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Paradoxical impact of cholesterol on lipid packing and cell stiffness

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1. ABSTRACT

Cell stiffness or deformability is a fundamental property that is expected to play a major role in multiple cellular functions. It is well known that cell stiffness is dominated by the intracellular cytoskeleton that, together with the plasma membrane, forms a membrane-cytoskeleton envelope. However, our understanding of how lipid composition of plasma membrane regulates physical properties of the underlying cytoskeleton is only starting to emerge. In this review, we first briefly describe the impact of cholesterol on the physical properties of lipid bilayers in model membranes and in living cells, with the dominant effect of increasing the order of membrane lipids and decreasing membrane fluidity. Then, we discuss accumulating evidence that removal of cholesterol, paradoxically, decreases the mobility of membrane proteins and increases cellular stiffness, with both effects being dependent on the integrity of the cytoskeleton. Finally, we discuss emerging evidence that oxidized modifications of low-density lipoproteins (oxLDL) have the same effects on endothelial biomechanical properties as cholesterol depletion, an effect that is mediated by the incorporation of oxysterols into the membrane.

2. INTRODUCTION

Cholesterol is one of the major lipid components of the plasma membrane in all mammalian cells, where it constitutes up to 45 mol% with respect to other lipids. Remarkably, there is a strong heterogeneity in cholesterol distribution between the plasma and the intracellular membranes, with the majority of cholesterol, up to 90%, found in the plasma membrane (1, 2). Furthermore, cholesterol is also distributed heterogeneously within the plasma membrane, giving rise to a concept of cholesterol-rich membrane rafts/domains (3, 4). The impact of cholesterol on the physical properties of the membrane lipid bilayer is well studied, both in pure lipid environments, such as liposomes or lipid monolayers, and in cellular membranes. Briefly, an increase in membrane cholesterol has been shown to increase lipid packing and decrease fluidity and deformability of lipid bilayers. However, an increasing number of studies, including by our group and other investigators, demonstrate that an increase in the rigidity and lipid packing of the bilayer does not translate into an increase in the overall stiffness of the cellular envelope, a bi-component system where the sub-membrane cytoskeleton underlies the membrane lipid bilayer. In contrast, it appears that there is an inverse relationship between the lipid order of the membrane bilayer and the stiffness of the cellular envelope that is dominated by the cortical cytoskeleton. The goal of this review is to discuss the current knowledge about the

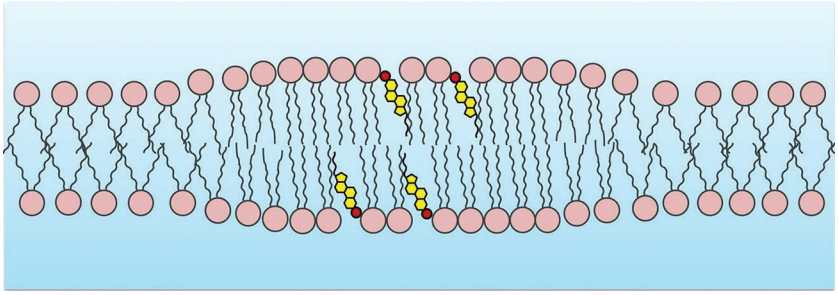

Figure 1. Schematic of membrane thickening and phospholipid tail alignment caused by inserted cholesterol molecules. The phospholipid molecules (pink spheres with grey tails) form a bilayer. Cholesterol molecules are represented by red spheres for hydroxyl groups with yellow steroid rings and a black hydrophobic tail.

impact of membrane cholesterol on cellular biomechanics and the implications of these effects on cellular function.

3. CHOLESTEROL-INDUCED ORDERING OF MEMBRANE PHOSPHOLIPIDS IN ARTIFICIAL MEMBRANES

The cholesterol molecule consists of a planar ring structure built of four fused steroid rings with a hydroxyl group and a hydrophobic tail. The molecule is oriented with the steroid rings parallel to the hydrocarbon chains of the membrane phospholipids: the hydroxyl group interacts with the polar head groups of the phospholipids while the rigid lipophilic steroid ring system interacts with the hydrocarbon chains, restricting their motion within the bilayer (2, 5).

At low cholesterol concentrations (below 15 mol%), the lateral order of artificial phospholipid membranes made up of saturated phospholipids such as DPPC or DMPC is initially disrupted, causing membrane fluidization at room temperature, an effect that is reversed as cholesterol concentration increases (e.g. (6, 7)). Addition of cholesterol also abolishes the phase transition from the liquid to the gel phase of the model membrane (8). At the same time, however, above the phase transition temperature in membranes, the presence of cholesterol causes condensation of the phospholipids and a decrease in membrane fluidity. As discussed in detail below, the predominant effect of cholesterol in biological membranes is a decrease in membrane fluidity. The mechanism of the condensation effect is related to the energetic requirement of the membrane to shield the hydrophobic portions of cholesterol molecules from the external polar environment. For membrane phospholipids, their hydrophobic tails are shielded from the external polar environment by their large hydrophilic headgroups. Cholesterol, however, possesses a smaller hydrophilic headgroup than phospholipids; therefore, when present in a membrane, cholesterol is unable to shield the hydrophobic region as efficiently as phospholipids. To better shield the hydrophobic interior of the membrane in the presence of cholesterol, the phospholipid tails become more aligned as they pack closer together, and the area they occupy decreases (9). Thus, an increase in membrane cholesterol concentration has an ordering effect on the membrane phospholipid tails, which also manifests itself as a decrease in membrane fluidity due to hindering of the lateral motion of membrane phospholipids, as described in earlier studies (e.g. (10-12)).

