Significance of sterol structural specificity: Desmosterol cannot replace cholesterol in lipid rafts

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Desmosterol is an immediate precursor of cholesterol in the Bloch pathway of sterol synthesis and an abundant membrane lipid in specific cell types. The significance of the difference between the two sterols, an additional double bond at position C24 in the tail of desmosterol, is not known. Here, we provide evidence that the biophysical and functional characteristics of the two sterols differ, and that this is because the double bond at C24 significantly weakens the sterol ordering potential. In model membranes, desmosterol was significantly weaker than cholesterol in promoting the formation or stability of ordered domains and in mammalian cell membranes, desmosterol associated less avidly than cholesterol with detergent-resistant membranes. Atomic-scale molecular dynamics simulations showed that the double bond gives rise to additional stress in the tail, creating a rigid structure between C24-C27 and favoring tilting of desmosterol distinct from cholesterol. Functional effects of desmosterol in cell membranes were assessed upon acutely exchanging ∼70% of cholesterol to desmosterol. This led to impaired raft-dependent signaling via the insulin receptor while non-raft dependent protein secretion was not affected. We suggest that the choice of cholesterol synthesis route may provide a physiological mechanism to modulate raft-dependent functions in cells.

In model membranes, cholesterol associates preferentially with long, saturated acyl chains, such as those in sphingolipids, thus reducing the area per lipid molecule (1,2). There is substantial evidence to suggest that ordered lipid domains (rafts) composed of sterol and saturated lipids also exist in eukaryotic cell membranes and play important roles in numerous biological processes (3,4). Lipid rafts are considered to exist in a liquid-ordered (Lo) state characterized by tight ordering but relatively high lateral mobility of lipids, and operationally often defined as detergent resistant membranes (DRMs) (5,6). Instead, unsaturated phospholipids are loosely packed, forming a liquid disordered (Ld) membrane that is solubilized upon addition of mild detergents. At least in model membranes, cholesterol is able to promote the separation of Lo and Ld domains (7-9). In cells, cholesterol levels influence the domain partitioning and biological activity of proteins that co-isolate in detergent resistant membranes (DRMs) (10,11).

Taken the postulated critical role for cholesterol in raft formation and the diversity of sterols in biological materials,
the sterol structural requirements for promoting ordered domain formation are highly relevant. Until now, the effects of sterol/steroid structure have mostly been addressed in model membranes. Slight modifications of the cholesterol structure, e.g. a shift of the double bond in the sterol ring, or alteration of the 3-OH group, change the domain forming properties of the molecule (12-14). Among the structurally closest relatives of cholesterol are its immediate biosynthetic precursors. The only difference between cholesterol and its immediate precursor desmosterol is an additional double bond between carbon atoms 24 and 25 in the tail of desmosterol (Fig. 1). Recently, the influence of desmosterol on lipid order was found to be similar to that of cholesterol (15).

Cholesterol biosynthesis can proceed from lanosterol via two intersecting routes, the Kandutsch-Russell or Bloch pathways. The choice of pathway is determined by the stage at which the double bond at position C24 in the sterol side chain is reduced. If this double bond is retained until the last reaction, cholesterol synthesis proceeds via desmosterol (Bloch pathway) whereas early reduction leads to the formation of lathosterol and 7-dehydrocholesterol (Kandutsch-Russell pathway). The biological significance of this complexity remains enigmatic. We have shown that in cellular membranes, lathosterol associated with DRMs at least as efficiently as cholesterol (16). Accordingly, also in model membranes, lathosterol formed rafts that were at least as detergent-resistant as, and even more thermally stable than cholesterol-containing rafts (13). Moreover, 7-dehydrocholesterol was found to be more strongly domain-promoting than cholesterol (17).

In contrast to many relatives of cholesterol, desmosterol is an abundant structural membrane component in mammalian cells such as spermatozoa and astrocytes (18,19). Inability to convert desmosterol to cholesterol – lack of functional 24-dehydrocholesterol reductase – leads to the human disorder desmosterolosis (20). This rare malformation syndrome is characterized by severe developmental defects and cognitive impairment (21). Instead, 24-reductase knock-out mice exhibit a much milder phenotype, being viable although small and infertile (22).

In the present work, we compared the roles of desmosterol and cholesterol in membrane organization by employing spectroscopic methods in model membranes and detergent solubility in model and cell membranes. In addition, we addressed the functional effects of exchanging the majority of membrane cholesterol to desmosterol in living cells, by analyzing both raft-dependent and -independent parameters. We found that the biophysical and functional properties of desmosterol differ considerably from those of cholesterol. These studies were complemented by detailed atomistic simulations to shed light on the origin of these differences, which initiates from the hydrocarbon tail region in the two sterols.

**Experimental procedures**

**Multilamellar vesicle (MLV) preparation.** Lipids were from Avanti Polar Lipids except for desmosterol (Steraloids) and cholesterol (Sigma). Desired lipid mixtures were prepared from stock solutions (Table I). All MLVs were prepared containing 15 mol % cholesterol, desmosterol or no sterol. Samples that were measured by fluorescence also included 0.5% 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes). Lipids were dried under N$_2$, and redissolved in 20 µl chloroform. After redrying under N$_2$ and further drying for 1 h at high vacuum, the lipids were dissolved in the desired volume of PBS. To ensure uniform dispersion of the vesicles, the samples were incubated at 55 ºC for 15 min during which each sample was vortexed 4 times 30 s. The vesicles were incubated at for at least 1 h at the desired temperature before experiments.

