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Lysis of Platelets and Erythrocytes by the Incorporation of a Unique Oxygenated Sterol: 22R-Hydroxycholesterol

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Summary. We have found that an oxygenated sterol, 22R-hydroxycholesterol [(22R)-5-cholestene-3β,22-diol], lyses not only platelets but also erythrocytes in a dose-dependent manner. The lysis of platelets and erythrocytes were evidenced by the release of intracellular proteins, lactate dehydrogenase and hemoglobin, respectively. Their morphological change was shown by scanning electron microscopy. Elevated temperature was required for the lysis, probably to redistribute the sterol in the lipid bilayers in the plasma membranes. When the sterol was incorporated at low temperature, the temperature had to be raised to readily lyse cells. This lytic effect was, surprisingly enough, restricted to the R-isomer; the S-isomer was only marginally effective. Furthermore, sitosterol and other oxygenated sterols, with a hydroxyl group at different positions in the side chain of cholesterol, were much less lytic, regardless of the configuration of the hydroxyl group introduced.

A possible mechanism for this interesting phenomenon will be discussed in relation with a structural alteration in lipid bilayers in plasma membranes brought about by the incorporation of this unique oxygenated sterol.

Key Words 22R-hydroxycholesterol · platelet · erythrocyte · hemolysis · lateral diffusion · clustering

Introduction

Various oxygenated sterols have been suggested to be important regulators of biological functions including chemotaxis of polymorphonuclear leukocytes [3]. These effects are not necessarily explained by the well-known effect of these compounds on cholesterol biosynthesis [8]. We may have to consider the structural constraint in lipid bilayers imposed by the insertion of these sterols into plasma membrane [20]. We have also shown that several oxygenated sterols rapidly modulate platelet aggregation in plasma [16]. In a previous publication [5], we have investigated the interaction between dipalmitoylphosphatidylcholine and various cholesterol analogues, with altered length of side chain and with a hydroxyl group introduced at the C-22 position. One of the striking findings was that R- and S-isomers of 22-hydroxycholesterol[5-cholestene-3β,22-diol] behaved very differently; the R-isomer had very weak interaction with the phospholipid.

In the present communication, we wish to demonstrate that lysis of platelets and erythrocytes is brought about specifically with the incorporation of 22R-hydroxycholesterol into the plasma membrane.

Materials and Methods

Materials

Cholesterol and sitosterol were purchased from Tokyo Kasei Co. (Tokyo, Japan) and Tama Biochemical Co. Ltd. (Tokyo, Japan), respectively. Sterols with hydroxyl group on the side chain and 20-methyl-5-pregnen-3β-ol were synthesized as previously described [7, 9–12]. Other sterols were purchased from Steraloids, Inc. (Witon, N.H.). All other chemicals were of analytical grade.

Preparation of Platelet and Erythrocyte

Bovine blood was obtained at a local slaughter house and anticoagulated with Acid Citrate Dextrose (ACD). Platelet rich plasma (PRP) was prepared by a previously described procedure [14]. PRP and erythrocytes were washed with Tris-ACD buffer (pH 7.35) for three times.

Measurement of Lysis of Platelets and Erythrocytes

Sterols were dissolved in ethanol and were added to the Tris-ACD buffer to reduce the alcohol concentration. Equal volume of the alcohol-containing Tris-ACD buffer and platelet or erythrocyte suspension were mixed and the final concentration of ethanol was 1%. Platelet (5×10^8/ml) and erythrocyte (1×10^8/ml) suspensions were centrifuged ($1200 \times g$, 5 min) and supernatants were collected after incubation at various temperatures for different periods. Platelet lysis was assessed by measuring the activity of lactate dehydrogenase (LDH) in the supernatant by a

