Loss of membrane cholesterol affects lysosomal osmotic stability

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Abstract. Lysosomal destabilization is critical for the organelle and living cells. Using methyl-β-cyclodextrin (MβCD) to selectively deplete lysosomal membrane cholesterol, we investigated the effect of cholesterol on the organelle osmotic stability. The results show that loss of membrane cholesterol caused changes in the lysosomal osmotic properties. The lysosomes lost the ability to resist osmotic shock and became more sensitive to osmotic stress. As a result, the lysosomes lost membrane integrity rapidly. Microscope observation showed that the lysosomes were liable to swell in the hypotonic sucrose medium. It is presumably due to an enhancement of the lysosomal permeability to water caused by the loss of membrane cholesterol. The results indicate an important role of cholesterol in the maintenance of lysosomal stability.

Key words: Lysosome — Membrane — Cholesterol — Osmotic stability — Water permeability

Introduction

Lysosomes carry out a variety of activities. Loss of lysosomal integrity is critical for the organelle and living cells. The leakage of lysosomal protons can cause elevation of intralysosomal pH and loss of membrane potential. These changes may lead to lysosomal dysfunction. The most serious result of lysosomal destabilization is the leakage of their enzymes. The leaked lysosomal enzymes can cause necrosis and apoptosis (Brunk et al. 1997). The enzymes leaked into the cytoplasm may bring about harmful effects in the pathogenesis of many diseases, such as prion encephalopathies (Laszlo et al. 1992), Alzheimer's disease (Nixon et al. 1992) and others. Since lysosomal destabilization can cause many detrimental effects, a number of studies have focused on the mechanisms by which lysosomes are destabilized.

The lysosome acts as an intracellular osmometer, being thus susceptible to osmotic destabilization (Lloyd and Forster 1986). As demonstrated previously, lysosomes can be osmotically destabilized by the oxidation of their membrane thiol groups (Wan et al. 2002) and changes in the membrane physical state (Yang et al. 2000). The lysosomal membrane is a barrier responsible for its integrity. By now, little is known about the role of each component of lysosomal membranes on the organelle osmotic stability. Cholesterol is an essential component of membranes. This class of lipid, among others, regulates various membrane properties such as membrane fluidity, permeability, stability and contour (Mukherjee and Maxfield 2004). As reported previously, cholesterol is abundant in lysosomal membranes (Gallegos et al. 2002). It is therefore of interest to study whether membrane cholesterol could affect lysosomal stability.

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Material and Methods

Materials

1,6-diphenyl-1,3,5-hexatriene (DPH), methyl-β-cyclodextrin (MβCD), 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (UMBG) and acridine orange were from Sigma (St. Louis, MO, USA). Percoll was purchased from Amersham (Uppsala, Sweden). Cholesterol assay kit was from Biosino Bio-Technology and Science Inc. Other chemicals were of analytical grade from Beijing Chemical Factory.

Preparation of lysosome

Male Wistar rats were starved for 24 h and killed by decapitation. Rat liver lysosomes were isolated as described previously

(Wang et al. 2006). Briefly, rat liver was homogenized in 0.25 mol/l sucrose and centrifuged at 3000 × *g* for 8 min. The supernatant was incubated at 37°C for 5 min in the presence of 1 mmol/l $CaCl_2$ to promote separation of lysosome from mitochondria (Yamada et al. 1984). Then, the supernatant was centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in sucrose and mixed with Percoll (2 : 1 by vol.), and centrifuged at 40,000 × *g* for 90 min. The lower 1/4 volume of the gradient (lysosomal fraction) was pooled and mixed with 10 volumes of 0.25 mol/l sucrose and centrifuged at 10,000 × *g* for 13 min to remove Percoll. The purified lysosomes were resuspended in 0.25 mol/l sucrose medium at 1.35 mg membrane protein per milliliter for use. All performances were carried out at 4°C. In this study, we used about 100 rats. Principle of laboratory animal care and all procedures were in accordance with the guidelines of the China government concerning the use of animals scientific research.

Treatment of lysosomes with MβCD

MβCD was dissolved in 0.25 mol/l sucrose through vigorous stirring for 30 min. Purified lysosomes were treated with MβCD at indicated concentration in 0.25 mol/l sucrose or 0.1 mol/l sucrose at 37°C for the indicated time. The lysosomes in the treatment medium are at 1.35 mg membrane protein/ml.

