A double bond in ring B of cholesterol is not required for the membrane stability of human erythrocytes

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Abstract

In cholesterol-membrane interactions, the role of the double bond in the ring B of the sterol remains to be revealed. In model membranes composed of sterols and phospholipids, the behavior of 7-dehydrocholesterol or β-cholestanol is different from that of cholesterol. However, it is not clear whether similar results are observed in biological membranes. So, we examined effects of these sterols using human erythrocytes. The hemolysis of the erythrocytes under high hydrostatic pressure (200 MPa) or hypotonic buffer was suppressed by cholesterol, 7-dehydrocholesterol, and β-cholestanol. Moreover, the detachment of cytoskeletal proteins from the membrane under low ionic strength conditions was also suppressed by 7-dehydrocholesterol and β-cholestanol, as well as cholesterol. These results suggest that the double bond in the ring B of cholesterol plays no important role in the membrane stability of human erythrocytes.

Keywords: bilayer-cytoskeleton interaction, cholesterol, β-cholestanol, 7-dehydrocholesterol, hemolysis, mass spectrum

1. Introduction

Cholesterol is an important component of biological membranes [1] and also a precursor of steroid hormones [2] and bile salts [3]. In particular, the cholesterol-membrane interaction is of interest in that cholesterol regulates the stability and function of the membrane [4]. For instance, the human erythrocyte membrane is stabilized by cholesterol [5]. To obtain further information on the cholesterol-erythrocyte membrane interaction, it is useful to compare with results from other sterols. So, determining the role of the double bond in ring B of cholesterol, we have selected the two sterols such as 7-dehydrocholesterol and β-cholestanol (dihydrocholesterol) (Fig. 1). 7-Dehydrocholesterol is a precursor of vitamin D [6]. Compared with cholesterol, 7-dehydrocholesterol possesses an additional double bond in ring B. Reduction of 7-dehydrocholesterol by the enzyme 3β-hydroxy-sterol-$\triangle^7$-reductase results in cholesterol [7, 8]. The default of this enzyme leads to the Smith-Lemli-Opitz syndrome (SLOS), which exhibits the reduction of cholesterol content and enhancement of 7-dehydrocholesterol level in the membrane [7,8]. Patients with SLOS are characterized by similar facial features such as a small upturned nose and cleft palate, mental retardation, and developmental delay [8]. From a clinical point of view, the action of 7-dehydrocholesterol in the biomembrane is significantly different from that of cholesterol. To understand such difference at molecular level, the behaviors of both sterols have been investigated using model membranes. For instance, 7-dehydrocholesterol hinders a tightly-packed arrangement with dipalmitoylphosphatidylcholine (DPPC) due to a slight torsion of the ring and leads to less domain formation than cholesterol [9]. On the other hand, β-cholestanol lacks a double bond in ring B of the sterol nucleus (Figure 1) and all three of the six-membered rings are relatively unstrained chair conformation compared with cholesterol [10]. β-Cholestanol is a minor sterol of the animal body [11]. Cholesterol displays preference for DPPC over egg-PC, but β-cholestanol interacts equally with both phospholipids [12].

Hemolysis is expressed by hemoglobin release from the erythrocyte membrane under various stress conditions

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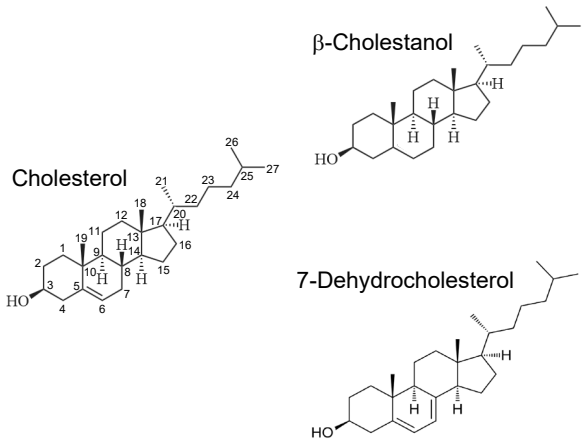

