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EFFECTS OF P-CHLOROMERCURIBRNZOATE AND N-ETHYLMALEIMIDE ON HEMOLYSIS UNDER HYDROSTATIC PRESSURE OF HUMAN ERYTHOCYTES

The relation between high-pressure (200mpa)-induced hemolisis and spectrin tetramer-dimer equilibrium was examined by using human erythrocytes treatedwith N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMB). The values of hemolysis at 200 Mpa of NEM-treated erythrocytes increased upon mild modification of membrane SH groups, but decreased upon severe modification. In both cases, the membrane structure of NEM-treated cells became more stable against high pressure upon further incubation at 37^0C in reagent-free buffer. In PCMB-treated erythrocytes, the cells were hemolyzed completely at 200 Mpa. On the other hand, the concentration of spectrin dimers increased upon treatment with NEM, but remained constant for further incubation at 37^0C. In the case of PCMB-treated cells, spectrin tetramers did not dissociate into dimers as with NEM. These results suggest that hemolytic properties at 200 Mpa in erytrocytes treated with SH-reactive reagents are inexplicable in terms of the dissociation only of spectrin.

1. INTRODUCTION

When human erythrocytes are exposed to high pressure, hemolysis [1] and vesiculation [2] start to occur at about 130 Mpa. The degree of hemolysis and vesiculation increases gradually at higher pressures. In such hemolyzed cells, parts of spectrin are detached from the membrane [3]. Spectrin is a cytoskeletal protein. Spectrin heterodimers self-associate in a head-to-head fashion to from tetramers [4]. The spectrin tetramer-dimer equilibrium is affected by several factors such as temperature, salts, and chemical reagents. In the native membrane, most of spectrin are tetramers and play an important role in the maintenance of the membrane mechanical stability. We have demonstrated that the hemolytic properties of human erythrocytes under high pressure provide useful information on the membrane structure [1-3]. Thus, it is of unterest to examine how the membrane stability under high pressure is modulated by the dissociation to spectrin dimers. Here, using SH-reactive reagents, we describe the relation between high-pressure-induced hemolysis and the spectrin tetramer-dimer equilibrium. A preliminary report of some of these data has been published [5].

2. MATERIALS AND METHODS

2. 1. MATERIALS

Compounds were obtained from the following sources; N-ethylmaleimide (NEM), Wako Pure Chemicals; p-chloromercuribenzoate (PCMB), Nacalai Tesque. All other chemicals were of reagent grade.

2. 2. TREATMENT OF ERYTHROCYTES WITH SH-REACTIVE REAGENTS

Human blood that was drawn in citrate/mannitol/adenine/phosphate solution was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged at 750 x g for 10 min at 4^0C. The plasma and buffy coat were removed carefully. The erythrocytes were washed three times in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7,4). For chemical modifications of erythrocytes, the red cells suspended at a 20 %

hematocrit in PBS were incubated for 30 min at 15 or 37^{0}C with NEM (2mM) and PCMB (100 μM). After incubation, the erythrocytes were washed three times in chilled PBS. Parts of NEM-treated cells were furthermore incubated for 1 h at 37^{0}C in PBS.

2. 3. HEMOLYSIS

Intact erythrocytes and chemically modified ones were subjected to a high pressure, as described previously [3]. Briefly, 3 ml of the erythrocyte suspension (0,3 % hematocrit) in PBS was inserted into a syringe-type cell and set in the pressure bomb. The samples were compressed at a rate of 20 Mpa/min, incubated for 30 min at 200 Mpa and 37^{0}C, and then decompressed up to atmospheric pressure at a rate of 40 Mpa/min. The erythrocyte suspension was centrifuged at 750 x g for 10 min at 35^{0}C. The concentration of hemoglobin in the supernatant was estimated from the absorbance at 542 nm. One hundred percent hemolysis was carried out by adding 10 μl of 5 % Triton X-100 into the suspension.