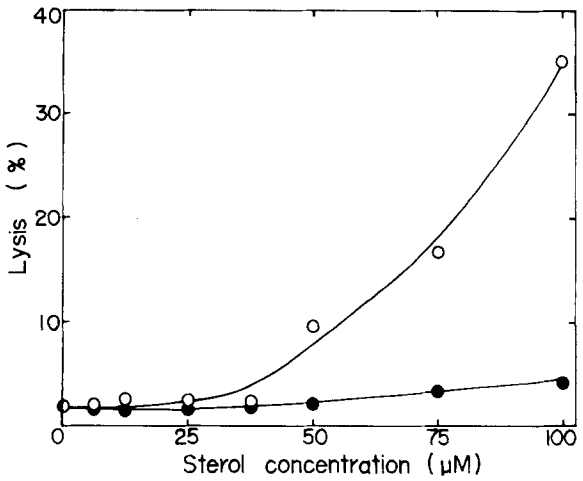

Fig. 1. Dose-dependent lysis of platelet with 22-hydroxycholesterols. Various concentrations of 22R- (-○-) and 22S-hydroxycholesterol (-●-) were added to platelet suspensions. They were incubated at 37°C for 20 min and platelet lysis was measured as described in Materials and Methods

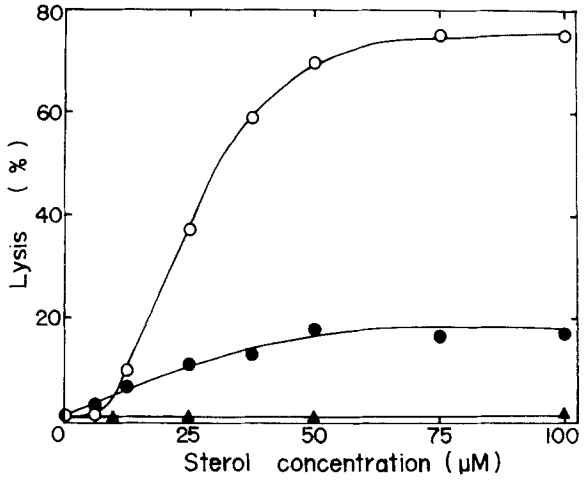

Fig. 2. Dose-dependent lysis of erythrocyte with 22-hydroxycholesterols. Various concentrations of 22R- (-○-), 22S-hydroxycholesterol (-●-) and cholesterol (-▲-) were added to erythrocyte suspensions. They were incubated at 37°C for 10 min and hemolysis was measured as described in Materials and Methods

previously described procedure [15]. Lysis of erythrocyte was determined from the amount of hemoglobin in the supernatant by measuring the absorbance at 410 or 577 nm. Degree of lysis was expressed as relative to the value obtained with sonicated platelets or erythrocytes.

Lipid Contents

Lipids were extracted with chloroform and methanol [2]. The amounts of phospholipids were determined after ashing using dipalmitoylphosphatidylcholine as the standard [1]. Sterol contents were determined by a Shimadzu gas chromatograph Model GC-7A equipped with 1% OV-17 column using 20-methyl-5-pregnen-3β-ol as the internal standard.

Scanning Electron Microscopy

Platelets (100 μl) or erythrocytes (100 μl) were added to 2 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, in a vial in which a piece of small glass pretreated with poly-L-lysine was put at the bottom. This was kept at room temperature for 30 min to fix the structure, and centrifuged for 5 min at 1500 × *g*. The slide glasses, to which fixed platelets or erythrocytes were attached, were washed with the phosphate buffer. They were then post-fixed with 1% osmium tetroxide in the same buffer for 30 min at 4°C, and were dehydrated with graded ethanols for 10 min each at room temperature. Samples on slide glasses were further dried with isoamyl acetate for 10 min for three times at room temperature. They were finally dehydrated in carbon dioxide with a Hitachi HCP-2 critical point dryer, and were coated with gold with an Eiko IB-3 ion coater. They were observed under a JEOL scanning electron microscope JSM-T200 at 25 kV.

Results and Discussion

During the course of investigations on the effect of oxygenated sterols on platelet aggregation, it was found that the mere supernatant of washed platelet suspensions preincubated specifically with 22R-hydroxycholesterol (50 μM) at 37°C for 1 hr was able to aggregate platelets in plasma in the presence of calcium ion (*data not shown*). The extent of aggregation, however, was diminished, if platelets in plasma were pre-exposed to either ADP or 5HT (5-hydroxytryptamine) to make them desensitized specifically to these agonists [6]. These data strongly indicate that washed platelets excrete small molecules like ADP and 5HT after they are incubated with this oxygenated sterol.