In terms of membrane elasticity, cholesterol has been shown to increase the elastic compressibility modulus of lipid bilayers, making the membrane effectively more rigid and less deformable (13, 14). Moreover, the heterogeneous distribution of membrane cholesterol, influenced by its differential interactions with phospholipids with varied degrees of acyl tail saturation, results in a non-monotonous change in the membrane elasticity modulus as cholesterol concentrations are increased (15-17). The decrease in in-plane membrane elasticity with increasing cholesterol concentration has been found to also depend on the degree of phospholipid acyl chain saturation and headgroup identity. Phospholipid membranes with greater tail saturation have greater decreases in elasticity when exposed to higher cholesterol concentrations (18, 19). This effect results in the formation of a liquid ordered phase in which phospholipid acyl tails appear to be extended and tightly packed, leading to the formation of membrane ordered domains (e.g. (20-22)). The ordering effect of cholesterol is also associated with increased thickness of the hydrophobic core of the membrane. For example, the thickness of saturated DPPC bilayers was shown to increase with increasing cholesterol concentration from 57.0 Å in bilayers without cholesterol, to 59.6 Å and 60.2 Å in bilayers containing 9% and 28% cholesterol respectively (23). This ordering effect is also attributed to an increase in *trans* configurations of the hydrocarbon chains in the case of unsaturated phospholipids. Accordingly, cholesterol-rich ordered membrane domains are expected to have a higher thickness than the rest of the membrane (Figure 1). Accumulating evidence suggests that cholesterol-rich membrane domains that are formed in cellular plasma membranes play an important role in the regulation of cellular stiffness.

4. IMPACT OF CHOLESTEROL ON PHYSICAL PROPERTIES OF PLASMA MEMBRANE LIPID BILAYERS

4.1. Experimental tools for modulating cellular cholesterol

A transition from studying effects of cholesterol on the properties of artificial membranes to cellular membranes presented an immediate challenge of modulating cell membrane cholesterol in a specific and reproducible way. Prior to the development of β-cyclodextrin (βCD)-based cholesterol extraction, the means to modify cellular cholesterol in a controlled way were limited. It was possible to incubate cells with liposomes containing either low or high cholesterol concentrations, which would respectively decrease or increase cellular cholesterol levels upon fusion to cell membranes. Alternatively, it was possible to serum starve the cells by maintaining them in media containing little to no serum. Clearly, both approaches introduce multiple non-specific effects. A major advance in the field was the development of a method to specifically modulate membrane cholesterol in cellular membranes in a precise and reproducible way by exposing cells to β-cyclodextrins (βCDs) (24, 25), which led to an explosion of studies addressing the role of cholesterol in a variety of cellular functions.

Cyclodextrins, oligosaccharides consisting of α-(1-4)-linked D-glycopyranose units, have long been utilized as carriers of hydrophobic drugs due to their high solubility in water and the presence of a hydrophobic cavity within the molecule (26). The size of the cavity depends on the degree of the polymerization and provides relative specificity for different hydrophobic loads (26), (27). Specifically, heptamers, or β-cyclodextrins, were shown to be the most efficient at extracting cholesterol, whereas hexamers, or α-cyclodextrins are more efficient at extracting phospholipids (28). In terms of extraction mechanism, Rothblatt and colleagues (29) proposed that, based on the kinetic and energetic analysis of cholesterol efflux, a cyclodextrin molecule diffuses into the immediate proximity of the plasma membrane bilayer and cholesterol molecules diffuse directly into the cyclodextrin hydrophobic cavity without having to dissolve into the aqueous phase of the cytosol.

It is important to note that, while numerous studies established that β-cyclodextrins can be used for precise and reproducible cholesterol extraction, enrichment or replenishment in multiple cell types and tissues, the specificity of cholesterol extraction for cholesterol-rich vs. cholesterol-poor membrane domains remains controversial. Briefly, some specificity was suggested by studies showing that short exposures (≤ 2 min) or very low concentrations (≤ 1 mM) of MβCD result in selective extraction of cholesterol from cholesterol-rich membrane domains (30, 31), however, the preponderance of studies show that MβCD is capable of removing cholesterol from both types of membrane domains (27). A more detailed analysis of the impact of cholesterol depletion and enrichment on membrane fluidity and local lipid order of plasma membranes is presented below.

4.2. Cholesterol effects on the fluidity of plasma membranes

Membrane fluidity, an inverse of viscosity, can be estimated by measuring the motion of different moieties within the bilayer. The two most common methods to measure membrane fluidity are *fluorescence anisotropy,* based on the rotational diffusion of fluorescent lipid probes, and *FRAP* (Fluorescence Recovery After Photobleaching), an approach that measures the rate of lateral diffusion of a fluorescent moiety into a region that was previously photobleached.

Fluorescence anisotropy is based on the incorporation of small fluorescent lipophilic probes into the membrane bilayer that orient themselves with the hydrocarbon chains of the phospholipids. Membrane viscosity is estimated from the rotational diffusion coefficients of the probes, which is hindered by the neighboring lipids (32-34). The probes differ in their selectivity for plasma vs. intracellular membranes and also in their specific position within the bilayer. For example, DPH (1,6-diphenyl-1,3,5-hexatriene) is incorporated into the hydrophobic core of the bilayer and has poor selectivity between the plasma and intracellular membranes, whereas its derivative TMA-DPH (4-trimethyl-ammonio-1,6-diphenyl-1,3,5-hexatriene) is incorporated in the outer leaflet close to the membrane surface and is more selective to the plasma membrane (32, 35). Universally, the effect of cholesterol removal is an increase in membrane fluidity, as measured by DPH-based fluorescence anisotropy. Specific examples include a significant decrease in fluorescence anisotropy corresponding to increased membrane fluidity in cholesterol depleted HEK293 cells (36, 37), membrane vesicles isolated from a basophilic leukemia cell line (38), human glioblastoma cells (39), primary cerebellar granule cells (40) and sperm (41).

