INCORPORATION OF CHOLESTEROL IN SPHINGOMYELIN-EGG YOLK PHOSPHATIDYLCHOLINE VESICLES HAS PROFOUND EFFECTS ON DETERGENT-INDUCED PHASE TRANSITIONS: A TIME-COURSE STUDY BY CRYO-TRANSMISSION ELECTRON MICROSCOPY

Antonio Moschetta, Peter M. Frederik, Piero Portincasa, Gerard P. vanBerge-Henegouwen, Karel J. van Erpecum.

submitted
Abstract

Vesicle ↔ micelle transitions are important phenomena during bile formation and intestinal lipid processing. The hepatocyte canalicular membrane outer leaflet contains appreciable amounts of phosphatidylcholine (PC) and sphingomyelin (SM), and both phospholipids are found in the human diet. We therefore studied detergent-induced phase transitions in SM-PC vesicles. Methods: Phase transitions were evaluated by spectrophotometry and cryo-transmission electron microscopy (cryo-TEM) after addition of taurocholate (3-7 mM) to SM-PC vesicles (4 mM phospholipid, SM/PC 40%/60%, without or with 1.6 mM cholesterol). Results: After addition of excess (5-7 mM) taurocholate, SM-PC vesicles were more sensitive to micellization than PC vesicles. As shown by sequential cryo-TEM, addition of equimolar (4 mM) taurocholate to SM-PC vesicles induced formation of open vesicles, then (at the absorbance peak) multilamellar and fused vesicular structures coinciding with thread-like micelles, and finally transformation into an uniform picture with long thread-like micelles. Incorporation of cholesterol in the SM/PC bilayer changed initial vesicular shape from spherical into ellipsoid and profoundly increased detergent resistance. Disk-like micelles and multilamellar vesicles, and then extremely large vesicular structures were observed by sequential cryo-TEM under these circumstances, with persistently increased absorbance values by spectrophotometry. These findings may be relevant for bile formation and intestinal lipid processing.

Introduction

Micelle ↔ vesicle phase transitions have been studied extensively during the past decades for various reasons. For example, to incorporate membrane proteins into phospholipid vesicles, in general, mixed micelles containing surfactant, phospholipid and the protein of choice are first constructed, and functional insertion of the protein within the bilayer of the vesicles is subsequently obtained by inducing micelle \( \rightarrow \) vesicle transitions through removal of the surfactant. Also, several phase transitions occur during the process of bile secretion. Nascent bile within the canaliculus is generally believed to contain cholesterol-phospholipid vesicles (1). Upon progressive bile concentration in the bile ducts and in
the gallbladder, these vesicles are largely transformed into mixed bile salt-phospholipid-cholesterol micelles. During this process, cholesterol crystal formation (an essential step in gallstone formation) may occur in case of cholesterol supersaturated bile, possibly after aggregation of small unilamellar vesicles (2). Alternatively, primordial and multilamellar vesicles have been visualized by cryo transmission electron microscopy during the crystallization process (3-5).

After a meal, the gallbladder empties, and dilution of bile upon entering the intestine induces micelle → vesicle phase transitions. Enzymes within the intestinal lumen such as phospholipase A₂ may then induce the reverse process again with formation of open vesicles, bilayer fragments and micelles (6). The intermediate structures formed during vesicle → micelle transition, and the vesicles and micelles themselves, are thought to be important for optimal activities of various digestive enzymes, and for intestinal absorption of various lipids. Vesicle ↔ micelle transitions have therefore been studied in some detail by turbidity measurements (7;8), nuclear magnetic resonance (8;9) and cryo-transmission electron microscopy (10;11).