In order to investigate whether this excretion is limited to small molecules, we have determined extracellular activity of an enzyme, lactate dehydrogenase, which is normally found only inside cells [15]. Washed platelets were incubated with varied concentrations of 22R-hydroxycholesterol at 37°C for 20 min and were quickly centrifuged. The activity of the enzyme obtained in the supernatant was determined. As demonstrated in Fig. 1, platelets became very leaky if they were pre-incubated with 40 μM 22R-hdyroxycholesterol (shown by -○-). About 20% of the enzyme molecules were excreted to the medium after washed platelets were preincubated with 75 μM of this sterol. This lytic effect was, however, relatively small with the S-isomer (shown by -●-). This enormous difference between these two isomers could not be attributed to their amounts incorporated in cells; as shown later in Table 2 they were incorporated to comparable extents.

The lytic effect of this sterol was even more obvious with erythrocytes. Erythrocytes washed and suspended in Tris-ACD buffer were incubated

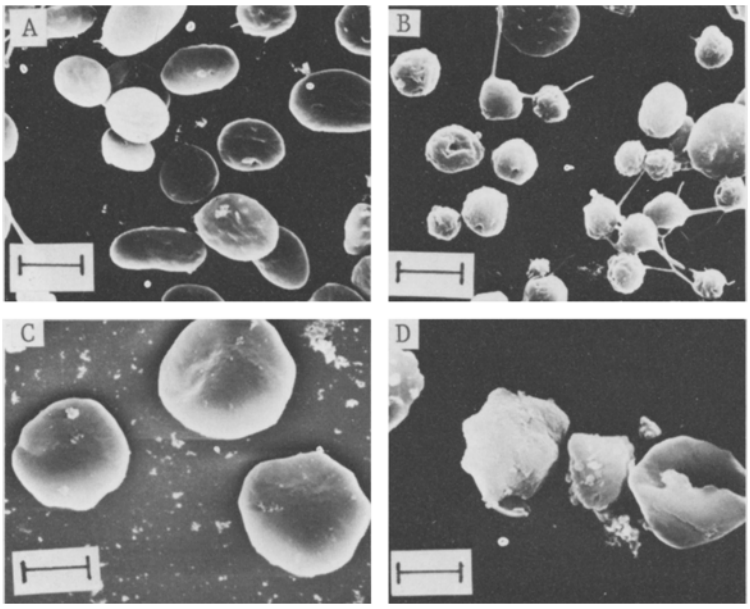

Fig. 3. Scanning electron micrographs of platelets and erythrocytes. Platelets and erythrocytes were treated with either 1% ethanol (control) or 50 μM 22R-hydroxycholesterol at 37°C for 30 min. *A*, control platelets; *B*, platelets treated with 22R-hydroxycholesterol; *C*, control erythrocytes; *D*, erythrocytes treated with 22R-hydroxycholesterol; bar, 2 μm; 7500×

with varied concentrations of 22-hydroxycholesterols at 37°C for 10 min. They were then centrifuged and the amounts of hemoglobin excreted to the medium were determined. As convincingly demonstrated in Fig. 2, the R-isomer (shown by -○-) was again very effective in making erythrocytes leaky. Fifty μM of the sterol was enough to make them excrete about 70% of hemoglobin. On the other hand, the S-isomer was again much less effective (shown by -●-). Cholesterol was totally ineffective (shown by -▲-). This lytic effect was indeed very specific for 22R-hydroxycholesterol, as demonstrated in Table 1. All the oxygenated sterols tested and sitosterol were much less effective than 22R-hydroxycholesterol in inducing the excretion of hemoglobin. Other effective sterols, 20S- and 24-hydroxycholesterols, were only one-fourth as effective as 22R-hydroxycholesterol. However, even the effect of the 22R-isomer was very nominal with erythrocytes in plasma, probably due to the binding of sterols to lipoproteins [19]. We have not further investigated the effect of plasma in this study.

The morphological alteration by the incorporation of the 22R-isomer was shown by scanning electron microscopy. As demonstrated in Fig. 3, normal platelets with flat disc shape (shown by *A*) shrank and became spheroidal with wrinkles on the surface and with some pseudopod-like protrusions (shown by *B*) after the incubation with the 22R-isomer. More drastic deformation was noted with erythrocytes. Compared to the normal erythrocytes (shown by *C*), the treated erythrocytes (shown by *D*) were collapsed and looked as if the contents were released.