Assay of lysosomal integrity

Lysosomal integrity was assessed by measuring lysosomal marker enzyme latency. The enzyme latency refers to the percent of intact lysosomes as revealed by the inability of substrate to reach the lysosomal enzyme until the organelles are deliberately ruptured (Greene and Schneider 1992). UMβG, the substrate of lysosomal β-hexosaminidase, was used at 1 mmol/l to measure the enzyme activity (Bird et al. 1987). The liberated 4-methylumbelliferone was determined by measuring its fluorescence (excitation 365 nm, emission 444 nm) on a Hitachi F-4010 fluorescence spectrophotometer. Activities of the enzyme measured in the absence and presence of 0.36% Triton X-100 was designated the free activity and the total activity, respectively. Percentage free activity was calculated as (free activity/total activity) ×100. Loss of lysosomal integrity was determined as increased percentage free activity.

Steady-state fluorescence anisotropy measurement

Lysosomal membrane fluidity was assessed as described previously (Zhang et al. 2000). DPH labeling solution (10 μmol/l) was prepared by diluting the tetrahydrofuran-dissolved, DPH stocking solution (2 mmol/l) with 0.1 mol/l PBS buffer (pH 7.4) containing 0.1 mol/l sucrose and stirring vigorously. For labeling, lysosomes were stirred and incubated in the labeling solution at 37°C for 60 min. After labeling we washed lysosomes by centrifugation to delete extra DPH. Then the lysosomes were resuspended in 0.25 mol/l sucrose medium. Fluorescence was measured on Hitachi F-4010 fluorescence spectrophotometer with excitation and emission at 350 and 452 nm, respectively. Steady-state fluorescence anisotropy (*r*) was calculated according to the equation:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with the excitation polarizer in the vertical position and the analyzing emission polarizer mounted vertically and horizontally, respectively. $G = I_{HV}/I_{HH}$ is the correction factor. High degrees of fluorescence anisotropy indicate higher degrees of membrane order or lower degrees of membrane fluidity, and *vice-versa*.

Lysosomal membrane preparation

Control or treated lysosomes were centrifuged at 40,000 × *g* for 10 min. The washed lysosomes were lysed for 30 min in distilled water. Then, lysosomal membranes were collected by a centrifugation at 40,000 × *g* for 30 min. Lysosomal membrane protein was determined according to Lowry and coworkers (1951). All performances were carried out at 4°C.

Assay of lysosomal membrane cholesterol

Lysosomal membrane cholesterol was measured as described by Heider and Boyett (1978). In short, isopropyl alcohol was mixed with lysosomal membranes at 0.3 ml/mg membrane protein and sonicated for 10 min to extract membrane cholesterol. Then, the sample was centrifuged at 20,000 × *g* for 5 min. The supernatant was used for cholesterol analysis. Cholesterol content was quantified using cholesterol kit (Biosino Bio-Technology and Science, Inc.).

Microscopic observation of lysosomes

To study the effects of MβCD on lysosomal size, lysosomes were stained with 50 μmol/l fluorescence dye acridine orange for 5 min. The lysosomes were treated with MβCD at 37°C for 1 min and incubated in 0.1 mol/l or 0.25 mol/l sucrose medium for 2 min. Then, the lysosomes were visualized using a fluorescence microscope (Olympus IXT1, excitation 490 nm, emission 620 nm).

Results

Lysosomes are sensitive to osmotic stress. To study the effect of membrane cholesterol on lysosomal osmotic stability, we

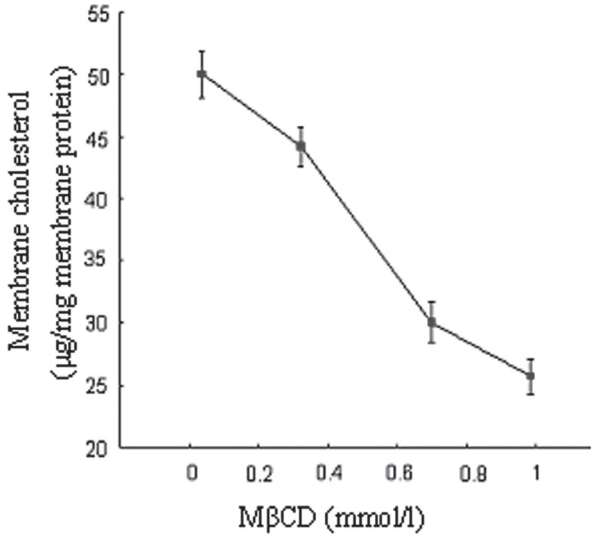

Figure 1. MβCD treatment decreased lysosomal membrane cholesterol. Lysosomes were treated with MβCD at indicated concentration for 1 min. Then, lysosomal membrane cholesterol was measured as described in Materials and Methods. Values are means ± S.D. of three separate measurements.