Fig. 1 Chemical structures of sterols

[13]. The cell volume is influenced by stresses such as high hydrostatic pressure (simply, pressure) and osmotic pressure [14]. Cell shrinkage is induced by pressure, whereas swollen cells are observed in hypotonic buffer [14]. From hemolytic properties, pressure-induced hemolysis is mainly characterized by the bilayer-cytoskeleton interaction, whereas hypotonic hemolysis by the surface area-to-volume ratio [14]. Hemolysis under pressure or hypotonic buffer is suppressed upon loading of cholesterol into the membrane [5].

In the present work, we have examined the effect of these sterols on the hemolysis under stress conditions and the bilayer-cytoskeleton interaction in the human erythrocyte membrane. Sterol contents in the erythrocyte membrane have been estimated from peak intensities on mass spectra. The results obtained show that the double bond in ring B of cholesterol plays no important role in the stabilization of the human erythrocyte membrane.

2. Materials and Methods

2. 1 Materials

Compounds were obtained from the following sources: cholesterol, Nacalai Tesque; β-cholestanol and 2, 5-dihydroxybenzoic acid (2, 5-DHB), Tokyo Kasei; 7-dehydrocholesterol and methyl-β-cyclodextrin (CD), Sigma-Aldrich. All other chemicals were of reagent grade.

2. 2 Hemolysis of human erythrocytes

Human erythrocytes were obtained from the Fukuoka Red Cross Blood Center. The erythrocytes were washed three times with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). For the loading of sterols such as cholesterol, β-cholestanol, and 7-dehydrocholesterol, the erythrocytes were incubated for 1 h at 37˚C at a 20 % hematocrit in a 0.5% CD/1.3 mM sterol complex-containing PBS, which was preincubated for 1~2 h at 37˚C [5]. After the incubation, the erythrocytes were washed with PBS. For the pressure treatment, the erythrocytes in PBS were incubated for 30 min at 200 MPa and 37˚C, and decompressed up to atmospheric pressure [5,13]. Such pressure-treated erythrocyte suspensions were centrifuged for 1 min at 1,000 × g and room temperature (~25˚C), and the absorbance of hemoglobin in the supernatant was measured at 542 nm [5,13]. For the hypotonic hemolysis, the erythrocytes were suspended in 10 mM sodium phosphate (pH 7.4) containing 54 mM NaCl, incubated for 10 min at 37˚C, and centrifuged for 1 min at 1,000 × g [14]. The degree of hemolysis was estimated, as mentioned above.

2. 3 Analysis of erythrocyte membrane sterols

Membrane lipids were extracted from erythrocytes by using a mixed solvent of chloroform and methanol (v/v = 2:1) [15]. For the detection of 7-dehydrocholesterol, cholesterol, and β-cholestanol by a matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) in linear positive-ion mode, 2, 5-DHB (10 mg/mL) was dissolved in a mixed solvent of chloroform and methanol (v/v = 2:1) and used as a matrix [16]. As MS samples, equal volumes of lipids and 2, 5-DHB were mixed [16]. The measurement was carried out using an Autoflex speed MALDI-TOF mass spectrometer (Bruker). Cholesterol and β-cholestanol were detected as H^+ adduct ($M + H^+ - H_2O$) at m/z 368.9 and 370.9, respectively [17].

2. 4 Bilayer-cytoskeleton interaction

To detach the cytoskeletal proteins from the membrane, open ghosts, which were prepared from the sterol-loaded erythrocytes using 5 mM sodium phosphate, pH 8 (5P8) at 0˚C, were suspended in 7 volumes of 5P8 and incubated for 30 min at 37˚C [18]. To separate the detached proteins from the membrane, ghost suspensions were centrifuged for 20 min at 20,000 × g and 4˚C. The protein concentration in the supernatant was determined by the method of Lowry et al. [19].