**Detergent turbidity.** Detergent turbidity measurements were performed essentially as described in (17) except that the samples were remeasured at 1.5 h (instead
of 2 h) after detergent incubation at room temperature, using a Pharmacia LKB spectrophotometer.

**Fluorescence measurements.** Fluorescence emission spectra and polarization were recorded using a Varian Cary Eclipse fluorometer (Varian, Cary, NC) equipped with a thermostated cuvette holder. The excitation/emission wavelengths were 359 and 427 nm, respectively. Measurements were carried out in quartz cuvettes in which the samples were kept homogeneous by magnetic stirring and temperature inside the cuvette monitored during experimentation. For DPH quenching measurements, fluorescence was measured while heating the samples continuously from 21 °C to 56 °C and cooling back to 21 °C with a rate of 1 °C/min. The heating and cooling curves were similar. \( F/F_0 \) represents relative unquenched fluorescence, i.e. the ratio of fluorescence intensity in a 12-S LPC (1-palmitoyl-2-(12-doxyl)-stearylphosphatidylcholine) containing mixture (F) normalized relative to the fluorescence intensity in vesicles where the quencher lipid 12-S LPC was replaced by DOPC (dioleylphosphatidylcholine) (\( F_0 \)). The degree of DPPC (dipalmitoylphosphatidylcholine) – rich domain formation is given by \( \Delta F/F_0 \), the difference between the unquenched fluorescence in the DPPC/12-S LPC vesicles and DOPC/12-S LPC vesicles that do not form segregated lipid domains (i.e. \( F/F_0^{DPPC} - F/F_0^{DOPC} \)). To derive domain transition temperatures (\( T_m \)) and formation coefficients (\( a \), \( \Delta F/F_0 \) vs. temperature plots were fitted by a sigmoidal Boltzmann equation (23). The \( T_m \) is given by the half point of the curves, and the domain formation coefficient by the difference in the degree of domain formation (\( \Delta F/F_0 \)) before and after domain melting. This was calculated by subtracting the lower asymptote of the Boltzmann equation from the higher asymptote.

**Subcellular membrane fractionation.** Chinese hamster ovary CHO-K1 cells (ATCC CCL-61) were grown in 5% lipoprotein-deficient serum (prepared as in (24) to near confluence and scraped in PBS. All following steps were performed at temperatures between 0-4 °C. The cells were washed in PBS and resuspended in 400 μl buffer containing 140 mM KCl, 10 mM TrisHC1 pH 7.5, supplemented with protease inhibitors (chymostatin, leupeptin, antipain and pepstatin, at 25 μg/ml each). The cells were lysed by passing them 60 times through a 25 G needle after which the nuclei were spun down by centrifugation for 10 min at 1000 g. 400 μl of supernatant was mixed with 1600 μl 2.4 M sucrose and transferred to a SW28Ti ultracentrifuge tube. The sample was overlaid with a step gradient of 1.8, 1.6, 1.4, 1.2, 1.0, 0.8 and 0.6 M sucrose. After centrifugation for 24 h at 70,000 g at 4 °C, 2 ml fractions were collected and used for sterol determination, Western blotting or DRM isolation.

**DRM isolation.** The protocol used for DRM isolation is an adaptation of (25). All steps were performed at 0-4 °C. 10% TX-100 was added to the low-density fractions (−0.7-0.9M sucrose) to a final concentration of 0.5% (total volume 4.6 ml) followed by a 30 min incubation on ice. The samples were adjusted to 40% sucrose by adding 85% sucrose in TNE (25 mM TrisCl pH 7.5, 150 mM NaCl, 1 mM EDTA), transferred to a SW28Ti tube and overlaid with 3 ml 35 % sucrose TNE and 2 ml 5% sucrose TNE. After centrifugation for 18 h at 80,000 g, the DRMs at the interface between the 5% and 35% sucrose layers were separated from the solubilized membranes in the 40 % sucrose phase.

**Sterol determination.** Free sterols were quantified as described in (26). Briefly, lipids were extracted according to Bligh and Dyer (27). The free sterol fraction was isolated by TLC (petroleum ether/diethyl ether/acetate 60:40:2) and analyzed by silver-ion high performance liquid chromatography (Ag⁺-HPLC) using UV-detection.

**Sterol exchange.** Human hepatoma HuH7 cells were plated on gelatin-coated dishes two days prior to the experiments. To deplete cellular cholesterol, the cells were
incubated for 30 min on a shaking water bath (50 rpm) at 37°C in the presence of 10 mM methyl-β-cyclodextrin (MβCD). Then the cells were washed 3x with PBS containing Ca²⁺ and Mg²⁺ ions (PBS⁺) and incubated in the presence of either cholesterol-MβCD or desmosterol-MβCD complex for 1 h at 37°C without shaking (complex prepared as in (28); molar ratio desmosterol:MβCD = 1.0:8.7, used in a concentration 50 µg/ml of sterol). Thereafter, the complexes were removed, cells washed 3x with PBS⁺ and subjected to further experiments. Essentially no cells were lost during the procedure.

