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Forum Review

Lipid Rafts in Membrane–Cytoskeleton Interactions and Control of Cellular Biomechanics: Actions of oxLDL

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ABSTRACT

Membrane–cytoskeleton coupling is known to play major roles in a plethora of cellular responses, such as cell growth, differentiation, polarization, motility, and others. In this review, the authors discuss the growing amount of evidence indicating that membrane–cytoskeleton interactions are regulated by the lipid composition of the plasma membrane, suggesting that cholesterol-rich membrane domains (lipid rafts), including caveolae, are essential for membrane–cytoskeleton coupling. Several models for raft–cytoskeleton interactions are discussed. Also described is the evidence suggesting that raft–cytoskeleton interactions play key roles in several cytoskeleton-dependent processes, particularly in the regulation of cellular biomechanical properties. To address further the physiological significance of raft–cytoskeleton coupling, the authors focus on the impact of oxidized low density lipoproteins, one of the major cholesterol carriers and proatherogenic factors, on the integrity of lipid rafts/caveolae, and on the organization of the cytoskeleton. Finally, the authors review the recent studies showing that oxLDL and cholesterol depletion have similar impacts on the biomechanical properties of vascular endothelial cells, which in turn affect endothelial angiogenic potential. *Antioxid. Redox Signal.* 9, 1519–1534.

INTRODUCTION

In the last decade, numerous studies have demonstrated that multiple membrane proteins are not distributed randomly or homogenously in the cellular membranes but are concentrated in cholesterol-rich and sphingomyelin-rich membrane domains, termed "lipid rafts" or "membrane rafts." Even though the exact nature of these domains, including size, morphology, stability, and precise composition, are still controversial, lipid rafts are generally proposed to serve as scaffolding platforms for the association of signaling molecules and compartmentalization of cellular processes, as described in detail in several excellent reviews (1, 11, 29, 30, 110, 111) and summarized in the "consensus definition" of the recent Keystone Symposium on Lipid Rafts and Cell Function (March 23–28, 2006) (94). It is important to note, however, that there is still no universally accepted working definition of a raft domain. In most studies, "lipid rafts" are defined as membrane fractions that are resistant to cold detergents and/or have low density (high buoyancy) in sucrose gradients, even though recent studies have shown that detergent resistance may not be a valid criterion (4, 51, 71, 84). It also becomes increasingly clear that since cholesterol resides not only in rafts but also in nonraft membranes, sensitivity of a process to cholesterol depletion cannot be used as direct evidence for the involvement of the rafts (40, 41, 90). Despite these limitations, there is growing evidence suggesting that cholesterol-rich membrane domains play an important role in the regulation of membrane–cytoskeleton properties and cytoskeleton-dependent cellular processes, particularly in the control of cellular biomechanics. The primary goal of this review, therefore, is to review the studies that addressed the role of lipid rafts, as defined by the authors, in membrane–cytoskeleton interactions. In addition, we will also summarize the evidence suggesting that the integrity of lipid rafts may be disrupted by oxidized modifications of low density lipoproteins (oxLDL) and explore the impact of oxLDL on the properties of the cytoskeleton and cellular biomechanics.

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ROLE OF RAFTS IN MEMBRANE–CYTOSKELETON INTERACTIONS AND CYTOSKELETON-DEPENDENT PROCESSES

Two lines of evidence suggest that lipid rafts play an important role in the regulation of membrane–cytoskeleton interactions: (a) partitioning of the key cytoskeletal regulatory molecules, such as phosphatidylinositol 4,5 bisphosphate (PIP2), Rho-type GTPases, and integrins, into the raft complexes; and (b) direct association of several cytoskeletal proteins with the rafts. It is reasonable to expect, therefore, that rafts might be involved in regulating the cytoskeleton organization and thus regulate cytoskeleton-dependent cellular processes. Here we will review the studies demonstrating that the integrity of the rafts are important in regulating membrane–cytoskeleton interactions and discuss the evidence implicating membrane rafts in the regulation of several cytoskeleton-dependent processes. Finally, we will examine more closely the potential roles of rafts in regulating cellular biomechanics by affecting the cytoskeleton.

Association of cytoskeleton modulators with rafts: PIP2, Rho-GTPases, and integrins

PIP2. PIP2 is a minor lipid component of the plasma membrane that is known to regulate the organization of actin cytoskeleton and in particular the formation of actin–membrane linkages (142). PIP2 also exerts biological activity by providing the substrate for phospholipase C, yielding two second messengers, inositol triphosphate and diacylglycerol, that are involved in the regulation of numerous cellular functions. The involvement of PIP2 in a wide array of cellular processes, yet the relative specificity to which specific stimuli are coupled to different PIP2-dependent responses, suggests the existence of distinct PIP2 pools. Consistent with this view, Pike and Casey (93) showed that when A431 cell lysates were separated using sucrose gradient centrifugation, the level of PIP2 level in the low-density caveolin-rich fractions (lipid rafts) was significantly higher than in the high-density fractions (nonrafts). Treatment of A341 cells with either epidermal growth factor or bradykinin resulted in hydrolysis of PIP2 to inositol phosphates. Interestingly, while there was ~50% decrease in PIP2 in the caveolar fraction, there was no change in the PIP2 in the noncaveolar plasma membrane fraction, suggesting that in intact cells, agonist-induced turnover of PIP2 was localized to lipid rafts. This notion of spatial localization of signaling is consistent with the localization of other components to the relevant signal cascade to rafts including heterotrimeric G proteins and EGF receptor (95). Treatment of A431 cells with methyl-β-cyclodextrin (MβCD), a cyclic oligosaccharide with high affinity to cholesterol that is widely used to remove cholesterol from the cellular membranes (21) and disrupt lipid rafts abolishes the localization of PIP2, EGF receptor, and the heterotrimeric G protein Gq to the caveolar fraction—restoring cholesterol restores localization. The observation that cholesterol depletion blocks bradykinin- and EGF-induced PIP2 turnover suggests that this localization is required for proper signaling (95).

While the studies summarized above suggest that PIP2 localizes to rafts, two studies by van Rheenen *et al*. (126, 127) have challenged this notion. Using fluorescent microscopy to assess the distribution of PIP2 distribution in fixed cells, they conclude that PIP2 is homogenously distributed in the plasma membrane. They suggest that previously reported local enrichments of PIP2 observed using light microscopy are due to folding of the membrane (127). Based on correlations between plasma membrane sterols fluorescence in living cells and membrane topography, Wustner (137) proposed a similar explanation for the spatial heterogeneities of plasma membrane staining of sterols resolvable by light microscopy. This explanation, however, has in turn been challenged by a study of Golub and Caroni (43) showing that concentration of PIP2 in membrane patches in stimulated PC12 cells cannot be explained by membrane topography because the lipophilic dye DiD, which distributes homogenously throughout the lipid phase of cellular membranes, was not preferentially located at sights highlighted by raft markers, including PIP2. This study, therefore, supports the previous findings of Pike and Casey (93) demonstrating the nonhomogenous distribution of PIP2 in the plasma membrane.

Because individual rafts are estimated to be small, below the resolution of light microscopy, these observations by themselves do not address the localization of PIP2 to individual rafts. In a later study, van Rheenen *et al.* (126) demonstrate using two labeling techniques with the spatial resolution that would be required to resolve PIP2 clustering in rafts—fluorescence energy transfer (FRET) and ultrastructural analysis of immunotagged PIP2—that PIP2 did not cluster. Further, they demonstrate that very low concentrations of Triton X-100 promoted the clustering of PIP2 as assessed using FRET and fluorescent microscopy. This result led them to suggest that the association of PIP2 with rafts reported earlier may be due to the detergent-based extraction techniques used in the previous studies and that PIP2 may not have initially been associated with rafts. The observation that the addition of detergents can promote the clustering of PIP2 is an important finding and one that supports the notion that insolubility in a detergent is not a definitive proof that a compound associates with lipid rafts in living cells, a concept stressed by others (84, 107). However, it should also be noted, as emphasized by van Rheenen *et al*. (127), that the labeling-based techniques they used may fail to detect to PIP2 bound to proteins or other molecules. Thus, even though FRET has the spatial resolution to detect free PIP2 in rafts, it is possible that raft-associated PIP2 was not detected if it was bound to signaling molecules (*i.e.*, if it were active). Furthermore, the disagreement as to whether PIP2 clusters to rafts may result from the different model cell type employed by different investigators as the types of lipid domains present vary with cell type (*e.g.*, some but not all cell types contain caveolae). In summary, the fact that PIP2 is enriched in caveolin-rich plasma membrane fractions resulting from either detergent-based (93) or detergent-free (95) isolation techniques, as well as the association of PIP2 with micron-scale lipid domain in NGF-stimulated PC12 cells not exposed to detergent suggests that PIP2 is raft or caveolae associated, at least in some cell types, though this issue remains controversial.