These studies were generally performed with phosphatidylcholine as the phospholipid (often with phosphatidylcholine from egg yolk, which contains 16:0 acyl chains at the sn-1 position and mainly unsaturated (18:1>18:2>20:4) acyl chains at the sn-2 position). Although phosphatidylcholine is the exclusive (>95%) phospholipid in human bile (with an acyl chain composition similar to egg yolk phosphatidycholine (12)), considerable amounts of saturated phosphatidylcholines and sphingomyelins may occur in human food (13). Preliminary data in the mouse suggest that dietary sphingomyelins may reduce markedly intestinal cholesterol absorption (14). It should also be taken into account, that both phosphatidylcholine and sphingomyelin are the major
phospholipids of the hepatocyte canalicular membrane outer leaflet (15). Cholesterol has a high affinity for sphingomyelin (16-18) and is thought to be preferentially located together with this phospholipid in detergent-resistant rafts (19). We therefore studied effects of including sphingomyelin within egg yolk phosphatidylcholine containing-vesicles (with and without cholesterol) on vesicle → micelle phase transitions by means of spectrophotometry and by sequential state-of-the-art cryo-transmission electron microscopy. Whereas previous electron microscopy studies were often prone to artefacts such as evaporation, advances in technology now avoid these caveats with the aid of temperature- and humidity-controlled conditions (37°C, 100% humidity, see “Methods”).

MATERIALS AND METHODS

Materials

Taurocholate was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and yielded a single spot upon thin-layer chromatography (butanol-acetic acid-water, 10:1:1 vol/vol/vol, application of 200 µg bile salt). Cholesterol (Sigma) was ≥ 98% pure by reverse-phase HPLC (isopropanol - acetonitril 1:1, vol/vol, detection at 210 nm). Phosphatidylcholine from egg-yolk (EYPC; Sigma), and sphingomyelin from egg-yolk (EYSM; Avanti Polar-Lipids Inc., Alabaster, AL, USA) yielded a single spot on thin-layer chromatography (chloroform–methanol-water 65:25:4, vol/vol/vol, application of 200 µg lipid). Acyl chain compositions as determined by gas-liquid chromatography (20) showed a preponderance of 16:0 acyl chains for EYSM. As shown by reverse-phase HPLC, EYPC contained mainly 16:0 acyl chains at the sn-1 position and mainly unsaturated (18:1>18:2>20:4) acyl chains at the sn-2 position, similar to phosphatidylcholine in human bile (12). All other chemicals and solvents were of ACS or reagent grade quality.
The enzymatic cholesterol assay kit was obtained from Boehringer (Mannheim, Germany), 3α-hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentrations (21) from Sigma. The reverse-phase C18 HPLC column was from Supelco (Supelcosil LC-18-DB, Supelco, Bellefonte, PA, USA).

**Preparation of model systems**

Lipid mixtures containing variable proportions of cholesterol, phospholipids (both from stock solutions in chloroform), or taurocholate (from stock solutions in methanol) were vortex-mixed and dried at 45°C under a mild stream of nitrogen and subsequently lyophilized during 24 hrs, before being dissolved in aqueous 0.15 M NaCl plus 3 mM NaN₃. Tubes were sealed with teflon-lined screw caps under a blanket of nitrogen to prevent lipid oxidation and vortex-mixed for 5 min followed by incubation at 37°C in the dark. The final mol percentages cholesterol, phospholipid and bile salt did not differ more than 1% from the intended mol percentages.

**Lipid analysis**

Phospholipid concentrations in model systems were assayed by determining inorganic phosphate according to Rouser (22). Cholesterol concentrations were determined with an enzymatic assay (23), and bile salts with the 3α-hydroxysteroid dehydrogenase method (21).