Effects of cholesterol depletion on the lateral mobility of lipid probes or lipid components of biological membranes are mostly consistent with increased membrane fluidity reported by fluorescence anisotropy. Pucadyil and Chattopadhyay (42) reported that cholesterol depletion of hippocampal membranes resulted in a significant increase in lateral diffusion of two fluorescent lipids: $DiIC_{18}$, which partitions preferentially into ordered domains, and *FAST* DiI, which partitions into disordered membranes. Interestingly, the effect of cholesterol depletion on lipid diffusion was higher for ordered than for disordered phases. Cholesterol depletion was also reported to increase the lateral mobility of a fluorescent analogue of sphingomyelin (43). A recent study showed,

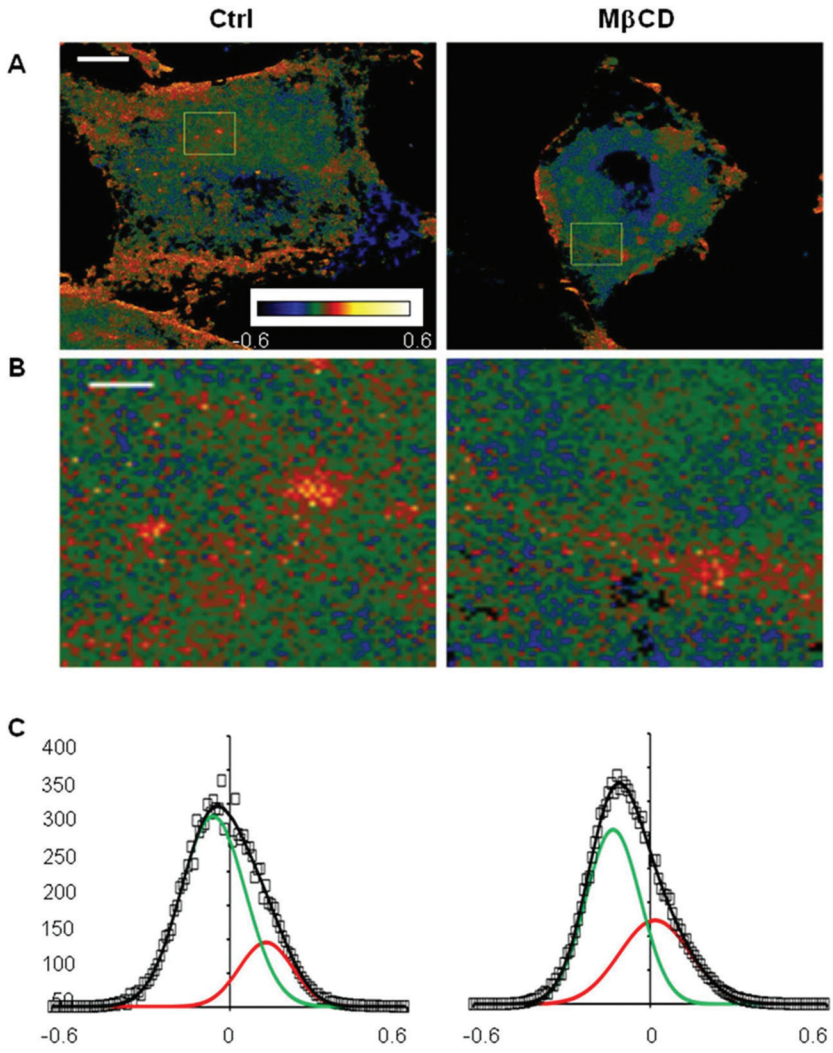

Figure 2. Impact of cholesterol depletion on lipid packing of membrane domains in endothelial cells. A: Typical GP images of control and cholesterol-depleted cells. Bar is 5.6 μm. B: The zoomed-in representative regions of the GP images shown above. Scale bar is 1μm. All images are shown in pseudo color with yellow and red corresponding to the higher GP values, presumably ordered domains, and green and blue corresponding to the lower GP values, presumably disordered domains. C: GP histograms fitted by a two-Gaussian distribution. Reproduced from Shentu *et al*., 2010, American J of Physiology Cell Physiology 299: C218-229.

however, that cholesterol depletion may also decrease the mobility of a lipid probe in primary hair cells isolated from guinea pigs, an effect that was specific to the basal membranes, while no effect was observed on the apical membranes (44). The source of this local heterogeneity is not clear. In any case, an increase in membrane fluidity is expected to be associated with increased lipid packing/ordering of the membrane.

4.3. Cholesterol effects on lipid packing of the plasma membrane bilayer

Lipid packing or ordering of biological membranes can be estimated by Laurdan two-photon microscopy, a dye that is sensitive to the polarity of the local environment and undergoes a red shift as the phase boundary changes from gel to fluid (45, 46). Changes in membrane order are estimated by calculating the general polarization (GP) ratio, a normalized ratio of fluorescence intensity of gel phase vs. fluid phase. Typically, plasma membranes are shown to be highly heterogeneous in their lipid order with GP values varying in a continuous manner and without a sharp, clear distinction between “ordered” and “disordered” domains. Nevertheless, GP histograms reflect the relative abundance of the membrane domains with more fluid or more ordered properties, providing a “map” of lipid order of individual cells. Figure 2 shows a typical example of a “domain map”, as detected by Laurdan two-photon microscopy.