While a number of studies have explored the regulation of the cytoskeleton by PIP2 (as reviewed in Ref. 142) the work of Kwik *et al.* (68) is of specific interest here as it connects de-

pletion of plasma cholesterol, PIP2 distribution, actin cytoskeleton organization, and membrane biomechanics. Cholesterol depletion resulted in a ~50% decrease in plasma membrane PIP2 in intact cells. Cholesterol depletion also altered the actin cytoskeleton, resulting in fewer and thinner stress fibers relative to control cells (Fig. 1A and B). Kwik *et al*. (68) explored mechanical interactions between membrane-bound proteins and the cytoskeleton by measuring the lateral movement of HLA molecules associated with the outer leaflet of the plasma membrane, using two complementary approaches, FRAP (fluorescence recovery after bleaching) and optical tweezers to move beads bound to the HLA molecules. Cholesterol depletion significantly decreased the mobile fraction of the HLA molecules as estimated by FRAP. Consistent with these observations, when HLA-bound beads were moved along the surface of the cell (*i.e.*, in the plane of the plasma membrane), beads were significantly more likely to be confined to their initial region of the membrane in cholesterol-depleted cells relative to beads in control cells. Disrupting actin network with cytochalasin D appeared to block both of the cholesterol-depletion effects. These results imply that the HLA molecules were confined by an elastic element of the cytoskeleton, preferentially in cholesterol-depleted cells.

On the surface, it appears paradoxical that actin depletion resulted in both destabilization of the actin cytoskeleton and greater actin-dependent confinement of HLA molecules in the plasma membrane. This apparent paradox could be resolved if cholesterol depletion had different effects on different portions of the actin cytoskeleton, specifically if cholesterol depletion destabilizes the actin stress fibers *(i.e.*, the actin structure observed by Kwik) but stabilizes the cortical actin, which lies just beneath the plasma membrane and likely mediates the observed changes in the confinement of HLA molecules within the plasma membrane. Consistent with this view, Fessler *et al*. (36) demonstrated that cholesterol depletion induces actin polymerization in the cortical rim in human polymorphonuclear leukocytes (Fig. 1C and D). An increased confinement of HLA molecules in cholesterol depleted cells is also consistent with our recent study showing that cholesterol depletion increases the stiffness of endothelial cells (14). We did not, however, observe any obvious changes in overall F-actin content or structure, though we did not specifically analyze cortical actin.

Kwik *et al*. (68) also demonstrated that the effects of depleting cholesterol on cytoskeleton structure and bead confinement can be mimicked by sequestering PIP2. While they did not investigate the mechanisms by which cholesterol depletion decreases plasma membrane PIP2, given that PIP2 is enriched in lipid rafts and cholesterol depletion disrupts rafts, a reasonable scenario is that the disruption of rafts by cholesterol results in loss of the PIP2 from the plasma membrane which in turn results in the observed cytoskeletal changes. Given the multiple pathways by which PIP2 affects the actin cytoskeleton and actin-membrane linkage (17, 142), PIP2 is an attractive candidate for the mechanism by which rafts can influence the structure of the underlying cytoskeleton. Further studies, however, are needed to test this hypothesis.

Rho family GTPases and integrins. Members of the Rho family of small GTP-binding proteins (Rho proteins) including Rho, Rac, and Cdc42, are well known to play the key roles in the organization of the submembrane cytoskeleton and its coupling to the plasma membrane (46, 99). Activation of Rho induces formation of stress fibers, Rac induces membrane ruffling, and Cdc42 stimulates the formation of filopodia (66, 89, 99). Furthermore, Rho-induced changes in the organization of the cytoskeleton regulate cell polarization and migration, trafficking, proliferation, gene expression, and ontogenetic transformation (111). In general, activation of Rho-GTPases involves their translocation from cytoplasm to the plasma membrane, where RhoA, Rac1 and CDC42 were shown to be concentrated in lipid rafts and caveolae (35, 67, 79). Activation and the translocation of activated Rho proteins from cytoplasm to the plasma membrane is controlled in part by integrin signaling (25, 26).

Del Pozo *et al.* (25, 26) presented two lines of evidence demonstrating that partitioning of Rac1 to lipid rafts is essential for Rac1 function. First, using 3T3 fibroblasts as a model system, they showed that in the absence of integrin signaling, which occurs in suspended cells or cells adhered to polylysine-coated surfaces, raft markers are lost from the plasma mem-

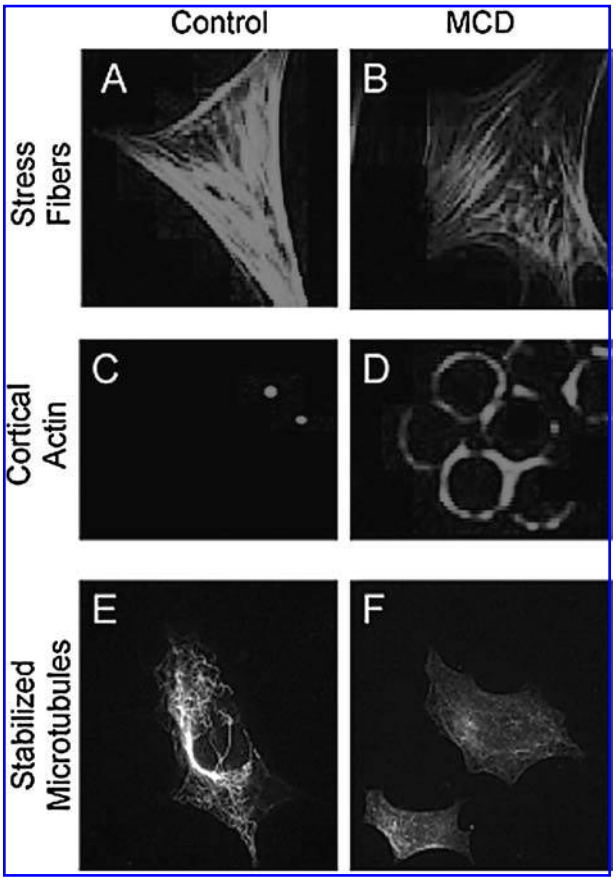

FIG. 1. Disrupting lipid rafts with methyl-β-cyclodextrin (MβCD) decreases the number and thickness of actin stress fibers (A and B), induces the polymerization of cortical actin (C and D), and blocks the stabilization of microtubules (E and F). Images are from references and used with permission (36, 68, 91).

brane and activated Rac1 does not translocate to the plasma membrane. They also showed that depletion of plasma membrane cholesterol causes the internalization of raft markers and blocks the translocation of activated Rac1 to the plasma membrane. Preventing the internalization of rafts by exposing cells to cholera toxin B (CTxB)-conjugated latex beads—CTxB binds the ganglioside G_{M1}, which is a structural component of lipid rafts—enabled activated Rac1 to translocate to the plasma membrane in suspended cells. Taken together, these data suggest that raft membrane domains, which are dependent on integrin signaling for their presence, are receptive regions for activated Rac1. In further support of this notion, activated Rac1 preferentially binds to the cholesterol- and cavelolin-1-enriched plasma membrane fraction relative to whole plasma membranes—no binding was detected to the cholesterol-depleted fraction. This preferential binding appears to be mediated at least in part by the biophysical properties of the lipids as activated Rac1 preferential bound to liposomes prepared from synthetic lipids that approximate a liquid-ordered state that may approximate that of lipid rafts relative to other lipid mixtures.