treated lysosomes with MβCD to selectively deplete their membrane cholesterol (Gimpl et al. 1997) and examined whether membrane cholesterol affects lysosomal osmotic stability.

First, we examined the effect of MβCD on the lysosomal membrane cholesterol. As shown in Fig. 1, lysosomal membrane cholesterol decreased from 50.5 μg/mg membrane protein to 27.0 μg/mg membrane protein along with increasing MβCD concentration (from 0 to 1.0 mmol/l). The lysosomal membrane cholesterol reduced by 11.0, 38.3 and 46.6% after treating the lysosomes with 0.3, 0.7 and 1.0 mmol/l MβCD, respectively. The results confirmed that MβCD is able to deplete lysosomal membrane cholesterol. It supports the assumption that increasing the membrane cholesterol loss could promote lysosomal destabilization. In our study, changes in lysosomal osmotic stability were assessed by the measurement of lysosomal enzyme latency after a brief incubation in a hypotonic sucrose medium. As shown in Fig. 2, the MβCD-treated lysosomes (line 2) exhibited greater percentage of free enzyme activity than the control lysosomes (line 1) at each hypotonic concentration of the sucrose medium. It indicates that MβCD-treated lysosomes lost their integrity more markedly than the control ones and that the treated lysosomes were more susceptible to the increasing osmotic imbalance across their membranes. Since MβCD can selectively deplete membrane cholesterol, the destabilization of MβCD-treated lysosomes might be attributed to the loss of membrane cholesterol, which was supported by an additional experiment. As shown in Fig. 3, the MβCD treatment caused lysosomal destabilization in the hypotonic sucrose. The lysosomes lost enzyme latency markedly when the MβCD concentration increased from 0.3 to 1.0

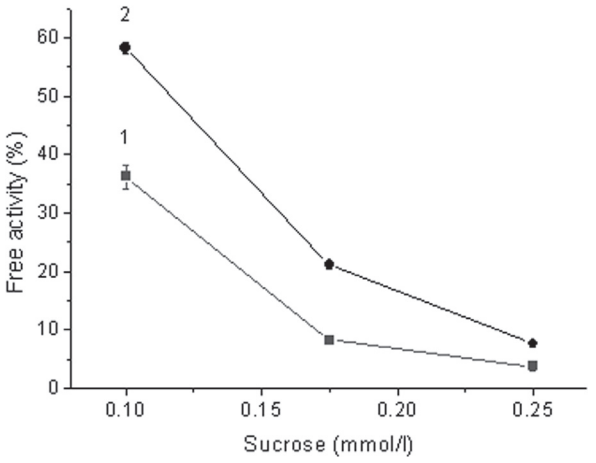

Figure 2. Effect of sucrose concentration on the osmotic stability of MβCD-treated lysosomes. Lysosomes were treated with 0.7 mmol/l MβCD at 37°C for 1 min. Then, the lysosomal sample was incubated in the sucrose medium at indicated concentration for 2 min. Lysosomal free enzyme activity was measured immediately after the incubation. Values are means ± S.D. of three separate measurements. Line 1, control lysosomes; line 2, MβCD-treated lysosomes.