Gaus *et al*. (45) were first to test the effects of cholesterol depletion and enrichment on the lipid order of membrane domains using Laurdan imaging. Their data

show that cholesterol depletion of macrophages results in a significant decrease in lipid order of both ordered and fluid domains, and a relatively small, but significant, shift in the total area of the cell membrane covered by ordered vs. fluid domains (about a 10-20% shift from ordered to fluid). Cholesterol enrichment, however, did not increase the order of either of the domains, but increased the area covered by the ordered domains (also by about 20%). A decrease in lipid order following cholesterol depletion was also observed in plasma membranes of multiple other cell types, including kidney cells (47), hippocampal membranes (48), and aortic endothelial cells (49), as well as in cell lines CHO (50) and HEK (37). Interestingly, MβCD-induced cholesterol depletion was observed not only to decrease lipid order, but also to decrease membrane heterogeneity (49).

In summary, it is well established that removal of cellular cholesterol increases membrane fluidity, as measured by the diffusion of lipid probes, and decreases the lipid order/packing of the membrane lipid bilayer of plasma membranes. This effect is based on the physical interactions between cholesterol molecules and phospholipids, and is fully consistent with the impact of cholesterol on fluidity and lipid packing of non-biological membranes.

An increase in membrane fluidity should be expected to promote the ability of membrane proteins to move within the membrane and form regulatory protein-protein complexes, which clearly should have major functional consequences. However, accumulating evidence demonstrates that cholesterol depletion actually inhibits the lateral motilities of membrane proteins in several systems, an effect that depends on the integrity of the sub-membrane cytoskeleton.

5. IMPACT OF CHOLESTEROL ON MEMBRANE-CYTOSKELETON INTERACTIONS

5.1. Impact of cholesterol on the lateral mobility of membrane proteins

The first surprising result came from the work of Edidin and colleagues, who used FRAP to determine the effect of cholesterol depletion on the lateral diffusion of a class of HLA (Human Leukocyte Antigen) molecules in fibroblasts and lymphoblasts (51). In contrast to an increase in membrane fluidity reported by fluorescence anisotropy of membrane probes, Kwik *et al*. showed that cholesterol depletion significantly decreased the mobility of the HLA molecules. A similar result was also observed for epidermal growth factor receptors in the same cells. A decrease in the lateral mobility of the membrane proteins in cholesterol-depleted cells was abrogated by depolymerizing F-actin, indicating that this effect depends on the stability of the cytoskeleton (51). A similar effect was observed for voltage-gated K^+ channels Kv1.4., with cholesterol depletion resulting in a significant decrease in the lateral mobility of the channels (52). At the same time, the mobilities of two other voltage-gated K^+ channels, Kv2.1. and Kv1.3., were either unaffected or slightly increased, leading to the conclusion that these channels partition into separate membrane domains. The exact mechanisms of the differential effects of cholesterol on Kv channels, however, remain unclear. A decrease in mobility following cholesterol depletion was also reported for several other proteins, including Serotonin$_{1A}$ receptors (53) and Nicotinic acetylcholine receptor (54). The major effects were a decrease in the mobile fraction and of the diffusion coefficients. Interestingly, Baier *et al*. also showed that depolymerization of F-actin resulted in partial recovery of receptor mobility in cholesterol-depleted cells (54). Taken together, these observations lead to two conclusions: lateral mobility of membrane proteins may be inversely related to the fluidity of lipid components of the membrane, and the mobility is critically dependent on the integrity of the cytoskeleton. These observations also imply that, surprisingly, the integrity/stability of the sub-membrane cytoskeleton and/or membrane-cytoskeleton adhesion is enhanced by cholesterol depletion. Direct evidence for this effect is discussed in the next section.

5.2. Cholesterol loss increases the strength of membrane-cytoskeleton adhesion

Earlier studies investigated the role of cholesterol-rich membrane domains in membrane-cytoskeleton interactions with accumulating evidence suggesting that these domains serve as focal points for these interactions. More specifically, several major regulators of the cytoskeleton function, such as phosphatidylinositol 4,5 bisphosphate (PIP_2) and Rho-GTPases were found to partition into the protein complexes found in raft domains (55). Cholesterol depletion, on the other hand, was shown to disrupt the association of PIP_2 with the raft domains (56), decrease the level of PIP_2 on the plasma membrane (51), and prevent membrane association and activation of Rac1 (57). These studies were performed in several cell types including fibroblasts and lymphoblasts, as well as in immortalized cell lines. Furthermore, multiple cytoskeleton proteins were identified in cholesterol-rich detergent-resistant membrane fractions in neutrophils using proteomic analysis (58). Based on these studies, the expectation was that cholesterol depletion resulting in the disruption of cholesterol-rich membrane domains should result in weakening of membrane-cytoskeleton interactions and dissociation of the sub-membrane cytoskeleton from the plasma membrane. Thus, studies of the effect of cholesterol depletion on the lateral mobility of membrane proteins and studies of the association of cytoskeleton proteins with cholesterol-rich domains created opposite expectations regarding the effect of membrane cholesterol on the strength of membrane-cytoskeletal adhesion. It was critical, therefore, to determine directly how changes in the level of membrane

cholesterol regulate the adhesion between the plasma membrane and the sub-membrane cytoskeleton.