In parallel to the work of del Pozo, Palazzo *et al*. (91) explored a similar model of integrin-regulated, raft-mediated Rho signaling in fibroblasts, specifically in the context of microtubule (MT) stabilization. The α-tubulin in stabilized MTs becomes detyrosinated, resulting in a newly exposed glutamate residue at the C-terminus, which facilitates the identification of stabilized (Glu tubulin) and unstabilized tubulin (Try tubulin). Integrin-mediated adhesion stabilizes MTs but treatment with MβCD blocked MT stabilization but did not interfere with unstabilized tubulin or cell spreading (Fig. 1E and F). This inhibition was reversed by restoring cholesterol. The raft marker G_{M1} was enriched in the leading edge of migrating fibroblasts where stabilized MTs preferentially oriented. Taken together, these studies indicate the regulation of the cytoskeleton by integrins and Rho GTPases are at least in part modulated by membrane rafts.

Association of cytoskeletal and cytoskeleton-binding proteins with rafts

A number of cytoskeletal and cytoskeleton-binding proteins have been shown to associate with lipid rafts. Proteomic analysis of a caveolin-enriched Triton-insoluble membrane fraction containing raft membrane domains isolated from human endothelial cells revealed the presence of actin and vimentin (116). Using proteomic analysis, Nebl *et al.* (86) identified 19 major proteins in cholesterol-rich detergent-resistant membrane fractions isolated from bovine neutrophils. Of these 19 proteins, almost half were cytoskeletal or cytoskeleton-binding proteins, including vimentin, actin, fodrin (nonerythroid spectrin); myosin-IIA; myosin IG; the actin cross-linking proteins, α-actinin 1 and α-actinin 4; and the F-actin binding protein, supervillin. Based on this information and known activities of the proteins, they proposed a working model of the detergent resistant membrane skeleton (Fig. 2A). In other cell types that possess caveolae, caveolin-1-associated membrane domains may interact with the actin cytoskeleton via filamin, an F-actin cross-linking protein. Caveolin-1 binds filamin in biochemical studies. In intact cells, caveolin-1 and filamin colocalize at the level of confocal and electron microscopy suggesting a second mechanism by which membrane domains physically interact with the actin cytoskeleton (Fig. 2B) (117). Harder *et al.* (49) used an alternative approach of identifying proteins that may be involved in raft–cytoskeleton connections. Specifically, they identified a small group of proteins that were released from isolated membrane fractions following treatment with cholesterol binding agents. Annexin-II was the most abundant transmembrane or tightly associated peripheral protein identified, leading to the view that annexin-II acted as a linker between cholesterol-rich membranes and the other proteins they isolated—the cortical cytoskeleton proteins α-actinin, ezrin, and moesin, and membrane-associated actin (Fig. 2C). Harder *et al.* (49) speculated that annexin-II may serve to link cortical actin to plasma membrane rafts and early endosomes. Taken together, these studies suggest several different nonexclusive mechanisms by which rafts can associate with the cytoskeleton and cytoskeleton binding proteins, thereby mediating membrane-cytoskeleton interactions.

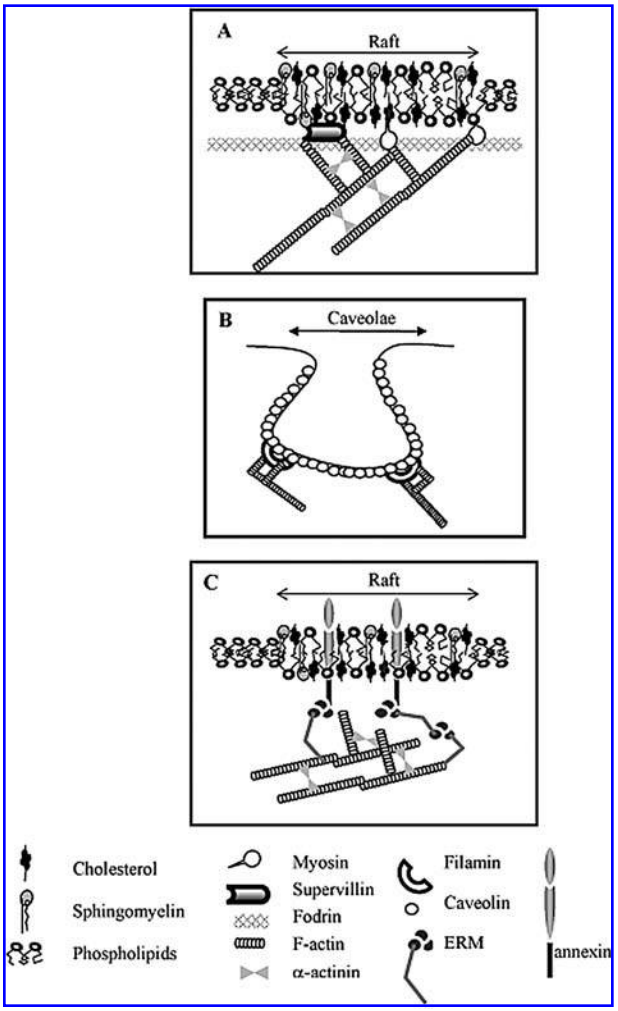

FIG. 2. Proposed mechanisms of raft–cytoskeleton interactions. (**A**) F-actin binds to the raft membrane by binding to supervillin and myosin; (**B**) F-actin binds to caveolin through filamin; (**C**) F-actin binds to the ERM proteins ezrin and moesin, which in turn bind to annexin II. Panels **A** and **B** are adapted from Refs. 86, 117.

Rafts regulate cytoskeleton-dependent processes

Studies discussed above indicate that cytoskeleton proteins and modulators of the cytoskeleton can both preferentially localize to lipid rafts and that cholesterol depletion alters the properties of the cytoskeleton and its interactions with the membrane. These studies suggest, therefore, that lipid rafts could potential play a role in regulating cytoskeleton-dependent cellular processes. The cytoskeleton plays a critical role in a large number of cellular processes of fundamental importance, including proliferation, trafficking, signaling, migration, and polarization. In this section, we will review the role of lipid rafts in two such processes: the formation of the immunological synapse in activated T-cells and cellular polarization/migration. These relatively well-studied examples illustrate that rafts can regulate the cytoskeleton and that interfering with these raft–cytoskeleton interactions blocks the relevant cellular function. Next we will summarize our recent findings indicating that rafts also play an important role in the regulation of cytoskeleton biomechanical properties, and how these properties are related to cell function.

One of the most dramatic examples of raft–cytoskeleton interactions occurs during the activation of T cells. Upon binding ligands on an antigen presenting cell (APC), T cell receptors segregate to lipid rafts while additional T cell receptors move to the T cell–APC interface. Subsequent changes, including those involving the actin cytoskeleton, result in the formation of a stable complex called the immunological synapse or the supramolecular activation complex, which is required to amplify and sustain the T cell response (60, 97). The early stage of cell activation includes the formation of an actin cap in the T cell below the region binding the APC. In the late stage of T cell activation, smaller rafts coalesce in an actin-dependent process (48, 78) and thereby bring together raft-associated signaling molecules. It is thought that these signaling molecules, including Src family kinases, in turn initiate signaling cascades that result in further reorganization of the underlying actin (77). In this one example, we see bidirectional interactions between the lipid rafts and the actin cytoskeleton: signaling molecules localized to the rafts are at least partially responsible for the reorganization of the actin cytoskeleton, while the reorganization of actin is required for coalescing of the rafts required for the formation of an immunological synapse. Consistent with this view, disruption of the rafts or interfering with actin structure or dynamics (48, 78, 136) attenuates T cell responses.

In addition to the membrane patches that form in activated T cells, raft–cytoskeleton interactions are known to occur in various other cell types as well. For example, during migration of polarized cells in response to chemoattractant gradients, raft domains appear to act as organizational domains for membrane receptors, signaling molecules, and regulators of the actin cytoskeleton (74). Reminiscent of the formation of the immunological synapse, prior to stimulation, raft domains are below the resolution of light microscopy, but when the T cells are stimulated to polarize and migrate, larger raft domains visible with light microscopy appear in specific regions of the cell, with G_{M3}-enriched rafts distributing to the leading edge while G_{M1}-enriched rafts distribute to the uropod (trailing edge) (44). Treatment with drugs that reduce membrane cholesterol block acquisition of a migratory phenotype and chemotaxis (44). Similar results were reported in the MCF-7 human adenocarcinoma cell line (75). Depletion of cholesterol or sphingolipids inhibited neurite outgrowth mediated by L1 and N cadherin, two cell adhesion molecules localized to rafts in neurons, but did not inhibit neurite outgrowth mediated by β1 integrin, which is excluded from rafts (85). Cholesterol depletion also blocked NGF-induced motility in PC12 cells (43). Acknowledging the possibility that cholesterol or sphingolipid depletion might exert effects by pathways independent of raft disruption, Nakai and Kamiguchi (85) applied micro scale chromophore-assisted laser inactivation (micro-CALI) to disrupt G_{M1}-enriched rafts. Micro-CALI of G_{M1} disrupted the integrity of rafts and when directed to the leading lamella of the growth cone, decreased neurite extension.