Insulin stimulation and Optiprep gradient. Sterol exchange was performed as above and 10 nM insulin (Actrapid®, Novo Nordisk) was added to the indicated samples for 5 min. Thereafter, the cells were processed for analyzing IR activity or IR DRM association using Optiprep-gradient fractionation in the presence of 0.1% Triton X-100 as described (29,30).

Albumin secretion and ¹²⁵I-insulin binding. For analyzing albumin secretion, the cells were incubated for 1 h at 37°C in serum-free medium containing 25 mM mevalonate and 10 µM lovastatin after sterol exchange. The media were then collected and centrifuged briefly to remove any cells present. The proteins in the media were recovered by TCA-precipitation, and the amount of albumin analyzed by Western blotting. The albumin amounts were corrected with the amount of cellular protein in each sample. For analyzing ¹²⁵I-insulin binding, the cells were put on ice after the sterol exchange and incubated in the presence of 50 pM ¹²⁵I-insulin (specific activity 2000 Ci/mmol, Amersham Pharmacia) and 0, 0.1 nM, 1.0 nM, 10 nM or 100 nM unlabeled insulin for 30 min. The cells were washed 5 times, lysed in 0.05 N NaOH-0.1% SDS and radioactivity in the samples measured with Wallac 1470 Automatic Gamma Counter. Unspecific binding (determined as the radioactivity in samples containing 100 nM unlabeled insulin) was subtracted from the values, and ¹²⁵I-insulin binding expressed as CPM/mg protein.

Western blotting. Western blotting was performed as described previously (30). 20 µg of cellular protein or equal volumes from gradient fractions were used for analysis. Antibodies used were from Prof. Ari Helenius (anti-calnexin, rabbit polyclonal), Transduction laboratories (anti-IRβ; rabbit polyclonal), DAKO (anti-human albumin, rabbit polyclonal), Upstate Biotechnology (anti-pTyr; mouse monoclonal, clone 4G10) and Santa Cruz (anti-caveolin, rabbit polyclonal). Analysis of IR activation and quantification of Western blotting results were performed as described (29,30).

Fluorescence microscopy. Cells grown on cover slips were subjected to the sterol exchange protocol, fixed with 4% PFA, quenched with 50 mM NH₄Cl and stained with filipin (Sigma) and FITC-lentil lectin (Sigma). Images were captured with an Olympus IX71 inverted microscope equipped with a CCD camera, and similar exposure times were used for all the conditions.

Atomic-scale simulations. We studied three different bilayers composed of lipid and cholesterol/desmosterol molecules hydrated with 3600 waters. The first bilayer comprised of 128 DPPCs. The second and third bilayers had 128 DPPCs together with 32 cholesterol and desmosterol molecules, respectively. The simulations were performed using the GROMACS suite (31). The parameters for bonded and nonbonded interactions for DPPC molecules were taken from a previous study for a pure DPPC bilayer (32), and the partial charges are from the underlying model description (33). The cholesterol force field was taken from (34). For desmosterol we employed the same force field except for the hydrocarbon tail region, in which the double bond was described by the standard Gromacs force field at www.gromacs.org. For water, the SPC model was used (35). Each simulation covered a time scale of 100 ns including the equilibration period of 20 ns prior to analysis. Due to the
extensive time scale of the simulations, statistical difference in the structural results was very small (SD < 1%). Periodic boundary conditions were applied in all three directions and the LINCS algorithm (36) was employed to constrain the bond lengths including the hydrogen atoms. The time step was set to 2 fs. The simulations were carried out at constant pressure (1 atm) and temperature (323 K). The Berendsen thermostat and barostat (37) were applied with time constants of 0.5 and 0.6 ps, respectively. The temperatures of the solute and solvent were controlled independently. A semi-isotropic scheme for pressure control was employed and the particle-mesh Ewald method (38) was used for electrostatics. A more detailed description of the simulation protocol used in this study can be found elsewhere (39,40).

Results

Effects of desmosterol and cholesterol on the formation and stability of ordered domains in model membranes

The level of ordered domain formation in the presence of cholesterol vs. desmosterol was studied using a detergent-insolubility assay. In this method, the light scattering remaining after the addition of Triton X-100 to a multilamellar vesicle preparation was measured by optical density (OD). A 1:1 mixture of DPPC and DOPC with or without 15% sterol was used. The main phase transition temperatures of pure fully hydrated DPPC and DOPC membranes are about 42 °C and –20 °C, respectively. In the vesicles, DPPC-rich ordered domains coexist with DOPC-rich fluid domains at 23 °C, and the percent OD remaining after detergent addition roughly approximates the fraction of DRMs present in the preparation (41). In agreement with earlier results, we found that addition of cholesterol to the liposomes significantly decreased solubility as indicated by higher remaining ODs in the presence of cholesterol as compared to absence of sterol (Fig. 2 a) (41). However, desmosterol was much less efficient than cholesterol in promoting insolubility by using the same criteria (Fig. 2 a). The differential solubility of cholesterol and desmosterol containing liposomes was also observed in liposomes containing 30 mol % of sterol (data not shown).