Sun *et al*. (59) investigated the impact of cholesterol on the adhesion strength between the cytoskeleton and the membrane by pulling membrane tethers from the surface of aortic endothelial cells using Atomic Force Microscopy (AFM). This approach, described in detail by (60), measures the tether force (*F*), recorded by the deformation of the AFM cantilever, as the cantilever is pulled away from the cells at different speeds. The dependence of the tether force and the pulling speed is described by $F = F_0 + 2\pi\eta_{eff}v$ (61), where F_0, the threshold force, reflects the amount of force needed to initiate a tether formation and is a function of the membrane-cytoskeleton interaction, η_{eff} is the effective membrane surface viscosity, and v is the speed at which the cantilever is pulled away from the cell. Using this approach, we found that cholesterol depletion significantly increased the threshold force that is required to pull the tether, which is directly related to the membrane-cytoskeleton interaction (59). An increase F_0 in was abrogated by depolymerization of F-actin, further suggesting that this effect should be attributed to the stabilization and strengthening of the sub-membrane cytoskeleton. To isolate the contribution of actin-cytoskeleton in determining the F_0 of generating the tether, we defined the membrane-cytoskeleton adhesion, F_{ad} as the difference in F_0 before and after latrunculin treatment, i.e. $F_{ad} = F_0 - F_0^{lata}$ Cholesterol depletion results in almost a 2-fold increase in F_{ad}, whereas cholesterol enrichment significantly decreases the threshold tether force F_0 so that it becomes almost equivalent toc $F_0^{lata.}$ These observations led to the conclusion that cholesterol depletion increases membrane-cytoskeleton adhesion, whereas cholesterol enrichment has the opposite effect. Furthermore, cholesterol depletion also increases the variance of tether force, suggesting that cholesterol depletion makes tethers much more heterogeneous, presumably due to the enhanced membrane-cytoskeleton adhesion. In contrast, cholesterol enrichment reduces the membrane-cytoskeleton adhesion and makes the bilayer easier to detach from and flow on the cytoskeleton.

In terms of the mechanism, our more recent study suggests that cholesterol depletion enhances membrane-cytoskeletal interactions by causing dispersion and redistribution of PIP_2 (62). Similarly to the previous study, we used aortic endothelial cells to show that while PIP_2 appeared in discrete patches in control endothelial cells, cholesterol depletion resulted in the loss of discrete PIP_2 domains and almost uniform distribution across the cell membrane. Most importantly, while addition of exogenous PIP_2 had no effect on the tether force in control cells, it significantly increased the tether force in cholesterol-depleted cells indicating stronger membrane-cytoskeleton adhesion. We hypothesized that cholesterol depletion may strengthen membrane-cytoskeleton interactions by increasing the number of focal points enriched with the PIP_2 linker. We could not completely exclude the possibility, however, that cholesterol depletion makes it easier for the exogenous PIP_2 to be incorporated into the plasma membrane, thus more studies are needed to evaluate this point. Interestingly, changes in PIP_2 distribution were also shown to be critical for the effect of cholesterol depletion on constraining the lateral mobility of membrane proteins reported earlier (51), but in that case, cholesterol depletion resulted in the loss of PIP_2 from the plasma membrane. The authors suggested that the loss of PIP_2 results in re-organization and stabilization of the sub-membrane cytoskeleton. Clearly, more studies are needed to provide better understanding of these mechanisms.

6. A LOSS OF CHOLESTEROL RESULTS IN CELL STIFFENING

6.1. Cell membrane deformability

As described above, the first indication that cholesterol removal may result in the stabilization of the cytoskeleton came from a study by Kwik *et al*. (51) showing that cholesterol depletion of human fibroblasts constrained the mobility of a class of transmembrane proteins, an effect that was fully dependent on the integrity of F-actin. Furthermore, Kwik *et al*. proposed that cholesterol depletion resulted in stabilization of the sub-membrane cytoskeleton. Since the overall stiffness of the membrane-cytoskeleton complex is well recognized to be dominated by the rigidity of the sub-membrane cortical cytoskeleton (63), these observations implied that a loss of membrane cholesterol may have significant implications for the biomechanical properties of the membrane-cytoskeleton complex.

Byfield *et al*. (64) provided the first direct evidence that cholesterol depletion results in significant stiffening and loss of deformability of vascular endothelial cells by measuring progressive membrane deformation in response to negative pressure applied by a glass micropipette (Figure 3). Exactly the same approach was used earlier to determine the effect of cholesterol on membrane stiffness of liposomes, showing that an increase in membrane cholesterol results in bilayer stiffening (13). However, in contrast to its effect on the bilayer stiffness, removal of membrane cholesterol either by a cholesterol-depleting agent, methyl-β-cyclodextrin (MβCD), or by serum starvation resulted in the slower rate of membrane deformation and smaller maximal deformation in response to the same level of negative pressure, indicating an increase in endothelial stiffness (64). Cholesterol depletion-induced endothelial stiffening was accompanied with a significant change in endothelial morphology and spreading (65). Enriching the cells with cholesterol had no effect. Importantly, a typical membrane area that is aspirated into the pipette in these experiments is rather large (the tips of the pipettes are ~5 μm in diameter) and the membrane is clearly