Golub and Caroni recently published an eloquent demonstration of the reciprocal interactions between the rafts and the cytoskeleton using PC12 rat pheochromocytoma cells as a model system (43). In recently plated but not quiescent well-adhered cells, the raft-associated components: cholesterol, GAP43, and PIP2 were localized to micron-scale patches at cell surface ruffling lamellae, which were intensely stained for F-actin. Acute stimulation of adhered cells with NGF resulted in the rapid (~20 s) appearance of dynamic PIP2-rich patches in areas of the edge—within 60 sec, intensely labeled F-actin structures appeared, and within 3 min, 90% of the patches went on to exhibit lamellipodial motility. Treatment with cyclodextrin dramatically inhibited patch formation and NGF-induced motility. The formation of these patches in NGF-stimulated cells was dependent on Cdc42, N-WASP, PIP2, and an intact actin cytoskeleton. In addition to interacting with the actin cytoskeleton, these patches capture and stabilize microtubules via the recruitment of IQGAP1 to the patches. Disruption of microtubules with nocodazole blocked the ability of raft to assemble in to large clusters and blocked sustained lamellipodial movement in favor of rapidly alternating protrusive mobility.

In addition to the aforementioned roles, the cytoskeleton is the primary determinant of many biomechanical properties of a cell (96, 102, 104, 135). A cell's biomechanical properties are important determinants of how a cell responds to applied force and the forces a cell may generate—both of which can affect overall cellular function. For example, the ability of circulating white blood cells (WBC) to travel along narrow capillaries is affected by the blood cell stiffness. Increasing WBC cell stiffness increases their retention in capillaries (31) and some conditions that increase WBC retention have been shown to increase their stiffness (28)—in both cases, disrupting F-actin blocks the effect. Cellular force generation is critical for various cellular processes, including migration and trafficking, with recent studies indicating that increased cellular stiffness is correlated with the magnitude of forces exerted by a cell on its substrate (133).

In purified lipid bilayers, decreasing cholesterol content decreases the stiffness of the bilayer (34, 87). In living cells, a major determinant of the plasma membrane stiffness is the underlying cytoskeleton (96, 102, 104, 135). Given the dual functions of cholesterol in affecting lipid bilayer stiffness and affecting cytoskeleton structure via lipid raft, it is not clear *a priori* how cholesterol depletion would change cell stiffness. To address this question, we have used micropipette aspiration to determine how the stiffness of plasma membrane in endo-

thelial cells is affected by altering the levels of cellular cholesterol (31). Enriching cells with cholesterol did not alter stiffness but depleting cholesterol ~50% by treatment with MβCD increased membrane stiffness by ~65%. The cholesterol depletion-induced stiffening was blocked by disrupting F-actin, suggesting that the actin cytoskeleton is responsible for the observed stiffening. Using an independent particle-tracking technique, we also showed that cholesterol depletion increased the stiffness of the "deep" cytoskeleton, the part of the cytoskeleton 2–4 μm into the cell and away from the cortical cytoskeleton. A functional significance of this increased stiffening is suggested by the observation that it was accompanied by an increased activation of volume-regulated anion channels (VRAC) (15). The increased channel activity is consistent with the view that membrane tension can regulate VRAC activity and the fact that increasing membrane stiffness results in a proportional increase in membrane tension for a given increase in membrane area. Furthermore, more recently the study of Sun *et al*. (121) has shown that cholesterol depletion significantly increases the adhesion energy between the membrane and the cytoskeleton, as assessed by pulling membrane tethers using AFM. More specifically, depletion of membrane cholesterol resulted in an increase in the rigidity of the membrane, estimated by the amount of force needed to pull a tether. This observation is consistent with cholesterol depletion-induced increase in cellular stiffness demonstrated in our studies (14, 16). Furthermore, similar to the depletion-induced increase in cell stiffness, an increase in the rigidity of the membrane was fully abrogated by depolymerization of F-actin. Later in this review, we present an additional example of the relationship between lipid rafts, cell biomechanics, and functional outcomes when we present recent evidence indicating the exposure of endothelial cells to oxLDL or cholesterol-depleting conditions disrupts lipid rafts, increases cellular stiffness, increases cellular force generation, and potentiates capillary morphogenesis.

In this section, we surveyed the evidence indicating that lipid rafts regulate the structure and function of the cytoskeleton and membrane–cytoskeleton linkages, and that disruption of lipid rafts by depleting or sequestering cholesterol by cholesterol-binding pharmacological agents alters membrane–cytoskeleton interactions and controls cellular biomechanics. As one begins to consider the broader implications of the *in vitro* data discussed above, it is important to address the question of whether disruption of lipid rafts may occur under normal and pathophysiological conditions *in vivo.* In the next section we review the evidence suggesting lipid rafts/caveolae may be disrupted by exposing the cells to oxidized modification of one of the major physiological cholesterol carriers, low density lipoproteins (oxLDL), and the impact of this effect on the organization of cellular cytoskeleton and cell biomechanics.

IMPACT OF oxLDL ON THE INTEGRITY OF LIPID RAFTS

Elevated levels of oxLDL are considered to be one of the major risk factors for the development of coronary artery disease (CAD) and plaque formation (reviewed in refs. 5 and 27). Indeed, oxLDL is present in atherosclerotic lesions in human and in rabbit arteries (143) and elevation of plasma levels of oxLDL is associated with an increased risk of CAD (54, 56, 123). It is also well known that elevation of oxLDL is associated with plasma hypercholesterolemia, both in humans (18, 128) and in the animal models of atherosclerosis (52, 55). It was proposed, therefore, that oxLDL is the primary lipoprotein particle responsible for the buildup of cholesterol in macrophages and for the transformation of macrophages into foam cells (118). However, the evidence to support this hypothesis remains controversial. Some studies have shown that oxLDL indeed results in a strong accumulation of cellular cholesterol in different types of macrophages, whereas other studies have shown only a moderate or no increase of cellular cholesterol (12, 100, 125, 138). No significant changes were found in the cholesterol level of vascular endothelial cells exposed to oxLDL (16, 61). It was suggested that the discrepancy between the studies can be attributed to the different degrees of LDL oxidation state and/or to the difference between the specific cell types (138). Specifically, it was proposed that, since in heavily oxidized LDL particles, a substantial amount of cholesterol turns into different oxysterols, it may be expected that oxidized LDL may not be efficient as a cholesterol loading agent. Consistent with this idea, Yancey and Jerome (138) showed that mildly oxidized LDL (5–10 TBARS) is more efficient in loading macrophages with cholesterol than heavily oxidized LDL used in other studies. In the last decade, however, a growing number of studies have suggested that oxLDL may actually result in *partial depletion* of cholesterol from the caveolae fractions of the membrane rather than in the accumulation of cellular cholesterol (14, 16). Furthermore, it has been suggested that exposure to oxLDL results in disruption and/or internalization of cholesterol-rich sphingomyelin-rich lipid raft domains and that oxLDL-induced disruption of lipid rafts is one of the major mechanisms responsible for oxLDL-induced endothelial dysfunction. In general, two lines of evidence suggest that oxLDL disrupts lipid rafts/caveolae in endothelial cells: (i) oxLDL depletes cholesterol from caveolin-rich fractions, induces internalization of lipid raft markers, and the effects of oxLDL on several cellular functions were shown to be simulated by cholesterol depletion, and (ii) oxLDL result in hydrolysis of sphingomyelin, a second major component of lipid rafts.