To further investigate the potential differences between desmosterol and cholesterol in the degree to which they promote ordering, we measured the steady-state DPH fluorescence polarization in 1:1 DPPC/DOPC vesicles, with or without 15% sterol. The higher the DPH fluorescence polarization, the more closely packed (ordered) is the lipid environment (42). We found that DPH polarization was significantly higher in cholesterol than in desmosterol containing liposomes (Fig. 2 b), suggesting higher order in cholesterol containing lipid mixtures. Yet, desmosterol increased DPH polarization as compared to the situation in the absence of sterol (Fig. 2 b). This supports the idea that desmosterol is capable of tightening lipid packing (ordering) albeit less efficiently than cholesterol.

Next, the contribution of the two sterols in domain formation and the relative stability of the ordered domains was assessed in fluorescence quenching experiments. Vesicles were prepared of a 1:1 mixture of DPPC and a nitroxide-labeled phosphatidylcholine (12-SLPC, a fluorescence quenching lipid with phase behavior similar to DOPC) with or without 15% sterol, and trace amounts of DPH. DPPC and 12-SLPC tend to form separated DPPC-rich ordered and 12-SLPC-rich disordered domains. DPH displays equal partitioning between both phases. Moreover, perturbations induced by DPH are weak and of local nature, and do not affect thermotropic behavior in any significant manner (43). In the 12-SLPC domain, DPH fluorescence is quenched but DPH molecules remaining in the DPPC-rich domain have a low probability of interacting with quencher molecules and give higher fluorescence intensity. In this domain forming lipid mixture, the temperature dependence of quenching can reveal the thermal stability of ordered domains: upon increasing temperature, the domains start to "melt" and DPH fluorescence starts to decrease because of
increasing contact with 12-SLPC molecules. The midpoint of a quenching vs. temperature curve, i.e. the apparent melting temperature, is a measure of domain stability (17,41).

Fig. 2 c shows the sterol and thermal dependence of DPH fluorescence obtained with this method. In agreement with previous studies, there is domain formation at 23 °C in the absence of sterol and the degree of domain formation decreases upon increasing the temperature, being abolished by 37 °C (41) (Fig. 2 c, no sterol). Also consistently with earlier data, domain formation was enhanced by cholesterol, as indicated by the increase in the initial fluorescence at 20-23 °C (Fig. 2 c) and calculated formation parameter (23) (Fig. 2 d). Importantly, in the presence of desmosterol the initial fluorescence was more moderately increased, suggesting decreased domain formation as compared to cholesterol (Fig. 2 c,d). To assess domain stability, the average transition temperatures (T_m) were calculated (23). The T_m obtained for cholesterol containing vesicles was significantly higher than for vesicles containing no sterol, as shown previously (23) (Fig. 2 e). However, the T_m for desmosterol containing vesicles was essentially the same as for no sterol samples, suggesting that desmosterol exhibited very little if any domain stabilizing effect (Fig. 2 e). Together, these results suggest that in model membranes, the potential of desmosterol to form and stabilize ordered domains is weaker than that of cholesterol.

Association of desmosterol with cellular membranes
To compare the distribution of desmosterol and cholesterol in cell membranes, we analyzed their partitioning in subcellular membranes of different equilibrium densities. CHO cells were used for the experiment because they synthesize cholesterol efficiently via desmosterol (44). The cells were cultivated in lipoprotein-deficient serum for 7 days to upregulate cholesterol biosynthesis and increase the amount of endogenous desmosterol (to 5-10% of total sterol). Postnuclear supernatant was prepared and fractionated in a sucrose gradient, and desmosterol and cholesterol analyzed from the gradient fractions by Ag⁺-HPLC. The distribution of desmosterol and cholesterol in the fractions was found to be highly similar (Fig. 3 a). The sterols were depleted from high-density fractions that contained the majority of the rough endoplasmic reticulum (ER) as assessed by calnexin immunoblotting (data not shown). Both sterols peaked at relatively low-density fractions which comigrated with a pool of caveolin-1 (not shown) and presumably contained the majority of the plasma membrane, as well as endocytic organelles and Golgi membranes. These data are in agreement with the findings of Phillips and coworkers who reported the desmosterol/cholesterol ratio to be closely similar in whole cells and isolated plasma membrane (45).

Next, the detergent solubility of desmosterol vs. cholesterol in cellular membranes was analyzed. The low-density membranes (corresponding to 0.7-0.9 M sucrose) were extracted with cold Triton X-100, and soluble and insoluble material separated on a discontinuous sucrose density gradient as described previously (25). DRMs were recovered from the interphase between 5 and 35% sucrose layers. The amount of cellular cholesterol and desmosterol was then analyzed from the DRMs and soluble membranes by Ag⁺-HPLC. We found that the desmosterol/cholesterol ratio was >3 times higher in the soluble membranes than in DRMs (Fig. 3 b). Together, these observations suggest that the endogenously synthesized desmosterol largely resides in the same membranes as cholesterol but partitions into DRMs less efficiently than cholesterol.