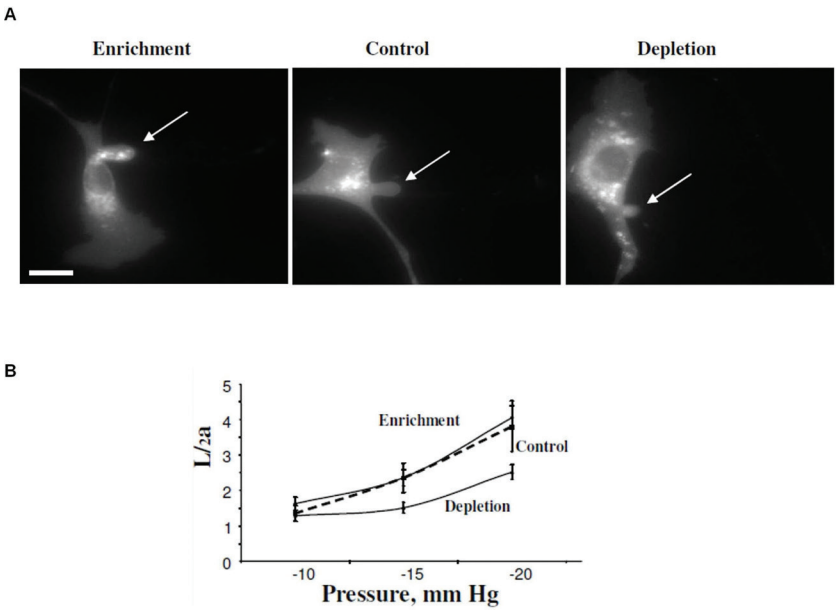

Figure 3. Cholesterol depletion increases stiffness of vascular endothelial cells. A: Typical images of membrane deformation in response to negative pressure in cells depleted or enriched with cholesterol as compared to a control cell. Membrane is visualized with a fluorescent dye Di_{18}; bar is 10 μm. B: Maximal membrane deformation normalized to a pipette diameter as a function of applied pressure. Reproduced from Byfield *et al*., 2004, Biophys J. 2004 Dec;87(6):3850-61.

undergoing deformation together with at least some of the sub-membrane cytoskeleton. The stiffening effect induced by cholesterol depletion is fully abrogated by the depolymerization of F-actin. The latter observation is consistent with the notion that integrity of F-actin plays the dominant role in determining cellular biomechanical properties. Most of these studies were performed using bovine aortic endothelial cells as a cellular model. Importantly, an increase in cell stiffness upon cholesterol depletion was also shown in Chinese Hamster Ovary cells (CHO K1) demonstrating that this effect is not unique to endothelial cells (66). Clearly, more studies are needed to determine the relationship between membrane cholesterol and cellular stiffness in other vascular tissues, particularly in vascular smooth muscle cells.

6.2. Stiffness of the intracellular "deep" cytoskeleton

Another rather unexpected observation came from the analysis of correlated motion of fluorescent beads that were phagocytosed by endothelial cells (67). The motion of internalized particles is indicative of the material properties of a cellular milieu, with greater motion indicating a softer material (68). Furthermore, movement of individual particles deforms the cytoskeletal network around them, resulting in displacement of other particles in their vicinity (69). Thus, analysis of the correlated motion of particles within a cell allows estimation of the stiffness of the intracellular cytoskeleton (70, 71). Using this approach, we showed that cholesterol depletion results in a significant (~50%) decrease in the correlated motion of particles within endothelial cells, indicating an increase in the intracellular stiffness. It is also important to note that a decrease in the correlated motion of the particles may also be indicative of a decrease in motor activity. In either case, these observations indicate that cholesterol depletion regulates the material properties of the intracellular cytoskeleton.

6.3. Force generation

An increase in cellular stiffness in cholesterol depleted endothelial cells correlates with an increase in endothelial force generation. First, it was established that decreasing the level of cellular cholesterol facilitates the ability of aortic endothelial cells to induce gel contraction (72), which is indicative of an increase in force generation on the cell-substrate interface. Indeed, these observations are consistent with earlier studies that demonstrated that increased cell stiffness correlates with the magnitude of forces that cells exert on substrates (73). To gain further insights into the effect of cholesterol loss on endothelial force generation, we analyzed the local forces generated by endothelial cells using Traction Force Microscopy (TFM), as described previously (74). In this study, we showed that consistent with the gel contraction assays, TFM analysis revealed that cholesterol depletion results in an almost 2-fold increase in force exerted by the cells on their substrates (75). Similar to the impact of cholesterol on endothelial stiffness, while cholesterol depletion increased endothelial force generation, cholesterol enrichment had no effect. Force generation effects of cholesterol depletion, however, can be rescued

by replenishing cellular cholesterol. Thus, it appears that for both endothelial stiffness and force generation, there is a critical cholesterol level that is required to maintain the normal parameters, so that addition of cholesterol over this level has no further effect.

7. INVERSE EFFECTS OF OXLDL AND OXYSTEROLS ON LIPID PACKING AND CELL STIFFNESS

With an increased realization that the loss of membrane cholesterol has opposite effects on the lipid order of the membrane vs. on membrane-cytoskeleton interactions and cellular stiffness, the question arises of how this effect is related to physiological and pathological conditions in dyslipidemia. The main concern is usually an increase in cholesterol load rather than cholesterol deficiency. Our studies, however, provide multiple lines of evidence that exposing endothelial cells to oxidized low-density lipoproteins (oxLDL) results in the same effects on endothelial biomechanics as cholesterol depletion. Specifically, these effects include disruption of lipid packing (49), increase in cell stiffness (49, 72), increase in force generation (72, 76), and enhanced sensitivity to shear stress (77). Furthermore, we also showed that exposing cells to 7-ketocholesterol, one of the major components of oxLDL, also disrupts lipid order and increases endothelial stiffening (78). All of these studies focused on aortic endothelial cells (bovine and human) because of the high prevalence of atherosclerosis in these arteries. It would be very interesting and important to compare the biomechanical properties of endothelial cells across different vascular beds. A more detailed discussion of the current studies is provided below.