oxLDL-induced cholesterol depletion of caveolae

Blair *et al.* (7) were first to present evidence that oxLDL disrupts endothelial caveolae, a morphologically distinct subpopulation of lipid rafts known to contain multiple signaling complexes and to play major roles in the regulation of cell signaling (1, 112, 115). Blair *et al.* (7) have shown that a short (30 min) exposure of porcine aortic endothelial cells to a relatively low dose (10 μg/ml) of oxLDL results in a dramatic, virtually total decrease of cholesterol level in the caveolae fractions of these cells. At the same time, oxLDL but not native LDL, was shown to act as cholesterol acceptor. Blair *et al.* (7) have also shown that oxLDL induces reversible internalization of caveolin, a major structural element of caveolae and one of the major caveolae markers. Since both the structure and the function of caveolae critically depend on membrane cholesterol (19, 101), Blair *et al.* (7) concluded that oxLDL disrupts endothelial caveolae. Interestingly, Smart *et al.* (114) had shown earlier that caveolin

is also internalized and translocated from the plasma membrane to the Golgi in response to cholesterol oxidation. In this case though, the loss of caveolin from the plasma membrane did not affect the number and morphology of endothelial caveolae. It is not clear whether cholesterol oxidation may play a role in oxLDL-induced cholesterol depletion and internalization of caveolin. A later study by the same group has shown that the level of caveolae cholesterol in oxLDL-treated endothelial cells can be preserved by exposing the cells to high density lipoproteins (HDL), which in this case serves as cholesterol donor (124). Most importantly, Kincer *et al.* (62) have shown that diet-induced hypercholesterolemia in apoE-deficient mice (apoE$^{-/-}$), a mouse model for atherosclerosis, results in a dramatic decrease of the cholesterol level in caveolae isolated from whole blood vessels. While it is definitely counterintuitive that hypercholesterolemia results in the depletion of plasma membrane cholesterol, this observation is consistent with the earlier studies showing that oxLDL can induce cholesterol depletion in endothelial caveolae (7). It is also important to note that since endothelial cells constitute only a single cell layer on the inner surface of the blood vessels, while the majority of the vessel cells is contributed by smooth muscle cells, a decrease in the level of caveolae cholesterol in the whole vessel homogenate indicates that this effect is observed not only in endothelial cells but also in the smooth muscle cells. This study demonstrates that depletion of caveolae cholesterol may occur both *in vitro* and *in vivo*.

Consistent with these studies, we have also shown that exposing human aortic endothelial cells to oxLDL decreases the surface expression of G_{M1} (16), another major lipid raft marker (92). Furthermore, we have shown that depletion of endothelial cholesterol with methyl-β-cyclodextrin (MβCD) has a similar effect. However, in contrast to the observations by Blair *et al.*, we did not detect any significant changes in the levels of cholesterol, neither in caveolin-rich nor in caveolin-poor membrane fractions in response to oxLDL exposure (16). In terms of oxLDL concentration, oxidation state, and the time of exposure, the two studies were similar. The major difference, however, between the two studies appears to be the nature of raft membrane fractions isolated in the two cases, as evidenced by the relative amount of cholesterol recovered from these fractions. Blair *et al.* (7) isolated caveolin-rich membrane fractions from endothelial plasma membranes using a method that was shown earlier to represent only ~4% of total cellular cholesterol (113). This is strikingly lower than the amount of cholesterol recovered from the buoyant membrane fractions isolated in our study, which represents ~75% of total cellular cholesterol (16). In the majority of the earlier studies, the amount of cholesterol recovered from the buoyant (raft) membrane fractions varied between 30–50% (13, 40, 41, 69, 103). Thus, based on the relative amount of membrane cholesterol, caveolae fractions isolated in the study of Blair *et al*. (7) appears to represent a small subpopulation of caveolin-rich membrane fractions. Interestingly, whereas caveolae represent only 2–7% of the plasma membrane surface (101), the surface area occupied by cholesterol-rich membrane domains appears to constitute a much larger area of the plasma membrane surface ranging from ~10–15% (39,105) to ~40–80% (42, 122, 146). Thus, clearly caveolae constitute only a small portion of a larger population of lipid raft domains. Therefore, even a complete loss of cholesterol from the pool that constitutes only ~4% of total cellular cholesterol would not result in a significant change in the cholesterol level on the whole cell or total plasma membrane levels. Importantly, earlier studies have shown that membrane cholesterol may exist in distinct kinetic pools and it was suggested that these pools represent "raft" and "nonraft" cholesterol (50, 103, 139). It is remarkable, however, that only the caveolae fraction of the membrane appears to be highly sensitive to oxLDL-induced cholesterol depletion. These observations suggest that multiple cholesterol pools may exist within the raft domains of the membrane.

Cholesterol depletion can be induced not only by oxLDL but also by oxidized phospholipids, one of the active components of minimally-modified oxLDL (141). Specifically, oxidized products of palmitoyl-arachidonyl-phosphatidyl choline (oxPAPC), which were identified previously as active components of oxLDL, (134), also induced internalization of endothelial caveolin (130) and depletion of cholesterol from the buoyant caveolin-enriched membrane fractions (141). Furthermore, the major active phospholipid in oxPAPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC) also induced cholesterol depletion from the same fractions. Similarly to oxLDL, both oxPAPC and POVPC acted as cholesterol acceptors. In this case, however, there was only a moderate (30%) decrease in the cholesterol level of the buoyant caveolin-rich membrane fractions. In this study, it was not specified how much cholesterol was recovered from the buoyant fractions relative to the total amount of cholesterol in the plasma membrane. It is not clear, therefore, whether the buoyant fractions isolated in this study represent the same subpopulation of lipid rafts as the caveolae fractions characterized by Blair *et al.* (7). Thus, the difference between the relative ability of oxPAPC and oxLDL to deplete cholesterol from endothelial caveolin-rich membrane fractions might be attributed to the differences in the nature of the membrane domains isolated in the two studies. Alternatively, oxPAPC may be a weaker cholesterol acceptor as compared to oxLDL.

Further support for the notion that oxLDL depletes endothelial cholesterol comes from the observations showing that a number of oxLDL/oxPAPC-induced effects can be simulated by depleting cellular cholesterol with MβCD. Specifically, oxLDL-induced inhibition of endothelial nitric synthase (eNOS) was shown to be simulated by MβCD-induced cholesterol depletion (7) and abrogated by maintaining cellular cholesterol at a constant level (124). Similarly, oxPAPC-induced production of an inflammatory cytokine interleukin-8 was also simulated by MβCD-cholesterol depletion and prevented by cholesterol loading (141). Furthermore, Yeh *et al*. (141) showed that oxPAPC also results in a sustained activation of sterol regulatory element-binding protein (SREBP) and induction of SREBP-targeted genes (LDLR and HMG CoA synthase). Since it is well known that SREBPs are regulated by the level of cellular cholesterol and activated by cholesterol depletion (57), the ability of oxPAPC to activate SREBP is consistent with the observation that oxPAPC induces cholesterol depletion. Our recent observations provide further evidence for the link between oxLDL and cholesterol depletion by showing that oxLDL-induced changes in endothelial biomechanics are also simulated by MβCD-induced and serum starvation-induced cholesterol depletion (16). In summary, oxLDL/oxPAPC-induced impact

on several endothelial functions appears to be cholesterol dependent.

Taken together, these studies demonstrate that oxLDL and its active component oxPAPC may serve as cholesterol acceptors and remove cholesterol from caveolae. An interesting and important implication of these studies is that caveolae represent a separate cholesterol pool that does not equilibrate quickly with the rest of the membrane. The mechanisms, however, that could be responsible for the poor exchange between the cholesterol pools of caveolae and the rest of the membrane are not clear. It is also not clear what is the mechanism that underlies the specificity of cholesterol efflux only from a small subpopulation of endothelial lipid rafts. Further studies are needed to address these issues.

oxLDL-induced hydrolysis of sphingomyelin

Several studies have shown that oxLDL induces hydrolysis of sphingomyelin (SM) (2, 24, 32, 47, 63), a second major lipid component of lipid rafts (reviewed in Ref. 8). Hydrolysis of sphingomyelin and generation of ceramide results in the formation of large ceramide-rich microdomains (platforms) that are characterized by tight lipid packing and implicated in the regulation of cell signaling via reorganization and clustering of a variety of signaling molecules (reviewed in Ref. 8). oxLDL-induced SM hydrolysis was demonstrated in a variety of cell types, including vascular smooth muscle cells, macrophages, and endothelial cells. As expected, oxLDL-induced hydrolysis of sphingomyelin is accompanied with a concomitant increase in the level of cellular ceramide (2, 24, 32, 47, 63). Furthermore, Grandl *et al.* (45) have recently shown that oxLDL results in the formation of ceramide-rich lipid rafts in human macrophages. It was also shown that oxLDL-induced hydrolysis of sphingomyelin can be mediated by both neutral and acid sphingomyelinases (SMase) (47, 63).