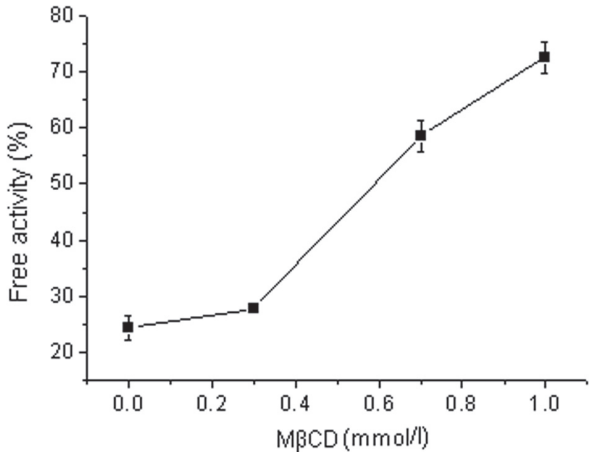

Figure 3. Effect of MβCD concentration on the lysosomal osmotic stability. Lysosomes were treated with MβCD at indicated concentration in 0.25 mol/l sucrose medium at 37°C for 1 min. Then, the lysosomal samples were incubated in 0.1 mol/l sucrose medium for 2 min. Lysosomal free enzyme activity was measured immediately after the incubation. Values are means ± S.D. of three separate measurements.

mmol/l. The results show that increasing MβCD concentration produced greater loss of membrane cholesterol and therefore further destabilized lysosomes. In addition, a slight loss of membrane cholesterol (such as a 10% loss by 0.3 mmol/l MβCD, *cf.* Fig. 1) did not markedly affect the lysosomal stability.

We further studied the dynamics of MβCD-induced changes in the lysosomal osmotic stability. As shown in Fig. 4, MβCD-treated lysosomes lost enzyme latency more rapidly and markedly than control lysosomes in the hypotonic sucrose medium. It indicates that MβCD-treated lysosomes lost the ability to resist hypotonic pressure and become more sensitive to osmotic stress. The results further establish that membrane cholesterol plays an important role in the maintenance of lysosomal osmotic stability.

As shown above, loss of membrane cholesterol could osmotically destabilize lysosomes. Theoretically, an increase in the lysosomal osmotic sensitivity should induce influx of water into the lysosomes in an osmotic stress. Thus, the organelle should swell and lose its integrity. To obtain direct evidence concerning the effect of MβCD on lysosomal osmotic stability, we examined changes in the size of MβCD-treated lysosomes after a brief incubation in hypotonic sucrose medium. The lysosomes were visualized by the fluorescence of acridine orange using a fluorescence microscope. As shown in Fig. 5, control lysosomes only slightly swelled during a 2-min incubation in 0.1 mol/l sucrose medium (average lysosomal diameters of Fig. 5A and B were 0.6 and 0.9 μm, respectively). It suggests that normal lysosomes have an ability to maintain their osmotic stability. The organelle can resist an osmotic stress in a short period. In contrast to the control ones, MβCD-treated lysosomes enlarged markedly under the same incubation conditions (average lysosomal diameters of Fig. 5C and D are 1.3 and 1.6 μm, respectively). Increasing the MβCD concentration from 0.5 to 1.0 mmol/l enlarged the lysosomes markedly. The results morphologically reinforce the above conclusion that loss of membrane cholesterol could osmotically destabilize lysosomes.

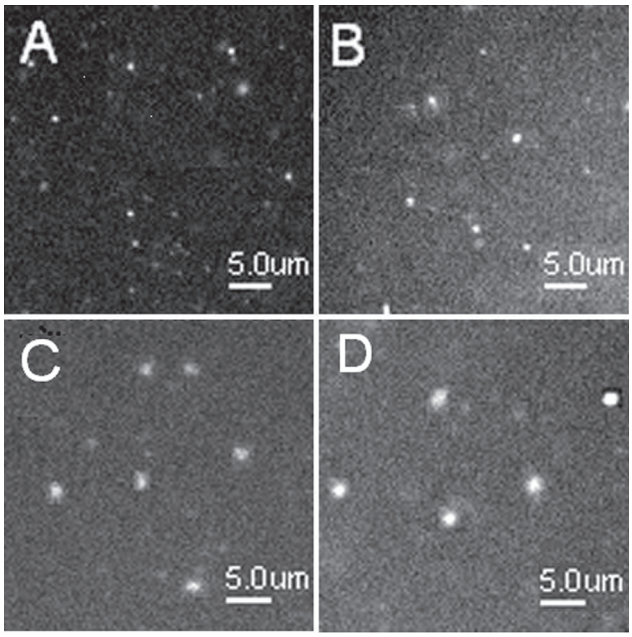

Figure 5. Effects of MβCD on the lysosomal size. Lysosomes were stained with 50 μmol/l acridine orange for 5 min and treated with MβCD at 37°C for 1 min. Then, the samples were incubated in 0.1 mol/l or 0.25 mol/l sucrose medium for 2 min. Lysosomes were visualized using fluorescence microscope: **A.** control lysosomes in 0.25 mol/l sucrose medium; **B.** control lysosomes in 0.1 mol/l sucrose medium; **C.** 0.5 mmol/l MβCD-treated lysosomes in 0.1 mol/l sucrose medium; **D.** 1.0 mmol/l MβCD-treated lysosomes in 0.1 mol/l sucrose medium. A typical result out of three experiments is shown.

