Interaction of Carbodiimide with Human Erythrocytes: Hemolytic Properties Induced by High Pressure, Heating, and Hypotonic Medium

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The effects of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) on the hemolysis of human erythrocytes were examined. To hemolyze, the erythrocytes were exposed to high pressure (200 MPa), heating (52 °C), or a hypotonic medium. Upon EDC treatment, the hemolysis induced by high pressure or heating was completely suppressed, whereas osmotic hemolysis was enhanced. In EDC-treated erythrocytes, high-molecular-weight aggregates of membrane proteins were formed by cross-linking of the membrane with the cytoskeleton, in addition to that of membrane proteins themselves. In such a cross-linking reaction, however, iron(II) in hemoglobin and thiol groups in membrane proteins remained stable. Although phosphate transport via band 3 was considerably suppressed by EDC, the intracellular K^+ concentration was almost constant. These results suggest that high-pressure- or heat-induced hemolysis, compared with osmotic hemolysis, is suppressed by a stabilization of the cytoskeletal network.

The membrane structure of human erythrocytes has been studied as a prototype of the biological membrane. The erythrocyte membrane mainly comprises proteins and phospholipids. The stability and deformability of the erythrocyte membrane are controlled by the cytoskeletal network.[1] The cytoskeleton consists of spectrin, protein 4.1, and actin.[2] Further, it also contains minor components such as adducin and tropomyosin.[2] Ankyrin and protein 4.1 bind to the transmembrane proteins such as band 3 and glycophorin C, respectively.[2,3] These linking proteins attach the cytoskeleton to the red cell plasma membrane. Thus, membrane protein–protein interactions play an important role in the structure and function of the erythrocyte membrane. The defect in these interactions causes a destruction of the membrane, i.e., hemolysis. Therefore, a study of hemolysis provides useful information concerning the interactions among membrane components. Compared with osmotic hemolysis, high-pressure-induced hemolysis is unfamiliar. We have investigated the membrane structure of the erythrocyte exposed to high pressure.[4,5] The membrane dynamics is significantly different in both membranes.[4] For instance, the hole size in high-pressure-treated erythrocyte membranes alters reversibly for changes in temperature and pH. On the other hand, a resealed membrane prepared by osmotic hemolysis is stable to both temperature and pH. The different responses in both membranes have been ascribed to the difference in the cytoskeletal structure.

The cross-linking of the membrane proteins may be fruitful for evaluating their roles in hemolysis. Water-soluble carbodiimide, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), has been used to cross-link membrane proteins.[6,7] EDC activates the caboxyl groups of proteins. The activated carboxyl groups react with the amino groups of the same or other proteins to give cross-linked products.[8] Because EDC is a zero-length cross-linker, cross-linked polypeptide residues must be in contact with each other. In the present work, we describe the effect of cross-linking of membrane proteins on hemolysis induced by high pressure, heating, and a hypotonic medium. The hemolytic properties due to these methods are discussed on the basis of the stability of the cytoskeleton and ion transport.

Experimental

Materials. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and *N*-ethylmaleimide (NEM) were purchased from Wako Chemicals. All other chemicals were of reagent grade.

Chemical Modification of Erythrocytes. Human blood was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged at 750*g* for 10 min at 4 °C. The plasma and buffy coat were carefully removed. The erythrocytes were washed three time in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4, M = mol dm^{-3}). The erythrocytes in PBS (at 20% hematocrit) were treated with EDC (1—10 mM) or NEM (2 mM) for 30 min at 37 °C, unless otherwise noted. For the experiment of phosphate transport, washing and chemical modification of erythrocytes were carried out in Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.4). EDC- or NEM-treated erythrocytes were washed three times in PBS or Tris buffer.

Hemolysis. For high-pressure-induced hemolysis, the erythrocytes were suspended at 0.3% hematocrit in PBS and subjected to a pressure of 200 MPa for 30 min at 37 °C.[4] After decompression, the suspension was centrifuged for 5 min at 750*g* and 35 °C. Hemoglobin release into the supernatant was measured at 542 nm. The degree of hemolysis was determined as previously described.[4]

For hypotonic hemolysis, erythrocytes (6 μl) were added to 2 ml

of a hyporonic medium (10 mM sodium phosphate, 46.9 mM NaCl, pH 7.4), incubated for 10 min at 37 °C, and then centrifuged for 10 min at 750*g*. The degree of hemolysis was similarly determined.

For hemolysis by heating, the erythrocyte suspension (0.3% hematocrit) in PBS was incubated for 15 min at 52 °C and centrifuged for 10 min at 750*g*. The degree of hemolysis was similarly determined.