SM hydrolysis was observed in response to both heavily-oxidized and mildly-oxidized LDL (2, 24, 32, 47, 63). When compared in the same settings, an increase in ceramide production was similar for heavily-oxidized LDL obtained by copper oxidation and for mildly-oxidized LDL obtained by incubating the particles with confluent endothelial cells (63). It is also important to note that activation of SMases were also observed in response to oxidized phospholipids (oxPC), including oxPAPC and POVPC that are typically present in minimally- and moderately-oxidized LDL (72, 131). In terms of the mechanism of oxLDL-induced activation of sphingomyelin/ceramide pathway, Auge *et al*. (3) demonstrated that in smooth muscle cells, oxLDL induces the release of activated matrix metalloproteinases-2 (MMP-2) and that blocking the activity of MMP-2 inhibited oxLDL-induced activation of neutral SMase activity and ceramide production, as well as activation of the downstream signaling events through the Erk phosphorylation pathway. Auge *et al.* (3) proposed that extracellular MMP-2 may be the link between oxLDL and SMase activation. In a commentary to this article, Izgi *et al.* (59) suggested further that the link between oxLDL and SMase activation may be not the extracellular but membrane-type-1 MMP-2 that partitions predominantly to plasma membrane caveolae (38). oxLDL was also shown to provide the substrate for the endogenous SMases by enriching the cells with sphingomyelin (63). Finally, Kitatani *et al*. (64) showed that oxLDL may also stimulate the *de novo* synthesis of ceramide without altering the level of membrane sphingomyelin. In all cases, the increase in the membrane ceramide levels is expected to have a strong impact on the integrity of membrane domains. It is important to note, however, that in contrast to the studies described above, Hundal *et al*. (58) reported that oxLDL inhibited SMase activity and blocked ceramide production in bone marrow-derived macrophages. Hundal *et al*. (58) suggested that the difference between their observations and earlier studies can be attributed either to a relatively low oxLDL concentration used in their study or to the differences between the cell types.

Hydrolysis of sphingomyelin and an increase in the cellular ceramide levels were implicated in several of oxLDL/oxPC-dependent cellular processes, such as oxLDL-induced proliferation of vascular smooth muscle cells (2) and apoptosis of vascular endothelial cells (47) and macrophages (24), and oxPC-induced synthesis of an inflammatory cytokine interleukine-8 (131). It is also interesting to note that oxLDL-induced inhibition of SMase activity in macrophages was shown to be associated with the inhibition of apoptosis in bone marrow-derived macrophages (58). Specific pathways, however, involved in the diverse ceramide-induced cellular effects have been summarized in a number of excellent reviews and are beyond the scope of this review (119, 140).

In summary, oxLDL may induce the disruption and reorganization of lipid rafts by two different mechanisms: (a) depletion of caveolae cholesterol, and (b) hydrolysis of sphingomyelin resulting in the formation of ceramide-enriched membrane platforms, as illustrated in Fig. 3. Furthermore, the two effects may be mutually dependent. Specifically, hydroly-

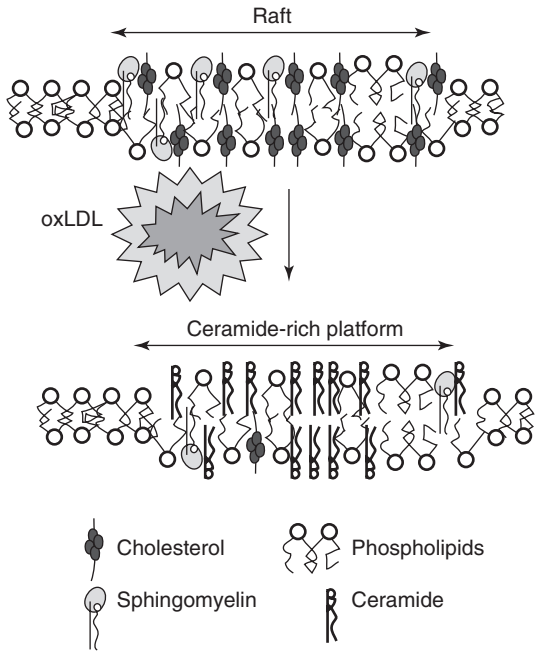

FIG. 3. Mechanisms implicated in oxLDL-induced disruption of lipid rafts:cholesterol depletion and hydrolysis of sphingomyelin, resulting in the formation of ceramide-rich membrane platforms.

sis of sphingomyelin was shown to induce translocation of cholesterol from the plasma membrane to the intracellular pools (120), suggesting that depletion of plasma membrane cholesterol can be in part due to its translocation to the intracellular membranes. In summary, disruption of lipid rafts was suggested to constitute one of the major mechanisms underlying the impact of oxLDL on cellular functions. Next, we will review the studies investigating the impact of oxLDL on the organization of cellular cytoskeleton and cellular biomechanics.

oxLDL AND ORGANIZATION OF CYTOSKELETON: IMPLICATIONS FOR CELLULAR BIOMECHANICS

As discussed above, the cytoskeleton constitutes the mechanical scaffold of all mammalian cells and is the major determinant of the cellular biomechanics. Therefore, changes in the organization of the cytoskeleton and particularly an increase in the polymerization state of the major cytoskeletal networks are expected to have a significant impact on cell deformability. In this section, we will summarize what is known about the impact of oxLDL on the organization of the cytoskeleton and cell biomechanics and discuss the evidence for the role of lipid rafts in these effects.

oxLDL-induced reorganization of the cytoskeleton

Several studies have shown that exposure to oxLDL induced rapid polymerization of actin, resulting in formation of filamentous actin (F-actin) and actin stress fibers (33, 80–82). More specifically, oxLDL was shown to induce actin polymerization/formation of stress fibers in vascular endothelial cells (33), monocytes (82), and of several types of macrophages, including peritoneal macrophages and a macrophage-like cell line J774 (80, 81). Consistent with these studies, we have also observed an increase in F-actin stress fibers in aortic endothelial cells exposed to oxLDL (Byfield *et al*., unpublished observations). This effect, however, was not universal, as demonstrated by a lack of oxLDL-induced formation of stress fibers in RAW cells, another macrophage cell line (81). In all of the studies where the effect was observed, oxLDL-induced actin polymerization developed within 1–10 min of the exposure (33, 80–82). It appears that these effects were relatively independent of the degree of LDL oxidation. In one study, minimally oxidized LDL (mmLDL) and strongly oxidized LDL (oxLDL) induce virtually identical increase in F-actin (80), and in another study the effect of mmLDL was ~10–25% stronger than that of oxLDL (81). No effect was observed in both studies in response to the native (nonoxidized) LDL (80, 81). It is important to note that, although the effects of mmLDL and oxLDL on actin polymerization were similar, mmLDL binds to native LDL receptor and not to the scavenger receptor that is responsible for the binding of oxLDL (81). Actin polymerization was also observed in response to POVPC, one of the biologically active phospholipid oxidation products of minimally modified LDL (80).

More complicated patterns of F-actin reorganization were described after longer exposures to oxLDL. Zhao *et al*. (145) showed that exposing endothelial cells (HUVECs) to oxLDL for 24 h resulted in massive redistribution of F-actin with the partial disappearance of individual stress fibers and clustering of F-actin in the peripheral regions of the cells. The total amount of F-actin was not quantified in this study and no obvious differences were apparent in the presented images. In macrophages, the pattern of F-actin re-arrangement appears to be slightly different.