**Preparation of small unilamellar vesicles**

Small unilamellar vesicles were prepared by sonication. Lipids, from stock-solutions in chloroform, were vortex-mixed, dried under a mild stream of nitrogen and subsequently lyophilized during 24 hrs. The lipid film was dissolved in nitrogen-flushed aqueous 0.15 M NaCl plus 3 mM
NaN₃, and thereafter, the suspensions were probe-sonicated during 30 min. at 50°C (above the main transition temperatures of the phospholipids). After sonication, the suspension was centrifuged during 30 min. at 50000 x g at 40°C, in order to remove potential remaining vesicular aggregates and titanium particles. The resulting small unilamellar vesicles were stored at temperatures above 40°C, and used within 24 hrs. Small unilamellar vesicles were prepared with 100% PC, or SM 40% / PC 60% as the phospholipid. Final phospholipid concentration was 4 mM. Vesicles were either prepared without or with cholesterol (cholesterol / phospholipid ratio 0 or 0.4).

**Interactions of small unilamellar vesicles with taurocholate**

Interactions of small unilamellar vesicles with various taurocholate solutions (final concentrations varying between 3 and 7 mm) were followed by measuring optical density (OD) at 405 nm every min. during 80 min. at 37°C, in a thermostated Benchmark microplate reader (BioRad, Hercules, CA, USA). The solutions were stirred for 3 seconds prior to each measurement. During this period, the time course of various phase transitions was visualized by performing cryo-transmission electron microscopy at several time points during the incubation. In the case of cholesterol-containing vesicles, at the end of the incubation, the mixtures were also examined by polarizing light microscopy, in order to examine whether liquid or solid cholesterol crystals had formed.

**Cryo-transmission electron microscopy (cryo-TEM):** sample preparation for cryo-TEM was done in a temperature and humidity controlled chamber using a fully automated (pc-controlled) vitrification robot (Vitrobot, patent applied). This system was recently developed in collaboration with one of the authors (PMF) based on the work by Bellare et al. (24) and Frederik et al. (25). Within an environmental
chamber (temperature controlled and equipped with an ultrasonic device generating a mist to attain a relative humidity of ~100%), a specimen grid is dipped into a suspension, withdrawn and excess liquid is blotted away between two filter papers backed by foam pads. Thin films are formed between the bars of the grid, and to vitrify these thin films, the grid is ‘shot’ into melting ethane placed just outside the chamber and accessed through a shutter. Once a thin film is formed, it has a large surface to volume ratio, which makes heat and mass exchange fast processes. About dew point temperature will be attained in 0.1 sec. and further evaporation may be substantial at this point. At normal room conditions (24 °C, 40% relative humidity) a thin film may lose 50% of its water within 2 seconds and osmotic effects therefore have to be considered when not working at a 100% relative humidity (see also Hubert et al. and Frederik et al., Conference proceedings EUREM Brno 2000 and submitted). All the experiments are conducted at 37 °C with 100% relative humidity. When the vitrification robot is set up (vial with suspension in place, filter papers mounted, all parameters set) a forceps with grid is loaded from outside and melting ethane is prepared and for the rest the preparation/vitrification process runs automatically under PC command to end with a grid in melting ethane. The grids with vitrified thin films were analysed in a CM-12 transmission microscope (Philips, Eindhoven, The Netherlands) at –170°C using a Gatan-626 cryo-specimen holder and cryo-transfer system (Gatan, Warrendale, PA/USA). The vitrified films were studied at 120 kV with a pressure lower than 0.2x10⁻³ Pa., and at standard low-dose conditions, micrographs were taken.
RESULTS

Resistance of phospholipid vesicles against detergent bile salts in the absence of cholesterol

As shown in Figure 1A-C, vesicles without cholesterol and containing PC as the sole phospholipid tended to be rather resistant against the detergent effects of taurocholate, as indicated by the relatively slow decrease of absorption values during the time period studied (conditions: vesicular phospholipid 4 mM final concentration, addition of taurocholate at 7-5 mM final concentration, 37°C). Partial replacement of vesicular PC by SM, without inclusion of cholesterol, led to significant vesicular destabilization, as evidenced by low absorption values upon addition of taurocholate. Figures 1D-E show the results obtained upon incubation of the same vesicle population with progressively decreasing concentrations of taurocholate. At taurocholate concentration of 4 mM (Fig. 1D) added to PC-containing vesicles, a small increase of the absorbance can be observed. In case of SM-PC vesicles, there is a large but reversible increase of absorbance after addition of 4 mM taurocholate. After addition of taurocholate at a concentration of 3 mM (Fig. 1E), increased absorbance appears not to be reversible during the experiment, especially in case of SM-PC vesicles.