Exchange of cholesterol with desmosterol in cellular membranes
To investigate whether substitution of cholesterol by desmosterol has functional effects on proteins, we set up a protocol to acutely exchange the majority of cellular cholesterol with desmosterol in living cells. We used HuH7 hepatoma cells in which we have earlier studied raft-
dependent signaling (30). HuH7 cells normally contain cholesterol as the major structural sterol, with no desmosterol detectable (Fig. 4 a). First, ~70% of cholesterol was depleted in 30 min using MβCD (Fig. 4 a). Immediately thereafter, the cells were replenished with either desmosterol or cholesterol from a MβCD complex during 1 h. Ag⁺-HPLC analysis of sterols revealed that upon either cholesterol or desmosterol replenishment, the sterol amount returned to the starting level (Fig. 4 a). Accordingly, staining of the cells with the fluorescent sterol binding antibiotic filipin revealed a significant reduction in fluorescence upon cholesterol depletion, and recovery of the initial staining pattern and intensity upon both sterol repletions (Fig. 4 b).

The structural and functional organization of the Golgi apparatus has been reported to be dependent on cholesterol levels (46,47). We found that in the cholesterol depleted HuH7 cells, the morphology of the Golgi complex was slightly perturbed as judged by the more diffuse FITC-lentil lectin staining. However, the perinuclear fluorescence pattern typical of untreated cells was restored upon both sterol replenishments (Fig. 4 b). To analyze if the secretory function of the Golgi was affected by the sterol exchange procedure, albumin secretion from HuH7 cells during 1 h was analyzed after the repletion. We have earlier shown that ~50% of the newly synthesized albumin is secreted from HuH7 cells in 1 h (16). Anti-albumin Western blotting of proteins from the medium showed that similar amounts of albumin were secreted from untreated, cholesterol repleted and desmosterol repleted cells (Fig. 4 c). At the end of the 1-h period, the sterol levels were analyzed again to assess how much of the desmosterol was converted to cholesterol during the experiment. The proportion of cholesterol was increased but the majority, ~63%, of cellular sterol was still desmosterol (desmosterol 22.9±5.11 nmol, cholesterol 13.1±3.90 nmol). This suggests that the lack of effect on albumin secretion was not due to the low level of desmosterol but rather, that albumin secretion was not sensitive to the structural difference between the two sterols. Furthermore, the result shows that the exogenous desmosterol introduced was being metabolized in HuH7 cells. Indeed, hepatic cells are known to convert biosynthetic precursors of cholesterol efficiently to the end product (16,44).

**Effect of exchanging cholesterol with desmosterol on insulin receptor activation and raft association**
We have earlier shown that in HuH7 cells, insulin signaling takes place in cholesterol-sphingolipid rafts. The inactive IR was detergent soluble but upon insulin addition, the receptor was recruited into DRMs. Cholesterol depletion inhibited insulin signaling and impaired the DRM association of the active IR, and upon cholesterol repletion, IR raft association was restored (30).

Having confirmed that both the cholesterol and desmosterol repleted HuH7 cells are viable and functional, we analyzed insulin signaling in these cells. IR activation was measured as described previously, using anti-phosphoTyr and anti-IR immunoblotting (29,30). We found that, in contrast to albumin secretion, IR activation was markedly attenuated in desmosterol repleted compared to cholesterol repleted cells (Fig. 5 a). This defect was comparable to that observed previously upon cholesterol depletion (30). However, the IR levels were similar and insulin binding equally efficient after cholesterol and desmosterol repletion, as assessed by Western blotting and [¹²⁵I]-insulin binding experiments, respectively (Fig 5 a,b).

To study whether desmosterol interfered with the association of the ligand-occupied IR with DRMs, we stimulated cholesterol or desmosterol repleted HuH7 with insulin, lysed the cells in cold Triton X-100 and fractionated the material in an Optiprep gradient as described previously (30). In this method, DRMs are recovered at the top of the gradient in fractions containing 0-20% Optiprep. We found that, in contrast to cholesterol, desmosterol failed to support the DRM association of the active IR as evidenced by the recovery
of the receptor almost exclusively from the bottom fractions of the gradient (Fig. 5c). Quantitation of the Western blots showed a significantly higher fraction of the IR in DRMs in cholesterol-repleted compared to desmosterol-repleted cells (5.6 ± 0.6% vs. 1.7 ± 0.04% of total IR in DRMs, respectively, n = 6, p < 0.001). Together, these results indicate that desmosterol cannot functionally replace cholesterol in supporting IR activation. This may be related to the weaker membrane ordering properties of desmosterol as suggested by the selective association of the ligand-bound IR with DRMs in the presence of desmosterol.

Atomic-scale molecular dynamics simulations
To better understand why the biophysical and functional properties of desmosterol in membranes differ from those of cholesterol, we performed atomic-scale molecular dynamics simulations. We studied the properties of DPPC-cholesterol and DPPC-desmosterol bilayers at a sterol concentration of 20 mol% in the fluid phase and compared them to those of a pure DPPC bilayer. Below, they are referred to as PC (pure DPPC), PC-CHOL (a mixture of DPPC and cholesterol), and PC-DESMO (a mixture of DPPC and desmosterol). To our knowledge, previous atomic-scale simulation studies of lipid-desmosterol systems are not available.