Zerbinatti and Gore (144) showed that exposure of J774A.1 cells to oxLDL decreased the amount of total actin and simultaneously shifted the equilibrium from monomeric G-actin to F-actin. Thus, even though the amount of F-actin did not change, oxLDL induced a marked increase in the relative amount of F-actin. In contrast to HUVECs, the shift in G-actin/F-actin equilibrium was accompanied with the formation of bundles of F-actin stress fibers distributed throughout the cytoplasm (144). A 24 h exposure to oxLDL also resulted in a marked decrease in the expression of α-actin, smooth muscle myosin heavy chain-1, and calponin, suggesting that the contractile response of smooth muscle cells is impaired (22). The equilibrium between G and F-actin and the spatial distribution of the filaments was not analyzed in this study (22). A more prolonged incubation of smooth muscle cells to oxLDL (3–6 days) resulted in a decrease in actin content, a striking disorganization of actin-myosin filaments, and collapse of the network (76). Thus, it appears that acute and prolonged exposures to oxLDL may have different effects on the organization of the cytoskeleton in different cell types. Clearly, though, it is hard to compare the observations obtained on different time scales in different cell types, and it is necessary to examine these effects systematically in cell-specific manner in further studies.

In terms of the mechanism, two signaling pathways were shown to be involved in oxLDL-induced actin polymerization. In endothelial cells, Essler *et al*. (33) showed that oxLDL-induced formation of actin stress fibers depends on the activation of Rho A/Rho kinase pathway. Consistent with these observations, Siebold *et al.* (106) demonstrated that oxLDL induced rapid (1–30 min) activation of Rho A in HUVECs, as estimated by the translocation of Rho A from the cytosol to the membrane. Similarly, oxLDL-induced activation/membrane translocation of Rho A was observed in platelets (98) and in smooth muscle cells (37). Indeed, it is well known that activation of Rho A, associated with its translocation to the membrane, results in actin polymerization and formation of the stress fibers (46, 99). Rho A-induced formation of actin stress fibers is also implicated in generation of intercellular gaps in the endothelial monolayer and increase in endothelial permeability in response to thrombin (53). It is likely, therefore, that oxLDL-induced activation of Rho A is responsible for formation of actin stress fibers in vascular endothelial cells, resulting in the disruption of endothelial permeability barrier. Indeed, intercellular gaps were observed after both, the short (minutes) and the prolong (24 h) exposures of vascular endothelial cells to oxLDL and reorganization of F-actin was proposed to play a central role in these changes (33, 145).

Another mechanism that was implied in oxLDL-induced actin polymerization is activation of phosphoinositide 3-kinase (PI3K)/Akt pathway (80, 81). The evidence, however, is more controversial. First, several studies showed that mmLDL induced activation of PI3K with the subsequent phosphorylation of Akt in peritoneal macrophages (6, 80, 81). It was also shown

that PI3K-specific inhibitors (wortmannin and LY294002) efficiently inhibited mmLDL-induced actin polymerization in peritoneal macrophages (80). This mechanism was proposed to play a critical role in mmLDL-induced macrophages spreading and regulation of phagocytosis (80). However, even though, as described above, mmLDL and oxLDL had similar effects on actin polymerization in macrophages, significant PI3K activation was observed only in response to mmLDL but not oxLDL (81). Furthermore, POVPC that also induced actin polymerization had no effect on PI3K activity (81). Interestingly, mmLDL-induced PI3K activation protected macrophages from oxLDL-induced apoptosis, showing that the signaling pathways activated in these cells by mmLDL and by oxLDL are clearly distinct (9). In addition, it was shown that incubation of endothelial cells with oxLDL either had no effect on PI3K/Akt activity (88) or resulted in Akt dephosphorylation (10). Taken together, these observations suggest that PI3K/Akt pathway may play a role in mmLDL-induced actin polymerization in macrophages, but other mechanisms are involved in other cell types and in response to different modification of oxLDL.

Little information is available about the impact of oxLDL on the other major cytoskeletal networks. Malorni *et al*. (73) showed that oxLDL induces partial depolymerization and disorganization of microtubule network in natural killer cells (NK), and it was suggested that this effect may be partially responsible for the inhibitory effect of oxLDL on the killing ability of NK cells. Muller *et al.* (83) showed that oxLDL-induced apoptosis of macrophages was accompanied with a collapse of the vimentin network, disappearance of vimentin filaments, and accumulation of vimentin in large granules near the cell center, whereas nonapoptotic macrophages retained normal vimentin network. Further studies are needed to investigate molecular mechanisms underlying oxLDL-induced changes in microtubules and intermediate filaments networks.

oxLDL-induced changes in cell deformability and force generation

In general, since cell deformability depends primarily on the submembrane cytoskeleton (96, 104), oxLDL-induced increase in F-actin stress fibers is expected to make cells stiffer, less deformable. As expected, activation of Rho A, which is well known to induce formation of F-actin stress fibers, results in an increase in cell stiffness (65). Consistent with this expectation, our recent study (16) has shown that exposing human aortic endothelial cells to oxLDL results in a strong increase of the cellular stiffness, as estimated by micropipette aspiration (Fig. 4A and B). Importantly, this effect could be simulated in the same cells by the depletion of cellular cholesterol induced either by MβCD or by serum starvation (16). Exposure to oxLDL also resulted in a decrease of the plasma membrane expression of G_{M1} (16), one of the major lipid raft markers, an observation consistent with the earlier studies showing that oxLDL induces internalization of caveolin-1 (7). Furthermore, we have shown that an increase in endothelial stiffness is also observed in aortic endothelial cells isolated from hypercholesterolemic pigs, as compared with the cells isolated from control animals. Changes in membrane deformation under different dyslipidemic conditions are summarized in Fig. 4C. While the level of caveolae cholesterol was not measured in this study, previous studies have demonstrated that hypercholesterolemia may induce cholesterol depletion and disruption of endothelial caveolae (23, 62). We proposed, therefore, that oxLDL-induced increase in endothelial stiffness may be attributed to the disruption/internalization of lipid rafts.

One of the important implications of an increase in cell stiffness is an increase in the ability of the cells to generate force on cell–substrate interface (133). Consistent with this expecta-

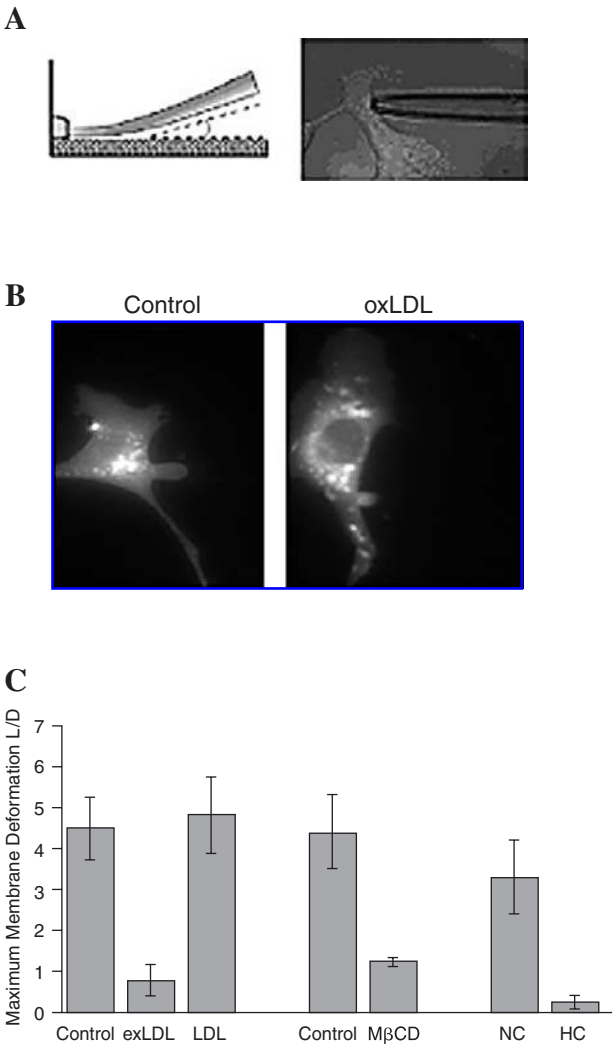

FIG. 4. OxLDL/cholesterol depletion increases the stiffness of endothelial cells. (**A**) Schematic side view (**A**) and bright contrast image (**B**) of a micropipette approaching a cell. *Inset*, micropipette. (**B**) Typical images of membrane deformation of a control cell and an oxLDL-treated cells at −5 mm Hg (pipettes are invisible because they don't fluoresce, bar is 30 m*M*). (**C**) Maximum membrane deformation (L/D) for control, oxLDL, and cholesterol-depleted endothelial cells in culture and for endothelial cells freshly isolated from the aortas of normal (NC)and hypercholesterolemic (HC) pigs. (L/D: L is the aspirated length of membrane projection and D is the inner diameter of the pipette). The pressure is maintained for 180 s. A decrease in the L/D parameter indicates a decrease in membrane deformability and an increase in membrane stiffness (Adapted from Ref. 15).