In Figure 2, the time-course of SM-PC vesicle → micelle transitions after addition of equimolar (4 mM) taurocholate is visualized by sequential cryo-transmission electron microscopy (TEM). Initial vesicles before addition of the detergent are spherical (Fig. 2A: time point a in Fig. 1D). During the uphill part of the absorbance curve (time point b in Fig. 1D), multiple open vesicles coexist with globular micelles. Maximal vesicular sizes have increased from 60 nm to 100 nm diameter (Fig. 2B). At the absorbance peak (time point c in Fig. 1D), globular micelles,
multilamellar and fused vesicular structures are present. Vesicles have further increased in size. Also, at this time point, some thread-like micelles have formed (Fig 2C). During the downhill part of the absorbance curve (time point d in Fig 1D: results not shown) and at the end of the experiment (time point e in Fig 1D), an uniform picture of long thread-like micelles is present (Fig 2D). In contrast, cryo-TEM after addition of excess (7 mM) taurocholate to SM-PC vesicles revealed globular micelles at the end of the experiment (time point a in Fig. 1A: results not shown).

**Resistance of cholesterol-containing phospholipid vesicles against detergent bile salts**

As shown in Figure 3A, incorporation of cholesterol in SM-PC vesicles prevents the destabilizing effect of SM (conditions: vesicular phospholipid 4 mM final concentration; vesicular SM/PC 40%/60%; vesicular cholesterol/phospholipid ratio 0.4; addition of taurocholate at 7 mM final concentration; 37°C). Absorbances of these cholesterol-enriched vesicles were stable in case of PC as the sole vesicular phospholipid, but increased markedly in the case of incorporation of SM in the vesicles. The same happened with incubation at lower taurocholate concentrations (6 mM, 5mM, 4mM and 3 mM; figures 2B, 2C, 2D and 2E, resp.).
Figure 1: Effects of taurocholate on sonicated small unilamellar vesicles without cholesterol, composed with 100% PC or with 40% of PC replaced by SM (final phospholipid conc. 4 mM, 37°C). As shown by the decrease of absorbance values (OD 405nm), in case of excess taurocholate, vesicles exhibit enhance sensitivity to detergent when SM is also included in the bilayer. (final taurocholate conc. 7mM, 6mM and
5mM, A, B and C, resp.). At lower taurocholate concentrations (4 mM in D; 3 mM in E) an increase of the absorbance values can be observed. ■ = EYPC; ▲ = EYSM.

Figure 2

Figure 2: Cryo-transmission electron microscopic images after addition of equimolar taurocholate (4 mM final conc.) to sonicated small unilamellar vesicles composed with 40% SM and 60% PC without cholesterol (final phospholipid conc. 4 mM, preparation at 37°C, 100% relative humidity). A: Sphere-like vesicles (max size ~ 60 nm) at initiation of the experiment (time point a in Fig. 1D). B: during the uphill part of the absorbance curve (time point b in Fig. 1D), there are some open vesicles (max size ~ 100 nm). C: at the absorbance peak (time point c in Fig. 1D), multilamellar and fused vesicles (max size < 290 nm), globular and thread-like micelles are present. D: at the end of experiment, there are large numbers of “thread”-like micelles (time point e in Fig. 1D). Bar represents 100 nm.
In Figure 4, the time-course of phase transitions after addition of 4 mM taurocholate to cholesterol-containing SM-PC vesicles is followed by sequential cryo-TEM. As shown in Figure 4A, initial vesicles often appear ellipsoid (time point a in Fig. 3D). During the uphill part of the absorbance curve (time point b in Fig 3D), large numbers of multilamellar vesicles are observed, together with disk-like micelles (Fig. 4B). At the end of the experiments (time point c in Fig. 3D), extremely large vesicular structures are present, precluding adequate visualization by electron microscopy. Concomitant light microscopy revealed numerous aggregated and fused large vesicular structures.