To assess the sterol-dependent ordering of lipid hydrocarbon chains we determined the molecular order parameter, SCD, often measured in nuclear magnetic resonance experiments. Profiles of the order parameter for the sn-2 chain of DPPC in PC, PC-CHOL and PC-DESMO systems are shown in Fig. 6a. While both sterols were found to increase the order of the chain compared to the pure PC case, cholesterol was more effective in this respect.

The increase of membrane order is associated with changes in the number of gauche defects in acyl chains and in the tilt angles of hydrocarbon chains with respect to the membrane normal. Also, according to these parameters cholesterol was found to be more effective than desmosterol in ordering the bilayer (Table II). Fig. 6b shows that the hydrocarbon chains of DPPC lie, on average, almost parallel to the bilayer normal in the presence of cholesterol, while the effect of desmosterol is less prominent. Similar conclusions can be drawn from the structural properties describing the condensing effect: cholesterol reduces the average surface area per DPPC and increases membrane thickness more effectively than desmosterol (Table II). We also determined the orientation of the sterols in the bilayer by considering the tilt angle between the sterol ring axis (vector between carbon atoms C3 and C17) and the bilayer normal. The average tilt angles of cholesterol and desmosterol were found to be clearly different, about 20° for cholesterol and 27° for desmosterol (Table II).

The above results indicate that simulated bilayers with desmosterol are more fluid-like than those including cholesterol. To study the molecular mechanisms responsible for the different actions of the two sterols, we focused on the conformations of the sterol tails and their interactions with the hydrocarbon chains of the DPPC molecules. The molecular order parameters of the tail segments of the two sterols are shown in Fig. 6c. While the cholesterol tail is strongly ordered, standing almost upright in the membrane normal direction, the ordering of the desmosterol tail is significantly lower and decreases strongly after the first segment of the chain. This decrease is associated with the different conformations of the first two torsion angles in the chain (torsion around the bonds C17-C20 and C20-C22). To illustrate this, the populations for conformations of the torsion angle around the bond C17-C20 are shown in Fig. 6d.

Importantly, the double bond in the tail of desmosterol gives rise to a major difference compared to cholesterol and changes the shape and flexibility of the end of the tail – the last four atoms lie on a surface and create a rigid structure. Consequently, the van der Waals
interactions of the desmosterol tail with the hydrocarbon chains of the DPPCs should be different from those of cholesterol. To elucidate the effect of the double bond on the packing of atoms in the hydrocarbon core and van der Waals interactions, we carried out a similar analysis as Róg and Pasenkiewicz-Gierula (48). We observed that van der Waals interactions of the last four atoms of the tail with DPPC hydrocarbon chains are stronger in the case of desmosterol than cholesterol. At the beginning of the tail, close to the steroid ring structure, the effect was found to be opposite. Here, the hydrocarbon chains of DPPC pack better around the upper part of the cholesterol tail and thus interact more favorably with this part of cholesterol than desmosterol.

In previous studies, the hydroxyl group of epicholesterol was shown to reside deeper in the water phase than that of cholesterol (49). We therefore studied the location of the sterol hydroxyl group as well as its interactions with DPPC at the water-membrane interface. Results of these analyses did not, however, reveal significant differences between cholesterol and desmosterol at the membrane-water interphase (not shown). Together, the simulations show that the double bond in the hydrocarbon tail of desmosterol gives rise to an additional stress in the tail, thus changing its conformation at the beginning of the tail compared to cholesterol. This seemingly minor difference has rather profound implications for various structural properties of the bilayer, in particular fluidity.

Discussion

Our study provides the first evidence to suggest that the biophysical and functional characteristics of desmosterol containing membranes differ considerably from those of corresponding cholesterol containing membranes. Experimental data from model membranes suggest that the potential of desmosterol to promote the formation and stabilization of ordered domains is weaker than that of cholesterol. Studies in cell membranes show that desmosterol has lower affinity for DRMs, and fails to support the DRM association and activation of the raft-dependent signaling receptor, IR, as effectively as cholesterol. Finally, simulations in model membranes support the idea that several properties of the bilayer differ in a desmosterol vs. cholesterol containing membrane: cholesterol is superior in ordering, i.e. reducing the number of gauche defects in acyl chains and reducing the average surface area occupied by phospholipids.

The atomistic simulations suggest that the difference in sterol orientation is largely responsible for the weaker effects of desmosterol on bilayer properties. The tilt of the steroid ring with respect to DPPC membrane normal was found to be 27º for desmosterol and 20º for cholesterol. The reason why the tilt of desmosterol is distinct from cholesterol lies in the tail region of the two sterols. In desmosterol, the double bond in the hydrocarbon tail changes its conformation compared to cholesterol, hence perturbing the membrane structure and tilting desmosterol. Figure 7 shows the structural and orientational differences of desmosterol and cholesterol observed through molecular dynamics simulations. These snapshots of the bilayer systems clearly illustrate the flexibility of the desmosterol tail and the propensity of desmosterol to be more tilted than cholesterol with respect to membrane normal.