Phosphate Transport and Intracellular K^+ Concentration in EDC-Treated Erythrocyte. Phosphate transport in EDC-treated erythrocytes was measured by using the ^{31}P NMR, as previously described.[9] Briefly, EDC-treated erythrocytes prepared from 1.5 ml of intact cells were mixed with 1.5 ml of transport buffer (30 mM sodium phosphate, 160 mM sucrose, 22 mM sodium citrate, pH 6.2). After incubation for 1 h at 25 °C, the ^{31}P NMR spectra of the suspension were run at 161 MHz on a JEOL GSX-400 spectrometer.

Intracellular K^+ concentration in EDC-treated erythrocytes was determined by the ^{39}K NMR. EDC-treated cells (1.5 ml) were mixed with the same volume of PBS. The ^{39}K NMR spectra were recorded at 25 °C with a NMR tube containing both the erythrocyte suspension (about 3 ml) and a small glass capillary filled with saturated KCl (reference signal). The ^{39}K NMR spectra were run at 18.50 MHz on a JEOL GSX-400 spectrometer with the following instrument settings: 8,192 data points, a 2-kHz spectral width, a 2-s pulse repetition, 200 scans, and a 90 °C flip angle.

ESR Measurement. ESR spectra of EDC (2—10 mM)-treated erythrocytes were recorded on a JEOL JES-RE-1X spectrometer using liquid nitrogen. The changes in the oxidative states of iron of hemoglobin due to EDC were studied by inserting samples into a quartz tube of 5 mm diameter and freezing them at −196 °C.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) of Membrane Proteins. Ghost membranes were prepared from EDC-treated erythrocytes according to the method of Dodge et al.[10] SDS-PAGE of membrane proteins in ghosts was performed by using 2.5% (stacking gel) and 8% (separation gel) acrylamide, according to the method of Laemmli.[11] Gel was stained with Coomassie blue.

Results

Hemolytic Properties of EDC-Treated Erythrocytes by High Pressure, Heating, or Hypotonic Medium. Human erythrocytes were treated with EDC (2—10 mM) and subjected to a pressure of 200 MPa. The degree of hemolysis greatly decreased upon increasing the concentration of EDC, i.e., at 10 mM of EDC, the hemolysis was almost completely suppressed (Fig. 1). In addition, heat-induced hemolysis, in which the changes were small, was also suppressed by EDC. On the other hand, the degree of hypotonic hemolysis increased upon EDC labeling of erythrocytes. To check the possibility of the reaction of membrane SH-groups with EDC,[12] the erythrocytes were pretreated with a SH-reactive agent such as NEM and then exposed to EDC. The suppressive effect of EDC on high-pressure-induced hemolysis was observed in NEM-treated erythrocytes (Table 1).

Effects of EDC on Ion Transport. Anion transport in human erythrocytes is mediated by band 3.[13] A reaction of band 3 with EDC was estimated by measuring the phosphate transport in EDC-treated erythrocytes. Phosphate transport was gradually inhibited by EDC, i.e., about 30% inhibition was observed with 10 mM EDC (Fig. 2). Previously, we showed that high-pressure-induced hemolysis is affected by

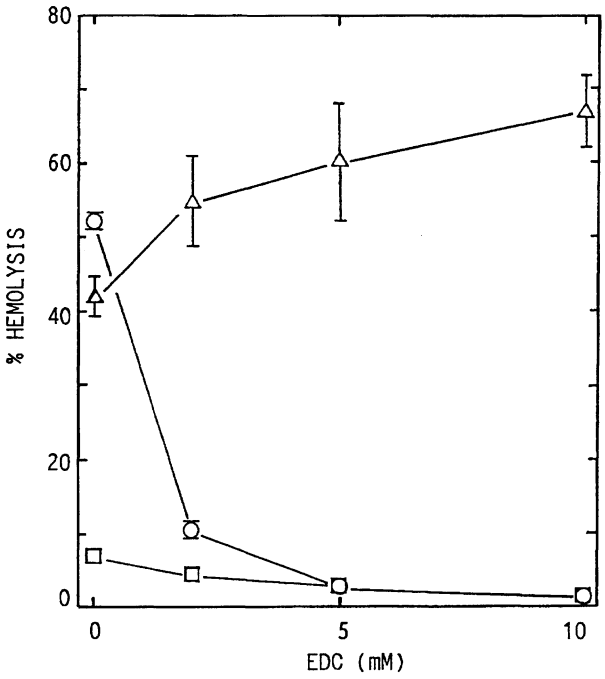

Fig. 1. Effect of EDC on hemolysis. EDC-treated erythrocytes were subjected to a high pressure (200 MPa) (○), hypotonic medium (△), or heating (52 °C) (□).