2. 5 Statistics

All values are expressed as mean ± standard deviation (SD). Where appropriate, Student's t-test for paired data was used to assess the significance of difference.

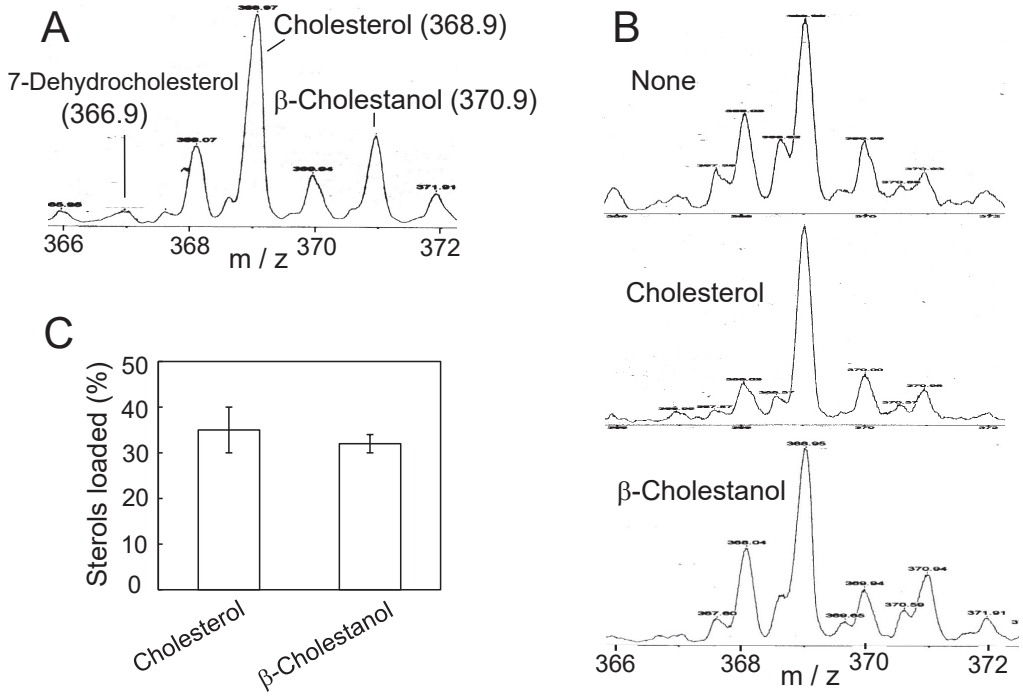

Fig. 2 MALDI-TOF mass spectra of sterols and sterol contents loaded into erythrocyte membranes. (A) A mixture of cholesterol, 7-dehydrocholesterol, and β-cholestanol was dissolved in chloroform. (B) Lipids were extracted from intact membranes (none), cholesterol- or β-cholestanol-loaded ones. (C) Sterol contents estimated from peak intensities. Values are means ± SD for three independent experiments.

