphoryl-choline; PSM, *N*-palmitoyl-D-erythro-sphingosylphosphorylcholine; DOPE-Rho, 1,2-dioleoyl-*sn*-glicero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); TMA-DPH, 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; TUDCA, Tauroursodeoxycholic acid; UDCA, Ursodeoxycholic acid

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occur. However, little is known regarding the effects of BA interaction with lipid membranes at submillimolar concentrations. The aim of this study was the detailed characterization of these effects on membranes with different lipid compositions. In particular, we focused on the importance of cholesterol (Chol), since membrane rafts are a key structure in the extrinsic pathway of apoptosis [13]. In fact, the apoptotic effects of cytotoxic BAs were suppressed upon Chol depletion and DCA has been shown to induce a significant change in both concentration and distribution of Chol in the plasma membrane of living cells [14,15]. UDCA, a cytoprotective BA, on the other hand, did not exhibit this effect, suggesting that BAs might have different effects on lipid membranes depending on their molecular properties [14]. Also, given the influence of cytotoxic BAs on death receptor activation in the plasma membrane [2], BA-induced apoptosis could be the effect of alterations on plasma membrane structure.

The objective of this work was to determine if cytotoxic or cytoprotective BAs, at physiologically active concentrations, were able to modulate the biophysical properties of lipid membranes to an extent which could translate into changes in the apoptotic propensity of cells. Two putative mechanisms for the cytoprotective role of more hydrophilic BAs were studied here: i) cytoprotective BAs could disrupt partition of cytotoxic BAs to lipid membranes and ii) changes in the biophysical properties of lipid membranes induced by cytoprotective BAs could oppose changes induced by cytotoxic BAs. Any of these mechanisms could potentially result in a prevention of membrane compromise, leading to cell survival. Here, DCA and CDCA were used as models for cytotoxic BAs, and both UDCA and TUDCA were used as models for cytoprotective BAs. In order to characterize differences in lipid membrane binding properties for the two classes of BAs, nitro-2-1,3-benzoxadiazol-4yl (NBD) fluorescent derivatives DCA-NBD and UDCA-NBD were synthesized (Fig. 1A). Model membranes were used throughout this study in order to characterize the effects of BA in different lipid compositions, namely in the presence or absence of liquid ordered (l_o), *raft-like*, membranes.

In this study, we show that while cytotoxic BAs display a higher affinity for lipid membranes than the cytoprotective molecules, both classes of BAs have higher affinity for liquid disordered (l_d) membranes than for l_o. All BAs studied are found to have limited insertion in the lipid membrane upon binding, i.e., a more superficial location in the bilayer. Additionally, cytoprotective BAs are shown to have no effect on cytotoxic BA interaction with lipid membranes. Importantly, the cytotoxic BAs DCA and CDCA are found to prevent the rigidification of lipid membranes induced by Chol and to significantly expand membrane surface area. These effects are not associated with the removal of Chol from the membrane. On the other hand, cytoprotective BAs at physiologically active concentrations had no effect on membrane properties.

From the absence of effects of cytoprotective BAs on the properties of lipid membranes, we conclude that the cytoprotective properties of these molecules are not associated with modulation of lipid membrane structure. On the other hand, given the observed significant effects of cytotoxic BAs on membrane rigidity, it is concluded that the apoptotic activity of these molecules could potentially be associated with changes in plasma membrane organization, e.g. raft formation, or with an increase in mitochondrial membrane affinity for specific apoptotic proteins.

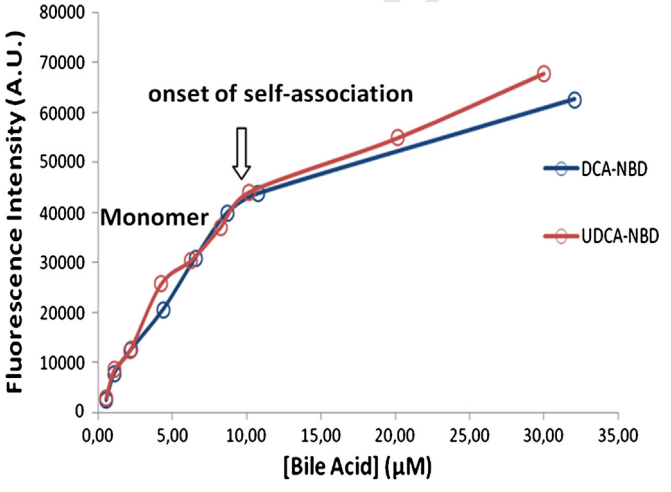

Fig. 1. Labeled bile acid fluorescence intensity emission as a function of concentration in buffer. Labeled bile acids were excited at 460 nm and emission was recovered between 480 and 700 nm. Intensity values obtained were corrected for inner-filter effects.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glicero-3-phosphocholine (POPC), *N*-palmitoyl-D-erythro-sphingosylphosphorylcholine (PSM) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-biotinyl (DPPE-biotin) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and Chol was from Sigma-Aldrich (St. Louis MO, USA). All lipids were kept in Uvasol grade chloroform (Sigma-Aldrich). Unlabeled DCA, CDCA and TUDCA in sodium salt form, HEPES, NaCl and Avidin were purchased from Sigma-Aldrich. UDCA sodium salt was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) and 1-[2-hydroxy-3-(N,N-di-methyl-N-hydroxyethyl)ammoniopropyl]-4-[β-[2-(di-n-butylamino)-6-napthyl] vinyl]pyridinium dibromide (di-4-ANEPPDHQ) were purchased from Invitrogen (Carlsbad, CA, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (DOPE-Rho) and 1,2-dipalmitoyl-*sn*-glycero-3phosphoethanolamine-N-(7-nitro-2-1,3-benzoxa.diazol-4yl) (DPPE-NBD) were obtained from Avanti Polar Lipids. Dehydroergosterol (DHE) was from Sigma-Aldrich. All fluorescent probes were kept in UVasol-grade solvents at −26 °C.

2.2. Synthesis of fluorescent derivatives of BAs

All reagents were acquired from Sigma-Aldrich, ^{1}H-NMR spectra were recorded on a Bruker 400 Ultra-Shield Spectrometer, and Mass Spectra were recorded in a Micromass Quattro Micro API Spectrometer, with ESI +/− source.

DCA-NBD:

a) Synthesis of the fluorophore with diamine linker, NBD-NH$(CH_2)_3NH_2$. To a solution of 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD-Cl, 100 mg, 0.5 mmol) in DMF (5 mL) was added 1,3-propanediamine dropwise (42 μL, 0.5 mmol). The reaction mixture was stirred at room temperature for 30 min, concentrated in vacuum and the product isolated by precipitation from DMF/diethyl ether, leading to the desired fluorophore with the attached linker in 82% yield (97 mg). ^{1}H-NMR (MeOD) δ (ppm): 2.12 (br t, 2H, NH_2), 2.73, 2.89, 3.62 (3×br s, 6H, $3CH_2$), 6.43 (d, 1H, NBD), 7.95 (s, 1H, NH), 8.50 (br d, 1H, NBD).

b) The amino acid coupling reaction was performed by activating DCA (100 mg, 0.24 mmol), using TBTU (93 mg, 0.29 mmol) as coupling agent in DMF (5 mL), in the presence of diisopropylamine (51 μL, 0.29 mmol) at room temperature. Then, NBD-NH$(CH_2)_3NH_2$ (69 mg, 29 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. The mixture was poured into water and extracted with EtOAc (3×), the organics were combined, dried over Na_2SO_4, filtered and concentrated in vacuum. The target compound was purified by silica-gel column chromatography with n-Hex/

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