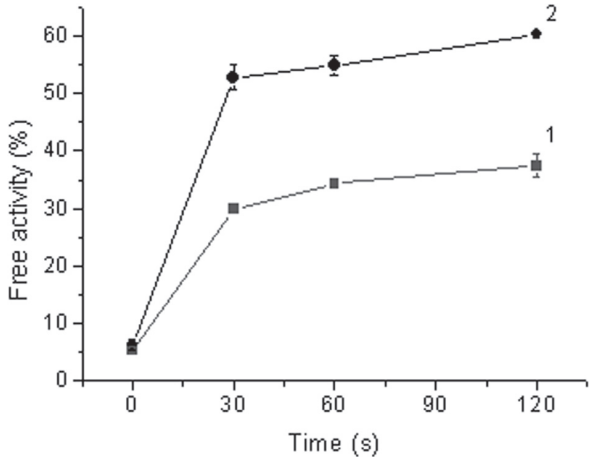

Figure 4. Effect of incubation duration on the osmotic stability of MβCD-treated lysosomes. Lysosomes were treated with 0.7 mmol/l MβCD at 37°C for 1 min. Then, the lysosomal sample was incubated in 0.1 mol/l sucrose medium for indicated time. Lysosomal free activity was measured immediately after the incubation. Line1, control lysosomes; line 2, MβCD-treated lysosomes. Values are means ± S.D. of three separate measurements.

Since cholesterol can modulate membrane physical state (Mukherjee and Maxfield 2004) and the membrane fluidity affects osmotic stability of lysosomes (Yang et al. 2000), we further examined whether the MβCD treatment could affect the lysosomal membrane fluidity. The results show that the treatment of lysosomes with MβCD reduced the fluorescence anisotropy of DPH (Table 1). It suggests that the lysosomal membranes were fluidized.

Table 1. Effect of MβCD treatment on lysosomal membrane fluidity

Treatment of lysosomes	Anisotropy	*p*
Control	0.188 ± 0.005	–
0.3 mmol/l MβCD	0.179 ± 0.004	<0.05
0.7 mmol/l MβCD	0.172 ± 0.002	<0.05
1.0 mmol/l MβCD	0.160 ± 0.003	<0.05

Lysosomes were treated with MβCD at indicated concentration for 1 min. Then, control and MβCD-treated lysosomes were labeled with DPH. Lysosomal membrane fluidity was assessed by measuring DPH fluorescence anisotropy. All procedures were as described in Materials and Methods. Values are means ± S.D., $n = 3$. Statistical analysis was performed using Student's *t*-test.