2. 4. NONDENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS OF SPECTRIN OLIGOMERS

Ghost membranes were prepared from chemically modified erythrocytes, according to the method of Dodge et al. [6]. To extract cytoskeletal proteins from the membranes, one volume of the ghosts was mixed with one volume of 0,1 mM EDTA, 0,1 mM PMSF, pH 8.0, and incubated overnight at 0^{0}C. After incubation, the ghost suspension was centrifuged at 42,000 x g for 30 min at 4^{0}C. The electrophoresis was carried out using 2,5 % acrylamide gel. Gels were stained with Coomassic Blue and the bands of spectrin tetramers and dimers were analyzed using an Advantic DM-303 scanning densitometer.

3. RESULTS

The effect of NEM on high pressure-induced hemolysis was examined (Table 1). When intact cells were treated with 2 mMNEM at 15^{0}C or 37^{0}C, the volue of hemolysis at 200 Mpa was 85,9 or 33,7 %, respectively. These values are largely different from that of intact cells, i.e., the membrane structure becomes more fragile against high pressure upon mild modification of membrane thiol groups, whereas it becomes more stable upon severe modification. Iinterestingly, when these NEM-trrreated erythrocytes were incubated in reagent-free buffer for 1 h at 37^{0}C, thecell membrane in both cases became more stable against high pressure. Next, the effect of NEM on spectrin tetramer-dimer equilibrium was examined (Table 1). Thhe equilibrium of spectrin shifted to dimers upon modification with NEM. However, the contents of spectrin dimers remained constant for ferther incubation at 37^{0}C. Additionally, the effect of PCMB on hemolysis at 200 Mpa was examined. The erythrocytes treated with 100 μM PCMB were hemolyzed completely under high pressure. However, spectrin tetramer-dimer equilibrium remained almost constant (data not shown).

Table 1

Effects of NEM on hemolysis at 200 Mpa and spectrin tetramerdi mer equilibrium.

Reagent (treatment)	Reagent-free Incubation at 37°C	Equilibrium (%)		% Hemolysis
		Dimer	Tetramer	
None	none	12,7	87,3	45,9
None	1h	13,6	86,4	41,4
2mM NEM (15ºC, 30min)	none	61,4	38,6	85,9
2mM NEM (15^{0}C, 30 min)	1h	60,4	39,6	78,8
2mM NEM (37^{0}C, 30 min)	none	72,4	27,6	33,7
2mM NEM (15^{0}C, 30 min)	1h	73,2	26,8	16,0

4. DISCUSSION

High-pressure-induced hemolysis is sensitive to the chemical modifications [1] and enzymatic digestion [7] of membrane proteins. In the present work, we have shown that the membrane stability of human erythrocytes at 200 Mpa is modulated by chemical modifications of membrane Shgroups. The values of hemolysis at 200 Mpa of NEM-treated erythrocytes increase upon mild modification of membrane SH groups, but decrease upon severe modification or further incubation at 37^0C. Such a decrease of hemolysis at 200 Mpa may be associated with the reorganization of membrane proteins in chemically modified erythrocytes [8]. Data of spin labeling demonstrate that such a reorganization occurs above 30^0C [8]. In diamide-treated erythrocytes, spectrins are intra- and intermolecularly cross-linked [1]. The hemolytic properties at 200 Mpa of such red cells are similar to those of NEM-treated cells[8]. It seems likely that the shift to spectrin dimer makes the membrane structure more unstable. So, it is interesting to examine the effect of SH-reactive reagents on spectrin tetramer-dimer equilibrium. The rate of spectrin dimer increases upon modification of membrane SH groups, but does not change upon further incubaiton at 37^0C. Thus, thetetramer-dimer equilibrium of spectrin is incapable of explaining the hemolytic properties at 200 Mpa of SH-modified erythrocytes. This klea is also supported by the results from PCMB-treated erythrocytes.

Interestingly, the hemolysis at 200 Mpa of PCMB-treated erythrocytes is enhanced dramatically. Mercurials such as PCMB and $HgCl_2$ react with water channels and inhibit the water transport [9]. Further experiments are necessary to determine whether the membrane fragility under high pressure of PCMB-treated erythrocytes is associated with the inhibition of water channels.

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