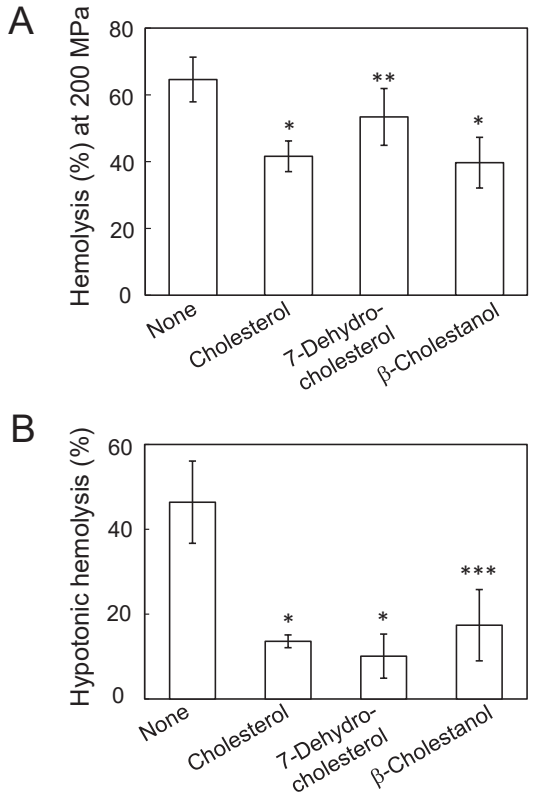

Fig. 3 Hemolysis of sterol-loaded erythrocytes under stress conditions. Intact erythrocyte membranes (none) were loaded with cholesterol, 7-dehydrocholesterol, or β-cholestanol. These sterol-loaded erythrocytes were exposed to a pressure of 200 MPa (A) or hypotonic stress (B). Values are means ± SD for three independent experiments. *,**,*** $p < 0.01$, 0.08, 0.05 vs. none, respectively.

3. Results

3. 1 Effects of the double bond in ring B of cholesterol on membrane stability of human erythrocytes

Figure 2A shows a MALDI-TOF mass spectrum of a mixture of cholesterol, 7-dehydrocholesterol, and β-cholestanol dissolved into organic solvent, where each sterol concentration after mixing was 10 μM. Cholesterol and β-cholestanol were able to be identified as a molecular ion peak, but 7-dehydrocholesterol was not [20].

Cholesterol, 7-dehydrocholesterol, or β-cholestanol was additionally loaded into the intact erythrocyte membrane using a sterol/CD complex, as previously described in plant sterol loading (Fig. 2B) [15]. Judging from the peak intensities on a mass spectrum, about 30% of original cholesterol level was additionally loaded for cholesterol or β-cholestanol (Fig. 2C).

Effects of 7-dehydrocholesterol and β-cholestanol on the membrane stability of erythrocytes were examined using these sterol-loaded cells. Hemolysis under a pressure of 200 MPa was suppressed in 7-dehydrocholesterol-or β-cholestanol-loaded erythrocytes, as just seen in cholesterol-loaded ones (Fig. 3A). Similar results were observed in the hypotonic hemolysis of these sterol-loaded erythrocytes (Fig. 3B). These results reveal that the double bond in ring B of cholesterol is not essential for its contribution to the

membrane stabilization of human erythrocytes.

3. 2 Effects of the double bond in ring B of cholesterol on detachment of cytoskeletal proteins from the erythrocyte membrane under hypotonic buffer

When erythrocyte ghosts are exposed to a hypotonic buffer, cytoskeletal proteins such as spectrin and actin are detached from the membrane [18, 21]. Such detached proteins are released into the buffer. So, we examined the sterol effect on such protein detachment using a sterol-loaded erythrocyte membrane. Such protein detachment was suppressed by cholesterol, 7-dehydrocholesterol, and β-cholestanol (Fig. 4).

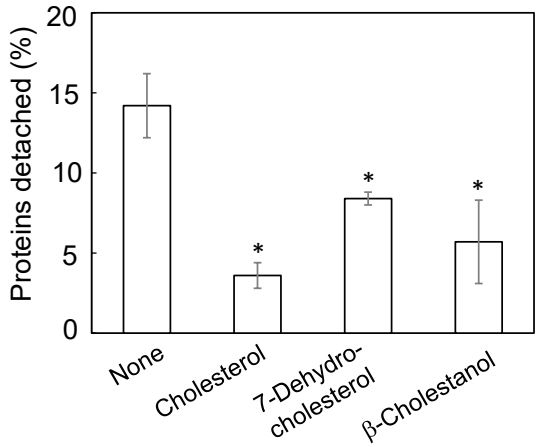

Fig. 4 Protein detachment from sterol-loaded erythrocyte membranes exposed to hypotonic buffer. Intact erythrocyte membranes (none) were loaded with cholesterol, 7-dehydrocholesterol, or β-cholestanol. Open ghosts prepared from these sterol-loaded erythrocytes were incubated in 5P8 for 30 min at 37 ˚C. Values are means ± SD for three independent experiments. *$p < 0.05$ vs. none.

