ELSEVIER

Biophysical Chemistry 131 (2007) 105–110

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Atomic force microscope observation on biomembrane before and after peroxidation

Jin-Ye Wang [a,*], Li-Ping Wang [b], Qiu-Shi Ren [b]

[a] *Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Fenglin Road, Shanghai 200032, China*
[b] *School of Life Science and Biotechnology, Shanghai Jiaotong University, 1954 Huashan Road, Shanghai 200030, China*

Received 20 June 2007; received in revised form 25 September 2007; accepted 25 September 2007
Available online 2 October 2007

Abstract

Atomic force microscope (AFM) has been used to visualize the morphological change on the surface of erythrocyte membrane before and after oxidation. A smooth surface of intact erythrocyte cell was observed, while treatment by ferrous ion and ascorbate induced hemolysis of intact erythrocytes, generated many holes with average size of 146.6±33.2 nm in diameter (n=28) on membrane surface as seen by AFM. Ghost membrane and its inside-out vesicles were also used for the experiment. Skeleton structure and protein vesicles could be observed on the surface of an intact erythrocyte membrane before oxidation. Sendai virus induced fusion of inside-out vesicles seemed suppress peroxidation, while no such effect was observed in ghost membrane and erythrocyte systems.

Keywords: Asymmetry; Atomic force microscope; Erythrocyte; Fusion; Peroxidation; Sendai virus

1. Introduction

An asymmetric distribution of phospholipids between the inner and outer monolayer is a common feature of biological membranes [1]. Generally, most of phosphatidylcholine (PC) and sphingomyelin are distributed in the outer layer of the membrane, while phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner layer of the membrane. Unlike membrane proteins, the asymmetric distribution of phospholipids is dynamically changed, which is called "flip-flop" and correlated with translocase [2–4]. Although the real physiological function of the asymmetric distribution is not very clear, it has been recognized that this is important for vesicular trafficking, including vesicle budding and fusion, molecular recognition and sorting between cells [5]. A direct evidence that PE as well as PS is exposed onto the cell surface during early stages of apoptosis, resulting in the total loss of asymmetric distribution of aminophospholipids in the plasma membrane bilayer has been proposed [6]. PE is redistributed at the cleavage furrow of dividing cells during cytokinesis, which may play a pivotal role in mediating a coordinate movement between the contractile ring and plasma membrane to achieve successful cell division [7]. On the other hand, membrane fusion is reported to stimulate phospholipid flip-flop between the external and internal monolayers, leading to a loss of membrane phospholipid asymmetry [8]. Another important rearrangement of phospholipids in biomembranes is bilayer-to-inverted hexagonal transition. Presence of the non-lamellar structures may be involved in membrane processes, either temporarily, like in membrane fusion or locally, e.g. to affect the activity of membrane-bound protein [9]. The fact that some amphiphiles which raise the bilayer to hexagonal phase transition temperature of PE inhibit Sendai virus-induced hemolysis of erythrocytes and fusion between ghosts and liposomes also implies the existence of nonbilayer intermediate [10]. We have shown in liposomal systems that the inverted hexagonal phase is more sensitive to hydroperoxidation than the multilamellar phase [11], and the higher content of aminophospholipid in the exofacial layer seems to be related with the higher rate of the

Abbreviations: AAPH, 2,2′-azobis (2-amidino-propane) dihydrochloride; AFM, atomic force microscope; MGO, DL-glycerin-monooleate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TBARS, thiobarbituric acid reactive substances; TNBS, 2, 4, 6-trinitrobenzenesulfonic acid.

* Corresponding author.
E-mail address: jywang@mail.sioc.ac.cn (J.-Y. Wang).

0301-4622/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2007.09.010

ferrous ion-induced peroxidation of multilamellar vesicles [12]. In the present study, human erythrocyte, which is a typical example that aminophospholipids distribute more in the inner leaflet, and its inside-out vesicles were used to compare the peroxidizability, as they have different amount of aminophospholipids in outer surface of the membrane. Erythrocytes fused by Sendai virus, ghosts fused by Sendai virus and lipid fusogen were also used for comparison with their unfused ones in ferrous ion induced peroxidation. Atomic force microscope (AFM) was used for observation of the membrane structure of both intact erythrocytes and ghost membranes before and after oxidation.

2. Experimental section

2.1. Materials

2, 4, 6-trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma (St. Louis, MO, USA). Ferrous sulfate and other reagents such as antioxidants, buffers, organic solvents were of analytical grade.