DISCUSSION

The present study points to a key role of cholesterol in formation of pathophysiologically relevant phosphatidylcholine plus sphingomyelin-containing bilayers and in modulating interactions between those vesicles and detergent bile salts. We have obtained a time-course of bile salt-induced phase transitions with the aid of state-of-the-art cryo-TEM. By vitrification from 37°C with 100% relative humidity, osmotic and temperature-induced artefacts are prevented, thus allowing observation of lipid-rich structures close to their original state: at the moment of vitrification by ultra-rapid cooling (10^{-5} sec.), the vapour pressure reduces, all supramolecular motions are arrested, thereby preserving microstructures and avoiding any artifacts related to crystallization of water and other compounds (24;25).
Figure 3: Effects of taurocholate on sonicated small unilamellar vesicles composed with fixed amounts of cholesterol (chol/PL ratio = 0.4) and with 100% PC or with 40% of PC replaced by SM (final phospholipid conc. 4 mM, 37°C). Incorporation of cholesterol prevents the destabilizing effects of SM (taurocholate final conc. 7 mM in A; 6 mM in B; 5 mM in C; 4 mM in D; 3 mM in E).

■ = EYPC; ▲ = EYSM.
Figure 4: Cryo-transmission electron microscopy images after addition of taurocholate (4 mM final conc.) to sonicated small unilamellar SM-PC vesicles (final phospholipid conc. 4 mM, SM/PC ratio 40/60, chol/phospholipid ratio 0.4, 37°C, 100% humidity). A: Some vesicles have an ellipsoid shape at the initiation of the experiment (time point a in Fig. 3D). B: During the uphill part of the absorbance curve (time point b in Fig. 3D), large numbers of multilamellar vesicles are observed, together with disk-like micelles (arrows). C: At the end of the experiment (time point c in Fig. 3D), extremely large vesicular structures are present. Bar represents 100 nm.

Previous studies have examined in detail phase transitions induced by addition of detergent to egg yolk phosphatidylcholine-containing vesicles or by dilution of micellar solutions, with formation of long cylindrical
micelles as intermediate structures (10;11;26;27). In the present study, we have focused on phase transitions of SM-EYPC vesicles—with or without cholesterol incorporated in the bilayer—after addition of various amounts of taurocholate. In the absence of cholesterol, and after addition of excess taurocholate (final taurocholate/phospholipid ratio >1), SM-containing vesicles exhibited increased sensitivity to the detergent, compared to vesicles composed exclusively with EYPC, as shown by spectrophotometry (Fig. 1A-C). These data are in line with previous reports (28-30). At equimolar taurocholate-phospholipid ratios, there was a strong but transient increase of absorbance values. Sequential cryo-transmission electron microscopy revealed in the early stages after addition of the detergent open vesicles, then (coinciding with the absorbance peak) multilamellar and fused vesicles and finally (coinciding with low absorption values) an uniform picture of thread-like micelles. Open vesicular structures that we visualized in the early stages after addition of the detergent have been described before (6) and may indicate initiation of transition toward micellar phases. Upon addition of low amounts of taurocholate (taurocholate/phospholipid ratio <1), increased absorbance values were not reversible during the experiment. After addition of excess taurocholate (taurocholate/phospholipid ratio >1), the resulting bile salt-phospholipid mixtures plot in the one-phase zone (only micelles) of the equilibrium ternary phase diagram (31;32) and vesicle → micelle transitions progress at extremely fast rates, thus precluding visualization of intermediate structures. In contrast, after addition of equimolar amounts of taurocholate (taurocholate/phospholipid ratio =1), resulting model systems plot near or at the border of the one-phase (micellar) zone and the right two-phase (micelles and vesicles-containing) zone (31;32), and vesicle → micelle transitions progress at slow rates, thus allowing visualization of intermediate structures.
Incorporation of cholesterol in the vesicular bilayer had profound effects on detergent-induced phase transitions. In the absence of the sterol, and in the earliest stages, spherical vesicles were visualized, but in presence of the sterol the vesicles often had an ellipsoid shape (Fig. 2A and 4A). The changes in bilayer two-dimensional conformation observed in presence of cholesterol may be due interactions between aliphatic chains of two sterol molecules (one in each monolayer) in cholesterol-sphingomyelin microdomains (33), thus inducing a local decrease in bilayer curvature. Interestingly, Crawford et al. (1;34) with the aid of electron microscopy could visualize in the bile canaliculi mostly ellipsoid non-spherical unilamellar vesicles, which probably contained cholesterol and phospholipid. It has been postulated that the non-spherical shapes of vesicles with decreased curvatures at the lateral sides may have relevance for interactions of cholesterol with detergent bile salts and subsequent solubilization in mixed micelles (34).