Notably, an increase of the tilt can also be associated with reduced ordering effect of sterol as in the case of monounsaturated POPC bilayers: in a POPC bilayer the tilt for cholesterol is 33º (Róg and Pasenkiewicz-Gierula, submitted). Thus, it is possible that the double bond in the tail of desmosterol does not play a major role in a matrix of POPC or other unsaturated lipids, but rather its effects are more specifically characteristic to domains including large amounts of saturated lipids. The differences between desmosterol and cholesterol are hence expected to be most pronounced in ordered domains. This may explain why cholesterol and desmosterol were found to
increase the order of POPC chains in a quite similar manner (15). However, the lateral diffusion rate of desmosterol in a DPPC matrix was found to be higher than that of cholesterol, in agreement with our findings (50).

Importantly, our study suggests, by using insulin signaling as a read-out, that desmosterol does not fulfill the role assigned for cholesterol in raft-dependent functions. Considering that 7-dehydrocholesterol, the alternative penultimate precursor of cholesterol, was found to be more strongly domain-promoting than cholesterol (17), a possible dichotomy emerges: the Kandutsch-Russell pathway proceeding via raft-promoting and Bloch pathway via less raft-promoting sterols to cholesterol. Interestingly, there is evidence for differential use of the pathways, for instance during aging. In young mice, brain cholesterol is synthesized preferentially via the desmosterol pathway, in old mice via the lathosterol pathway (51). Fine-tuning of the sterol composition by alternative use of the pathways could perhaps be employed to optimize raft-dependent processes, for instance by compensating for age-related impairments.

References
Footnotes

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Abbreviations: chol, cholesterol; desmo, desmosterol; DMRs, detergent-resistant membranes; DOPC, dioleylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPH, 1,6-Diphenyl-1,3,5-hexatriene; HPLC, high performance liquid chromatography; IR, insulin receptor; L\text{\textsubscript{O}}, liquid-ordered; L\text{\textsubscript{D}}, liquid-disordered; M\text{\textbeta}CD, methyl-\textbeta-cyclodextrin; OD, optical density; PC, phosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; 12-SLPC, 1-palmitoyl-2-(12-doxyl)-stearoylphosphatidylcholine; Tm, transition temperature

Figure legends

Figure 1. Structures of cholesterol and desmosterol. Carbon atoms in cholesterol are numbered.

Figure 2. Effect of desmosterol on detergent solubilization, DPH polarization and DPH quenching in multilamellar vesicles. A. Detergent solubilization of MLVs composed of DPPC/DOPC (1:1) with and without 15% sterol. The % OD\text{\textsubscript{400}} represents the optical density remaining after incubating the vesicles in the presence of 0.5% TX-100. Values = average ± SD, n = 3. B. DPH polarization of MLVs composed of DPPC/DOPC (1:1) with and without 15% sterol at 30 °C. Chol = cholesterol, desmo = desmosterol, no = no sterol containing vesicles. Values = average ± SD, n = 4. C. Quenching curves of MLVs composed of DPPC/12-SLPC (1:1) with and without 15% sterol were measured as a function of temperature. \Delta F/F\text{\textsubscript{0}} represents relative unquenched fluorescence. D, E. Domain formation coefficients (a) and transition temperatures (T\textsubscript{m}) derived from DPH quenching curves. T\textsubscript{m} is given by the half point of the curves, the temperature at which melting is half complete, and a by the difference in the degree of domain formation (\Delta F/F\text{\textsubscript{0}}) before and after domain melting. Averages of 5 replicate samples from two independent experiments, error bars = SD, * = p<0.05 between chol and desmo in A, B, D, E (two-tailed Student’s t-test).

Figure 3. Association of desmosterol with subcellular membranes. A. Postnuclear supernatant from CHO cells was fractioned in a sucrose density gradient and free sterols in the fractions determined. B. Light membrane fraction (0.7 – 0.9 M sucrose) was incubated in ice-cold 0.5% TX-100 for 30 min. Soluble and insoluble membranes were separated on a sucrose density gradient. Free sterols in the DRM and soluble membrane (sol.mem.) fraction were analyzed and the sterol ratio calculated.

Figure 4. Sterol exchange, cell morphology and albumin secretion. A. HuH7 cells were cholesterol depleted by using M\text{\textbeta}CD and replenished with cholesterol or desmosterol in the presence of sterol-M\text{\textbeta}CD complex. The amounts of free sterol analyzed from each condition. Average of 8 replicate samples from 3 independent experiments, error bars = SD. B. HuH7 cells were subjected to the sterol exchange protocol as above, fixed and stained with filipin and FITC-lentil lectin. Ctrl = control, M\text{\textbeta}CD = cells fixed after cholesterol depletion, chol = cells repleted with cholesterol, desmo = cells repleted with desmosterol. Scale bar 10 \mu m. C. After cholesterol or desmosterol replenishment, cells were incubated for 1 h, the media collected and proteins recovered by TCA-precipitation. The amount of albumin secreted was analyzed by Western blotting and expressed as % of untreated samples (control). A representative immunoblot and quantitation of the data are shown. Values = average ± SEM, n = 3-4.
Figure 5. **IR activation and DRM association after sterol exchange.** A. Cholesterol or desmosterol replenished cells were stimulated with insulin or left untreated for 5 min. The amount of IR in the samples was detected by immunoblotting with anti-IRβ antibody and the active receptor with anti-pTyr antibody. IR activation is expressed as the ratio of band intensities (pTyr/IR). Representative immunoblots and quantitation of the data are shown. Values = average ± SEM, n = 10, * = p < 0.001. B. Binding of $[^{125}]$-insulin onto sterol replenished cells in the presence of the indicated amounts of unlabeled insulin. Values are averages of duplicate samples from a representative experiment. C. Sterol replenished and insulin stimulated cells were subjected to Optiprep-density gradient in the presence of 0.1 % Triton X-100 and the amount of IR in the fractions analyzed by Western blotting. Representative blots are shown.