Table 1. Effect of various sterols on hemolysis

Sterols[a]	Lysis (%)[b]
Cholesterol	1.8
Sitosterol	2.4
7-Ketocholesterol	2.1
6β-Hydroxycholestanol	1.6
6-Ketocholestanol	4.9
5α,6β-Dihydroxycholestanol	3.0
5α-Hydroxy-6-ketocholestanol	2.6
5α,6α-Epoxycholestanol	2.3
20R-Hydroxycholesterol	5.5
20S-Hydroxycholesterol	17.7
22R-Hydroxycholesterol	71.0
22S-Hydroxycholesterol	16.0
24R-Hydroxycholesterol	15.9
24S-Hydroxycholesterol	18.2
25-Hydroxycholesterol	1.5

[a] Chemical nomenclatures of sterols are listed below: *cholesterol*, 5-cholesten-3β-ol; *sitosterol*, stigmast-6-en-3β-ol; *7-ketocholesterol*, 5-cholesten-3β-ol-7-one; *6β-hydroxycholestanol*, 5α-cholestane-3β,6β-diol; *6-ketocholestanol*, 5α-cholestan-3β-ol-6-one; *5α,6β-dihydroxycholestanol*, cholestane-3β,5α,6β-triol; *5α-hydroxy-6-ketocholestanol*, cholestane-3β,5α-diol-6-one; *5α,6α-epoxycholestanol*, 5,6α-epoxy-5α-cholestan-3β-ol; *20R-hydroxycholesterol*, (20R)-5-cholestene-3β,20-diol; *20S-hydroxycholesterol*, (20S)-5-cholestene-3β,20-diol; *22R-hydroxycholesterol*, (22R)-5-cholestene-3β,22-diol; *22S-hydroxycholesterol*, (22S)-5-cholestene-3β,22-diol; *24R-hydroxycholesterol*, (24R)-5-cholestene-3β,24-diol; *24S-hydroxycholesterol*, (24S)-5-cholestene-3β,24-diol; *25-hydroxycholesterol*, 5-cholestene-3β,25-diol.

[b] Sterols (50 μM) were added to erythrocyte suspensions. They were incubated for 30 min and centrifuged to collect the supernatant. Hemolysis was measured as described in Materials and Methods.

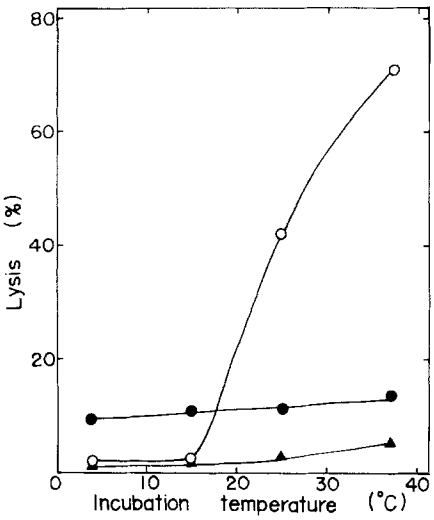

Fig. 4. Effect of incubation temperature on hemolysis induced by 22-hydroxycholesterols. Fifty μM of 22R- (-○-), 22S-hydroxycholesterol (-●-) and cholesterol (-▲-) were added to erythrocyte suspensions at various temperatures. They were incubated for 30 min and hemolysis was determined as described in Materials and Methods

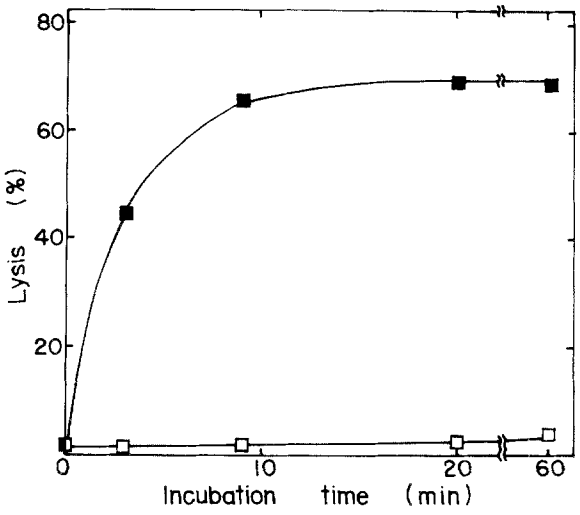

Fig. 5. Rapid hemolysis of 22R-hydroxycholesterol-incorporated erythrocyte by temperature shift. Erythrocytes were incubated with 50 μM 22R-hydroxycholesterol for 5 min at 4°C and resuspended in Tris-ACD buffer at either 4°C (-□-) or 37°C (-■-) after washing. They were centrifuged at various times and hemolysis was determined as described in Materials and Methods