Sphingomyelin-phosphatidylcholine vesicles with cholesterol incorporated in the bilayers were highly resistant against detergent-induced micellar solubilization. Intermediate multilamellar vesicles, disk-like micelles and –at the end of the experiments- large vesicular aggregates were formed upon addition of the detergent. SM exhibits a much higher gel to liquid crystalline phase transition temperature \( T_m \) than EYPC: whereas EYPC has a \( T_m \) below 0 °C (35), we previously found \( T_m \) of hydrated EYSM to be 36.6 °C (30). Pure phospholipids exist in a solid, ordered gel phase below a melting temperature \( T_m \) that is characteristic of each lipid, and in a liquid disordered (also called "liquid crystalline") phase above \( T_m \). Note that the \( T_m \) of EYSM is close to the incubation temperature in our experiments including preparation for cryo-EM. For this lipid species it is particularly essential to attain 100% humidity (as in the present study) during cryo-preparation to prevent a
temperature drop (“dew-point” effect) below $T_m$, which is known to change the shape of vesicles. The phase behaviour around $T_m$ is influenced by the presence of cholesterol: lipids with a high $T_m$ in the pure state (e.g. disaturated PC species and sphingomyelins) may form a so-called "liquid-ordered" phase around $T_m$ (36-38). This liquid-ordered phase has properties intermediate between the gel and liquid-crystalline phases: Like in the gel phase, tight acyl-chain packing and relatively extended acyl chains characterize the liquid-ordered phase. On the other hand, like lipids in the liquid-crystalline phase, lipids in the liquid-ordered phase exhibit relatively rapid lateral mobility within the bilayer. Recent data indicate that in bilayers containing more than one phospholipid, in the presence of cholesterol, phase separation of the phospholipids with the higher $T_m$ (such as SM) into cholesterol-rich liquid-ordered domains occurs, and that such a phase separation is a prerequisite for detergent-resistance (37;38). We propose that, when present together with cholesterol in liquid ordered domains, SM becomes relatively resistant to the micellizing effects of detergent bile salts.

In conclusion, we have shown that incorporation of sphingomyelin in egg yolk phosphatidylcholine vesicles enhances vesicle → micelle phase transitions, with formation of intermediate open, multilamellar and fused vesicular structures. When cholesterol is also included in the bilayer, sphingomyelin-egg yolk phosphatidylcholine vesicles appear resistant against bile salt-induced micellar solubilization. Instead, multilamellar and large aggregated vesicles are formed. These findings may have implications for canalicular bile formation and intestinal lipid solubilization.
References


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