7.1. OxLDL-induced fluidization and stiffening of endothelial membranes

Oxidative damage of low-density lipoproteins (LDL) is well known to be a major risk factor for the development of cardiovascular disease. The levels of oxLDL increase in hypercholesterolemia in both humans and animal models, and it is found in atherosclerotic lesions (79-81). It is important to note, however, that the term oxidized LDL is used to describe a wide variety of LDL modifications ranging from minimally or mildly-oxidized to strongly-oxidized, which may differ significantly in their composition and biological effects. This variability can be attributed to the fact that LDL oxidation *in vivo* is mediated by a variety of free radicals including superoxide, hydroxyl radicals and nitric oxide produced by enzymatic action of several enzymes, such as NADPH oxidase (NOX), NO synthase and lipoxygenase (82). The most frequently used oxidation route *in vitro* is exposure to free transition metals, particularly copper, although this pathway is not significant in the physiological oxidation of LDL *in vivo*. The rationale to use copper-oxidized LDL as a molecular model for oxidative modifications of LDL *in vivo* is that it is recognized by the cellular scavenger receptors and results in significant endothelial dysfunction. One of the major postulates in the field regarding oxLDL has been that oxLDL is detrimental to endothelial function because by virtue of being recognized by the scavenger receptors, it should be more efficient in loading cells with cholesterol. This notion was challenged by the studies of Smart and colleagues, who showed that rather than enriching endothelial cells with cholesterol, oxLDL depleted cholesterol from caveolae (83). Following this study, we tested the impact of oxLDL on the deformability of aortic endothelial cells and found that, similarly to cholesterol depletion, exposure to oxLDL resulted in a significant increase in endothelial stiffness (72). The impact of oxLDL on endothelial stiffening correlated positively with the degree of LDL oxidation (78). These studies were conducted using copper-oxidized LDL. More recently, we have also determined that exposing endothelial cells to enzymatically oxidized LDL also results in endothelial stiffening (manuscript in preparation). Furthermore, the effect of oxLDL on endothelial stiffness was verified in endothelial cells freshly-isolated from hypercholesterolemic pigs demonstrating that it is highly physiologically relevant (72).

OxLDL-induced endothelial stiffening was accompanied with a decrease in lipid order/fluidization of endothelial membranes, as determined by Laurdan imaging described above (49). Notably, both oxLDL-induced fluidization of the membrane domains and the stiffening of the cellular envelope could be reversed by increasing cholesterol content of the membrane. Thus, both of these effects are clearly not mediated by an increase in membrane cholesterol. However, since we also did not detect any significant decrease in cholesterol content, efflux or internalization in oxLDL-treated cells, we proposed an alternative hypothesis that oxLDL-induced fluidization of the membrane and endothelial stiffening are mediated by the membrane insertion of oxysterols.

7.2. 7-ketocholesterol-induced fluidization and stiffening of endothelial membranes

OxLDL contains an array of oxidative lipids that are bioactive under pathological conditions. Our studies showed that oxLDL-induced endothelial stiffening can be accounted for by the oxysterol fraction of the lipoprotein, with the most prominent effects caused by 7-ketocholesterol. We also found increased incorporation of 7-ketocholesterol in arterial tissues of dyslipidemic mice (78). Figure 4 shows a shift in the distribution of endothelial elastic moduli to higher values in cells exposed to 7-ketocholesterol, indicating an increase in cell stiffness. At the same time, the lipid order parameters of endothelial membranes shift to a more fluid, less ordered phase, as indicated by a relative decrease in highly-ordered (yellow) domains and an increase in less ordered (blue-green) domains. In addition, 27-hydroxycholesterol, which was also