2.2. Ghost membrane from human blood

Preparation was carried out at 4 °C. The fresh, heparinized human blood was centrifuged at 2000 ×*g* for 10 min, and erythrocytes were separated from plasma and buffy coat and washed three times in a physiological saline. White ghosts were prepared in phosphate buffer by the method of Steck and Kant with some modifications [13]. i.e., instead of alkaline buffer (pH 8.0) with lower concentration (5 mM), neutral buffer (pH 7.4) with a little higher concentration (10 mM) was used to prevent fragmentation of the ghost membranes. Half of the white ghost was further treated by the buffer with lower ion strength (0.5 mM, pH 8.0) to prepare inside-out vesicles [13]. Procedures of density barrier and ultracentrifugation were omitted. The concentration of the membrane was confirmed by phosphorus determination [14] after transferred the phosphate buffer into a physiological saline solution.

2.3. Membrane asymmetry analysis

Asymmetry of aminophospholipids in ghost membranes was investigated by TNBS labeling method [15]. Briefly, freshly prepared 0.01 M TNBS in the same buffer as membranes was added to the membrane solution in the presence or absence of 2% Triton X 100. The final concentration of TNBS was 0.6 mM. The reaction mixture was incubated at room temperature in the dark for 1 hour. Absorbance at 420 nm was analyzed.

2.4. Peroxidation analysis

Thiobarbituric acid reactive substances (TBARS) in ghost membrane and its inside-out vesicles were quantified by measuring malonyldiadehyde-thiobarbituric acid adducts formed by acid hydrolysis in boiling water for 15 min and then immediately cooled. After centrifuged at 8000 rpm for 5 min, absorbance at 535 nm was measured [16]. Oxygen consumption was monitored polarographically with a Clark-type oxygen probe (Rank Brothers, Cambridge, England). An incubation vessel, containing about 1.5 ml of membrane solution, was equipped with a magnetic stirrer, and the temperature was maintained at 37 °C in a circulating water bath. The solution was saturated with air prior to the addition of ferrous sulfate.

2.5. Fatty acid analysis

Total lipids were extracted from the ghost membrane by the method of Folch et al. [17]. Organic solvent (chloroform–methanol 2:1 by volume) contained 0.0002% of t-butyl hydroxyanisole as an antioxidant and a proper amount of heptadecanoic acid as an internal standard. Methyl esters of total lipids were prepared with 0.6 N methanolic–HCl as described by Kates [14]. Separation of fatty acid methyl esters was performed using a fused silica HR-SS-10 capillary column (25 m × 0.25 mm I.D.; Shinwa, Kyoto, Japan) with a splitless mode (Shimadzu GC 18A). Helium was used as a carrier gas. The initial oven temperature was 50 °C, increased to 160 °C at 20 °C /min, followed by an increase in temperature to 210 °C at 2 °C /min and then held constant for 10 min. The injector and detector temperature was 250 °C.

2.6. Atomic force microscope observation of biomembranes

Erythrocyte cells before and after oxidation with ferrous ion was diluted to about 2×10^5 cells/ml, fixed with 5% glutaraldehyde for 5 min (post fixation with 1% OsO_4 was needed for ghost membranes), deposited on a poly-lysine treated cover glass for adsorption of the cells. The extra solution was removed with filter paper, then a drop of sodium cacodylate buffer (20 mM, pH 7.4, 80 mg dextran 60C/ml, dextran can prevent the hemolysis of erythrocytes derived from washing) was added to wash and to remove sodium chloride of the sample, as sodium chloride will crystallize after drying the sample and disturb the observation. Subsequently most of the solution was removed by filter paper. The glass coverslip with attached cells was glued to a steel disc and placed on the stage of the atomic force microscope (AFM). AFM observation was carried out with Olympus OMPM-AFM-01 by a constant height mode in the air at room temperature. The sample was scanned under a sharpened pyramidal tip (silicon nitride) with a diameter of 40 nm (Olympus OMCL-TR400PS-2). The scan speed was 0.2 s/line. The size of holes on the membrane surface was randomly measured in the AFM image with Image-Pro Discovery Version 4.5 (Media Cybernetics Inc., USA).

2.7. Fusion with Sendai virus

Sendai virus (Z strain) was obtained kindly from Dr. Y. Kaneda, Osaka University, and grown in allantoic sac of embryonated chicken eggs (10-day old) and harvested after an 84 h incubation at 36 °C. After purification, hemagglutinating units (HAU) was measured against chicken erythrocytes. The mixture of erythrocytes and Sendai virus was first incubated at 4 °C for 10 min to allow agglutination, then moved to 37 °C and