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Dynamic Response of a Cholesterol-containing Model Membrane to Oxidative Stress

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It is important to study the effects of lipid oxidation on biological membranes and how this affects physiological functions and causes disease. We investigated the vesicular dynamics of a biomimetic membrane system consisting of an unsaturated phospholipid, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and cholesterol (Chol) under oxidative stress. We found that lipid vesicles with a DOPC:Chol ratio of 50:50 are more susceptible to oxidative stress than homogeneous DOPC lipid vesicles.

Studies of cellular membrane oxidation are important because such oxidation is believed to contribute to human aging and the deterioration of disease through the disruption of structural organization, lipid packing, and, ultimately, membrane function.[1] Lipid peroxidation is a biomarker of cellular oxidative stress that has long been recognized to contribute to both oxidative damage resulting from inflammatory processes and chronic diseases such as atherosclerosis and cancer.[2] Within bilayer membranes, cholesterol double bonds and unsaturated lipids are vulnerable to oxidation.[3] Cholesterol, one of the main constituents of membrane lipid clusters (lipid rafts) that concentrate most signaling receptors,[4] is oxidized spontaneously (autoxidation) or enzymatically.[5] Oxidized cholesterol influences membrane stability and cell death by altering membrane properties such as fluidity and permeability.[6–8] During low density lipoprotein (LDL) oxidation, cholesterol is reportedly oxidized only after most of the unsaturated lipids have been oxidized.[9] In contrast, cholesterol has been shown to be more susceptible to oxidation than unsaturated lipids in cultured cells.[10] It has also been reported that cholesterol could be protected from oxidation by sphingomyelin[11] and that the oxidation of unsaturated lipids is promoted by cholesterol.[12] However, very little is known about the stability and/or dynamics of membrane structures under oxidative stress.

Recently, cell-sized lipid vesicles (>10 μm) have been actively studied as cell models because they are similar to natural cell structures with regard to size and membrane composition.[13] Since they are large enough to allow the direct microscopic observation of the membrane behavior of individual vesicles, there have been several studies on their morphological dynamics in response to external stimuli, such as pH,[14] surfactants,[15] photoisomerization,[16] and proteins.[17] Along these lines, it has recently been found that photoinduced oxidation caused lipid vesicles consisting of unsaturated lipids to exhibit morphological changes.[18] However, to our knowledge, there have been no previous experimental studies on the effects of cholesterol within a lipid bilayer on oxidative membrane dynamics. In this letter, we report on the microscopic observation of the dynamic response of phospholipid/cholesterol membranes to oxidative stress, such as the exposure to hydrogen peroxide and energy transfer from a photosensitive molecule.

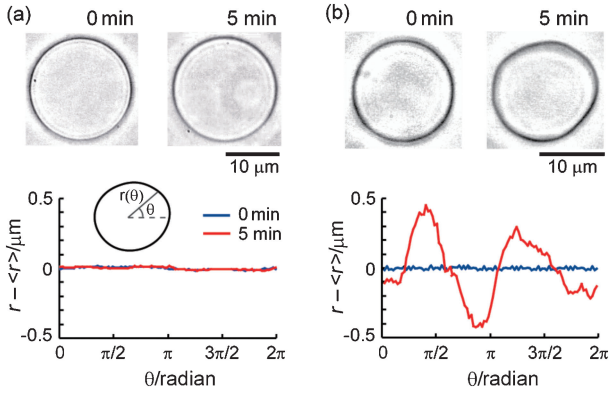

Figure 1. Membrane fluctuation of DOPC (a) and DOPC:Chol = 50:50 (b) lipid vesicles induced by hydrogen peroxide. (upper) Typical microscopic images at 0 and 5 min after exposure to 1 M hydrogen peroxide. (bottom) Spatial fluctuation in the radius (r) around the contour.[21]

First, we investigated the stability of membranes, in terms of membrane fluctuation, after exposure to hydrogen peroxide. Two types of lipid vesicles were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and cholesterol (Chol): DOPC vesicles and DOPC:Chol = 50:50 vesicles. We used a natural swelling method from dry lipid films.[19] Equal volumes of the lipid vesicle solution and hydrogen peroxide solution (1 M) were poured into a test tube and gently mixed by soft tapping.[20] We then observed any changes in membrane morphology with a phase-contrast microscope (Olympus BX50, Japan) at RT. Figure 1 shows typical microscope images of the DOPC and DOPC:Chol = 50:50 membrane systems, together with the degree of membrane fluctuation.[16,17] There were no changes in the structure of DOPC vesicles after 5 min of exposure to hydrogen peroxide (Figure 1a). In contrast, DOPC:Chol = 50:50 vesicles exhibited membrane fluctuation almost immediately after exposure to the pro-oxidant (Figure 1b). Such large vesicular fluctuation requires excess membrane area.[16] The absence of fluctuation in the periphery of the DOPC vesicles suggests that the membrane area remains steady to fit the spherical surface of the inner liquid phase. Figure 2 shows the responsiveness of membrane fluctuation to oxidative stress ($n = 15$). Although virtually all of the DOPC:Chol = 50:50 lipid vesicles fluctuated (93%), fluctuation