4. Discussion

In the present work, we have used MALDI-TOF MS to detect the sterols in the erythrocyte membrane. The hydroxyl group at C-3 of sterols used here is important in the ionization of the molecules. In MALDI-TOF MS the hydroxyl group at C-3 is attacked by H^+ from matrix and then detached as H_2O so that the sterol is detected as a $[M+H - H_2O]^+$ [17]. This reaction is expected to be influenced by the structural properties around the hydroxyl group. The number of double bonds in ring B of sterols is altered in β-cholestanol and 7-dehydrocholesterol. Such changes in the number of double bonds are expected to influence the ionization of the hydroxyl group at C-3 of sterols in MALDI-TOF MS. The low sensitivity of 7-dehydrocholesterol, compared with cholesterol and β-cholestanol, suggests that the additional double bond in ring B inhibits the ionization reaction [20]. Thus, 7-dehydrocholesterol is different from cholesterol and β-cholestanol on ionization of the hydroxyl group at C-3. Mass spectra show that when the intact erythrocyte membrane is loaded with β-cholestanol using a CD/sterol complex, cholesterol content of the membrane remains unaltered and the content of loaded sterol is about 30% of original cholesterol level.

In the bilayer-cytoskeleton interaction, the transmembrane proteins such as band 3 are linked to cytoskeletal proteins such as spectrin via anchor proteins [22]. Cholesterol induces the tight association of bilayer with cytoskeleton, perhaps via band 3-cholesterol interaction [15]. In the present study, the hemolysis under high pressure or hypotonic conditions and the detachment of cytoskeleton from the membrane are suppressed by 7-dehydrocholesterol and β-cholestanol, as well as cholesterol. Here, it is of interest to compare present results with previous ones, which were obtained using plant sterols such as β-sitosterol and stigmasterol [15]. Chemical structure of β-sitosterol and stigmasterol is different from cholesterol only in side chain, i.e., both plant sterols have one additional ethyl group at C-24 [15]. Moreover, stigmasterol has a double bond between C-22 and C-23 in addition to this ethyl group [15]. Although both plant sterols are loaded at the same level as β-cholestanol into intact erythrocyte membranes using a same method as present one, the plant sterols do not exhibit the suppression of hemolysis and protein detachment [15]. These data suggest that the side chain of cholesterol plays an important role in the membrane stability of erythrocytes, but the double bond in ring B of cholesterol does not.

There are functional differences between cholesterol and 7-dehydrocholesterol, as seen in SLOS. For instance, the ligand binding activity of solubilized hippocampal serotonin1A receptor, where the cholesterol level is reduced, restores by reloading of cholesterol, but not by 7-dehydrocholesterol [23]. Similar results are observed in the measurement of the membrane dipole potential. Here, the membrane dipole potential is the potential difference generated from the alignment of the dipolar components of the phospholipids and interface water within the lipid bilayer [24]. In DPPC vesicles, the effect of cholesterol on the membrane dipole potential is much larger than that of 7-dehydrocholesterol [25]. On the other hand, interestingly, the ordering of phospholipids in solubilized membrane, in which serotonin1A receptor is contained, is restored by reloaded cholesterol and 7-dehydrocholesterol [23]. These data suggest that the side chain of cholesterol affects main-

ly the overall membrane properties such as hemolysis and phospholipid ordering, whereas the double bond in the ring B of cholesterol plays an important role in the specific interaction of the sterol with adjacent phospholipids and integral proteins. The present work reveals that the addition rather than the decrease of the double bond in the ring B of cholesterol induces the subtle modulation of the action of cholesterol.

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