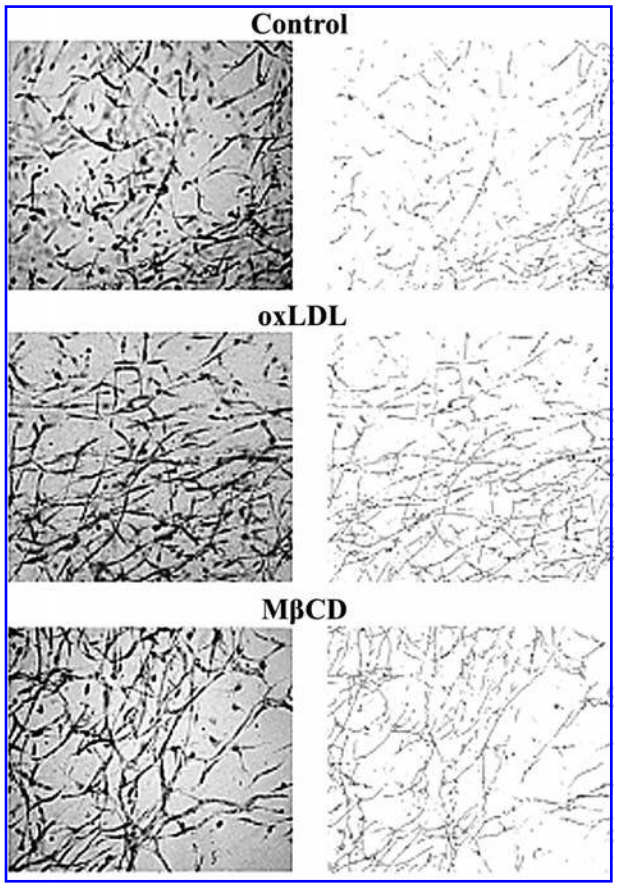

FIG. 5. oxLDL/cholesterol depletion facilitates the formation of EC networks. *Left:* Images of HAECs network for control, depleted, and oxLDL treated cell populations grown within collagen gels for 48 h. *Right:* Skeletonized version of the images shown on the left (Adapted from Ref. 15).

tion, we have shown that oxLDL not only increases endothelial stiffness but also increases endothelial force generation as assessed by their ability to compact collagen gels. This effect was also fully simulated by MβCD-induced cholesterol depletion (16). A different effect of oxLDL, however, was observed by Zerbinati and Gore (144) who demonstrated that exposure to oxLDL resulted in a significant decrease in the ability of macrophages to generate force, as estimated by confining the cells in a magnetic trap and measuring the isometric force generated by the cells during their attempts to move out of the trap. Interestingly, this effect was accompanied with oxLDL-induced cholesterol loading that was not observed in our study, suggesting that the difference between the two results may be attributed to the difference in the cholesterol levels. Alternatively, since cellular force was measured by different approaches, it is also possible that two different parameters were measured in the two studies.

There are multiple implications of oxLDL-induced changes in cellular biomechanics. Earlier studies have shown that the ability of cells to generate force play an important role in the regulation of cell motility (70, 132). Our studies demonstrated that an increase in endothelial cell force generation facilitates the ability of endothelial cells to form endothelial networks (108, 109), an essential step in the formation of new capillaries. Consistent with this notion, we have shown that oxLDL facilitates endothelial network formation, an effect that was also simulated by MβCD-induced cholesterol depletion (Fig. 5) (16). These data led to the speculation that oxLDL-induced facilitation of the angiogenesis process may contribute to neovascularization of atheromatous lesions, one of the major complications of advanced atherosclerotic lesions (20, 129). Other stiffness/force-related cellular responses may include changes in the ability of the cells to sense and respond to mechanical stimuli, changes in cell morphology and spreading, which in turn may lead to changes in cell proliferation, and differentiation. Though the study of the impact of oxLDL on cellular biomechanics is just beginning, several dramatic affects on cellular function already have been uncovered. While it appears that oxLDL-induced disruption of rafts mediated these processes, future studies are needed to better understand mechanistic details and to evaluate potential relevance to the pathophysiology of oxLDL *in vivo*.

CONCLUSIONS

In this review, we have explored lipid rafts/caveolae and membrane–cytoskeleton interactions on three interrelated levels: molecular, mechanical, and cellular (Fig. 6). Disruption of lipid rafts alters cytoskeletal structure, including the extent of polymerization, stabilization, crosslinking, and membrane association. These molecular level processes change a set of intercoupled biomechanical properties in a cell. For example, increased force generation by the cytoskeleton results in a

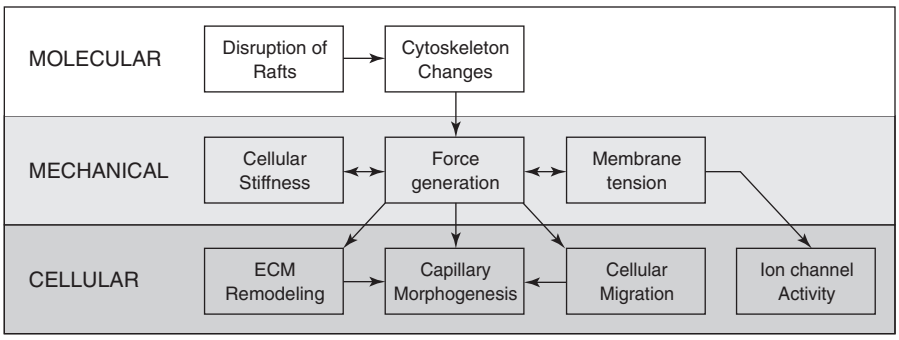

FIG. 6. Summary of molecular, mechanical, and cellular interactions discussed in the review.

stiffening of the cytoskeleton and hence stiffening of the cell and plasma membrane. Increased force generation as well as increased stiffness can increase membrane tension. Changes in these biomechanical properties in turn impact cellular level functions. For example, altered force generation by a cell influences its ability to migrate and remodel the extracellular matrix, which both affect capillary morphogenesis. In addition, changes in membrane tension influence the activity of various mechanosensitive ion channels, including VRAC. While many of these relationships have been elucidated *in vitro* using cholesterol sequestering agents to disrupt rafts, oxLDL has also been demonstrated to disrupt lipid rafts and to result in similar biomechanical and cellular changes as cholesterol depletion. The parallel between cholesterol-depletion-induced and oxLDL-induced effects suggests that raft disruption may play a role in processes *in vivo*, including those related to the pathophysiology of oxLDL.

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ABBREVIATIONS

APC, antigen presenting cell; AFM, atomic force microscope; apoE, apolipoprotein E; CTxB, cholera toxin B; ECM, extracellular matrix; EGF, epidermal growth factor; F-actin, filamentous actin; FRAP, fluorescence recovery after bleaching; FRET, fluorescence energy transfer; HDL, high density lipoprotein; HLA, human leukocyte antigen; HMG CoA synthase, 3-hydroxy-3-methyl-glutaryl-CoA synthase; HUVECs, human umbilical vein endothelial cells; IQGAP1, IQ motif containing GTPase activating protein 1; LDL, low density lipoproteins; LDLR, LDL receptor; MMP, matrix metalloproteinases; MβCD, methyl-β-cyclodextrin; MT, microtubule; mmLDL, minimally-oxidized LDL; NGF, Nerve growth factor; N-WASP, neural Wiskott–Aldrich syndrome protein; oxLDL, oxidized LDL; oxPAPC, oxidized palmitoyl-arachidonyl-phosphatidyl choline; PI3K, phosphoinositides 3-kinase; PIP2, phophatidylinositol 4,5 bisphosphate; 1-POVPC, palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine; SM, sphingomyelin; SMase, sphingomyelinases; SREBP, sterol regulatory element-binding protein; TBARS, thiobarbituric acid reactive substances; VRAC, volume-regulated anion channels.

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