Figure 6. **Atomic-scale molecular dynamics simulations.** Results are shown for PC (black line), PC-CHOL (gray line), and PC-DESMO (dashed line) systems. A. The molecular order parameter ($S_{CD}$) profiles calculated for the sn-2 chain of DPPC. Small segment numbers correspond to carbons close to the glycerol group. B. Distributions of tilt angles for the sn-2 chain of DPPC. C. The molecular order parameter profiles calculated for the short hydrocarbon tails of the sterols. Segment 1 corresponds to C13-C17-C20, segment 2: C17-C20-C22, 3: C20-C22-C23, 4: C22-C23-C24, 5: C23-C24-C25, 6: C24-C25-C26(or C27). D. Torsion angle distribution. Populations of the torsion angle around the C17-C20 bond in the sterol molecules are shown.

Figure 7. **Snapshots of molecular dynamics simulations for the bilayer systems studied.** On the left, there is a DPPC-cholesterol bilayer surrounded by water. Hydrocarbon chains of DPPC are shown in green, cholesterol molecules are depicted in yellow. On the right, there is a corresponding membrane composed of DPPC and desmosterol molecules. Desmosterol is shown in red.
Table I. Composition of MLVs used in the assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>DPPC</th>
<th>DOPC</th>
<th>12-SLPC</th>
<th>Total lipid conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent turbidity</td>
<td>1</td>
<td>1</td>
<td>50</td>
<td>500</td>
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<tr>
<td>DPH quenching</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F_{\text{DPPC}} )</td>
<td>1</td>
<td>1</td>
<td>50</td>
<td></td>
</tr>
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<td>( F_{0,\text{DPPC}} )</td>
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<td>50</td>
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<tr>
<td>( F_{\text{DOPC}} )</td>
<td>1</td>
<td>1</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>( F_{0,\text{DOPC}} )</td>
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<td>1</td>
<td>50</td>
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<tr>
<td>DPH polarization</td>
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<td>50</td>
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</table>
Table II. Effects of sterols on bilayer properties. The molecular order parameter ($S_{CD}$) is an average over the $S_{CD}$ order parameter profiles of the sn-1 and sn-2 chains, including all carbons in the chains, to characterize overall ordering in a bilayer. The chain tilt angle is averaged over the sn-1 and sn-2 chains as defined in (52). Average values for the number of gauche states per acyl chain, the lifetime of trans conformations, and the membrane thickness (53) of PC, PC-CHOL, and PC-DESMO bilayers are given. The average surface area (SA) was determined by dividing the average total area of the bilayer by the number of DPPC molecules, i.e., by 64 in all three systems (note that there are 128 molecules in PC and 160 molecules in PC-CHOL and PC-DESMO bilayers).

<table>
<thead>
<tr>
<th>Membrane</th>
<th>PC</th>
<th>PC-CHOL</th>
<th>PC-DESMO</th>
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<tbody>
<tr>
<td>$S_{CD}$</td>
<td>0.15</td>
<td>0.28</td>
<td>0.23</td>
</tr>
<tr>
<td>Tilt (°)</td>
<td>DPPC 23.7</td>
<td>Sterol 19.8</td>
<td>27.0</td>
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<tr>
<td>No. gauche</td>
<td>3.0</td>
<td>2.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Lifetime (ps)</td>
<td>86</td>
<td>117</td>
<td>101</td>
</tr>
<tr>
<td>Thickness [nm]</td>
<td>3.92</td>
<td>4.69</td>
<td>4.22</td>
</tr>
<tr>
<td>SA [nm$^2$]</td>
<td>0.66</td>
<td>0.60</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A

% OD

chol desmo no

0 10 20 30

B

polarization

chol desmo no

0.00 0.05 0.10 0.15 0.20 0.25

C

ΔF/F₀

Temp (°C)

chol desmo no

20 24 28 32 36 40 44 48 52 56

D

B

chol desmo no

0.05 0.10 0.15 0.20 0.25

E

T_m (°C)

32 31 30 29 28 27

- cholesterol
- desmosterol
- no sterol

* indicates statistical significance.
Figure 3
Figure 4

A

![Graph showing cholesterol and desmosterol levels with MβCD sterol conditions.

B

Staining images with filipin and FITC-lentil lectin showing different treatments.

C

Western blot analysis with anti-albumin antibody showing a band at 53.5 kD.
Figure 5

A

anti-pTyr

anti-IRβ

insulin  

chol desmo chol desmo

83 kD

B

C

0 0.1 1 10

unlabeled insulin (nM)

chol desmo

83 kD

DRMs

0% 20% 25% 30% 35% 40% OP

83 kD

0% 20% 25% 30% 35% 40% OP

83 kD

chol desmo
Figure 6
Figure 7