Table 1. Effects of NEM[a] and EDC[b] on High-Pressure-Induced Hemolysis[c]

Treatment		% Hemolysis
1st	2nd	
None	None	46.4±1.2
None	EDC	6.3±3.2
NEM	None	59.1±3.8
NEM	EDC	1.8±0.6

a) Erythrocytes were treated with 2 mM NEM for 10 min at 37 °C. b) Erythrocytes were incubated with 10 mM EDC for 30 min at 23 °C. c) Erythrocytes were subjected to a pressure of 200 MPa for 30 min at 37 °C.

Ca^{2+}-activated K^+ efflux.[14] Thus, the effect of EDC on the intracellular K^+ concentration was examined. On the basis of the signal intensity of ^{39}K NMR, we found that the intracellular K^+ concentration in EDC -treated erythrocytes remained almost constant (Fig. 2).

SDS-PAGE of EDC-Treated Erythrocyte Membranes. Intact erythrocytes were treated with EDC (1—10 mM) and ghost membranes were prepared from these red cells. SDS-PAGE of these membranes is shown in Fig. 3. With increasing the EDC concentration, hemoglobin became bound to the membrane, so that we could not prepare hemoglobin-free ghosts. In SDS-PAGE of such ghosts, bands corresponding to spectrin and band 3 disappeared, and diffuse bands and new band due to high-molecular-weight aggregates were observed.

ESR Measurement. Previously, we showed in H_2O_2-treated erythrocytes that the iron(II) contained in hemoglobin is oxidized to iron(III)[15] and that active oxygens produced

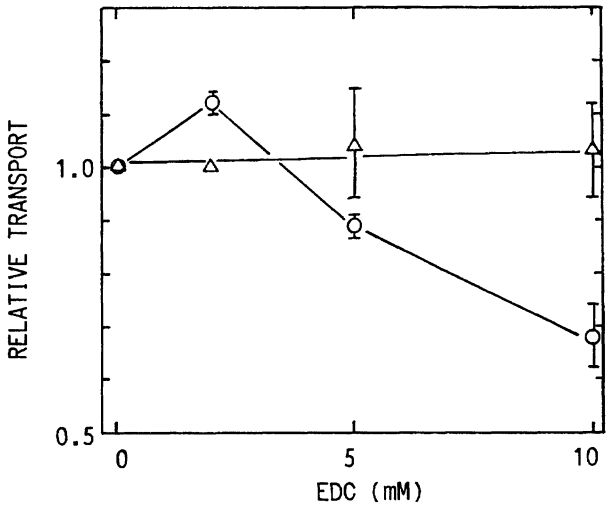

Fig. 2. Effect of EDC on ion transport. Phosphate influx (○) or K^+ efflux (△) in EDC-treated erythrocytes was measured by using ^{31}P NMR or ^{39}K NMR, respectively.

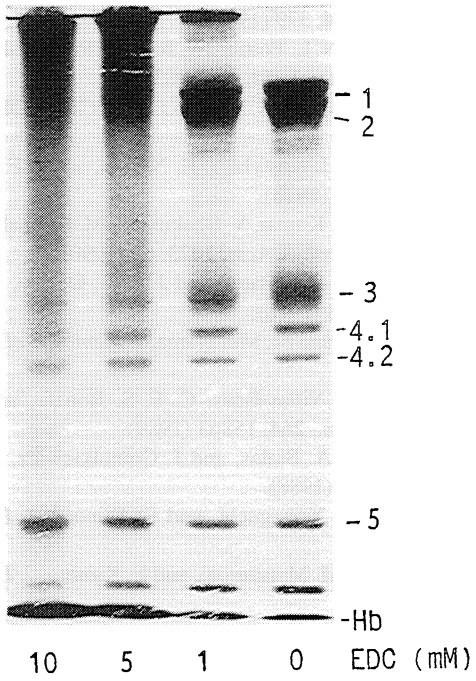

Fig. 3. SDS-PAGE of membrane proteins in EDC-treated erythrocytes. Erythrocytes (20% hematocrit) were treated with EDC (1—10 mM) for 30 min at 37 °C. Hb: hemoglobin.

from the reaction of nonheme iron with H_2O_2 cause the covalent binding of hemoglobin to the cytoskeleton of the membrane.[16,17] Thus, in order to examine whether the iron-(II) of Hb is oxidized by EDC, the ESR spectra of EDC (2—10 mM)-treated erythrocytes were measured at −196 °C. There was no ESR signal due to the oxidation of Hb (data not shown). This indicates that EDC is a mild cross-linker.