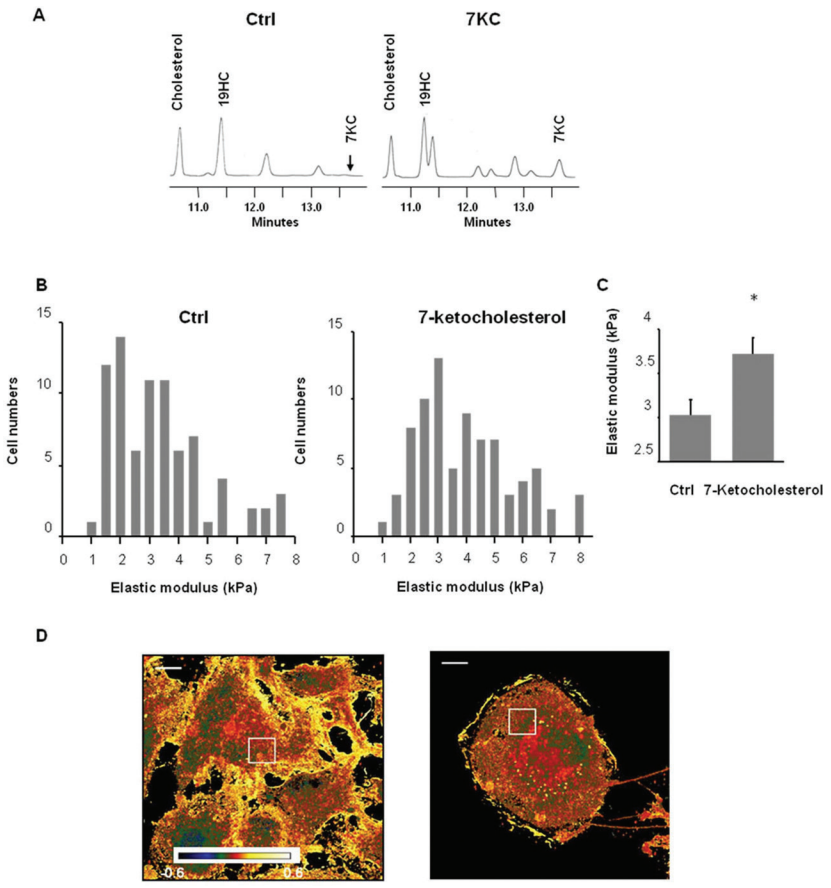

Figure 4. 7-ketocholesterol has inverse effects on lipid order and endothelial stiffness. A: Typical GC chromatographs of oxysterols in lipid extracts isolated from control cells; cells exposed to 10 g/ml 7-ketocholesterol. B: Histograms of elastic modulus measured in control (Ctrl) and 7-ketocholesterol-treated cells. C: Average elastic modulus for control (Ctrl) and 7-ketocholesterol-treated cells. (mean ± SEM, n=80 cells for each experimental condition). D: Typical GP images of control cells (Ctrl), 7-ketocholesterol-treated cells. Scale bar is 11.2 μm.

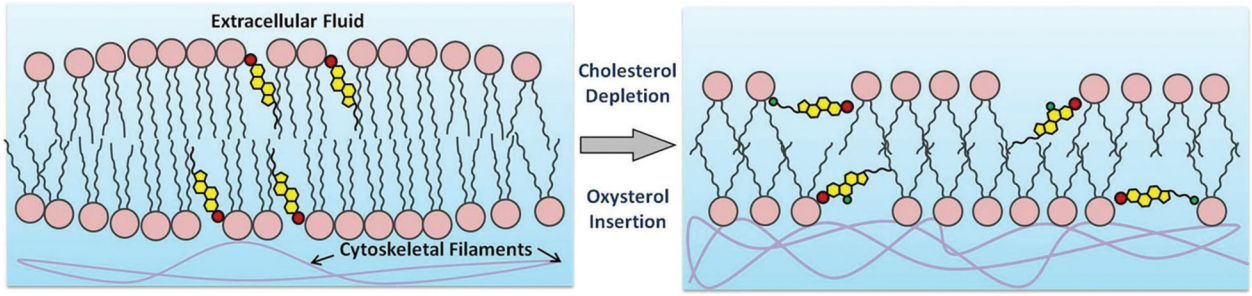

Figure 5. Schematic of membrane fluidization and stabilization of sub-membrane cytoskeleton following cholesterol depletion and oxysterol insertion. Coloring follows that of Figure 1, with the addition of oxysterols containing extra polar groups represented by green spheres. Sub-membrane cytoskeleton filaments are represented by violet strands.

found in atherosclerotic lesions, had a similar effect. The stiffening effects induced by both 7-ketocholesterol and 27-hydroxycholesterol were fully reversed by enriching the cells with cholesterol (78). In terms of the lipid order effect, similarly to oxLDL, exposure to 7-ketocholesterol also resulted in a significant shift of endothelial membranes to a more fluid/less ordered state. The latter is consistent with earlier studies showing that 7-ketocholesterol disrupts lipid order of model membranes (84). It is important to note that an inverse relationship between fluidization of the membrane and endothelial stiffening was also observed for another sterol, coprastanol (49), indicating this effect is not unique to the oxysterol components of oxLDL.

8. CONCLUDING REMARKS AND IMPLICATIONS FOR CELLULAR FUNCTION

In conclusion, multiple lines of evidence demonstrate that removal of membrane cholesterol or incorporation of specific oxysterols into the plasma membrane result in fluidization of the membrane lipid bilayer, and at the same time, lead to the strengthening of membrane-cytoskeleton interactions and increase in cell stiffness. Furthermore, membrane incorporation of oxysterols appears to be responsible for endothelial stiffening when exposed to oxLDL. The mechanisms, however, that couple fluidization of the membrane to increases in cell stiffness are still poorly understood. One mechanism that contributes to strengthening of membrane-cytoskeleton adhesion and stabilizing of the sub-membrane cytoskeleton is a loss or re-distribution of the regulatory phospholipid, PIP_2 (51, 62). A schematic summary is shown in Figure 5. Other possible mechanisms may include activation of Rho-GTPases or other signaling molecules that regulate the stability of the cytoskeleton. Further studies are needed to unravel these mechanisms.

In terms of functional significance, an increase in cell stiffness is likely to affect a plethora of cellular functions. More specifically, an increase in endothelial stiffness was shown to impair generation of nitric oxide in response to shear stress forces generated by blood flow (85), but also to facilitate flow-induced realignment of endothelial cells, a hallmark of their sensitivity to flow (77) and enhance transmigration of neutrophils through the endothelial layer (76). A series of studies also implicate endothelial stiffness as an important factor in angiogenesis (49, 73, 86, 87). Furthermore, an increase in smooth muscle stiffness has been proposed to underlie the stiffening of blood vessels in hypertension and aging (88, 89). Thus, changes in cellular cholesterol/oxysterol compositions may have profound effects on all of these properties. Functional significance should also be further assessed by exploring the impact of different modifications of oxLDL and particularly glycation of LDL that occurs in diabetes promoting further oxidation of LDL and formation of other oxidized lipids. The relationship between the lipid composition of the membrane and cellular stiffness can be an important factor in multiple pathological conditions.

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