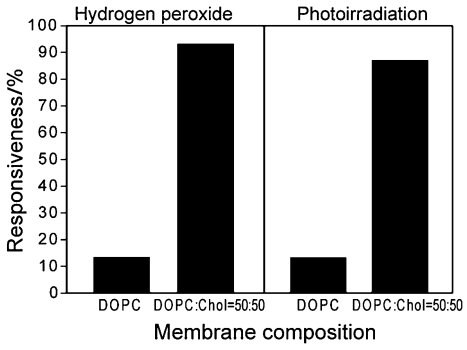

Figure 2. Responsiveness of membrane fluctuation to oxidative stress. Responsiveness was defined as the number of vesicles that showed membrane fluctuation 5 min after the application of oxidative stress, divided by the total number of observed vesicles ($n = 15$).

was rarely observed with DOPC liposomes (13%). Thus, cholesterol-containing lipid vesicles were less structurally stable upon exposure to hydrogen peroxide than DOPC vesicles.

Next, we investigated the response of lipid vesicles to photoirradiation as an another type of oxidative stress, using DOPC or DOPC:Chol = 50:50 vesicles with 1 mol % *N*-(rhodamine red-X)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rho-PE; $\lambda_{ex} = 560$ nm, $\lambda_{em} = 580$ nm). It has been reported that lipid oxidation in vesicles is caused by the generation of reactive oxygen species by irradiation of a fluorescence reagent in vesicles.[18,22] Lipid vesicles were irradiated with light by a dichroic mirror unit (WIG, 530–550 nm) with an extra-high-pressure mercury lamp. We observed the membrane fluctuation of lipid vesicles upon photoirradiation, similar to exposure to hydrogen peroxide as shown in Figure 1. We also measured the responsiveness to photoinduced oxidation. Again, while only a small percentage of DOPC vesicles fluctuated (13% of the total $n = 15$), most of the DOPC:Chol = 50:50 lipid vesicles showed fluctuation (87%). Thus, photoinduced oxidation showed the same trend in membrane fluctuation as exposure to hydrogen peroxide; i.e., cholesterol-containing lipid vesicles are more susceptible to oxidative stress than unsaturated lipid vesicles.

Our results suggest that under oxidative stress non-enzymatic cholesterol oxidation products, possibly 7α,β-hydroxycholesterol and 7-ketocholesterol,[5] are more readily generated than unsaturated lipids within bilayer membranes. We confirmed that the major oxidative products of the DOPC:Chol = 50:50 lipid vesicles were 7-ketocholesterol and 7β-hydroxycholesterol using HPLC[10] (data not shown). Further experiments on the quantitative analysis of the cholesterol oxidation products are underway. In agreement with our findings, Ma et al. reported that cultured cells exhibited membrane dynamics such as exocytosis through the uptake of oxidized cholesterol.[23] These dynamics may result from changes in the physicochemical properties of membranes such as molecular packing, which was reported for a Langmuir monolayer with oxidized cholesterol.[24] It is known that membrane fluctuation is enhanced by an increase in the effective area (molecular packing) of membrane-constituting molecules.[16] Previously, oxidative vesicular dynamics was examined with model membranes that only contained unsaturated lipids.[18] This is the first report of the direct observation of the dynamic response of lipid vesicles containing cholesterol to oxidative stress, and our results clarify that cholesterol plays an important role in membrane dynamics. These results may lead to a better understanding of biological membrane dynamics under oxidation.

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19 Lipid mixtures (DOPC and Chol) were dissolved in chloroform/methanol (2:1, v/v) in a glass test tube and were dried under vacuum for 3 h to form thin lipid films. The films were then hydrated overnight with deionized water. The final concentration was 0.2 mM lipid.
20 We confirmed that a lower concentration of hydrogen peroxide (100 mM) induced essentially the same membrane fluctuation. However, the onset of membrane response is longer (ca. 1 h). We, therefore, used a higher hydrogen peroxide concentration of 1 M for faster response.
21 The radii were calculated as follows: 1) The edge of the lipid vesicle was extracted from the image data. 2) The centroid position from the edge was calculated. 3) The distance from the centroid to the outer edge was calculated for every $\pi/50$ radians. 4) The distance data were presented as a function of the angle.
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