Discussion

In this paper, we have described the hemolytic properties of EDC-treated erythrocytes by high pressure, heating, or hypotonic medium. Here, it is useful to compare the properties of these hemolytic methods. In hypotonic hemolysis, water flows into the erythrocytes and causes them to swell and burst. This method is widely used to prepare the plasma membrane because membrane proteins remain stable. On the other hand, heating of the erythrocytes above 45 °C induces enzyme inactivation,[18] the denaturation of cytoskeletal proteins such as spectrin,[19] and gross changes in the cellular morphology.[20] Thus, the membrane structure becomes unstable so that hemolysis occurs.[21] In addition, the oxidation of hemoglobin by heating suggests that the complicated reactions occur within the erythrocyte membrane. As with heating, exposure of the erythrocytes to high pressure also induces the hemolysis. Spectrin detachment from the membrane demonstrates that the cytoskeletal network is destroyed by high pressure. Compared with heating, no oxidation of hemoglobin occurs in high-pressure-treated erythrocytes (T. Yamaguchi et al. unpublished observations).[22] Therefore, the high-pressure method seems to be more appropriate for analyzing the contribution of cytoskeletal proteins to the membrane stability.

Our interesting finding in this study is that the EDC-treated erythrocytes become unstable to hypotonic stress. In such erythrocytes, membrane proteins are cross-linked and anion transport is partially inhibited. A similar instability to hypotonic stress is observed in erythrocytes treated with anion transport inhibitors such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS),[23] although membrane proteins are not cross-linked. Here, it is of interest to consider the osmotic fragility of diamide-treated cells in which membrane proteins are cross-linked, but the active site of band 3 is not modified. The membrane structure of such erythrocytes remains stable to hyporonic stress.[24] These data suggest that the membrane instability of EDC-treated erythrocytes to hypotonic stress is due to anion transport inhibition rather than cross-linking of membrane proteins.

When intact erythrocytes are exposed to a heating of 52 °C, hemolysis and vesiculation[20] are occurred. At 52 °C, spectrin is dissociated from tetramers to dimers[19] so that the cytoskeletal network becomes more fragile. However, the present work demonstrates that the membrane structure of EDC-treated erythrocytes is stabilized against heating at 52 °C.

The membrane stability of the erythrocyte under high pressure has been studied using chemically[4,9] or enzymatically[5] modified cells. These results indicate that high-pressure-induced hemolysis is more sensitive to membrane protein–protein interactions, compared with hypotonic hemolysis. Previously, we showed that high-pressure-induced hemolysis is suppressed in Ca^{2+}-A23187-treated erythrocytes.[14] In these erythrocytes, Ca^{2+}-activated K^+ efflux occurs and intracellular viscosity is increased. We thus examined whether the K^+ efflux occurs in EDC-treated cells. However, upon EDC treatment there was no efflux of intracellular K^+.

High-pressure-induced hemolysis is suppressed by anion transport inhibitors.[25] Bulky ligands such as DIDS and 4-

acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate (SITS) bind covalently to band 3 and induce the conformational changes of band 3.[26] Thus, the interaction of the cytoplasmic domain of band 3 with the cytoskeletal proteins via ankyrin is tightened[27] so that the membrane structure becomes more stable to high pressure.[25] EDC binds covalently to band 3 and inhibits anion transport.[6] Therefore, high-pressure-induced hemolysis may be partially suppressed by the binding of EDC to band 3, as seen in DIDS and SITS.

Cytoskeletal proteins such as spectrin and actin are associated with transmembrane proteins via linking proteins. The driving force for the association between these components may be hydrophobic, ionic, and van der Waals interactions.[28] It is well known that these interactions are largely modulated by high pressure.[29] In high-pressure-treated erythrocytes, spectrin is partially detached from the membrane.[4] Such detachment is suppressed by cross-linking of spectrin with diamide,[4] which is an oxidant of SH-groups. Previously, we showed that high-pressure-induced hemolysis is considerably suppressed by cross-linking (i.e., disulfide bond) of transmembrane proteins with the cytoskeletal ones.[24] Such a unique cross-linking occurs upon diamide treatment under high pressure (100 MPa), but not under atmospheric pressure. Here, the active site of band 3 is not blocked with diamide. In the present work, on the other hand, high-pressure-induced hemolysis is completely suppressed by the mild treatment of erythrocytes with EDC at atmospheric pressure. In this case, there is no contribution of membrane SH-groups for such suppression. SDS-PAGE of EDC-treated erythrocyte membranes demonstrates that high-molecular-weight aggregates are formed by peptide linkage of transmembrane proteins with cytoskeletal ones. Perhaps, junctional proteins such as actin, adducin, protein 4.1, spectrin, and tropomyosin may be also cross-linked each other. Additionally, the active site of band 3 is blocked with EDC. Thus, the membrane structure in EDC-treated erythrocytes becomes extraordinarily stable to high pressure by both cross-linking of membrane proteins and EDC binding to band 3. We thus suggest that a combination of high-pressure technique and cross-linkers such as EDC provides unique information concerning the study of interactions between membrane proteins.

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