Table 2. Sterol contents of erythrocytes[a]

Incubation with:	Temp. (°C)	Sterol/phospholipids (molar ratio)	
		Cholesterol	22R- or 22S-Hydroxycholesterol
Ethanol	4	0.67	—
Ethanol	37	0.71	—
22R-	4	0.71	0.32
22R-	37	0.68	0.29
22S-	4	0.65	0.31
22S-	37	0.67	0.29

[a] 22-Hydroxycholesterols (50 μM) and ethanol (1%) were added to erythrocyte suspensions (1×10^8/ml). They were incubated for 30 min either at 4 or 37°C. Lipids were extracted and were determined as described in Materials and Methods.

The following experiments were performed in order to obtain some understandings about the mechanism of this lytic effect. Erythrocytes were incubated with 22-hydroxycholesterols and cholesterol at various temperatures for 30 min, and the amounts of hemoglobin excreted were determined as above. To our very surprise, the lysis was totally dependent on the incubation temperature. As unambiguously demonstrated in Fig. 4, when the incubation temperature was below 15°C the effect of R-isomer (shown by -○-) was almost as low as that obtained by the S-isomer and cholesterol (shown by -●- and -▲-, respectively). The effect of the R-isomer increased very sharply when the incubation temperature was increased to 25°C. When the incubation time was increased (or decreased) from 30 min, the curve for the R-isomer shifted to the left (or to the right). The effects of the S-isomer and cholesterol, of course, never became noticeably high even when the temperature was elevated.

This effect of incubation temperature cannot be explained by difference of the amounts of the R-isomer incorporated at different incubation temperatures. The amounts of the R-isomer incorporated by erythrocytes at 4 and 37°C were indeed not much different as revealed in Table 2. It is also noted in the table that cholesterol normally present in the membrane is not replaced by these isomers. The next experiment strongly indicates that the elevated temperature is required to redistribute the R-isomer in the lipid bilayers of plasma membrane. Erythrocytes were incubated with 22R-isomer (50 μM) at 4°C for 5 min, and were resuspended in Tris-ACD buffer either at 4 or 37°C after washing at 4°C. At various times they were centrifuged and the amounts of hemoglobin in the supernatants were determined. As clearly depicted in Fig. 5, hemoglobin was quickly excreted after erythrocytes were incubated at 37°C (shown by -■-). Due to the technical difficulty in rapid centrifugation, the earliest time point was 3 min, at which time the lysis had almost reached to the maximum. Qualitatively speaking, the lysis was obvious immediately after

the temperature shift. When they were incubated at 4°C, on the other hand, the excretion never became appreciable during this time scale (shown by -□-). Much longer incubation time, in the order of several hours, was needed to get appreciable excretion at 4°C (*data not shown*).

A possible explanation for this interesting phenomenon can be deduced from physical studies including our own [5, 18]; 22R-hydroxycholesterol has very weak interaction with a phospholipid. This is probably due to the "kink" in the side chain resulting from the introduction of a hydroxyl group with R configuration specifically at the C-22 position, as demonstrated by the study with nuclear magnetic resonance [13, 17]. It is conceivable that this sterol tends to get together and to make segregated clusters in the plasma membrane, once it is incorporated in lipid bilayers. On the other hand, the S-isomer and cholesterol would mix with other lipids in the membrane better than the R-isomer. Although high concentration of cholesterol in the membrane is known to make clusters and induce cold-induced hemolysis [4], the effect should be more benign than that of the R-isomer. When the R-isomer is incorporated at 4°C, it is incorporated rather randomly in the membrane and the lateral diffusion is needed for the segregation to occur. The shift of the temperature from 4 to 37°C will increase the fluidity and facilitate the lateral diffusion of the sterol for the cluster formation in the lipid bilayer. If this happens, the hemolysis can be explained by either one of the following two reasonings. 1): Phase separation occurs and makes drastic gap in fluidity at the boundary between fluid phase and less fluid sterol clusters, and makes plasma membrane leaky. 2): The segregated sterol clusters cannot locally retain the bilayer structures. More investigations are in progress to clarify the cluster formation.

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