Discussion

The lysosomes *in vivo* maintain their integrity to carry out various activities. Lysosomal destabilization may cause serious results such as leakage of lysosomal protons, loss of their membrane potential and even dysfunction of the organelle. The most remarkable alteration in destabilized lysosomes is the loss of their enzyme latency. Leakage of cathepsins (a family of lysosomal proteases) into the cytoplasm could induce apoptosis (Brunk et al. 1997). Lysosomal hydrolases can also cause necrosis if leakage of the enzymes is extensive and serious (Brunk et al. 1997). In addition, the released lysosomal enzymes can bring about harmful effects in the pathogenesis of many diseases. Since lysosomal integrity is critical for living cells, (particular) attention should be paid to the factors and events that are detrimental to lysosomal stability. Generally, lysosomes can be destabilized by two kinds of mechanisms. One type mechanism correlates to the alterations in lysosomal membranes. In this respect, the effect of membrane lipid peroxidation on lysosomal stability was extensively studied (Fong et al. 1973). Another mechanism for lysosomal destabilization has been attributed to the occurrence of osmotic stress. Lysosomes are named intracellular 'osmometer' owing to their susceptibility to osmotic stress (Lloyd and Forster 1986). An increase in the lysosomal osmotic sensitivity can cause the organelle to become more susceptible to osmotic shock, i.e. the lysosomes are prone to osmotic swelling and lysis. Our previous studies have demonstrated that photooxidation of lysosomal membrane thiol groups and changes in the physical state of lysosomal membranes can increase lysosomal osmotic sensitivity (Yang et al. 2000; Wan et al. 2002). In the living cells, some events may cause osmotic stress to lysosomes. The lysosomes of mammalian cells are surrounded by a high concentration of cytoplasmic K^+ (140 mmol/l). Abnormal entry and accumulation of K^+ in lysosomes may osmotically destabilize lysosomes (Ruth and Weglicki 1982). In addition to K^+, glucagons may produce an osmotic shock to lysosomes in cellular autophagy (Deter and de Duve 1967). In the past years, a number of studies focused on the mechanisms by which lysosomes are destabilized. By now, little is known about the role of each component of lysosomal membranes on the organelle stability. Cholesterol is an essential component of lysosomal membranes (Gallegos et al. 2002). This class of sterols can regulate various membrane properties such as membrane fluidity, permeability, stability and contour (Mukherjee and Maxfield 2004). The purpose of this study was to elucidate whether membrane cholesterol could affect lysosomal stability.

A previous study showed that treatment of living cells with MβCD could destabilize the lysosomes (Jadot et al. 2001). The way of MβCD affecting on the intracellular lysosomes in culture medium was not explained. Since the lysosomal membrane cholesterol was not measured in the study, it is unclear whether the lysosomal destabilization correlates to the cholesterol. In this work, we found that loss of membrane cholesterol may osmotically destabilize lysosomes. The above mentioned microscope observation provided direct evidence that loss of membrane cholesterol promoted influx of water into the lysosomes in our experimental conditions. As proposed previously, cholesterol can reduce the passive water permeability in direct proportion to its level in the membrane (Haines 2001). It supports the assumption that loss of membrane cholesterol may increase lysosomal membrane permeability to water.

Several mechanisms have been proposed to explain how cholesterol reduces membrane water permeability. Cholesterol modulates membrane physical state. It decreases the membrane order in the gel-phase membrane and increases the membrane order in the liquid-crystalline phase (Wustner 2007). The phase transition of lysosomal membrane occurs at about 15°C (Ruth and Weglicki 1982). Thus, the loss of membrane cholesterol is expected to induce an increase in the lysosomal membrane fluidity at the temperature above 15°C. In this study, we observed similar behaviour (of the membranes). Our previous study established that increasing lysosomal membrane fluidity could enhance osmotic sensitivity of the lysosomes (Yang et al. 2000). It may be attributed to the increases in the membrane permeability to water. We suppose that the loss of the membrane cholesterol may increase lysosomal water permeability through the membrane fluidization.

Solubility-diffusion theory has been proposed to account for water permeation of membranes. To cross a membrane by this mechanism, Born energy is required to transfer water from the high-dielectric aqueous phase (dielectric constant is about 80) to the low-dielectric membrane interior (dielectric constant is about 2) because the electrostatic energy of the water medium is much lower than that of the bilayer interior (Orme et al. 1988). Since cholesterol causes a significant drop in the dielectric constant in the region of the ester carbonyl

groups of phospholipids (Starke-Peterkovic et al. 2006), a relatively larger Born energy is required for water to enter the membrane. Therefore, cholesterol can reduce membrane permeability to water. Loss of the membrane cholesterol may increase the dielectric constant of membrane interior and thus decrease the Born energy required for water to cross the membrane. Thus, it becomes easier for water to pass through a membrane when its cholesterol content is reduced.

Transient defects of membranes have been proposed as a pathway for water permeation of membranes (Deamer and Bramhall 1986). Since membrane cholesterol can reduce the incidence of such defects in the membranes (Yeagle 1985), it is more difficult for water molecules to cross the membrane containing abundant cholesterol. Loss of the membrane cholesterol may increase the membrane defects and enhance the membrane permeability to water.

Finally, loss of the lysosomal membrane cholesterol perturbed the membrane lipid order what may cause changes in the membrane properties and increase the membrane fragility. Thus, the membranes may lose the ability to resist osmotic stress and become susceptible to osmotic destabilization.

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